INFLUENCE OF MAGNESIUM ON SALIVARY GLAND SECRETION: PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES

by

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ABSTRACT

The divalent abundant cation Magnesium (Mg^{2+}) has been known to play an important regulatory role in the stimulus-secretion coupling events in a number of epithelial secretory cells including the exocrine pancreas, lachrymal and the parietal cells. Since the salivary glands acinar cells have a similar structure and function as the exocrine pancreas, the stomach and the lachrymal gland, it was decided to investigate specifically the effect of perturbation of extracellular magnesium ($[Mg^{2+}]_o$) on both basal and secretagogue-evoked amylase secretion and total protein output and intracellular free calcium concentrations ($[Ca^{2+}]_i$) in the rat submandibular and parotid glands using spectrofluorimetry and spectroscopic techniques. The results have shown that both zero and elevated (5 and 10 mM) $[Mg^{2+}]_o$ can significantly ($P<0.01$) inhibit basal amylase or protein output compared to normal (1.1 mM) $[Mg^{2+}]_o$. Either acetylcholine (ACh), noradrenaline (NA) or Phenylephrine (Phe) can elicit marked increases in amylase secretion and total protein output from the isolated parotid and submandibular gland segments respectively in normal $[Mg^{2+}]_o$. In contrast, in the presence of either zero or elevated (5 and 10 mM) $[Mg^{2+}]_o$ the ACh, NA and Phe-evoked amylase secretion and total protein output were markedly attenuated when compared to normal (1.1 mM) $[Mg^{2+}]_o$. The inhibitory effect of zero $[Mg^{2+}]_o$ was much more pronounced when compared to 10 mM $[Mg^{2+}]_o$. A perturbation of $[Mg^{2+}]_o$ had little or no effect on the Electrical Field Stimulation (EFS) -evoked amylase responses in the parotid gland. Isoprenaline-evoked amylase secretion revealed a different pattern of secretion in the presence of $[Mg^{2+}]_o$ perturbation. In Fura-2 loaded salivary acinar cells a perturbation of $[Mg^{2+}]_o$ had no significant effect on basal $[Ca^{2+}]_i$ in the parotid gland but elevated (5 and 10 mM) $[Mg^{2+}]_o$ attenuated $[Ca^{2+}]_i$ in acinar cells taken from submandibular gland. In acinar cells from both glands ACh (10^{-5} M), evoked a marked increase in $[Ca^{2+}]_i$ above basal level in $[Mg^{2+}]_o$. The response comprises of an initial rise (peak response) followed by a plateau phase (plateau response) before declining back and stabilising a little above control level. In the presence of either zero or elevated (5 and 10 mM) $[Mg^{2+}]_o$ the ACh evoked increases (both peak and plateau phases) in $[Ca^{2+}]_i$ was significantly ($P<0.05$) attenuated compared to normal (1.1 mM) $[Mg^{2+}]_o$. In a nominally free Ca^{2+} medium containing 1mM Ethyl Glycol Tetracetic Acid (EGTA), ACh evoked only the initial Ca^{2+} peak, which was sensitive to Mg^{2+} perturbation. Reperfusion of

acinar cells with normal Ca^{2+} medium resulted in marked increases in $[Ca^{2+}]_i$, which was also sensitive to perturbation of $[Mg^{2+}]_0$. These results indicate that Mg^{2+} is regulating cellular Ca^{2+} homeostasis. In Magfura 2-loaded parotid acinar cells an increasing perturbation of $[Mg^{2+}]_0$ resulted in a gradual increase in intracellular free Mg^{2+} concentrations ($[Mg^{2+}]_i$). Stimulation of the cells with ACh resulted in a significant ($P<0.01$) decrease in $[Mg^{2+}]_i$. Treatment of Magfura-2 loaded parotid acinar cells with either zero extracellular sodium ($[Na^+]_0$) (substituting it with N-Methyl-D-Glucamine (NMDG)), dinitrophenol (DNP) bumetanide, amiloride, quinidine or lidocaine resulted in significant ($P<0.01$) increases in $[Mg^{2+}]_i$. In the presence of these inhibitors ACh still stimulated a reduction in $[Mg^{2+}]_i$. Taken together, these studies on animal model have demonstrated marked interactions between Ca^{2+} and Mg^{2+} signalling during the stimulus-secretion coupling process in the salivary glands.

The study also employs male and female human subjects to investigate the rate of salivary secretion and the quality of saliva during normal healthy and such pathophysiolgical conditions such as ageing, diabetes and surgical procedures. The results have demonstrated that either the ageing process or type I and type II diabetes are associated with significant ($P<0.01$) decreases in salivary secretion. However, both ageing and type I (but not type II diabetes) are associated with increased levels of protein in the saliva. Both ageing and diabetes are associated with significant ($P<0.05$) increases in Ca^{2+} levels and significant decreases in Mg^{2+} and Zn^{2+} levels in saliva compared to age-matched controls. Ageing and diabetes are also associated with reduced levels of K^+ in the saliva. Although type II diabetic patients presented the same type of salivary changes these were less intense when compared to type I diabetic. Surgical procedures had little or no effect on the various salivary parameters measured except for small increase in resting salivary output and protein concentration. These results indicate that both ageing and diabetes can affect markedly the function of the salivary glands leading to decreased secretion in saliva and to a variation in its composition.

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My wife Cristina, the love of my life

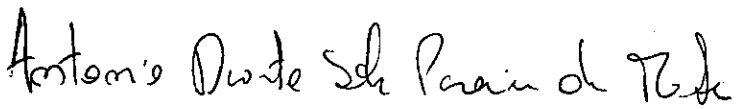
My parents Manuela and Américo, the references of my life

My son and daughter Gonçalo and Ines, the lights of my life

My brother Carlos, the brightness who has always inspired me

Declaration

I declare that while registered as a candidate for the degree for which this submission is made that I have not been a registered candidate for another award by any other awarding body. No material contained in this thesis has been used in any other submission for an academic award.

Signature: 

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Saliva definition properties, composition and functions

1.1.1 What is saliva?

Saliva may be generally defined as the fluid produced and secreted into the mouth by salivary glands (Jenkins, 1978). However, salivary glands are heterogeneous organs which produce different types of salivas with various composition and functions. Once in the mouth, the different types of salivas mix with each other and the mixture is contaminated with foreign elements, which are present in the oral cavity. These include: crevicular fluid slowly leaking from gingival crevice, oral microflora consisting of more than 500 bacterial species, fungi, viruses, and food debris among others (Whittaker *et al.*, 1996). Salivary glands have seasons and circadian rhythms of secretion for the overall flow and this is an important process for each of the components secreted by the gland (Shannon, 1966; Dawes, 1974). Unlike the plasma whose constituents are kept within a very tight compositional range, saliva composition is gender related. Moreover, it varies with gland stimulation nature, degree and thus with secretion flow (Baxter, 1933; Dawes, 1969; Dawes, 1984), and it has been shown to be highly variable among individuals and even in the same individual under different conditions (Dawes, 1987). This diversity precludes the existence of a plain definition of saliva and has brought over the years the need for the establishment of a nomenclature system. More recently, a set of guidelines for saliva nomenclature and collection has been established by several authors (Pellerin & Pellat, 1986; Atkinson *et al.*, 1993)

1.1.1.1 Whole saliva

Whole saliva is the fluid obtained from the mouth by expectoration. It contains the secretory products from all the salivary glands but also desquamated cells from oral epithelia, crevicular fluid, oral microflora specimens and food and drink residues following their ingestion (Edgar, 1992; Atkinson *et al.*, 1993). Draining, spitting, suction or swabbing methods can collect it. For assessment of overall salivary gland dysfunction whole saliva is superior and clinically more relevant (Navazesh, 1993; Navazesh *et al.*, 1992).

1.1.1.2 Mixed saliva

Mixed saliva refers to whole saliva which has been submitted to post collecting centrifugation in order to clear all contaminating elements (Pellerin & Pellat, 1986). The transformation of whole saliva into mixed saliva and has nowadays become an essential procedure for salivary analytical purposes and these terms tend to be used without distinction.

1.1.1.3 Pure Saliva

Pure saliva refers to saliva, which is collected directly from a salivary gland duct orifice, e.g. parotid saliva. When speaking about submandibular saliva objective confirmation should be made (e.g.. Sialography) that sublingual glands do not secrete fluid in the same duct. When collecting saliva from minor salivary glands the location of these should be indicated (e.g. labial, palatine) because there are differences in the secretions (Atkinson *et al.*, 1993)

1.1.1.4 Unstimulated and stimulated saliva

Unstimulated saliva refers to saliva secreted in the absence of exogenous stimulation of any nature. It corresponds to the basal secretion of the gland. On the other hand, stimulated saliva refers to saliva secreted in response to either mechanical, pharmacological or gustatory stimulation (Atkinson *et al.*, 1993)

1.1.1.5 Serous and mucous saliva

At the cellular level saliva production can be either serous or mucous in nature. Serous saliva is watery and rich in electrolytes and proteins of various natures. Mucous saliva is more viscid and rich in mucins, which are heavy O-glycosilated proteins forming a jelly like blanket coat known as mucus covering on all mucosa surfaces of the body (Tabak *et al.*, 1982; Tabak, 1995). Although this distinction between serous and mucous saliva is not usually made in salivary classifications, it is our belief that it should be so because

there are striking differences at the compositional and functional levels between these two types of saliva.

1.1.2 Composition and properties of saliva

1.1.2.1 Saliva composition

Saliva is composed of 99.4 to 99.5% of water, 0.32 % of organic compounds and 0.18% of inorganic matter (Pellerin & Pellat, 1986).

1.1.2.1.1 Organic composition of saliva

In spite of a very small concentration (2-3 $g.l^{-1}$) when compared to plasma, proteins account for the major organic constitution of saliva. The salivary protein family is very heterogeneous and its components play a major role in diversified salivary functions.

1.1.2.1.1.1 Proline Rich Proteins

In spite of being previously reported to exist in small amounts in tissues like pancreas or respiratory tract, Proline-rich proteins (PRP) constitute a particular family of salivary proteins, which are produced by serous acinar cells of major salivary glands (Ito *et al.*, 1983; Warner and Azen, 1984; Ito *et al.*, 1984). They are characterised by a proline content varying from 25-40% and are rich in glutamate and glycine all of these amino acids accounting for 75-80% of all residues (Bennick, 1982). Although very heterogeneous in nature PRP's can be grouped in three major types: acid, basic and glycosylated constituting 30, 23 and 17% respectively, of all salivary proteins (Kaufman & Keller, 1979).

Acid PRPs exist in four types namely: PRP1, PRP2, PRP3 and PRP4. Type 3 is obtained from cleavage of type one and types 2 and 4 are quite similar. Each one of them contains 11 residues of glutamic acid and two phosphoserine residues (Lazzary, 1983). The biological function of these proteins is fully linked to the structural features described, which give these proteins a remarkable affinity for calcium binding (Bennick *et al.*, 1981). Thus, these proteins are involved in calcium transport in saliva,

sialolitiasis prevention, inhibition of hydroxyapatite and dental calculus formation (Moreno *et al.*, 1979), maintenance of supersaturating of calcium and phosphate in saliva, buffering and remineralization of dental tissues (Fox, 1989).

The intrinsic affinity for hydroxyapatite is also responsible for a high attraction towards dental enamel or teeth surface to which they adhere strongly (Moreno *et al.*, 1982) taking part in the acquired dental pellicle (Bennick *et al.*, 1983). The acquired dental pellicle (ACDP) is a salivary derived proteinaceous film, which forms physiologically and spontaneously at the enamel surfaces after tooth brushing (Sonju & Rolla, 1973; Lamkin *et al.*, 1996). This protein film has been demonstrated to be important for the integrity of tooth enamel by exhibiting lubricating properties, perm-selectivity (which delays acid diffusion) (Zahradnick *et al.*, 1976) and by providing a medium through which fluoride, calcium and phosphate are delivered during remineralization (Zahradnick *et al*, 1977). In addition, the proteins constituting this pellicle act as natural ligands for many surface receptors in oral bacteria (Whittaker *et al.*,1996) and thus play a major role in premature steps of dental plaque formation and oral flora modulation, Acid PRP's participate in every one of these processes (Gibbons & Hay, 1988; Yao *et al.*, 1999).

Basic PRPs exist in four types, PRB 1, 2, 3 and 4. They differ from acidic PRP by a higher proline content. The scientific information on characterisation, structure and functions of this group is diminished compared to acidic proteins. However, it seems that functional properties share some homology with acidic PRP (Yao *et al.*, 1999).

Glycosylated PRPs have the same protein structure as basic PRP. Glycosyl residues mainly fucose, mannose and sialic acid account for 40% of the whole molecule. Glycosylated PRPs have lubricating properties (Hatton *et al.*, 1985) and bind some oral microorganisms thus playing a major role in modulation of oral microflora (Levine *et al.*, 1987).

1.1.2.1.1.2 Statherin

Statherin is a 43-residue tyrosine rich protein from serous origin, containing 10 residues of glutamic acid and 2 phosphoserines. For sometime, it was erroneously thought to be PRP 2. However, it has been demonstrated that Statherin belongs to a unique super family of proteins (Schlesinger & Hay, 1977; Jensen *et al.*, 1991). It has a major role in

inhibiting spontaneous precipitation of calcium and phosphate from saliva and secondary growth of calcium phosphate salts on tooth surface, helping to maintain a stable environment for the teeth (Hay, 1973; Hay & Gron, 1976; Raj *et al.*, 1992).

1.1.2.1.1.3 Histatins

Histatins are a group of salivary histidine-rich proteins of serous origin that were originally purified by numerous authors (Balekjian & Longton, 1973; Peters & Azen; 1977; Mckay *et al.*, 1984 a, b; Oppenheim *et al.*, 1986). Initially called Histidine rich proteins (HRP), they have been renamed by Oppenheim as Histatins from which at least seven types have been isolated. They constitute a independent unique family which shares some common features with the PRPs (Oppenheim *et al.*, 1988). These proteins undergo proteolytic cleavage after secretion mainly by salivary kallikrein, giving rise to smaller members of the family (Azen, 1973). Functionally, these proteins adhere strongly to hydroxyapatite, participate in ACDP, inhibit crystal growth and dental calculus formation (Hay, 1975), enhance glycolitic activity of salivary microorganisms (Holbrook & Molan, 1975) and have antimicrobial and anti fungal activity (McKay *et al.*, 1984 a, b; Pollock *et al.*, 1984). Anti fungal properties seem to be the most important ones. Development of synthetic delivery systems for these peptides and their use as therapeutic agents could be a relevant issue in future (Tsai *et al.*, 2000).

1.1.2.1.1.4 Cystatins

Cystatins are small protease inhibitors whose presence in saliva has been known for over nearly two decades (Shomers *et al.*, 1982 a, b; Hiltke *et al.*, 1999). Hitherto, three members of this family have been identified in human saliva. They include Cystatin-S (Isemura *et al.*, 1984), Cystatin-SN (Isemura *et al.*, 1986) and Cystatin-AS (Isemura *et al.*, 1987), all of acidic nature with exception of Cystatin-SN. These proteins share the ability of competitively and reversibly inhibiting cysteine proteases of microorganisms and viruses. They are very important in preventing tissue penetration and infection by microorganisms (Barret *et al.*, 1986; Bobek & Levine, 1992). They can adsorb on to hydroxyapatite (Shomers *et al.*, 1982 a, b) and thus, participate in ACDP, and inhibition of transformation of dicalcium phosphate dihydrate (Isemura *et al.*, 1984) in more

precipitable forms of calcium phosphate. The later is very important for prevention of dental calculus formation. Some recent studies have reported an increase of salivary cystatin production and secretion in peridontitis patients (Henskens *et al.*, 1996).

1.1.2.1.1.5 Amylase

α-Amylase is the most prevalent digestive enzyme present in saliva. It occurs in parotid saliva at concentration of 60-120 mg .100 ml^{-1} and submandibular saliva of approximately 25 mg .100 ml^{-1} (Edgar, 1992). Human salivary amylase hydrolyses α-1-4 glycoside bonds in starch (Pellerin & Pellat, 1986), yelding maltose, maltotriose, α-limit dextrins and glucose as final products. In spite of playing the same role as pancreatic amylase and having similar composition and immunological activity (Liang *et al.*, 1999), these enzymes have different isoelectric points, molecular weights and catalytic properties (Liang *et al.*, 1999). Salivary amylase exists in two families: family A is glycosylated with average molecular weight of 62000 Da while family B is non-glycosylated with 56000 Da. At least six izoenzymes have been identified (Liang *et al.*, 1999). Although participating in the initial digestion of starch (Tseng *et al.*, 1999), the importance of salivary amylase in digestion has been shown to be secondary compared to pancreatic, as people who lack it fail to show any digestive perturbations. However, salivary amylase has several important intra-oral functions such as affinity for hydroxyapatite, participation in ACDP and modulation of intra-oral microflora (Scannapieco *et al.*, 1995; Gong *et al.*, 2000). The catalytic activity of salivary amylase also plays an important role in degradation of sticky starch rich foods which are retained in dental surfaces and their transformation in slow glucose releasing devices which may play quite a role in dental decay pathogenesis (Tseng *et al*, 1999).

1.1.2.1.1.6 Salivary Peroxidases

Human whole saliva has been shown to contain different groups of peroxidases with acidic and basic isoelectric points (Morisson & Allen, 1963). Salivary peroxidases catalyses the oxidation of tiocyanates (SCN-) present in saliva into the acid/base pair hypotiocyanite (OSCN-) and hypotiocyanous (HOSCN) ions. Hydrogen peroxide acts as a co-factor in this reaction. OSCN/HSCN act as broad spectrum anti bacterial agents

(Iwamoto & Matsumura, 1966; Tenovuo & Kurkijarvi, 1981). Salivary peroxidases have been detected adsorbed to bacteria, enamel surfaces and participates in ACDP (Tenovuo & Kurkijarvi, 1981; Cole *et al.*, 1981).

1.1.2.1.1.7 Lactoferrin

Lactoferrin is a red iron binding protein with anti-bacterial activity, synthesized by acinar epithelial cells and neutrophils. It binds ferric ions (Fe^{3+}), thus depriving microorganisms of this essential nutrient. Lactoferrin was first isolated in milk (Lazzari, 1983) but its presence in salivary gland secretions was soon demonstrated (Masson *et al.*, 1966). In spite of resembling human transferin in its iron binding mechanisms (Bluard-Deconinck *et al.*, 1974), lactoferrin maintains iron affinity at lower pH, which may be responsible for keeping its anti-microbial properties even under dental plaque acid producing conditions. In its iron free state, lactoferrin also has agglutination and bactericidal effects, which require binding to cell surface (Fine *et al.*, 2002).

1.1.2.1.1.8 Lyzozyme

Lysozyme, also known as muramidase is an enzyme which hydrolyses the β- 1, 4 glycosydic bonds N acetyl-muramic acid and N-acetylglucosamine in peptidoglucan structure of cell wall of micro-organisms leading to cell lysis in sensitive species. Lysozyme is constituted by a monomer chain of 131 amino acids with a molecular weight of 14 KDa and its presence in saliva has been known for several decades (Petit & Jolles, 1966; Osserman *et al*, 1974). Its anti-bacterial importance is thought to be reduced as many bacterial species which have adapted to oral cavity conditions can resist the actions of the enzyme at concentrations many times greater than those occurring in saliva (Gibbons *et al*, 1966). However, lysozyme could play an important role in oral flora modulation through its interactions and binding to bacterial surfaces (Lazzari, 1983).

1.1.2.1.1.9 Carbonic anhydrase

Saliva also contains an isoenzyme of carbonic anhydrase, which is numbered VI. It plays a major role in regulation of salivary pH as bicarbonate is the major buffering system in saliva. However, salivary carbonic anhydrase seems to protect teeth from caries via mechanisms other than direct regulation of pH and buffering capacity. Recently, carbonic anhydrase VI has been identified in ACDP at enamel surface suggesting that it may accelerate the removal of acid by function locally in the pellicle layer in dental surfaces (Leinonen *et al.*, 1999).

1.1.2.1.1.10 Mucins

Salivary mucins are high molecular weight glycoproteins composed of a degenerate protein backbone (apomucin) enriched in hydroxyaminoacids (threonine and/or serine) and proline. In addition, hundreds of carbohydrate side chains (oligosaccharides) are linked with O-glycosyl bonds to many but not necessarily all of the hydroxiaminoacids. Salivary mucins are synthesised by the mucus acinar cells of the paired submandibular and sublingual glands, as well as minor salivary glands, which are, distributed through palatal and bucal mucosa. Serous acinar cells, such as those found in human parotid gland do not contribute to production of salivary mucins (Tabak, 1995).

Human saliva contains two types of salivary mucins. Human type 1 salivary mucin (MG1) is composed of multiple covalently bound subunits and has very elevated molecular weight (Loomis *et al*, 1987). Human type 2 salivary mucin (MG2) is composed of a single chain being smaller and lighter than MG1 (Prakobphol *et al*, 1982). Mucins play a role in lubrication, tissue coating, digestion, food bolus formation, swallowing, and microbial interactions with bacteria, fungi and viruses (Cohen & Levine, 1989; Levine, 1993). MG1, which is heavier tends to sediment and exerts its functions though tissue coating, MG2 acts as a fluid phase agglutinin (Liu *et al.*, 1998). Mucin oligomer formation is apparently required for these molecules to exert its lubrication and viscoelastic properties. These molecules may form homotypic complexes or end to end oligomers with themselves via interchain disulphide bonds (Strous & Dekker, 1992). Mucins that coat various tissue surfaces can also form heterotypic complexes with other salivary molecules like, amylase, PRP, statherin,

histatins, IgA, Lysozyme and cystatins. These complexes are mediated primarily by non-covalent ionic forces and function to concentrate antimicrobial molecules at several tissue interfaces, as a repository for precursors of ACDP, a mechanism against proteolysis and a physiologic delivery system (Biesbrock *et al.*, 1991; Iontcheva *et al.*, 1997).

1.1.2.1.1.11 Immunoglobulins.

Besides innate anti-microbial systems, saliva also contains immunoglobulins as effectors of acquired immunity against potential pathogenic species. The major Salivary immunoglobulin (Ig) is secretory IgA (S-IgA), which occurs at ranging concentrations in whole saliva approximately of 100-300 $\mu g \cdot ml^{-1}$ in the adult. (Russel *et al.*, 1999). It is produced in salivary glands by mucosal plasma cells which secrete polymeric IgA, and is taken up and transported by a receptor, secretory component expressed on basolateral surface of glandular epithelial cells and released into the saliva as S-IgA (Mesteki *et al.*, 1991). S-IgA is the main product of the common mucosal immune system (CMIS) exceeding all other isotypes together (for a complete review of the CMIS refer to Ogra *et al.*, 1998). Human IgA occurs in two subclasses IgA1 & IgA2 (Mesteky & Russel 1986); and both occur in saliva usually with predominance IgA1 (60%). IgA1 is unique in having an elongated rich proline and O-glycosylated hinge region that is susceptible to cleavage by bacterial proteases (Killian & Russel, 1998). Humans and primates have high levels (2-3 $mg \cdot ml^{-1}$) of circulating monomeric IgA (90% IgA1) contrasting to most mammals which have a dimeric circulating form of IgA in concentrations lower than $1 mg.ml^{-1}$ (Russel *et al.*, 1999). Past studies have revealed important functions for salivary IgA including: inhibition of microbial adherence by blocking adhesins (Hajishengallis *et al.*, 1992), neutralization of toxins or viruses and virulence factors (Smith *et al.*, 1985), protection against mucosal pathogens such as *Streptococcus pyogenes, Vibrio cholerae and* influenza Virus (Russel *et al.*, 1998).
In addition, the oral cavity receives Igs derived from the circulation by transudation through the gingival crevice including IgM, IgG, and Ig A roughly to their proportion in blood plasma ($< 15 \mu g \cdot ml^{-1}$) which are minimal but can be elevated in gingivitis or peridontitis (Russel *et al.*, 1999).

1.1.2.1.1.12 Growth Factors

During the past four decades a large number of growth factors have been progressively identified in salivary secretions in a wide range of animal species. The list of biologically active peptides includes: epidermal growth factor (EGF) (Cohen, 1962; Gresik 1994), nerve growth factor (NGF) (Barka, 1980; Gresik 1994), transforming growth factor alpha (TGFα) (Humphreys-Beyer *et al.*, 1994), insulin (Murakami *et al.*, 1982; Kerr *et al.*, 1995), insulin-like growth factors I and II (IGF-I & IGF-II)(Ryan *et al.*, 1992), transforming growth factor beta (TGFβ) (Jaskoll *et al.*, 1994) and fibroblast growth factor (Hiramatsu *et al.*, 1994). The origin of these molecules has been quite controversial. Initially, thought to derive from plasma serum or crevicular fluid (Fekete *et al.*, 1993), there are now overwhelming evidences that at least some of these molecules (TGFα, EGF, NGF, Insulin) are synthesised by ductal cells in salivary glands (Purushotam *et al*, 1995). A detailed description on structure and function for all of these factors is clearly beyond the scope of this work (see review Zelles *et al.*, 1995). However, in a general way these molecules are thought to play a critical role in wound healing, the control of gastric acid secretion, cytoprotection and maintenance of oral and GI tract integrity (McKay *et al.*, 1992; Oxford *et al.*, 1998; Pedersen *et al.*, 2002). Oral mucosa healing rate is known to occur faster than skin (Zelles *et al.*, 1995). This may have to do with a strong presence of these molecules in saliva as submandibular gland produces more EGF and NGF than any other organ in the body (Rall *et al.*, 1985).

1.1.2.1.1.13 Other proteins

The proteins presented above constitute the main bulk of proteinaceous organic secretion in saliva. However saliva contains other proteins, some of them have received less attention by investigators in past years while others are just now being discovered.

1.1.2.1.2 Inorganic composition of saliva

Inorganic species concentration is about 2 g . l^{-1}, the range values in table reflect individual and stimulation status variations (Pellerin & Pellat, 1986).

IONS	SALIVARY CONCENTRATION (mM) Range/mean	PLASMA CONCENTRATION (mM)
$[Ca^{2+}]$	0.50-2.80/ 1.75	2.40-2.60
$[PO_4^{3-}]$	1.94-22.61/ 5.50	0.96-1.30
$[F^-]$	0.00052 – 0.015/0.015	
$[Na^+]$	2.50-10.00/ 6.25	145.00
$[K^+]$	17.00-40.00/ 28.50	4.60
$[Cl^-]$	25.00-33.00/ 29.00	104.00
$[HCO_3^-]$	1.00-30.00/ 3.00	23.00
$[Mg^{2+}]$	0.10-0.50/ 0.30	0.74-0.92
$[I^-]$	0.01-0.02/ 0.01	0.55-1.18
$[NO_3^-]$	0.02-3.7/ 0.1	
pH	5.00-8.00	7.14
$[CNO^-]$	1.50	

Sodium, potassium and chloride are the most important ions in maintenance of the ionic strength of saliva (Tylstrup & Fejerskov, 1994). Ionic strength of saliva may play a role in avoiding enamel dissolution but it is a small one compared to pH variations. However, supersaturation of saliva in Ca^{2+} and PO_4^{3-} towards enamel tissue is critical for permanent remineralization of this dental tissue

Saliva is hypotonic in nature when compared to plasma due to the heavy resorption of sodium during salivary secretion. This fact is important in facilitating taste perception.

1.1.3 Salivary Functions

1.1.3.1 Mucosal protection

Saliva plays a major role in mucosal protection (Tabak, 1995). This functional aspect is directly linked to its lubrication and tissue repair properties. The lubrication of oral soft and hard surfaces by mucins and glycoproteins is critical to maintenance of its integrity during mastication, speech and other mechanical stresses exerted during oral functions

(Tenovuo, 1998). The tissue healing properties of saliva lie on the presence of growth and other immune factors, which have been discussed in this work.

1.1.3.2 Microbial Control

Salivary antibodies are mainly of the IgA class, whose principal action is to aggregate specific microorganisms and prevent their adhesion and colonisation of hard and soft tissues. Bacterial lysis due to complement activation may occur in the gingival crevice as a result of the effects of other Immunoglobulins (e.g. IgG, IgM) in the crevicular fluid, which may also opsonise bacteria to assist removal by phagocyte cells. Microbial modulation of oral flora (especially at the bacteria level) may also occur through adhesive interactions between salivary macromolecules and microbial surface receptors known as adhesins. Mucous coat, and ACDP will therefore determine the resident commensal flora of soft and hard tissues respectively; while soluble proteinaceous salivary content play an important role in aggregation, swallowing and elimination of other micro-organisms present in saliva (Whittaker *et al.*, 1996; Tenovuo, 1998).
The action of sialoperoxidase, lactoferrin, lysozyme and histatins help to modulate the oral flora in a non-specific manner. Although saliva contains a very large microbial population (indicating that these systems are incapable of controlling many of the normal residents of the mouth), they may be of importance in preventing colonisation by transient pathogens (Könönen, 2000).

1.1.3.3 Remineralization of teeth

Saliva is supersaturated in calcium and phosphate ions in respect to dental enamel. This is responsible for preventing teeth from naturally dissolving in saliva and to promote the growth of hydroxyapatite crystals during the remineralization phase of the caries process (Lagerlof, 1998; Dowd, 1999).

1.1.3.4 Neutralisation and buffering

The acid nature of some food intake and final metabolic products of some oral bacteria may have deleterious effects on oral tissues. Natural pH of saliva is always elevated when compared to the pH at which dental hard tissues start to dissolve, namely critical pH (5.5 for hydroxyapatite, 4.5 for fluorapatite). To achieve this tight regulation of pH saliva relies on some buffering systems. The principal constituent responsible for these properties (especially in stimulated saliva) is the bicarbornate and carbonic anhydrase system. Besides bicarbonate, phosphates and proteins present in saliva also contribute to buffering, especially in unstimulated saliva where the concentration of bicarbonate is very diminished when compared to the post-stimulation state (Lagerlof, 1998; Dowd, 1999).

1.1.3.5 Alimentation

Saliva plays a fundamental role in nutrition of individuals. The lubrication properties of saliva are very important for speech, mastication, formation of food bolus, swallowing and translocation across oesophagus (Pedersen *et al.*, 2002). Organic glycoprotein material of saliva is responsible for these lubricating properties (Tabak, 1995).
Another effect of the water content of saliva is the dilution of substances introduced into the mouth and their subsequent removal by swallowing or spitting. Dissolving solid food stuffs is also fundamental for taste perception (Ten Cate, 1998).

1.1.3.6 Digestion

Saliva participates in the initial phase of digestion of fat and complex carbohydrates. Digestion of fat starts in saliva with a lipase produced by Ebner's glands of the tongue. This lipase degrades triglicerides with short-chained fatty acids, its importance is small since it is not the only existing pre duodenal lipase (Gastric lipase). Salivary amylase initiates carbohydrate digestion especially starches, yelding maltose, maltotriose and α-limit-dextrins as final products. This enzyme is rapidly inactivated as food enters stomach because of acid and proteolytic degradation of the enzyme, and thus it has a limited action when compared to pancreatic amylase (Dowd, 1999).

1.2 Anatomy of the Salivary glands

Saliva is produced by salivary glands. In mammals, salivary glands occur as three major pairs, parotid, submandibular and sub-lingual which are located outside the oral cavity, capsulated and with extended duct systems to discharge their secretions and are responsible for 95% of saliva production. The remaining saliva is produced and secreted by minor salivary glands. Several hundreds of these glands are distributed throughout the oral mucosa and they include labial, lingual, palatal bucal, glossopalatine and retromolar (Ten Cate, 1998).

1.2.1 Parotid gland

Parotid glands are the most volumous of major salivary glands. In human, it is a pyramidal triangular shaped, paired gland weighting averagely 25 grams. It is divided in two main lobes by a fibrous plane, which gives way to the passage of facial nerve, external jugular and parotid communicating veins. The surface is lobulated and the colour is grey-yellowish. Parotids are located in a space limited anteriorly by the posterior edge of the ascending ramus of the mandible and the temporo-mandibular joint, posteriorly by the mastoid process and anterior edge of sterno-cleido mastoid muscle, superiorly by external audition conduct and inferiorly by a line extending from the inferior mandibular edge to the anterior portion of sterno-cleido mastoid muscle (Figure. 1.1).

Branches of the external carotid provide arterial irrigation and posterior auricular arteries, venous branches of external jugular and parotid communicating veins drive blood supply. Innervation is provided through auriculo-temporal, superficial cervical plexus and sympathetic nerves. Saliva produced inside the gland is collected in a main excretory duct, 4 cm long and 3 mm wide and it is named the Stensen's duct. Stensen's duct originates in gland depth and exits through its anterior portion, runs across facial region and opens in the oral cavity in the inner cheek at first upper molar level (Rouvière & Delmas, 1990; McMinn *et al.*, 1990).

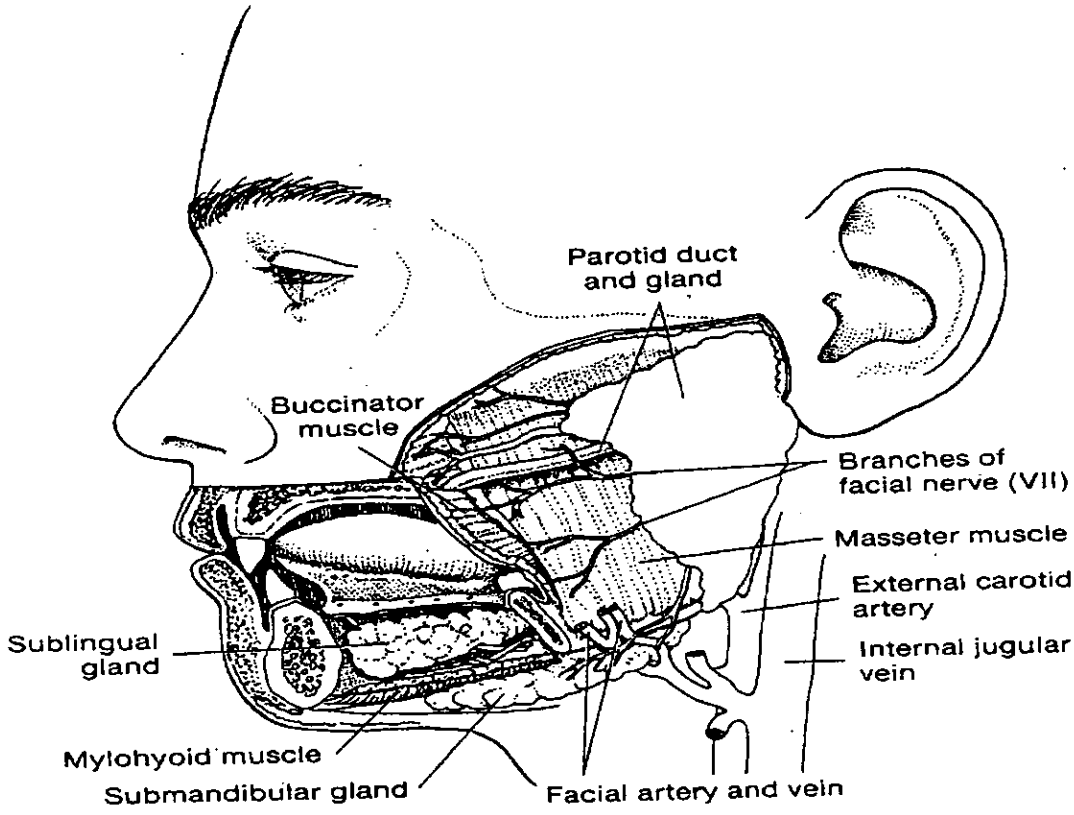


Figure: 1.1- Anatomy Of Major Salivary Glands (Taken from Avery, 1992)

1.2.2 Sub-mandibular gland

Sub-mandibular, weighting 7 grams, is a small almond shaped, rose coloured paired gland. It is located in a space sited in the upper side hyoid region related anteriorly with the sub-mandibular fossa in the internal face of mandibular body (Figure 1.1). An anterior process emerges from the gland's deeper portion runs over the milo hyoid muscle ending at the posterior side of the sub-lingual gland. Branches of facial and sub-mentonian arteries, which originate the venous drainage through the facial vein, provide irrigation. Innervation comes from the lingual nerve. Saliva produced internally in the gland is driven into the oral cavity by Wharton's duct which opens in the floor of the mouth near the lingual frenum (Rouvière *et al.*, 1990; Mc Minn *et al.*, 1990).

1.2.3 Sub-lingual gland

Sub-lingual is a small pair gland-weighing 3 grams, which locates in the floor of the mouth behind the mandibular body underneath alveolar mucosa, near the lingual frenum (Figure 1.1). Arterial irrigation is assured by branches from the sub-lingual arteries which originate deep lingual and ranin veins responsible for venous blood flow. Sub-lingual, which is a branch from the lingual nerve provides innervation. Main secretory duct is called *Rivinus or bartholin* goes side by side with Warthon's duct and opens externally to it at mouth floor level. Short accessory ducts *(Walther ducts)* are also present (Rouviére *et al.*, 1990; McMinn *et al.*, 1990).

1.2.4 Minor salivary glands

The minor salivary glands are important components of the oral cavity. They exist as individual glands located in sub-mucosa between muscle fibers and consist of groups

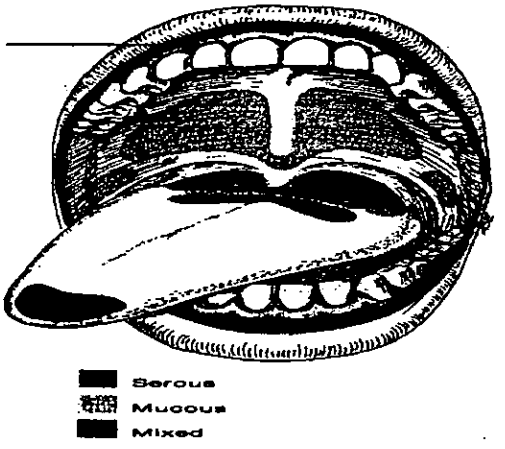


Figure 1.2 Location and Type of Minor Salivary Glands (Taken from Avery, 1992)

of secretory end pieces made up of mucous acinar cells and serous or seromucous demilune cells. The ductal systems comprise intercalated ducts, intra-lobular ducts and excretory ducts opening directly though the mucosa (Hand *et al.*, 1999).

These glands are located throughout the oral cavity (Figure 1.2) and are named after their location. The glands of cheeks and lips are termed the bucal and labial glands. The glands of both posterior soft and hard palate are called palatine. Glossopalatine refers to

the glands located in tonsil folds. Lingual glands are located in several regions of the tongue (Avery, 1992).

1.3 Salivary Gland Structure

The salivary glands are organised like grapes on a vine (Figure 1.3). The grapes represent secretory end pieces known as acini and the stalks represent the ductal system which modifies and conducts primary saliva produced in the acini (Ten Cate, 1998).

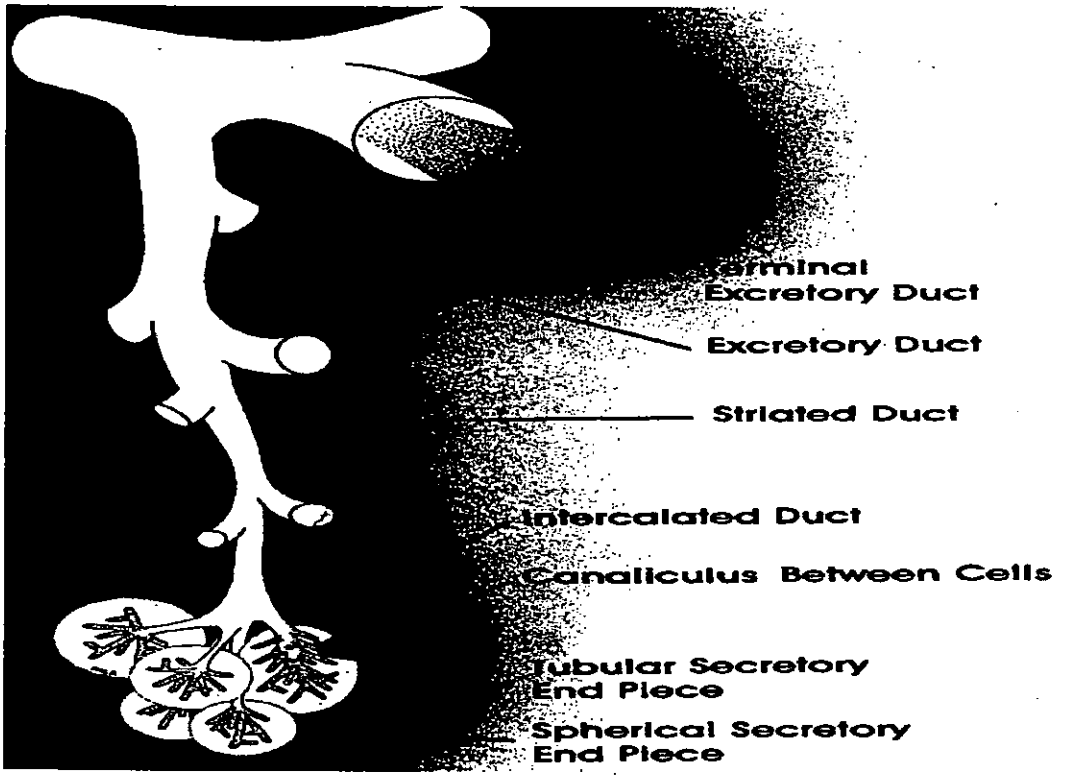


Figure 1.3- Internal Organisation of Salivary Glands (Taken from Ten Cate, 1998)

1.3.1 Acini

The functional unit of the salivary gland tissue is the alveolus or acinus. An acinus is a cluster of pyramid-shaped cells, either mucous, serous or a combination of both, that secrete into a terminal collecting duct. Both the major and minor glands are composed of many acini, through the larger glands contain more acini or units arranged in lobules and lobes. The outer edge or base of the cells rests on a basement membrane between the cells and connective tissue. Within this connective tissue are the nerves and blood vessels necessary to the various aspects of cellular activity. This connective sheet separates every acinus, lobules and lobes. The apex of the cells faces the hollow centre (lumen) of the acinus. Acinar cells are responsible for the major salivary protein and fluid volume output (Brand & Isselhard, 1998).

Regardless of their differences in volume and type of saliva secreted, salivary glands are composed of the same acinar cell types either serous or mucous or a combination of both called serous demilunes (Figure 1.4). Some glands consist of pure or nearly pure serous acini (parotid), whereas others are pure mucous (sublingual) or a mixed combination of serous and mucous cells (sub-mandibular) (Brand & Isselhard,1998).

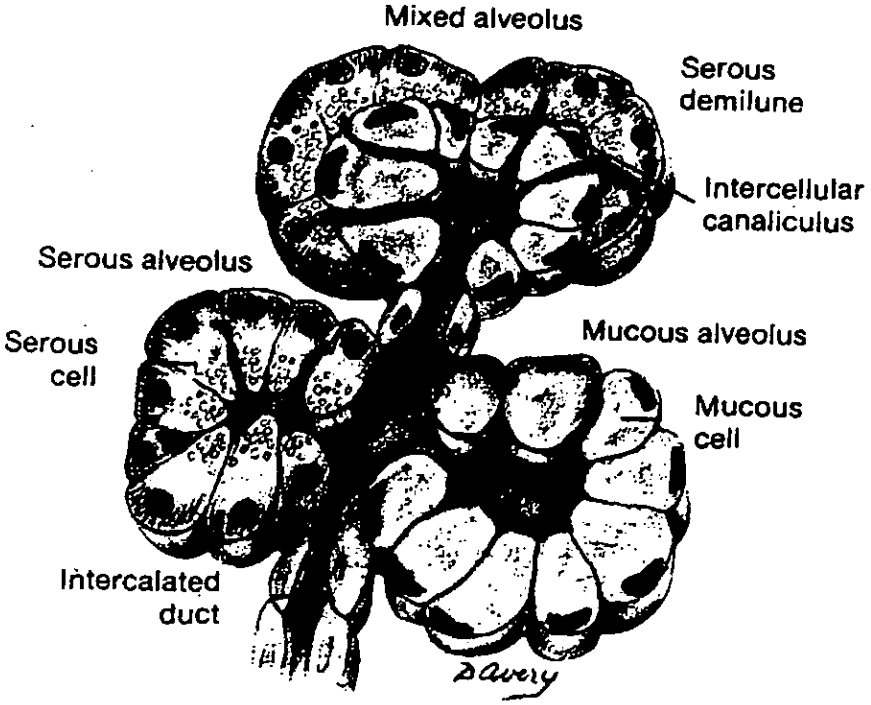
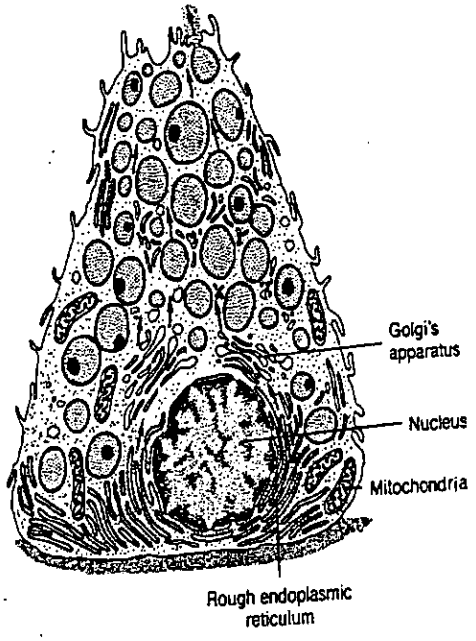


Figure 1.4- Structure of Acini (Taken from Avery, 1992)

1.3.1.1 Serous acini



Serous acini are composed of serous cells (Figure 1.5). These are pyramid-shaped cells with all features of secretory cells: well-developed rough endoplasm reticula and Golgi and secretory granules where proteins are packed, the nucleus is round and close to the base of the cell. Serous secretion is a thinner watery secretion, rich in electrolyte and low glycosylated proteins as enzymes. Serous cells are the main source of amylase, PRP, Histatins among other (Avery, 1992; Junqueira & Carneiro, 1990).

Figure 1.5- Serous Acinar Cell (Taken from Avery, 1992)

1.3.1.2 Mucous acini

Mucous acini are composed of mucous cells (Figure 1.6). These are classic secretory cells as serous ones in which the main proteinaceous products are the extremely heavy glycosylated proteins called mucins. Although mucous secretion is also composed of 99% of water and electrolytes, the presence of mucins is responsible for a much higher viscosity of this type of secretion. Mucous acini are more tubular and have larger lumen than serous ones. The nucleus of mucous cells is flattened and lies against basal part of the cell pushed by enormous secretory granules filled with heavily packed mucins (Junqueira & Carneiro, 1990).

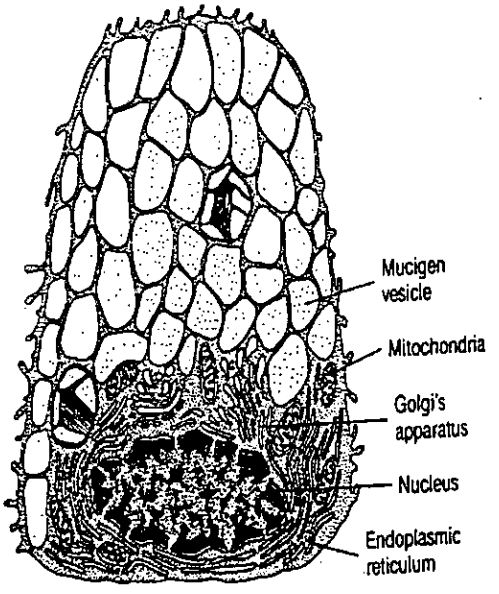


Figure 1.6- Mucous cell (Taken from Avery, 1992)

1.3.1.3 Seromucous acini

In glands that have both mucous and serous components, it is possible to see the separate types of acini, or see them together as mixed or serous mucous acini (Figure. 1.4). In those structures the mucous cells are disposed in a tube-like fashion with a half-moon cluster of serous cells at the end. These are known as serous demilunes. These hybrid acini produce both serous and mucous saliva (Ten Cate, 1998).

1.3.2 Ductal system

The ductal system of salivary glands is a varied network of ducts divided in three classes: intercalated, striated and terminal (Figure 1.7). Besides the function of conducting saliva produced in the acini into the oral cavity, ducts also actively participate in its production and final modulation (Brand & Isselhard, 1998).

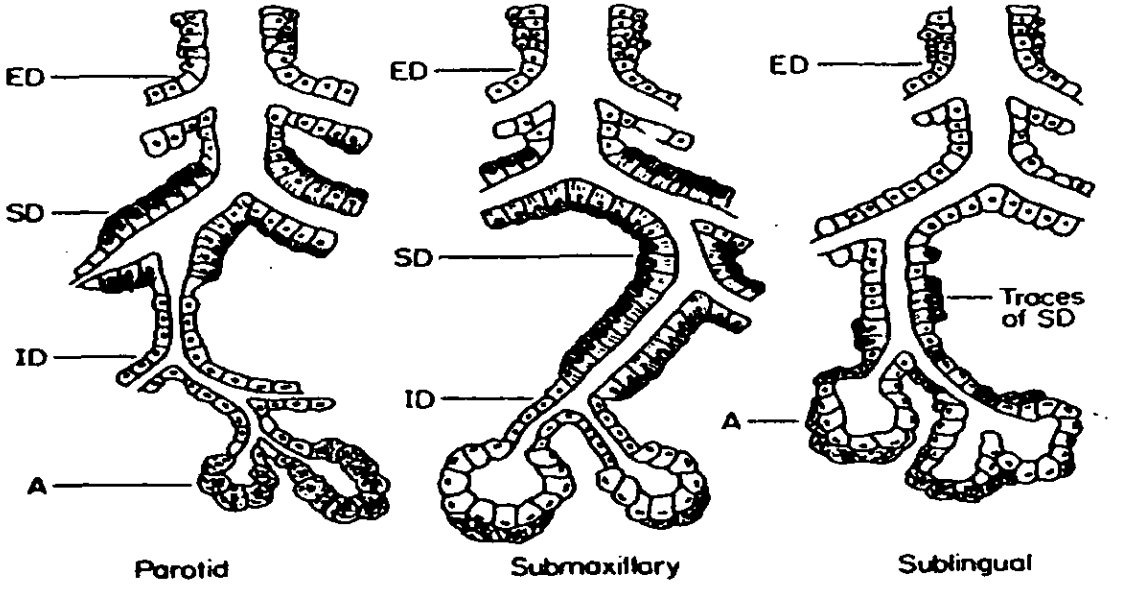


Figure 1.7- Duct System. ID- Intercalated Duct, SD-Striated Duct, ED-Excretory Duct (Taken from Jenkins, 1975).

1.3.2.1 Intercalated ducts

Intercalated ducts are small intralobular ducts that directly drain the acini (Figure 1.7). Short, cubical cells line them with centrally placed nuclei. A high degree of structural pleomorphism has been described in these ducts with respect to arrangement, location, diameter, length and epithelial thickness. In major glands they are short while in some minor glands they are longer and convoluted. Intercalated ducts draining from serous acini are more prominent when compared to mucous ones (Figure. 1.7).

The function of these ducts is simply to carry the acinar secretions unchanged to the next set of ducts within the lobule (Denny *et al.*, 1997).

1.3.2.2 Striated (secretory) ducts

The intercalated duct passes into the striated ducts (Figure 1.8), which are named so because the bases of the cells within appear to be striped. The basal cell membrane has infoldings in which mitochondria become trapped. These mitochondria can be stained giving a striped appearance in histological preparations. Striated ducts are always surrounded by a number of longitudinally oriented small blood vessels.

The specialised structure of the striated ducts implies a particular function: They modify the secretions passing through them. When produced in the acini saliva is a isotonic fluid compared to plasma, as it passes the striated duct its cells convert it in to a marked hypotonic fluid. The massive infoldings of the basal plasma membrane along with the numerous mitochondria are thought to reflect the sodium-pumping capacity of the cell wall in this location. A concentration gradient is created which favours sodium resorption from the luminal fluid. Bicarbonate and potassium ions are actively secreted into the lumen for electric compensation.

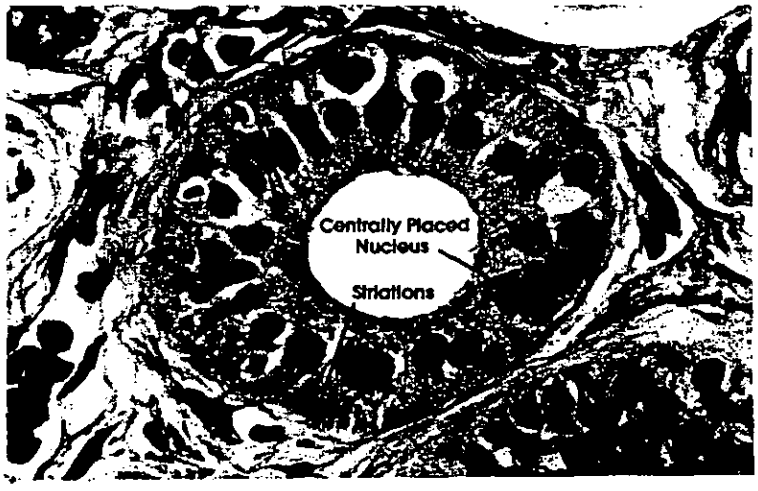


Figure 1.8- Striated ducts (Taken from Avery 1992).

Because under normal conditions of flow these cells do not absorb water, but instead these ionic changes result in formation of hypotonic solution (Valdez & Turner, 1991; Ten Cate, 1998).

1.3.2.3 Terminal excretory ducts

Terminal excretory ducts lead salivary fluid to the oral cavity (Figure.1.7). The histology of these ducts varies from pseudo stratified with tall columnar cells to real stratified epithelia as the duct merges with oral mucosa at ductal orifice. Their participation in saliva production has been controversial as some authors describe them as mere conduits while others defend their capacity in turning saliva even more hypotonic (Denny *et al.*, 1997).

1.3.2.4 Myoepithelial cells

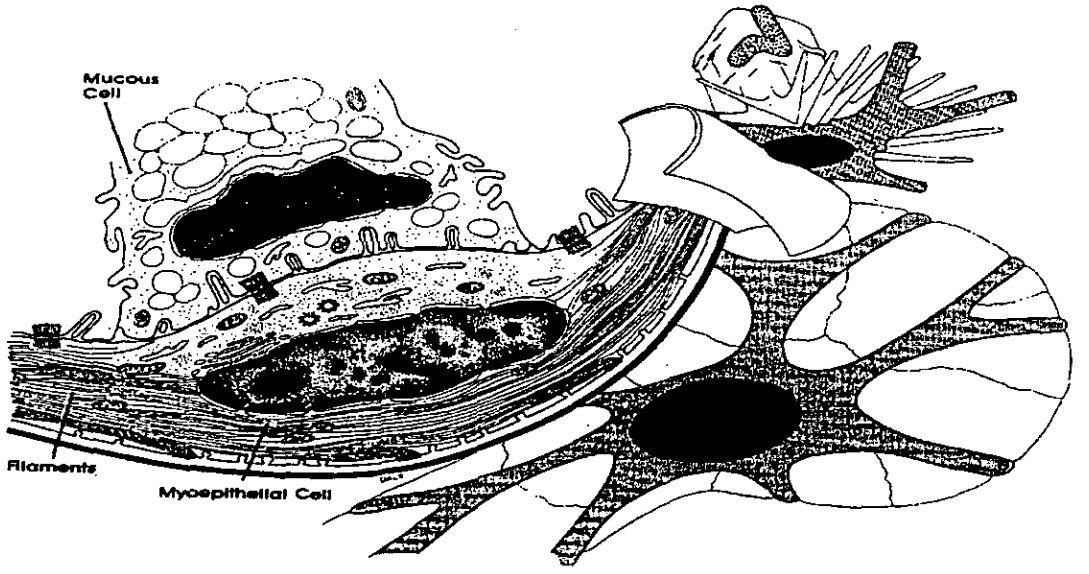


Figure 1.9- Myoepithelial Cell (Taken from Ten Cate, 1998)

Myoepithelial cells are found close to the terminal secretory end piece associated with basal portions of acini or intercalated ducts (Figure 1.9). The ultrastructural features of mioepilthelial cells are very similar to those of smooth muscle cells, with same types of microfilaments, desmosomal attachments and dark body. They have several functions all related with its contraction abilities. Support for secretory cells, prevention of

cellular overdistension and widening of intercalated duct, aid rupture of mucous acinar cells and squeezing of the acinus aiding salivary output (Valdez & Turner, 1991; Ten Cate, 1998).

1.4 Control of salivation

Unlike endocrine cells, salivary exocrine cells are involved in two different but integrated cellular processes, the production of the secretory fluid (water and electrolytes) and the secretion of protein material by exocytosis.

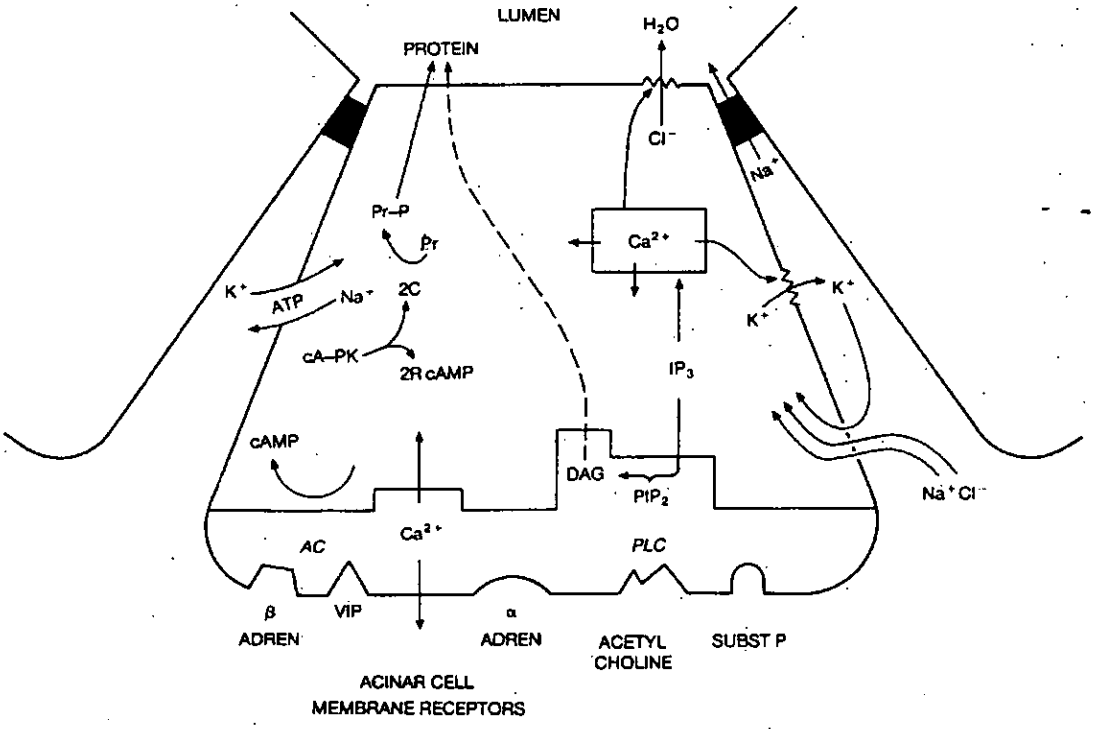
The regulation of both aspects of secretion in salivary glands is controlled through the autonomic nervous system. Sympathetic and parasympathetic branches provide innervation to the glands. Sympathetic supply in man originates in T1-T4 preganglionic fibres, which continue through effector post ganglion fibres, derived from superior sympathetic ganglion. Parasympathetic nerves innervate salivary glands through the seventh and ninth cranial nerves (De Bias, 1990). Nervous stimulation of the glands is initiated by a number of events such as sight, smell or thought of food, food in the mouth, chewing, contraction of myoepithelial cells, increased activity of acinar and ductal cells and an increased blood flow and vasodilatation in order to maintain secretion (Fergusson, 1988). In general parasympathetic stimulation predominates, and moreover, it is always present in some degree and leads to vasodilatation and production of watery saliva at high flow rates with low glycoprotein content. Sympathetic stimulation is present intermittently and it leads to vasoconstriction and much slower rate of highly viscous glycoprotein-rich saliva (Schneyer *et al.*, 1972; Quissell *et al.*, 1992).

1.4.1 Stimulus-secretion coupling

The regulation of salivary function through the autonomic nervous system is achieved *via* a co-ordinated sequence of signal transduction and intracellular signalling events. These include, activation of salivary basolateral cell membrane receptors and associated signal transduction proteins by neurotransmitters released by nerves, generation of intracellular second messengers and activation of cell physiological response pathways (Ambudkar, 2000). This type of regulated secretion is usually called stimulus-secretion

coupling. Douglas and Rubin first employed this term in 1961, when they were studying the role of calcium in mediating secretion by chromaffin cells.

Figure 1.10- A schematic diagram of Stimulus-Secretion Coupling events in salivary acinar cells (Taken from Edgar, 1992).



It is useful to divide stimulus-secretion coupling in membrane receptors and regulatory proteins, intra-cellular messengers or second messengers (Figure 1.10) and effector for better understanding purposes (Hersey *et al.*, 1990). Receptors are membrane proteins that bind to the extracellular ligands, which behave like first messengers (acethylcholine (ACh), noradrenaline (NA), Substance P (Sub P) etc.) and carry the signalling capacity. Intracellular messengers are "information molecules like ions (e.g. Ca^{2+}), cyclic nucleotides (adenosine 3', 5' cyclic monophosphate (cAMP)) or phospholipid products (inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG)) which convey a message from the plasma membrane to the inside of the cell (Takuma, 1990; Berridge, 1993, Hancock, 1998). Effectors are then the systems activated directly or indirectly by intracellular messengers that bring about cellular response, examples include enzymes such as the protein kinases, phosphatases, ATPases and specific membrane ion channels among others (Hancock, 1998). In the case of protein secretion, regulatory proteins on

the zymogen granules are phosphorylated and the end result is the migration, docking and direct fusion of the zymogen granule with the luminal plasma membrane and subsequently exocytosis (Burgoyne, 1990). For fluid secretion, the end result is the net translocation of water, sodium, chloride and other osmotically active solutes into acinar and ductal lumen (Nauntofte, 1992).

1.4.1.1 Membrane receptors and neurotransmitters in salivary glands

Salivary glands cells have receptors for a number of neurotransmitters, which are located in the basolateral membrane of the cell and possess extracellular binding sites which are specific for their respective ligands (Figure 1.10) (Petersen, 1992).

1.4.1.1.1 Muscarinic receptors

Muscarinic receptors are present in a number of tissues and are known to be coupled to biochemical effector systems such as stimulation of phosphoinositides (PI) turn-over, inhibition of adenylate cyclase, activation of guanylate cyclase and activation of ion channels (Nathanson, 1987). ACh, which is the neurotransmitter released by parasympathetic postganglionic nerves of the cholinergic system is the physiological ligand for muscarinic receptors (Cook *et al.*, 1994).

In salivary glands muscarinic receptors are linked to membrane G proteins and are of major importance in fluid secretion regulation through activation of intracellular calcium signalling mechanisms (Putney & Bird, 1993; Petersen *et al.*, 1994).

Muscarinic receptor subtypes (M1, M3 or both) have been identified in rat sub-mandibular and parotid glands, human sub-mandibular gland and porcine parotid and they have been shown to act via stimulation of intracellular Ca^{2+} and inhibition of cAMP (Quissel *et al.*, 1992; Culp *et al.*, 1996; Watson *et al.*, 1996).

1.4.1.1.2 Alpha adrenergic receptors

Alpha-adrenergic receptors are classified as $\alpha 1$ or $\alpha 2$ and have been subdivided further into other subtypes (Raymond *et al.*, 1990). Alpha-receptors were first identified in salivary glands by Batzri *et al.*, (1973). Alpha-1 receptors are located post synaptically,

whereas α2 receptors are located both pre and post synaptically. Interaction of NA with α1 receptors leads to activation of phospholipase C (PLC) and PI metabolism and is one of the major pathways in fluid secretion in salivary glands (Baum, 1987). Postsynaptic α2 receptors presence has been demonstrated in rat parotid and sub-mandibular glands but its functional significance remains unclear (Bylund *et al.*, 1982; DeHaye *et al.*, 1985). Presynaptic α2 receptors are involved with neural control of NA and will not be discussed here since they are beyond the scope of this work.

1.4.1.1.3 Beta adrenergic receptors

In rat salivary glands, evidence now exists stating that major beta-adrenergic receptors are of β1 type (Quissel *et al.*, 1992). β adrenergic receptor sub-types have been described and cloned as β1, β2 and β3. It is well established that activation of β adrenergic receptors in salivary glands by either adrenaline or NA is the major stimulus for protein exocytosis. This process seems to be mediated mainly by an cAMP dependent process (Takuma, 1990).

1.4.1.1.4 Substance P receptors

Substance P is a member of a family of peptides termed *tachykinins,* whose biological actions comprehend induction of smooth muscle contraction and stimulation of salivary flow (Quissel *et al.*, 1992). Substance P receptors have been identified in rat parotid and submandibular glands and have been shown to have direct effects on amylase and mucin release from rat parotid slices and sub-mandibular cells, respectively. These effects were found to be independent of α and β adrenergic stimulation (Rudich & Butcher, 1976; Fleming *et al.*, 1984). Substance P receptors are coupled in a guanosine triphosphate (GTP) manner to the activation of phospholipase C (PLC) and intracellular calcium metabolism (Taylor *et al.*, 1986; Fleming *et al.*, 1987). In rat submandibular gland substance P receptors have been shown to be G protein coupled (Hershey & Kraus 1990). PLC and calcium metabolism are the major pathway of fluid secretion, however, the role of substance P in sustained fluid secretion is poorly understood (Ambudkar, 2000).

1.4.1.1.5 Vasoactive Intestinal Peptide (VIP) receptors

VIP is a neuropeptide released along with ACh from post-parasympathetic neurones and appears to potentiate ACh induced salivation (Lundberg *et al.*, 1980). VIP receptors have been identified in both rat parotid and submandibular gland (Dehaye *et al.*, 1985; Turner and Bylund, 1987). These receptors were shown to be coupled to the activation of adenylate cyclase system, leading to an increased secretion of protein upon activation in parotid and submandibular (Inoue *et al.*, 1983; Turner and Camden, 1990).

1.4.1.1.6 Purinergic receptors

In 1971 Burnstock proposed that extracellular purines could bind to specific plasma membranes receptors. P_1 receptors are responsive to adenosine and its analogues while P_2 type receptors are sensitive to other nucleotides (Quissel *et al.*, 1992).

Best known functional aspects of purinergic receptors come from its actions as cation channels in the non-adrenergic non cholinergic system (NANC) of smooth muscle cells (Burnstock *et al.*, 1978). However, it was observed in 1982 that ATP increased the permeability of the plasma membrane and induced a calcium dependent amylase secretion in a way that receptors of the non-adrenergic non-cholinergic system might be involved (Gallacher, 1982). These results have been confirmed and extended to rat parotid and submandibular acinar cells (Soltoff *et al.*, 1990; Hurley *et al.*, 1994; Métoui *et al.*, 1994).

Responses of purinergic receptors in rat parotid acinar cells were shown to be mediated by non-specific cation channels known as P_{ZZ} receptors (Slotoff *et al.*, 1992). Opening of these channels increased the permeability of the plasma membrane to calcium and sodium, which increased the intracellular concentration of these cations leading to depolarisation of the plasma membrane and secretory events. The efflux of chloride and potassium were also observed (McMillan *et al.*, 1993).

Since ATP is released with other neurotransmitters during nerve stimulation, it could participate in the modulation of neurotransmitters. In the parotid gland, the effect of ATP is additive to cholinergic stimulation.

Purinergic receptors could also play a major role in the ductal phase of pancreatic and salivary secretion since purinergic receptors coupled to calcium movements are present

on ductal cells from exocrine pancreas (Hug *et al.*, 1994). More recently, rat submandibular ducts have also been shown to possess two types of purinergic receptors, an ionotropic P_{2X4} receptor coupled to kallikrein secretion and a metabotropic receptor P_{2Y1} whose functions remain unknown (Amsallem *et al.*, 1996).

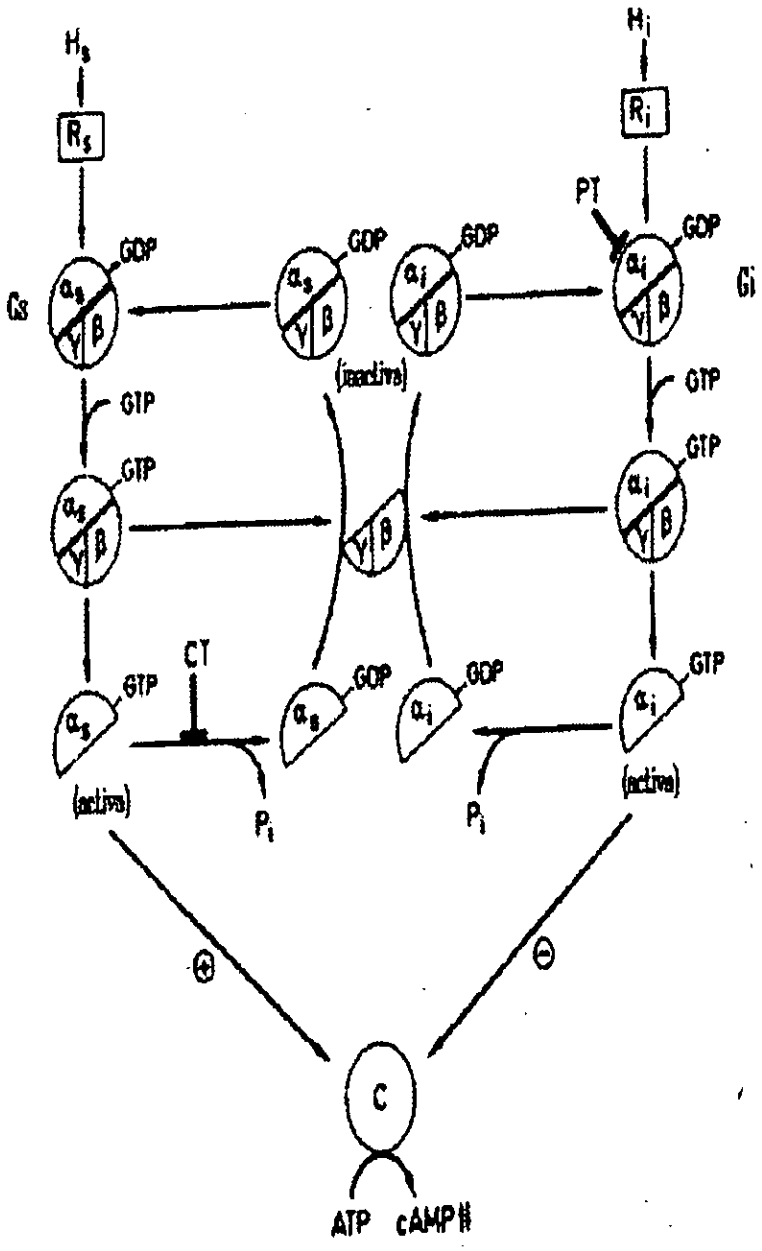
1.4.1.2 Membrane regulatory proteins and signal transducers

1.4.1.2.1 G-proteins

In salivary glands, the major receptors that regulate fluid and protein secretion belong to the G-protein-coupled receptor family (GPCR), which means that G proteins are the major elements coupling extracellular signals to intracellular effector of secretion (Ambudkar, 2000). G-proteins, (so called because they bind GTP), are a family of proteins located on cytoplasm surface of membranes, that physically transduce information from extracellular signals such as neurotransmitters into regulation of effector enzymes and ion channels (Neer, 1995). G-proteins exist as heterotrimers composed of three polypeptides: an α sub-unit that binds and hydrolyses GTP, a β and a γ subunits that usually exist as a β/γ complex that does not dissociated unless denatured and therefore behaves as a functional monomer (Neer, 1995).

Activation of G proteins (Figure 1.11) is based on the α sub-units possession of a guanine nucleotide-binding site, which in the resting state is occupied by GDP. The binding of an agonist to the receptor increases the affinity of the receptor to the G-protein complex. Receptor interaction changes the conformation of the G-protein, which decreases the affinity for GDP so that it comes off the active site. Because of the high GTP concentration in the cell GTP replaces GDP. Once the GTP is bound, the α subunit assumes its activated state and dissociates both from the receptor and the β/γ complex. The dissociated GTP-bound α-subunit of the G-protein is responsible for the activation of the effector enzyme (i.e. PLC or adenylate cyclase). The activated state lasts until GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α-subunits. All isoforms of the α-subunits are GTPases although the intrinsic rate of GTP hydrolysis varies greatly from one type to another (Catry *et al.*, 1990; Linder *et al.*, 1990). Once GTP is cleaved to GDP, the α and β subunits reassociate becoming inactive and returning to the receptor.

Figure 1.11- A schematic diagram showing the diferrent G proteins in salivary secretion (Taken from Mateos, 1996).



This system amplifies the signal because the lifetime of the GTP-complex is much longer than that of the neurotransmitter-receptor one.

The first receptor-effector system for which a role of guanine nucleotides was recognised was the adenylate cyclase system (Rodbell, 1980; Smigel *et al.*, 1984; Putney, 1988). G proteins regulating adenylate cyclase have been classified as stimulatory (Gs) and inhibitory (Gi), based on the presence of distinct alpha subunits αs and αi, which can also be distinguished on the basis of sensitivity to cholera and pertussis toxins. These toxins act by catalysing the ADP-ribosylation of αs and αi respectively. G proteins, which regulate phosphoinositide hydrolysis have been referred collectively as Gp (Yule, 1994). Other G proteins have been described, including transducin, a G protein which mediates the coupling of photon excitation to the activation of a cyclic GMP phosphodiesterase; and also Go (o for other), purified from the brain, whose function is not yet fully understood (Neer, 1995; Putney, 1988).

1.4.1.2.2 Membrane transducers

1.4.1.2.2.1 Adenylyl Cyclase (AC)

In mammals AC is a single membrane polypetide which role is the catalysation of cyclic AMP (cAMP) (an intracellular second messenger) from ATP. AC exists in several isoforms of which at least nine types have been reported and cloned, but they all share structural homology. In general, they contain two clusters of six transmembrane-spanning highly hydrophobic domains that separate two catalytic domains on the cytoplasmic side of the membrane (Hancock, 1997). AC is activated indirectly upon receptor binding by a ligand. However, AC regulation differs from one isoform to another (Watson *et al.*, 1998). The enzymes exhibit type specific stimulatory and inhibitory regulation by G protein α and $\beta\gamma$ subunits, Ca^{2+}, calmodulin, forskolin, P-site inhibitors and PCK (Iyengar, 1993; Premont *et al.*, 1996; Xia & Storm, 1996). In salivary glands, several types of AC have been demonstrated and constitute (through cAMP generation) the main pathway for stimulated protein exocytosis (Baum,1987; Ambudkar, 2000).

1.4.1.2.2.2 Phospholipase C (PLC)

Phospholipase C (PLC) is a group of enzymes present in all mammalian tissues. Several isotypes have been presently cloned and are divided in four famillies: α, β, δ & γ (Hanckock, 1997). Differrent mechanisms of PLC activation have been described depending upon the isoform (Spiegel *et al.*, 1996). PLC β, is activated through interaction with regulatory G proteins. Phosphorylation seems to be crucial for the activation of other isoforms as PLC γ whose activation depends on phosphorylation on tyrosine residues usually catalysed by tyrosine kinase receptors (Clapham, 1995). It has also been reported that Ca^{2+} may also been involved in PLC control. PLC δ contains one motif which confers Ca^{2+} binding potential to this protein, and therefore PLC δ may represent a Ca^{2+} controlled form of the PLC familly (Hancock, 1997).

The activation of PLC ultimately results in the hydrolysis of plasma membrane phospholipids, mainly phosphatidylinositol 4,5 biphosphate (PtdIns $(4,5)P_2$) which is

broken down into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which act as second messengers in this pathway (Berridge, 1993).

In salivary glands PLC is of chief importance in regulation of fluid secretion in acinar cells and ionic composition modulation of salivary flow by ductal cells (Ambudkar, 2000; Nautofte, 1992).

1.4.1.3 Second messengers and intracellular effector systems

1.4.1.3.1 Diacylglycerol and Protein Kinase C

Diacylglycerol (DAG) is one of the endogenous activators of protein kinase C (PKC). DAG is normally absent from cell membranes, but it is transiently produced from inositol phospholipids in response to extracellular signs (Yule & Williams, 1994).

Receptors linked to the activation of phospholipase C leads to the production of IP3 and DAG. However, DAG can also be formed from the hydrolysis of other membrane phospholipids, such as phosphatidylcholine (PC). It should therefore be seen not as a single chemical but a family of related compounds. The structure of which is determined by the acyl groups present in the original lipid that was hydrolysed by the phospholipase (Billah & Anthes, 1990). DAG is only transiently produced in membranes and can be further metabolised to other signalling compounds. Best-known route of DAG metabolism is its phosphorylation to phosphatidic acid (PA) by diacylglycerol kinase (DGK). This chemical stimulates inositol 4,5-biphosphate formation, activates PLC, acts as a cell mitogen, can be re-incorporated in cell membranes as phosphatidylinositol phosphates and it is subsequently degraded to arachidonic acid. The regulation of DAG breakdown pathways remains an open question but it seems to depend on the acyl groups involved (Hanckock, 1997).

DAG activates PKC by increasing its affinity to calcium without any changes in intracellular Ca^{2+} levels (Nishizuka, 1988), although an Ca^{2+} independent activation mechanism has also been postulated by some authors (Quissel *et al.*, 1992).

PKC was originally thought to be a single protein but in 1989 evidence suggested that it was in fact a family of close related proteins (Parker *et al.*, 1989). PCK can be activated by either Ca^{2+}, proteolytic cleavage or phospholipids such as DAG, depending on the isoform of this enzyme (Hanckock, 1997). PCK has been shown to play a pivotal role

in intracellular Ca^{2+} signalling by either enhancing or decreasing the activity of kinases that regulate PIP_2 formation and breakdown resulting in either IP_3 elevation or inhibition (Francis & Singh, 1990; Wolfe *et al.*, 1996).

In salivary glands PCK plays a role in protein exocytosis mediated by DAG, IP_3 and calcium-mobilising receptors (muscarinic, α1-adrenergic and substance P) which are totally independent from cAMP (Putney, 1986; Moller *et al.*, 1996). PCK has the ability to phosphorylate a wide range of intracellular proteins, however, the exact mechanisms underlying salivary PCK dependent exocytosis or its role in salivary secretion remain to be fully elucidated (Ambudkar, 2000).

1.4.1.3.2 Inositol triphosphate and calcium

1.4.1.3.2.1 Importance of calcium in fluid secretion

In 1963 Douglas and Poisner first demonstrated that calcium was involved in the regulation of fluid secretion in salivary glands.They showed that sustained salivary fluid released in the cat superfused salivary gland was only possible when calcium was present in the extracellular medium (Douglas & Poisner, 1963). Later, it was demonstrated that following stimulation with either α1 adrenergic or muscarinic agonists there was an increase in the rate of calcium uptake and efflux from salivary gland cells (Neilsen & Petersen, 1972). This fact led to the assumption that intracellular calcium homeostasis and cell membrane permeability to calcium was altered after agonist stimulation of salivary glands (Poggioli & Putney, 1982). It was suggested that calcium efflux was associated with immediate rise in fluid secretion while calcium uptake was involved in more prolonged salivation (Putney, 1986; Petersen & Gallacher, 1988; Petersen *et al.*, 1994).

Intracellular calcium was shown to be sequestered in an intracellular organelle (referred to as the internal calcium store), from which it was released following agonist stimulation (Aub *et al.*, 1982; Muallem *et al.*, 1988). Experiments with permeabilized cells demonstrated the presence of an ATP-dependent calcium uptake mechanism and a calcium store in the endoplasmic reticulum (ER) of exocrine gland cells (Berridge & Irvine, 1984; Muallem, 1989). The finding that IP_3 released calcium from the internal stores (Streb *et al.*, 1983) provided the link between calcium mobilisation and inositol

phospholipid turn-over following agonist stimulation previously demonstrated by Mitchel in 1975. During the past 20 years, the development of intracellular fluorescent probes for measuring intracellular calcium and other ions (Tsien *et al.*, 1982), has increased our knowledge on the sequential events of calcium signalling in salivary and other exocrine cell types.

Changes in intracellular calcium ($[Ca^{2+}]_i$) upon agonist stimulation in salivary cells are of biphasic nature characterised by an rapid and transient initial rise in $[Ca^{2+}]_i$ which decreases to a lower more prolonged plateau phase (Hellman *et al.*, 1987; Baum & Ambudkar, 1988; Mertz *et al.*, 1990). It also has been demonstrated that the initial elevation of $[Ca^{2+}]_i$ was independent of extracellular calcium concentration ($[Ca^{2+}]_0$) and this corresponded to calcium being liberated from the internal stores of endoplasmic reticulum (ER) (Merrit & Rink, 1987; Takemura & Putney, 1989). The plateau phase (secondary increase) was totally dependent on the presence of extracellular calcium and corresponded to calcium entering the cytosol from the extracellular side (Ambudkar *et al.*, 1992).

These findings have clearly set the links between $[Ca^{2+}]_i$ variations and salivary output. Moreover, workers have confirmed the critical importance of this ion on the secretory process as the most important second messenger involved in fluid and electrolyte secretion. In fact, the rise in intracellular calcium due to calcium exit from intracellular stores is the triggering factor in fluid secretion. Moreover, sustained stimulated and prolonged salivary output is totally dependent on cytosolic calcium elevation caused by its influx from the extracellular side of the acinar cells (Petersen, 1992; Cook *et al.*, 1994).

1.4.1.3.2.2 Regulation of calcium in salivary gland cells

Cytosolic resting Ca^{2+} in salivary gland cells (SGC) is around 100 nM compared to a $[Ca^{2+}]_0$ between 1.2 and 2.0 mM which forces the SGC to work against a heavy concentration gradient in order to maintain such low $[Ca^{2+}]_i$.Regulation of $[Ca^{2+}]_i$ in salivary gland cells is typical of others non-excitable cell types and depends on the co-ordinated action of several calcium channels and pumps localised in the different cellular membranes (Baum *et al.*, 1993).

The ER lumen is where most of intracellular calcium is stored, constituting an important intracellular pool from where it can be mobilised upon cell stimulation. Free calcium concentration inside ER lumen has been shown to vary between 70 μM and 300 μM depending on the authors and the methods of measurement (Montero *et al.*, 1995; Van de Putt & Elliott, 1997; Mogami *et al.*, 1998). Some authors have also found that there is an considerable Ca^{2+} buffering in the ER by proteins such as calreticulin, and that total calcium concentration inside the ER lumen is thus within the mM range (Clapham, 1995; Camacho & Lechleiter, 1995). Independently of what the exact concentration may be, it is certain that a strong concentration gradient exists between ER lumen and the cell cytosol.

When the cell is stimulated by agonists (Muscarinic or α1 adrenergic), IP_3 level rises and it diffuses through the cytosol at speeds of ~ 230 $\mu m.s^{-1}$ and occupies IP_3 receptors (IP_3R) located on the ER membrane for minutes before being degraded (Clapham, 1995; Sanderson, 1996). IP_3R is in fact a calcium channel which has been extensively characterised in the past years and it allows calcium to be rapidly mobilised from the ER lumen into the cytosol (Mikoshiba, 1993). IP_3R exists in three isoforms IP_3R1, IP_3R2 and IP_3R3 (Marshal & Taylor, 1993) and all of them have been identified in the salivary glands (Lee *et al.*, 1997a). Upon IP_3R activation by IP_3 $[Ca^{2+}]_i$ rise in the cytosol and activate a number of Ca^{2+}-dependent ion channels, which drive fluid secretion into salivary acinar lumen (see saliva formation).

Regulation of the IP_3R has been a subject of intense study in the past years, but still remains to be fully elucidated. It has been well established that Ca^{2+} stimulates IP_3 mediated Ca^{2+} release at lower concentrations and inhibits it at concentrations > 300 nM. The regulatory properties of $[Ca^{2+}]_i$ are totally dependent on IP_3 levels, as this increases the receptor becomes more sensitive to lower $[Ca^{2+}]_i$ ensuring an open state of the channel when $[Ca^{2+}]_i$ is low. In contrast, elevated $[Ca^{2+}]_i$ has a feed-back mechanism which diminishes Ca^{2+} exit from the ER (Berridge & Irvine, 1989; Beprozvanny *et al.*, 1991; Berridge 1993, 1997).

It has also been suggested that Ca^{2+} concentration inside the ER could itself regulate the open state of IP_3R. In permeabilised human salivary cell lines, depletion of the ER Ca^{2+} stores induces the IP_3R to be in the lower state of activity (Tanimura & Turner, 1996).

Inside the ER lumen, several proteins with calcium binding properties (e.g. calmodulin, calreticulin or glycoproteins) also help to buffer and control calcium concentration. A

third mechanism of IP_3R control has been suggested as these proteins could be seen as putative direct IP_3R modulators (Clapham, 1995; Berridge, 1997).

Refill of the ER Ca^{2+} is dependent on the activity of an Ca^{2+} -ATPase pump present in the ER membrane designed as SERCA for sarco-endoplasmic reticulum calcium pump, from which three isoforms are known to exist: SERCA2a, SERCA2b and SERCA3 (Wu *et al.*, 1995). From these SERCA2b and SERCA3 have been shown to be present in salivary gland cells (Lee *et al.*, 1997b; Meehan *et al.*, 1997).

The exact mechanisms regulating SERCA pump are not fully understood (Ambudkar, 2000). However, the studies reported to date clearly demonstrate that the SERCA pump can efficiently pump Ca^{2+} into the ER within the range of $[Ca^{2+}]_i$ induced in the ER and cytosol following stimulation of the cells (Baum & Ambudkar, 1988; Muallem, 1989; Mogami *et al.*, 1998). The Kd for SERCA has been reported as 30 nM reaching Vmax at about 100 nM of $[Ca^{2+}]_i$ which means that this pump is active even at resting cytosolic $[Ca^{2+}]_i$ (Mertz *et al.*, 1990b). On the other hand, recent experiments with intact pancreatic cells have demonstrated that upon depletion of internal stores SERCA activity is increased (Mogami *et al.*, 1998). So it appears from these results that SERCA activity is both regulated by an increase in $[Ca^{2+}]_i$ in the cytosol as well as by its decrease inside the RE lumen (Berridge, 1997).

Elevation of $[Ca^{2+}]_i$ also activates a Ca^{2+} pump located in the basolateral membrane of salivary gland acinar cells which promotes the efflux of cytosolic calcium into the extracellular medium in a ATP-dependent fashion (Ambudkar & Baum, 1988; Ambudkar *et al.*, 1989). This pump usually referred to as plasma membrane calcium ATPase (PMCA) and has been proven to be the most effective Ca^{2+} efflux pathway in parotid acinar cells (Baum & Ambudkar, 1988). Several authors have demonstrated that PMCA efficacy is directly proportional to $[Ca^{2+}]_i$. Kd for this channel have been proved to be somewhat between 100-200 nM and saturation point for $[Ca^{2+}]_i$ of over 500-700 nM, proving that this pump efficacy is maximal in stimulated cells.(Ambudkar & Baum, 1988; Camello *et al.*, 1995). PMCA activity has also been shown to be controlled and enhanced by direct interaction with calmodulin (Which could enable its activity at low $[Ca^{2+}]_i$), and to be sensitive to changes in membrane potential and intracellular pH (Ambudkar *et al.*, 1989; Ambudkar & Baum, 1988).

In conclusion it seems for some authors that PMCA has a more significant role in regulating $[Ca^{2+}]_i$ in activated cells (i.e. when $[Ca^{2+}]_i$ is higher), while the SERCA due

to its more elevated affinity for Ca^{2+}, is more effective at lower $[Ca^{2+}]_i$ (Ambudkar, 2000).

1.4.1.3.2.3 Capacitative Ca^{2+} entry (CCE)

In the middle eighties Putney verified that Ca^{2+} influx into cells was increased several fold upon stimulation with an agonist and that the influx was inactivated when the stimulus was removed. He called this effect "the capacitative Ca^{2+} entry" (CCE) or Ca^{2+} influx (Putney, 1986b). Today, it is widely accepted that CCE is the critical driving force for sustained salivary fluid output as it provides the necessary elevated $[Ca^{2+}]_i$ upon stimulation with muscarinic or α1 adrenergic agonists (Melvin *et al.*, 1991; Petersen *et al.*, 1994). However, in spite of the heavy efforts of research in this field in the past years, the molecular mechanisms involved in CCE regulation remain to be elucidated (Parekh & Penner, 1997; Lockwich *et al.*, 2000).

One of the earlier findings in CCE regulation came about as a result of the use of a drug called thapsigargin, a SERCA pump inhibitor. Once SERCA is inhibited and intracellular calcium stores are depleted, this then turns the stimulus to trigger CCE. These interesting observations indicate that Ca^{2+} entry was *via a* store operated channel (SOC) (Takemura *et al.*, 1989; Putney & Bird, 1993a). The first model of SOC was proposed by Putney (Putney, 1986b) and he predicted that this channel had some how a spanning domain into the ER to which it was physically linked. Ca^{2+} would therefore enter the ER lumen directly from the extracellular space and be exited in to the cytosol *via* the IP_3R channel (Putney, 1986b). Further studies by other authors using Mn^{2+} as Ca^{2+} surrogate have contradicted Putney's model and proved that after activation of CCE, Ca^{2+} was passing from the extracellular side directly into the cell cytosol before accumulation in the ER lumen (Mertz *et al.*, 1990b).

In the past few years several factors have been proposed as possible regulators of the SOC in non-excitable cells (Parek & Penner, 1997). 1) Nitric Oxide Synthase (NOS) has been proposed as regulator of SOC in submandibular gland (Xu *et al.*, 1997). 2) Reports on the activity of a protein kinase that may act as a SOC regulator on rat parotid cell have also been considered (Ambudkar, 1996; 1997). 3) Different levels of Ca^{2+} permeability in SOC have been noted suggesting the existence of several levels of CCE

activation that seem to be regulated by phosphorylation events. 4) some authors have even postulated the existence of different SOC in the same cell.

To date, none of these studies have been conclusive and presently, two models have been proposed for the gating of the SOC, although no data exist to support either conclusively (Ambudkar, 2000).

In the first model (Figure. 1.12) (named conformational coupling hypothesis, (CCH)), a physical coupling between a Ca^{2+} channel protein in the plasma membrane and another factor in the ER membrane has been postulated to occur upon cellular activation to control Ca^{2+} into the cytosol. More recently, Berridge has

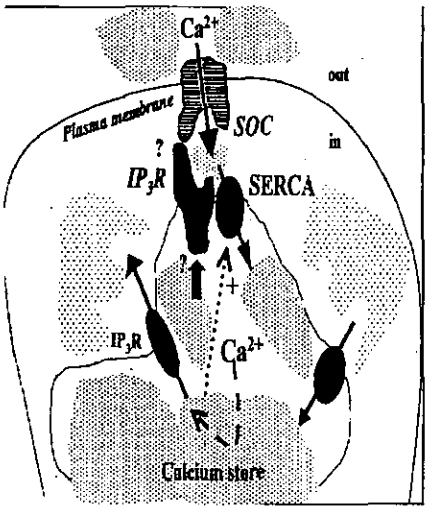
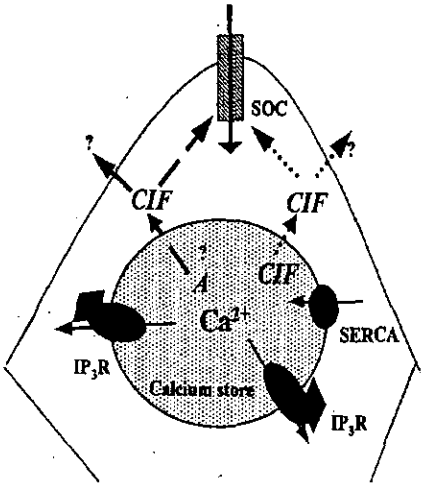


Figure 1.12- A shematic model for conformational coupling hypothesis (Taken from Ambudkar, 2000)



suggested that this coupling is mediated *via* an IP3R in the ER, probably IP3R3 (Berridge,1996; 1997). However, some studies have been published which are contradictory with this model (Khan *et al.* 1996).

Figure 1.13- A shematic model for Calcium Influx Factor hypothesis (Taken from Ambudkar, 2000)

Another hypothesis proposes the release of a metabolite, (a Calcium Influx Factor (CIF)) from the ER which would be involved in the activation of SOC (Figure 1.13). Activation could be produced by CIF directly or this could be in the cytosol and posteriorly activated by an activation factor released from the ER (Randriamampita & Tsien, 1993; 1995;).

Another important issue in understanding Ca^{2+} signalling is the spaceo-temporal aspect of $[Ca^{2+}]_i$ variations. Sub-maximal concentration of agonists have been shown to induced oscillatory $[Ca^{2+}]_i$ changes and wave propagation whose role in cell physiology is still under very active research (Berridge, 1997).

In general the models proposed to explain this phenomenon suggest that $[Ca^{2+}]_i$ oscillations are generated as a result of the regulation of either $[Ca^{2+}]_i$ release or $[Ca^{2+}]_0$ influx (Ambudkar, 2000). Oscillations caused by intracellular Ca^{2+} release could be originated in cells with only one type of IP_3R and intracellular calcium store which would be regulated by the biphasic effect of calcium on the activity of IP3R. Other authors have proposed the involvement of two different types of $[Ca^{2+}]_i$ stores, the classic one sensitive to IP_3 and another one with lower sensitive to IP_3 or even regulated by a ryanodyne receptor (RyR). In a third model, several authors have found that in some cell types $[Ca^{2+}]_i$ oscillations depend on the presence of extracellular Ca^{2+}, leading to the assumption that they are generated by the repeated activation and inactivation of Ca^{2+} influx (Foskett & Wong, 1994; Liu *et al.*, 1998a).

Both oscillatory as well as wave propagation of $[Ca^{2+}]_i$ has been described to occur in salivary gland cells, however, the exact role of these phenomena in cellular physiology remains to be fully elucidated (Gray, 1988; Foeskett & Wong, 1994; Lee *et al.*, 1997ab; Tojyo *et al.*, 1997).

1.4.1.3.3 Cyclic-3'5' adenosine monophosphate and protein kinase A

Cyclic-3'5' adenosine monophosphate (cAMP), is produced at the plasma membrane of cells by the enzyme complex known as adenylyl cyclase and it is released into the cytosol. From there, cyclic AMP can diffuse and act on the next part of the signalling process (Hancock, 1997). In salivary glands, the activation of β-adernergic receptors give rise to cAMP cytosolic levels and represent the most powerfull pathway for stimulated protein exocytosis (Baum, 1987). The cellular roles of cAMP are mediated exclusively via activation of cAMP activated protein kinase (PKA) a cytosolic widespead protein found in eukaryotes (Hancock, 1997). In the inactive state, i.e. in the absence of cAMP, PKA is found as a tetramer of two catalytic subunits (C) and two regulatory subunits (R). On activation cAMP binds to the (R) subunits causing the complex to dissociate. The regulatory subunits remain a dimer with the release of the two catalytic units with kinase activity and which are responsible for phosphorylation events and thus cellular responses. PKA is known to occur in two types I and II based on structural differences in the regulatory subunits (Hancock, 1997). In rat parotid salivary gland cells PKA activity seems to be 80% of type I and 20 % of type II

(Quissel, Deisher & Barzen, 1989, Hinkle & Soor, 1992). The main role of PKA in salivary secretion appears to be its interactions with protein granules vesicle membrane associated proteins (VMAP) which are responsible for the triggering of stimulated exocytosis (Takuma & Ichida, 1993; Fujita *et al.*, 1996). However, cAMP and PKA have been shown to be also involved in cell differentiation. Gene expression can be controlled through the phosphorylation and activation of transcription factors such as CREB, which binds to cAMP-response element (CRE) regions of the DNA (Hanckok, 1997). In rat salivary glands, cAMP has been shown to enhance some salivary protein gene expression (Zhou *et al.*, 1997).

1.4.2 Saliva formation

Saliva formation is purely under nervous control. Unlike other exocrine glands, in salivary glands fluid (water and ions) secretion and protein exocytosis pathways are regulated by totally separate signal transduction mechanisms (Ambudkar, 2000).

1.4.2.1 Fluid secretion

According to the model accounting for the formation of saliva proposed by Thaysen *et al.* in 1954 and later verified, secretion of saliva occurs in two main stages (Martinez *et al.*, 1966; Young & Shogel, 1966). The secretory end pieces of the salivary glands (the so-called acini) secrete a fluid (primary saliva), which is isotonic and resembles plasma in ionic composition. This fluid is then modified in a second stage, within the duct system by selective resorption of salt. Because the ducts have a low permeability to water, the final product that is secreted into the oral cavity is hypotonic with a salt concentration below that of primary secretion (Nauntofte, 1992).

It is well known that the secretion of saliva includes active transport of solutes and is not dependent on pressure filtration (Cook *et al.*, 1994). Lately much effort has been put into characterising the different cellular signalling and regulatory mechanisms, which are activated upon stimulation of the salivary glands by secretagogues. Detailed information of the multiple mechanisms involved in the formation of primary saliva from the acinar cells have emerged primarily from animal studies conducted on major salivary glands of rat, mouse, rabbit and cat (Cook *et al.*, 1994; Nauntofte, 1992;

Petersen, 1992). Indeed, the physiology of the duct system and in particular the myoepithelial cells is not as well understood (Ambudkar, 2000).

1.4.2.1.1 Secretion of primary saliva

The rate of salivary secretion to the oral cavity is a consequence of the rate of formation of the primary saliva by the acinar cells. The fluid is produced from the interstitial fluid that contains small molecules and resembles plasma ionic concentration. The acinar plasma membrane is permeable to lipid-soluble substances but is less permeable to water and much less to small ions. In addition, the tight junctions that separate the luminal fluid from the interstitial fluid are leaky to cations and allow for its transport from the interstitium to the lumen leading into the duct system (Turner, 1993a). Thus, specific membrane-transport mechanisms (ion channels, co transporters, exchange systems and pumps) mediate electrolyte and fluid secretion on receptor activation. The general principle behind the formation of primary saliva is that acinar cells lose K^+ to the interstitium (blood side) and Cl^- to the lumen. To preserve electroneutrality, Cl^- then drives Na^+ from the interstitium to the lumen probably via a paracellular pathway through tight junctions. Transepithelial water movements follow for osmotic reasons (Turner, 1993b).

On secretagogue-induced stimulation of the acini, the rapid and almost simultaneous increase in $[Ca^{2+}]_i$ throughout the acinar cell allows for simultaneous opening of K^+ and Cl^-, Ca^{2+} dependent channels on the basolateral and luminal membranes, respectively (Petersen, 1992; Dissing *et al.*, 1993). This results in virtually unchanged membrane potential of approximately –60 mV (Nauntofte & Dissing, 1988; Roberts *et al.*, 1978). The outcomes are a net loss of K+ (Nautofte & Dissing, 1988) and Cl^- (Melvin et al., 1989; Ishikawa & Cook, 1993), and water (Nautofte & Poulsen, 1986; King & Agre, 1996) from the acini and subsequently cell shrinkage where the minimum volume is attained within a few seconds (Nakahari *et al.*, 1990). The major part of the secretagogue-induced K^+ loss from the acinar cell occurs through Ca^{2+} sensitive high conductance channels located on the basolateral membrane (Liu *et al.*, 1998; Hayashi *et al.*, 1996). The Cl^- loss presumably occurs through Ca^{2+} -sensitive channels on the luminal membrane (Melvin *et al.*, 1992; Arreola *et al.*,1996 ab).

Concomitant with the Cl^- loss, a loss of HCO_3^- also takes place (Lee & Turner, 1991) in such a way that ratios between internal and external Cl^- and HCO_3^- remain unchanged (Nauntofte, 1992). Following the decrease in acinar Cl^- and HCO_3^- concentrations, the carbonic anhydrase catalyses a conversion of CO_2 and H_2O into HCO_3^- and H^+ resulting in a transient acidification (Nauntofte & Dissing, 1989). The loss of anions to the lumen is supposed to cause a lumen negative potential difference with respect to the interstitium.

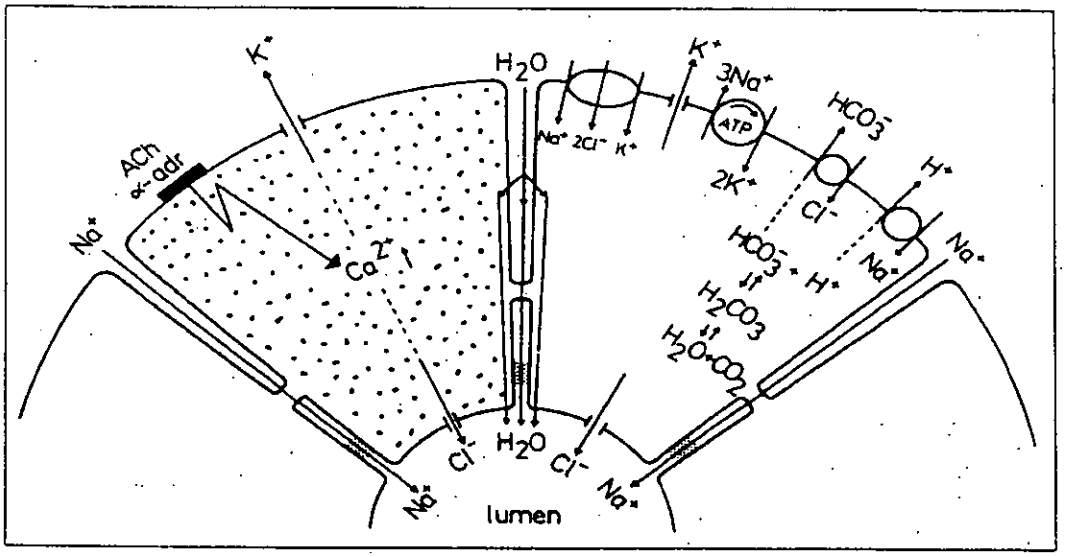


Figure 1.14- A shematic model for primary saliva formation (Taken from Nauntofte, 1992)

This now drives cations (mainly, Na^+) from the paracellular spaces to the luminal compartment through the cation-selective tight junctions to preserve extracellular electroneutrality (Turner, 1993 ab). Due to net loss of K^+ from the acinar cells to the paracellular spaces during this initial part of the secretory process, the K^+ concentrations build up to the usual plasma levels (Poulsen, 1978). This might be the reason for a considerable part of the primary fluid at the onset of secretion not only being rich in Na^+ and Cl^- but also in K^+ (Young & Martin, 1971). Furthermore, it was suggested that the presence of a luminal K^+ channel might contribute to the secretion K^+ to the primary fluid under the sustained secretion phase (Cook *et al.*, 1994).

After the initial secretory response induced by receptor activation, a markedly elevated intracellular Na^+ concentration is observed (Dissing & Nauntofte, 1990; Wong & Foskett 1991). This is mainly due to a Na^+ gain by activation of the Na^+/H^+ exchanger

which is responsible for the majority of the net Na^+ gain (Dissing & Nauntofte, 1990; Murakami *et al.*, 1990; 1991), and for the extrusion of H^+ generated from the metabolism and HCO_3^- production (Maganel & Turner, 1990; 1991). The whole process is required in maintaining a nearly constant intracellular pH (pH_i). The residual Na^+ gain is mediated by Na^+ coupled transport of Cl^- and K^+ by the Na^+,K^+ and Cl^- cotransporter that also accounts for 20-30% of the total K^+ uptake during secretion (Dissing & Nauntofte, 1990; Murakami *et al.*, 1990; 1991; Petersen, 1992).

The net uptake of ions into the cell causes uptake of water and the start of cell swelling toward the prestimulatory acinar cell volume. The elevated Na^+ concentration in turn stimulates the activity of the electrogenic Na^+/K^+ pump (Dissing & Nauntofte, 1990) , and thus the O_2 consumption is markedly increased (Nauntofte & Poulsen, 1986). Extrusion of the Na^+ by the Na^+/K^+ pump re-establishes the steep inwardly directed Na^+ gradient and a large part of the acinar K^+ concentration (Nauntofte, 1992). Following the initial K^+ and Cl^- losses, there is also a net Cl^- reuptake promoted by the $Na^+/2Cl^-/K^+$ co-transport system driven by the overall favourable inward-directed gradient for this electroneutral transport mechanism (Dissing & Nauntofte, 1990; Poulsen & Nauntofte, 1990). This is imposed by the cellular decrease in the concentrations of Cl^- and K^+. In spite of the acinar acidification caused by the loss of HCO_3^- and an enhanced metabolic activity, pH_i is able to recover to prestimulatory levels by the H^+ extrusion mediated by the Na^+/H^+ exchanger. The progressive cell alkalinization increases the intracellular HCO_3^-, which again helps the cellular Cl^- to rise via the Cl^-/HCO_3^- exchanger (Melvin & Tamer, 1992). Accordingly, the Cl^-/HCO_3^- exchange mechanism operating in parallel with the Na^+/K^+ pump and the $Na^+/2Cl^-/K^+$ mechanism constitute essential mechanisms in maintaining a high intracellular Cl^- concentration both in stimulated and unstiulated Cl^- reuptake. When the stimulus is removed and the $[Ca^{2+}]_i$ decreases, the electrolyte concentrations, cell volumes and pumps activities are brought back to prestimulatory levels. The acinar cells are able to secrete substantial amounts of primary saliva as long as sustained cell stimulation is present (Nauntofte, 1992).

1.4.2.1.2 Fluid modification in the ducts

The modification of the plasma-like isotonic primary fluid (Figure.1.15) starts from the beginning of the striated duct system. During passage through the duct system the ionic composition of primary saliva and synthesis and secretion of number of substances like kallikrein and growth factors is known to occur (Zelles, *et al.*, 1995).

The autonomic nervous innervation of the duct system provides control for the electrolyte transport systems. Like the acinar cells, these cell also have been shown to respond to muscarinic and α1-adrenergic stimulation by increasing $[Ca^{2+}]_i$ but in contrast, these cells seem to lack the capability of responding to substance P (Valdez & Turner, 1991; Dinudon *et al.*, 1993).

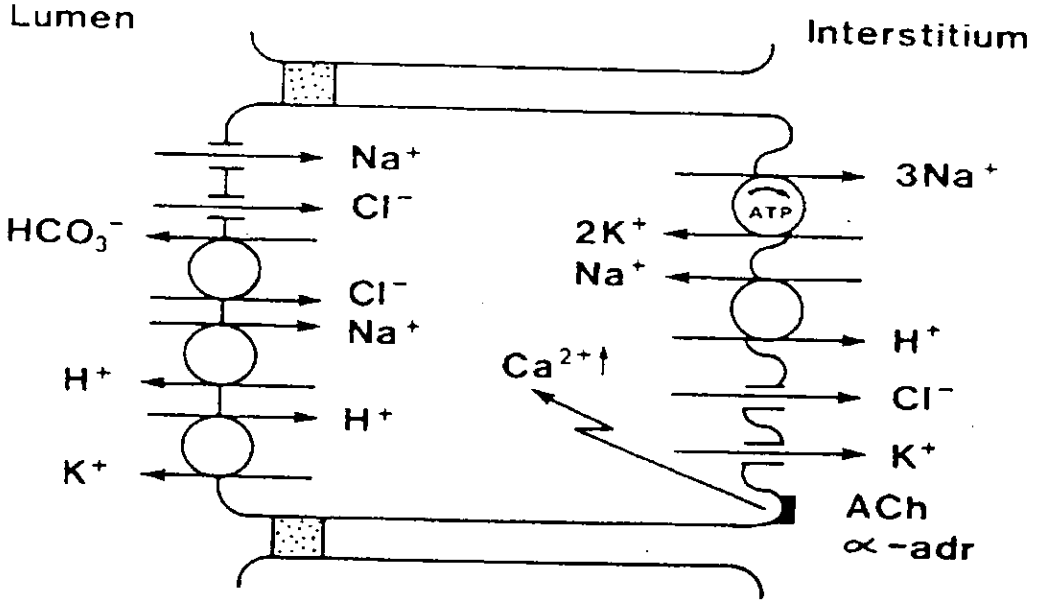


Figure 1.15- A schematic model for primary fluid modification in the striated ducts (Taken from Nauntofte, 1992)

In contrast to the acinar epithelium, the intercellular tight junctions in the duct system possess a very low permeability to ions. As the primary fluid passes through the duct system, it is modified with respect to the ionic composition by reabsorption of Na^+ and Cl^-. At the same time the ductal cells secrete K^+ and $HCO3^-$ into saliva. Because the ducts have a low permeability to water, the reabsorption of ions occurs without water making the final product secreted into the oral cavity hypotonic compared to the primary fluid. At low salivary secretion rates, some water reabsorption might occur through a transcellular or paracellular way driven by the steep transepithelial osmotic

gradient (Cook *et al.*, 1994). It seems that both the intralobular striated ducts and the excretory ducts absorb $Na^+$and Cl^- and secrete K^+ and HCO_3^- , although the striated ducts apparently are the major contributors to this process as they posses a larger luminal surface. The amount of the secreted K^+ does not match the amount of Na^+ reabsorpted because of the concomitant reabsorption of Cl^- to preserve electroneutrality. As secretion rates increase, reabsorption of Na^+ and Cl^- falls down making saliva less hypotonic. Accordingly, saliva secreted into the oral cavity is strongly dependent on flow rate (Thaysen *et al.*, 1954).

The primary driving force for an absorption by the salivary ducts comes from the energy consuming Na^+/K^+ pumps, located on the basolateral membrane of the duct cells. These phenomena maintain the inwardly directed Na^+ and outwardly directed K^+ gradients, by ductal extrusion of Na^+ to the interstitium and uptake of K^+ (Bundgard *et al.*, 1977).

The luminal membrane is postulated to contain at least two transport mechanisms for Na^+; a Na^+ channel and a Na^+/H^+ exchange mechanism, both being sensitive to the diuretic amiloride. These mechanisms are responsible for the ductal net uptake of Na^+ which in turn activates the electrogenic Na^+/K^+ pump that extrudes $3Na^+$ in exchange for $2K^+$ taken up by the cells across the basolateral membrane. It has been suggested that stimulatory action of the adrenal hormone aldosterone on Na^+ reabsorption and K^+ secretion is mediated by increasing the luminal an channel conductance and the activity of the luminal Na^+/H^+ exchanger (Gruber *et al.*, 1973). In contrast, VIP has been shown to inhibit Na^+ reabsorption in the ducts (Dennis & Young, 1978).

The activation of the Na^+/H^+ exchanger is not only important for the ductal Na^+ gain, but also for the pH_i regulation by extrusion of H^+ generated by cell metabolism and carbonic anhydrase activity. In parallel, the HCO_3^- produced drives a ductal Cl^- reabsorption and luminal secretion of HCO_3^- into saliva via a luminal membrane Cl^-/HCO_3^- exchange mechanism. Additional reabsorption of Cl^- is thought to exist via luminal Cl^- channels. The net cellular gain of Cl^- is proposed to be balanced by corresponding release of Cl^- to the interstitium via opening of basolateral Cl^- channels.

K^+ is transported from the interstitium to the cytosol by the Na^+/K^+ pump and is thought to enter the lumen through a K^+/H^+ exchange mechanism. This exchanger is suggested to extrude K^+ into the saliva under the concomitant uptake of H^+ to the cytoplasm. The uptake of H^+ then provides the substrate for the Na^+/H^+ exchanger mediating Na^+ reabsorption. Finally, the basolateral membrane is suggested to contain K^+ channels and a Na^+/H^+ exchange mechanism (Dinudon *et al.*, 1993; Turner, 1993 a, b).

1.4.2.2 Protein secretion

As in many other secretory cell types the protein secretion in salivary acinar cells is operated through the exocytosis phenomenon (Burgoyne, 1990). Exocytosis was a term first employed by Palade in 1959, after the observation that zymogen granules of secretory cells migrate toward the apical membrane of the secretory cell, bud and fuse with plasma membrane and empty their contents in luminal spaces (Geada, 1998).
In both resting and stimulated acinar cells, nearly all newly synthetized protein (More than 80-90%) follows the secretory pathway (Zastrow & Castle 1987). These proteins enter the endoplasmic reticulum (ER) during translation; most are transported to the Golgi complex where post-translational modifications are largely completed, and then routed into forming granules, where they are condensed for storage at concentrations that exceed 300 mg . ml^{-1} (Arvan *et al.*, 1984).
Two main pathways for exocytosis have been described in salivary acinar cells, an unstimulated and other one, which happens only upon cell stimulation (Castle, 1998).

1.4.2.2.1 Unstimulated secretion

In salivary acinar cells 85% of the newly synthesised proteins are stored in granules, which undergo maturation and must receive an intracellular signal in order to migrate and fuse with apical membrane in the exocytotic process. However, the remainder (15%) is released from the cell without storage (Castle & Castle, 1998). In unstimulated cells that have been pulse-labelled with radioactive amino acids and then subjected to chase incubation for extended periods of time, the discharge of newly synthesised proteins can be seen to begin around 40 min post pulse and peaks between 60 and 90 min (Arvan & Castle, 1987). The information obtained by the kinetic analysis of secretion has been interpreted as indicating that there are at least three pathways of unstimulated secretion in parotid acinar cells (Arvan & Castle, 1987; Zastrow *et al.*, 1989). One pathway corresponds to the classic constitutive pathway originating in trans Golgi-network and present in other secretory cell types. Pathway 2 is a vesicular constitutive like pathway thought to arise from maturing secretion granules and carry low dense proteins as cargo. The third pathway is the unstimulated exocytosis of mature granules. The presence of non- stimulated pathways in salivary glands, has to do with

its own non-stopping secretory nature, providing a route for continuous supply of functional salivary organic products into the oral cavity. However, the significance of this multi-pathway of non-stimulated secretion is presently unknown, although some authors (See Castle & Castle, 1998 for a review) have drawn some speculation in this issue.

1.4.2.2.2 Stimulated secretion

In salivary gland acinar cells, most of the intracellular transport process is devoted to the production of secretory granules, which are the carriers for the major regulated pathway- stimulated granule exocytosis (Castle & Castle, 1998). Among the various types of regulated secretory cells the salivary acinar cells are impressive in their ability to mount a massive exocytotic release of proteins in response to neural stimulation. Maximal stimulation by β adrenergic agonist like isoproterenol causes the release of 70-80% of the store secretion in an hour (Castle *et al.*, 1972).

As in other cell types that rapidly release a large fraction of store products upon stimulation, exocytosis is a compound process in which fusion of granules to the cell surface is also accompanied by the successive fusion of granules to other granules (Von Zastrow & Castle, 1987; Burgoyne, 1990).

The regulation of stimulated exocytotic process in salivary acinar cells is not fully elucidated (Ambudkar, 2000). β adrenergic stimulation and the consequent production of cAMP, are presently recognised as the signal most strongly linked to the secretion of salivary proteins that are stored in membrane bound secretory granules (Baum, 1987).

The events leading to formation of cAMP and PKA have been fully characterised. However, little is known about the events controlling the migration docking and fusion of the granules, in the exocytotic process (Quissel *et al.*, 1992). In the past two decades research in this field has tried to establish a link between PKA activity and the exocytotic process. It has been suggested that vesicle associated membrane proteins (VAMP) could constitute a substrate for PKA-dependent phosphorylation events (Fujista-Yoshigaki *et al.*, 1996).

Recent findings by Fujista-Yoshigaki *et al.*, (1996) have identified a vesicle associated membrane protein (VAMP2) which is located at the secretory membrane granule in parotid acinar cells and is involved in cAMP induced amylase secretion (Fujista-

Yoshigaki *et al.*, 1996). These authors suggest that the t-SNARE-binding region of VAMP2 is masked by some protein X and activation of PKA caused the dissociation of X from VAMP2. PKA would exert this effect indirectly through phosphorylation of some other cytosolic proteins, which remain to be identified (Fujita-Yoshigaki *et al.*, 1999).

1.4.3 Control of salivary flow and composition

Salivary flow is under autonomic nervous control.

1.4.3.1 Resting flow

Under resting conditions and when heavy salivary stimulation factors such as mastication are absent, there is a slow flow of saliva, which keeps oral tissues hydrated and lubricated. This unstimulated flow of saliva varies considerably during the day and is influenced by different factors.

1.4.3.1.1 Circadian variation

Each salivary gland has a daily pattern of unstimulated secretion, which is apparently independent of eating and sleeping behaviour. Unstimulated flow peaks at 5 am and attains the minimum flow at noon.

1.4.3.1.2 Hydration

Loss of body water and dehydration has been reported to cause a decrease and even stopping of salivary flow. This effect dries the mouth and participates in thirst mechanism of water drinking (Brunstrom, 2002).

1.4.3.1.3 Stress

Stress and fear situations are known to decrease salivary output. This was believed to be a secondary process to sympathetic stimulation present in flight or flee response

situations. However, more recently some authors have reported this effect to be under direct inhibitory effects from salivary hypothalamic nuclei.

1.4.3.2 Psychic Flow

In some animal species such as the dog, sight of food has proven to constitute a powerful stimulus for salivation. This is due to psychic stimulation of salivary glands on the anticipation of food and masticator stimulation. Although this mechanism is seeming experienced in man, objective increase in salivation caused by psychic flow has failed to be proven to occur. Although this mechanism is apparently experienced in man, objective increase in salivation caused by psychic stimuli has not been proven. It seems that momentaneous increased awareness of saliva present in mouth is most likely to occur (Jenkins, 1978).

1.4.3.3 Stimulated flow

1.4.3.3.1 Mastication gustatory and olfactory stimuli

Chewing savourless elements such as wax or gum elicit a three-fold increase in

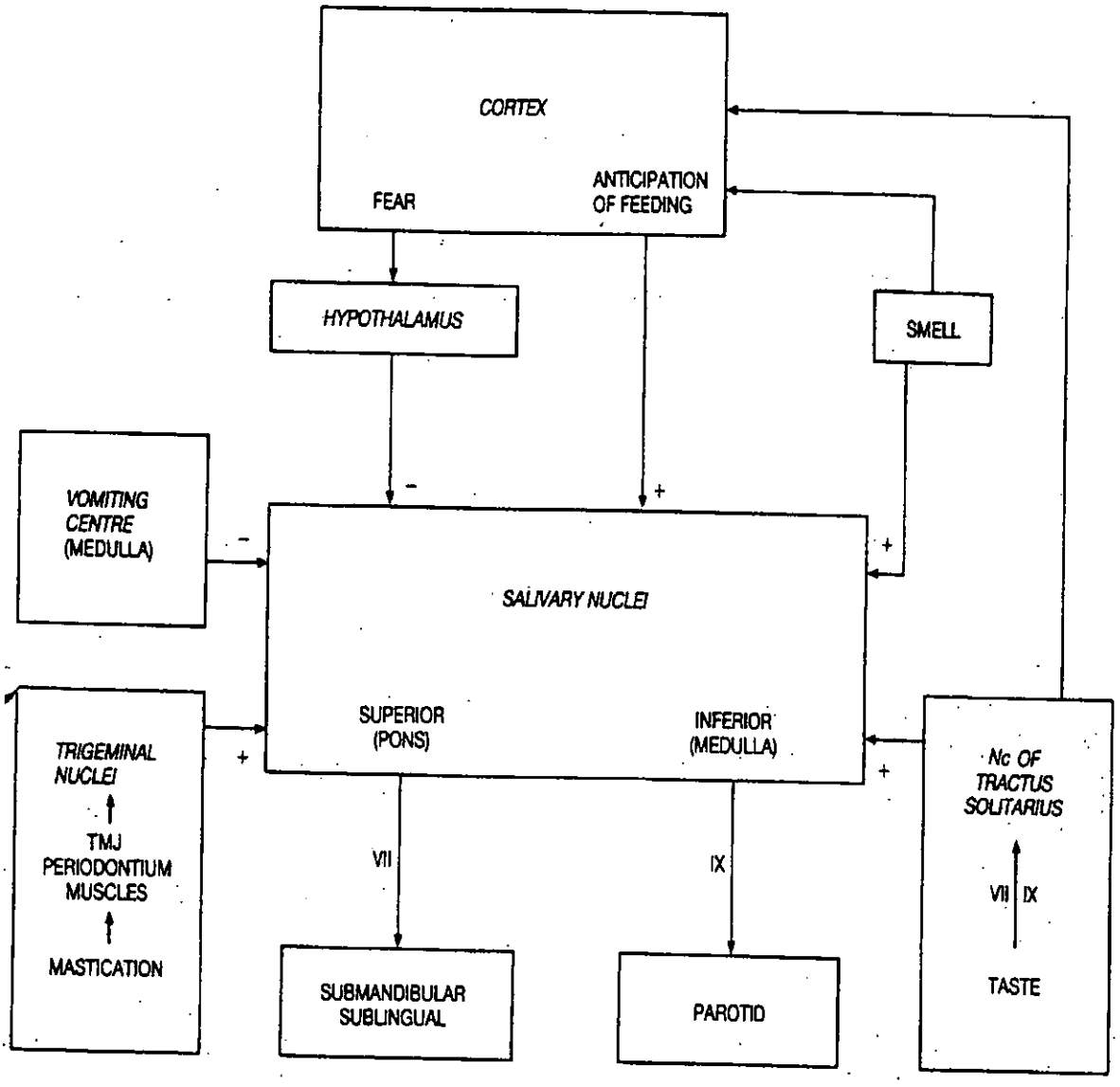


Figure 1.16- A schematic model showing the nervous control pathways of salivary secretion (Taken from Edgar, 1992)

salivary flow (Figure 1.16). This is reflex mechanism mediated through receptors, which are present in mastication muscles, periodontal ligament or mucosa via salivary nuclei and increase in parasympathetic secretomotor discharge.

Taste stimuli have been proved to be the most powerful stimulator for salivary flow, increasing flow by up to ten fold. Sour stimuli are the most effective followed by sweet, salt and bitter.

Most foods also elicit olfactory stimuli and direct responses to smell have been demonstrated.

decreased salivary output is known to exist after vomiting suggesting the existence of connections between vomiting and salivary centres in medulla and cortex, respectively.

1.4.4 Factors affecting composition of saliva

1.4.4.1 Flow rate

Variations in flow rate strongly affect saliva composition (Figure 1.17). As the flow rate increases upon stimulation of the glands salivary proteins, sodium, chloride, bicarbonate and calcium concentrations rise oppositely to those of phosphates and magnesium. With the increase in salivary flow, the time for ductal modifications to occur is diminished explaining these variations, and why final salivary composition tends to resemble that of acinar saliva.

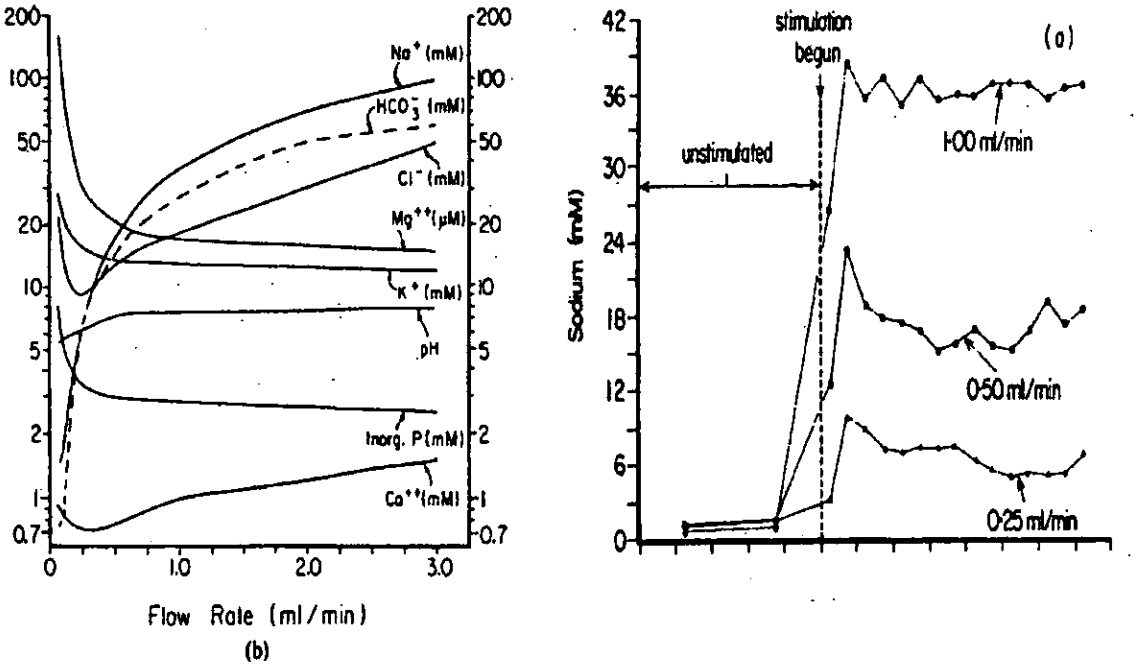
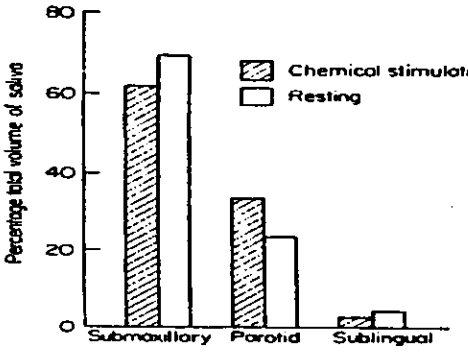


Figure 1.17- Salivary composition and flow rate (Taken from Jenkins, 1978)

1.4.4.2 Differential gland contribution



Glands do not contribute in the same proportions to whole saliva in resting and stimulated states (Figure 1.18). Also the composition of pure salivas is quite different among the existing glands. This factor affects the composition of whole saliva.

Figure 1.18- Differential gland contribution (Taken from Jenkins, 1978)

1.4.4.3 Circadian Rhythm

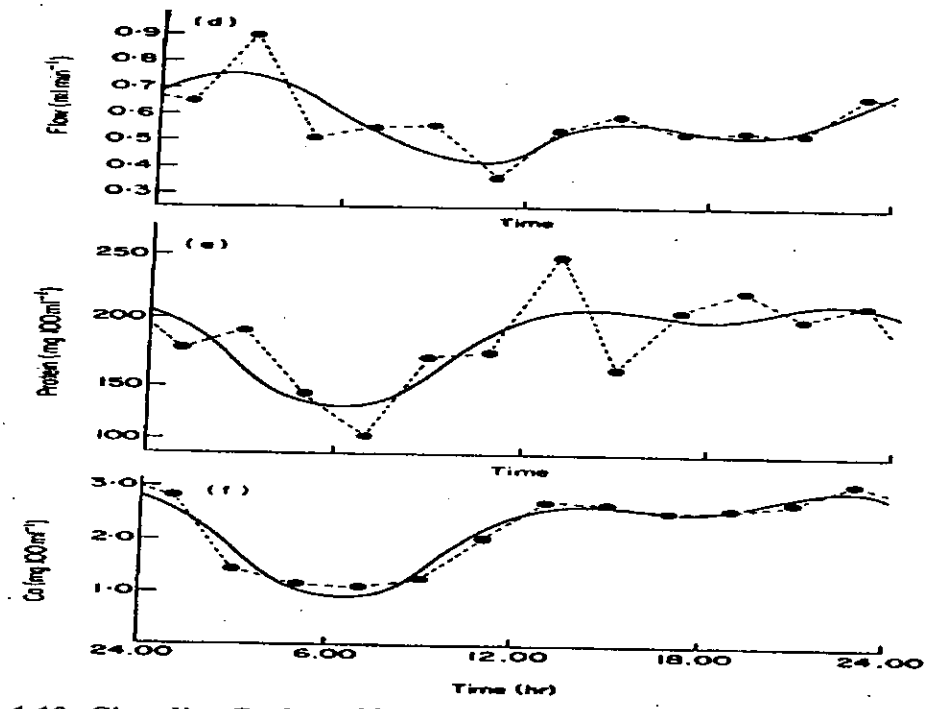


Figure 1.19- Circadian Rythm of Secretion (Taken from Jenkins, 1978)

Salivary constituents, like flow, displays a circadian rhythm of secretion (Figure.1.19). This factor influences the composition of saliva throughout the day.

1.4.4.4 Nature and duration of stimulus

For flow to be held constant, the composition of saliva may vary with the duration of the stimulus. Secretion elicited with either salt, sour, sweet or bitter stimuli shows no variation in electrolyte composition. However, salt stimulus has been reported to elicit an increase in protein secretion (Jenkins, 1978).

1.5 Pathological aspects of salivary gland function

Salivary dysfunction is defined as any alteration in the quantity and/or quality of salivary output (Navazesh, 1992). Dysfunction can include either an increase or decrease in salivary output and composition. Salivary dysfunctions with increased salivary output are rare and commonly secondary to either central or peripheral neurologic disorders rather than primary gland disturbance. On the other hand, there are numerous causes of salivary dysfunction with decreased salivary output. Xerostomia is defined as the subjective complaint of a dry mouth. This symptom may or may not be associated with measurable alterations in salivary function (Närhi, 1994).Where an objective diminution of salivary output is verified the term hyposialia is preferred although hyposialia and Xerostomia are usually used as synonyms in literature.

Patients with hyposialia have a an seriously impaired life quality presenting severe handicapping symptoms (Herrera *et al.*, 1988, Atkinson & Wu, 1994). Dry mouth usually does not become a clinical problem until the stimulated and resting flow rates fall respectively, below 0.1 and 0.7 $ml.min^{-1}$ (Ben-Aryeh *et al.*, 1981; Navazesh *et al.*, 1992; Sreenby, 1996). Patients usually complaint of a painful burning sensation in the mouth, difficulty swallowing dry food such as crackers, impairment of taste, increase in caries, painful ulcers of the mouth and increased fluid consumption (Atkinson & Fox, 1993). Hyposialy can produce changes in all of tissues in the mouth. It can cause fissures of the tongue, bucal membranes and lips, particularly in the corners of the mouth (Tabak, 1995) . It predisposes patients to dental caries which may be rampant and difficult to handle (Hay, 1995). Patients with dentures have difficulty in the use of their appliances because they frequently suffer from traumatic lesions in the oral cavity. In addition, patients have difficulty with mastication, deglutition and speech because of

a lack of lubrication results in sticking of the tongue and food bolus to the oral mucosa or denture surface (Tabak, 1995). These patients have been reported to have impaired tasting and smelling functions (Dysgueusia and Dysosmia), low masticator performance (Dusek, 1996) and inadequate nutritional intake (Ernst, 1993). Patients with dry mouth are predisposed to mucosal infections, including fungal infections that may be symptomatic and difficult to treat, probably related to the lack of the protective salivary barrier (Loesche, 1995).

1.5.1 Causes of Hiposialia

There are multiple causes of diminished salivary function, some of them result of primary defects of the gland and others are secondary to other factors (see table 1). Discussion of all aetiology of salivary dysfunction is clearly beyond the scope of this work, therefore only a brief overview of the major causes of salivary dysfunction will be given here.

Condition	Examples
Medications	Anticholinergics, tricyclic antidepressants, sedatives, tranquilizers, antihistamines, antihypertensives, cytotoxic agents, anti-Parkinsonian drugs, anti-seizure drugs, skeletal muscle relaxants
Oral diseases	Acute and chronic parotitis, sialolith, mucocele, partial/complete salivary obstruction
Systemic diseases	Mumps, Sjögren's syndrome, diabetes, HIV/AIDS, scleroderma, sarcoidosis, lupus, Alzheimer's disease, dehydration, graft versus host disease
Head and neck radiotherapy	Salivary gland malignant tumours, oral cancer, pharyngeal cancer

Table 1. Causes of salivary gland hypofunction (Taken from Ship, 2002)

1.5.1.1 Head and Neck Radiotherapy

A common treatment modality for oral and pharyngeal cancers is radiotherapy, and a clear relationship has been established between radiation and salivary dysfunction. There is a dose response relationship between radiation and salivary flow rates (Valdez *et al.,* 1993). Doses of radiation between 2100 to 4000 cGy have been known to cause severe damage to salivary glands, and since for most head and neck cancers radiation doses are greater than 4500 cGy, this is a common sequel of this kind of treatment (Parsons, 1994).

The serous acini of salivary glands are the most radiosensitive followed by mucous and ductal cells, and although the exact mechanism of cell damage is still not completely understood, the sequence of cell death is easily observed (Nagler *et al.,* 1997). In patients receiving high doses of radiation for 5 days a week, the stimulated salivary flow response drops 57% after the first week, and 76% after 6 weeks and 95% after three years (Nagler *et al.,* 1997).

1.5.1.2 Medication -induced salivary gland dysfunction

Drug-induced salivary dysfunction is very important to recognise because it is correctable. Over 400 drugs can affect salivary flow, classes of drugs which are frequently involved (See table 1) include anorexic drugs, antidepressants, antipsycotics, sedatives hypnotic, antihistamines, antiparkinsonism drugs, antihypertensive agents, diuretics, opiates, muscle relaxants, imunosupressive agents and medication for hiperacidity of stomach (Atkinson & Fox, 1992). Elderly peolple are a major target population for these kind of situations as they are often affected by pathological states and use a proportionally more elevated number of drugs than other age groups (Mulligan & Sobel, 1994). It is, however, not uncommon that these patients who are on medication do not perceive changes in either the amount or quality of saliva even if the medication is known to impair salivary function (Billings *et al.,* 1996). Thus, the absence of subjective complaints of oral dryness does not indicate adequate saliva production. Accordingly, diagnosis of medication induced hyposalivation require measurements of salivary output, and should be routinely performed in medicated patients (Navazesh, 1994).

Medications can affect the production of salivation in number of ways. It might influence the neural regulation central or peripherally via interactions with the autonomic nervous systems on the salivary gland tissue. It is likely that most frequently medication induced reductions in salivation are associated with peripheral interactions at the receptor level in the gland tissue. Thus, drugs that inhibit receptor systems like the muscarinic, alpha-adrenergic and beta-adrenergic may result in hyposalivation and compositional changes of the saliva. Other medications may cause effects indirectly via affecting the salt and water balance of the body or the hormonal or metabolic status. Furthermore, medication induced inhibition of the transport mechanisms responsible for the secretion of electrolytes, water and proteins in salivary cells may also result in reduced saliva output and compositional changes (Smith, 1994).

1.5.1.3 Systemic diseases affecting salivary gland function

The number of systemic conditions which salivary glands are involved with is overwhelming (see table 1). Usually they fall into one of these categories Infectious, metabolic, autoimmune, hormonal or neurological. In infectious diseases such as acute parotiditis (Mumps) the inflammatory infiltrate in the gland is responsible for the symptoms which are acute (gland swelling and pain) and disappear spontaneously upon cure of the disease. In autoimmune diseases such as Sjörgen syndrome chronic progressive lymphocyte infiltrate impairs salivary gland function. In metabolic diseases salivary involvement may be directly related with the disease like chloride defective transport mechanism underlying cystic fibrosis. However, more often salivary dysfunction is secondary to an altered metabolic state such as dehydration, or impaired protein synthesis. In hormonal diseases, the electrolyte-altered state or dehydration is the major causes of salivary dysfunction. Neurological diseases are characterised for lack of neural function, which controls the gland.

1.5.1.3.1 Diabetes and salivary secretion

Diabetes is probably the most frequent metabolic disease with salivary implications. In fact, salivary hypofunction and xerostomia have long been recognized features of diabetes mellitus type I and II, particulary when there has been dehydration and inadequate glucose blood control (Chavez *et al.*, 2002). Salivary impaired function has been demonstrated to occur more frequently in diabetic patients with previous history of neuropathy (Moore *et al.*, 2001). T cell lymphocitic infiltration in parotid gland parenchyma similar to the auto-imune inflamatory infiltrate present in the pancreas of type 1 diabetic patients has also been reported and accounted as a contribution factor in salivary alterations (Markopoulos *et al.*, 1998). In addition, auto-imune anti-bodies present in diabetic serum were recently demonstrated to be present in saliva of diabetic patients (Todd *et al.*, 2002).

Qualitatively, saliva of type I and II diabetic patients has been described to have, increased protein and glucose concentrations (Twetman *et al.*, 2002), diminished anti-oxidant capacity (Belce *et al.*, 2000) and EGF concentration (Oxford *et al.*, 2000).

These modification have been pointed responsible for the major incidence of oral infections such as caries (Twetman *et al.*, 2002), candida, periodontitis and xerostomic consequences in the diabetic patient (Oxford *et al.*, 2000).

1.5.2 Salivary function and ageing

Salivary gland dysfunction and xerostomia are common problems among the adults and tend to increase with age (Osterberg *et al.*, 1992). Actual estimation of xerostomia prevalence in elders over 65 years reaches 30% (Ship *et al.*, 2002). The idea that the ageing process in itself could be responsible for diminished salivary output over individual lifespan was brought in by earlier histomorphometric studies confirming salivary glands parenchyma changes in older populations (Scott, 1987). Loss of over 1/3 of acinar fluid producing cells replaced by adipose and fibrotic tissue and a proportional increase in ductal epithelial cells have been reported in parotid and submandibular glands in individuals of advanced age (Ariji *et al.*, 1994). However, studies measuring salivary output in healthy individuals are controversial. Cross-sectional evaluations of salivary flow rates revealing lower flow rates among older healthy individuals have

been reported by some authors (Navazesh, 1992; Cowman *et al.*, 1994). On the other hand, more recent studies suggest salivary glands to be relatively age-stable (Ship *et al.*, 2002) with cross-sectional (Jones & Ship, 1995) and longitudinal (Ship *et al.*, 1995) studies examining whole, parotid and submandibular flow rates in healthy subjects revealing no general diminution with age, which is contrast to what should be expected from histomorphometric studies (Scott, 1987). Is still not clear why a great number of functional studies demonstrate age stable output in the presence of reduced acinal cell volume across the human life-span. It has been hypothesized that young adults contain a substancial excess of fluid producing tissue, constituting a secretory reserve, which is lost and replaced by fat and connective tissue with aging. Thus, older people deplete their hypothesized secretory reserve (Fergusson, 1996; Ship *et al.*, 2002). It is theorized that adequate or young-adult levels of secretory function can still be achieved in the elderly as long as no further stress is placed on the system. Most older adults, however, are not free from disease and are likely to be at increased risk from many non-physiological or exogenous conditions that could further reduced or compromise the glands purported limited reserve capacity. Thus, the aging process would not be directly resposible for the prevalence of xerostomia, but for the greater vulnerability of salivary glands to external insults instead. However, further research is needed to prove the existence of a secretory reserve hypothesis and clarify the true influence of the aging process in salivary secretion which remains controversial.

1.5.3 Burning mouth syndrome

Burning Mouth Syndrome (BMS) is a condition of unknown aetiology, receiving wide-spreading attention in Dental Scientific community because of its life quality impairement potential. It is characterized by stomatodynia (pain and burning sensation of the oral mucosa) and it is not associated with remarkable organic objective causes. Among other factors saliva is thought to play a major role in BMS symptoms as it possesses specific rheological properties which are known essential for maintaining a balanced situation in the oral cavity. Recent litereature presents evidence that patients with BMS seem to have changes in salivary flow and composition (Chimenos-Kustner & Marques-Soares, 2002).

1.6 Magnesium

Magnesium is named after the Greek City, magnesia where large deposits of magnesium carbonate were found. Magnesium sulphate was isolated from Epson spring water in 1195 by *Grew.* Several forms of magnesium carbonate and oxide were isolated in the XVIII century by *Valentine* and *Black* respectively. In 1808, Sir *Humphrey Davy* became the first to isolate the impure metal form of magnesium. Pure form of magnesium was obtained in 1829 by *Bussy* (Mooren & Singh, 1997). Magnesium is the eighth element in order of abundance both terrestrially and cosmically (Emley, 1966).

Magnesium is extremely electropositive and readily loses two electrons to yield the divalent cation Mg^{2+}. Magnesium ions (Mg^{2+}) have an ionic radius approximately two thirds that of calcium and sodium ions and one half that of potassium ion (Williams, 1993). It is the most charge dense of all biological cations. This density results in having the largest hydrated radius and the smallest atomic radius among biological cations (Roof & Maguire, 1994). The small magnesium atom accommodates 6 oxygen ligands to form a stable Mg $(H_2O)_6^{2+}$ hydrate. These water molecules exchange very slowly with bulk water with exchanges rates three orders of magnitude less than those for Ca^{2+}, K^+ or Na^+. The hydrated form of Mg^{2+}, which is encountered by membrane transporters, has 300-350 times the volume of the unhydrated forms of either Ca^{2+}, K^+ or Na^+. The slow rate of water removal of hydration is the major rate limiting factor controlling the ability of Mg^{2+} to react with membrane transport systems (Jung & Brierley, 1994).

1.6.1 Biological Roles of Magnesium

The importance of extra and intracellular magnesium has become gradually recognised during the last century. At the present moment, pathologies such as diabetes, hyposecretion, cardivascular diseases and dyslipidemias are all associated with an altered magnesium metabolism (Sheehan, 1991; Altura& Altura, 1990; Geux *et al.*, 1993). Magnesium has also been used as an adjuvant in the therapeutics of cardio vascular and liver diseases among others (Sueta *et al.*, 1994; Weiss & Lasserre, 1994; Crippa & Giorgi-Pierfranceschi, 1997; Yago *et al.*, 2000).

In the body, most of magnesium is found mainly in bone (52%), followed by skeletal muscle (46%) and extracellular fluid (2%) (Elin, 1987). Serum levels of magnesium

account for less than 1% of total and is controlled by the gastro-intestinal tract and kidneys. Dietary intake is absorbed in jejunum and ileum in a proportion of 30-50% (Reinhart, 1988). Control of the absorption exists and depends on the amount and the type of diet (Gullestad *et al.*, 1994; Durlach, 1988). Excretion of magnesium is controlled at kidney level through variable reabsorption in the loop of Henle. Here, different hormones have been shown to control the reabsorption process determining the amount of magnesium being excreted in urine, examples include: calcitonin, glucagon, parathyroid and anti diuretic hormone all of which seem to favour magnesium reabsorption (Quamme, 1989; Rouffignac *et al.*, 1993).

Magnesium is essentially an intracellular ion, approximately 98% of non-skeletal magnesium being in the intracellular compartment. In fact, magnesium is the second most abundant intracellular divalent cation exceeded only by potassium (Reinhart, 1988). Within cell, magnesium plays a vital role in the regulation of numerous biochemical and physiological processes. It is involved in the synthesis of DNA and RNA as well as maintenance of their conformation (Henrotte *et al.*, 1993). Possibly in relation to its capacity to form complexes with phospholipids, magnesium has been demonstrated to affect membrane fluidity and permeability (Storch & Schachter, 1985; Beavis & Garlid, 1987). Intracellular magnesium has been associated with processes as important as the secretion of hormones including insulin (Ishizuka *et al.*, 1994) and prolactin (Kasahara, 1993). Many of the actions of magnesium are due to its role as a co-factor of a wide range of enzymes. It is well know that magnesium activates virtually all the enzymes involved in the metabolism of phosphorylated compounds, as well as many enzymes in the glycolytic and tricarboxilic acid pathways (Heaton, 1990). Magnesium has also been shown to be implicated in the regulation of several membrane transport systems such as Na^+/K^+ ATPase, Ca^{2+} pumps, $Na^+/Cl^-/K^+$, K^+/Cl^-, Na^+/H^+ and Cl^-/HCO_3^- co transporters (Heaton, 1993; Flatman, 1993).

Inside the cell 90-95%, of magnesium are bound to highly charged anionic ligands such as ATP, ADP, RNA, polyphosphates, proteins and citrate (See Birch, 1993 for review; Gunther, 1990; Flatman, 1991; Cohan, 1991; Wacker, 1993). Depending on the cell type, free and bound magnesium have been shown to be completely or partially exchangeable (Gunther, 1990). Intracellular distribution of magnesium varies accordingly to cell type (Griwold & Pace, 1956; Rosenthal *et al.*, 1956; George & Heaton, 1975; George & Polimeni, 1972; Clemente & Meldosi, 1975). In epithelial secretory cells such as pancreatic acinar cells magnesium seems to be distributed around

rough microsomes and inside zymogen granules (Clemente & Meldosi, 1975). This latter suggestion was supported by the fact that magnesium is released with enzymes in both saliva (Nielsen & Petersen, 1972) and pancreatic juice (Jansen *et al.*, 1980).

Cytosolic magnesium concentration of mammalian cells is regulated at a level way below that predicted by the Nerst's potential. Thus, magnesium is not passively distributed indicating that an active transport regulates the intracellular magnesium in order to maintain low levels in sublingual acini (Zhang & Melvin, 1995) and pancreatic cells (Lennard & Singh, 1992; Mooren *et al.* 2001) among others.

A sodium dependent efflux mechanism for magnesium has been demonstrated in numerous cell types (Vorman & Gunther, 1993; Mooren & Singh, 1997) including sub-lingual acini (Zangh & Melvin, 1995). This system has been shown to be quite heterogeneous depending on cell type (See Yago *et al.*, 2000 for a review).

Over the past few years research has provided evidence that the control of intracellular magnesium homeostasis is altered by a number of receptors, especially those that promote calcium mobilisation. Also, magnesium has been shown to play an important physiologial role in secretagogue-evoked secretory responses in some epithelial secretory cells such as acinar pancreatic cells and gastric parietal cells (Yago *et al.*, 2000; Mooren *et al.*, 2001). In the pancreas, the classical secretagogues such as ACh and CCK-8 can elicit marked and drastic changes in $[Mg^{2+}]_i$ which seem to be closely related to the Ca^{2+} signalling process (Lennard & Singh, 1992; Yago *et al.*, 2000; Mooren *et al.*, 2001). In rat and mouse pancreatic segments, raising $[Mg^{2+}]_0$ in the perfusate to 10 mM resulted in a marked inhibition of ACh, CCK-8 and EFS-induced protein, trypsinogen and amylase secretion as compared to responses obtained in (normal 1.1 mM and 0 mM) $[Mg^{2+}]_0$ (Juma *et al.*, 1996; Yago *et al.*, 2000). The inhibitory effects of high $[Mg^{2+}]_0$ on the secretory activity of the exocrine pancreas have been proven to be mediated through a disruption of the Ca^{2+} signalling events, possibly by Mg^{2+} acting at different stages of Ca^{2+} mobilisation process such as efflux from internal stores and capacitative Ca^{2+} entry (Mooren *et al.*, 1997, 2001; Lennard & Singh, 1992; Geada, 1998). These facts implicate magnesium to be under tight regulation, but also to exert important regulatory functions in the stimulus-secretion coupling events in pancreatic acinar cells.

Salivary glands are secretory organs of epithelial origin. The presence of Mg- dependent transport systems have been demonstrated in the rat sublingual acinar cells, similarly, Mg^{2+} efflux has been shown to be under the influence of muscarinic receptor

stimulation (Zhang & Melvin 1994; 1995). However, the cellular mechanisms of Mg homeostasis and its possible physiological role in the salivary secretory process remains to be elucidated. Thus, this study was designed to focus on the role of perturbation of $[Mg^{2+}]_0$ on salivary gland secretion.

1.7 Scope of this work

1.7.1 Objectives

The main objective of this work was to gain an understanding of some of the physiological aspects of salivary gland function and regulation, both at basic and clinical levels. The initial objective was to investigate the effect of a perturbation of $[Mg^{2+}]_0$ on basal and secretagogue-evoked salivary protein and amylase secretion and moreover to characterise Mg^{2+} transport system in parotid acinar cells. Very little is known about the possible roles of the magnesium ion in the stimulus secretion-coupling mechanisms responsible for primary acinar salivary production, this study was originally focused on a major part of the research undertaking the following investigations:

Specific aims-section A:

a) To examine the effects of perturbation of $[Mg^{2+}]_0$ on secretagogue-evoked protein secretion and $[Ca^{2+}]_i$ in the rat sub-mandibular gland.
b) To examine the effects of perturbation of $[Mg^{2+}]_0$ on secretagogue-evoked amylase secretion and $[Ca^{2+}]_i$ in the rat parotid gland in order to gain insight into the relationship between Mg^{2+} and Ca^{2+} signalling in the stimulus-secretion coupling process in parotid acinar cells.
c) To study the effects of secretagogues and transport inhibitors on $[Mg^{2+}]_i$ variations and on membrane transport in magnesium unloaded cells at the physiological level.

Specific aims-section B

The second part of the study employed human subjects to investigate the influence of age, gender, oral surgical recovery and diabetes (types I and II) on salivary flow rates and its compostion. The study measured salivary resting and stimulated flows, and levels of K^+, Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+} and Cl^- in the saliva. The rationale was to improve our knowledge about normal and pathophysiological composition of saliva since this is believed to be crucial and also of major importance for a better clinical practice in oral medicine.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All chemicals were purchased from Sigma (Spain), except collagenase, which was obtained from Worthington Biochemical Corporation, (New Jersey, USA), Fura-2 and Magfura 2 were obtained from Molecular Bioprobes Europe (The Netherlands).

2.2 Animal experimental procedures

All animal experiments were performed on either isolated parotid or submandibular glands taken from adult (250-350 g) male and female CD strain Wistar rats. The Ethics Committees of University of Central Lancashire in Preston, England, ISCS-Sul in Portugal and University of Extremadura in Spain approved all procedures. Animals were humanely and swiftly killed by blow to the head followed by cervical dislocation. An incision was made in the upper part of the neck. The parotid and submandibular glands were quickly removed and placed into a modified Krebs-Henseleit (KH) solution comprising of (mM): - NaCl, 103; KCl, 4.76; $CaCl_2$, 2.56; $MgCl_2$, (either 0, 1.1, 5 or 10); $NaHCO_3$, 25.0; NaH_2PO_4, 1.15; D-glucose, 1.8; Sodium Pyruvate, 4.9; Sodium Fumarate, 2.7 and Sodium Glutamate, 4.9. The solution was kept at pH 7.4 while being continuously gassed with a mixture of 95% O_2: 5% CO_2 and maintained at 37°C. In this study a concentration of 1.1 mM Mg^{2+} was used as the normal $[Mg^{2+}]_0$ since this is the physiological total plasma Mg^{2+} (Yago *et al.*, 2000) and moreover, other authors had previously used the same concentration to investigate the effects of changes of $[Mg^{2+}]_0$ on nerve-mediated and secretagogue-evoked secretory responses in the exocrine pancreas (Francis *et al.*, 1990; Lennard & Singh, 1992; Wisdom *et al.*, 1996; Mooren *et al.* 2001). In addition, both zero $[Mg^{2+}]_0$ and an elevated (5 and 10 mM) $[Mg^{2+}]_0$ were used for comparison in order to investigate the effects of $[Mg^{2+}]_0$. During the perturbation of $[Mg^{2+}]_0$, an equivalent concentration (mM) of NaCl was either removed or added to the modified KH solution to maintain a constant osmolarity.

2.3 Measurement of amylase output from parotid gland segments during perturbation of extracellular $[Mg^{2+}]_0$

The parotid glands were cut into small segments (10-25 mg) and a total weight of about 175-250 mg was placed in a Perspex flow chamber (Vol. = 1 ml) and superfused with oxygenated KH solution at 37°C at a flow rate of 1.8 ml min^{-1}. The effluent from the chamber passed directly to an on-line automated fluorimetric assay for the continuous measurement of amylase output. This method is a modification of the original technique described previously by Rinderknecht and Marbach (1970) and subsequently by Michalek and Templeton (1987). It depends essentially on the liberation of dialyzable fluorogenic products from the enzymatic breakdown of a modified starch (amylopectin anthranilate) used as substrate by amylase (see Appendix 1 for preparation of amylopectin anthranilate and preparation of substrate). Figure 2.1 shows a diagram of the set-up used in the present study. The effluent from the chamber was mixed with an air segmented substrate solution and subsequently passed through two glass mixing coils (27 and 14 turns respectively) submerged in a thermostatically heated water bath (37°C). The mixture was dialysed through a cuphrane membrane (Technicon part No. 933.0225.01) embedded in a 12 " dry operated dialyzer (Bran-Luebbe, Germany) with a recipient stream buffer (NaCl, 0.05 M and Triton-X-100, 0.1% v/v, at pH 7.0; see also Appendix 1 for recipient preparation). The air segmented recipient stream which contains the released fluorochrome was then pumped via a debubbler through a 37°C thermostated removable 750 µl quartz flowcell cuvette placed in a Perkin Elmer LS 50 B fluorimeter. The fluorescence was monitored with excitation and emission wavelengths of 343 nm and 414 nm, respectively. Excitation and emission slits were set at 5 nm. Continuous α-amylase output fluorescence intensity data were collected directly into a computer and processed via a Winlab Software.

α-amylase (Sigma type II-A) was used as a standard to calibrate the assay at both beginning and end of each experiment. One unit of amylase is defined as the amount of

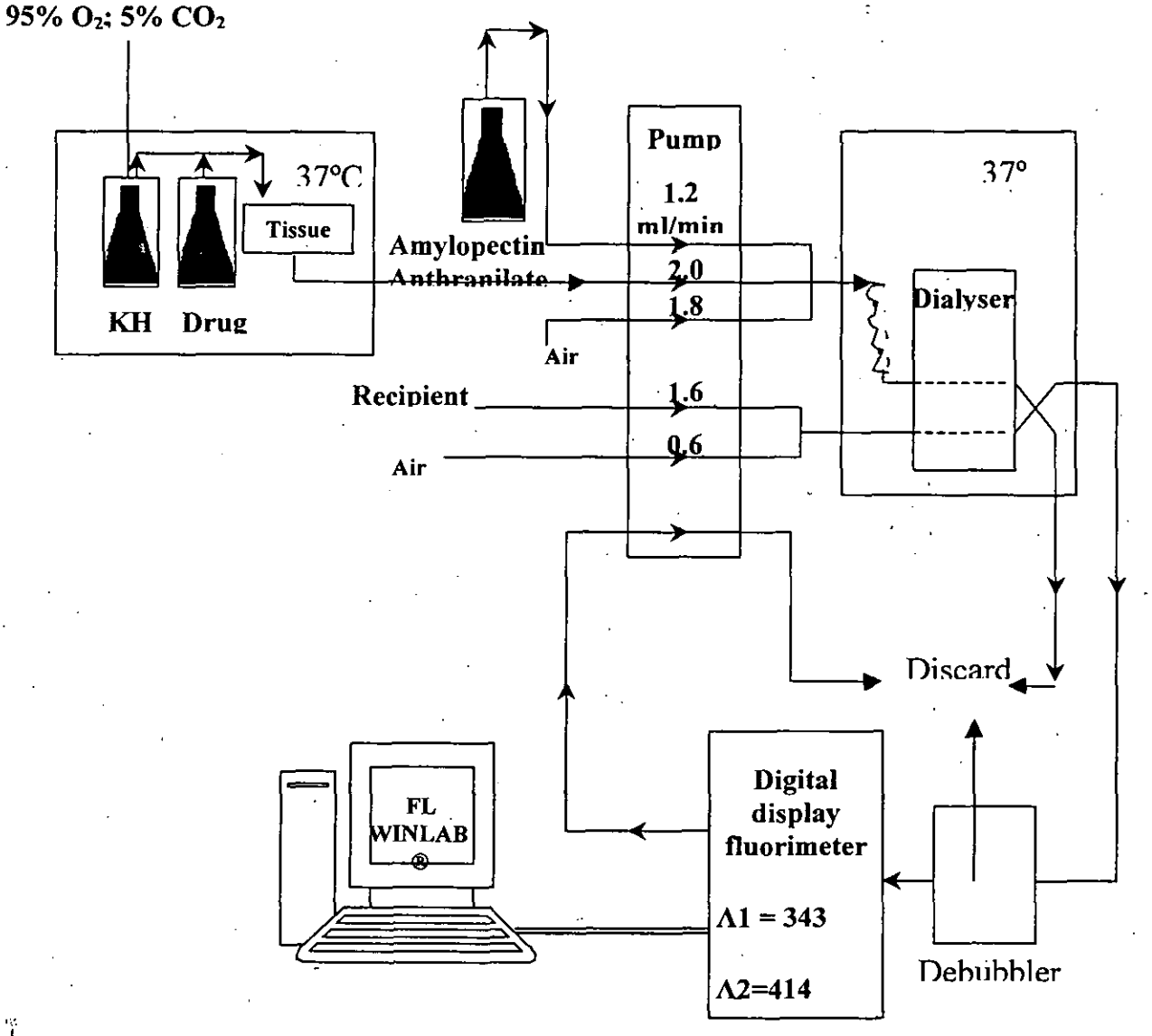


Figure 2.1- Diagram of the set up for on-line amylase measurements (Adapted from Michalek & Templeton, 1987).

amylase, which can liberate 1.0 g of maltose from starch in 3 minutes at pH 6.9 and 20°C. To test for linearity of the system several concentrations (0.1 - 60) units. ml^{-1} . α-amylase were tested (see Figure 2.2). Standards of 30 Units . ml^{-1} α-amylase were chosen for routine use throughout the experiments.

After each experiment the tissue was removed from the chamber, blotted dry and weighed. Conversion of fluorescence intensity was performed in order to express the results as Units of amylase. ml^{-1} . $(100\ mg\ of\ tissue)^{-1}$.

For the study of nerve-mediated secretion, a special Perspex tissue chamber containing silver wire electrodes for electrical field stimulation (EFS) was employed. The parameters of EFS were: amplitude, 50 V; frequency, 5, 10 or 20 Hz; pulse duration, 1 ms.

Basal amylase output was obtained after allowing tissue to stabilize for 40 minutes. During secretagogue stimulations, either acetylcholine (ACh), noradrenaline (NA), Isoprenaline (ISO) or phenylephrine (PHE)) was added directly to the superfusing chamber in known concentrations. Either EFS or secretagogue stimulation was always applied for a duration of 10 minutes. Furthermore, in those experiments involving the effects of the absence of extracellular calcium, Ethylene-Glycol-Tetracetic Acid (EGTA) (1 mM) was added thus making the solution nominally calcium free. Figure 2.2 shows a typical time course chart recording of amylase secretion from one experiment.

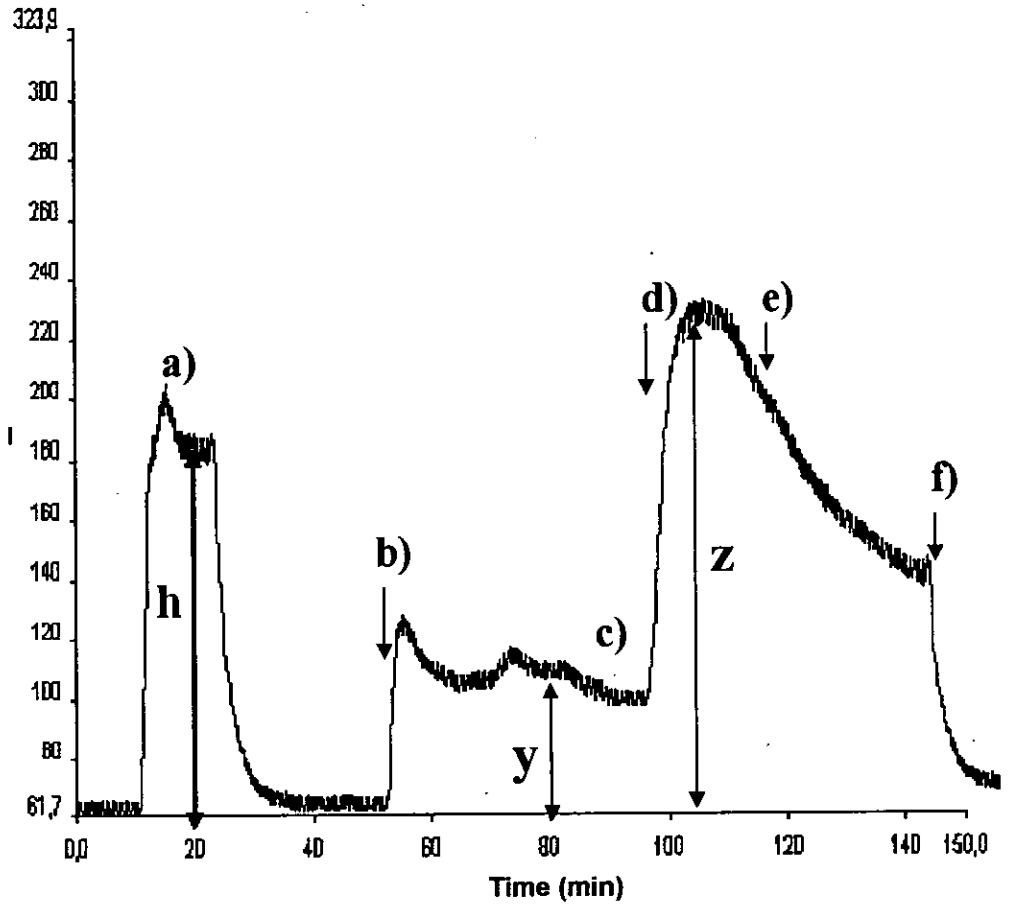


Figure 2.2 Time course of typical on-line experiment for the measurement of amylase output for superfused parotid segments. I in the ordinate axis means fluorescence intensity. (a) addition of amylase standard (30 U . ml^{-1}) to the perfusing chamber for 10 min, (b) addition of the tissue segments to the chamber (see arrow). After allowing tissue to stabilize for 40 minutes and basal amylase was maintained at a constant steady state level (c) the tissue was stimulated with either a secretagogue or EFS (10 Hz, 50 V, 1 ms) (see arrow) at (d) for 10 min. At the end of the stimulation period (e) the tissue was superfused in normal KH solution for another 30 – 40 min until amylase output decreased to a low level (f). At this point the tissue was removed from the chamber and weighed.

2.4 Measurement of total protein output from submandibular gland segments during perturbation of $[Mg^{2+}]_0$

The isolated submandibular glands were cut into small segments (10-20 mg) and the cut tissues were suspended in 20 ml oxygenated KH solution. The tissue segments were rinsed 3-4 times with KH solution (20 ml each) to remove any protein due to cutting of the tissues. Submandibular segments approximately (200 mg) were incubated for 30 min with either KH solution alone (control) or KH solution containing either 10^{-5} M ACh, 10^{-5} M NA or 10^{-5} M PHE, during perturbation (0 mM, 1.1 mM, 5 mM, 10 mM) of $[Mg^{2+}]_0$ in a shaking water bath at 37°C. Following incubation the tissues were removed, blotted dry and weighted. Total protein concentration in the effluent was measured using an established colorimetric method (Lowry *et al.*, 1951) with bovine serum albumin as a standard. Protein output was expressed as $\mu g \cdot ml^{-1}.(100 \text{ mg of tissue})^{-1}$.

2.5 Preparation of isolated acinar cells suspension from parotid and submandibular glands for measurements of either $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$ using both cell suspension and single cell techniques.

Adult male Sprague-Dawley rats weighing between 200 and 300 g were used throughout this study. Glands were obtained and treated as explained in the experimental procedure. Isolated acinar cells suspension from either parotid or the submandibular glands were obtained by a modification of an established method by (Baum *et al.*, 1990).

Glands from one animal were placed on a plastic Petri dish with 1 ml incubation medium comprising in [mM]-NaCl, 137; KCl, 5,4; $MgCl_2$, 1.1; $CaCl_2$, 1.28; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; Glucose, 5.6; Hepes, 33; pH 7.4; 0,1% w/v bovine serum albumin. Any remaining extraneous tissue was quickly removed and the glandular tissue was chopped with iris scissors (300 times) until a soft homogenate consistency was obtained. Minced tissue was then transferred to 50-ml propylene tube filled with 10-ml dispersion medium consisting of incubation medium to which 37 $U.ml^{-1}$ of collagenase and 0.1 $mg.ml^{-1}$ of soya bean trypsin inhibitor was added. Tissue suspension was then gassed with 95% O_2 + 5% CO_2 for 10 seconds capped and incubated in a shaking water

bath at 120 cycles.min^{-1} for 80 min. At 20 min intervals cell suspension was gassed for 10 seconds and pipetted through a plastic 10 ml pipette up and down for 10 times to aid dispersion. After 80 min, the resulting acinar cell suspension was centrifuged at 100 g for 1 min and resuspended in incubation medium. This procedure was repeated three times to wash off the collagenase. The cell suspension was then pipetted through a 1 ml plastic pipette and filtered through a double layered muslin gauze, centrifuged and resuspended in 10 ml of incubation medium containing 0.1 mg.ml^{-1} soy bean trypsin inhibitor, maintained at 37°C and gassed every 20 min before being used in the experiment.

Cell suspensions from either the parotid or submandibular glands were loaded with either 2 μM of Fura-2 acetylmethoxy ester (AM) or Magfura-2 (AM) for $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ measurements respectively in the presence of 0.025 % pluronic acid for 40 min at room temperature (20°C). After the loading procedure, cells were centrifuged, and resuspended in an incubating medium (comprising in (mM) - NaCl, 137; KCl, 5,4; $MgCl_2$, 1.1; $CaCl_2$, 1.28; Na_2HPO_4, 0,33; KH_2PO_4, 0.44; Glucose, 5.6; Hepes, 33; pH 7,4; 0,1% w/v bovine serum albumin) for 30 min at 37°C for complete desterification of the probe. Centrifugation and resuspension in incubation medium were performed before each experiment. In experiments where extracellular Mg^{2+} was perturbed, the cells were centrifuged and then resuspended in incubation medium containing the appropriate concentration of $MgCl_2$. Gassing was performed every 20 min. Cell viability was assessed using the blue trypan test and batches of cells with less than 95% viability were discarded.

2.6 Measurement of $[Ca^{2+}]_i$ in fura-2 loaded submandibular acinar cell suspension using spectrofluorimetry

Measurement of $[Ca^{2+}]_i$ was performed in Fura 2-AM loaded cells by a method described below. A volume of 2 ml of submandibular cell suspension, was added to an Hellma quartz cuvette in a LS 50 B (Perkin Elmer, Beaconsfield, Bucks, England) spectrofluorimeter; and stirred slowly at 37°C. Cell suspension was excited by a fluorescent xenon source at wavelength of 340 nm and 380 nm and fluorescence emission was read at 510 nm. Slits were set at 5 nm. After a initial period of 50 seconds, the basal $[Ca^{2+}]_i$ fluorescence intensity was read and stabilised. Thereafter, ACh (10^{-5}

M) was added directly to the cell suspension. At the end of each experiment and for calibration purposes, a maximum and minimum of fluorescence (Rmax & Rmin) was obtained by adding 5 μM digitonin and 10 mM EGTA to the cuvette, respectively. Fluorescence intensity was converted in $[Ca^{2+}]_i$ expressed in nM by established methods (Grienkiewicz *et al.*, 1985). Kd for Ca^{2+} was 224 and calibration calculations were performed through a Winlab software by Perkin Elmer, Beaconsfield, Bucks, England. These experiments were performed in different $[Mg^{2+}]_0$ (0 mM, 1.1 mM, 5 mM and 10 mM). Peak $[Ca^{2+}]_i$ values were chosen from raw data collection points as the highest value obtained. Plateau values were calculated with software from raw data as mean values between 100 sec and 200 sec after peak value.

2.7 Measurement of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ in Fura-2 and Magfura-2 (respectively) loaded single parotid acinar cells, using microspectrofluorimetry.

A small volume (250 μl) of parotid cell suspension in different $[Mg^{2+}]_0$ solutions was placed on a thin glass cover slip attached to a Perspex perfusion chamber. Thin glass cover slips were imbedded and coated with Polly-lysine and let to dry in open air, to promote cell attachment. Perfusion (approximately 1ml . min^{-1}) at room temperature (22° C) was started after a 2-min period to allow spontaneous attachment of the cells to the glass cover slip. The chamber was placed on the stage of an

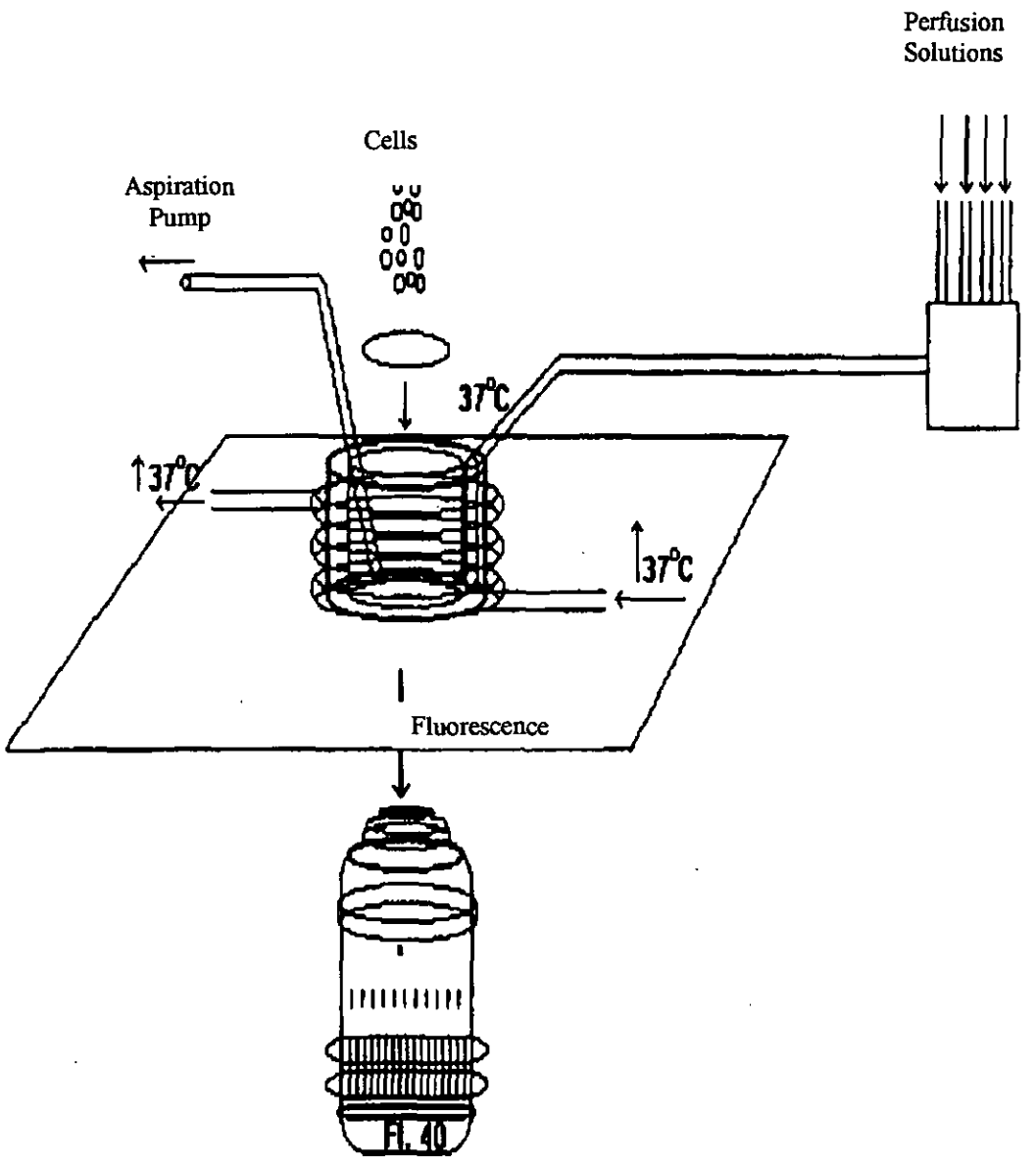


Figure 5.1- A schematic diagram of the microspectrofluorescence set up (Taken from Mateos, 1998).

inverted fluorescence-equipped microscope (Nikon Diaphot 300), equipped with a Fluor X40 fluorescence objective. Cells were excited at 340 nm and 380 nm (for both Fura – 2 and Magfura – 2) by a computer filter wheel, and the emission fluorescence was detected at 510 nM. The emitted images were captured by a cooled digital CCD camera (C-6790, Hamamatsu Photonics) and recorded using dedicated software (Argus-His Ca, Hamamatsu Photonics). Once a basal level of either $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$ was obtained cells were superfused with 10^{-5} M ACh in the incubation medium. Traces were expressed as 340/380 ratio fluorescence variations instead of calibrated traces, as this is now a generally accepted procedure (Pariente *et al,* 2003).

During the measurement of $[Ca^{2+}]_i$, Fura-2 AM loaded acinar cells were stimulated with ACh (10^{-5} M) in zero, normal and elevated $[Mg^{2+}]_0$. In some experiments extracellular calcium ($[Ca^{2+}]_0$) was removed from the superfusing medium which contained 1 mM EGTA. Cells were stimulated with 10^{-5} M ACh in zero, normal and elevated $[Mg^{2+}]_0$ to monitor its effects on Ca^{2+} liberation from intracellular stores. In these experiments following recovery to resting basal $[Ca^{2+}]_i$ levels, cells were then perfused with a medium containing 1.8 mM $[Ca^{2+}]_0$ as this is known to trigger capacitative calcium entry (CCE). These experiments were repeated in different $[Mg^{2+}]_0$ to study its effects on CCE. Final $[Ca^{2+}]_i$ value was expressed as 340/380 fluorescence ratio (Pariente *et al.*, 2003).

During the measurement of $[Mg^{2+}]_i$, Magfura-2 (AM) loaded acinar cells were perfused with different $[Mg^{2+}]_0$ media to obtain cytosolic Mg^{2+}. In other experiments, cells were stimulated with ACh (10^{-5} M) in different $[Mg^{2+}]_0$ and in the presence and absence of extracellular Na^+. In some experiments, extracellular Na^+ was removed and instead NMDG was added. The rationale was to characterise the mechanism of Mg^{2+} efflux. In another series of experiments, known concentrations of a number of transport inhibitors such as dinitrophenol, bumetanide, lidocaïne, amiloride and quinidine were also employed in the absence and presence of 10^{-5} M ACh. Final $[Mg^{2+}]_i$ value was expressed as 340/380 fluorescence ratio (Pariente *et al.*, 2003).

2.8 Measurement of human salivary secretion and its quality in normal and pathophysiological conditions

2.8.1 Subject selection

Fifteen human male and female subjects were selected for each group. Subjects were selected as volunteers and each signed a consent form. This procedure received ethical clearance and approval by local Ethic Committees at ICSS Portugal and at University of Central Lancashire in Preston. Age and gender were important criteria in order to investigate and establish an age and gender dependent relationship of salivary composition profile within the healthy groups. For pathophysiological studies either diabetic or oral surgery recovery patients were selected. Saliva was collected at either

the Military Hospital, the Portuguese Diabetes Association, and a private dental clinic in Lisbon owned by the PhD student

2.8.2 Saliva collection

Saliva collection was undertaken between 7 and 8 am, and subjects were instructed to being in a fasting state. For the study of the effects of surgery on salivary gland function, saliva collections were performed between 7 and 8 am on the day of the surgery and exactly 48 hours after the surgery. All surgical procedures were performed in the morning.

Both resting and stimulated whole saliva were collected by established methods (Navazesh, 1992). Unstimulated saliva was collected by spitting method. Subjects were instructed to swallow the saliva present in the mouth and a chronometer was started. At one-minute intervals, subjects were instructed to salivate into previously weighed 50-ml falcon tubes during 10 minutes. Stimulated saliva was collected by stimulation with citric acid method. Subjects were instructed to swallow the saliva present in the mouth and a chronometer was started. Four drops (0.17 g) of citric acid (0.1 M) were applied to tongue dorsum and subjects were instructed to roll the tongue in order to spread the citric acid. At one-minute intervals saliva was collected by spitting into previously weighed propylene 50-ml falcon tubes and more four drops of citric acid were applied. This procedure was repeated during 10 minutes. Immediately after collection, the salivas were weighed and stored at - 80°C until used for analysis procedures.

Rates of resting and stimulated secretions were expressed in $g \cdot min^{-1}$.

2.8.3 Ion analysis

Saliva collected was analysed for the concentrations of Cl^-, K^+, Na^+, Ca^{2+}, Mg^{2+} and Zn^{2+}. Saliva samples were defrosted at room temperature and then centrifuged at 6000 rpm for 10 minutes before beeing used in order to remove extrinsic contamination elements such as oral epithelia cells, microrganisms, and food debris among others. Concentration of Cl^- was determined with the use of a Chloride Titrator (Jenway England), and the results are expressed as $mMol \cdot l^{-1}$.

For the determination of the other ions, saliva was diluted at either 1/100 or 1/1000 and either K^+, Na^+, Ca^{2+}, Mg^{2+} or Zn^{2+} concentration was obtained in a Shimadzu AA 670 Atomic Absorbance Spectrophotometer, (Japan). Results were expressed as mg . l^{-1}. In order to minimise ionic contamination of salivary samples, propylene tubes of saliva collection were previously decontaminated by immersion in 60% w/v nitric acid during a two-week period before collection.

2.8.4 Total protein analysis

Saliva samples were defrosted at room temperature and the centrifuged at 6000 rpm during 10 min before use. Total protein concentration expressed as mg.l^{-1} was determined using established colorimetric methods (Lowry *et al.*, 1951) with the use of an Helios spectrophotometer by reading samples at 720 nm. Bovine serum albumin was used for calibration purposes.

2.9 Statistical analysis of all data

Data are presented either as original chart recordings or as Mean $\pm$ Standard Error of the Mean (S.E.M). Control and test values were compared using paired or unpaired Student's t-test and ANOVA or MULTIANOVA plus post Hoc test as appropriate (SPSS for Windows version 10.0). Values of $P<0.05$ were taken as significant while values of $P<0.01$ were taken as highly significant.

CHAPTER 3

EFFECT OF EXTRACELLULAR MAGNESIUM ON SECRETAGOGUE-EVOKED SECRETORY RESPONSES IN THE ISOLATED RAT SUBMANDIBULAR GLAND

3.1 Introduction:

Saliva is secreted mainly by three pairs of salivary glands and it performs a fundamental role in oral cavity homeostasis (Herrera *et al*, 1988). Salivary secretion is controlled entirely by the autonomic nervous system (Ambudkar, 2000). The autonomic neurotransmitters, ACh and NA act via different stimulus-secretion coupling pathways involving cellular calcium (Ca^{2+}) and adenosine 3; 5 cyclic monophosphate (cAMP) to elicit secretion (Baum, 1987). Recently, the divalent cation magnesium (Mg^{2+}) has been implicated in the exocytotic process of other exocrine gland such as the pancreas (Yago *et al*,2000). Mg^{2+} is believed to exert its effect on secretagogue evoked secretory response via cellular Ca^{2+} mobilisation (Francis *et al.*, 1990; Petersen, 1992). Since, the submandibular gland can secrete digestive proteins just like the exocrine pancreas, it was decided to investigate the effect of perturbation of extracellular $[Mg^{2+}]_o$ on ACh, PHE and NA evoked secretory responses on the rat submandibular gland. The rationale was to develop therapeutics strategies in the resolution of several diseased states usually associated with salivary gland function.

3.2 Materials and Methods

As described in Chapter 2.

3.3 Results

3.3.1 Measurements of total protein output

Figure 3.1 shows a family of histograms of basal protein output in zero, normal (1.1 mM) and elevated (5 mM and 10 mM) $[Mg^{2+}]_o$. The results show that both zero and elevated $[Mg^{2+}]_o$ can inhibit basal protein output compared to normal $[Mg^{2+}]_o$.

Figure 3.2 shows the effect of 10^{-5}M ACh on total protein out from submandibular segments during perturbation of $[Mg^{2+}]_o$. The control values during $[Mg^{2+}]_o$ are also shown in Figure 3.2 for comparison. The results show that ACh can elicit significant ($P<0.05$) increases in total protein output from submandibular segments compared to the respective controls. Maximal protein output occurred in the presence of normal (1.1 mM) $[Mg^{2+}]_o$. In both zero and elevated $[Mg^{2+}]_o$ the secretory effect of ACh was significantly ($P<0.05$) reduced.

Figure 3.3 shows the effect of 10^{-5} M PHE on total protein output from isolated submandibular segments, incubated for 30 min at 37°C during perturbation of $[Mg^{2+}]_o$. Like ACh, PHE can also elicit marked increases in total protein output. Both zero and elevated (5 mM and 10 mM) $[Mg^{2+}]_o$ can significantly ($P<0.05$) attenuate the PHE - evoked total protein output compared to 1.1 mM $[Mg^{2+}]_o$.

Since both ACh (muscarinic receptor agonist) and PHE (α1-adrenergic agonist) can stimulate total protein output from submandibular segments, it was decided to investigate the effect of sympathetic autonomic neurotransmitter, NA on protein output during perturbation of $[Mg^{2+}]_o$. NA is known to exert its secretory effects via both cyclic AMP metabolism and Ca^{2+} mobilisation. Figure 3.4 shows the effect of 10^{-5} M NA on protein output. The results show that NA can elicit maximal secretory effect at 1.1 mM $[Mg^{2+}]_o$. Both zero and elevated $[Mg^{2+}]_o$ attenuated the secretory responses to NA.

3.3.2 Measurements of $[Ca^{2+}]_i$ during perturbation of $[Mg^{2+}]_o$

Since a perturbation of $[Mg^{2+}]_o$ can have marked effects on secretagogue-evoked total protein output, it was relevant to understand the cellular mechanism of action of Mg^{2+}. In the exocrine pancreas Mg^{2+} acts by regulating Ca^{2+} mobilisation (Mooren *et al.*, 2001; Yago *et al.*, 2000). Like the pancreas it is possible that Mg^{2+} may act in a similar manner in the salivary glands. Thus, it was decided to measure $[Ca^{2+}]i$ during perturbation of $[Mg^{2+}]_o$.

Figure 3.5 shows original traces of $[Ca^{2+}]_i$ in submandibular Fura 2-loaded acinar cell suspension in 0 mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_o$ in basal condition and following stimulation with 10^{-5} M ACh. The results show that supramaximal doses of the muscarinic agonist can elicit a marked transient increase in $[Ca^{2+}]_i$ within 5 - 10 seconds of addition of the secretagogue (peak response). This is followed by either a gradual decrease or elevation in $[Ca^{2+}]_i$ (plateau phase) depending on the $[Mg^{2+}]_o$. The basal $[Ca^{2+}]_i$, the initial peak phase (5 – 10 sec) and plateau phase (150 – 200 sec) after ACh addition following $[Mg^{2+}]_o$ perturbation were measured and analysed and the data are plotted as a family of histograms in Figure 3.6. The results show that both zero and elevated $[Mg^{2+}]_o$ can markedly attenuate $[Ca^{2+}]_i$ mobilised by ACh.

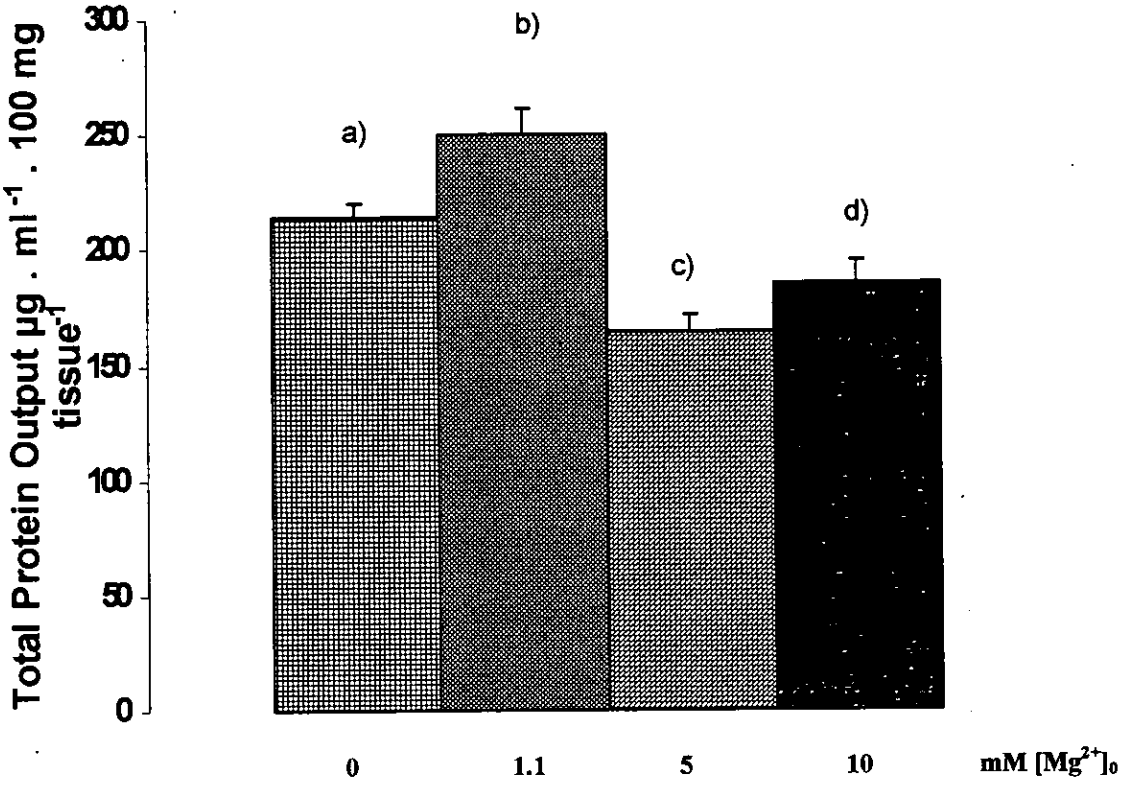


Figure 3.1 – Histograms showing basal total protein output from isolated submandibular segments in (a) zero, (b) 1.1 mM , (c) 5 mM and (d) 10 mM $[Mg^{2+}]_o$. Each point is mean ± SEM (n= 30-40) taken from 20 glands and 10 rats). Note that both low and high $[Mg^{2+}]_o$ significantly ($p< 0.05$) attenuated the basal protein ouput compared to normal (1.1 mM) $[Mg^{2+}]_o$.

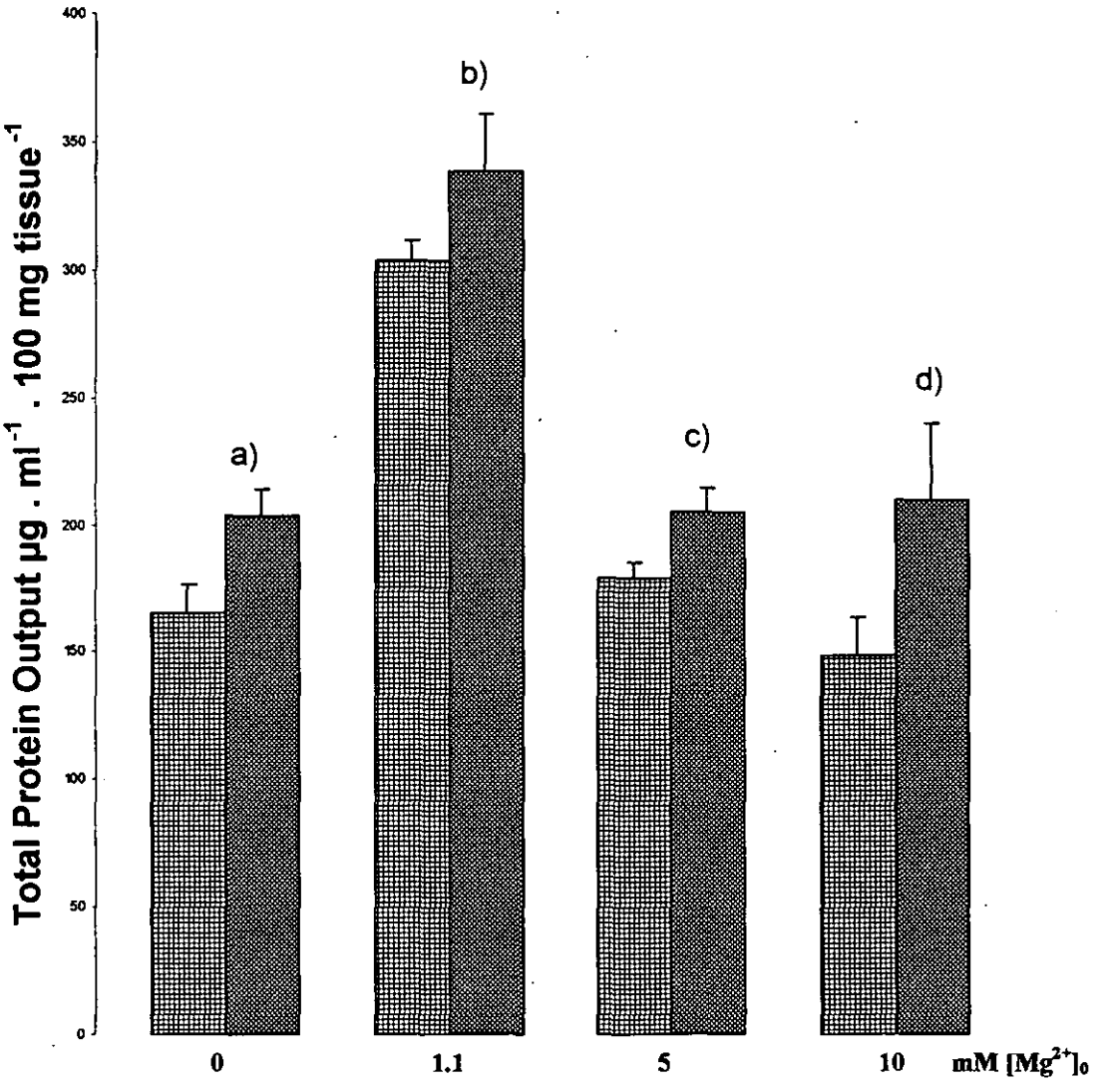


Figure 3.2- Histograms showing total protein output from submandibular gland segments in control conditions (light columns) and during stimulation with 10^{-5} M ACh (dark columns) following perturbation of $[Mg^{2+}]_0$ (a) 0 mM, (b) 1.1 mM, (c) 5mM and (d) 10 mM. Each point is mean ± SEM (n=8-12 taken from 8 glands and four rats). Note that both low and high $[Mg^{2+}]_0$ significantly ($p< 0.05$) attenuated the secretory effect of ACh compared to normal (1.1 mM) $[Mg^{2+}]_0$.

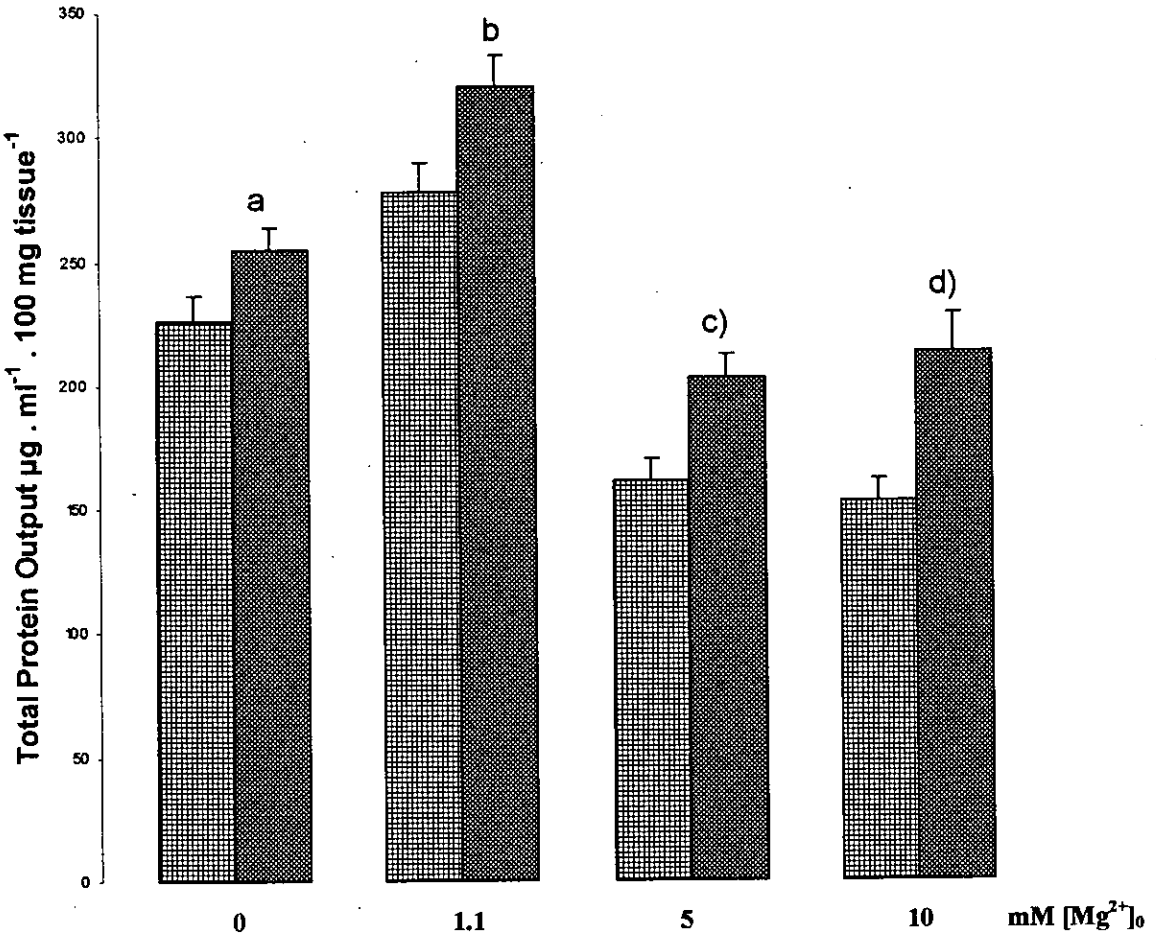


Figure 3.3- Histograms showing total protein output from submandibular gland segments in control conditions (light columns) and during stimulation with 10^{-5} M PHE (dark columns) following perturbation of $[Mg^{2+}]_0$ (a) 0 mM, (b) 1.1 mM, (c) 5mM and (d) 10 mM. Each point is mean ± SEM (n=8-12 taken from 8 glands and four rats). Note that both low and high $[Mg^{2+}]_0$ significantly ($p< 0.05$) attenuated the secretory effect of PHE compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$.

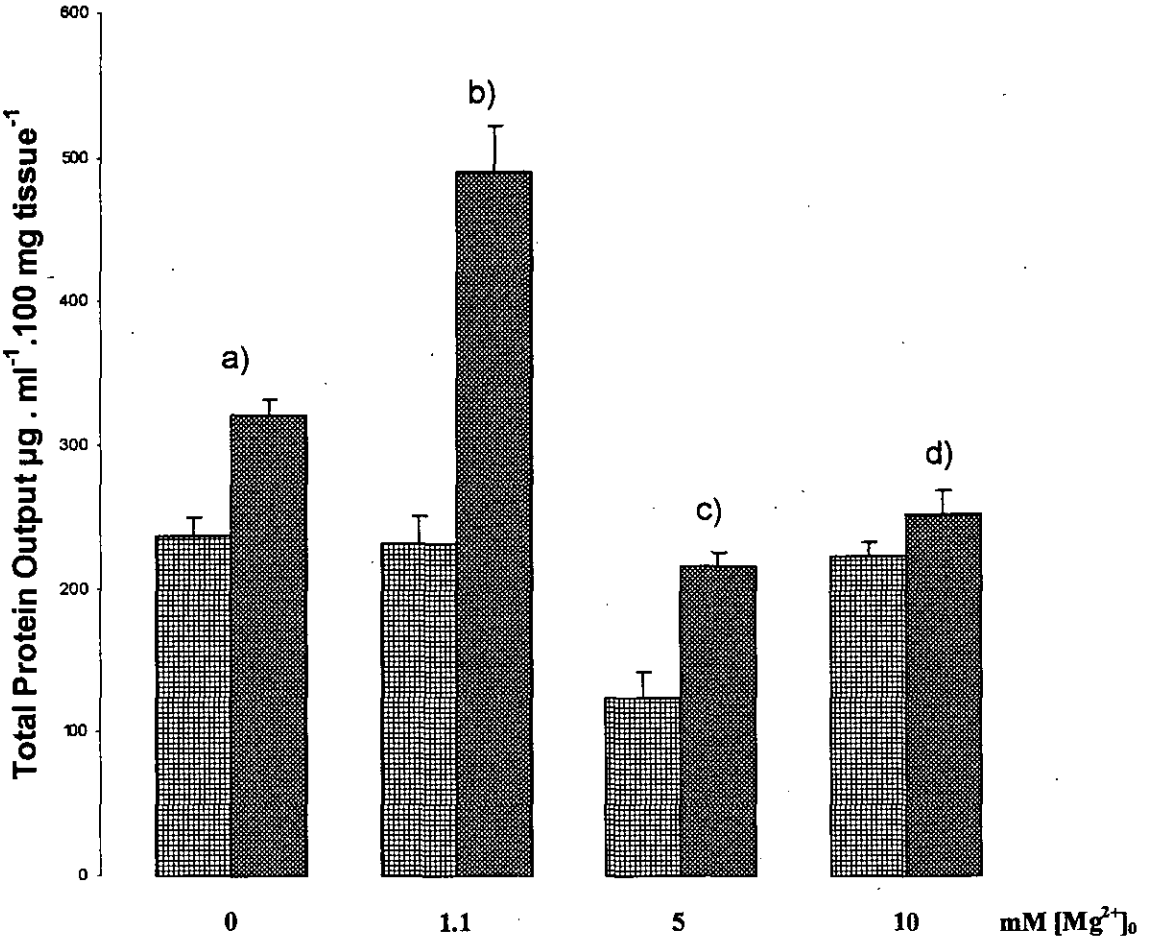


Figure 3.4- Histograms showing total protein output from submandibular gland segments in control conditions (light columns) and during stimulation with 10^{-5} M NA (dark columns) following perturbation of $[Mg^{2+}]_0$ (a) 0 mM, (b) 1.1 mM, (c) 5mM and (d) 10 mM. Each point is mean ± SEM (n=8-12 taken from 8 glands and four rats). Note that both low and high $[Mg^{2+}]_0$ significantly ($p< 0.05$) attenuated the secretory effect of NA compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$.

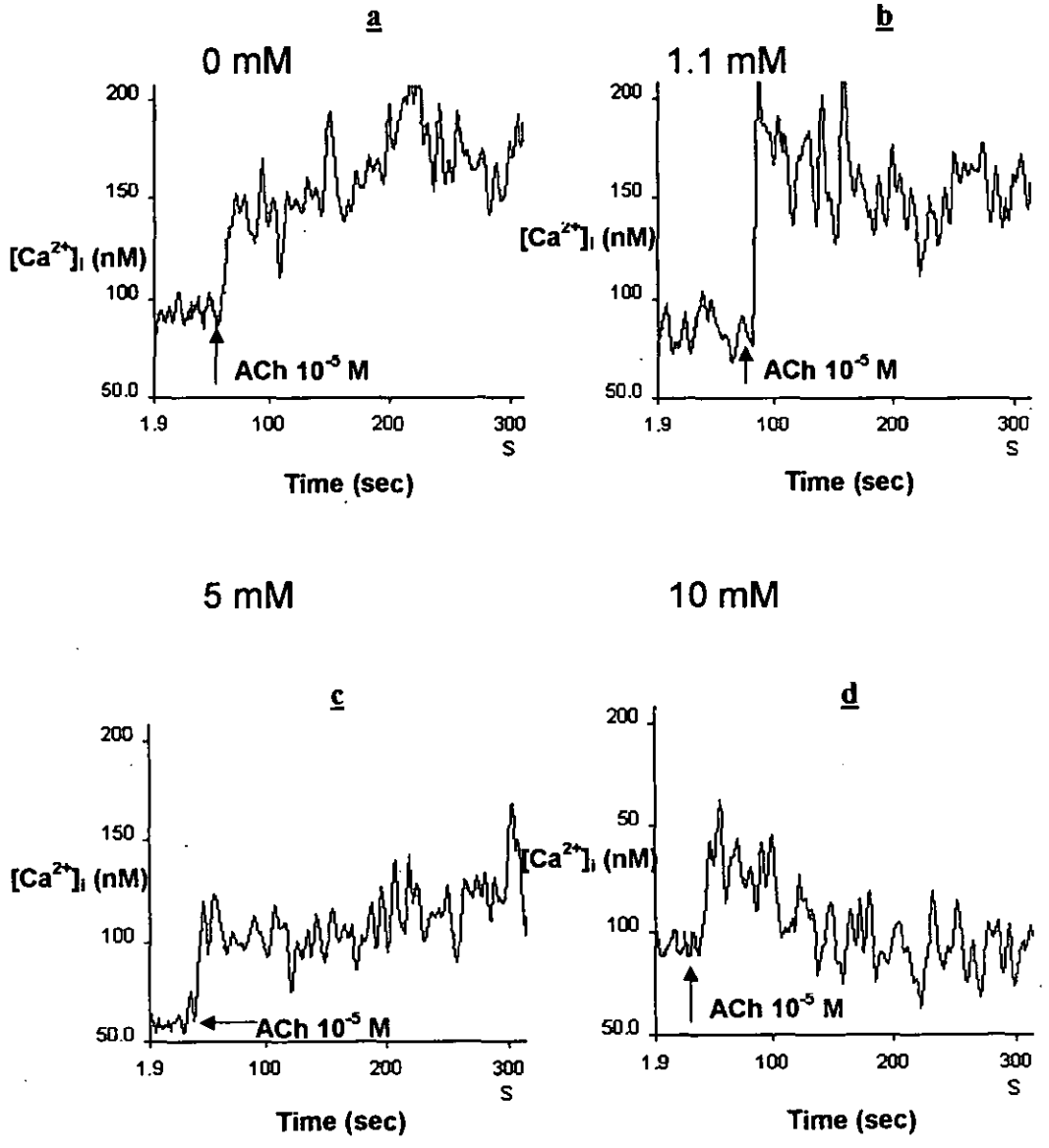


Figure 3.5- Time course of $[Ca^{2+}]_i$ in nM concentration in Fura-2 loaded submandibular cell suspension in a) 0 mM, b) 1.1 mM, c) 5 mM and d) 10 mM $[Mg^{2+}]_0$ following stimulation with 10^{-5} M ACh. Traces are typical of 6 – 20 such experiments taken from 10 animals. These results have been used to calculate the data shown in Figure 3.6. Vertical calibration is $[Ca^{2+}]_i$, horizontal calibration is time (seconds). The arrows indicate the time of ACh addition to the cell cuvette. Peak $[Ca^{2+}]_i$ values were chosen from raw data collection points as the highest value obtained. Plateau values were calculated with software from raw data as mean values between 100 sec and 200 sec after peak value.

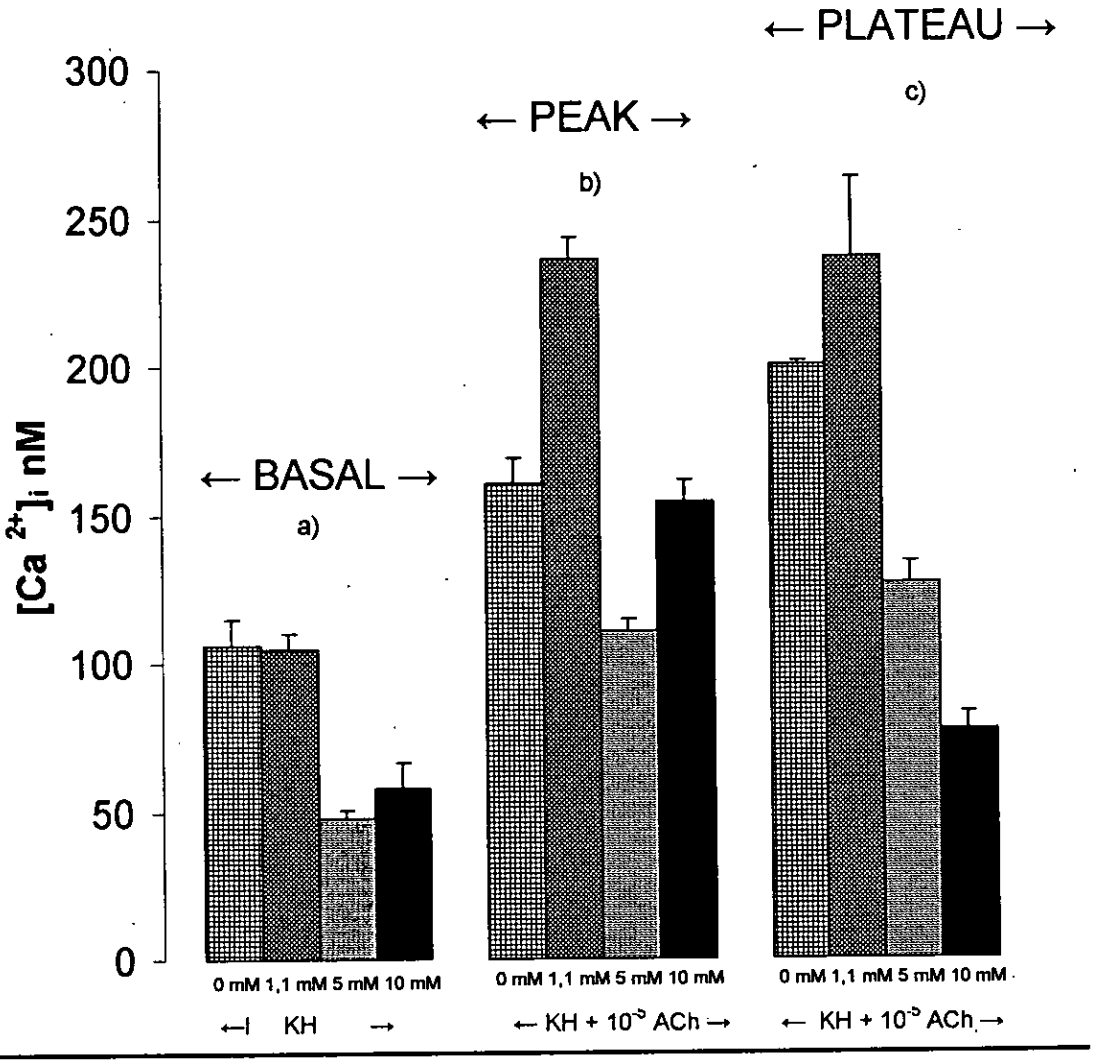


Figure 3.6- Histograms showing the mean (± SEM) (a) basal, ACh-evoked (b) peak and (c) plateau phases of the Ca^{2+} transient during perturbation of $[Mg^{2+}]_0$. The control responses are shown for comparison. Each point mean ± SEM (n between 6 and 20 taken from 10 animals). Peak $[Ca^{2+}]_i$ values were chosen from raw data collection points as the highest value obtained. Plateau values were calculated with software from raw data as mean values between 100 sec and 200 sec after peak value. Note that high $[Mg^{2+}]_0$ significantly ($p< 0.05$) attenuated the a) basal $[Ca^{2+}]_i$ compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$. In both b) the peak and c) plateau phase of the $[Ca^{2+}]_i$ mobilisation both low and high $[Mg^{2+}]_0$ significantly ($p< 0.05$) attenuated the $[Ca^{2+}]_i$ mobilisation compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$.

3.4 Discussion

The present study has demonstrated that the abundant divalent cation Mg^{2+} plays an important physiological role in the control of both basal and secretagogue-evoked total protein output from isolated submandibular gland segments. Either ACh, NA or PHE elicited marked increases in protein output in normal $[Mg^{2+}]_o$. The secretory effect of PHE was more pronounced compared to the responses obtained with either ACh or NA. However, perturbation of extracellular $[Mg^{2+}]_o$ employing either low or high concentrations of the divalent cation resulted in significant decreases ($P<0.05$) in both basal and secretagogue-evoked protein output. These results indicate that both hypo and hypermagnesemia are associated with decreased basal and secretagogue-evoked protein secretion. These results are in agreement with the finding of previous studies in which a modification in $[Mg^{2+}]_o$ also resulted in decreased secretory responses in both the parotid and the exocrine pancreas (Francis *et al*, 1990; Yago *et al.*, 1999; Singh *et al*, 1995; Yago *et al*, 2000)

The question, which now arises, is: how does Mg^{2+} act to reduce the secretory effect of either ACh, PHE or NA. It is now well known that Ca^{2+} plays a major role in the stimulus-secretion coupling process. Thus, it was relevant to measure $[Ca^{2+}]_i$ both at rest and during secretagogue stimulation The results have also shown that the muscarinic agonist ACh, elicited marked increases in $[Ca^{2+}]_i$ (both the initial peak and plateau phases) in submandibular acinar cells in normal $[Mg^{2+}]_o$. However, in low and elevated $[Mg^{2+}]_o$, the ACh-induced cellular calcium mobilisation, both peak and plateau phases were significantly ($P < 0.05$) reduced compared to control values. It is noteworthy that in a previous study, it was shown that a modification of $[Mg^{2+}]_o$ had no significant effect on basal $[Ca^{2+}]_i$ in parotid acinar cells (Yago *et al.*, 2002). Surprisingly, in this study, zero $[Mg^{2+}]_o$ had no effects on basal $[Ca^{2+}]_i$ compared to 1.1 mM $[Mg^{2+}]_o$. In contrast, both 5 mM and 10 mM attenuated basal $[Ca^{2+}]$ significantly ($P<0.01$) compared to 1.1 and zero $[Mg^{2+}]_o$. Taken together, these results suggest that Mg^{2+} plays a major role in the control of Ca^{2+} mobilisation both the release from intracellular stores and its entry into the cell since both the peak and plateau phases of the calcium transients correspond to release from intracellular stores and calcium influx from the extracellular medium, respectively (Metz *et al.*, 1990a,b).

The mechanism of action of Mg^{2+} in inhibiting secretagogue-evoked secretory responses in the submandibular gland is still unclear. However, it is possible that Mg^{2+} is required for the activation of important Ca^{2+} regulatory enzymes. In the absence of $[Mg^{2+}]_0$ and possibly a decrease in $[Mg^{2+}]_i$, the Ca^{2+} -dependent enzymes that modulate Ca^{2+} mobilisation are inactivated and thus, Ca^{2+} transport is attenuated. In contrast, in elevated $[Mg^{2+}]_0$, the divalent cation acts like an antagonist to inhibit Ca^{2+} mobilisation. It has been described previously that Mg^{2+} is a natural antagonist for cellular Ca^{2+} (Mooren *et al*, 2001) and moreover, previous studies have demonstrated that elevated $[Mg^{2+}]_0$ can attenuate the release of Ca^{2+} from intracellular stores and its influx into the cell (Mooren *et al.*, 2001; Yago *et al.*, 2000).

In conclusion, the results of this study have demonstrated that the abundant divalent cation Mg^{2+} can regulate basal and secretagogue-evoked submandibular salivary protein secretion possibly by controlling Ca^{2+} mobilisation.

CHAPTER 4

NERVE-MEDIATED AND SECRETAGOGUE-EVOKED AMYLASE SECRETION AND CALCIUM AND MAGNESIUM MOBILISATION IN THE RAT PAROTID GLAND

4.1 Introduction

Saliva, the secretory product of salivary glands, has been known to perform a fundamental role in oral cavity homeostasis (Herrera *et al.*, 1988). This fluid has many functional properties including food preparation for swallowing, digestion, lubrication and protection of the teeth and mucous membranes (Edgar, 1992). Pathophysiologic conditions which result in decreased salivary flow produce a "dry mouth condition" which is associated with a number of oral complications such as high caries index and increased incidence of fungal infections (Atkinson & Fox, 1993).

Salivary gland function is controlled mainly by the autonomic nervous system where sympathetic and parasympathetic nerves trigger a sequence of cellular signal transduction events resulting in intracellular cascades to generate such second messengers as calcium (Ca^{2+}) and adenosine 3,5 cyclic monophosphate (cyclic AMP) which in turn activate ion transport pathways, water and protein secretion (Putney, 1988; Baum, 1987; Petersen, 1992; Baum & Ambudkar, 1988; Petersen & Gallacher, 1988; Baum, 1993). However, the precise cellular mechanisms by which the second messengers regulate salivary gland function are still not fully understood (Ambudkar, 2000). Thus, knowledge in this field can help to develop therapeutic strategies for resolution of some of the diseased states usually associated with salivary gland dysfunction.

On the other hand, magnesium (Mg^{2+}) whose biological importance has become gradually recognised over the last century (Yago *et al.*, 2000), is the second most abundant intra-cellular divalent cation, exceeded only by potassium (Reinhardt, 1988). Mg^{2+} has been shown to be involved in numerous biological processes, including regulation of enzyme and hormone secretion and several membrane ion transport systems (*e.g.* ion channels, membrane ATPases, $Na^+/K^+/Cl^-$, Na^+/H^+, K^+/Cl^-) in epithelial secretory cells (Birch, 1993; Ambudkar, 2000). However, in the salivary glands very little is known about the possible regulatory effects of magnesium on both basal and secretagogue-evoked secretory responses. Thus, this study was designed specifically to investigate the effect of perturbation of extracellular Mg^{2+} ($[Mg^{2+}]_o$) on secretagogue-evoked amylase secretion in the parotid gland.

Moreover, $[Mg^{2+}]_o$ has been known to play a regulatory role in stimulus-secretion-coupling events in epithelial secretory acinar cells, especially where calcium mobilising

agents are concerned (Mooren & Singh, 1997; Mooren *et al*, 2001; Yago *et al.*, 2000). In fact, preliminary results of this work in rat submandibular glands (see chapter 3 of this study) have suggested that $[Mg^{2+}]_0$ may exert a controlling role in secretory mechanisms specially in those which are dependent upon $[Ca^{2+}]_i$ mobilisation (Mata *et al.*, 2001). Since Ca^{2+} is the main second messenger involved in salivary fluid secretion, it seemed logical (as a preliminary approach) to study the effects of perturbation of extracellular magnesium ($[Mg^{2+}]_0$) variations on ACh-evoked $[Ca^{2+}]_i$ mobilisation. In addition the interactions between Mg^{2+} and Ca^{2+}, this study also measured $[Mg^{2+}]_i$ and characterised the mechanisms of its transport during the stimulus-secretion coupling process.

4.2 Materials and Methods

As described in chapter 2.

4.3 Statistical analysis

As described in chapter 2.

4.4 Results

4.4.1 Amylase measurements

Figure. 4.1 shows the mean (+- SEM) basal amylase output from superfused parotid segments during perturbation of ($[Mg^{2+}]_0$). The results show that both low (0 mM) and elevated (5 & 10 mM) $[Mg^{2+}]_0$ significantly ($P<0.01$) inhibited basal amylase output compared to normal (1.1 mM) $[Mg^{2+}]_0$. Figure 4.2 A shows the original charts recording of amylase output in rat parotid gland segments when stimulated three consecutive times using electrical field stimulation (EFS) at 5, 10 and 20 Hz (50V, 1ms) to activate intrinsic secretomotor nerves in normal (1.1 mM) $[Mg^{2+}]_0$. The mean (+- SEM) amylase output above basal level for repeated (3 times) stimulations at 5 Hz, 10 Hz and 20 Hz is shown in Figure 4.2 B. The results show that amylase output from superfused parotid segments is frequency dependent during the first stimulation, with maximal effect occurring at 20 Hz although the value was not significantly different from the values obtained at 10 Hz. Subsequent stimulations at 5 Hz increased amylase output gradually compared to the first stimulation. In contrast, at 10 Hz and 20 Hz, subsequent

stimulations caused gradual decreases in amylase outputs compared to initial stimulation at each frequency.

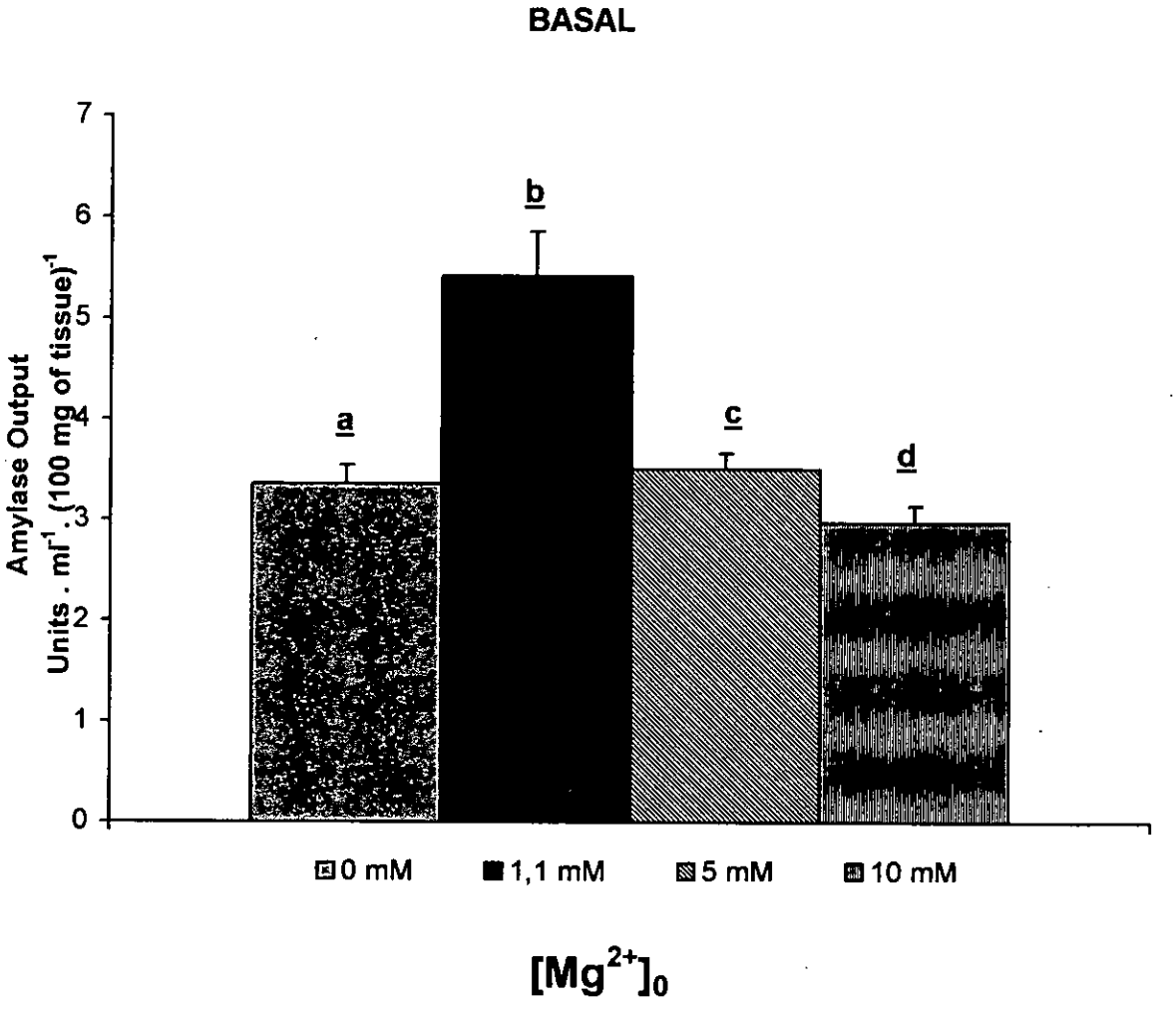


Figure 4.1. Histograms showing basal amylase secretion from isolated parotid gland segments in (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM $[Mg^{2+}]_o$. Each point is mean $\pm$ SEM, n=60-99. $P<0.01$ for a, c and d compared to b. Basal amylase output was obtained after allowing tissue to stabilize for 40 minutes.

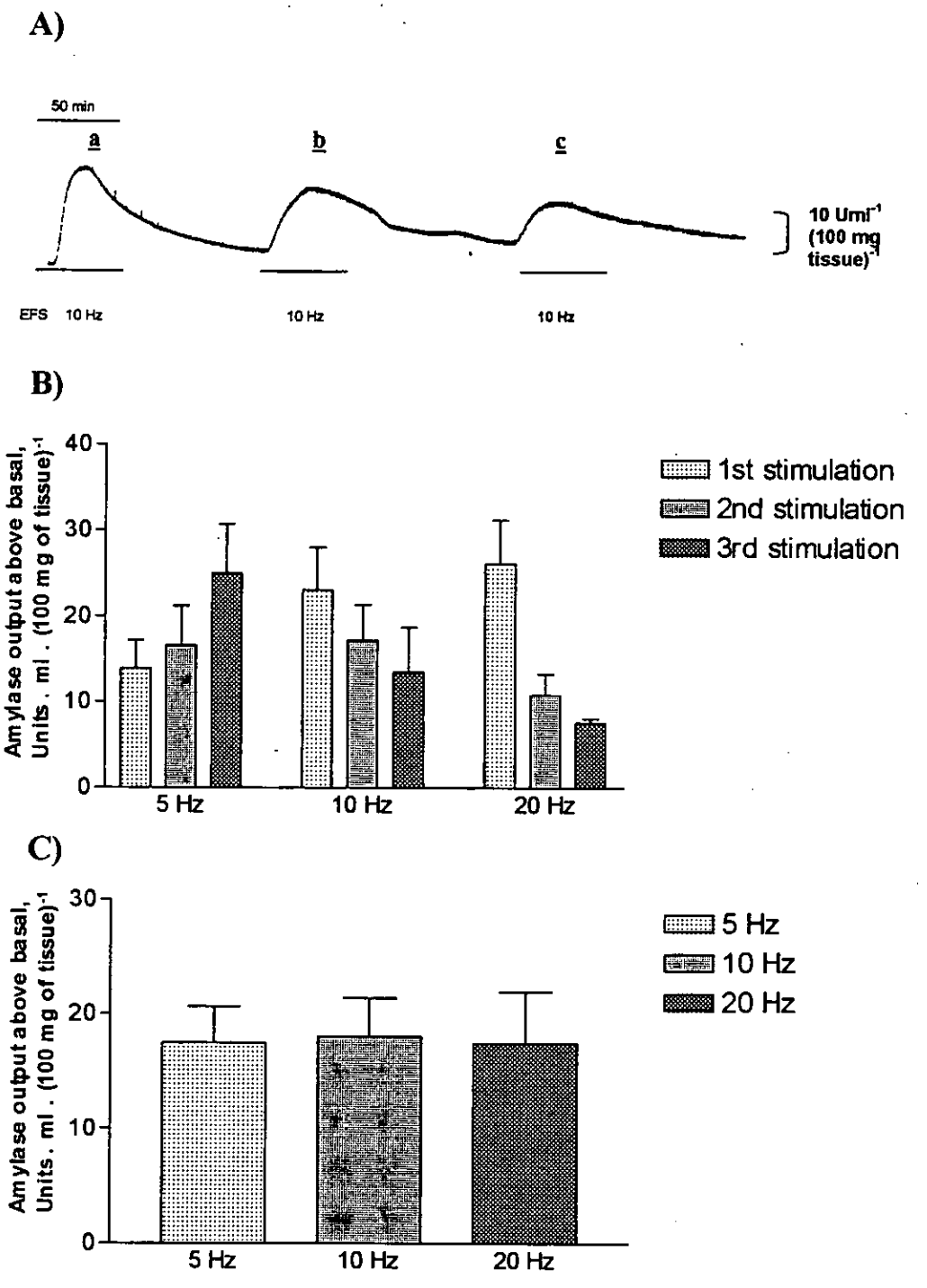


Figure 4.2 (A). Original chart recording showing the effect of repeated electrical field stimulation (EFS) at 10 Hz (amplitude of 50 V and 1 msec pulse width) on amylase output from superfused rat parotid segments in normal $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$. Traces are typical of 8 – 10 such experiments. **(B).** Histograms showing the mean (± SEM) amylase output above basal level for repeated EFS at 5 Hz, 10 Hz and 20 Hz, n= 8-10 and taken from 5 – 6 animals. **(C).** Histograms showing mean (± SEM) amylase output following combination of all the data for repeated EFS at each frequency.(n= 8-10), taken from Figure 4.2.B. Note that there is no significant difference in amylase output at the different frequencies when the repeated stimulation data are combined.

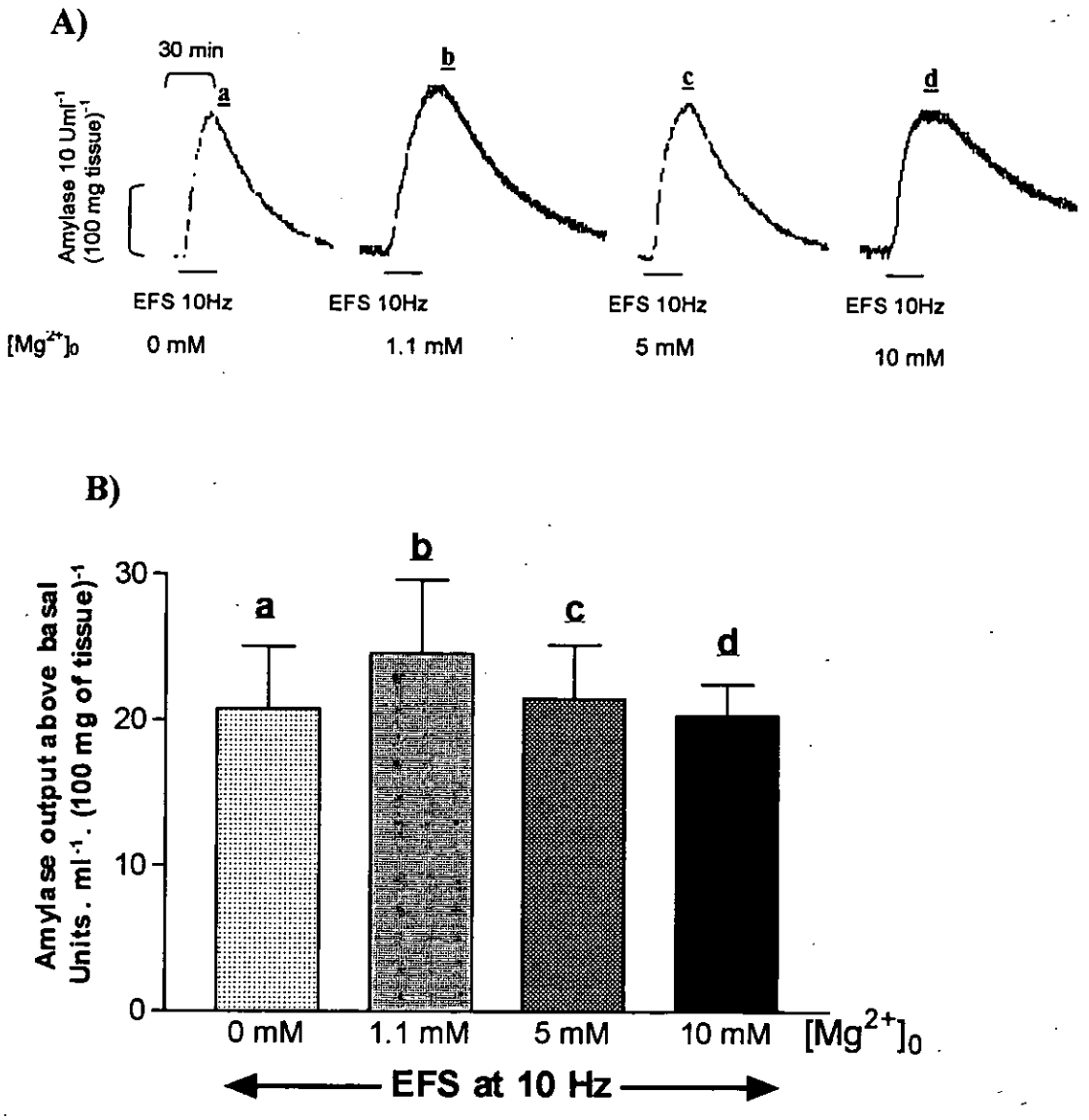


Figure 4.3 (A)- Original charts recordings of amylase output from isolated superfused parotid segments following single stimulations at 10 Hz EFS in 0 mM (a), 1.1 mM (b), 5 mM (c), and 10 mM (d) $[Mg^{2+}]_0$. Traces are representative of 8-15 experiments taken from 10 rats. **(B)** Histograms showing mean (+- SEM) amylase output above basal level for 10 Hz EFS during perturbation of $[Mg^{2+}]_0$, n = 8 –15. Note that there are no significant differences in amylase output following perturbation of $[Mg^{2+}]_0$ at 10 Hz EFS.

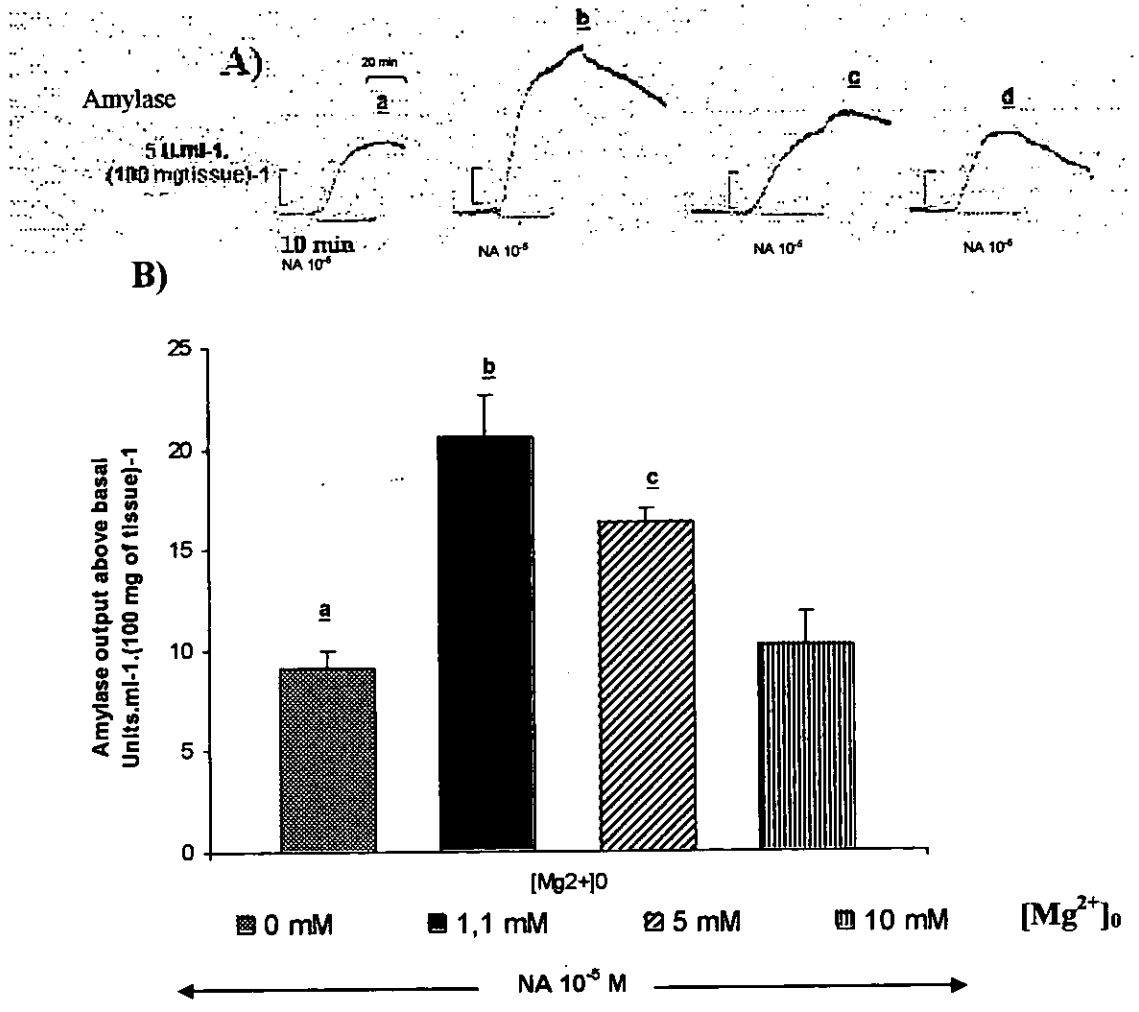


Figure 4.4 (A). Original chart recordings showing the effect of 10^{-5}M NA on amylase secretion from superfused isolated parotid gland segments in (a) 0 mM, (b) 1.1 mM, (c) 5 Mm and (d) 10 mM $[Mg^{2+}]_o$, Traces are typical of 8-12 such experiments for each concentration of $[Mg^{2+}]_0$ taken from 7 – 8 animals. Vertical and horizontal bars show the concentration of amylase output (Units . ml^{-1}. (100 mg of tissue)$^{-1}$) and time (in min) respectively. **(B)** Histograms showing the mean (± SEM) amylase output above basal level for 10^{-5}M NA stimulation in (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM $[Mg^{2+}]_o$, (n=8-12). $P<0.05$ for a,c and d compared to b. Note that both low and high (5 and 10 mM) $[Mg^{2+}]_0$ attenuated the NA-evoked amylase output.

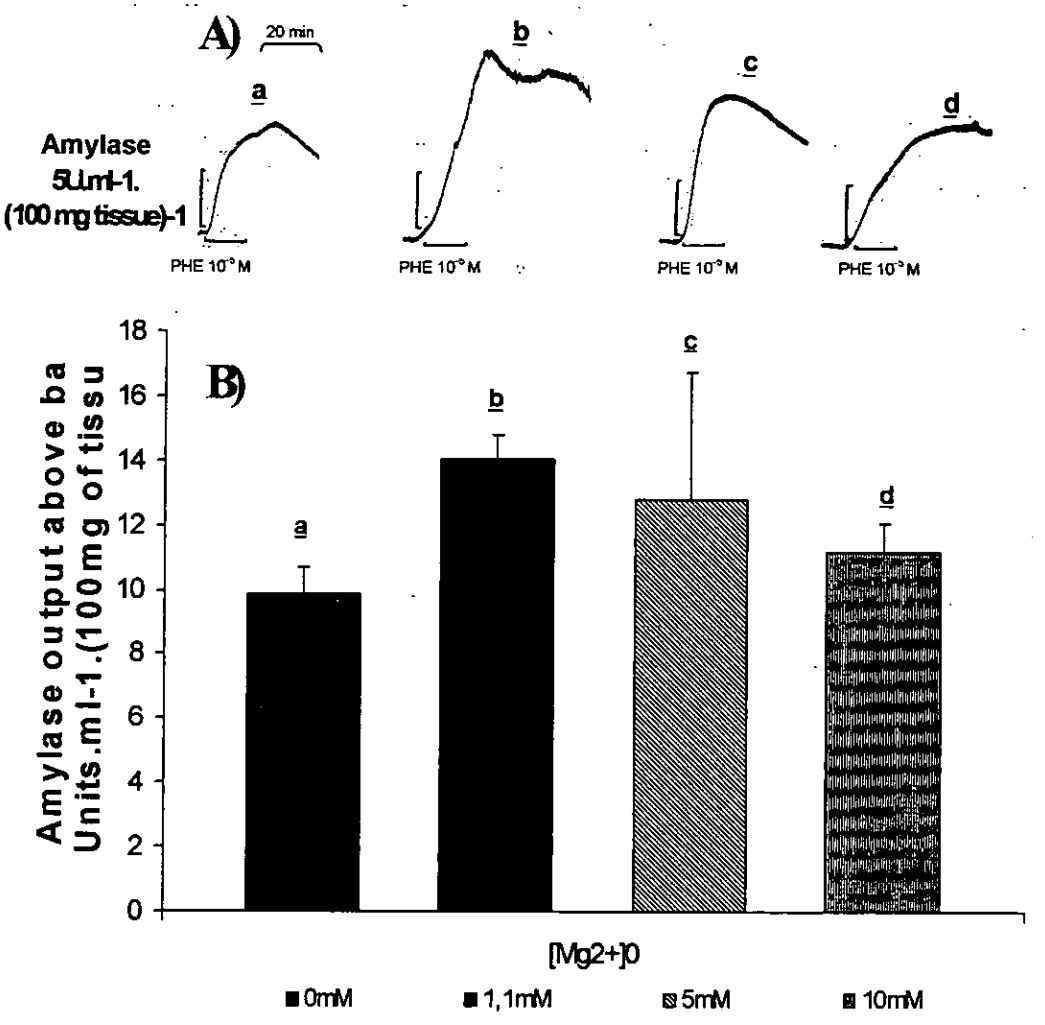


Figure 4.5 (A). Original chart recordings showing the effect of 10^{-5}M PHE on amylase secretion from superfused isolated rat parotid gland segments in (a) 0 mM, (b) 1.1 mM and (c) 10 mM $[Mg^{2+}]_o$. Traces are typical of 8-10 such experiments taken from 6 – 8 different rats. Vertical and horizontal bars show the concentration of amylase output (Units . ml^{-1}. (100 mg of tissue)$^{-1}$) and time (in min), respectively. **(B)** Histograms showing the mean (± SEM) amylase output above basal level following 10^{-5}M PHE stimulation in (a) 0 mM, (b) 1.1 mM and (c) 10 mM $[Mg^{2+}]_o$, (n=8-10). $P<0.05$ for a and d compared to b. However, there is no difference between b and c.

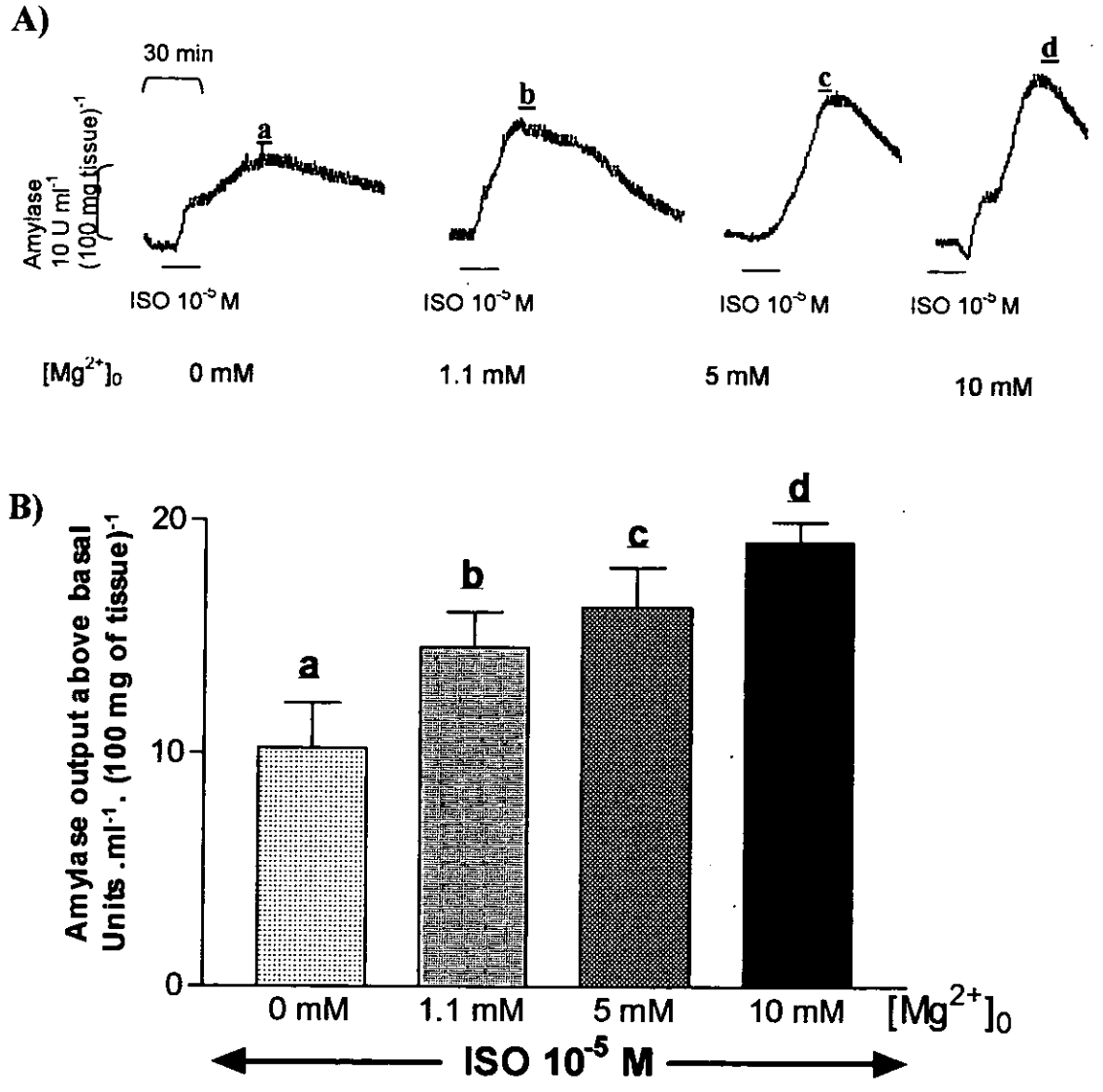


Figure 4.6- (A) Original charts recordings showing the effect of 10^{-5} M isoprenaline (ISO) on amylase output from isolated superfused rat parotid gland segments during perturbation of $[Mg^{2+}]_o$ (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM. Traces are typical of 8 – 10 experiments taken from the same number of animals. **(B)** Histograms displaying the mean (+- SEM) amylase output above basal level following ISO (10^{-5} M) stimulation in different $[Mg^{2+}]_o$ (n= 8 – 10). Note that increasing $[Mg^{2+}]_o$ resulted in increasing isoprenaline-evoked amylase output.

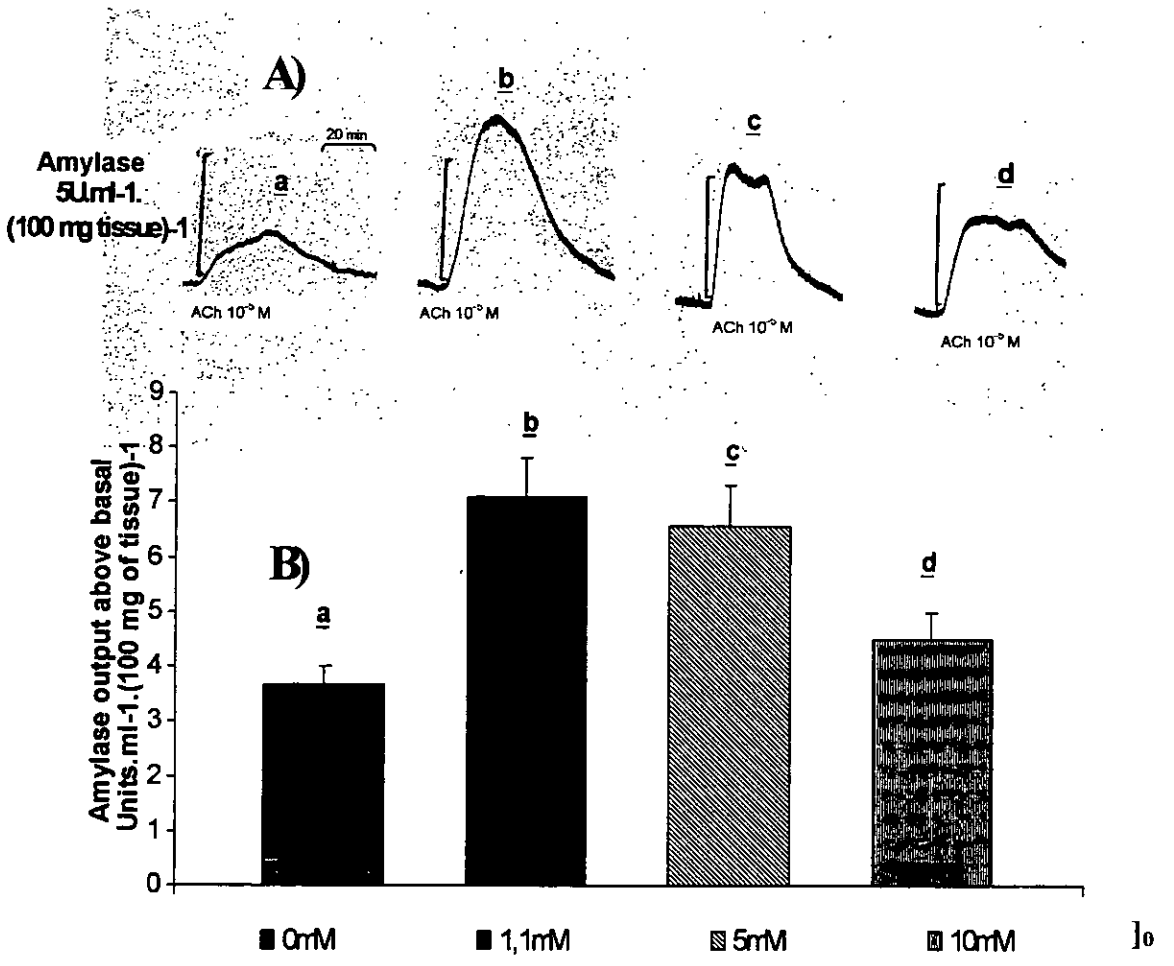


Figure 4.7 (A). Original chart recordings showing the effect of 10^{-5}M ACh on amylase secretion from isolated superfused rat parotid gland segments in (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM $[Mg^{2+}]_o$. Traces are typical of 8-15 such experiments taken from the same number of animals. Vertical and horizontal bars show the concentration of amylase output (Units . ml^{-1}. (100 mg of tissue)$^{-1}$) and time (in min) respectively. **(B)** Histograms showing the mean (± SEM) amylase output above basal level during stimulation with 10^{-5}M ACh in (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM $[Mg^{2+}]_o$, (n=8-15). $P<0.01$ for a and d compared to b. Note that b is not significantly different from c.

Figure 4.2 C shows the mean (+- SEM) amylase output for each frequency during repeated stimulations. These results indicate that when the data are combined for repeated stimulations, there was no significant difference in amylase output for each frequency. This is in contrast to the results obtained in initial stimulations for each frequency (see Figure 4.2 B).

Figure 4.3 shows the effect of 10 Hz, (50 V, 1 ms) EFS on amylase output during perturbation of $[Mg^{2+}]_0$. Original chart recordings of the responses are shown in Figure. 4.3 A and the mean (± SEM) amylase output above basal level is shown in Figure. 4.3 B. The results show that a perturbation of $[Mg^{2+}]_0$ had no significant effect on EFS-evoked amylase output at 10 Hz.

The effect of $[Mg^{2+}]_0$ on NA (10^{-5} M)-induced amylase secretion is shown in Figure. 4.4. Original chart recordings of the responses are shown in Figure. 4.4A and the mean (± SEM) amylase output above basal is shown in Figure. 4.4B. The results show that a perturbation of $[Mg^{2+}]_0$ has profound effect on NA-induced amylase output. Both zero and elevated $[Mg^{2+}]_0$ significantly ($P>0.01$) inhibited the NA-evoked amylase secretion compared to normal $[Mg^{2+}]_0$. Figure. 4.5 shows the effects of $[Mg^{2+}]_0$ on PHE (10^{-5}M)-induced amylase secretion. Original chart recordings of the responses and the mean (± SEM) amylase output above basal levels are shown in Figure. 4.5 A and B, respectively. Like NA, both low and elevated $[Mg^{2+}]_0$ significantly ($P<0.05$) inhibited PHE-evoked amylase output compared to normal $[Mg^{2+}]_0$. In contrast, 5 mM $[Mg^{2+}]_o$ had no effect on the PHE-induced secretory response compared to the response obtained in the presence of 1.1 mM $[Mg^{2+}]_o$.

Figure. 4.6 shows the effect of isoprenaline 10^{-5}M (ISO) on amylase output during perturbation of $[Mg^{2+}]_0$. Original chart recordings of the responses are shown in Figure. 4.6A and the mean (± SEM) amylase output above basal level is shown in Figure. 4.6B. The results show that a perturbation of $[Mg^{2+}]_0$ resulted in gradual increase in the isoprenaline-evoked amylase output with the lowest value at 0 mM and the highest at 10 mM which were significantly different from one another. Moreover, the results also showed that in the absence of $[Mg^{2+}]_0$ isoprenaline evoked a reduced response compared to the responses obtained in the presence of $[Mg^{2+}]_0$.

Figure. 4.7 shows the effect of 10^{-5}M (ACh) on amylase output during perturbation of $[Mg^{2+}]_0$. Original chart recordings of the responses are shown in Figure. 4.7A and the mean (± SEM) amylase output above basal level is shown in Figure. 4.7B. The results show that both low (0 mM) and elevated (10 mM) $[Mg^{2+}]_0$ can significantly ($P<0.05$)

inhibit the ACh-evoked amylase output from superfused parotid segments compared to normal $[Mg^{2+}]_0$. The inhibition at zero $[Mg^{2+}]_0$ was more pronounced compared to elevated $[Mg^{2+}]_0$.

In this series of experiments, the results have shown that a modification of $[Mg^{2+}]_0$ from 1.1 mM to 5 mM elicited qualitatively similar effect on the secretory response to either ACh, NA or PHE, all at a supra-maximal concentration of 10^{-5}M. In contrast, either zero or 10 mM $[Mg^{2+}]_0$ significantly ($p<0.05$) inhibited the secretory effects of either ACh, NA or PHE at the same concentration. On the other hand, isoprenaline evoked the maximal secretory effect at 10 mM $[Mg^{2+}]_0$ and the least at 0 mM $[Mg^{2+}]_0$. These differences in the results may be due to the fact that ACh, PHE and NA utilise cellular Ca^{2+} to mediate amylase output whereas ISO utilises cyclicAMP to mediate amylase output. The effect of EFS on amylase output during perturbation of $[Mg^{2+}]_0$ were similar to results obtained with ACh. PHE or NA but so small that no significant differences were found between the different $[Mg^{2+}]_0$.

4.4.1.1 Effects of different doses of secretagogues on amylase secretion

Experiments described above show the effect of supra-maximal dose of each secretagogue on amylase output during perturbation of $[Mg^{2+}]_0$. In another series of experiments, it was decided to ascertain whether $[Mg^{2+}]_0$ could also modify the effect of a lower concentration of the secretagogues.

Figure. 4.8 shows the mean (± SEM) amylase output above basal level for stimulation of superfused rat parotid segments with ACh at 10^{-8}, 10^{-7} and 10^{-6} M during perturbation of $[Mg^{2+}]_0$. The response to 10^{-5} M ACh is also shown for comparison. The results show that the effects of perturbation of $[Mg^{2+}]_0$ obtained with a supra-maximal dose of the secretagogue repeat themselves with even lower concentrations of ACh. Similar results are obtained when rat parotid gland segments were stimulated with a lower dose (10^{-6} M) of either NA or PHE following perturbation of $[Mg^{2+}]_0$ (Figure 4.9). Again, the results show that both low (0 mM) and elevated (5 mM) $[Mg^{2+}]_0$ can significantly ($P<0.05$) inhibit the secretory effects of the two salivary adrenergic secretagogues.

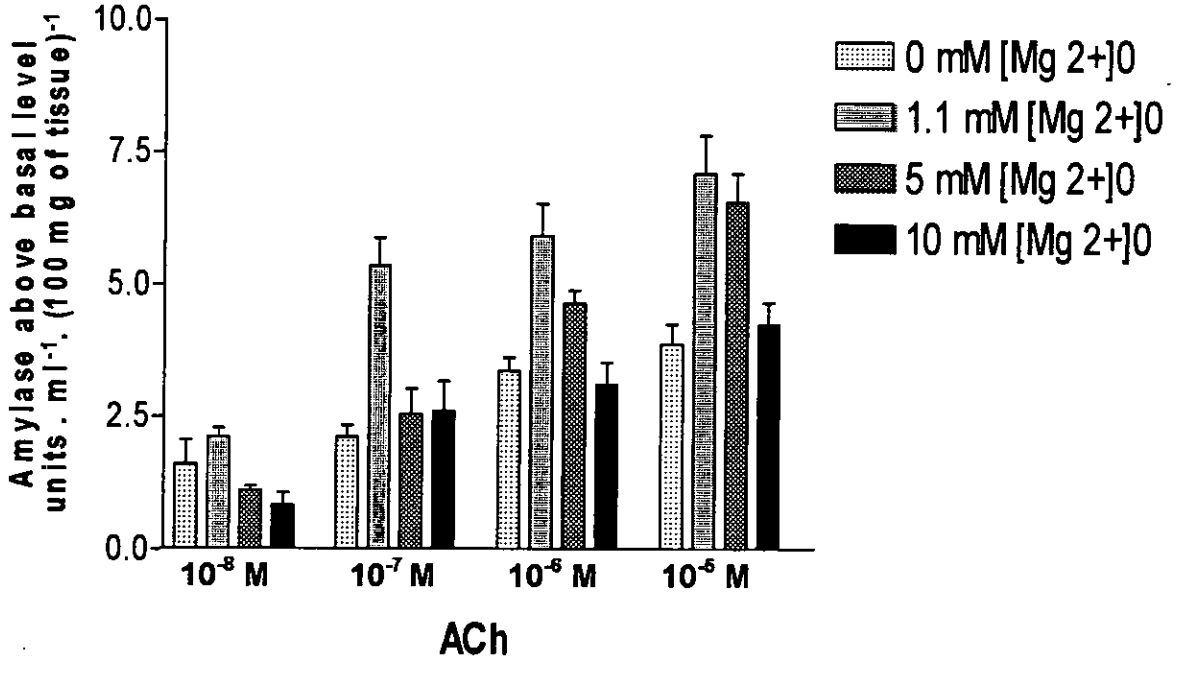


Figure 4.8- Histograms showing mean (+- SEM) amylase output above basal level following stimulation of isolated superfused rat parotid gland segments with different concentrations (10^{-8} – 10^{-5}) of ACh during perturbation of $[Mg^{2+}]_0$. n= 6 –15 taken from 6 rats.

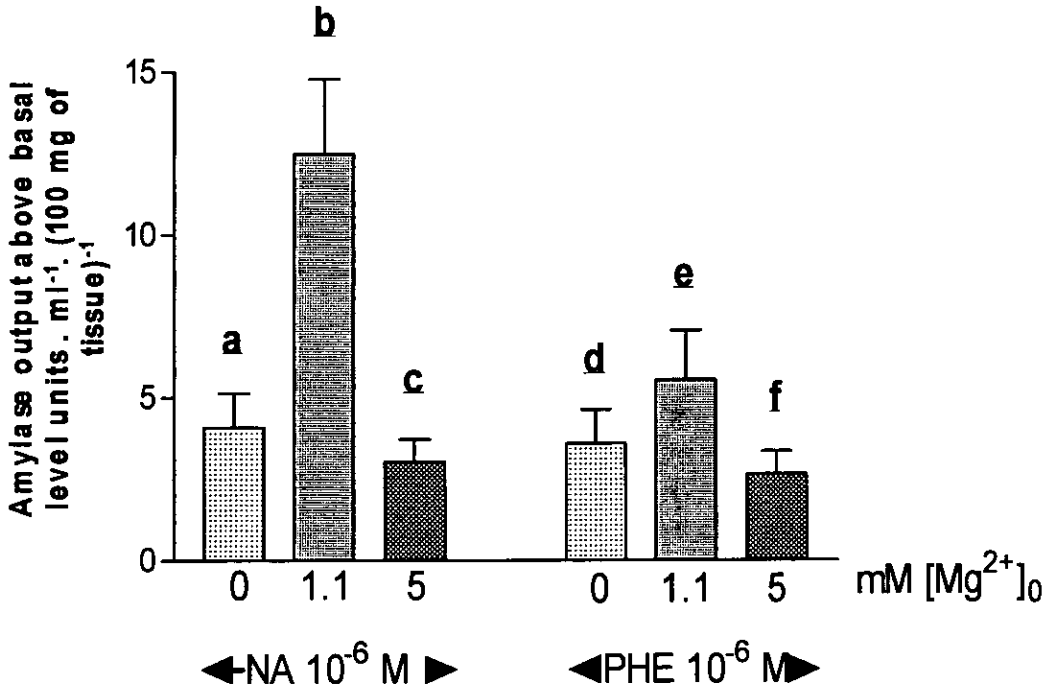
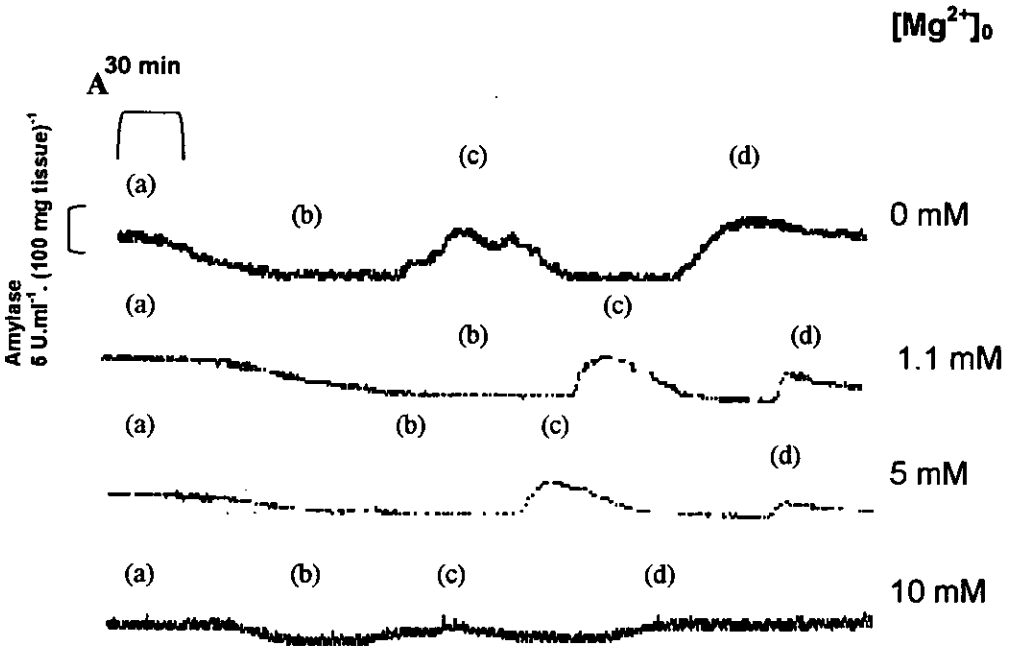


Figure 4.9- Histograms showing mean (+- SEM) amylase output from isolated superfused rat parotid gland segments following stimulation with either 10^{-6} M NA or 10^{-6} M PHE during perturbation (0, 1.1 and 5 mM) of $[Mg^{2+}]_0$. n= 6-8 different experiments taken from the same number of animals. Note that both zero and elevated $[Mg^{2+}]_0$ attenuated the secretagogue-evoked amylase output compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$.

4.4.1.2 Effect of perturbation of $[Mg^{2+}]_0$ on basal and ACh-evoked amylase release in normal and zero $[Ca^{2+}]_0$

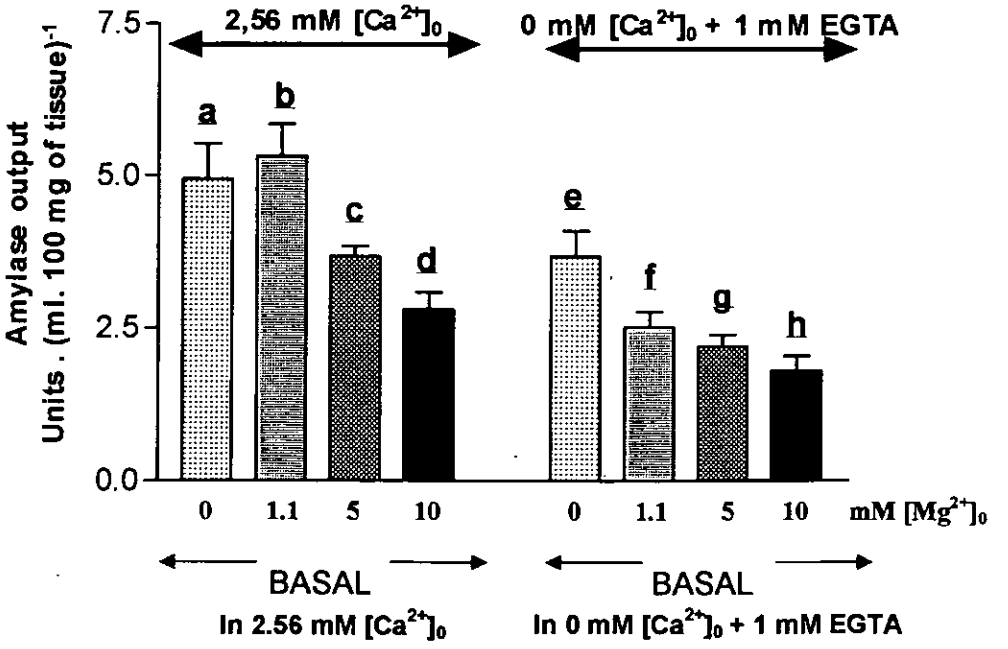
At this point in the study it seemed that the effects of $[Mg^{2+}]_0$ on secretagogue-evoked responses were more evident in the signalling mechanisms where intracellular calcium was involved as a second messenger. In fact, the experiments in which isoprenaline (known to act exclusively through cAMP) was used as secretagogue, it was shown that $[Mg^{2+}]_0$ failed to exert the inhibitory effects observed compared with other secretagogues (e. g. ACh, NA and PHE) which evoked their secretory effects via intracellular Ca^{2+}. Therefore, it seemed logical to investigate the effects of $[Mg^{2+}]_0$ on calcium-dependent amylase secretion at different levels of the intracellular calcium pathway, especially since there are two sources (extracellular and intracellular stores) of Ca^{2+} to mediate enzyme secretion (Takemura & Putney, 1989; Ambudkar *et al.*, 1992). Rat parotid segments were perfused with a media containing normal (2.56 mM) $[Ca^{2+}]_0$ (Figure 4.10 A (a)). When basal amylase secretion was stabilised, extracellular calcium was totally removed from perfusing medium and 1 mM EGTA was added (Figure 4.10 A (b)), parotid segments were then stimulated with 10^{-5} M ACh (Figure 4.10 A (c)). After peak recovery, parotid segments were again perfused with an extracellular medium containing (normal) 2.56 mM of $[Ca^{2+}]_i$, in the absence of the secretagogue, (Figure 4.10 A (d)). These experiments were repeated in the presence of different $[Mg^{2+}]_0$ namely: 0, 1.1, 5 and 10 mM. Figure 4.10 A (a – d) shows some original traces of the experimental protocol during perturbation of $[Mg^{2+}]_0$. Figure 4.10 B shows the mean (+- SEM) amylase secretion for basal in 2.56 mM $[Ca^{2+}]_i$, and for basal in 0 $[Ca^{2+}]_i$ + 1mM EGTA. Figure 4.10 C shows the ACh (10^{-5} M) –evoked mean (+- SEM) amylase above basal peak, in the absence of $[Ca^{2+}]_0$ and re-introducing normal (2.56 mM) $[Ca^{2+}]_0$ in 0, 1.1, 5 & 10 mM $[Mg^{2+}]_0$. The results show that: (a) elevated $[Mg^{2+}]_0$ can significantly ($P<0.05$) attenuate basal (in the presence and absence of $[Ca^{2+}]_0$), ACh-evoked amylase output in the absence of $[Ca^{2+}]_0$ and the secretory response elicited by re-introducing normal (2.56 mM) $[Ca^{2+}]_0$. (b) The results show that in the absence of extracellular $[Ca^{2+}]_0$, both basal and ACh-evoked amylase secretion from superfused parotid gland segments were significantly ($P< 0.01$) decreased at all levels of $[Mg^{2+}]_0$ compared to the responses obtained in normal $[Ca^{2+}]_0$ suggesting that

extracellular Ca^{2+} is required to maintain a high basal and secretagogue-evoked amylase secretion.



(a)	2.56 mM $[Ca^{2+}]_0$
(b)	0 mM $[Ca^{2+}]_0$ + EGTA
(c)	10^{-5} M ACh + 0 mM $[Ca^{2+}]_0$ + EGTA
(d)	2.56 mM $[Ca^{2+}]_0$

B)



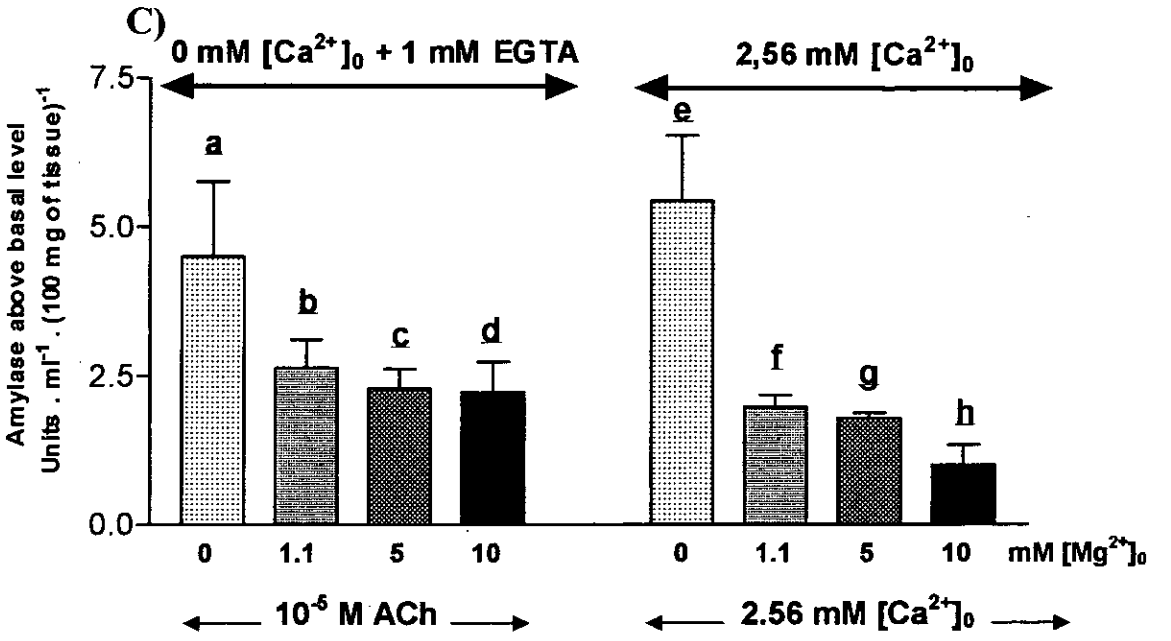


Figure 4.10 (A) Original charts recordings showing time course of amylase secretion from isolated superfused parotid gland segments in (a) normal (2.56 mM) $[Ca^{2+}]_0$, (b) a nominally free $[Ca^{2+}]_0$ containing 1 mM EGTA in the absence (c) presence of 10^{-5} M ACh in zero $[Ca^{2+}]_0$ + 1 mM EGTA and (d) following reperfusion with normal (2.56 mM) $[Ca^{2+}]_0$ alone during perturbation of $[Mg^{2+}]_0$. Traces are typical of 8 – 10 such experiments. (B) Histograms showing mean (+- SEM) basal amylase output in normal (2.56 mM) and in a nominally free $[Ca^{2+}]_0$ containing 1 mM EGTA during perturbation of $[Mg^{2+}]_0$. n= 8 – 10; (C) Histograms showing mean (+- SEM) amylase output above basal level from superfused parotid gland segments either during ACh (10^{-5} M) application in a nominally free $[Ca^{2+}]_0$ KH containing 1 mM EGTA and following reperfusion with normal (2.56 mM) $[Ca^{2+}]_0$ during perturbation of $[Mg^{2+}]_0$. (n= 8 – 10). In Figure 4.10 B, $P<0.01$ for c and d compared to a and b which are not significantly different from one another. Similarly, $P<0.01$ for f, g and h compared to e. In Figure 4.10 C, $P<0.01$ for b, c and d compared to a. Similarly, $P<0.01$ for f, g and h compared to e.

4.4.2 Secretagogue-evoked $[Ca^{2+}]_i$ in single rat parotid acinar cells

The results obtained with the experiments described above suggest that the effects of perturbation of $[Mg^{2+}]_o$ on the secretagogue-evoked amylase secretion seem to be exerted mainly in the processes that are dependent upon calcium mobilisation. Therefore, the next logical step in this study was to observe in detail the effects of perturbation of $[Mg^{2+}]_o$ on basal and secretagogue-evoked cellular calcium homeostasis. This study employs the technique of microspectrofluorimetry to measure $[Ca^{2+}]_i$ in Fura 2 -loaded single parotid acinar cells. Moreover, a concentration of 1.8 extracellular Ca^{2+} ($[Ca^{2+}]_o$) was used in single cells study as normal $[Ca^{2+}]_o$ compared to 2.56 mM in the amylase secretion measurements using parotid gland tissue, since several studies have employed this concentration to study the role of Ca^{2+} in the stimulus-secretion coupling process (Pariente *et al.*, 2003). Figure 4.11 shows basal $[Ca^{2+}]_i$ in 1.8 mM $[Ca^{2+}]_o$ and in a nominally free $[Ca^{2+}]_o$ Hepes solution containing 1 mM EGTA during perturbation of $[Mg^{2+}]_o$. All the results for $[Ca^{2+}]_i$ are expressed in ratio units as this is now an accepted procedure (Pariente *et al.*, 2003). The results show that elevated $[Mg^{2+}]_o$ can attenuate basal $[Ca^{2+}]_i$ in parotid acinar cells either in normal or in a nominally free $[Ca^{2+}]_o$ physiological salt solution containing 1 mM EGTA. In zero $[Mg^{2+}]_o$, $[Ca^{2+}]_i$ remains more or less the same in normal $[Ca^{2+}]_o$ compared to much reduced responses ($P<0.05$) in 5 and 10 mM $[Mg^{2+}]_o$. In contrast, in a nominally free $[Ca^{2+}]_o$, $[Mg^{2+}]_o$ in a range of 1.1 – 10 mM was markedly ($P<0.01$) decreased compared to the value obtained in zero $[Mg^{2+}]_o$. In addition, the results presented in Figure 4.11 show that in a nominally free $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ was significantly ($P<0.05$) reduced in the range of 1.1, 5 and 10 mM $[Mg^{2+}]_o$ compared to the data obtained in the presence of $[Ca^{2+}]_o$. Taken together, the results indicate that $[Ca^{2+}]_o$ is required to maintain a high basal level.

Figures 4.12 and 4.13 show original time courses traces of basal and ACh (10^{-5} M) evoked Ca^{2+} transient in normal (1.8 mM) (Figure 4.12) and in a nominally free (Figure 4.13) $[Ca^{2+}]_o$ during perturbation of $[Mg^{2+}]_o$, respectively. In normal $[Ca^{2+}]_o$, ACh evoked a marked transient increase in $[Ca^{2+}]_i$ comprising an initial rise (peak) followed by a gradual decrease which subsequently levels off to a plateau phase (plateau) above basal level. In the absence of $[Ca^{2+}]_o$, ACh evoked transient rise (peak) and subsequent decrease (plateau) reaching basal level. This plateau response was much smaller compared to the responses obtained in normal $[Ca^{2+}]_o$ (compare Figure 4.12 with Figure 4.13). In fact, the plateau phase was more or less basal value in the nominally free

$[Ca^{2+}]_o$ suggesting that normal $[Ca^{2+}]_o$ is required to maintain the plateau phase of the Ca^{2+} transient. In summary, both zero and elevated $[Mg^{2+}]_o$ attenuated these effects of ACh. These original traces were analysed and the mean (+- SEM) changes in peak and plateau $[Ca^{2+}]_i$ are depicted in subsequent Figures. Figure 4.14 shows the mean (+- SEM) peak and plateau increases in $[Ca^{2+}]_i$ following stimulation of Fura 2-loaded parotid acinar cells with 10^{-5} M ACh in different concentrations (0 mM, 1.1 mM, 5 mM and 10 mM) of $[Mg^{2+}]_o$. The cells were all perfused with a physiological salt solution containing 1.8 mM $[Ca^{2+}]_o$. The results show that both zero and elevated $[Mg^{2+}]_o$ can significantly ($P< 0.05$) attenuate the ACh- evoked increases in both the peak and the plateau of the Ca^{2+} transient. The effect of elevated (5 mM and 10 mM) was much more pronounced compared to zero $[Mg^{2+}]_o$. Figure 4.15 shows the mean (+- SEM) increases in $[Ca^{2+}]_i$ during ACh (10^{-5} M) stimulation during perfusion of single parotid acinar cells in a nominally free Ca^{2+} Hepes solution containing 1 mM EGTA and during perturbation of $[Mg^{2+}]_o$. The results show that only elevated (5 mM and 10 mM) $[Mg^{2+}]_o$ can significantly ($P<0.05$) attenuate the ACh –evoked increase in the initial peak phase of the Ca^{2+} transient. In the presence of zero $[Mg^{2+}]_o$ the initial peak phase of the Ca^{2+} transient evoked by ACh was slightly larger compared to the responses obtained in 1.1 mM $[Mg^{2+}]_o$. Moreover, the results also show that in the absence of $[Ca^{2+}]_o$, the ACh-evoked plateau phase of the Ca^{2+} transient was almost completely abolished suggesting that extracellular Ca^{2+} is required to maintain the plateau phase. Like the experiments on amylase secretion, these results indicate that elevated $[Mg^{2+}]_o$ is antagonising the mobilisation of both basal and secretagogue-evoked cellular Ca^{2+} mobilisation (Ca^{2+} release from intracellular stores and Ca^{2+} influx into the cell).

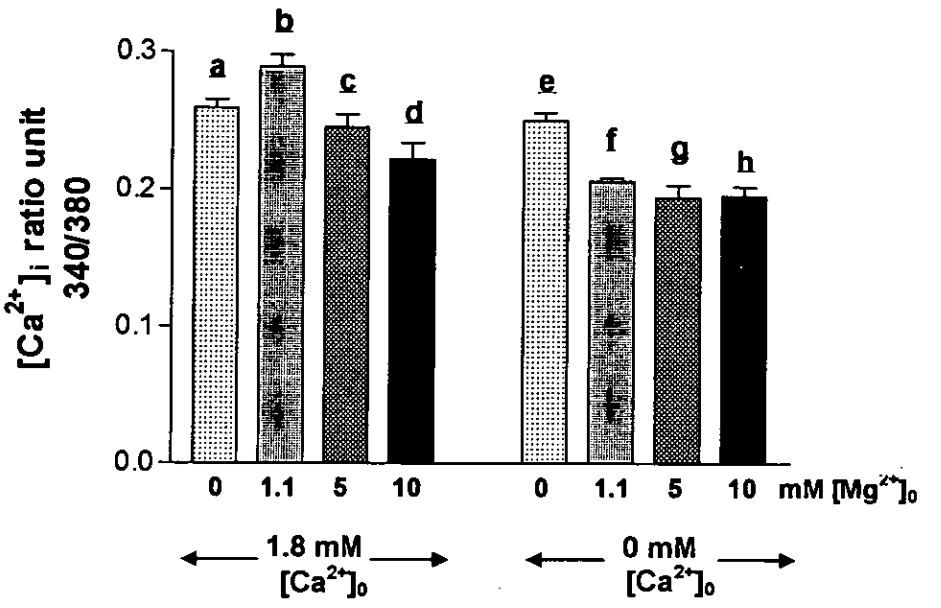


Figure 4.11- Mean (+- SEM) basal $[Ca^{2+}]_i$ in Fura 2-loaded parotid acinar cells in 1.8 mM and 0 mM $[Ca^{2+}]_o$ during perturbation of $[Mg^{2+}]_o$. Values are expressed as ratio units, n = 15 – 20 cells taken from 8 – 10 rats. $P<0.01$ for c and d compared to a and b. Similarly, $P<0.01$ for f, g and h compared to e.

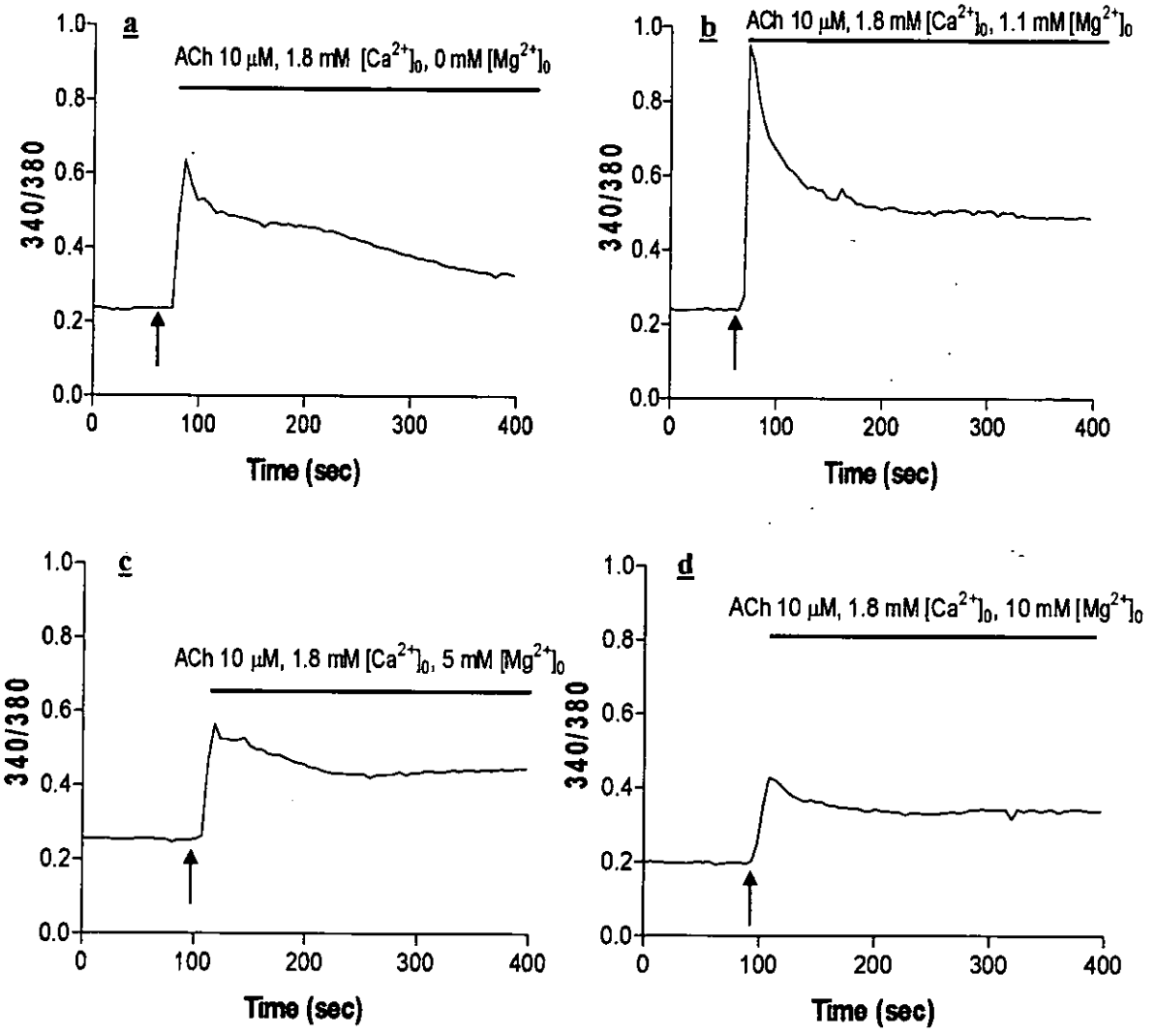


Figure 4.12- Original charts recordings showing the effect of perturbation of $[Mg^{2+}]_0$ (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM on ACh (10^{-5} M)-evoked changes in $[Ca^{2+}]_i$ in Fura 2-loaded single parotid acinar cells during perfusion with a physiological salt solution containing 1.8 mM $[Ca^{2+}]_0$. Traces are typical of 15 - 20 cells, taken from 6 – 8 different animals.

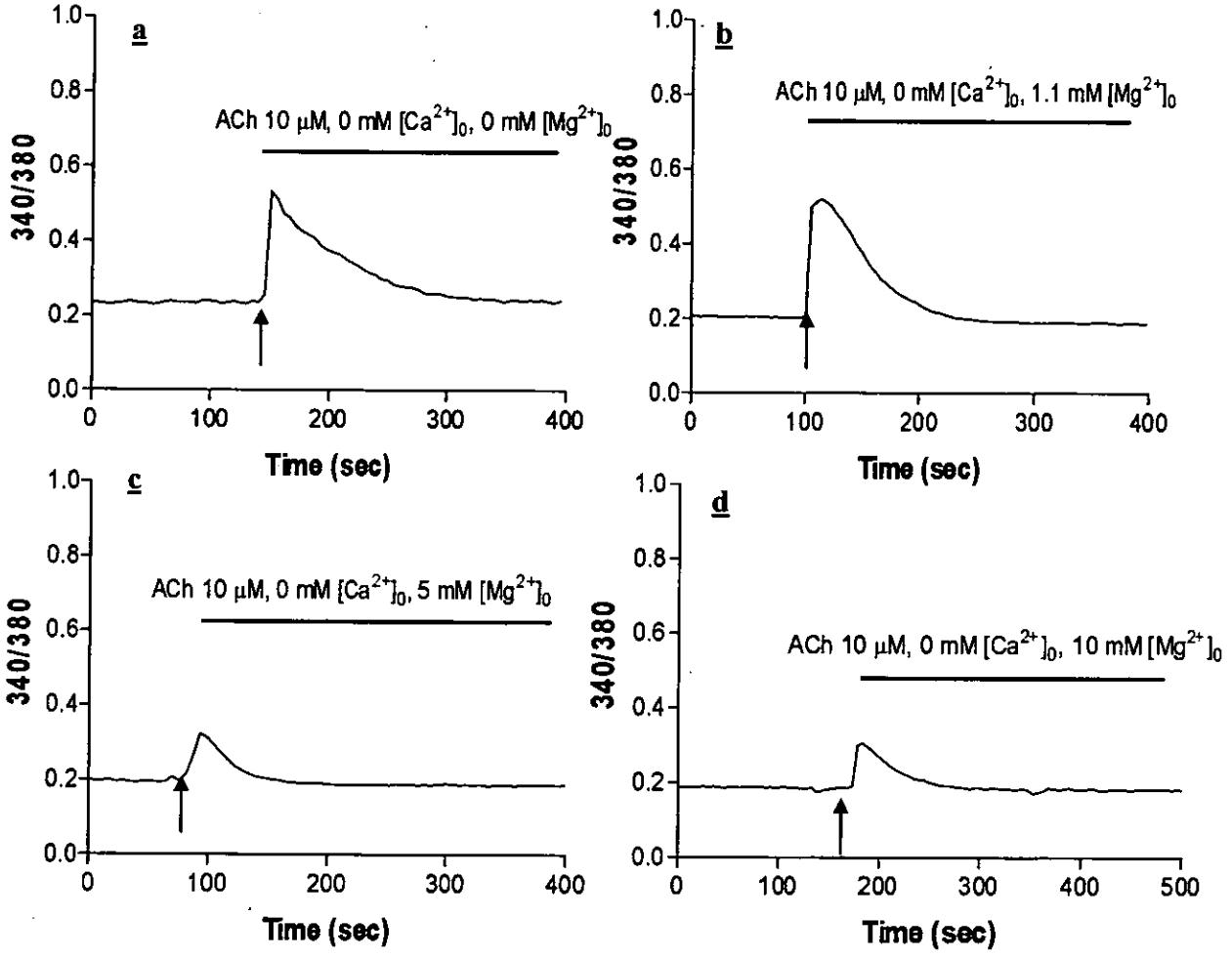


Figure 4.13- Original charts recordings showing the effect of perturbation of $[Mg^{2+}]_0$ (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM on ACh (10^{-5} M)-evoked changes in $[Ca^{2+}]_i$ in Fura 2-loaded single parotid acinar cells during perfusion with a physiological salt solution containing 0 mM $[Ca^{2+}]_0$ and 1 mM EGTA . Traces are typical of 15 - 20 cells taken from 6 – 8 different animals.

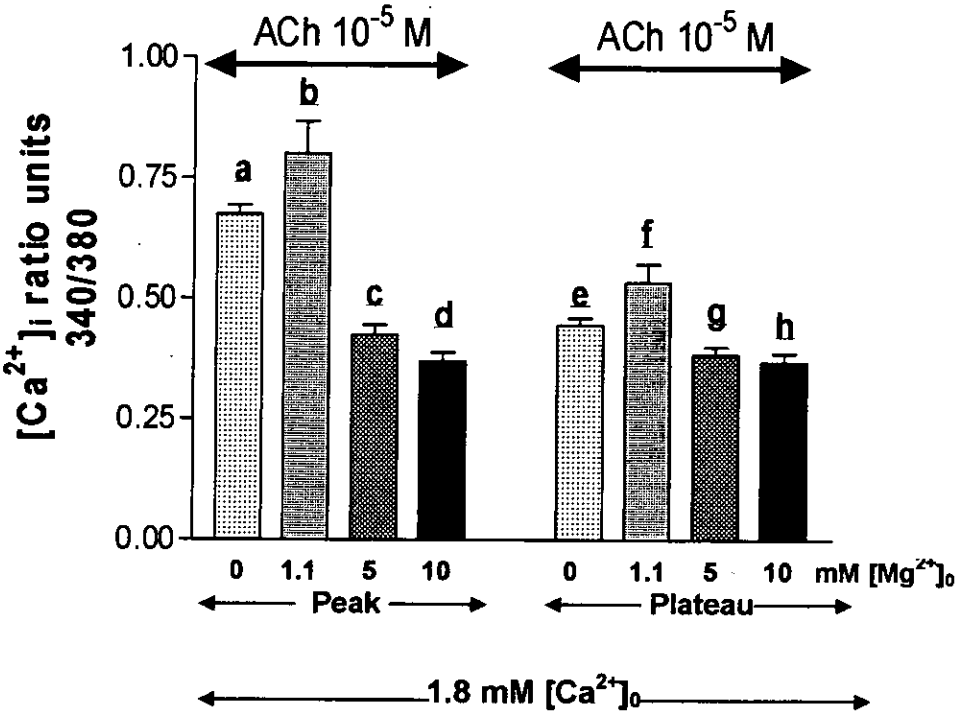


Figure 4.14- Mean (+-SEM) changes in the peak and plateau phases in the Ca^{2+} transient above basal level in Fura 2-loaded single parotid acinar cells evoked by 10^{-5} M ACh following perturbation of $[Mg^{2+}]_0$. The cells were perfused with a physiological salt solution containing 1.8 mM $[Ca^{2+}]_0$. n= 15 – 20 cells taken from 8 – 10 animals. Note that measurements were made 10 –15 seconds (peak) and 2 – 3 minutes (plateau) after ACh application. $P<0.01$ for a, c and d compared to b. Similarly, $P<0.01$ for e, g and h compared to f.

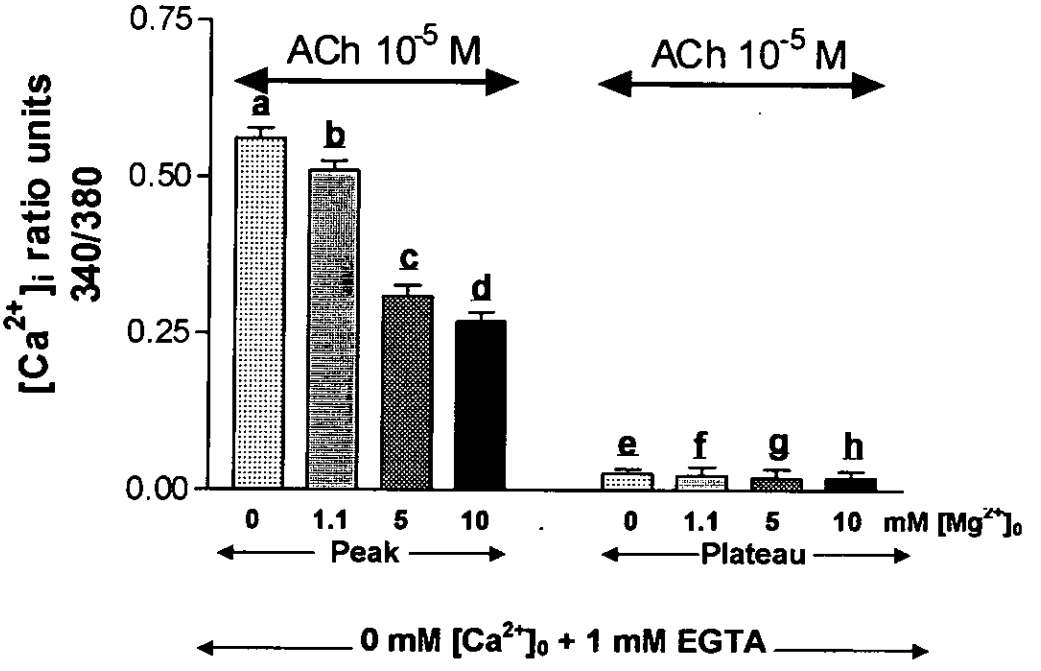


Figure 4.15- Mean (+-SEM) changes in the peak and plateau phases in the Ca^{2+} transient above basal level in Fura 2 loaded single parotid acinar cells evoked by 10^{-5} M ACh following perturbation of $[Mg^{2+}]_0$. The cells were perfused with a physiological salt solution containing 0 mM $[Ca^{2+}]_0$ and 1 mM EGTA. n= 15 – 20 cells taken from 8 – 10 animals. Note that measurements were made 10 –15 seconds (peak) and 2 – 3 minutes (plateau) after ACh application. The results clearly show that in the absence of $[Ca^{2+}]_0$, the ACh-evoked plateau phase of the Ca^{2+} transient was almost completely abolished. $P<0.05$ for b, c and d compared to a.

4.4.2.1. Effect of perturbation of $[Mg^{2+}]_0$ on capacitative calcium entry (CCE).

Stimulation of parotid acinar cells with ACh in the absence of $[Ca^{2+}]_0$ elicited a small, but rapid transient rise in $[Ca^{2+}]_i$ peak which rapidly returned to basal values. This type of signal is described as calcium exclusively exiting from intracellular stores. After peak recovery if cells are again perfused with a medium containing normal $[Ca^{2+}]_0$ a plateau shaped rebound in $[Ca^{2+}]_i$ is observed described as capacitative calcium entry (CCE), corresponding to calcium entering the cell from the extracellular side (Putney, 1988). CCE has been reported as the driving force for prolonged fluid secretion in salivary glands (Ambudkar, 2000). Therefore, an investigation was undertaken to determine the effects of a perturbation $[Mg^{2+}]_0$ (0, 1.1, 5 and 10 mM) on CCE.

Figure 4.16 shows original time course chart recordings of the protocol to measure CCE following perturbation of $[Mg^{2+}]_0$. Cells were superfused with a nominally free $[Ca^{2+}]_0$ Hepes solution containing 1 mM EGTA for 200 – 300 seconds. Thereafter, the cells were stimulated with 10^{-5} M ACh in a nominally free $[Ca^{2+}]_0$ medium containing EGTA. Following the peak and plateau phases, both ACh and the nominally free $[Ca^{2+}]_0$ medium containing 1 mM EGTA were replaced with normal Hepes solution containing 1.8 mM $[Ca^{2+}]_0$. The results for the ACh-evoked increases in the peak and the plateau phases of the Ca^{2+} transient in zero and during perturbation of $[Mg^{2+}]_0$ are shown in Figure 4.15. Figure 4.17 shows the time (see arrows) it takes for CCE to be activated following perfusion of Fura 2 loaded acinar cells with 1.8 mM $[Ca^{2+}]_0$ Hepes solution in different (0 mM, 1.1 mM, 5 mM and 10 mM) $[Mg^{2+}]_0$. The results show that in a physiological dose (e. g. 1.1 mM) of $[Mg^{2+}]_0$, CCE is extremely ($P<0,01$) rapid compared to delayed observations in zero and elevated $[Mg^{2+}]_0$. These effects were also associated with more or less similar time course in amylase secretion (see Figure 4.10 A). Figure 4.18 shows the maximal increases in $[Ca^{2+}]_i$ following the re-introduction of 1.8 mM Ca^{2+} Hepes to Fura 2-loaded acinar cells at 100 sec, 200 sec and 300 sec following activation of CCE in different $[Mg^{2+}]_0$. The results showed that in zero $[Mg^{2+}]_0$ there was a larger and sustained elevation in $[Ca^{2+}]_i$ after CCE activation. The effects of 1.1, 5 and 10 mM $[Mg^{2+}]_0$ on $[Ca^{2+}]_i$ were much smaller ($P<0.01$) compared to the responses obtained in zero $[Mg^{2+}]_0$. At least at these time points the results with elevated (5 mM and 10 mM) $[Mg^{2+}]_0$ were much less pronounced ($P<0.01$) compared to

normal (1.1 mM) $[Mg^{2+}]_0$. Taken together, these results indicate that $[Mg^{2+}]_0$ is regulating Ca^{2+} entry into parotid acinar cells and the level of Ca^{2+} influx is also dependent upon the concentration of $[Mg^{2+}]_0$. Low $[Mg^{2+}]_0$ seems to facilitate Ca^{2+} influx whereas elevated $[Mg^{2+}]_0$ has the opposite effect.

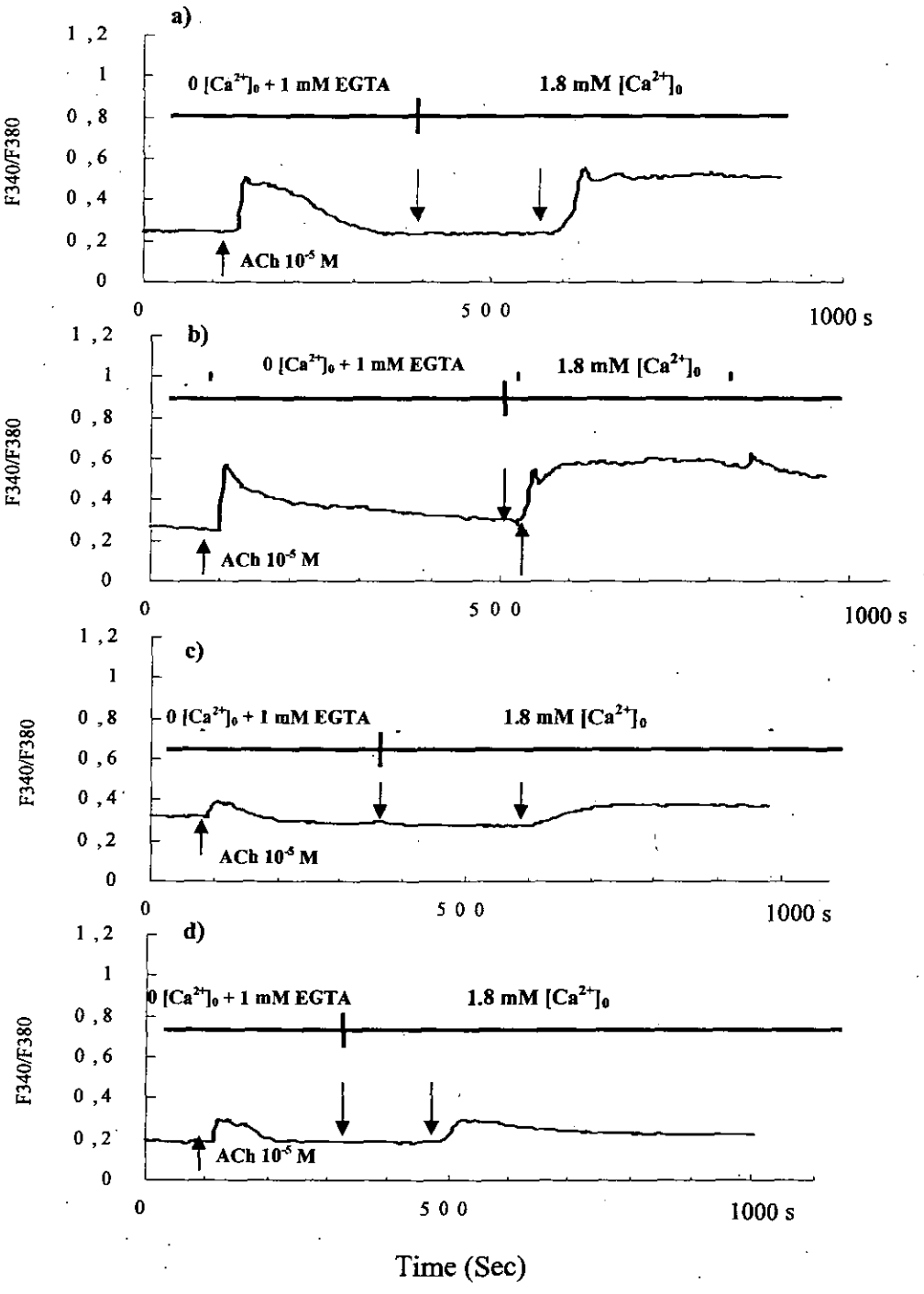


Figure 4.16- Time course of changes in $[Ca^{2+}]_i$ during perfusion of Fura 2-loaded parotid acinar cells with 0 $[Ca^{2+}]_0$ + 1 mM EGTA in absence and presence of 10^{-5} M ACh and following re-introduction of 1.8 mM $[Ca^{2+}]_0$ physiological salt solution in a) 0 mM , b) 1.1 mM , c) 5 mM and d) 10 mM $[Mg^{2+}]_0$. Traces are typical of 10 – 12 such cells taken from 5 – 8 rats. Traces have been used to analyse for data present in Figures 4.15, 4.17 and 4.18.

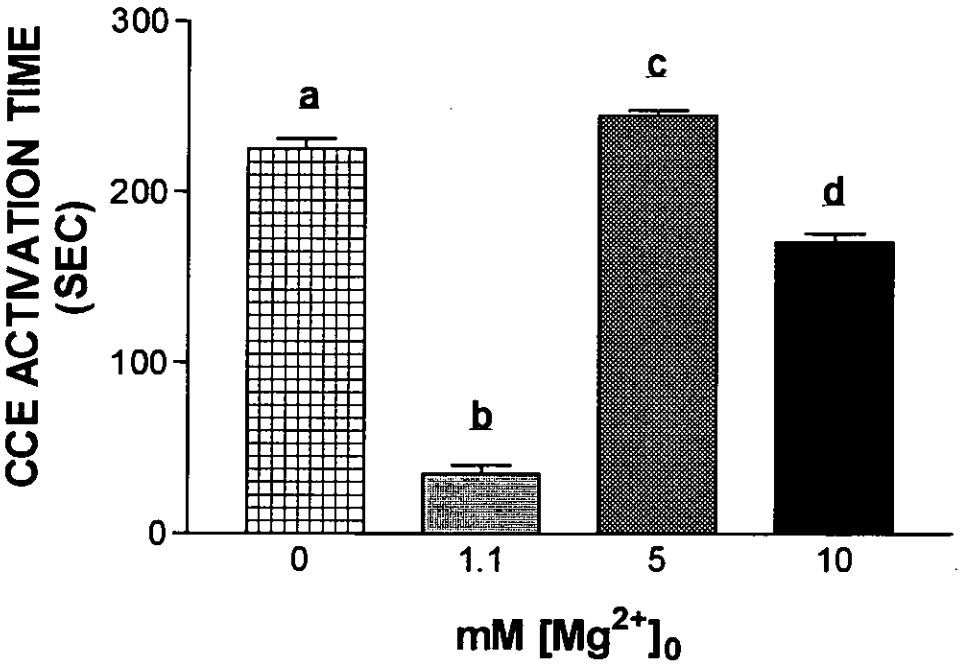


Figure 4.17- The mean (+- SEM) time (in seconds) taken for CCE to be activated in zero mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_0$ following perfusion of Fura 2-loaded parotid acinar cells with 1.8 mM $[Ca^{2+}]_0$ physiological salt solution. n = 10 – 12 cells taken from 5 – 8 animals. $P<0.01$ for b compared to a, c and d. Similarly, $P<0.01$ for d compared to a and c which are not significantly different from one another.

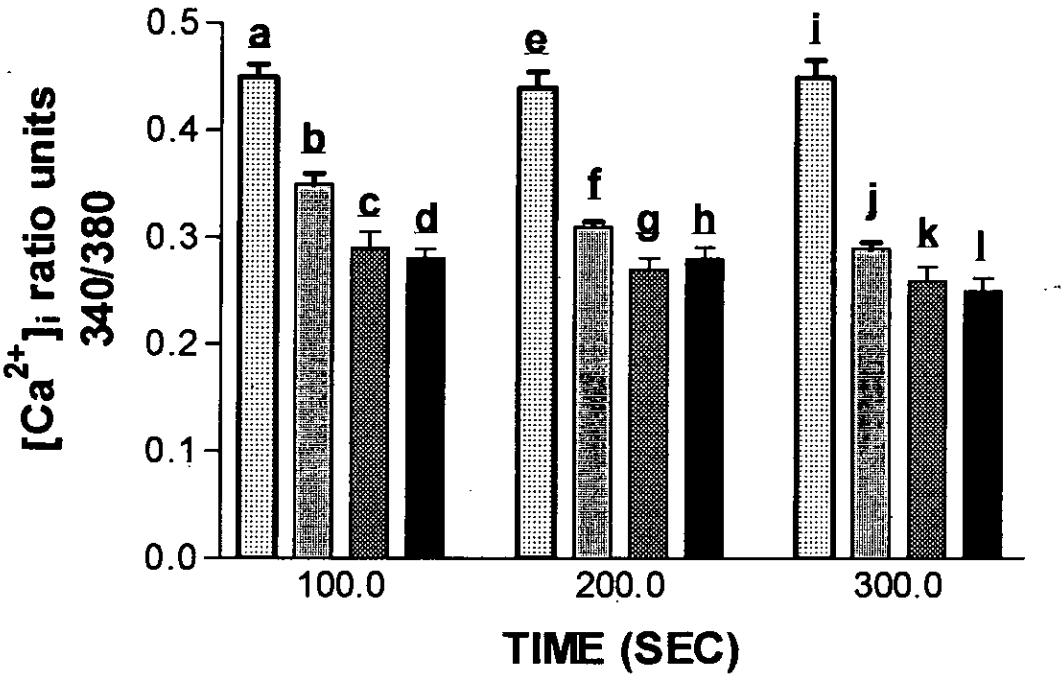
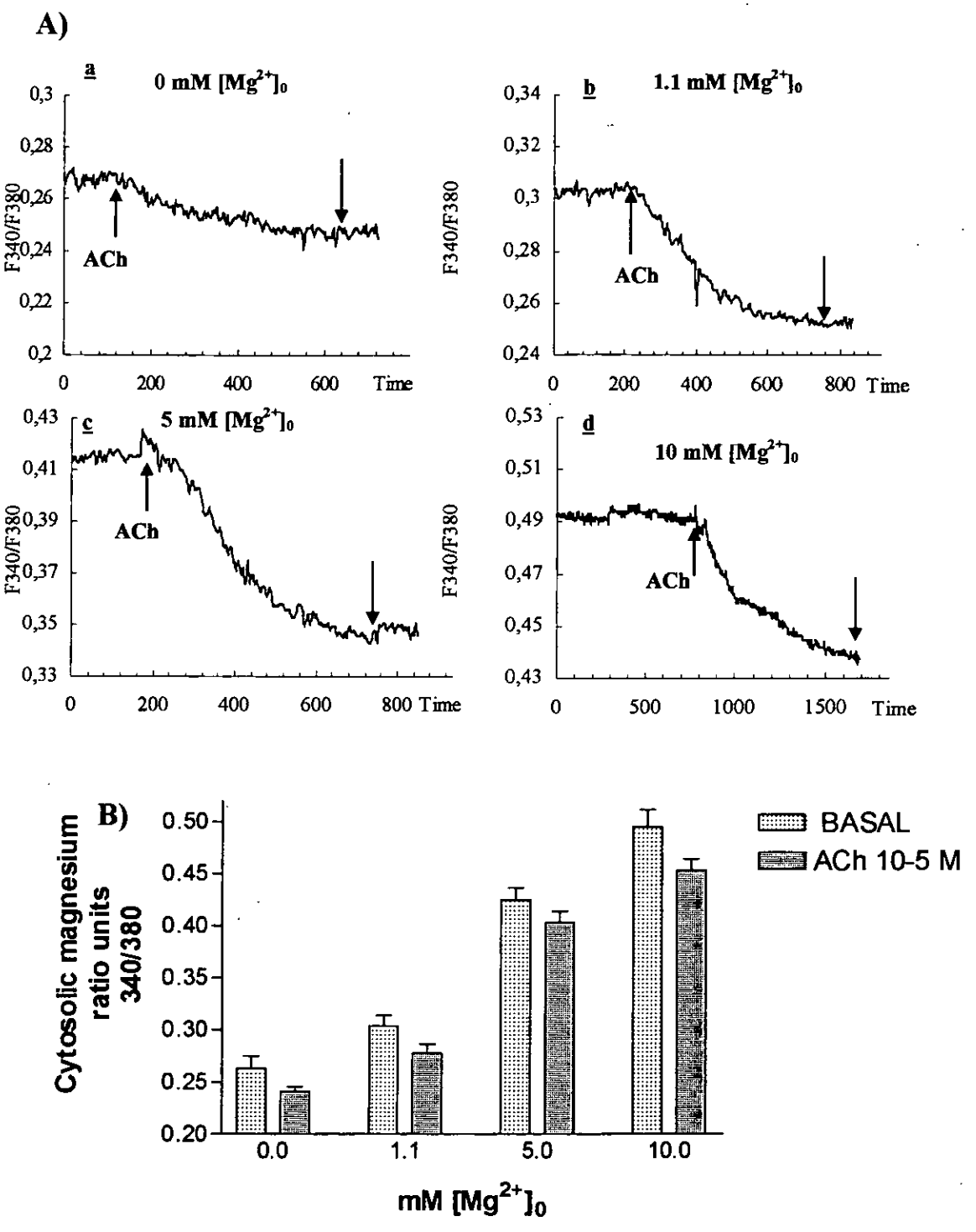


Figure 4.18- Mean (+- SEM) of the sustained elevation in $[Ca^{2+}]_i$ in Fura 2 loaded parotid acinar cells following activation of CCE with normal (1.8 mM $[Ca^{2+}]_0$) physiological salt solution during different (zero mM, 1.1 mM, 5 mM and 10 mM) $[Mg^{2+}]_0$. n= 8 – 12 experiments taken from 5 –10 animals. Note that elevated $[Mg^{2+}]_0$ is significantly ($P<0.01$) attenuating $[Ca^{2+}]_i$ at three different time points compared to zero $[Mg^{2+}]_0$ during reintroduction of normal $[Ca^{2+}]_0$. (a, b, c and d; e, f, g and h and i, j, k and l represent the $[Ca^{2+}]_i$ signal in zero mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_0$ respectively at 100, 200 and 300 seconds following CCE activation).

4.4.3 Secretagogue-evoked changes in $[Mg^{2+}]_i$ in Magfura 2 loaded parotid acinar cells

Since perturbation of $[Mg^{2+}]_o$ seems to have profound effect on both basal and secretagogue-evoked amylase secretion and $[Ca^{2+}]_i$, then it was pertinent to measure $[Mg^{2+}]_i$ during different $[Mg^{2+}]_o$ either alone or following perfusion of Magfura 2 loaded single parotid acinar cells with ACh. Figure 4.19 A shows original time course chart recordings of $[Mg^{2+}]_i$ during basal condition and following perfusion of parotid acinar cells with 10^{-5} M ACh in (a) zero, (b) 1.1, (c) 5 and (d) 10 mM $[Mg^{2+}]_o$. The results show that ACh can elicit a marked time-dependent decrease in $[Mg^{2+}]_i$ reaching a plateau level after 10 minutes of ACh application. Figure 4.19 B shows mean (+- SEM) basal (prior to ACh application) and ACh-evoked steady state decrease (10 min after ACh application) in $[Mg^{2+}]_i$ in zero mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_o$. The results show that basal $[Mg^{2+}]_i$ increase significantly ($P<0.05$) with elevated $[Mg^{2+}]_o$. Moreover, the ACh-evoked decrease in $[Mg^{2+}]_i$ was significantly ($P<0.05$) different from the respective control.

In another time course series of experiments the same Magfura 2-loaded acinar cells were perfused with different concentrations (0 mM, 1.1 mM, 5 mM and 10 mM) of $[Mg^{2+}]_o$ for 15 minutes each with increased concentration followed by stimulation with ACh (10^{-5} M) in the continuous presence of 10 mM $[Mg^{2+}]_o$. An original chart recording of the time-course changes in $[Mg^{2+}]_i$ is shown in Figure 4.20 A. The results show that $[Mg^{2+}]_i$ increases gradually following the application of different $[Mg^{2+}]_o$. Perfusion of the cell with 10^{-5} M ACh resulted in a rapid decrease in $[Mg^{2+}]_i$ reaching a plateau within 2 – 3 minutes of ACh application and remained at the same level even during the removal of ACh. The mean (+- SEM) data for $[Mg^{2+}]_i$ in different $[Mg^{2+}]_o$ in the absence and presence of ACh are shown in Figure 4.20 B. The results show that perfusion of parotid acinar cells with different concentrations of $[Mg^{2+}]_o$ resulted in a gradual and significant ($P<0.01$) increases in basal $[Mg^{2+}]_i$ compared to the concentration obtained in 0 mM $[Mg^{2+}]_o$. In the presence of 10 mM $[Mg^{2+}]_o$, ACh caused a marked and significantly ($P<0.05$) decrease in $[Mg^{2+}]_o$ suggesting that the ACh-induced decrease in $[Mg^{2+}]_i$ may be an energy-dependent process as Mg^{2+} has to move against its gradient.



4.19 (A)- Original charts recordings showing the effects of 10^{-5} M ACh on $[Mg^{2+}]_i$ in different $[Mg^{2+}]_0$ in Magfura 2-loaded parotid acinar cells. Traces are typical of 8 – 30 single cells taken from 7 – 15 different animals. **(B)**- Histograms showing the mean (+- SEM) basal $[Mg^{2+}]_i$ in different $[Mg^{2+}]_0$ prior to ACh and the steady-state (see downward arrows in Figure 4.18 (A)) $[Mg^{2+}]_i$ in the presence of 10^{-5} M ACh. n = 8 – 30 experiments taken from 7 – 15 different animals. Note that ACh evoked significant ($P<0.05$) decreases in $[Mg^{2+}]_i$ compared to basal for each concentration of $[Mg^{2+}]_0$.

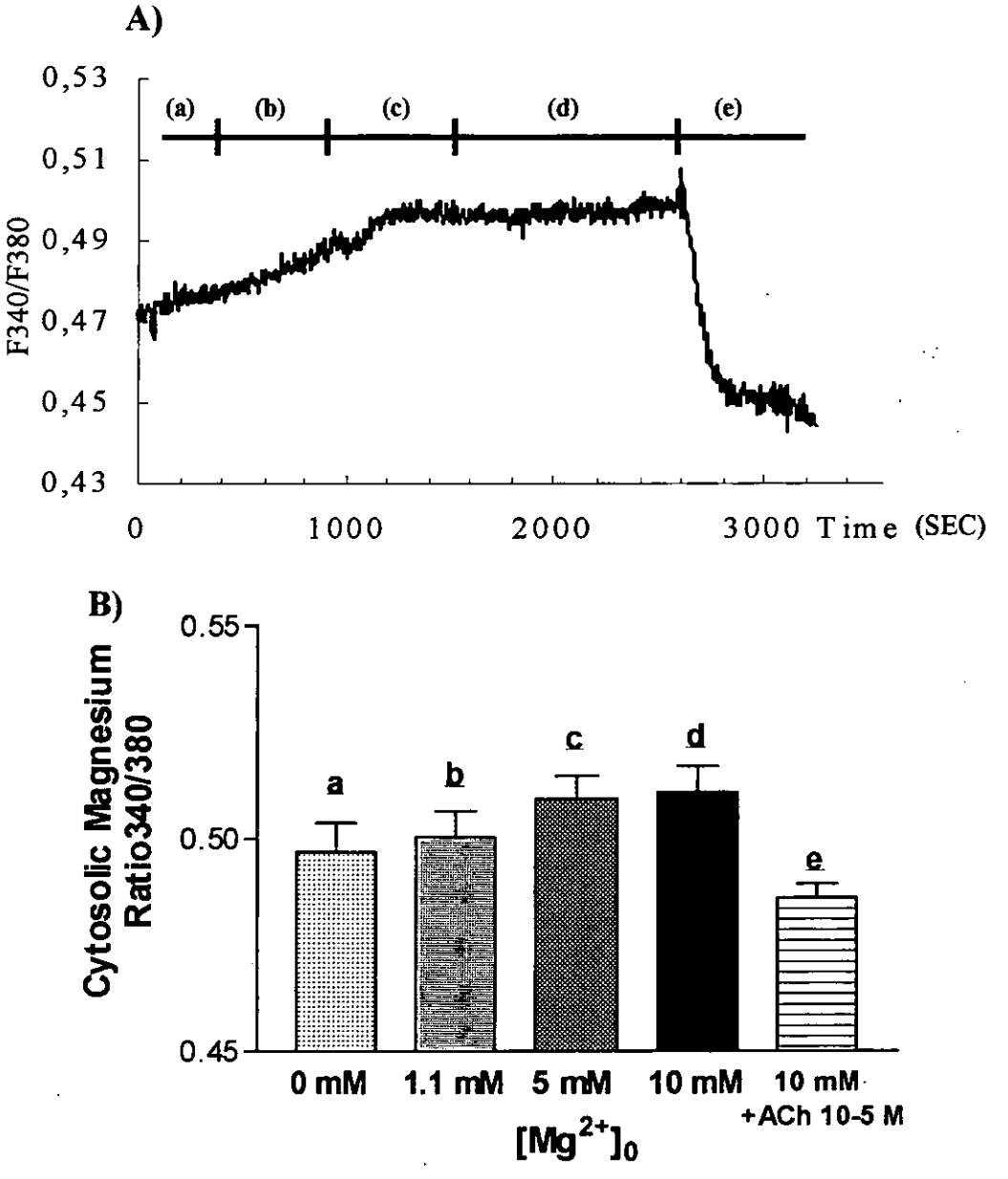


Figure 4.20- (A) Time course changes in $[Mg^{2+}]_i$ during perfusion of Magfura-2 loaded parotid acinar cells with different concentrations (a) 0 mM, (b) 1.1 mM, (c) 5 mM and (d) 10 mM $[Mg^{2+}]_0$ and subsequently, with (e) 10^{-5} M ACh. Trace is typical of 8 – 10 such cells taken from 5 – 8 animals. **(B)** Histograms showing mean (+- SEM) $[Mg^{2+}]_i$ during perfusion of Magfura-2 loaded acinar cells with different $[Mg^{2+}]_0$ and in the presence of 10^{-5} M ACh in 10 mM $[Mg^{2+}]_0$. n = 8 – 10 such cells taken from 5 – 8 animals. $P<0.05$ for e compared to d and a compared to d.

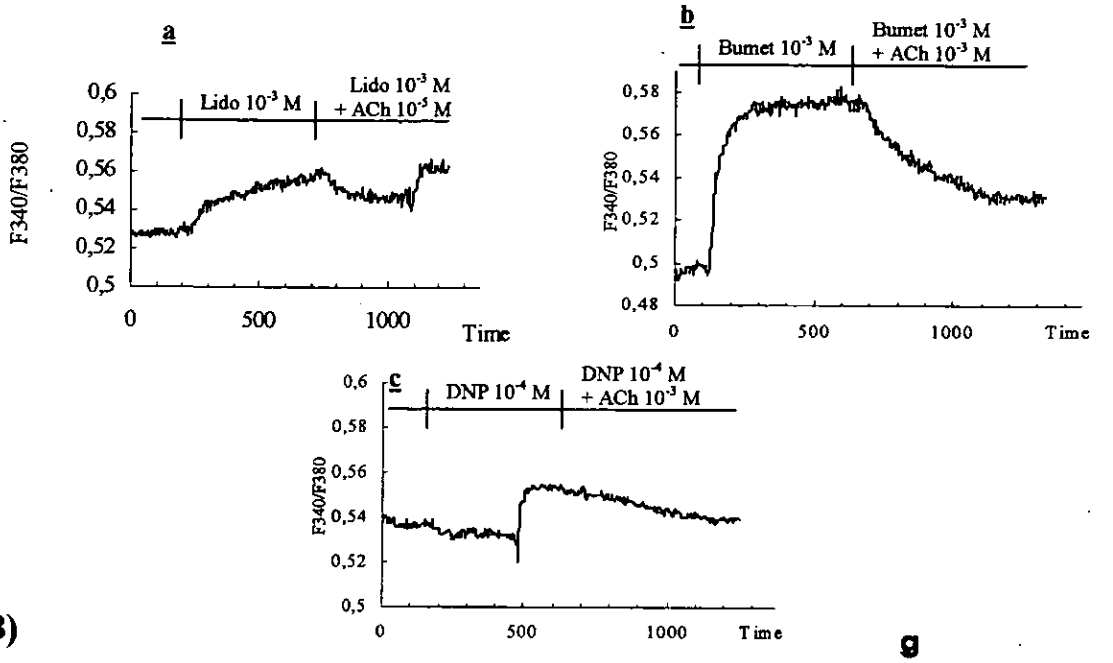
4.4.3.1 Effect of transport inhibitors on basal and ACh-evoked $[Mg^{2+}]_i$

Since ACh can elicit a marked decrease in $[Mg^{2+}]_i$ then it is pertinent to understand the mechanism of the ACh-induced Mg^{2+} transport. In this series of experiments a number of transports inhibitors were used to determine the mechanisms of ACh-evoked transport. Figure 4.21 shows the mean (+- SEM) changes in $[Mg^{2+}]_i$ in Magfura 2 loaded parotid acinar cells in 1.1 mM $[Mg^{2+}]_o$ either alone (basal) during perfusion with either 10^{-3} M lidocaine, 10^{-3} M amiloride, NMDG, 10^{-3} M quinidine, 10^{-4} M dinitrophenol or 10^{-3} M bumetanide or during perfusion with 10^{-5} M ACh in the continuous presence of each inhibitor. The results show that perfusion of Magfura 2 loaded acinar cells with either 10^{-3} M lidocaine, 10^{-3} M amiloride, NMDG, 10^{-3} M quinidine, 10^{-4} M dinitrophenol or 10^{-3} M bumetanide resulted in a marked and significant ($P<0.05$) elevation in $[Mg^{2+}]_i$ compared to the response (basal) obtained in the absence of these transport inhibitors. The effects of bumetanide and dinitrophenol were however, much more pronounced compared to either lidocaine, amiloride or NMDG. These results indicate that cytosolic $[Mg^{2+}]_i$ is somewhat Na^+ dependent and associated with Na^+/H^+ and Na^+/Mg^{2+} antiport systems and $Na^+/Cl^-/K^+$ co-transporter and that these processes are dependent upon ATP. Figure 4.21 A shows samples of original chart recordings of the effects of either 10^{-3} M lidocaine, 10^{-3} M bumetanide and 10^{-4} M dinitrophenol on $[Mg^{2+}]_i$ in absence and presence of 10^{-5} M ACh. The basal response prior to the application of each transport inhibitor is also shown for comparison.

Figure 4.22 A shows the effect of 10^{-5} M ACh on $[Mg^{2+}]_i$ in the presence and absence of the various membrane transport inhibitors. The control response in the presence of 1.1 mM $[Mg^{2+}]_o$ is also shown for comparison. The results show that in presence of 1.1 $[Mg^{2+}]_o$, ACh can elicit a small but significant ($P<0.05$) decrease in $[Mg^{2+}]_i$. This decrease in $[Mg^{2+}]_i$ was only partially blocked by either 10^{-3} M lidocaine or 10^{-3} M amiloride but not abolished completely. In contrast, either NMDG (a substitute for $[Na^+]_o$), 10^{-3} M quinidine, 10^{-4} M dinitrophenol or 10^{-3} M bumetanide had no effect on the decrease in $[Mg^{2+}]_i$ elicited by ACh. Figure 4.22 B shows the difference in the ACh-evoked decrease (or changes) in $[Mg^{2+}]_i$ in control and in the presence of the various blockers. The results show that the ACh-evoked decrease in $[Mg^{2+}]_i$ was much larger

($P<0.01$) in the presence of bumetanide. Taken together, the results reveal that the decreases in $[Mg^{2+}]_i$ elicited by ACh were insensitive to either bumetanide, DNP, quinidine or NMDG and only partially by either amiloride or lidocaine. Moreover, results also suggest that ACh-evoked decrease (or Mg^{2+} efflux) is not dependent upon either Na^+ or associated with either the Na^+: K^+: Cl^- co-transporter, or ATP. It is most likely to be associated with the Na^+ channel since it is inhibited by lidocaine, a local anaesthetic. In addition, the rise in $[Mg^{2+}]_i$ following treatment of the different transport inhibitors and sodium removal suggest that these substances are either inhibiting Mg^{2+} influx into intracellular stores or inhibiting its efflux from the cytoplasm to the extracellular medium.

A)



B)

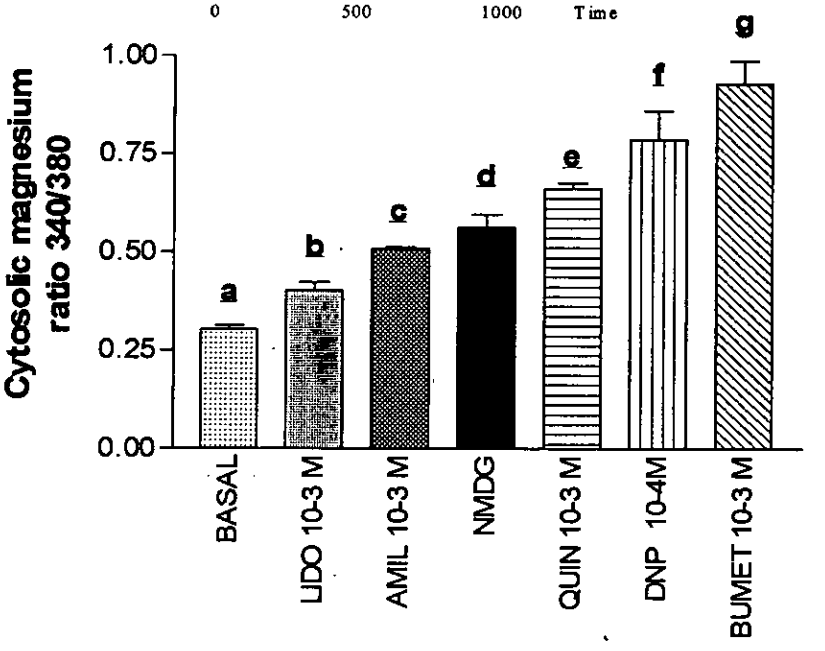
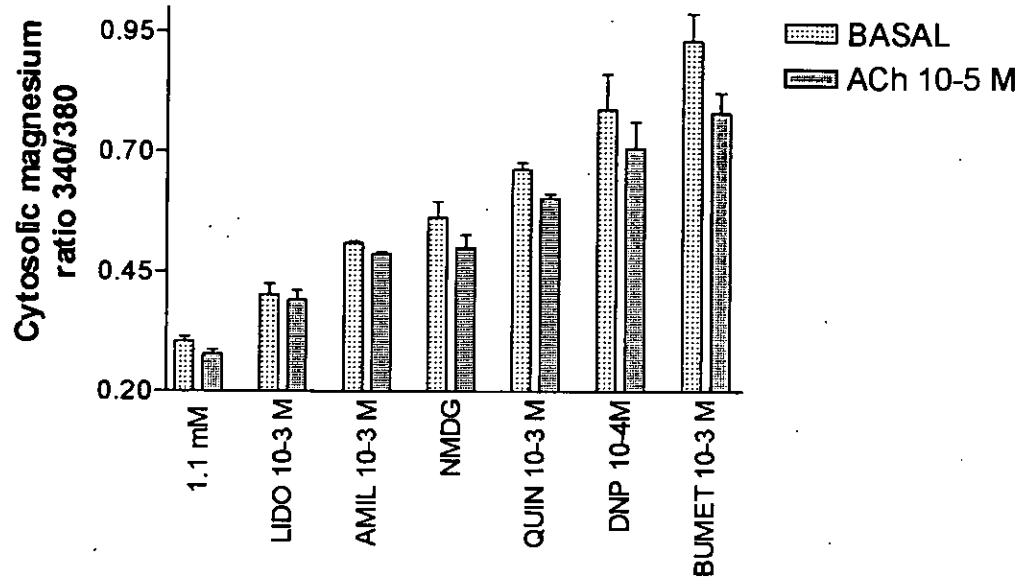


Figure 4.21 (A)- Original charts recordings showing the effect of either (a) 10^{-3} M lidocaine, (b) 10^{-3} M bumetanide or, (c) 10^{-4} M dinitrophenol on $[Mg^{2+}]_i$ in Magfura 2-loaded parotid acinar cells in the absence and presence of 10^{-5} M ACh. Traces are typical of 8 –12 experiments from 5 – 6 rats. (B) Histograms showing mean (+- SEM) basal $[Mg^{2+}]_i$ in 1.1 mM $[Mg^{2+}]_0$ and the increases in $[Mg^{2+}]_i$ in the presence of either 10^{-3} M lidocaine (LIDO), amiloride (AMIL), NMDG, quinidine (QUIN), bumetanide (BUMET) or 10^{-4} dinitrophenol (DNP). n = 8 – 12 experiments taken from 5 – 6 rats. $P<0.01$ for b, c, d, e, f and g compared to a.

A)



B)

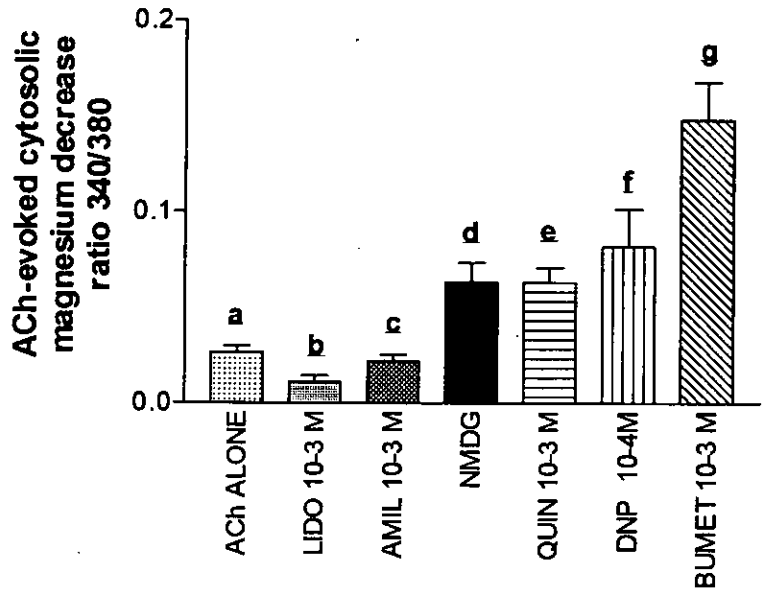


Figure 4.22 (A)- Histograms showing mean (+- SEM) changes in $[Mg^{2+}]_i$ following perfusion of Magfura-2 acinar cells with either 1.1 mM $[Mg^{2+}]_o$ physiological salt solution alone or with physiological salt solution containing either of 10^{-3} M lidocaine (LIDO), 10^{-3} M amiloride (AMIL), NMDG, 10^{-3} M quinidine (QUIN), 10^{-3} M bumetanide (BUMET) and 10^{-4} M dinitrophenol (DNP) in the absence and presence of ACh 10^{-5} M. N = 8 – 12 experiments taken from 5 – 6 rats. (B) Histograms showing mean (+- SEM) of the ACh-evoked decreases in $[Mg^{2+}]_i$, either alone or in the presence of each inhibitor, n= 8 – 12.

4.5 Discussion

The results of this study have shown that activation of intrinsic secretomotor nerves in the parotid gland can elicit frequency dependent increases in amylase output. EFS is a useful physiological tool to elicit the release of endogenous neurotransmitters from exocrine glands (Davison *et al.*, 1980; Wisdom *et al.*, 1996) which in turn stimulated enzyme secretion. Repeated stimulations of the same tissue caused a gradual increase in amylase secretion at 5 Hz. However, at 10 Hz and 20 Hz, there was a gradual decrease in amylase output. When the data for repeated stimulation either at 5 Hz, 10 Hz or 20 Hz were combined, there were no significant changes in amylase output for either. This interesting observation indicates that caution has to be taken when data are collected and considered during repeated stimulations at a particular frequency. The results also suggest that it would be better to stimulate the tissue once in order to obtain both a constant and maximal result.

The present results have also shown that stimulation of superfused parotid segments with either ACh, NA, PHE or ISO can result in marked increases in amylase secretion. A perturbation of extracellular Mg^{2+} $[Mg^{2+}]_0$ can have a profound effect on both basal and on secretagogue-evoked amylase secretion in the isolated rat parotid gland segments. Both zero and elevated $[Mg^{2+}]_0$ can significantly inhibit both the basal as well as the secretory effects of either ACh, NA or PHE compared to the responses obtained in normal $[Mg^{2+}]_0$. However, the effect of perturbation of $[Mg^{2+}]_0$ on EFS-evoked secretory responses was much less pronounced compared to the responses obtained with either ACh, NA or PHE. The responses obtained with ISO show a completely different pattern with amylase output, these increased proportionally as $[Mg^{2+}]_0$ is perturbed with increasing concentration. Since either ACh, NA or PHE utilise cellular Ca^{2+} as a mediator these results indicate that that EFS and ISO may utilise different intracellular mediators (e.g cAMP) to elicit enzyme secretion. The inhibition in respect to either ACh, NA and PHE was more marked in zero $[Mg^{2+}]_0$ compared to 5 and 10 mM $[Mg^{2}]_0$. However, when supra-maximal concentration (e.g. 10^{-5} M) of secretagogue was used, 5 mM $[Mg^{2+}]_0$ had no significant inhibitory effect on amylase secretion compared to normal $[Mg^{2+}]_0$. In contrast, at a lower concentration (e.g. 10^{-7} and 10^{-6} M) of the three salivary secretagogues, 5 mM and 10 mM $[Mg^{2+}]_0$ attenuated the secretory responses, but again the effect was less pronounced compared to zero $[Mg^{2+}]_0$. The results obtained

in this study employing isolated parotid gland segments are somewhat similar to the data obtained with the pancreas in response to either ACh or cholecystokinin-octapeptide during perturbation of $[Mg^{2+}]_o$ (Francis *et al.*, 1990; Wisdom *et al.*, 1996).

The question we now need to answer is: how does a modification in extracellular $[Mg^{2+}]_o$ lead to an inhibition of secretory effects of the classical salivary secretagogues such as ACh, NA and PHE but not so with ISOP and only to a small extent with EFS? In order to answer this question, it is important to understand the cellular mechanism of the stimulus-secretion coupling process and the physiological role of Mg^{2+} during cellular regulation.

The parotid gland is innervated with autonomic nerves. Stimulation of the autonomic nervous system results in the release of the two main endogenous neurotransmitters acetylcholine (ACh) and noradrenaline (NA). These in turn stimulate their respective receptors (cholinergic muscarinic for ACh and beta and alpha adrenergic for NA) on parotid acinar plasma membrane to elicit enzyme (digestive amylase) and fluid secretion (Petersen & Gallacher, 1987; Baum, 1993; Ambudkar, 2000). ACh and α-adrenergic agents (e.g. NA and PHE) act via cellular Ca^{2+} to elicit enzyme secretion whereas beta adrenergic activator lead to the elevation in endogenous cyclic AMP (Baum, 1987; Putney, 1986; Putney Jr; 1986[a]; Putney Jr & Bird, 1993) which in turn mediates enzyme secretion. The present results have shown that a modification of $[Mg^{2+}]_o$ had a completely different effect on isoprenaline-evoked amylase output and there is only a small change in the response to EFS. These observations suggest that a perturbation of $[Mg^{2+}]_o$ seem to have a different effect on the regulation of the stimulus-secretion coupling pathway involving cyclic AMP.

On the other hand, a perturbation of $[Mg^{2+}]_o$ had profound effects on Ca^{2+} mobilising secretagogues (e.g. ACh and PHE). Therefore, it was pertinent to investigate the relationship between the perturbation of $[Mg^{2+}]_o$ and the Ca^{2+} signalling during the stimulus-secretion coupling process.

The results of this study have shown that basal $[Ca^{2+}]_i$ is dependent upon $[Ca^{2+}]_o$. In a nominally free $[Ca^{2+}]_o$ and in the presence of 1 mM EGTA, basal $[Ca^{2+}]_o$ was significantly decreased compared to the values obtained in the presence of 1.8 mM $[Ca^{2+}]_o$. Perturbation of $[Mg^{2+}]_o$ had different effects on basal cellular calcium depending on the concentration of $[Mg^{2+}]_o$. In zero $[Mg^{2+}]_o$, basal $[Ca^{2+}]_i$ remained more or less the same in both normal and in a normally free $[Ca^{2+}]_o$. Elevated (5 and 10 mM)

seem to suppress basal $[Ca^{2+}]_o$ in both normal and in a nominally free $[Ca^{2+}]_o$ compared to values obtained in zero and 1.1 mM $[Mg^{2+}]_o$.

Perfusion of Fura-2 loaded parotid acinar cells with a supra-maximal dose of ACh in normal extracellular $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ resulted in a large transient increase in $[Ca^{2+}]_i$ reaching a maximum within 10-15 seconds (peak phase) followed by a decline in the Ca^{2+} signal reaching a steady-state plateau above basal level after about 2-3 minutes (plateau phase) of ACh application. When parotid acinar cells were perfused with ACh in the presence of either zero, 5 mM or 10 mM $[Mg^{2+}]_o$, there was a significant decrease in both the transient peak and the plateau phase of the Ca^{2+} signal compared to the responses obtained in 1.1 mM $[Mg^{2+}]_o$. The inhibitory effect of elevated (5 mM and 10 mM) $[Mg^{2+}]_o$ on the ACh-evoked $[Ca^{2+}]_i$ was much more pronounced compared to the decreases obtained in zero $[Mg^{2+}]_o$. In contrast, in a nominally free $[Ca^{2+}]_o$, ACh stimulation resulted in a significantly smaller Ca^{2+} signal (only the initial transient peak) compared to result obtained with ACh in normal $[Ca^{2+}]_o$. Thereafter, the Ca^{2+} signal returned to the basal level suggesting that $[Ca^{2+}]_o$ is required to maintain the plateau phase of the Ca^{2+} signal. Again, elevated (5 mM and 10 mM) $[Mg^{2+}]_o$ significantly attenuated the ACh-evoked increases in the transient peak phase of the Ca^{2+} signal compared to the responses obtained in normal (1.1mM) $[Mg^{2+}]_o$. In contrast, in zero $[Mg^{2+}]_o$ the ACh-evoked transient peak phase of the Ca^{2+} signal was much larger compared to the responses obtained in 1.1 mM $[Mg^{2+}]_o$. Taken together, these results suggest that $[Mg^{2+}]_o$ (both zero and elevated) are regulating secretagogue-evoked cellular Ca^{2+} mobilisation in parotid acinar cells.

It is now well established that the Ca^{2+} which is required for the stimulus-secretion coupling process comes mainly from two sources, one from the internal stores (e.g. endoplasmic reticulum) and the other from the extracellular medium (Petersen & Gallacher, 1987; Baum, 1993; Ambudkar, 2000). Since elevated $[Mg^{2+}]_o$ can attenuate both the transient peak and the plateau phase of the Ca^{2+} signals evoked by ACh, then it is tempting to suggest that Mg^{2+} is exerting its inhibitory effect on Ca^{2+} by blocking its influx (capacitative calcium entry) into the cell and its release from the internal stores (e.g. endoplasmic reticulum).

In order to test this interesting hypothesis, parotid acinar cells were firstly perfused with a nominally free $[Ca^{2+}]_o$ in presence of 1 mM EGTA and subsequently stimulated with ACh. The cholinergic agonist evoked a small transient peak compared to a much larger increase in normal $[Ca^{2+}]_o$. Moreover, in the absence of $[Ca^{2+}]_o$, the plateau phase seen

normally in the presence of $[Ca^{2+}]_0$ returned quickly to basal level. Elevated (5 mM and 10 mM) $[Mg^{2+}]_0$ significantly attenuated the initial transient peak suggesting that it is regulating Ca^{2+} release from the stores.

In contrast, elevated $[Mg^{2+}]_0$ had no significant effect on the plateau phase of the Ca^{2+} signal compared to the response obtained in 1.1 mM $[Mg^{2+}]_0$ in the presence of ACh. This may be due to the fact no Ca^{2+} is entering the cell due to its absence in the extracellular medium. Surprisingly, in zero $[Mg^{2+}]_0$ the ACh-evoked initial peak phase of the Ca^{2+} signal (see Figure 4.14) was much larger than the response (see Figure 4.15) obtained in normal (1.1 mM) Mg^{2+}. Taken together, these observations suggest that extracellular Mg^{2+} is behaving like an antagonist for the mobilisation of cellular Ca^{2+}. Furthermore, when Mg^{2+} is absent from the extracellular medium, more Ca^{2+} seem to be released from internal stores. Previous studies have suggested that Mg^{2+} is a natural antagonist for Ca^{2+} (Yago *et al.*, 2000; Mooren *et al.*, 2001).

The results so far have clearly indicated that Mg^{2+} can regulate Ca^{2+} release from the internal stores and moreover, the divalent cation may also control Ca^{2+} influx into the cell during the capacitative calcium entry process. The experiments presented in Figures 4.15-4.17 were done to test this interesting hypothesis. The results have demonstrated that normal (1.1 mM) $[Mg^{2+}]_0$ can rapidly activate the CCE compared to the delayed activation times in zero, 5 mM and 10 mM $[Mg^{2+}]_0$. In addition, the data have also shown that maximal elevation in $[Ca^{2+}]_i$ in parotid acinar cells occur in zero $[Mg^{2+}]_0$ compared to significantly less Ca^{2+} entry in the presence of elevated $[Mg^{2+}]_0$, especially in the presence of 5 mM and 10 mM $[Mg^{2+}]_0$. The results of this study are in total agreement with the data obtained in both the pancreas and parotid gland in which elevated $[Mg^{2+}]_0$ inhibited both Ca^{2+} release from intracellular stores and Ca^{2+} influx from extracellular medium (Francis *et al*, 1990; Yago *et al*, 2000; Wisdom *et al*, 1996; Yago *et al*, 1999; 2002).

Since Mg^{2+} can regulate both the release of Ca^{2+} from internal stores and the CCE, then it is vitally important to understand the mechanism of action of the divalent cation in exerting its effect on secretagogue-evoked secretory responses. Mg^{2+} is an important abundant divalent cation which is required in cellular regulation (Reinhardt, 1988). It is also a major co-factor in the activation of a number of enzymes (Birch, 1993). Elevated Mg^{2+} is known to act like a natural antagonist for such ions as Ca^{2+}, K^+ and Na^+ (Yago *et al.*, 2000; Birch, 1993). In contrast, reduced $[Mg^{2+}]_0$ may have reverse effects on the activities of several enzymes. In this study, it has been clearly demonstrated that

elevated $[Mg^{2+}]_o$ is exerting its inhibitory effect on ACh, (and possibly NA and PHE)-evoked amylase secretion by blocking Ca^{2+} mobilisation (Yago *et al.*, 2000; Francis *et al.*, 1990; Wisdom *et al.*, 1996). These three secretagogues are known to mobilise cellular Ca^{2+} (Putney, 1988; Baum, 1987; Petersen, 1992; Baum & Ambudkar, 1988; Petersen & Gallacher, 1988; Baum, 1993; Ambudkar, 2000); which in turn mediates parotid amylase secretion.

In contrast, the effect of low $[Mg^{2+}]_o$ on both basal and secretagogue-evoked parotid amylase secretion is less understood. In the exocrine pancreas low $[Mg^{2+}]_o$ enhanced the secretory effects (both amylase output and Ca^{2+} mobilisation) of either ACh or CCK-8 (Francis *et al.*, 1990; Wisdom *et al.*, 1996) whereas in the parotid gland low $[Mg^{2+}]_o$ inhibited the secretory responses to either ACh, NA and PHE.

Moreover, this study has also demonstrated that low Mg^{2+} can inhibit ACh-evoked Ca^{2+} mobilisation (both the peak and the plateau phases) but not the basal Ca^{2+} transport in either normal or in a nominally free extracellular calcium physiological salt solution. These results are in agreement with other studies (Yago *et al* 1999; 2001). In contrast, in a nominally free Ca^{2+} medium, ACh-evoked a much longer Ca^{2+} transient in low $[Mg^{2+}]_o$ compared to the responses obtained in normal $[Mg^{2+}]_o$. In addition, CCE was much slower in its activation but the steady-state elevation in $[Ca^{2+}]_i$ was much larger compared to the responses obtained with normal $[Mg^{2+}]_o$. Taken together, these results suggest that low $[Mg^{2+}]_o$ may not be acting like a Ca^{2+} channel antagonist compared to elevated $[Mg^{2+}]_o$, but it may exert its effects directly in the activities of Mg^{2+}-dependent enzymes which are associated with Ca^{2+} transport. Another possible explanation is that extracellular Mg^{2+}, but not intracellular Mg^{2+}, is apparently required to inhibit cellular Ca^{2+} mobilisation.

Mg^{2+} is associated with the metabolism of inositol trisphosphate (IP_3) (Petersen, 1992; Ambudkar, 2000; Yago *et al.*, 2000; Wisdom *et al.*, 1996). In the absence of $[Mg^{2+}]_o$, it is tempting to suggest that an inhibition of the Mg^{2+}-dependent enzyme may lead to reduce levels of IP_3 resulting in decreased cytoplasm Ca^{2+} concentration during secretagogue application. In relation to Ca^{2+} release from intracellular stores and the CCE, the absence of $[Mg^{2+}]_o$ would facilitate Ca^{2+} release from the stores and influx into the cell especially since Mg^{2+} is acting like a natural antagonist during the mobilisation of $[Ca^{2+}]_i$.

Mg^{2+} transport:

Since a perturbation of $[Mg^{2+}]_0$ resulted in an inhibition of cellular Ca^{2+} homeostasis and subsequently amylase release, it was pertinent to understand the transport mechanism of this important divalent cation during both basal and stimulated conditions. The results of this study have shown that perfusion of single Magfura-2 loaded parotid acinar cells with physiologic salt solution contaning different concentrations of $[Mg^{2+}]_0$ resulted in a gradual increase in $[Mg^{2+}]_i$. Moreover, stimulation of acinar cell with ACh in different $[Mg^{2+}]_0$ resulted in a gradual decrease in $[Mg^{2+}]_i$ reaching a plateau phase within 5 - 8 minutes. These findings suggest that ACh can mobilise cellular Mg^{2+}. The decrease in the Mg^{2+} signal may be due to the fact that Mg^{2+} is either leaving the cell or it is entering intracellular stores. If it is exiting the cell, then it has to move against a gradient since the secretagogue-evoked decrease was obtained in elevated (5 mM and 10 mM) $[Mg^{2+}]_0$ suggesting that this movement is dependent upon energy. These results of ACh are in agreement with the data obtained in previous studies employing the pancreatic acinar cells (Wisdom *et al*, 1996; Yago *et al*, 2000; Mooren *et al*, 2001; Mateos, 1998) and submandibular acinar cells (Zangh & Melvin, 1992; 1994).

It has been demonstrated, at least in pancreatic acinar cells, that the secretagogue-evoked decreases in Mg^{2+} signal were due to Mg^{2+} leaving the cell and that the efflux of Mg^{2+} was dependent upon extracellular Na^+ (Yago *et al*, 2002; Wisdom *et al*, 1996; Mateos, 1998). These studies were done employing the techniques of atomic absorbance spectroscopy and Magfura 2 tetrapotassium salt to measure Mg^{2+} release (Yago *et al.*, 2000). However, in the parotid acinar cells no previous study has attempted to characterise either basal or secretagogue-evoked Mg^{2+} transport. The results of this study have also shown that either lidocaine (a Na^+ channel blocker), amiloride (an inhibitor of Na^+/H^+ exchanger), NMDG (a substitute for $[Na^+]_0$), quinidine (an inhibitor of the Na^+/Mg^{2+} antiport), dinitrophenol (an inhibitor of ATP) and bumetanide (an inhibitor of the Na^+:K^+:Cl^- co-transporter) can all increase cytosolic Mg^{2+} concentration with the same order of potency, with lidocaine eliciting the least increase and bumetanide the maximal increase. In the presence of any of these inhibitors, ACh evoked a decrease in $[Mg^{2+}]_i$. The magnitude of the ACh-evoked decrease in $[Mg^{2+}]_i$ was much bigger in the presence of bumetanide and much more smaller in the presence of either lidocaine or amiloride. The decrease in $[Mg^{2+}]_i$ in response to ACh may be due to the fact that Mg^{2+} is leaving the cell (efflux) or it is sequestered in the stores. In pancreatic acinar cells it was demonstrated that Mg^{2+} was leaving the cell during ACh

stimulation. Since parotid acinar cells are similar to pancreatic acinar cells, it is tempting to suggest that ACh is indeed stimulating Mg^{2+} efflux. If this is the case, the results have shown that this "ACh-induced Mg^{2+} efflux" is insensitive to either Na^+ removal (substituting it for NMDG) or to either quinidine, dinitrophenol or bumetanide but only partial sensitive to lidocaine and amiloride. Taken together, the results suggest that the "ACh-evoked Mg^{2+} efflux" may partially be associated with the sodium channel activity since lidocaine the local anaesthetic which is known to inhibit the Na^+ channel activity can decrease the response to ACh. In addition, the "ACh-evoked Mg^{2+} efflux" may also be associated with the Na^+/H^+ antiport. Further experiments are required to characterise precisely how Mg^{2+} is being mobilised following ACh stimulation.

On the other hand, the transport inhibitors themselves and sodium substitution (with NMDG) can result in significant elevation in $[Mg^{2+}]_i$ compared to the basal value. These transport inhibitors may exert effects in increasing cellular Mg^{2+} via a different number of mechanisms. They may act by either facilitating Mg^{2+} release from intracellular stores, preventing its efflux from the cytosol or enhancing its influx from the extracellular medium. Since $[Mg^{2+}]_i$ rises in the presence of NMDG, it is tempting to suggest that cytosolic Mg^{2+} elevation is sensitive to extracellular Na^+. The same is true for bumetanide. In the presence of the loop diuretic, there was a marked elevation in $[Mg^{2+}]_i$. Bumetanide is known to inhibit the Na^+:K^+:Cl^- co-transport. The results with DNP indicate that the rise in $[Mg^{2+}]_i$ may be dependent on an energy process since DNP is known to inhibit ATP production. Another possible explanation for the effect of DNP is that basal Mg^{2+} efflux is dependent upon ATP and once inhibited with DNP, this resulted in an elevation of $[Mg^{2+}]_i$. Similarly, it can be argued that Mg^{2+} uptake into internal stores is ATP-dependent and this process could then be inhibited by DNP, resulting in a rise in $[Mg^{2+}]_i$. Furthermore, quinidine can also elevate $[Mg^{2+}]_i$ and this substance is known to inhibit the Na^+/Mg^{2+} antiport. Like NMDG, quinidine would inhibit Na^+ influx into the cell hereby facilitating cellular Mg^{2+} elevation.

In conclusion the results have shown that:

1. Either ACh, NA, PHE, ISO and EFS can evoke marked increases in amylase secretion in isolated rat parotid segments.
2. Elevated and zero $[Mg^{2+}]_o$ can attenuate ACh, NA or PHE (but not EFS or ISO) evoked amylase secretion.
3. ACh can elicit marked increases in $[Ca^{2+}]_i$ and this response is dependent upon $[Mg^{2+}]_o$.

4. An elevation in $[Mg^{2+}]_0$ can lead to an increase in $[Mg^{2+}]_i$, which in turns regulates cellular Ca^{2+} mobilisation.
5. The ACh-evoked decrease in $[Mg^{2+}]_i$ is insensitive to a number of transport inhibitors and to extracellular Na^+ removal but only partial sensitive to amiloride and quinidine.
6. Drugs which can inhibit Na^+ transport and ATP production can result in a marked elevation in $[Mg^{2+}]_i$.
7. Substitution of extracellular Na^+ with NMDG can lead to an elevation in $[Mg^{2+}]_i$.

Taken together, these observations indicate that Mg^{2+} transport (influx or efflux) is a complex process, which requires further attention.

CHAPTER 5

EFFECTS OF GENDER, AGE AND PATHOLOGICAL CONDITIONS ON HUMAN SALIVARY GLAND FUNCTION

5.1 Introduction

The understanding of salivary function in promoting a healthy oral condition has become a topic of major importance for the nowadays-oral clinicians. In order to understand the role of each salivary component in the oral cavity homeostasis, it is crucial to perceive how its changes or absence may be linked with pathological conditions (Mandel, 1993). In fact, several systemic diseases such as cystic fibrosis, HIV infection or autoimune diseases among others have been reported to produce marked and identifiable salivary changes (Fox, 1993). In addition, oral pathological conditions like candidiasis (Hicks *et al,* 1998), dental caries (Kholer & Bjarnasson, 1992), peridontitis (Kaufman & Lamster, 2000), oral cancer, chemotherapy and radiotherapy-induced mucositis were reported to induce palpable salivary alterations (Dumbrigue *et al,* 2000). An excellent plasma/saliva correlation has now been established for a wide number of steroid hormones including cortisol, aldosterone, estradiol, and progesterone (Forcella *et al.*, 2003) suggesting a great number of different possible salivary analytical procedures for the study of stress, aldosteronism, ovulation or pregnancy. Similarly to other body fluids (i.e., serum, urine and sweat), saliva has been proposed for the monitoring of a great number of systemic levels of therapeutic or abuse drugs from digoxin and caffeine to ethanol, cocaine, marijuana or opioids (Drobitch & Svensson, 1992). Thus, saliva offers an excellent alternative to serum as a biological fluid that can be analysed for diagnostic purposes. This is of great biomedical importance since individuals with modest training can collect whole saliva in a non-invasive manner, including patients. This may facilitate the development of screening tests that can be performed by individuals at home. Analysis of saliva can offer a cost-effective approach for screening of large populations, and may represent an alternative for patients when their blood is difficult to obtain or when compliance is a problem (Kaufman & Lamster, 2002).

However, knowledge integration between saliva and oral pathology is far from being complete. The study of salivary function has been overwhelmingly difficult by the enormous naturally occurring variability of this fluid when compared to others (like the plasma) in which the composition's regularity has permitted to undoubtedly separate physiological from pathological. Therefore, it is of critical importance to establish which salivation patterns and concentration ranges of each salivary component are to be

considered as normal in order for the clinician to diagnose altered salivary phenotypes possibly linked to pathological systemic or oral conditions.

This study was designed specifically to study some physiological and pathophysiological conditions, which can affect human salivary gland secretion and its composition. This study investigates the possible effects of ageing, diabetes (types I and II) and surgical recovery on salivary fluid secretion and composition compared to healthy age-matched controls.

5.2 Methods

As described in chapter 2.

5.3 Statistical analysis of data

In this study a multi anova test was employed to test analyse mean differences and interactions between factors. SPSS 10.0 was used as a statististical package and only $P<0.05$ were considered as significant values.

5.4 Results

5.4.1 Effects of age and gender on salivary secretion in healthy subjects

Figures 5.1 and 5.2 show the effect of age on salivary gland secretion rates in male (Figure 5.1 A) and female (Figure 5.2 A) during resting and stimulated conditions and the differences (secretory capacity) in the two parameters for male (Figure 5.1 B) and female (Figure 5.2 B) subjects. The results show that in both male and female subjects, basal and stimulated salivary secretion rates decreased significantly ($P<0.05$) with age. This was more pronounced in male subjects when comparing 24-34 year old males with 45-75 years of age. In female subjects the decrease in salivation was more or less the same for age groups 35-44, 45-54, 55-64 and 65-75 compared to 20-34 years of age. When the various basal, stimulated or salivary secretion rates were tested by multianova analysis for gender related differences, there were no significant results suggesting that

gender on its own is not a variability factor. However, in the multianova testing, the interactions between gender and age proved to be significant ($P<0.05$) for the various resting, stimulated and salivary secretion rates suggesting that male and female salivary secretion is not affected by age in a similar way.

Figures 5.3 A and 5.4 A show the effect of age on total protein output in the saliva for male and female, respectively in resting and stimulated conditions. The difference (secretory capacity) between these two physiological parameters for male and female subjects are shown in Figure 5.3 B and 5.4 B, respectively. The results show that protein output decreases with stimulation for both genders. Resting protein output increased with age for both male and female subjects whereas stimulated protein output remained more or less the same for male and female subjects with age. In relation to protein secretion, the amounts decreased significantly ($P<0.01$) with age for both male and female when comparing 20-34 years of age with either 55-64 or 65-75 years. Multianova testing for differences for the variable gender as for interactions between the variables gender and age failed to show statistical significance for the variables resting stimulated and protein secretion. These results suggest that there are no gender-related differences in these variables and that male and female protein secretion are affected by age in the same way. Taken together, the results indicate that the decrease in salivary secretion rates with age is associated with a concomitant increased concentration of basal salivary proteins but decreased protein secretion capacity.

Figures 5.5 and 5.6 show the effect of age on resting and stimulated Ca^{2+} concentration and their differences in saliva for male (Figures 5.5 A and B) and female (Figures 5.6 A and B) subjects. The results show that for male subjects basal, stimulated and secretory Ca^{2+} levels increase with age, but the differences between resting and stimulated values were significant ($P<0.05$) only between age groups of 20-34 years and 65-75 years. With female subjects, basal Ca^{2+} remains more or less the same whereas stimulated and secretory Ca^{2+} increased with age differences being significant ($P<0.01$) for age groups 55-64 and 65-75 years compared to 20-34 years of age. Multianova testing showed gender to be a significant factor for variable basal Ca^{2+} concentration suggesting that female have lower values for this variable. For various stimulated and secretory Ca^{2+}, there were no significant gender related differences. Multianova testing for interactions between variable gender and basal stimulated or secretory Ca^{2+} concentrations failed to show any significant statistical values indicating that both genders are affected in the same way by the age-factor induced changes.

Figures 5.7 and 5.8 show the concentrations of Mg^{2+} in saliva in basal and stimulated conditions for male (Figure 5.7 A and B) and female (Figure 5.8 A and B) subjects. The results show that Mg^{2+} levels in both basal and stimulated conditions significantly ($P<0.05$) increased with age for male subjects. For female subjects, basal and stimulated Mg^{2+} increased significantly ($P<0.05$) only for age groups of 55-64 years and 65-75 years when compared to 44-54 years of age and there were no other significant differences between any age groups. At all aged groups, stimulation induced marked decreases in Mg^{2+} concentrations with a maximal decrease at ages 55-64. With regards to female subjects, the same is true compared to male subjects. There were large decreases in stimulated Mg^{2+} levels except for 65-75 years of age. Multianova testing for gender related differences and gender age interactions proved to be significant ($P<0.05$) only for resting and stimulated saliva indicating that generally, female have lower Mg^{2+} concentration than male subjects and are affected differently by the ageing process. There were no significant gender related or gender versus age interactions differences in secretory Mg^{2+} indicating that for this ion the secretory capacity of the salivary glands are affected in the same way.

The levels of Zn^{2+} in basal and stimulated saliva for male and female subjects are shown in Figures 5.9 and 5.10, respectively. The results show that the concentrations of Zn^{2+} in resting and stimulated conditions decreased significantly ($P<0.01$) beyond 35 years of age when compared to subjects of 20-34 years of age. Stimulated Zn^{2+} output was more or less the same for each age group except for 65-75 years of age where there was only a small decrease suggesting a diminished secretory capacity for advanced aged individuals. Multianova analysis for gender related differences was significant ($P<0.01$) for the variables basal and stimulated secretion indicating that female have less Zn^{2+} in saliva than male subjects. There were no gender-related differences for the variable secretory Zn^{2+}. There was no significant gender and age interactions in any of the variables suggesting that gender does not influence the age-induced changes for this ion.

Figures 5.11 and 5.12 show the levels of basal and stimulated Cl^- concentrations in the saliva of male and female subjects, respectively. The results show that in male subjects resting Cl^- levels increase with age, whereas stimulated Cl^- concentrations remain more or less the same. When analysing the differences between basal and stimulated Cl^- (Figure 5.11 B), the results reveal marked decreases in Cl^- output in male subjects of 55-64 and 65-75 years of age when compared to subjects of 20-54 years of age. In female subjects, both basal and stimulated Cl^- levels in saliva remain more or less the same for

each age group but basal Cl^- output was always higher compared to stimulated Cl^- level. When comparing the differences between stimulated and basal Cl^- levels for each age group, older female subjects (e.g. 55-64 and 65-75 years of age) show a greater decrease in stimulated saliva Cl^- output compared to female subjects of 20-34 years of age. Multianova testing for the effect of the variable gender proved to be significant ($P<0.01$) only for basal Cl^-. When testing for gender and age interactions the multi-Anova testing was significant for basal, stimulated and secretory Cl^- indicating that male and female subjects are affected by age in different ways.

Figures 5.13 A and 5.14 A show the basal and stimulated levels of Na^+ in male (see Figure 5.13) and female (see Figure 5.14) subjects. The differences in male and female subjects Na^+ outputs are shown in Figure 5.13 B and 5.14 B, respectively. The results show that young male subjects (20-34 and 35-44 years of age) have an increased Na^+ secretory capacity compared to aged male subjects (45-54, 54-65 and 65-75 years of age). With respect to female subjects, the young females secrete more Na^+ in saliva compared to reduced Na^+ secretion at 45-54, 55-64 and 65-75 years of age. Multianova testing for the effects of the variable gender and interactions between the variables gender and age were considered significant ($P<0.05$) only for basal and stimulated Na^+ concentration suggesting that male and female subjects have different basal and stimulated Na^+ salivary concentration are affected by age in different ways. There were no statistical significant differences for gender and gender versus age interactions when Na^+ secretory output was considered indicating that where Na^+ secretory capacity was considered there were no differences between male or female subjects and that both were influenced by age in similar ways.

The levels of K^+ in basal and stimulated saliva for male and female subjects are shown in Figures 5.15 A and Figure 5.16 A, respectively. The results show that as male and female subjects aged, they secreted more or less the same basal salivary K^+ but stimulated K^+ concentration tended to decrease and was significantly ($P<0.05$) lower in 54-65 and 65-75 year old male and female subjects when compared to 20-34 and 35 44 years of age. Figures 5.15 B and 5.16 B show the differences between stimulated and basal K^+ levels in saliva, for male and female subjects, respectively. The results reveal that both male and female subjects secrete significantly ($P<0.05$) less stimulated K^+ in saliva as they aged (45-75 years of age) compared to young subjects (20-44 years of age). The decrease was much more pronounced in female subjects at age groups 55-64 and 65-75 years of age compared to 20-54 years of age. Multianova testing for

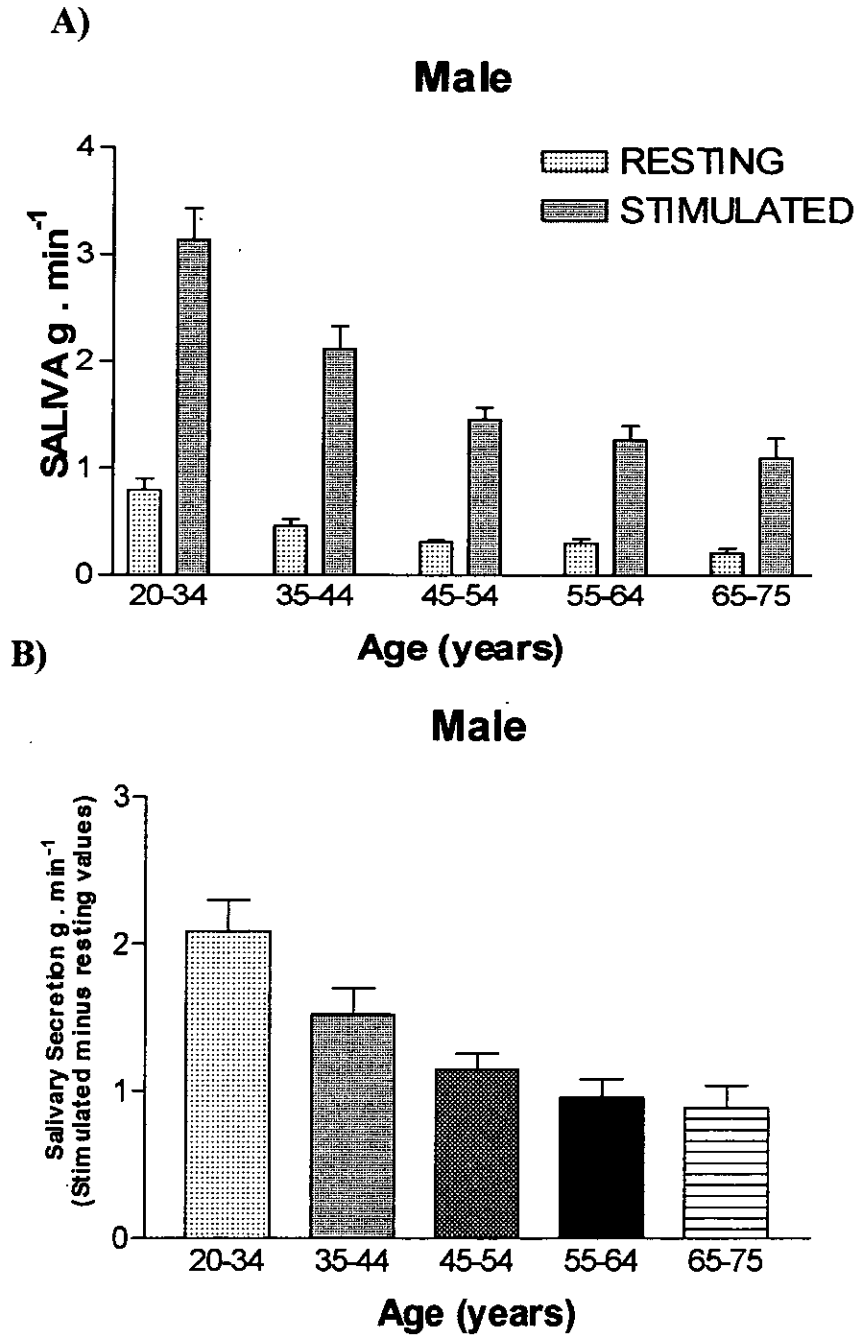


Figure 5.1 A)- Histograms showing the mean (+- SEM) for resting and stimulated salivary flow whole saliva in different age groups for male subjects. B) Histograms showing the mean (+- SEM) for salivary secretory capacity (stimulated minus resting salivary flow) in different age groups for male subjects. Note that ageing is associated with a decrease in stimulated salivary output. In this and up to Figure 5.16, n=15 for each age group.

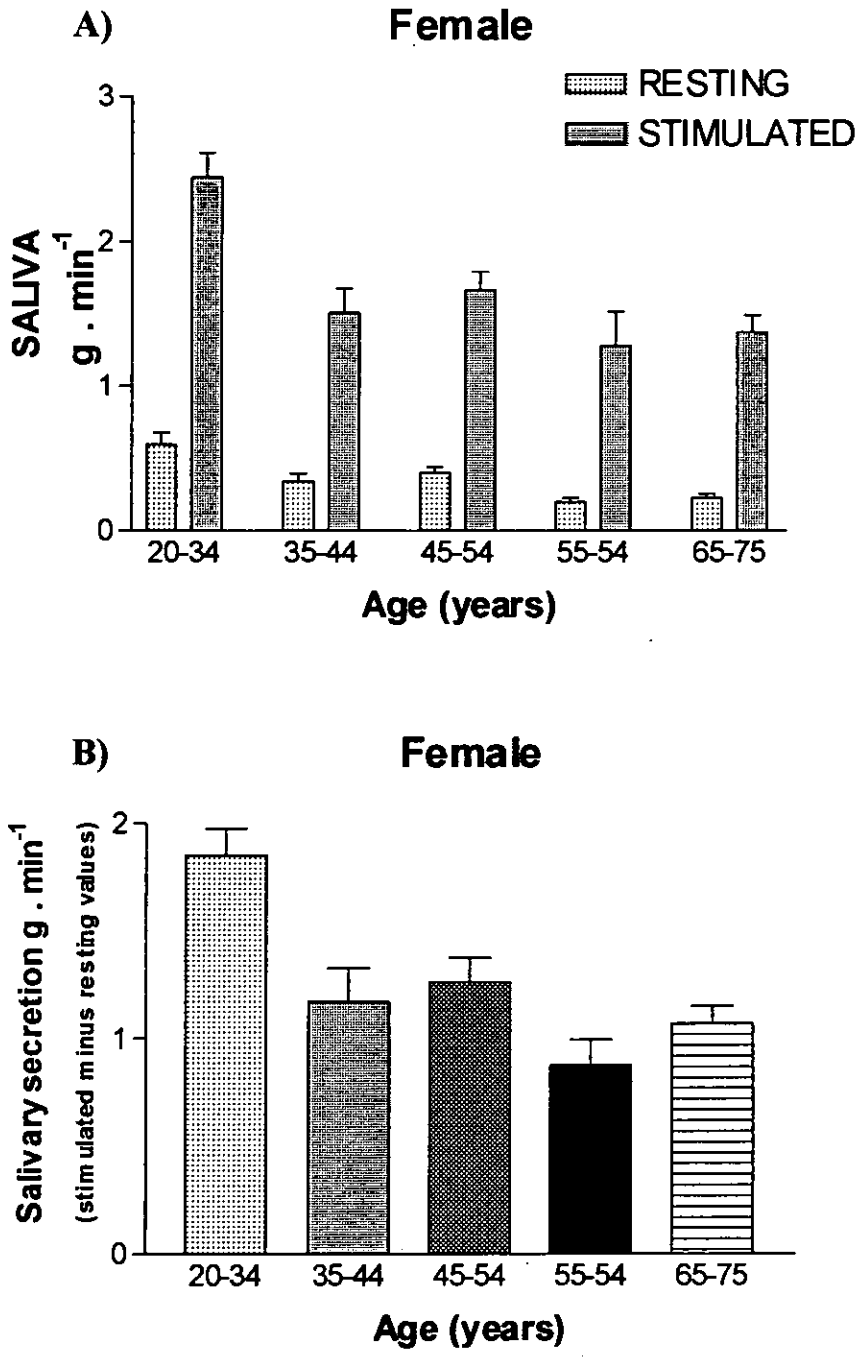


Figure 5.2 A)- Histograms showing the mean (+- SEM) for resting and stimulated salivary flow in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for salivary secretory capacity (stimulated minus resting salivary flow) in different age groups for female subjects. Note that ageing is associated with a decrease in stimulated salivary output; n=15 for each age group.

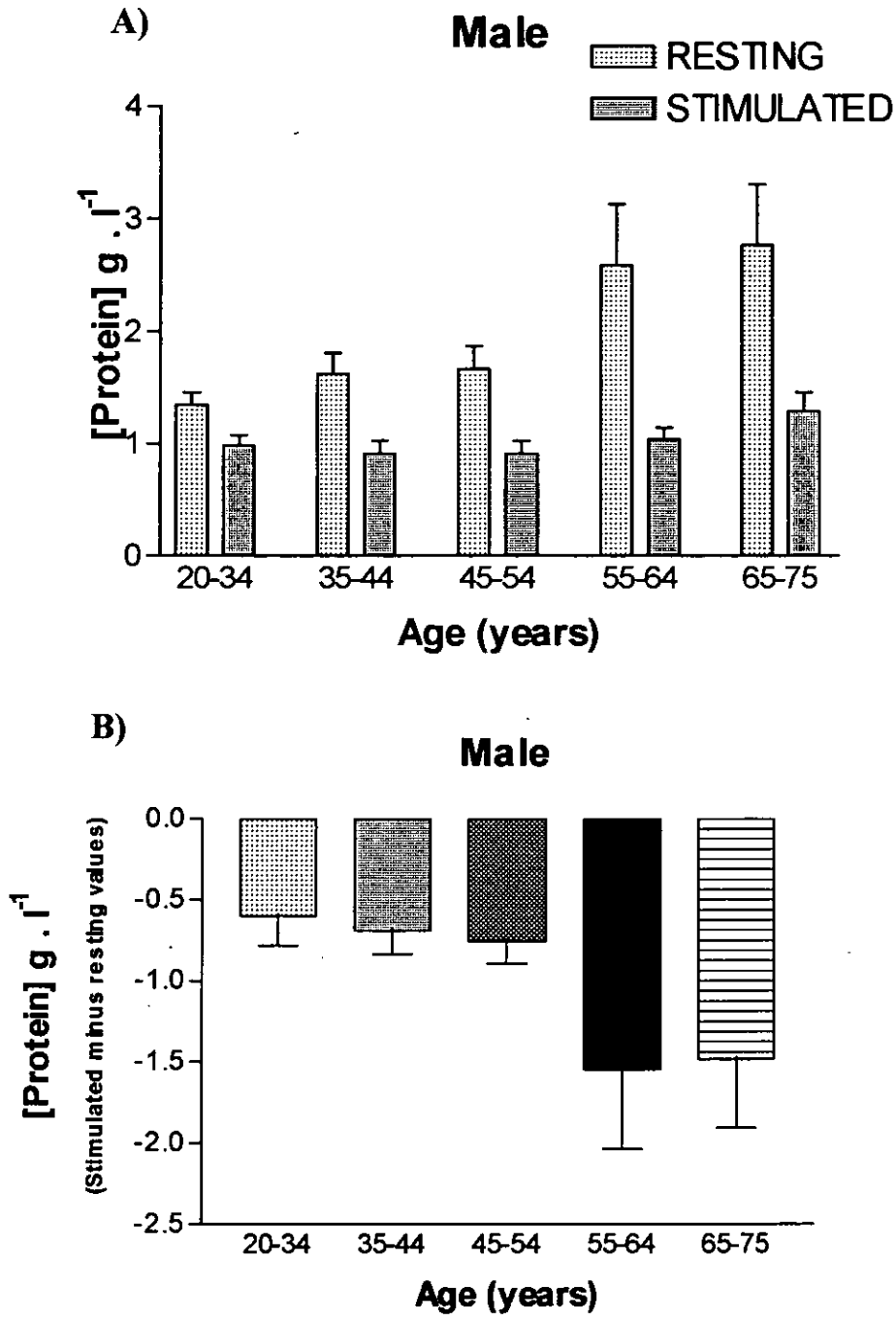


Figure 5.3 A)- Histograms showing the mean (+- SEM) for resting and stimulated total protein concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total protein salivary secretory capacity (stimulated minus resting salivary protein concentration) in different age groups for male subjects. Note that protein output for resting protein output is increased with age. (n=15).

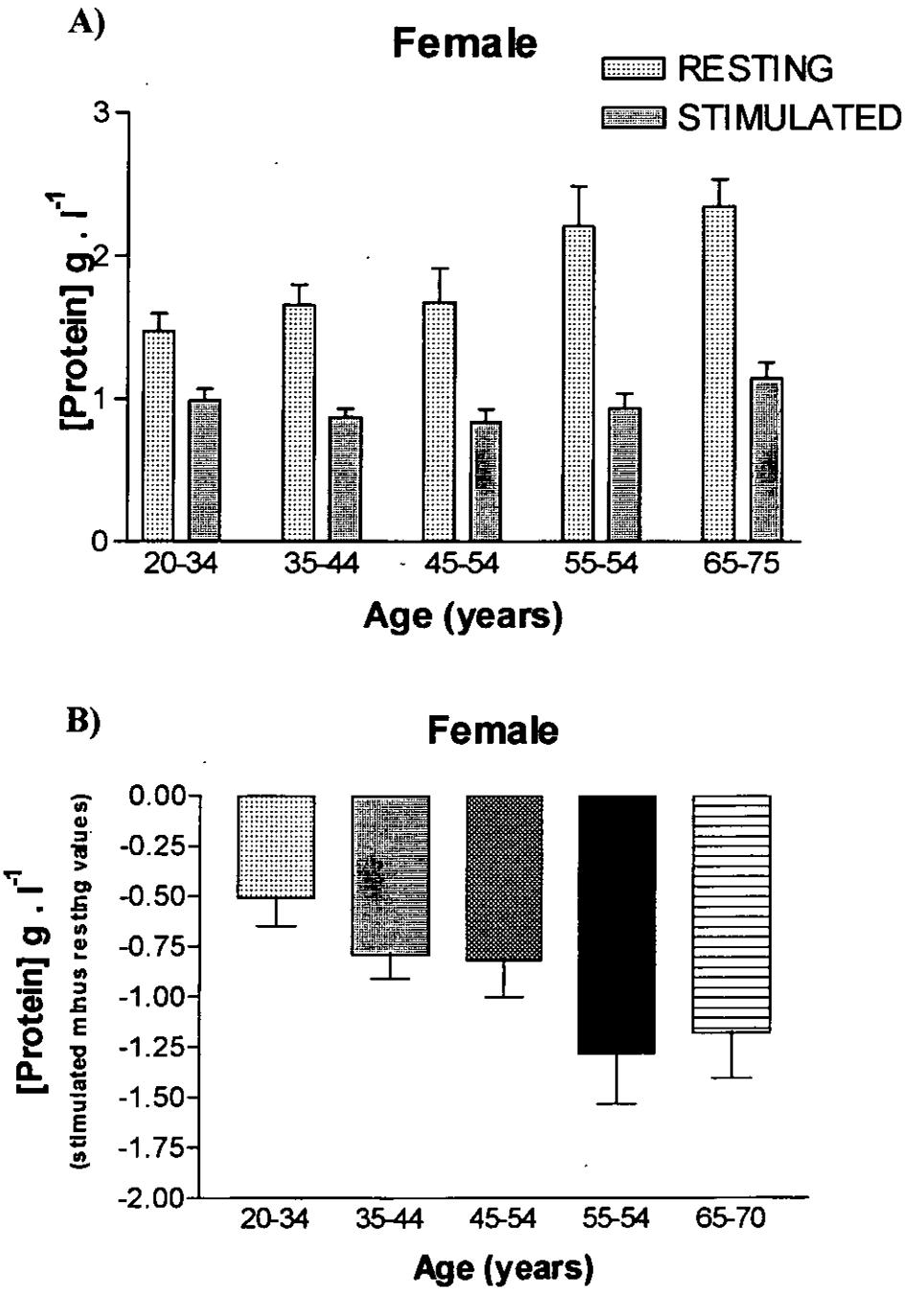


Figure 5.4 A)- Histograms showing the mean (+- SEM) for resting and stimulated total protein concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total protein salivary secretory capacity (stimulated minus resting salivary protein concentration) in different age groups for female subjects. Note the increase in protein output with age. (n=15).

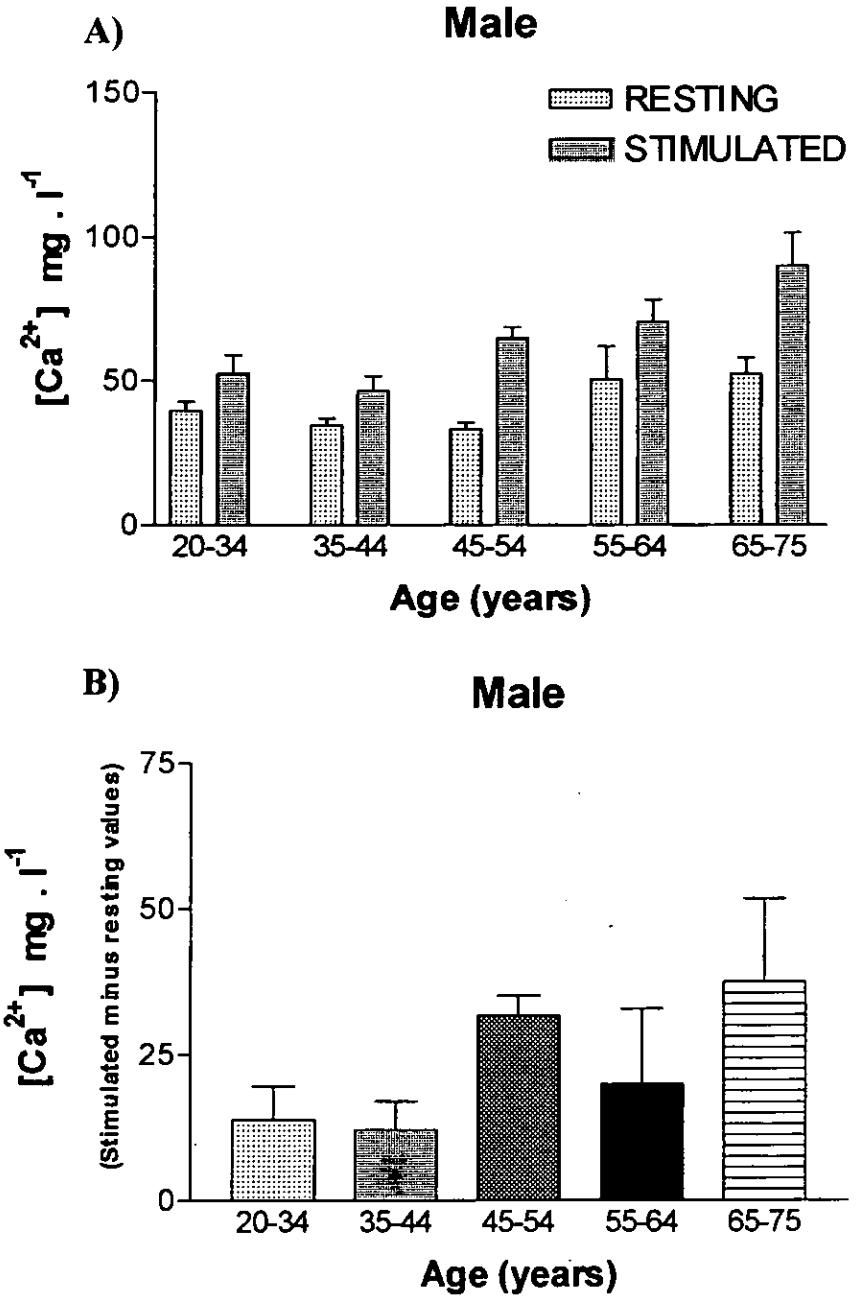


Figure 5.5 A) Histograms showing the mean (+- SEM) for resting and stimulated total calcium concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total salivary calcium secretory capacity (stimulated minus resting salivary calcium concentration) in different age groups for male subjects. (n=15).

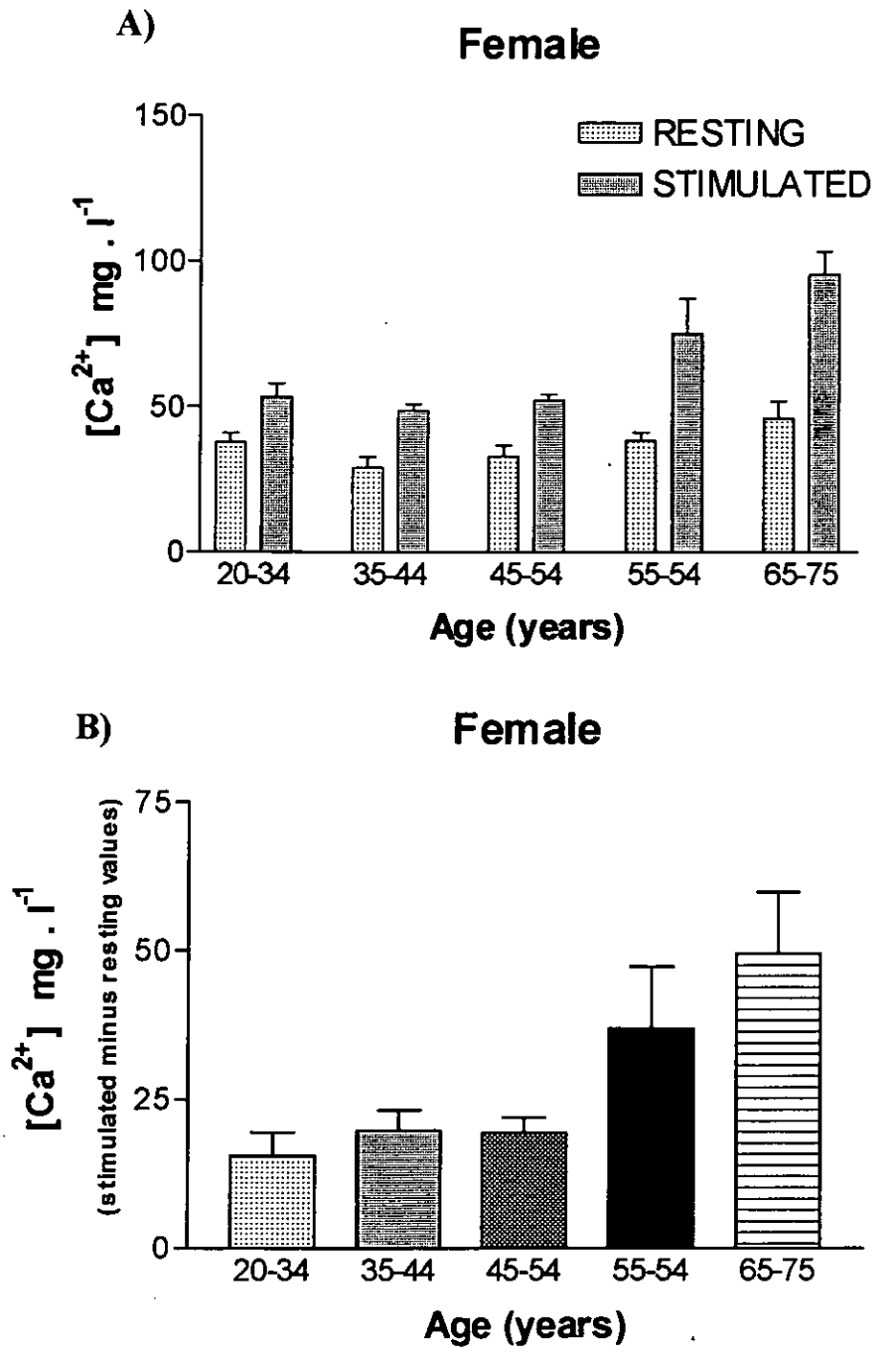


Figure 5.6 A)- Histograms showing the mean (+- SEM) for resting and stimulated whole saliva total calcium concentrations in different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary calcium secretory capacity (stimulated minus resting salivary calcium concentrations) in different age groups for female subjects. (n=15).

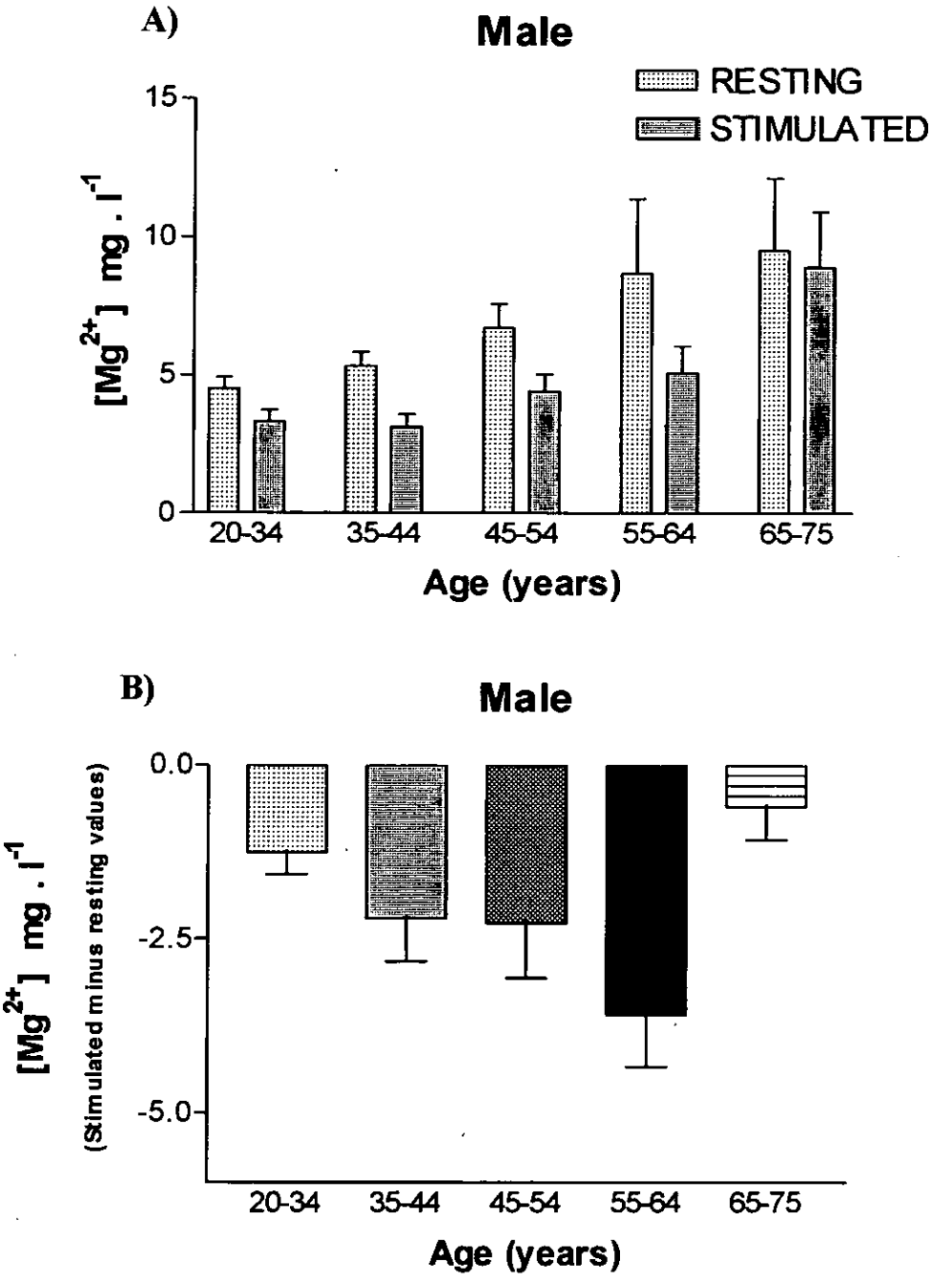


Figure 5.7 A)- Histograms showing the mean (+- SEM) for resting and stimulated total magnesium concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total salivary magnesium secretory capacity (stimulated minus resting salivary magnesium concentration) in different age groups for male subjects. (n=15).

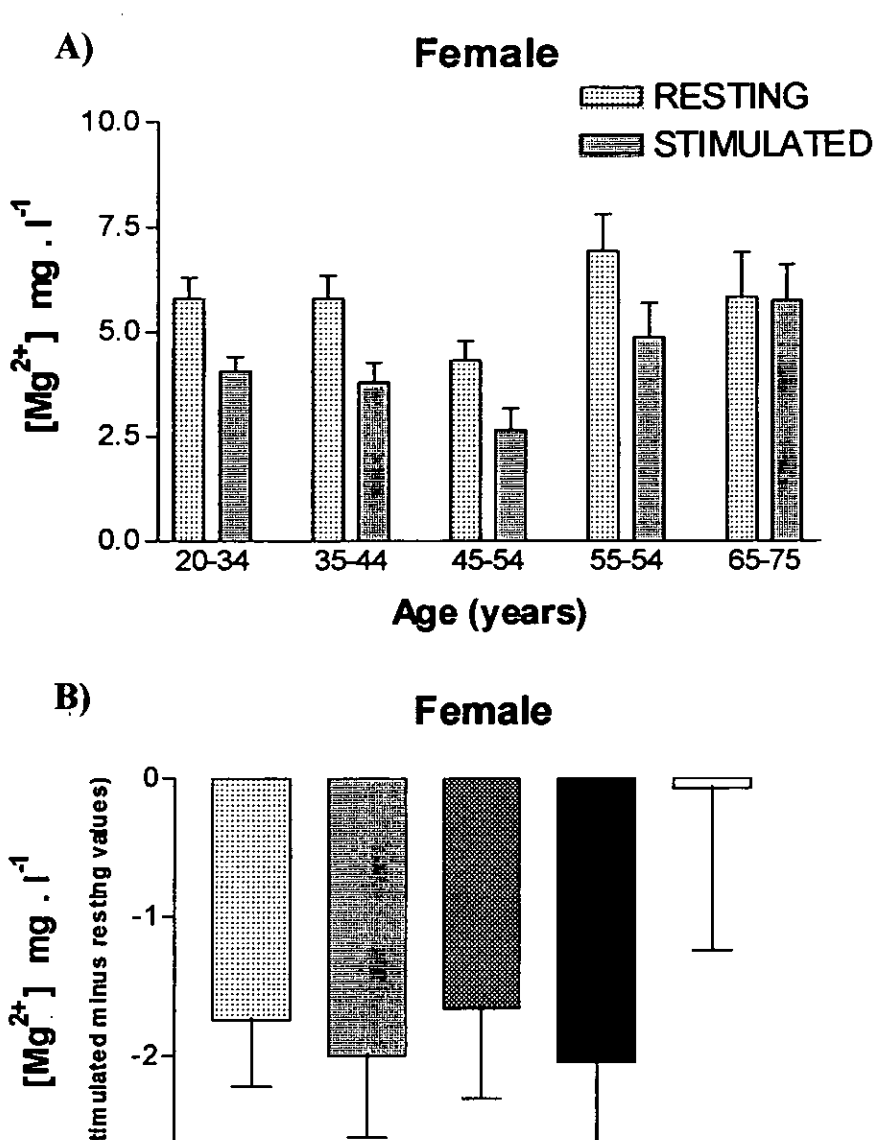


Figure 5.8 A)-Histograms showing the mean (+- SEM) for resting and stimulated total magnesium concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary magnesium secretory capacity (stimulated minus resting salivary magnesium concentration) in different age for groups female subjects. (n=15)

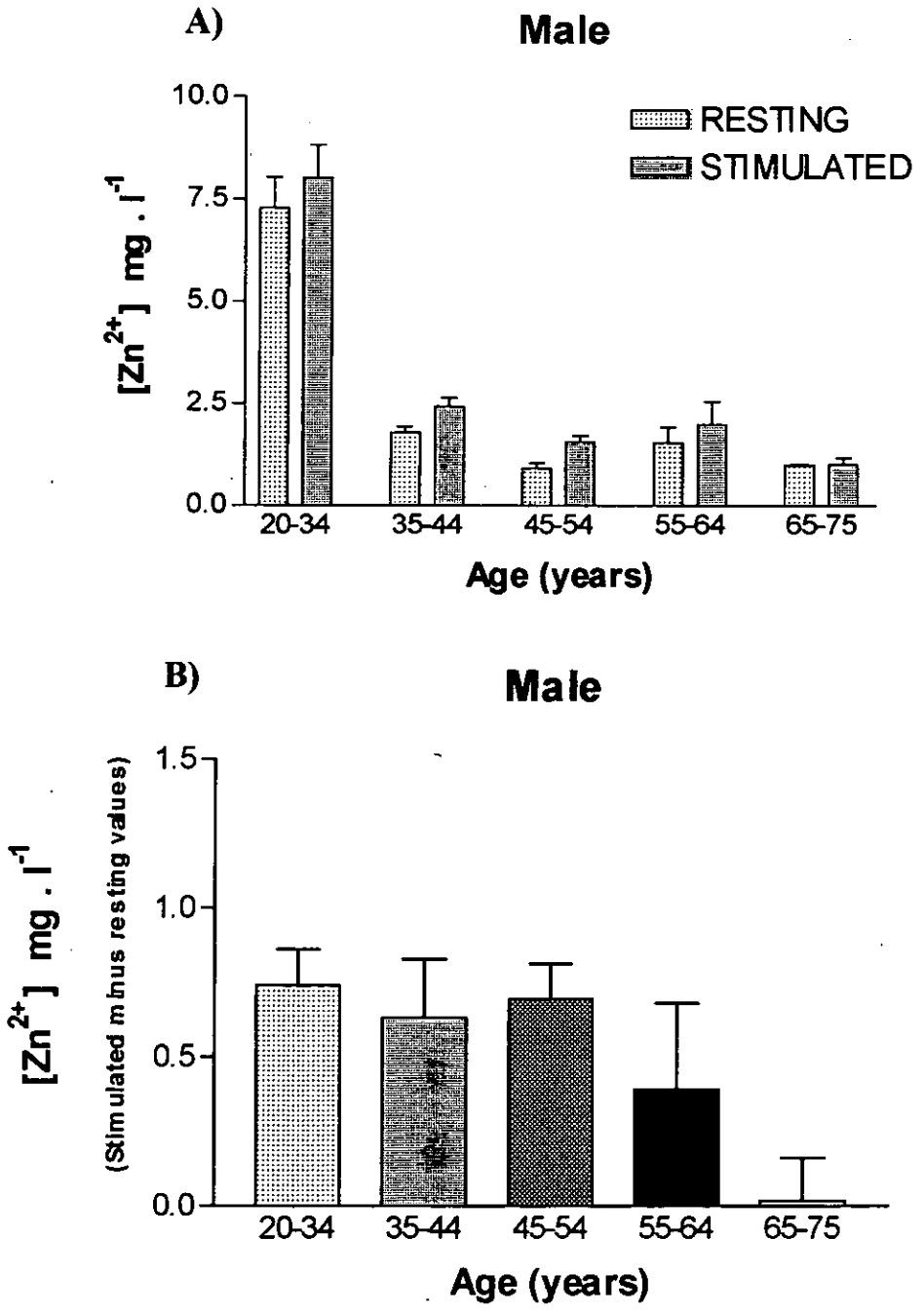


Figure 5.9 A)- Histograms showing the mean (+- SEM) for resting and stimulated total zinc concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total salivary zinc secretory capacity (stimulated minus resting salivary zinc concentration) in different age groups for male subjects. (n=15).

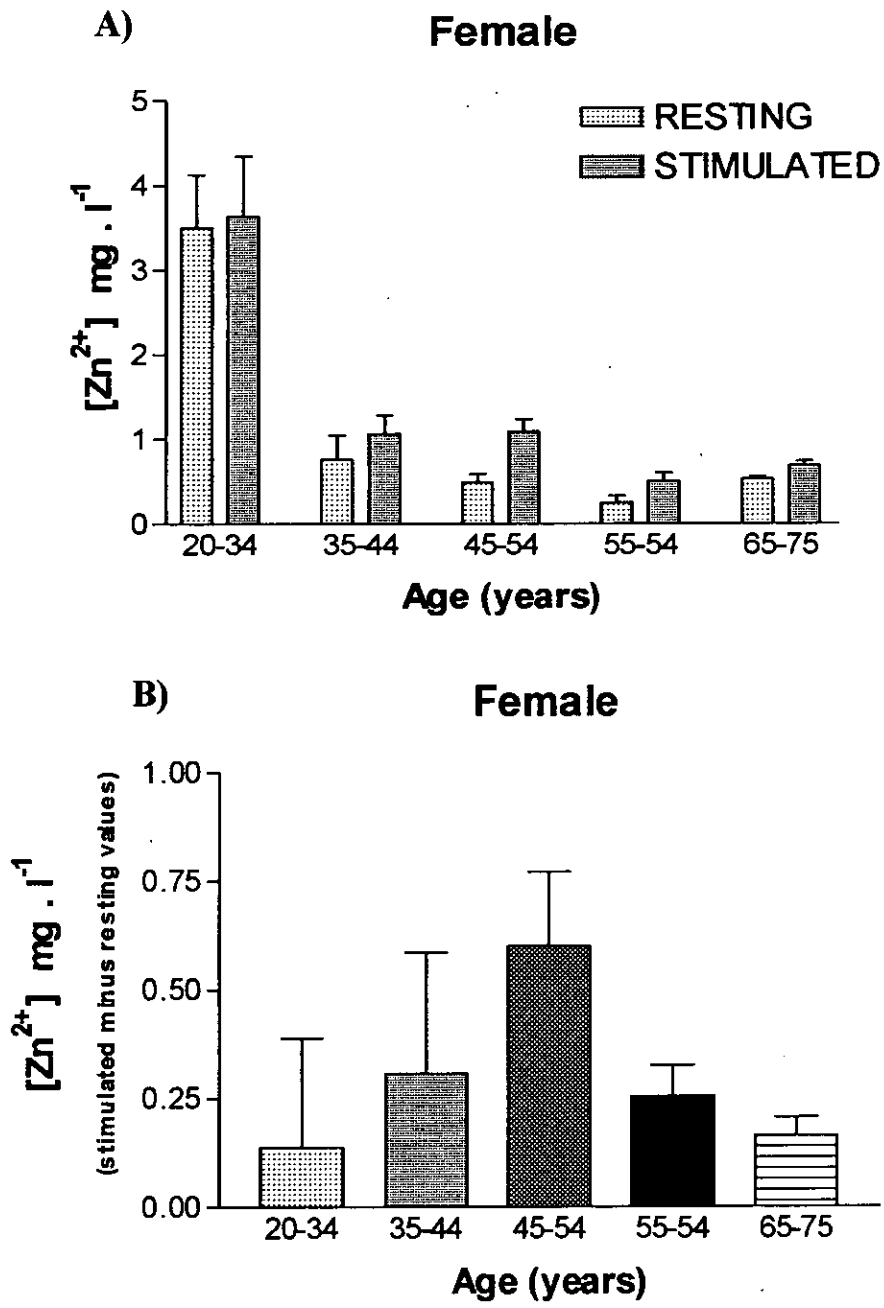


Figure 5.10 A)- Histograms showing the mean (+- SEM) for resting and stimulated total zinc concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary zinc secretory capacity (stimulated minus resting salivary zinc concentration) in different age groups for female subjects. (n=15).

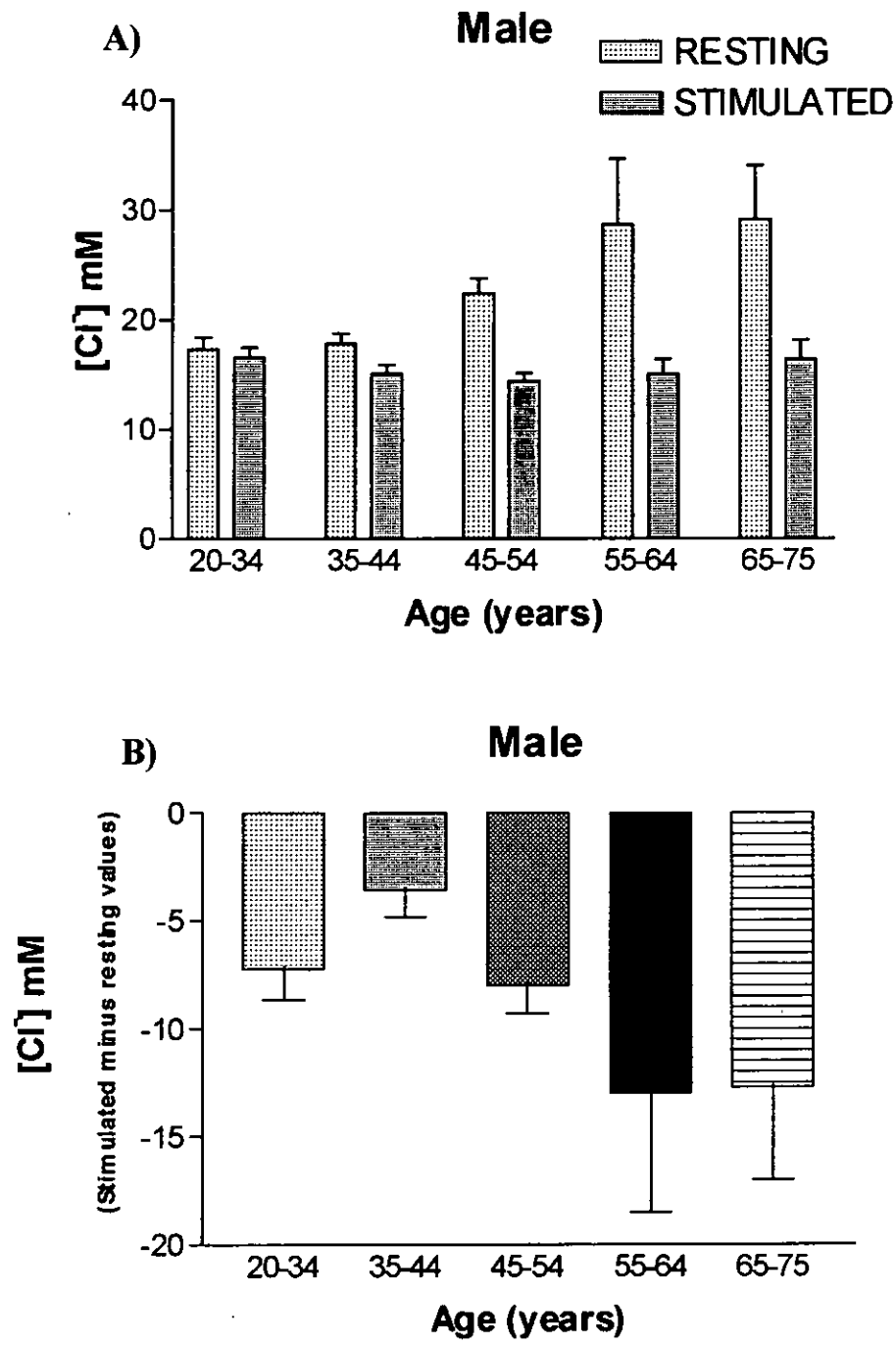


Figure 5.11 A)- Histograms showing the mean (+- SEM) for resting and stimulated total chloride concentrations in whole saliva of different age groups for male subjects. B) Histogram showing the mean (+- SEM) for total salivary chloride secretory capacity (stimulated minus resting salivary chloride concentration) in different age groups for male subjects. (n=15).

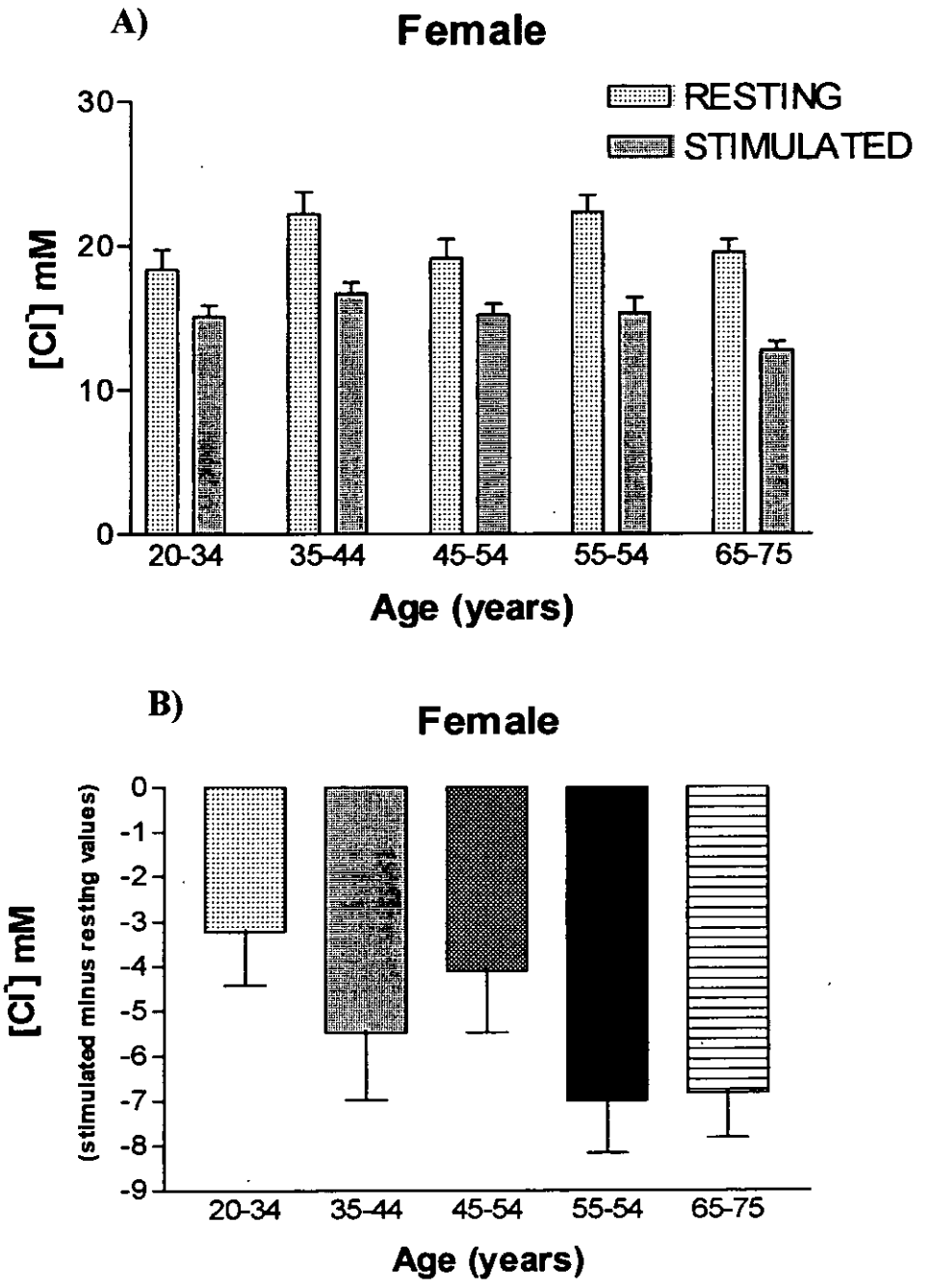


Figure 5.12 A)- Histograms showing the mean (+- SEM) for resting and stimulated total chloride concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary chloride secretory capacity (stimulated minus resting salivary chloride concentration) in different age groups for female subjects. (n=15).

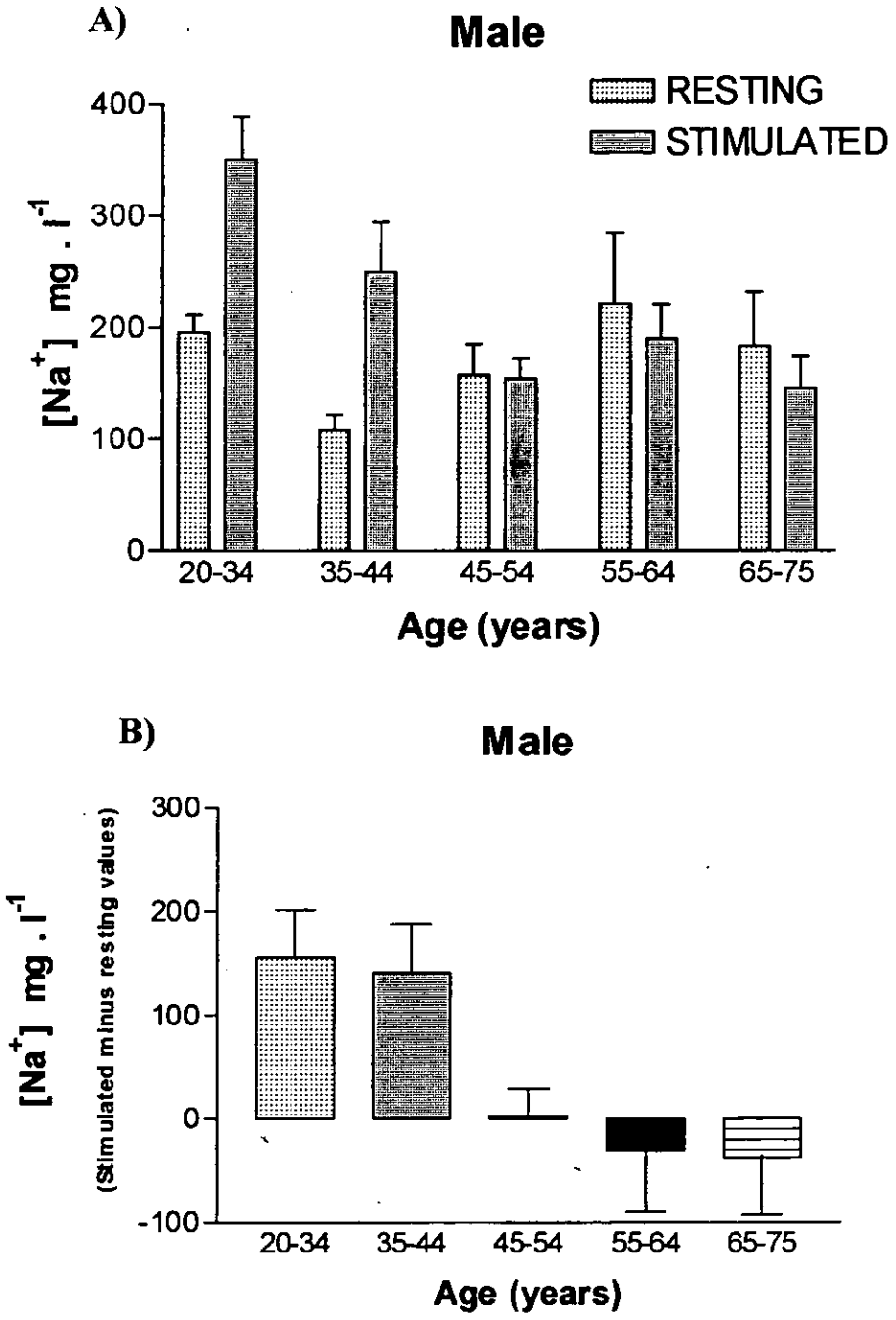


Figure 5.13 A)- Histograms showing the mean (+- SEM) for resting and stimulated total sodium concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total salivary sodium secretory capacity (stimulated minus resting salivary sodium concentration) in different age groups for male subjects. (n=15).

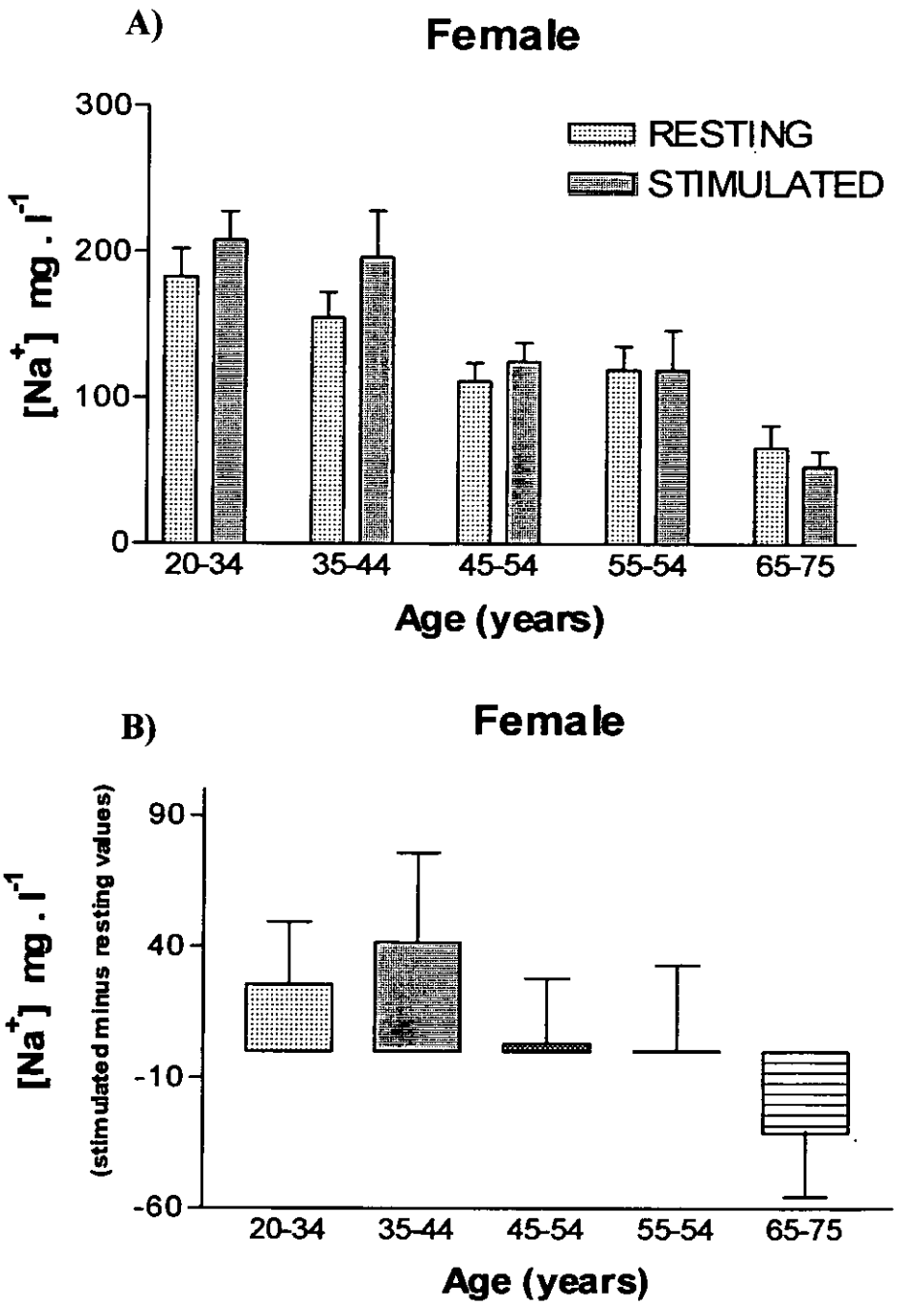


Figure 5.14 A)- Histograms showing the mean (+- SEM) for resting and stimulated total sodium concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary sodium secretory capacity (stimulated minus resting salivary sodium concentration) in different age groups for female subjects. (n=15).

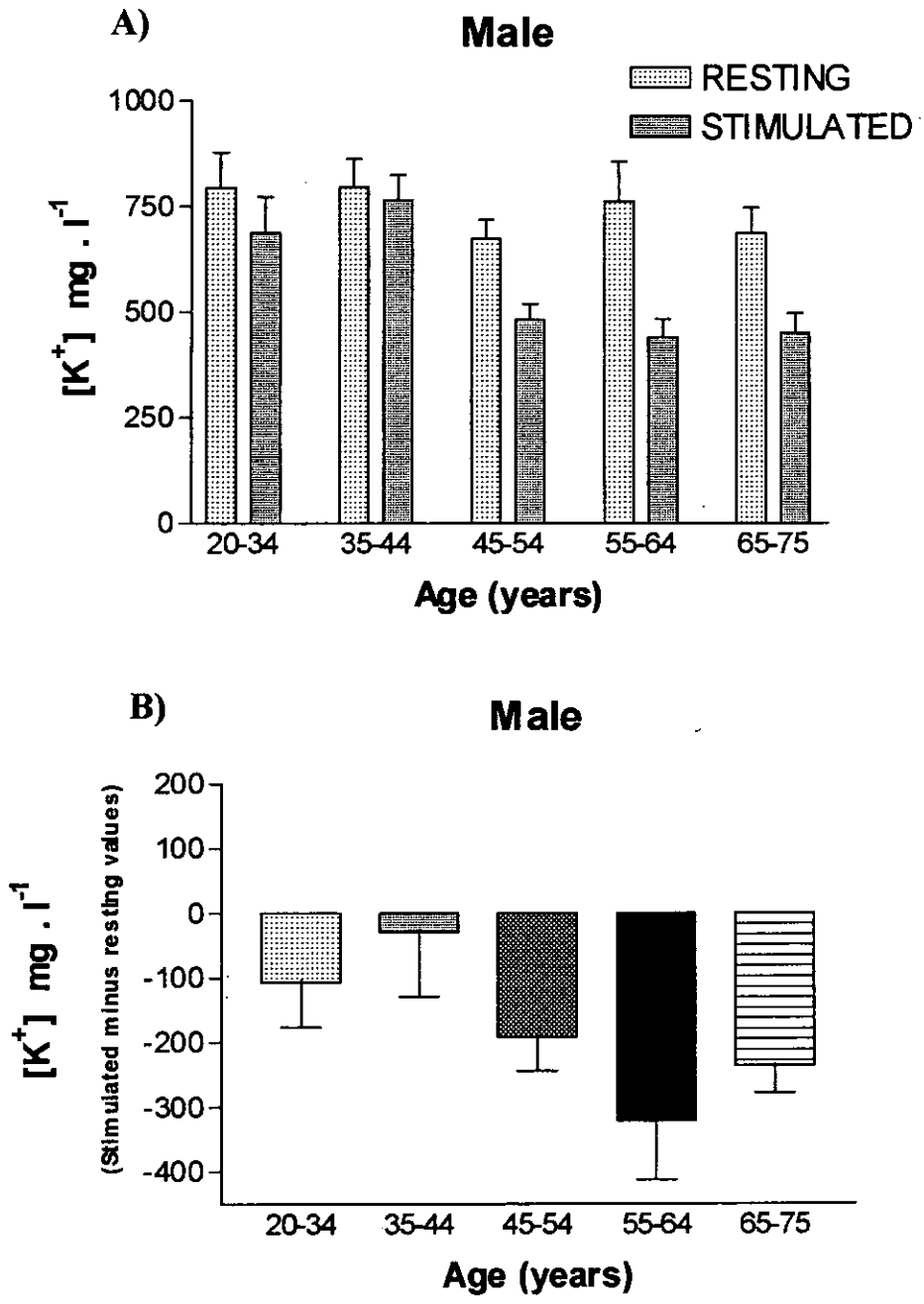


Figure5.15 A)- Histograms showing the mean (+- SEM) for resting and stimulated total potassium concentrations in whole saliva of different age groups for male subjects. B) Histograms showing the mean (+- SEM) for total salivary potassium secretory capacity (stimulated minus resting salivary potassium concentration) in different age groups for male subjects. (n=15).

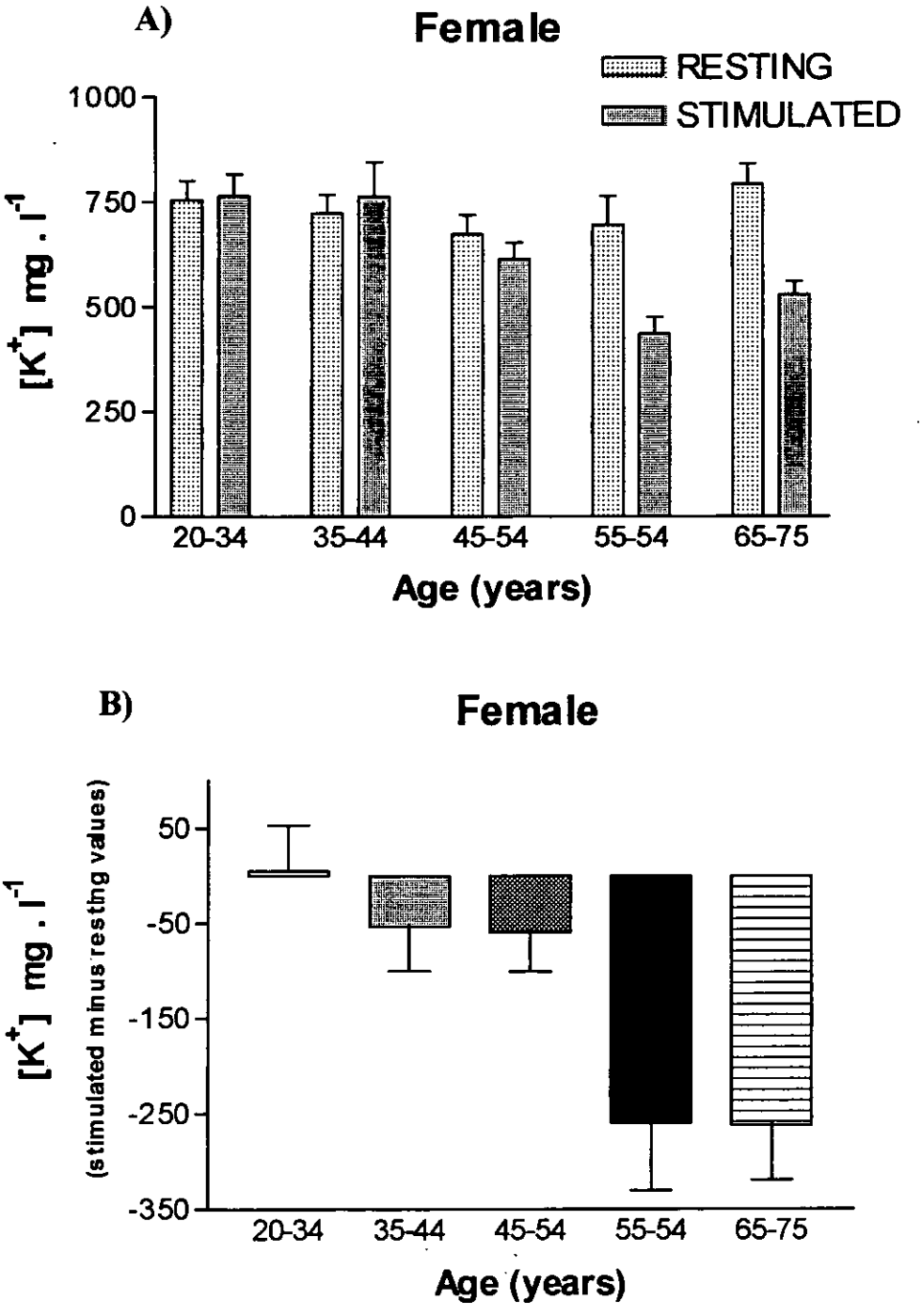


Figure 5.16 A)- Histograms showing the mean (+- SEM) for resting and stimulated total potassium concentrations in whole saliva of different age groups for female subjects. B) Histograms showing the mean (+- SEM) for total salivary potassium secretory capacity (stimulated minus resting salivary potassium concentration) in different age groups for female subjects. (n=15).

differences between different genders and interaction between gender and age failed to demonstrate any statistical differences for the variables basal, stimulated and secretory K^+ suggesting that there are no gender-related differences and that male and female subjects are affected in the same way by the age factor.

Taken together, the results show marked changes in the ions in saliva at each age group with the increase of some ions and decrease of the others.

5.4.2 Effects of types I and II diabetes mellitus on human salivary secretion

In this series of experiments, an investigation was made of the effects of type I and type II diabetes on human salivary secretion, and the quantity and quality of saliva compared to the respective age-matched controls for each group. For both type I and type II diabetic subjects an equivalent number of age-matched controls (e.g. range of years 20-30 years for type I) and (range of age 40-55 years for type II) subjects were employed.

Figure 5.17 A shows basal and stimulated flow rates in age-matched healthy controls and diabetic patients. The results show that basal and stimulated secretory rates saliva were significantly ($P<0.05$) decreased for type I and type II diabetic patients when compared to age-matched controls. However, the magnitude of the pathologic decrease in basal and stimulated saliva was less pronounced in type II diabetes compared to type I diabetes for both basal and stimulated saliva. The differences (secretory capacity) between stimulated and basal salivary secretory rates are shown in Figure 5.17 B. The results revealed significant ($P<0.05$) decreases in salivary output for both type I and Type II diabetics compared to their respective controls with a more pronounced decrease in type I diabetes.

Figure 5.18 A shows the amount of protein in the saliva of control and diabetic patients following basal and stimulated secretion. The results show that diabetic patients have significantly ($P>0.05$) more proteins in basal saliva compared to stimulated saliva. In addition, diabetic patients have significantly ($P<0.05$) more proteins in their basal and stimulated salivas compared to the respective healthy age-matched controls. The differences between stimulated and basal protein in saliva for the four groups are shown in Figure 5.18 B. The results reveal significant ($P<0.05$) decreases in stimulated protein output in type I and type II diabetes. Type I diabetes showed a significant ($P<0.01$)

decrease in protein output compared to age-matched controls while there were no differences between type II diabetes and their respective controls for protein output.

Figure 5.19 A shows the levels of Ca^{2+} in basal and stimulated saliva in the respective age-matched controls and type I and type II diabetic patients. The results revealed that the levels of Ca^{2+} increase significantly ($P<0.05$) in both basal and stimulated saliva for type I and type II diabetic patients. In relation to the differences between basal and stimulated saliva, type I diabetics produce significantly ($P<0.05$) more Ca^{2+} in saliva compared to the control. With regards to type II diabetics, both control and diabetic secrete the same amount of Ca^{2+} in saliva (Figure 5.19 B).

Figure 5.20 shows the level of Mg^{2+} in basal and stimulated saliva for age-matched controls and diabetic patients. The results show that basal and stimulated Mg^{2+} concentration are significantly ($P<0.05$) elevated in type I and type II diabetic patients compared to respective healthy age-matched controls. However, when considering the Mg^{2+} output, both type I and type II diabetics secrete significantly ($P<0.01$) less Mg^{2+} in saliva (Figure 5.20 B). Taken together, the results show a reciprocal relationship between the outputs of Ca^{2+} and Mg^{2+}. There was an increase in Ca^{2+} and a decrease in Mg^{2+} outputs in the saliva of diabetic patients.

The levels of Zn^{2+} in saliva during basal and stimulated conditions for healthy age-matched controls and diabetic patients are shown in Figure 5.21 A. The results show that diabetics secrete less Zn^{2+} in saliva during basal and stimulated conditions. The inhibitory effect of diabetes was much more pronounced for the type I diabetic compared to type II. The differences between stimulated and basal Zn^{2+} levels in saliva for control and diabetic patients are shown in Figure 5.21 B. Again the results confirm that diabetics secrete less Zn^{2+} in saliva.

Figure 5.22 shows the levels of K^+ in basal and stimulated saliva for age-matched healthy controls and diabetic patients. The results show that diabetics secrete more ($P<0.05$) K^+ in basal and stimulated saliva than their age-matched controls. In addition, the results show that diabetics secrete more K^+ in basal saliva compared to stimulated conditions. When comparing the differences between stimulated and basal K^+ concentrations in saliva for control and diabetic patients (see Figure 5.22 B), the results revealed a significant ($P<0.05$) decrease in K^+ output in the saliva of diabetic patients compared to their respective age-matched controls.

Figure 5.23 shows the levels of Na^+ in basal and stimulated saliva for age-matched controls and diabetic patients. The results show that for type 1 diabetics and their

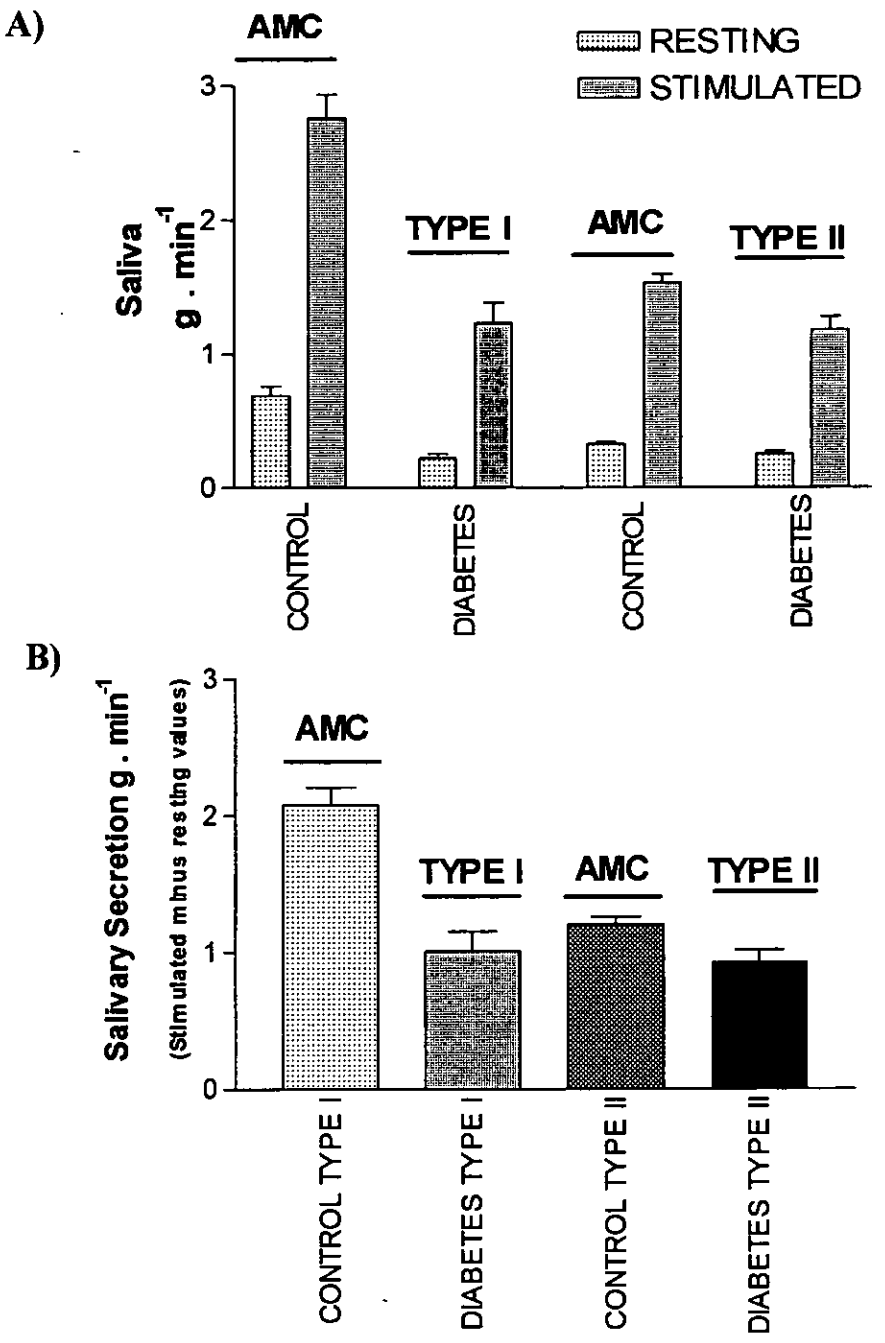


Figure 5.17 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary flow in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary secretory capacity (stimulated minus resting salivary flow) in type I and type II diabetic patients and their respective age-matched controls. In Figures 5.17 to 5.24 n=15 in each control and diabetic group.

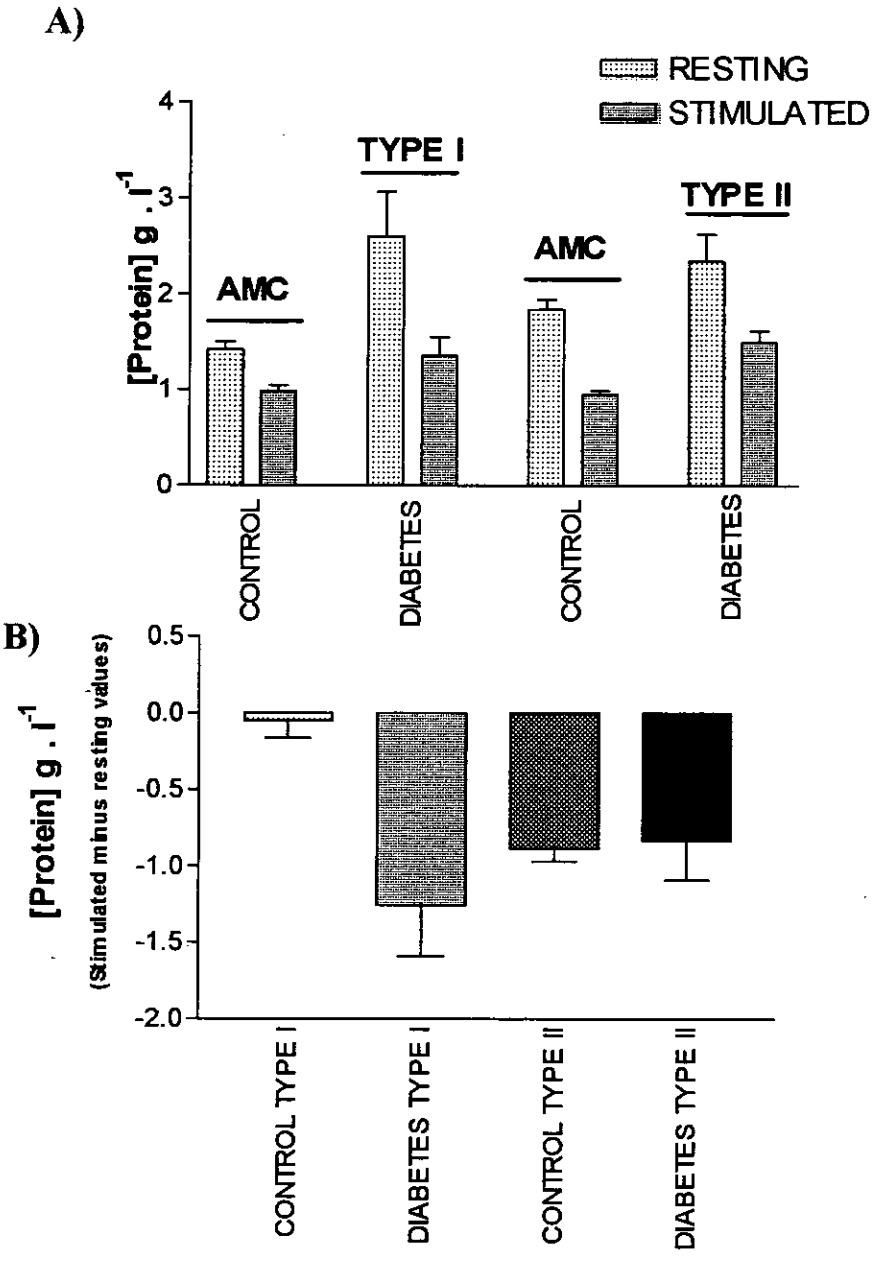


Figure 5.18 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary protein secretion in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary protein secretory capacity (stimulated minus resting salivary protein) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

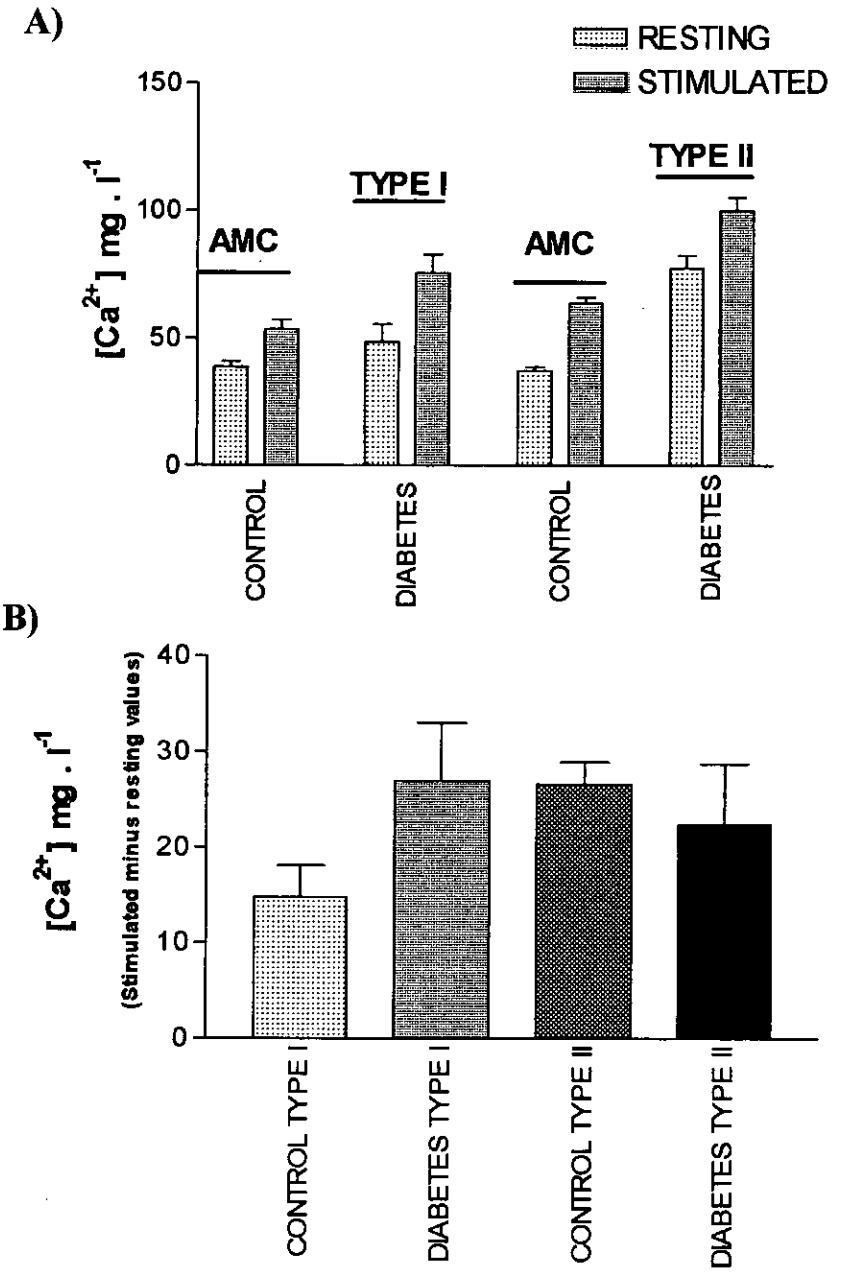


Figure 5.19 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary calcium concentrations in whole saliva of type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary calcium concentrations (stimulated minus resting salivary calcium) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

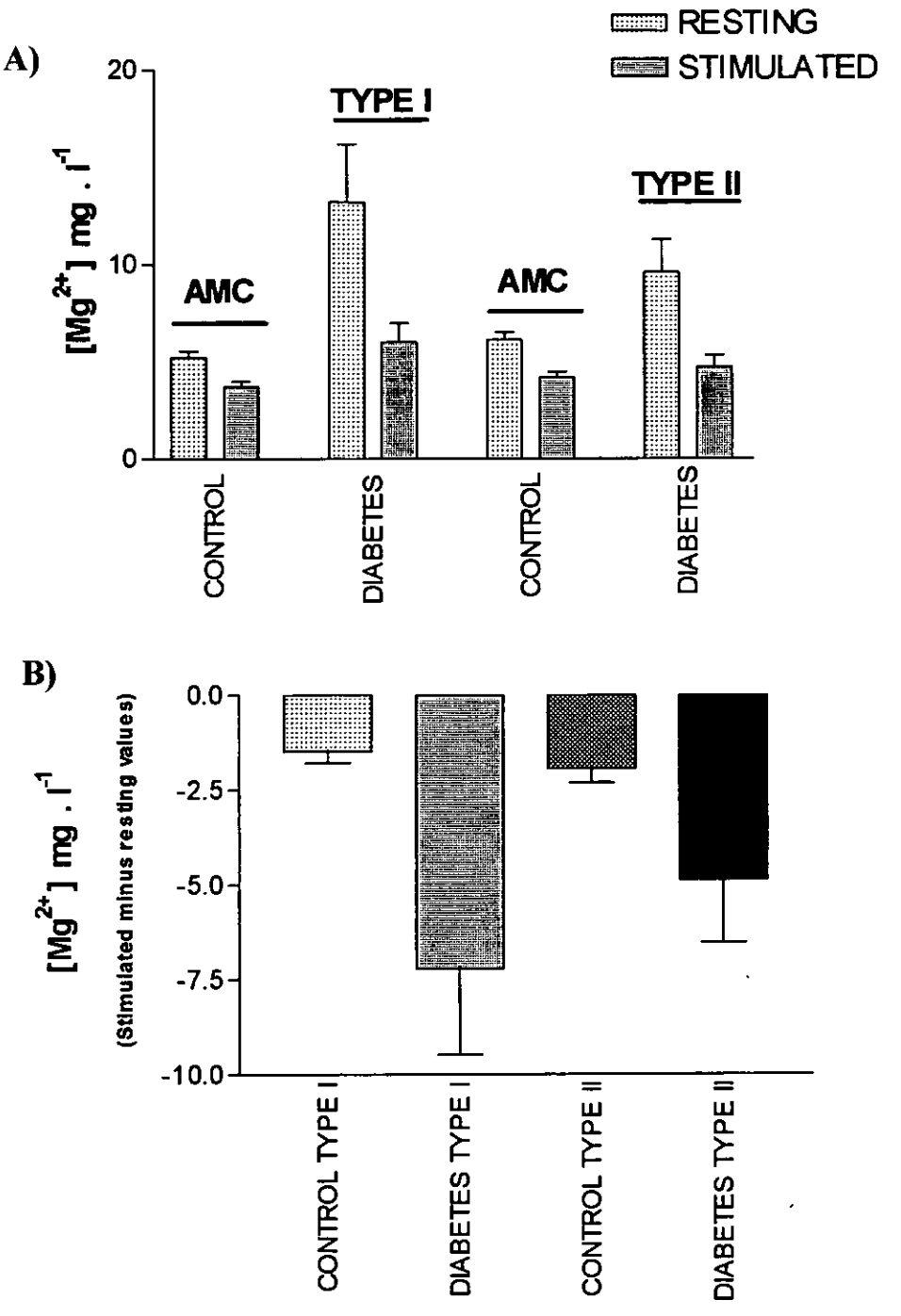


Figure 5.20 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary magnesium concentrations in whole saliva of type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary magnesium concentration (stimulated minus resting salivary magnesium) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

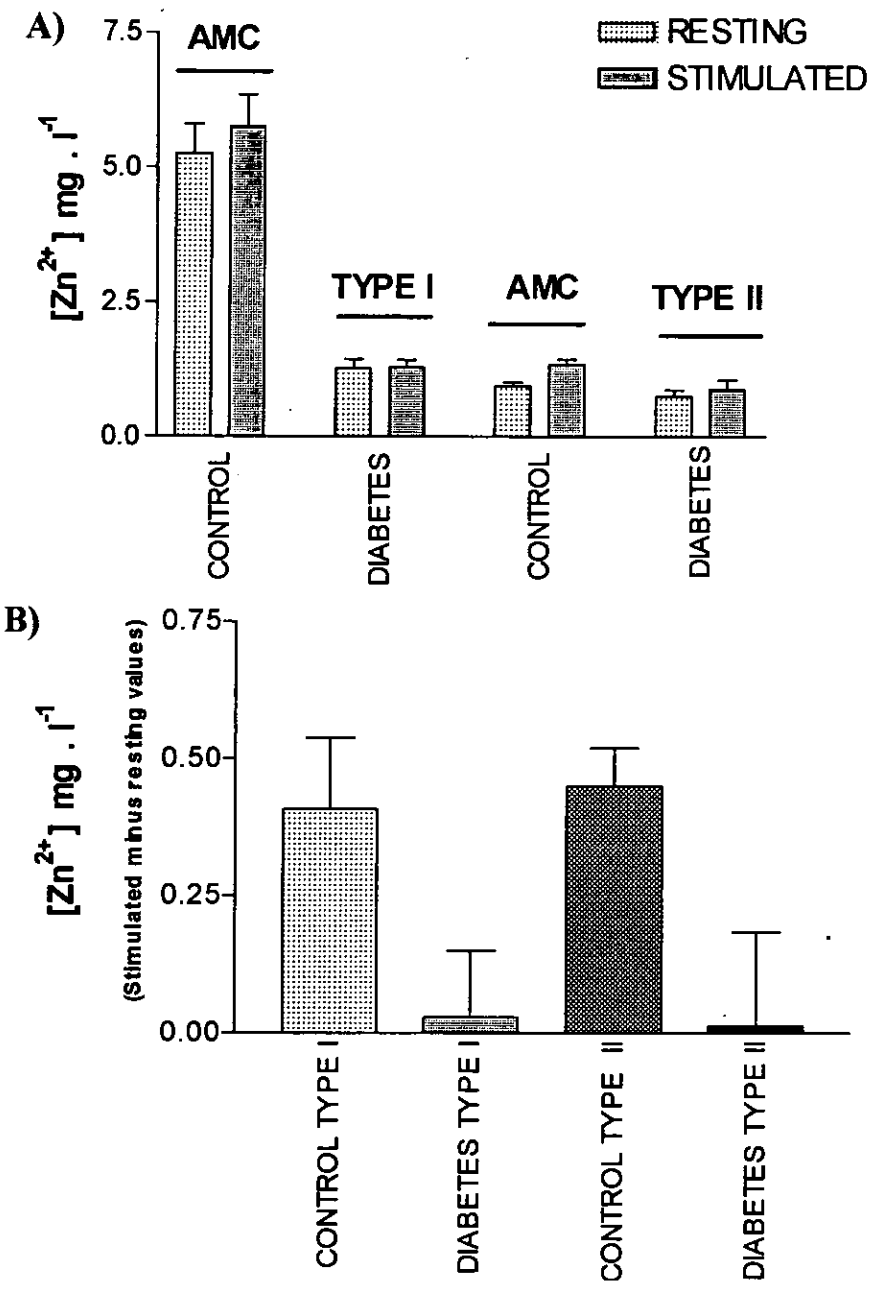


Figure 5.21 A)-Histograms showing the mean (+- SEM) of resting and stimulated zinc concentrations in whole saliva in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary zinc concentrations (stimulated minus resting salivary zinc) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

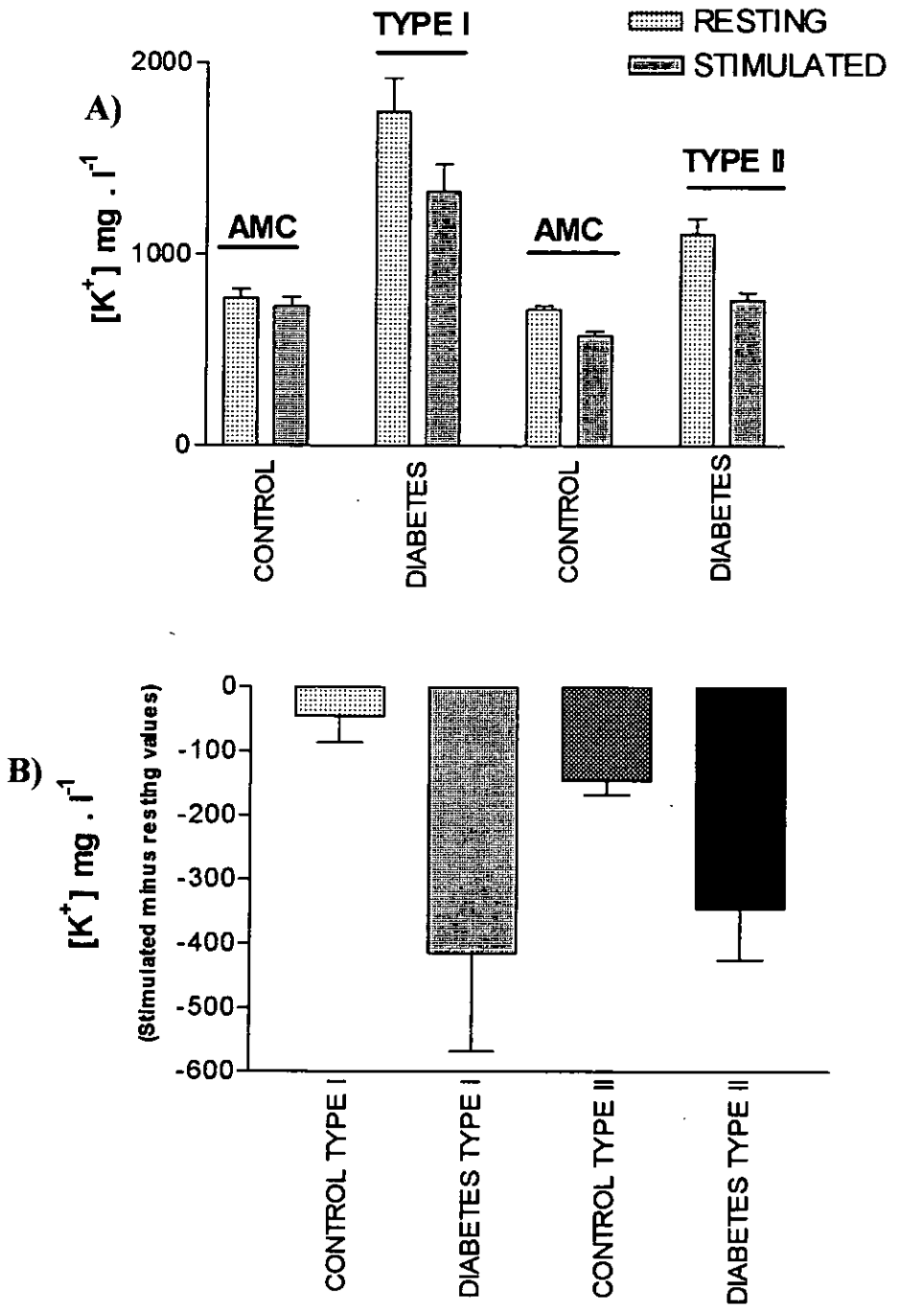


Figure 5.22 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary potassium concentrations of whole saliva in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary potassium concentrations (stimulated minus resting salivary potassium) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

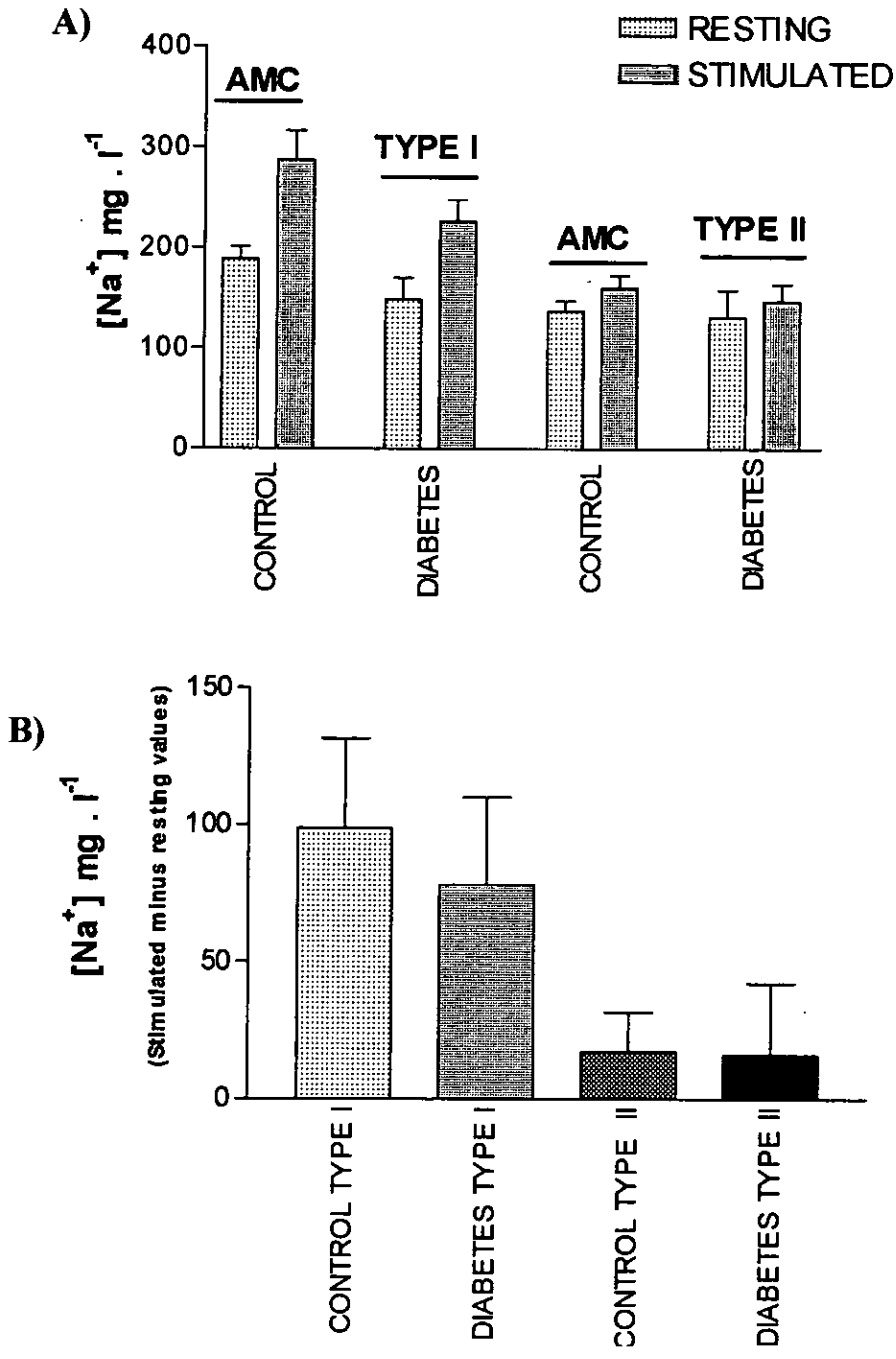


Figure 5.23 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary sodium concentrations of whole saliva in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary concentrations capacity (stimulated minus resting salivary sodium) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

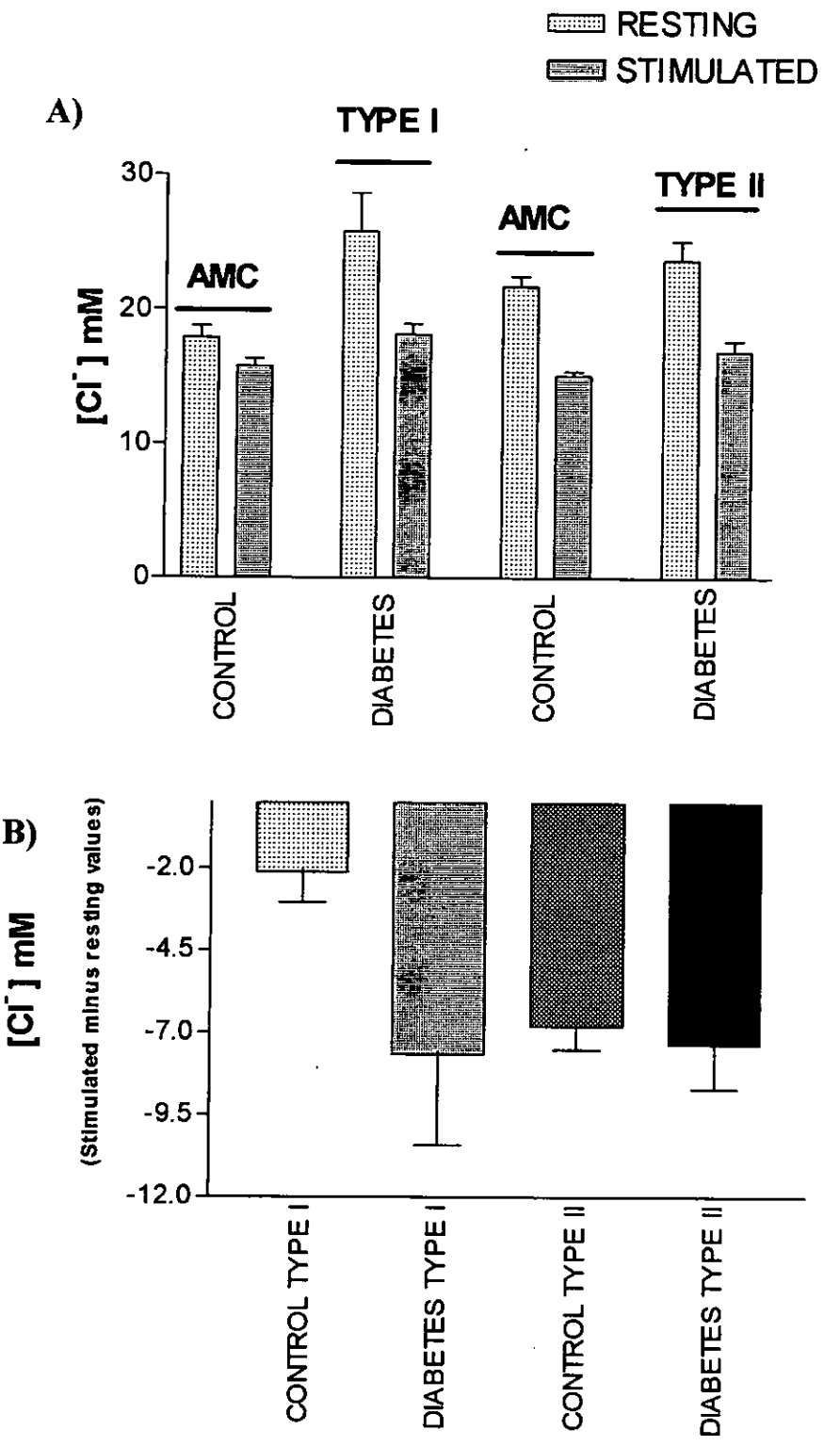


Figure 5.24 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary chloride concentrations of whole saliva in type I and type II diabetic patients and their respective age-matched controls (AMC). B)- Histograms showing the mean (+- SEM) of the salivary chloride concentrations (stimulated minus resting salivary chloride) in type I and type II diabetic patients and their respective age-matched controls. (n=15).

respective healthy controls, there was more Na^+ in stimulated saliva compared to basal saliva. However, there were no significant differences for either basal, stimulated or their differences in Na^+ salivary concentrations for type 1 diabetics when compared to their age-matched controls. With respect to Type 2 diabetics, they produce more or less the same amount of Na^+ in basal and stimulated saliva, similarly to their respective control.

Figure 5.24 shows the levels of Cl^- in basal and stimulated saliva for the respective healthy age-matched controls and diabetic patients. The results show that stimulated saliva contains less Cl^- than basal saliva. For type I diabetics salivary Cl^- was significantly ($P<0.05$) elevated in basal, stimulated (see Figure 5. 24 A) and secretory (see Figure 5.24 B) salivary concentrations compared to their healthy age-matched controls. For type II diabetics there were no significant differences for any of the salivary parameters tested when compared to their age-matched controls.

5.4.3 Effects of oral surgery on salivary gland secretion

In these series of experiments, an investigation was made of the effects of removal of a wisdom tooth (oral surgery procedure) on salivary gland secretion before and after the operation employing patients 20-24 years. Figure 5.25 A shows the salivary resting and stimulated flow rates before and after the surgical procedure.

The results show that basal salivary flow is slightly, but significantly ($P<0.05$) increased after the surgical procedure. There were no statistical differences in stimulated salivary flow either before or after surgery. The differences between stimulated and basal salivary flow (figure 5.25 B) show no statistical differences in the secretory capacity of the gland before and after surgery.

Figure 5.26 A shows the concentration of protein in saliva in resting and stimulated conditions before and after the surgery. There were no significant differences between basal salivary protein concentration despite these being slightly elevated after the surgery compared to the values obtained before the surgical procedure. However, there was a significant ($P<0.05$) increase in stimulated salivary protein concentration after the surgery. The difference in protein output (stimulated protein concentration minus basal protein concentration) is shown in Figure 5.26 B. The difference in the protein output was marginally significantly ($P=0,07$) elevated after the surgery compared to before.

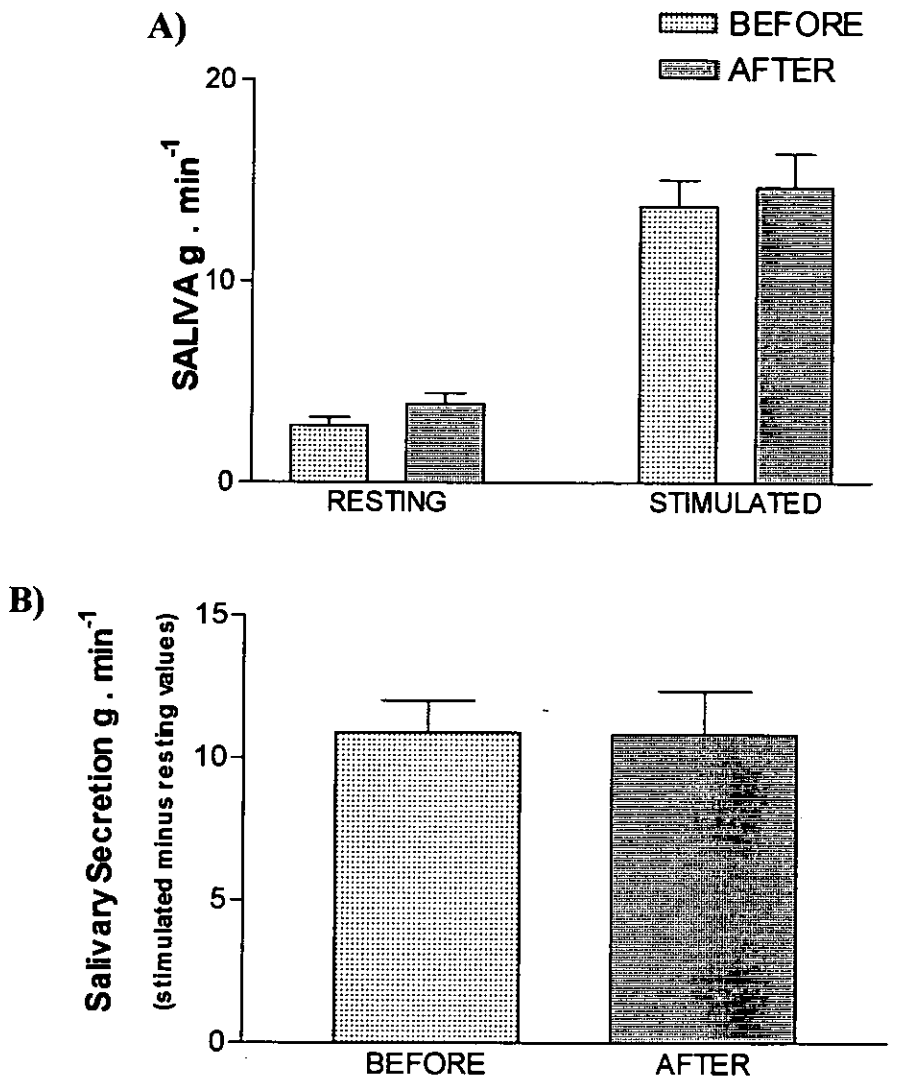


Figure 5.25 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary flow rates in oral surgery patients comparing the results obtained before and after the surgical procedure. B)- Histograms showing the mean (+- SEM) of the salivary secretory rates (stimulated minus resting salivary flow) in oral surgery patients comparing the results obtained before and after the surgical procedures. In Figure 5.25 to Figure 5.29, n= 8 for each group.

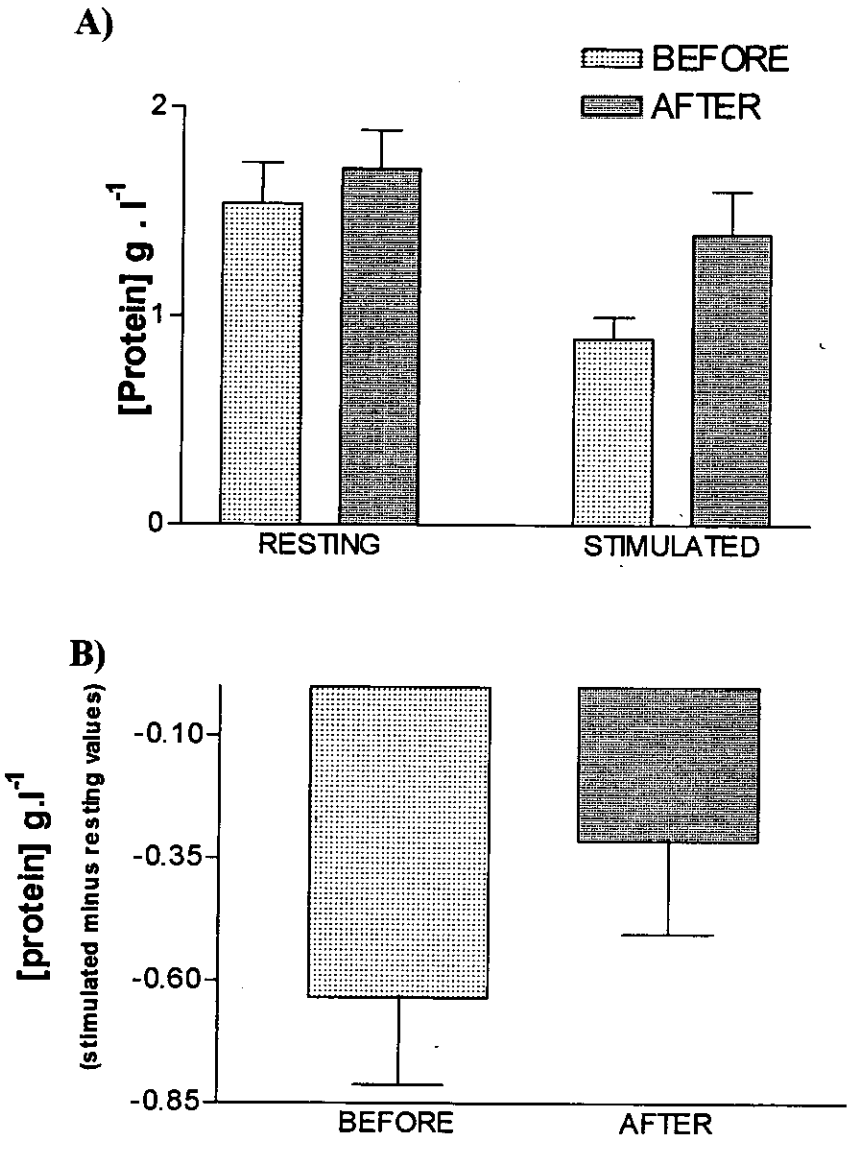


Figure 5.26 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary protein outputs in oral surgery patients comparing the results obtained before and after the surgical procedure. B)- Histograms showing the mean (+- SEM) of the salivary protein outputs (stimulated minus resting salivary protein secretion) in oral surgery patients comparing the results obtained before and after the surgical procedures. (n=8).

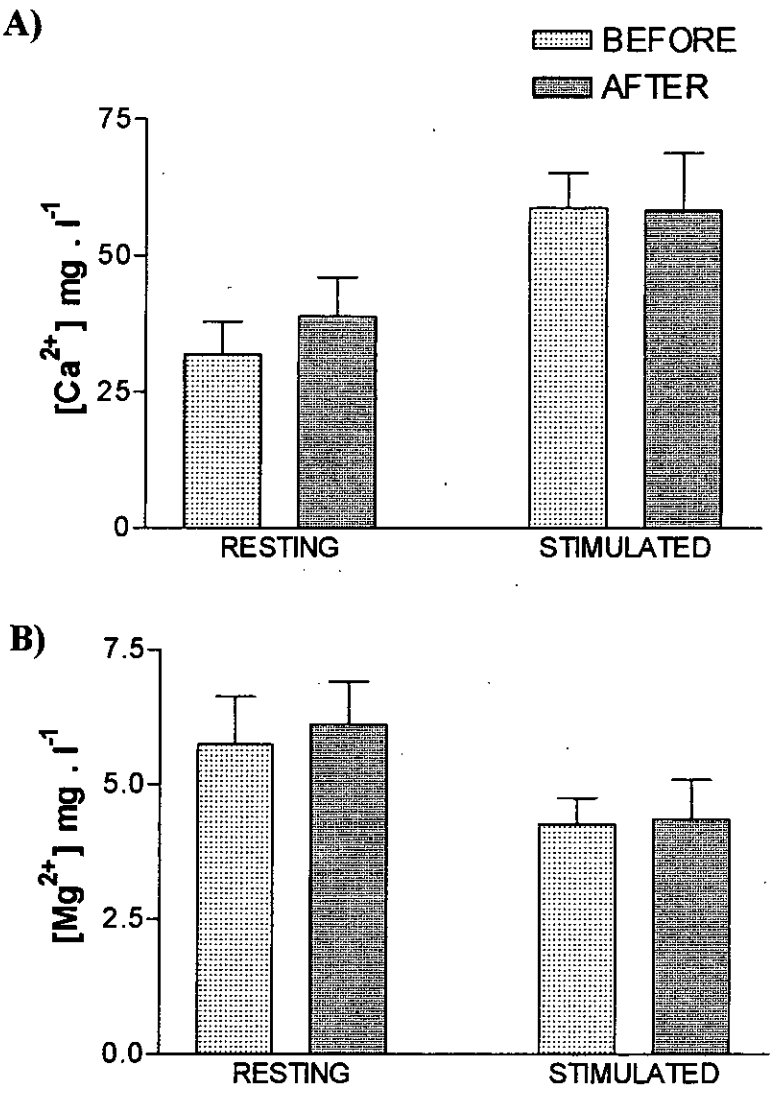


Figure 5.27 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary calcium concentration in whole saliva of oral surgery patients comparing the results obtained before and after the surgical procedure. B)- Histograms showing the mean (+- SEM) of resting and stimulated salivary magnesium concentration in whole saliva of oral surgery patients comparing the results obtained before and after the surgical procedure. The differences between stimulated and resting secretory capacity graphs are not shown since there were no differences in the results obtained before and after the surgical procedures. (n=8).

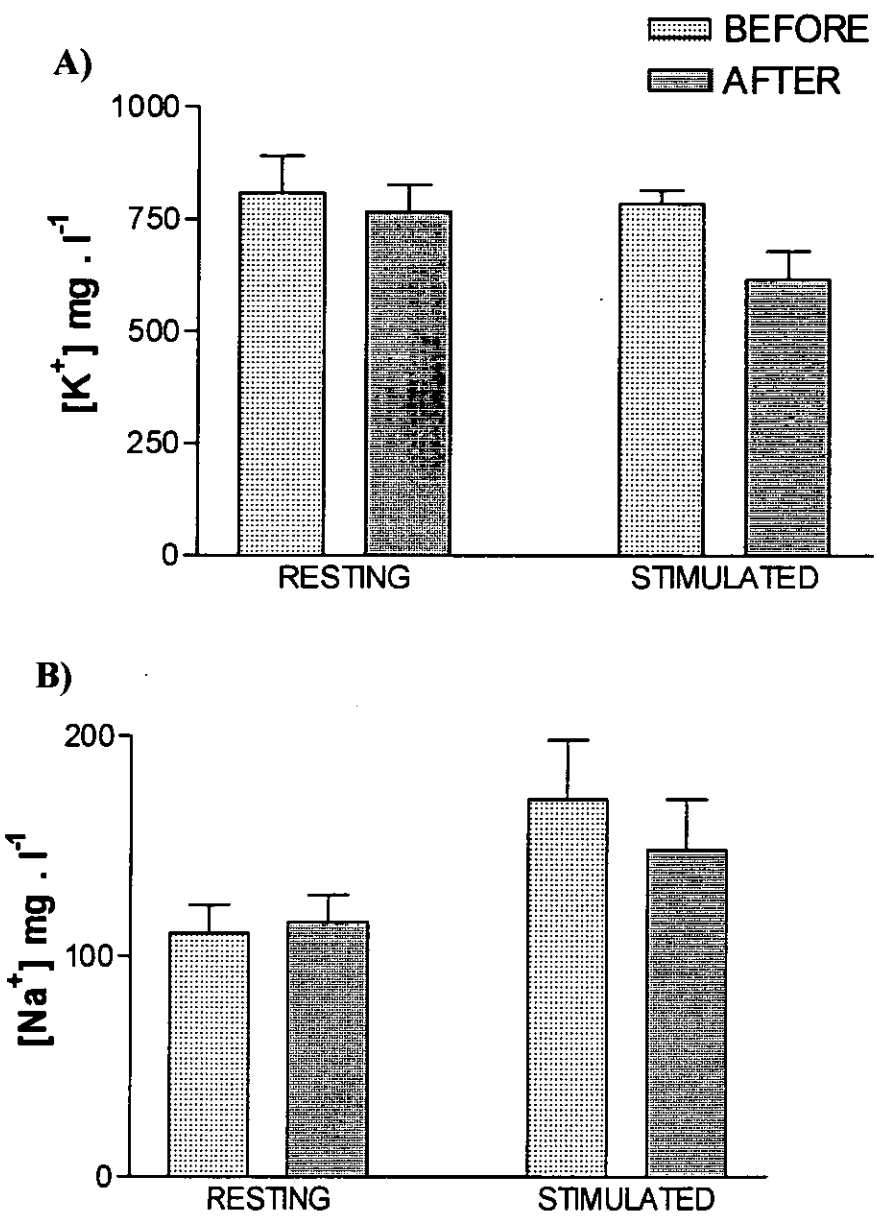


Figure 5.28 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary potassium concentrations in whole saliva of oral surgery patients comparing the results obtained before and after the surgical procedure. B)- Histograms showing the mean (+- SEM) of resting and stimulated salivary sodium concentrations in whole saliva oral surgery patients comparing the results obtained before and after the surgical procedure. Differences between stimulated and resting values of secretory capacity graphs are not shown since there were no differences in the results obtained before and after the surgical procedures. (n=8).

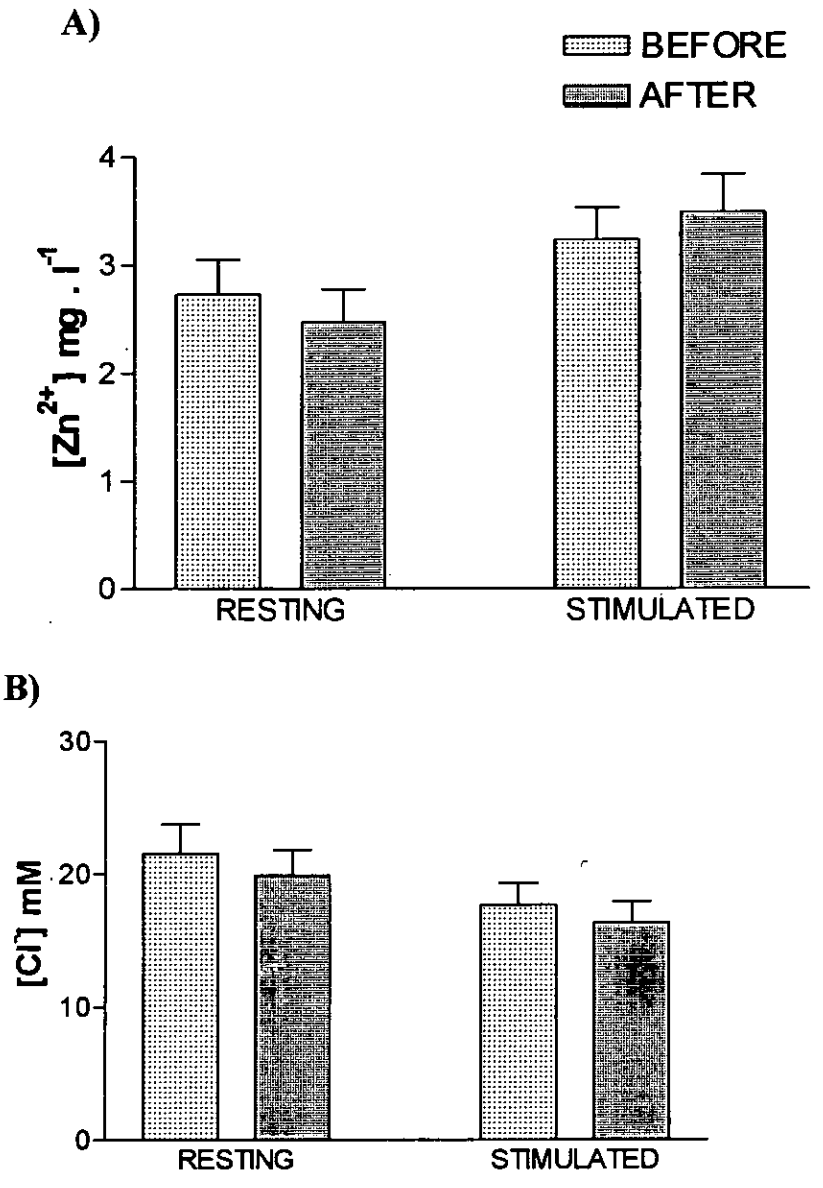


Figure 5.29 A)-Histograms showing the mean (+- SEM) of resting and stimulated salivary zinc concentrations in whole saliva of oral surgery patients comparing the results obtained before and after the surgical procedure. B)- Histograms showing the mean (+- SEM) of resting and stimulated salivary chloride concentrations in whole saliva of oral surgery patients comparing the results obtained before and after the surgical procedure. Differences between stimulated and resting secretory capacity graphs are not shown since there were no differences in the results obtained before and after the surgical procedures. (n=8).

Figure 5.27 shows the level of Ca^{2+} (Figure 5.27 A) and Mg^{2+} (Figure 5.27 B) in saliva for basal and stimulated conditions before and after the surgery. The results show no change in either Ca^{2+} or Mg^{2+} levels either before or after the surgery comparing basal values before and after the surgical procedures. Figure 5.28 shows the levels of K^+ (Figure 5.28 A) and Na^+ (Figure 5.28 B) in basal and stimulated salivation either before or after surgery. With regards to K^+, the results show no change in K^+ in either basal or stimulated condition before surgery. However, after the surgery K^+ level decreased in stimulated condition compared to basal after surgery and to stimulated before surgery in a significant ($P<0.05$) way suggesting that after the surgery there is less stimulated K^+. The stimulation of salivary secretion elicited a significant ($P<0.05$) increase in Na^+ concentration. Nevertheless, the results also show that there were no significant differences for basal and stimulated Na^+ before and after the surgery. Figure 5.29 shows the level of Zn^{2+} (Figure 5.29 A) and Cl^- (Figure 5.29 B) in saliva for both basal and stimulated salivation either before or after the surgery. The results indicate that there was a small increase in Zn^{2+} level in stimulated conditions after surgery compared to the values obtained before surgery. However, despite this tendency for greater Zn^{2+} secretion in saliva after the surgery, these results shown no significance when tested for mean differences. Resting and stimulated salivary Cl^- concentration was found to be slightly decreased after the surgical procedure. However, the differences found were not statistically significant when tested for mean differences.

Taken together, the results suggest that surgery increases basal salivary secretion and protein concentration in saliva while for the ionic composition, there were hardly any significant changes.

5.5 Discussion

5.5.1 Effects of gender and age on salivary gland function

The results of this study have demonstrated the existence of profound age and gender related effects on salivary gland function. Basal and stimulated salivary flow decreased significantly with age for both male and female subjects. Moreover, the difference between stimulated and basal salivary flow, which measures the secretory capacity of the gland, was also significantly decreased in advanced age groups compared to younger individuals of both gender. It is now, widely accepted that salivary gland dysfunction and xerostomia are common problems among older individuals and tend to increase with age (Osterberg *et al.*, 1992; Ship *et al.*, 2002). It is known that histomorphometric parenchyma changes as loss of acinar cells and replacement by adipose and connective tissue occur generally in the salivary gland ageing process (Ariji *et al.*, 1994). This loss of functional tissue is postulated to deplete a secretory reserve present in young adults turning older people salivary glands more vulnerable to external insults (Fergusson, 1996). However, it is theorised by some authors that adequate or young-adult levels of secretory function can still be achieved in the elderly as long as no further stress is placed on the salivary system (Ship *et al.* 2002). Thus, the ageing process would not be responsible for the prevalence of xerostomia, but for the greater vulnerability of salivary glands to external insults (which are more common in advanced age groups) instead (Ship *et al.*, 2002). However, this study employed healthy subjects with no complaints of xerostomia. This was confirmed by the present results since even in advanced age groups with decreased salivary function no signs of objective hyposalivation were found. Nevertheless, the results obtained in this study suggest that even healthy subjects experience an age-dependent decrease in salivary gland function. These findings are not in conflict with the secretory reserve depletion theory since this study has not excluded or even quantified the presence of external gland insults in the patients observed. Therefore, further studies would be of help in trying to associate the presence of a diminished salivary function in the elderly with the age process or the external gland insults.

No gender related differences were found for basal, stimulated or differences between stimulated and resting secretions (secretory capacity) in this study. However, interactions between the factor age and gender have proved to be significant indicating

that male and female subjects are not affected by age in a similar way. In fact, female subjects were found to have a smaller age-dependent decrease in salivary flow parameters when compared to male subjects. The age dependent decrease in female was attenuated from the moment females reached the age of 45 until 70 years of age. From these results it can only be speculated that this could reflect post-menopausal-induced changes, which would be interesting to analyse in further studies.

Concerning salivary protein production, the results of this study have shown that resting protein concentration increased with age, whereas stimulated protein concentration remain more or less the same. Consequently, salivary secretory protein capacity was found to diminished with age. Resting protein output is mediated mainly by a constitutive protein secretion pathway, which probably shows some stability over life span (Castle, 1992) (see general discussion for details). Therefore, the increase in resting protein concentration with ageing can be due to the age-dependent decrease in resting salivary fluid output that was verified to occur in this study. These results also suggest that resting saliva in older subjects has an increased viscosity and diminished hydrating properties.

On the other hand, stimulated salivary protein concentration showed some stability over measured age span. Since stimulated salivary flow was found to decrease with the ageing process, it can therefore be assumed that the quantity of stimulated protein must also decrease with age otherwise its concentration would not show any stability. There were no gender-related differences and neither significant gender and age interactions where salivary protein production was concerned, showing that male and female have the same amount and pattern of salivary production and are similarly affected by the ageing process.

The concentration of the salivary Ca^{2+} was shown to increase in an age-dependent manner for the parameters basal, stimulated and secretory capacity. Thus, older individuals of both genders have more Ca^{2+} in the saliva and demonstrate an increased secretory capacity for this ion. These results may be partially linked to the facts that fluid secretion diminishes with ageing and that most of the salivary Ca^{2+} is bound to salivary proteins and these also become more concentrated with the ageing process (Osterberg *et al.*, 1992). The functional significance of this fact may be an increased tendency for dental calculus formation in older individuals since salivary Ca^{2+} concentration has been found to be a major factor in this process (Pellerin & Pellat, 1986). The results of this study also demonstrated that female subjects have

significantly less salivary Ca^{2+} compared to male subjects. However, there were no significant interactions between the variables gender and age for this ion suggesting that both male and female be affected by age in the same way.

In this study, basal and stimulated salivary Mg^{2+} concentration increased in advanced age groups for both genders. This increase in Mg^{2+} concentration is probably due to age dependent fluid decrease since Mg^{2+} secretory capacity also diminished with the ageing process for male and female subjects. It is interesting to note that while secretory capacity for Ca^{2+} increased with the ageing process, it diminished for the Mg^{2+} ion suggesting an inverted and antagonistic behaviour for these two divalent cations. The results also have suggested that similarly to Ca^{2+}, female subjects have generally less basal and stimulated salivary Mg^{2+} compared to male subjects.

Basal, stimulated and secretory capacity of salivary Zn^{2+} levels were diminished in advanced age groups for both male and female subjects when compared to younger subjects. However, for this ion, the age-associated changes appeared very early comparing to the other ions. In fact, the biggest differences in basal and stimulated salivary Zn^{2+} were found between age groups of 20-34 and 35-44 years. This was most unusual and no explanation was found for this fact. However, the secretory capacity was more diminished in the 65-75 age groups for both male and female, suggesting a age-dependent salivary gland loss of function for this ion. Salivary Zn^{2+} plays an important role in dental calculus (Jin & Hak-Kong, 2002) formation and thus, aged patient could be more susceptible to this condition. Gender-related differences suggested that female had generally less basal and stimulated salivary Zn^{2+}. There were no genders and age interactions indicating that both gender are affected in the same way by the ageing process. Salivary Zn^{2+} concentration has been positively correlated with defence against dental calculus formation and therefore, these results may suggest that older individuals are more prone to salivary calculus formation than younger ones. For male subjects basal salivary Cl^- concentration was shown to increase with age while stimulated salivary Cl^- concentration remained more or less the same for every age group. Female subjects showed no age-related differences in basal or stimulated salivary Cl^-.

Basal, stimulated and secretory salivary Na^+ are significantly reduced in advanced male and female age groups compared to younger subjects. These results suggest an age-dependent decrease in salivary Na^+ function. Altered taste perception (Dysgueusia) has been shown to be more prevalent in older individuals and may be associated with age-

dependent salivary modifications since Na^+ has been described as a participating ion in taste perception (Spielman, 1990; Sato; 2002).

There were no age-related differences in basal salivary K^+ for either male or female subjects. However, stimulated and secretory salivary K^+ shown an age-dependent decrease for both male and female subjects indicating a decrease in salivary function for this ion. Salivary K^+ functions are not very well known, so no functional conclusions can be drawn over the age-dependent salivary alterations for this ion. There were no gender-related differences or any interactions between gender and age variables suggesting that both male and female subjects may be affected in the same way.

5.5.2 Effects of types I and II diabetes mellitus on human salivary secretion

In this study basal, stimulated and secretory fluid capacity (differences between stimulated and resting values) of diabetic individuals was found to be decreased for type I and II diabetic patients when compared to their healthy age-matched controls. These results indicate the presence of diabetes-induced impairment of salivary gland function. Similar findings have been previously described in the literature and may be associated with diabetes-induced neuropathy changes in the salivary gland parenchyma and presence of auto-immune lymphocytic gland infiltrate similar to the one occurring in the pancreas of these patients (Markopoulos & Belazi, 1998). These findings have been reported to be more frequent in uncompensated diabetic patients (Ship *et al.*, 2002). However, in this study, patients were selected from the Portuguese Association of Diabetic Patients and were screened to be well controlled for the disease. Therefore, diminished salivary function is not exclusive of poorly controlled disease but is also present in well-controlled patients. On the other hand, no quantitative comparisons had been made up to date between the two types of the disease. The results of the study demonstrated that type II diabetes also have a diminished salivary fluid output which was however, found to be less intense when compared to type I diabetes. The diminished salivary fluid output in these patients is believed to be responsible for a major incidence of oral infections like caries, candidal or peridontitis (Twetman *et al.*, 2002). Basal and stimulated salivary protein concentrations were found to be increased in type I and type II diabetic patients when compared to their respective age-matched

controls. Again this increase was more marked in type I diabetic patients when compared to type II. Salivary protein secretory capacity was found to be decreased in Type I diabetes but not in Type II. These results may indicate that in Type II diabetes the increase in basal and stimulated protein concentration are probably only due to the reduced salivary fluid secretion. Despite the fact that increased protein concentration in diabetic patients saliva has been described before by other authors (Twetman *et al.*, 2002), there are also reports of diminished antioxidant capacity and salivary peroxidase activity, suggesting that while some proteins may experience an enhanced output others may be diminished (Belce *et al.*, 2000). Therefore, it would be interesting to study qualitative alterations of salivary proteins associated with diabetes in order to gain a better understanding of its possible oral implications.

With respect to the Ca^{2+} ion, basal and stimulated salivary concentrations of this ion were found to be increased compared to age-matched controls for Type I and Type II diabetic subjects. However, the Ca^{2+} secretory capacity of the gland was enhanced only for type I diabetic patients while no statistical differences were found for the Ca^{2+} secretory capacity for the type II diabetics. Once again, these results suggest that in type I diabetes there is an increased Ca^{2+} resting stimulated and secretory enhanced capacity while in type II diabetes there is probably only an increase in basal and stimulated Ca^{2+} concentration resulting from the diminished fluid output and increased protein concentration. Nevertheless, both types I and II diabetic patients have more Ca^{2+} in their resting and stimulated salivas and may consequently are more prone to dental calculus formation and peridontitis.

Resting and stimulated salivary Mg^{2+} concentration were found to be elevated for type I and type II diabetic patients when compared to their age-matched controls. This increase in salivary Mg^{2+} concentration is probably due to the verified diminished salivary fluid output in these patients. When the secretory capacity for Mg^{2+} was evaluated, it was found to be decreased in both type I and II diabetic patients when compared to their age-matched controls. Hypomagnesemia has been found to be quite a common feature among type I and type II diabetic subjects (Tosiello, 1996), and it is caused mainly by ionic hyperglycosuric-induced renal depletion. On the other hand, Mg^{2+} depletion or hypomagnesemia has been held at least in part to be responsible for such complications as neuropathy and microangipathy in type I diabetes (Leeuw, 2001). In previous studies (see chapters 3 and 4) it was demonstrated that hypomagnesemia attenuated the secretagogue-evoked and nerve-mediated secretory responses in the rat

submandibular and parotid gland (Mata *et al.*, 2001; Yago *et al.*, 2002). These effects were mediated at the cellular Ca^{2+} mobilisation level. In rat parotid acinar cells zero levels of extracellular Mg^{2+} led to decreased levels of intracellular Mg^{2+} which were found to be responsible for enhanced IP3 induced Ca^{2+} exit from intracellular stores and enhanced but delayed capacitative calcium cytosolic entry from the extracellular side. It was suggested from those studies that probably hypomagnesemia led to a diminished intracellular Mg^{2+} concentration which impaired the correct function of Mg^{2+}-dependent enzymes regulating salivary secretion (Mata *et al.*, 2001; Yago *et al.*, 2002). In fact, both basal ACh and PHE-evoked secretory responses were found to be significantly decreased in the absence of extracellular Mg^{2+}. In this study it was found the diabetic patients to have diminished salivary output, diminished salivary proteins secretory capacity, enhanced resting and stimulated salivary Ca^{2+}secretion, increased Ca^{2+} secretory capacity and decreased Mg^{2+} secretory capacity. It is noteworthy that this reciprocal relationship between the Ca^{2+} and Mg^{2+} outputs, and the fluid and protein secretion modifications observed in the diabetic patients are strikingly similar to the effects of hypomagnesemia on rat salivary function found in our previous studies (Mata *et al.*, 2001; Yago *et al.*, 2002). Therefore, it would be extremely interesting to investigate the presence of hypomagnesemia in future studies of human diabetes-induced salivary changes.

Type I and type II diabetic patients have less resting, stimulated and secretory capacity for salivary Zn^{2+}. These effects were found to be more severe in type I diabetic patients compared to type II. Salivary Zn^{2+} plays a major role in preventing dental calculus formation (Jin & Hak-Kong, 2002). Thus, diabetic patients could be more prone to dental calculus formation.

For type I diabetic patients resting, stimulated and secretory salivary Cl^- were significantly elevated compared to age-matched controls. For type II diabetes there were no significant differences for any of the parameters tested compared to their age-matched controls. No changes were found in resting stimulated or secretory salivary Na^+ in type I and type II diabetic patients when compared to their age-matched controls. Resting and stimulated salivary K^+ concentrations were found to be increased in type I and type II diabetic patients when compared to their age-matched controls. The elevation was more severe in type I diabetes compared to type II. However, salivary K^+ secretory capacity was decreased in type I and type II diabetic patients when compared to their age-matched controls. Again, the diminishment in this parameter was more

sustained in type I diabetes compared to type II. Resting and stimulated elevation in diabetic saliva is probably secondary to diabetes-induced decrease in salivary fluid output.

5.5.3 Effects of oral surgery on salivary gland secretion

In this series of experiments an investigation was made on the effects of an oral surgical procedure (wisdom tooth removal) on the human salivary function. Basal salivary flow rate was found to be significantly elevated after the surgical procedure. This could be an effect of the healing process itself or be provoked by an increased oral movement caused by post-operative discomfort and leading to a permanent degree of stimulation. No significant changes were found for stimulated and secretory fluid capacity of saliva for collections undertaken before and after the surgical procedure.

Resting salivary protein concentration was slightly, but not significantly elevated after the surgical procedure. Stimulated salivary protein concentration and salivary protein secretory capacities were also elevated after the surgical procedure. Increased salivary protein concentration after the surgical procedure can originate from two sources. It can correspond to surgical wound plasma protein contamination, or it may derive from enhanced salivary gland secretion. Further studies including qualitative analysis of salivary proteins, would be of interest to clarify this issue. Except for stimulated salivary K^+, which was found to be decreased after the surgical procedure, there were no significant changes in any of the other ions measured.

Taken together, the results of this study indicate that:

1) There are marked age-dependent changes in salivary output and also age induced organic and inorganic salivary composition changes
2) Diabetes is pathological condition associated with profound quantitative and qualitative salivary changes which are more pronounced in type I diabetes when compared to type II diabetes. Moreover, the results suggested that these diabetes associated salivary changes could somehow be associated to hypomagnesemia.
3) Except for basal salivary output, there were no significant changes in salivary function in oral surgery recovery of the healthy young subjects employed in this part of the study.

CHAPTER 6

GENERAL DISCUSSION

6.1 Animal physiology

The results of this study have clearly demonstrated that a perturbation of $[Mg^{2+}]_0$ has profound effects on salivary gland function. In this study, it has been found that $[Mg^{2+}]_0$ plays a significant role in the stimulus-secretion coupling process leading to exocytosis and fluid secretion in rat salivary glands acinar cells. In chapter 3 of this thesis it was observed that when $[Mg^{2+}]_0$ was altered employing either elevated or low concentrations of the divalent cation this resulted in a significant decrease in both basal and ACh, PHE and NA-evoked total protein output in the rat submandibular gland segments. These decreases in protein output were associated with concurrent reductions in secretagogue-evoked cytosolic Ca^{2+} levels indicating a close relationship between Ca^{2+} mobilisation and protein output.

In chapter 4 of this study, the effects of perturbation of $[Mg^{2+}]_0$ on nerve-mediated (with EFS) and secretagogue-evoked (ACh, PHE, NA and ISO) amylase secretion in rat parotid gland segments were investigated. Again, the results have demonstrated that a perturbation of $[Mg^{2+}]_0$ (both zero and elevated) resulted in significant reductions in basal as well as ACh, PHE and NA-evoked amylase output. This inhibitory effect of $[Mg^{2+}]_0$ was also seen during stimulation of parotid segments with different concentrations (10^{-6} M and 10^{-5} M for PHE and NA and 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M for ACh) of secretagogues. Although the effects of a perturbation of $[Mg^{2+}]_0$ on EFS-evoked amylase secretion in rat parotid gland segments led to the same inhibitory pattern found with previous secretagogues, $[Mg^{2+}]_0$ perturbation-induced changes were much less pronounced and not significantly different from the amylase secretion obtained with normal (1.1 mM) $[Mg^{2+}]_0$. On the other hand, ISO-evoked amylase secretion elicited a different pattern during perturbation of $[Mg^{2+}]_0$. ISO-evoked amylase secretion increased gradually with increasing concentrations (0 mM, 1.1 mM, 5 mM and 10 mM) of $[Mg^{2+}]_0$. Taken together, the results suggest that Mg^{2+} is exerting a regulatory effect on secretagogue-evoked protein output in submandibular gland and amylase secretion from the parotid glands. Moreover, the results also indicate that this effect was more evident when secretion was provoked with secretagogues known to act via cellular Ca^{2+} mobilisation. In chapter 3 it was shown that in submandibular acinar cell suspensions loaded with Fura-2 AM for the measurement of $[Ca^{2+}]_i$, a perturbation of $[Mg^{2+}]_0$ led to profound changes in ACh-evoked cellular Ca^{2+}mobilisation. Zero and

elevated $[Mg^{2+}]_0$ inhibit Ca^{2+} mobilisation, both the initial peak and plateau phases in submandibular acinar cell suspensions. The results on Ca^{2+} mobilisation, shown in chapter 3 repeated themselves in chapter 4 during perturbation of $[Mg^{2+}]_0$ using single cell microspectrofluorimetric techniques to measure $[Ca^{2+}]_i$. Once again, zero and elevated $[Mg^{2+}]_0$ produced diminished peak and plateau phases of the Ca^{2+} transient when compared to the results obtained with normal $[Mg^{2+}]_0$.

In order to understand better the role of $[Mg^{2+}]_0$ in the Ca^{2+}-dependent secretion process in rat parotid gland, several experiments were conducted to characterise the interactions between Ca^{2+} and Mg^{2+} signalling. In some experiments amylase secretion was continuously monitored in rat parotid segments. Tissues were initially perfused with a physiological salt solution containing normal $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$ (2.56 mM and 1.1 mM, respectively). At some point the Ca^{2+} was removed from the perfusing medium and 1.1 mM EGTA, was added. Gland segments were then stimulated with 10^{-5} M ACh and after the elicited peak recovery, segments were again perfused with physiological salt solution containing normal $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$. These procedures were repeated in 0 mM, 5 mM and 10 mM $[Mg^{2+}]_0$. In another series of experiments the same procedure was repeated in Fura 2-loaded rat parotid acinar cell suspensions while monitoring $[Ca^{2+}]_0$ using described microspectrofluorimetric techniques to measure $[Ca^{2+}]_i$. These procedures helped to understand the effects of a perturbation of $[Mg^{2+}]_0$ in the different components of the Ca^{2+} signal (Ca^{2+} exiting from intracellular stores and capacitative calcium entry (CCE) from the extracellular side). The results of these studies have demonstrated that extracellular calcium is required to maintain sustained and prolonged basal and ACh-evoked parotid amylase secretion, which is in agreement with previous findings by other authors (Douglas & Poisner, 1963; Putney Jr, 1986a,b). In addition, the results indicate further that extracellular Mg^{2+} is apparently regulating cellular Ca^{2+} mobilisation, both its release from intracellular stores and capacitative Ca^{2+} entry from the extracellular medium during ACh-stimulation. In elevated (5 mM and 10 mM) $[Mg^{2+}]_0$, ACh-evoked Ca^{2+} release from intracellular stores was found to be diminished. Moreover, CCE activation was delayed and Ca^{2+} influx from the extracellular side was found to be significantly attenuated when $[Mg^{2+}]_0$ was elevated. These results strongly suggest that elevated $[Mg^{2+}]_0$ is acting like a direct antagonist on cellular Ca^{2+} mobilisation sites, which is in perfect agreement with previous results described by others in pancreatic acinar cells (Geada, 1998). Take together, the results indicate that in elevated (5 mM and 10 mM) $[Mg^{2+}]_0$, the antagonising effects of $[Mg^{2+}]_0$ are

responsible for a diminished stimulated Ca^{2+} exit from intracellular stores, less Ca^{2+} influx from the extracellular medium and delayed CCE which led to the decreased secretagogue-evoked protein and amylase output observed in submandibular and parotid glands segments.

The results obtained with the experiments employing zero $[Mg^{2+}]_0$, are somewhat more difficult to explain. In both submandibular and parotid gland segments either ACh; PHE or NA-evoked total protein and amylase outputs were markedly reduced when $[Mg^{2+}]_0$ was removed from the superfusing medium. Similarly, in Fura-2-loaded submandibular and parotid cells, the ACh-evoked calcium transient was diminished in both peak and plateau phases in the experiments that employed zero $[Mg^{2+}]_0$ but kept the $[Ca^{2+}]_0$ within normal ranges. In contrast, in a nominally free Ca^{2+} medium, ACh-evoked a much longer Ca^{2+} transient in low $[Mg^{2+}]_0$ compared to the responses obtained in either normal or elevated $[Mg^{2+}]_0$. In addition, CCE was much slower in activation, but the steady state elevation in $[Ca^{2+}]_i$ was much larger compared to the responses obtained with either normal or elevated $[Mg^{2+}]_0$. Similarly, in rat parotid segment studies where $[Ca^{2+}]_0$ was removed and 1 mM EGTA was added to the superfusing medium, the ACh-evoked amylase peak was found to be elevated in low $[Mg^{2+}]_0$ when compared to the results obtained with normal or elevated $[Mg^{2+}]_0$. In these experiments, reperfusion of parotid segments with a physiological salt solution containing normal $[Ca^{2+}]_0$ and zero $[Mg^{2+}]_0$ elicited a CCE-dependent rebound in amylase secretion which was found to be more elevated and sustained in zero $[Mg^{2+}]_0$ when compared to either normal or elevated $[Mg^{2+}]_0$.

Therefore, it seems that the effect of extracellular Mg^{2+} on Ca^{2+} release from intracellular stores can be considered as pure antagonism since maximal release is found in zero $[Mg^{2+}]_0$ but less Ca^{2+} comes out of the stores as $[Mg^{2+}]_0$ rises. The effects of $[Mg^{2+}]_0$ on CCE are more complex. The mechanisms of regulation of CCE in acinar salivary gland cells are still poorly understood and several theories have been proposed (see Ambudkar, 2000 or general introduction for review). From the present results it seems that extracellular Mg^{2+} is antagonising Ca^{2+} entry in the cytoplasm from the extracellular side since CCE is inversely proportional to changes in $[Mg^{2+}]_0$. However, it is also true that $[Mg^{2+}]_0$ strongly influences the Ca^{2+} channel opening velocity. In low (zero mM) and elevated (5 mM and 10 mM) $[Mg^{2+}]_0$, CCE triggering is significantly delayed when compared to normal (1.1 mM) $[Mg^{2+}]_0$. Some authors have proposed that CCE triggering is dependent upon Ca^{2+} channel phosphorylation events (Sakai &

Ambudkar, 1996; 1997). It is known that phosphorylation events are strongly dependent on Mg^{2+}, and therefore, it seems logical that the Ca^{2+} channel responsible for the CCE requires an optimal $[Mg^{2+}]_0$ in order to open quickly.

However, the question which now arises is: why do secretagogues evok diminished amylase or protein secretion in parotid and submandibular glands when $[Mg^{2+}]_0$ is low but $[Ca^{2+}]_0$ is normal? One possible explanation is that in normal (1.1 mM) $[Mg^{2+}]_0$, secretagogue-evoked elevation in protein and amylase secretion is driven by Ca^{2+} exiting intracellular stores and also by a very rapidly activated CCE. In contrast, in low (0 mM) $[Mg^{2+}]_0$ a delayed CCE could be responsible for a diminished initial protein and amylase secretion peak originating only in Ca^{2+} exiting from intracellular stores. Another possible hypothesis is that $[Mg^{2+}]_0$ is not only acting like a Ca^{2+} channel antagonist, but it may exert its effects directly on the activities of Mg^{2+}-dependent enzymes which are associated with Ca^{2+} transport, diacylglicerol and protein kinase C activity or exocytotic vesicles transport, budding and fusing with cell membrane regulation events (Yago *et al.*, 2000).

In order to understand better the interactions between Mg^{2+} and Ca^{2+} in the stimulus-secretion coupling events controlling salivary gland function, a series of experiments have been performed using Magfura 2-loaded rat parotid acinar cells where intracellular Mg^{2+} ($[Mg^{2+}]_i$) was measured employing a microspectrofluorimetric technique.

The results have shown that perfusion of single Magfura-2 loaded parotid acinar cells with a physiological salt solution containing increasing concentrations of $[Mg^{2+}]_0$ resulted in a gradually elevation in $[Mg^{2+}]_i$. Thus, it is plausible to assume that these variations in $[Mg^{2+}]_i$ may be responsible for most of the effects observed in intracellular Ca^{2+} mobilisation, which is known to be modulated by a number of Mg^{2+}-dependent enzymes. Cytosolic Mg^{2+} concentration of mammalian cells is regulated at a level well below that predicted by the Nerstian potential (Geada, 1998). Thus, Mg^{2+} is not passively distributed, indicating that an active transport regulates the $[Mg^{2+}]_i$ in sublingual acini (Zhang & Melvin, 1992), squid axons (Flatman, 1991), human red blood cells (Ferray & Garay, 1986) and pancreatic acinar cells (Lennard & Singh, 1992).

The results of this study have also demonstrated that a number of transport inhibitors can markedly elevate $[Mg^{2+}]_i$ in Magfura2-loaded parotid acinar cells. Firstly, it was shown that $[Mg^{2+}]_i$ homeostasis in resting parotid acinar cells is probably due to an energy-dependent process, since the presence of DNP (an ATP inhibitor) leads to an

increase in $[Mg^{2+}]i$. Second, in rat parotid acinar cells resting $[Mg^{2+}]_i$ seems to be associated with Na^+ transporters and highly dependent on extracellular Na^+. In fact, the presence of either lidocaine (a Na^+ channel blocker), amiloride (an inhibitor of Na^+/H^+ exchanger), NMDG (a substitute for $[Na^+]_0$), quinidine (an inhibitor of the Na^+/Mg^{2+} antiport), and bumetanide (an inhibitor of the $Na^+:K^+:Cl^-$ co-transporter) in the extracellular medium led to a significant increase in $[Mg^{2+}]_i$ in Magfura 2-loaded single parotid acinar cells. These transport inhibitors may exert their effects in increasing cellular Mg^{2+} via a different number of mechanisms. They may act by either facilitating Mg^{2+} release from intracellular stores, thus preventing its efflux from the cytosol or enhancing its influx from the extracellular medium. However, studies performed by other workers using sublingual acinar cells, have demonstrated that when $[Mg^{2+}]_0$ concentration is deliberately increased by loading cells with Mg^{2+}, a Mg^{2+} efflux originates in which this ion leaves the cell through a Na^+-Mg^{2+} antiport system (Zhang & Melvin,1994). In addition, several studies have demonstrated the existence of a membrane Na^+-Mg^{2+} antiport system which is believed to play a major role in the regulation of cytosolic Mg^{2+} (Flatman, 1991). Like these previous studies, it is tempting to suggest that in parotid acinar cells, a membrane Na^+ dependent system for the regulation of cytosolic Mg^{2+} exist on which these transport inhibitors are probably acting by preventing cellular Mg^{2+} efflux. However, more studies are needed in order to confirm this interesting hypothesis.

The results of the studies undertaken and presented in chapter 4 also shown that ACh can mobilise cellular Mg^{2+}. In fact, when stimulated with ACh, acinar cells experienced a slow but detectable decrease in $[Mg^{2+}]_i$, which reached a stable plateau phase after 5 – 8 minutes. The results have shown that the $[Mg^{2+}]_i$ decrease is insensitive to either Na^+ removal (substituting it for NMDG) or to either quinidine, dinitrophenol or bumetanide but only partial sensitive to lidocaine and amiloride. The results also suggest that the decrease in $[Mg^{2+}]_i$ may partially be associated with the sodium channel activity since lidocaine, the local anaesthetic that is known to inhibit the Na^+ channel activity, can decrease the response to ACh. In addition, the $[Mg^{2+}]_i$ decrease may also be associated with the Na^+/H^+ antiport, since the ACh-evoked response was also partially sensitive to amiloride. The decrease in $[Mg^{2+}]_i$ in response to ACh may be due to the fact that Mg^{2+} is leaving the cell (efflux) or it is sequestered in the stores. In pancreatic acinar cells, it was shown that secretagogue-evoked decrease in $[Mg^{2+}]_i$, corresponded to either Mg^{2+} leaving the cell (Yago *et al,* 2000), or Mg^{2+} entering intracellular stores (Mooren, 2001).

Nevertheless, there is a general agreement in the scientific community that, $[Mg^{2+}]_i$ is mobilised in a stimulus-secretion coupling dependent manner and that the slower changes in $[Mg^{2+}]_i$ occur in a way that is completely anti-parallel to the rapid Ca^{2+} signalling in acinar cells (Lennard & Singh, 1992; Mooren, 2001). Similarly, in this study it was demonstrated that $[Mg^{2+}]_i$ responds with a slow decrease upon ACh stimulation and despite the fact that the present findings seem somehow to favour the "ACh-induced Mg^{2+} cellular efflux" hypothesis, further experiments are required to characterise precisely how Mg^{2+} is being mobilised following secretagogue stimulation.

6.2 Human physiology

The results of the study involving the effects of ageing, diabetes and oral surgery recover on human salivary secretion are presented and discussed in chapter 5. Data show that the ageing process has profound effects on both the rate of salivary secretion and the quality of the saliva. This study employed healthy human subjects and despite the fact that generally no xerostomia was found in the elderly, advanced age groups experienced significant decreases in basal, stimulated and salivary secretory capacity (difference between stimulated and basal secretion) when compared to subjects from young aged groups. Except for salivary Ca^{2+} secretory capacity, for all other organic and inorganic salivary components (proteins, Mg^{2+}, Zn^{2+}, Na^{2+}, K^+ and Cl^-) the measured secretory capacity was found to be diminished in an age-dependent manner. Although this study has not investigated the presence of all possible salivary gland external insults, it is clear from the current work that in the elderly, there is an increased vulnerability expressed by a decrease in salivary function with ageing. Experiments involving the use of animals to study the ageing process of the salivary glands have clearly demonstrated that as the animals get old (from 5 to 12, to 16, to 20 and subsequently to 24 months) there are time-dependent changes in the morphology of the salivary parotid gland. This in turn is associated with significant decreases in amylase secretion and a derangement in cellular Ca^{2+} homeostasis (Mahay *et al*, 2002). If it is possible to extrapolate the findings of animal pathophysiological conditions to human, then it is clear that the ageing process is indeed affecting the secretory capacity of the salivary glands. Studies employing human parotid tissue have also shown the existence of profound age-associated histomorphological changes characterised by loss of

functional acini and substitution by connective and adipose tissue (Scott, 1987). However, it seems that in the human, there is an excess of secretory tissue, which constitutes a secretory reserve and allows old people to maintain adequate of salivary secretion levels as long as no further stress is placed on the gland (Ship *et al.*, 2002). In fact, in this study, older people shown decreased levels of salivary secretion rates when compared to younger age groups, however, no signs of xerostomia were found in the elderly suggesting that an adequate salivary function was maintained in these individuals.

In this study, it was also demonstrated that both type I and type II diabetic patients experienced profound modifications in the salivary gland function when compared to their healthy (non-diabetic) age-matched controls. The results show that diabetic patients have an overall impairment of salivary gland function, which is in agreement with previous reports made by other authors (Ship *et al.* 2002). It is now well established that diabetics have increased susceptibility to oral infections and diminished response in the wound healing process (Twetman *et al.* 2002).

A new approach undertaken in this study was to specifically compare the salivary profiles of type I and type II diabetic patients. It was shown that type II diabetic patients experience the same qualitative modifications in salivary function compared to type I diabetes, but these are quantitatively less severe. This is probably due to a later onset of the disease in these patients. Generally, diabetic patients have diminished salivary output, impaired salivary protein secretory capacity, enhanced resting and stimulated salivary Ca^{2+} secretion, increased Ca^{2+} secretory capacity and decreased Mg^{2+} secretory capacity. In a recent study employing streptozotocin-induced type I diabetes in rats, it was demonstrated that diabetes was associated with marked morphological changes in the salivary parotid gland including hyperplasia, and abundant infiltration of lipid vacuoles (Patel *et al.*, 2002). These morphological changes were associated with significant secretagogue-evoked dose-dependent increases in amylase secretion and cellular Ca^{2+} mobilisation (Mahay *et al.*, 2002). Again, if it is possible to extrapolate these findings of animal to human then it is clear that diabetes is indeed impairing salivary gland function. In the human, diabetes has shown to produce histomorphological changes comparable to the ones occurring in the pancreas of diabetic patients (Markopoulos & Belazi, 1998).

These findings are very interesting since they share striking similarities with the effects of hypomagnesemia in salivary gland function found in animal studies presented in

chapters 3 and 4 of this work. Hypomagnesemia is a frequent feature in diabetic patients and some authors have suggested that it could be partially responsible for the installation of neuropathy (Tosiello, 1996; Leeuw, 2002). It is also know that diabetic patients with neuropathy have more pronounced salivary modifications. Therefore, future studies should aim to investigate the existence of any positive relationship between hypomagnesemia, diabetes and salivary function impairment. If this association is proven to exist, oral prescription of Mg^{2+} supplements could be an easy way of decreasing oral infection susceptibility experienced by these patients in order to improve their oral condition.

The effects of oral surgery recovery on salivary gland function were also investigated in this part of the study. Except for stimulated salivary K^+, no other changes were perceptible in ionic salivary function for these patients. No functional significance could be inferred from this finding. Basal salivary secretion is elevated in these patients and could reflect a salivary gland response to bring an increased quantity of healing growth factors know to occur in saliva to the surgical wound in order to accelerate the healing process (Zelles *et al.*, 1995; Pedersen *et al.* 2002). However, this can also be only derived from the presence of mechanical stimulation caused by post-surgical discomfort. Further studies should try to bring new evidence to this issue. Salivary proteins are slightly elevated and it would be interesting to investigate this further especially if this corresponds to enhanced healing factors or derives from the presence of contaminating blood factor originated by the surgical wound.

6.3 Conclusion

The main conclusions from this study employing the rat model to study parotid gland secretion are outlined in the schematic models of Figures 6.1 and 6.2. The first model represents the relationship between secretagogue-evoked changes in the Ca^{2+} and Mg^{2+} signalling during the stimulus-secretion coupling process. Secretagogues activate their respective receptors on parotid acinar cells leading to the metabolism of PIP2 resulting in the formation of IP3 and DG. IP3 in turn stimulates the IP3 receptor on the ER resulting in the release of Ca^{2+} from the ER. This release of Ca^{2+} from the ER results in the activation of CCE allowing Ca^{2+} to enter the cell (probably both the cytoplasm and the ER). The elevated $[Ca^{2+}]_i$ activates calmodulin (CD) which in turn phosphorylates

regulatory proteins on the secretory granules. The granules now take in fluid and subsequently swell, thereby allowing them to migrate towards the luminal membrane where they dock and fuse. During this process, exocytosis occurs resulting in protein and enzyme secretion in to the lumen of salivary gland acini. In the schematic model of Figure 6.1 elevated $[Mg^{2+}]_o$ (and probably high $[Mg^{2+}]_i$) blocks the release of Ca^{2+} from the ER and CCE leading to a reduction in cellular Ca^{2+} and thus reduced protein and enzyme secretion. It is also proposed that Mg^{2+} may have effects on a number of enzymes in acinar cells. These include PLC, the kinases, PMCA, SERCA pump and others especially since Mg^{2+} is an important co-factor for the activation of enzymes (Birch, 1993). In low $[Mg^{2+}]_o$ (and probably low $[Mg^{2+}]_i$) the activities of most of the enzymes (eg PLC) are attenuated resulting in reduced cellular Ca^{2+} levels which in turn leads to a decrease in protein and amylase secretion.

In Figure 6.2 A, it is proposed that either sodium removal (using NMDG instead) or a number of membrane transport inhibitors (eg bumetanide, DNP, amiloride, quinidine, and lidocaine) can elevate $[Mg^{2+}]_i$ via a number of mechanisms. They may act to either inhibit the Na^+:Mg^{2+} exchange and the uptake of Mg^{2+} into internal stores and stimulated the uptake of Mg^{2+} into the cytoplasm resulting in an elevation in $[Mg^{2+}]_i$. The precise mechanism of action of the effects of the various inhibitors still need to be elucidated. Figure 6.2 B is a model to explain the ACh-evoked decrease in $[Mg^{2+}]_i$. In this model, it is proposed that ACh can stimulate a decrease in $[Mg^{2+}]_i$ by either facilitating its efflux and/or its uptake into internal stores. These processes may also be related to cellular Ca^{2+} homeostasis. However, further experiments are required to determined precisely how ACh is acting to decrease $[Mg^{2+}]_i$

In relation to human salivary secretion, the results have demonstrated clearly that both ageing and diabetes can have marked effects on both the output of saliva and the quality of the saliva when compared to their healthy age-matched controls. However, surgical procedures seem to have very little effect on salivary gland function.

Figure 6.1

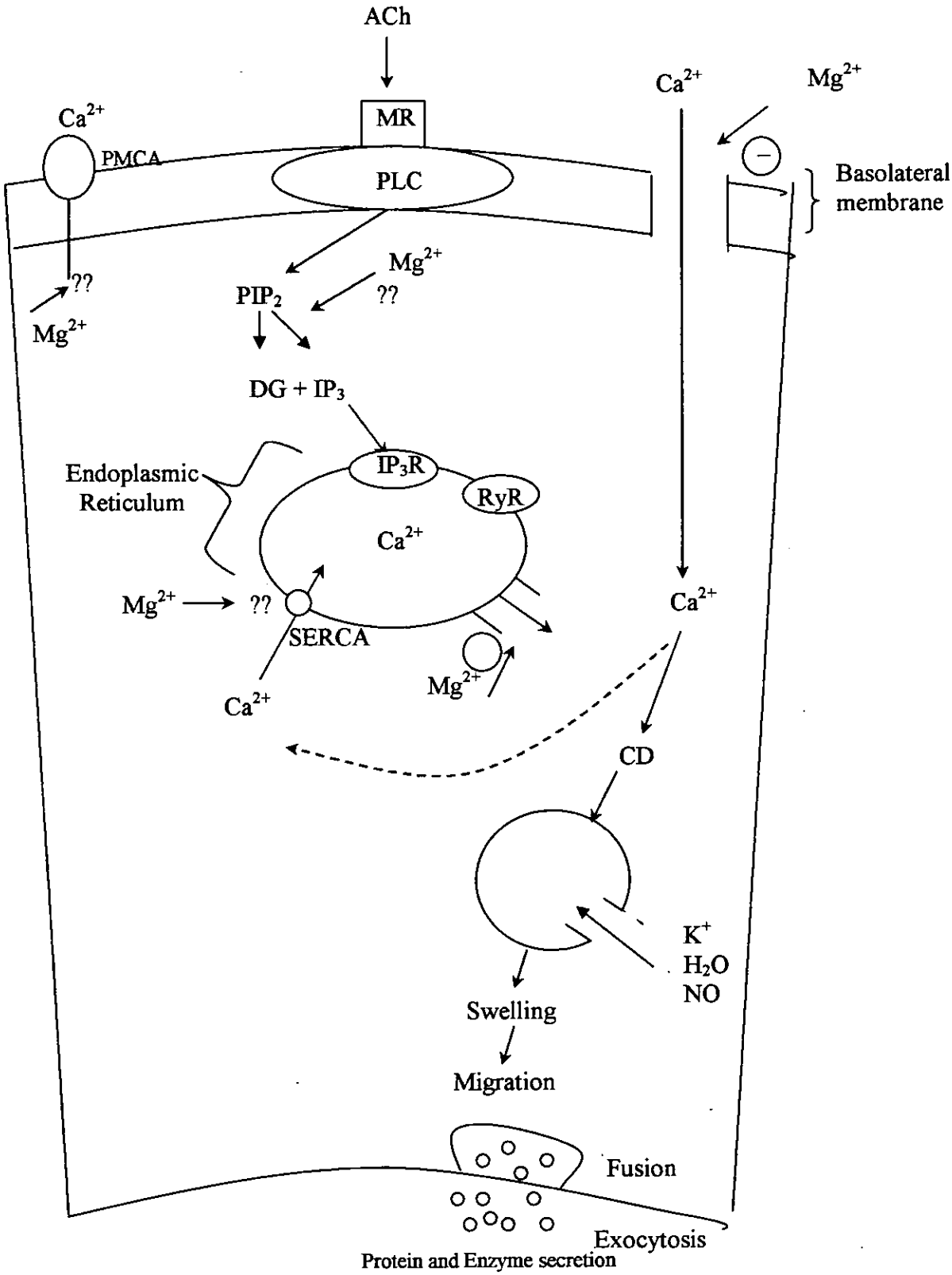

ACh
MR
PLC
PMCA
Ca^{2+}
Mg^{2+}
??
PIP_2
DG + IP_3
IP_3R
RyR
Endoplasmic Reticulum
SERCA
Basolateral membrane
CD
K^+
H_2O
NO
Swelling
Migration
Fusion
Exocytosis
Protein and Enzyme secretion

Figure 6.1- Schematic model illustrating the relationship between Mg^{2+} and Ca^{2+} signalling during amylase secretion in parotid acinar cells in response to such agonists as ACh. Secretagogues evoked an increase in $[Ca^{2+}]$ from the endoplasmic reticulum which in turn activates CCE leading to the stimulation of calmodulin (CD), Ca^{2+} - CD activates the phosphorylation of regulatory proteins on the salivary protein granules resulting in the influx of ions and water and subsequent swelling of the granules. The granules then migrate towards the luminal pole where they dock and fuse with the luminal membrane to bring about exocytosis and secretion. It is proposed that Mg^{2+} can regulate the metabolism of IP_3, Ca^{2+}- ATPase pumps (SERCA and PMCA) in the endoplasmatic reticulum (ER) and plasmic membrane respectively, Ca^{2+} release from the ER, and Ca^{2+} influx from the extracellular medium. High $[Mg^{2+}]_0$, and subsequently high $[Mg^{2+}]_i$, seems to attenuate Ca^{2+} release from the ER and its entry into the cell whereas low $[Mg^{2+}]_0$ and subsequently $[Mg^{2+}]_i$ has effects on enzymes which regulate cellular Ca^{2+} homeostasis. PLC=phospholipase C; IP_3=inositol trisphosphate; PIP_2=phosphatidyl inositol biphosphate; DG=diacylglycerol; CCE=capacitative calcium entry; IP_3R=IP_3 receptor; RyR=ryanodine receptor

Figure 6.2

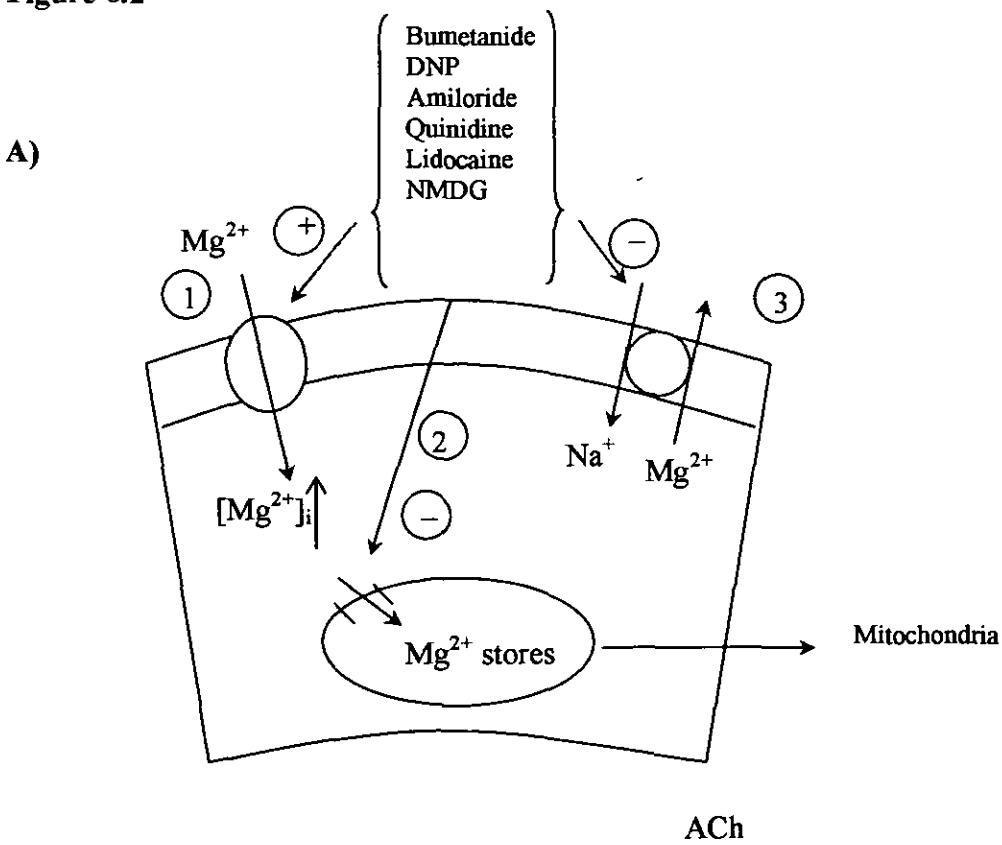


Figure 6.2- Schematic model of the events in the regulation of Mg^{2+} homeostasis in parotid acinar cells. In figure 6.2 A it is proposed that either Na^+ removal (substituting with NMDG) or such membrane transport inhibitor as bumetanide, dinitrophenol (DNP), amiloride, quinidine and lidocaine either inhibit the Na^+:Mg^{2+} exchange (3) and the uptake of Mg^{2+} into internal stores(2) and/or stimulate Mg^{2+} influx into the cell (1) resulting in an elevation in $[Mg^{2+}]_i$. In figure 6.2 B it is proposed that acetylcholine (ACh) stimulation leads to the activation of the transport processes which are responsible for Mg^{2+} movement either (1) from the cytoplasm to the extracellular medium and for the uptake of cytoplasmic Mg^{2+} into internal stores(2).

REFERENCES

Altura, B.M. & Altura, B.T. (1990). Role of magnesium in pathogenesis of high blood pressure: Relationship to its actions on cardiac and muscular smooth muscle. In: *Hypertension: Pathophysiology, Diagnosis and Management.* Eds: Laragh JH, Brenner BM, Raven Press, NY.

Ambudkar, I.S. (2000). Regulation of Calcium in Salivary gland secretion. *Crit. Rev. Oral. Biol. Med.* **11(1)**, 4-25.

Ambudkar, I.S. & Baum, B.J. (1988). ATP-dependent calcium transport in rat parotid basolateral membrane vesicles is modulated by membrane potential. *J. Membr. Biol.* **102,** 59-69.

Ambudkar, I.S.; Horn, V.J. & Baum, B.J. (1989). ATP-dependent calcium transport in rat parotid basolateral membrane vesicles is regulated by calmodulin. *Arch. Biochem. Biophys.* **268,** 576-584.

Ambudkar, I.S.; Lockwich, T.P.; Hiramatsu, Y. & Baum, B.J. (1992). Ca^{2+} entry in rat parotid acinar cells. *Mol. Cell. Biochem.* **114,** 73-77.

Amsallem, H.; Métioui, M.; VandenAbeele, A.; Elyamani, A.; Moran, A. & Dehaye J.P. (1996). Presence of a metabotropic and ionotropic purinergic receptor on rat submandibular ductal cells. *Am. J. Physiol.* **271** (*Cell Physiol.* **40**), 1546-1555.

Ariji, Y.; Ariji, K.; Araki, S.; Nakamura, S. & Kanda, S. (1994). Studies on quantitative computed tomography of normal parotid and submandibular glands. *Dentomaxillofac. Radiol.* **23**, 29-32.

Arvan, P.; Rudnick, G. & Castle, J.D. (1984). Osmotic properties and internal pH of isolated rat parotid scretory granules. *J. Biol. Chem.* **259,** 13567-13572.

Arvan, P. & Castle, D. (1987). Phasic release of newly synthesised secretory proteins in the unstimulated rat exocrine pancreas. *Cell Biol.* **2,** 327-331.

Atkinson, J.C.; Dawes, C.; Ericson, T.; Fox, P.C.; Gandara, B.K.; Malamud, D.; Mandel, I.D.; Navazesh, M.; Tabak, L.A. (1993). Guidelines for saliva nomenclature and collection. In: *Saliva As A Diagnostic Fluid.* Eds. Ann NY Acad. Sci; 694: xi. New York

Atkinson, J.C. & Fox, P.C. (1993). Salivary gland dysfunction. *Clin. Geriatr. Med.* **8,** 499-511.

Atkinson, J.C. & Wu, A.J. (1994). Salivary gland dysfunction: Causes, symptoms, treatment. *J. Amer. Dent. Assoc.* **125,** 409-416.

Aub, D.L.; Mckinney, J.S. & Putney, J.W. Jr. (1982). Nature of the receptor regulated calcium pool in rat parotid gland. *J. Physiol.* **331,** 557-565.

Avery, J.K. (1992). Salivary glands and tonsils. In: *Essentials Of Oral Histology And Embriology.* Mosby, St Louis. Missouri, pp: 184-195.

Azen, E.A. (1973). Properties of salivary basic proteins showing polymorphism. *Biochem. Genet.* **9,** 69-86.

Balekjian, A.Y. & Longton, R.W. (1973). Histones isolated from parotid fluid. *Biochem. Biophys. Res. Commun.* **50,** 676-682.

Barrett, A.J.; Rawlings, N.D.; Davies, M.E.; Machleidt, W.; Salvesen, G. & Turk, V. (1986). Cysteine proteinase inhibitors of the cystatin superfamily. In: *Proteinase Inhibitors,* AJ Barrett and G Salvesen, Eds., Amsterdam: Elsevier Science publishers, pp. 515-569.

Barka, T. (1980). Biologically active polypeptides in the submandibular gland. *J Histochem. Cytochem.* **28,** 836-859.

Batzri, S.; Selinger, Z.; Shramm, M. & Robinovitch, R. (1973). Potassium release mediated by the epinephrine α-receptor in rat parotid slices. *J. Biol. Chem.* **248,** 361-368.

Baum, B.J. (1987). Neurotransmitter control of secretion. *J. Dent. Res.* **66,** 628-632.

Baum, B.J. (1993). Principles of salivary secretion. In: *Saliva as a diagnostic fluid.* Eds. *Ann NY Acad. Sci*; New York, **694,** pp 17-23

Baum, B.J. & Ambudkar, I.S. (1988). Regulation of calcium handling by rat parotid acinar cells. *Mol. Cell. Biochem.* **82,** 67-73.

Baum, B.J.; Ambudkar, I.S.; Helman, J.; Horn, V.J.; Melvin, J.E.; Mertz, L.M.; Turner, R.J.; (1990). Dispersed Salivary Gland Acinar Cell Preparations for Use in Studies of Neuroreceptor-Coupled Secretory Events. *Methods in Enzymology.* **192,** 26-37.

Baum, B.J.; Ambudkar, I.S. & Horn, V.J. (1993). Neurotransmitter control of calcium mobilisation. In: *The Biology of Salivary Glands.* Dobrosielski-Vergona K, editor. Boca-Raton, FL: CRC-Press.,pp. 153-179.

Baxter, H. (1933). Variations in the inorganic constituents of mixed and parotid gland saliva activated by reflex stimulation of the dog. *J. Biol. Chem.* **102,** 203.

Beavis, A.D. & Garlid, K.D. (1987). The mitochondrial inner membrane anion channel. Regulation by divalent cations and protons. *J. Biol. Chem.* **262,** 15085-15093.

Belce, A.; Uslu, E.; Kucur, M.; Umut, M.; Ipbuker, A. & Seymen, H.O. (2000). Evaluation of salivary sialic acid level and Cu-Zn superoxide dismutase activity in type 1 diabetes mellitus. *Tohoku J. Exp. Med.* **192(3),** 219-225.

Ben-Aryeh, H.; Spielman, A.; Szargel, R. ***et al.*** (1981). Sialochemistry for diagnosis of Sjorgen's Syndrome in xerostomic patients. *Oral Surg.* **52,** 487-490.

Bennick, A.; McLaughlin, A.C.; Grey, A.A. & Madapallimattam, G. (1981). The location and nature of calcium binding sites in salivary acidic proline rich proteins. *J. Biol. Chem.* **256,** 4741-4746.

Bennick, A. (1982). Salivary proline rich proteins. *Mol. Cell. Biochem.* **45,** 83-99.

Berridge, M. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361,** 315-325.

Berridge, M.J. & Irvine, R.F. (1989). Inositol trisphosphates and cell signalling. *Nature* **314,**197-205.

Berridge, M.J. (1996). Capacitative Ca^{2+} entry- sifting through the evidence for CIF. *Biochem. J.* **314,** 1055-1056.

Berridge, MJ. (1997). Elementary and global aspects of calcium signalling. *J. Physiol.* **499,** 291-306.

Berridge, M.J. & Irvine, R.F. (1984). Inositol trisphosphate, a novel secondary messenger in cellular signal transduction. *Nature* **314,** 197-205.

Bezprozvanny, I.; Watras, J. & Ehrlich, B.E. (1991). Bell-shaped calcium responses curves of Ins (1,4,5) P_3 and calcium gated channels from endoplasmic reticulum of cerebelum. *Nature.* **351,** 751-754.

Biesbrock, A.R.; Reddy, M.S. & Levine, M.J. (1991). Interaction of a salivary mucin secretory imunoglobulin A complex with mucosal pathogens. *Infect. Immun.* **59,** 3492-3497.

Billah, M. & Anthes, J.C. (1990). The regulation and cellular functions of phosphatidylcholine. *J. Biol. Hem.* **269** (2), 281-291.

Billings, R.J.; Proskin, H.M. & Moss, M.E. (1996). Xerostomia and associated factors in a community dwelling adult population. *Community Dent. Oral Epidemiol.* **24,** 312-316.

Birch, N.J. (1993). *Magnesium And Cell.* Pub. Academic Press. London.

Bluard-Deconinck, J.M.; Masson, P.L.; Osinski, P.A. & Heremans, J.F. (1974). Amino acid sequence of cysteic peptides of lactoferrin and demonstration of similarities between lactoferrin and transferrin. *Biochim. Biophys. Acta.* **365**, 311.

Bobek, L.A. & Levine, M.J. (1992). Cystatins-inhibitors of cysteine proteinases. *Crit. Rev. Oral Biol. Med.* **3,** 307-332.

Brand, R.W. & Isselhard, D.E. (1998). Salivary glands. In: *Anatomy Of Orofacial Structures.* Mosby. St Louis. Missouri, pp: 119-125.

Brunstrom, J.M. (2002). Effects of mouth dryness on drinking behaviour and beverage acceptability. *Physiol. Behav.* **76(3)**, 423-429.

Bundgaard, M.; Moller, M. & Poulsen, J.H. (1977). Localization of sodium pumps sites in cat salivary glands. *J. Physiol. Lond.*; **273,** 339-53.

Burgoyne, R.D. (1990). Secretory vesicle-associated proteins and their role in exocytosis. *Ann.Rev.Physiol.* **52,** 647-659.

Burnstock, G. (1971). Neural nomenclature. *Nature Lond.* **229,** 282-283.

Burnstock, G.; Cocks, T.; Kasakov, L. & Wong, H. (1978). Direct evidence for ATP release from non adrenergic noncholinergic ("purinergic") nerves in guinea pig taenia coli and bladder. *Eur. J. Pharmacol.* **49,** 145-149.

Bylund, D.B.; Martinez, J.R. & Pierce, D.L. (1982). Regulation of autonomic receptors in rat submandibular gland. *Mol. Pharmacol.* **21,** 27-35.

Camacho, P. & Lechleiter, J.D. (1995). Calreticulin inhibits repetitive intracellular calcium waves. *Cell.* **82,** 765-771.

Camello, P.; Gardner, J.; Petersen, O.H. & Tepekin, A.V. (1995). Calcium dependence of calcium extrusion and calcium uptake in mouse pancreatic acinar cells. *J. Physiol. (Lond).* **490,**585-593.

Carty, D.J.; Padrell, E.; Codina, J.; Birnbaumer, L.; Hildebrandt, J.D. & Iyengar, R.J. (1990). Distinct nucleotide binding and release properties of the three Gi proteins. *J. Biol. Chem.* **265,** 6268-6273.

Castle, D. & Castle, A. (1998). Intracellular transport and secretion of salivary proteins. *Crit. Rev. Oral Biol. & Med.* **9(1),** 4-22.

Castle, J.D.; Jamieson, J.D. & Palade, G.E. (1972). Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit. *J. Cell Biol.* **53,** 290-311.

Chavez, E.M.; Borrell, L.N.; Taylor, G.W. & Ship, J.A. (2002). A longitudinal analysis of salivary flow in control subjects and older adults with type 2 diabetes. Oral *Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **91(2),**166-173.

Chimenos-Kustner, E. & Marques-Soares, M.S. (2002). Burning mouth and saliva. *Med. Oral* **7(4),** 244-253.

Clapham, D.E. (1995). Calcium signalling. *Cell.* **80,** 259-268.

Clemente, F. & Meldosi, J. (1975). Calcium and pancreatic secretion: Subcellular distribution of calcium and magnesium in the exocrine pancreas of the guinea pig. *J. Cell. Biol.* **65,** 88-102.

Cohen, R.E. & Levine, M.J. (1989). Salivary Glycoproteins *In* Human Saliva: Clinical Chemistry and Microbiology. Volume 1. J. O. Tenovuo, Ed: CRC Press Boca Raton, Florida pp101-130.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eye lid opening in the new born animal. *J. Biol. Chem.* **237,** 1555-1562.

Cole, M.F.; Hsu, S.D.; Baum, B.J.; Bowen, W.H.; Sierra, L.I.; Aquirre, M. & Gillespie, G. (1981). Specific and non specific immune factors in dental plaque from young and old populations. *Infect. Immun.* **31,** 998.

Cook, DI; Van Lennep, E.W.; Roberts, M.L. & Young, J.A. (1994). Secretion by the major salivary glands. In: Physiology of The Gastrointestinal Tract. Johnson LR, editor. New York. Raven Press, pp. 1061-1117.

Cowman, R.A.; Frisch, M.; Lasseter, C.J. & Scarpace, P.J. (1994). Effcts of beta-adernergic antagonists on salivary secretory function in individuals of different ages. *J. Gerontol: Biol. Sci.* **49**, B208-B214.

Crippa, G. & Giorgi-Pierfimceschi, M. (1997). Magnesium in cirrhosis of the liver. In: *Advances in Magnesium Research: Magnesium in Cardiology.* Ed: Smetana R, John Libbey, London, pp 294-302.

Culp, D.J.; Luo, W.; Richardson, L.A.; Watson, G.E. & Latchney, L.R. (1996). Both m1 and m3 receptors regulate exocrine secretion by mucous acini. *Am. J. Physiol.* **261,** C1963-C 1972.

Dawes, C. (1969). The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human parotid saliva. *Arch. Oral. Biol.*

Dawes, C. (1974). Rythms in salivary flow rates and composition. *Int. J. Chronobiol.* **2:** 253-279.

Dawes, C. (1984) Stimulus effects on protein and electrolyte concentrations in parotid saliva. *J. Phisiol.* **346,** 579-588.

Dawes, C. (1987). Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. *J. Dent. Res.* **66,** (spec. Issue): 648-653.

De Bias, D.A. (1990). Introduction to the autonomic nervous system. *In Basic Pharmacology in Medicine.* Edited by J. R. Di Palma & G.J. Di Gregorio, McGraw Hill, New York, pp 79-92.

DeHaye, J.P.; Cristophe, J.; Ernst, F.; Polaczek, P. & Van Bogaert, P. (1985). Binding *In Vitro* of vasoactive intestinal peptide on isolated acini of rat parotid glands. *Arch. Oral Biol.* **30,** 827-832.

Denny, P.C.; Ball, W.D. & Redman, R.S. (1997). Salivary glands: A paradigm for diversity of gland development. *Crit. Rev. Oral Biol. & Med.* **8(1),** 51-75.

Dinudon, A. ; Poronnik, P. ; Allen, G.D. et al. Control of intracellular Ca 21 by adrenergic and muscarinic agonists in mouse mandibular ducts and endpieces. *Cell Calcium* 1993a; **14,** 631-638.

Dissing, S.; Gromada, J.L.; Jorgensen, K.; *et al.* (1993). Spatio-temporal aspects of calcium signalling in acinar cells. *News in Physiological Sciences.* **8,**103-107.

Dissing, S. & Nauntofte, B. (1990). Na^+ transport properties of isolated rat parotid acini. *Am J Physiol,* **259,** G 1044-G 1055.

Douglas, W.W. & Poisner, A.M. (1963). The influence of calcium on the salivary response of the submaxillary gland to the acetylcholin or noradrenaline. *J. Physiol.* **165,** 528-541.

Douglas, W.W. & Rubin, R.P. (1961). The role of calcium in secretory response of the adrenal medula to acetylcholine. *J. Physiol,* **159,** 40.

Dowd, F.J. (1999). Saliva and dental caries. *Dent. Clin. North. Am.* **43(4),** 579-597.

Drobitch RK, Svensson CK. (1992). Therapeutic drug monitoring in saliva. An update. *Clin Pharmacokinet.* **23.** 365-379.

Dumbrigue, H.B.; Sandow, P.L.; Nguyen, K.T. & Humphrey-Beher, M.G. (2000). Salivary epidermal growth factor decrease in patients receiving radiation therapy to the head and neck. *Oral Surg Oral Med Oral Pathol* **89,** 710-716.

Durlach, J. (1988). Magnesium in Clinical Practice. John Libbey, London .

Dusek, M.; Simmons, J.; Buschang, P.H. & Al-Hashimi, I. (1996). Masticatory function in patients with xerostomia. *Gerodontology* **13,** 3-6.

Edgar, W.M. (1992). Saliva: Its secretion composition and functions. *Br. Dent. J.* **172,** 305-312.

Elin, R.I. (1987). Assessment of magnesium status in cells, tissues and body. In: *Magnesium in Cellular Processes and Medicine*. Eds: Altura B. Durlach J, Seelig M, Karger. Basel.

Emley, E.F. (1966). In: *Principles of Magnesium Technology.* Pergamon press. London.

Ernst, S.L. (1993). Diatery intake, food preferences, stimulated salivary flow rate and masticatory ability in older adults with complete dentures. *Spec. Care. Dent.* **13,**102-106.

Fekete, Z.; Korec, R.; Feketeova, E.; Murty, V.L.; Piotrowski, J.; Slomiany, A. (1993). Salivary and plasma insulin levels in man. *Biochem. Molec. Biol. Int.* **30,**623-629.

Fergusson, D.B. (1988). Control of digestion. *In Physiology for Dental Students.* Edited by: Wrigth, Butterworth & Co, Ltd.England. Ch 21.

Fergusson, D.B. (1996). The flow rate of unstimulated human labial gland saliva. *J. Dent. Res.* **75,** 980-985.

Fine, D.H.; Furgan, D. & Beydouin, F. (2002). Lactoferrin iron levels are reduced in saliva of patients with localized agressive periodontitis. *J. Periodontol.* **73(6),** 624-630.

Flatman, P.W. (1993). The role of magnesium in regulating ion transport. In: *Magnesium and the Cell.* Ed: Birch NJ, Academic Press, London, pp 197-216.

Fleming, N.; Bilan, P.T. & Graham, K.W. (1984). Effect of substance P on exocrine secretion by rat submandibular glands. *J. Dent. Res.* **63,** 1022-1027.

Fleming, N.; Bilan, P.T.; Sliwinsky-Lis, E. & Carvalho, V. (1987). Muscarinic, α1 adrenergic and peptidergic agonists stimulate phosphoinositides hydrolisis and regulate mucin secretion in rat submandibular gland cells. *Pfluger's Arch.* **409,** 416-421.

Forcella, S.; Mata, A.D.; Mesquita, M.F. & Spotto, G. (2002) Saliva and the biochemical aspects of oral homeostasis In: *Oxford Handbook Of Applied Dental Sciences.* Scully, C. Eds. Oxford University Press. Oxford pp 363-368.

Foskett, J.K. & Wong, D. (1994). $[Ca^{2+}]_i$ inhibition of Ca^{2+} release-activated Ca^{2+} influx underlies agonist-and tapsigargin-induced $[Ca^{2+}]_i$ oscillations in salivary acinar cells. *J. Biol. Chem.* **269,** 31525-31532.

Fox, P.C. (1989). Saliva Composition and its importance in dental health. *Compend Contin Educ Dent* Suppl 13, S456-S460.

Fox P.C. (1993). Salivary monitoring in oral diseases. *Ann NY Acad Sci* 694, 234-237.

Francis, L.P & Singh, J. (1990). Effects of phorbol esters and related compounds on acetylcholine evoked $[H^3]$ leucine labeled protein secretion in permeabilised rat pancreatic acini. *Exp. Physiol.* **75** (4), 537-546.

Francis, LP; Lennard, R & Singh, J. (1990) Mechanism of action of magnesium on acetylcholine evoked secretory responses in the isolated rat pancreas. *Exp Physiol* **75,** 669-680.

Fujita, Y.J.; Dohke, Y.; Hara, Y.M.; Kamata, Y.; Kozaki, S.; Furuyama, S. & Sugiya, H. (1996). Vesicle associated membrane protein 2 is essential for cAMP regulated exocytosis in rat parotid acinar cells. The inhibition of cAMP-dependent amylase release by botulinum neurotoxin B. *J. Biol. Chem.* **271(22),** 13130-13134.

Gallacher, G.V. (1982). Are there purinergic receptors on parotid acinar cells? *Nature Lond.* **296,** 83-86.

Geada, M.R. (1998). Molecular Mechanisms of Magnesium Transport in Epithelial Cells. PhD Thesis. University of Central Lancashire, Preston. UK.

George, G.A. & Heaton, F.W. (1975). Changes in cellular composition during magnesium deficiency. *Biochem. J.* **152,** 609-615.

Gibbons, R.J. & Hay, D.I. (1988). Human salivary acidic proline rich proteins and statherin promote the attachment of *Actinomyces Viscosus* LY7 to apatitic surfaces. *Infect. Immun.* **56,** 439-445.

Gibbons, R.; Stoppelar, J.D. & Harder, L. (1966). Lysozyme insensitivity of bacteria indigenous to the oral cavity of man. *J. Dent. Res.* **45,** 877.

Gong, K.; Mailloux, L. & Herzberg, M.C. (2000). Salivary film expresses a complex macromolecular binding site for *Streptococcus sanguis. J. Biol. Chem.* **275(12),** 8970-8974.

Gray, P.TA. (1988). Oscillations of free cytosolic Ca^{2+} evoked by cholinergic and catecholaminergic agonists in rat parotid acinar cells. *J. Physiol.* **406,** 35-53.

Gresik, E.W. (1994). The granular convoluted tubule (GCT) cell of rodent submandibular glands. *Micros. Res. Techn.* **27,** 1-24.

Griwold, R.L. & Pace, N. (1956). The intracellular distribution of metal ions in rat liver. *Expt Cell Res* **11,** 362-367.

Gueux, E.; Cubizolles, C.; Bussiere, L.; Mazur, A. & Rayssiguier, Y. (1993). Oxidative modification of triglyceride-rich lipoproteins in hypertriglyceridemic rats following magnesium deficiency. *Lipids* **28,** 573-575.

Gullestad, L.; Nes, M.; Ronnerberg, R.; Mitvedt, K.; Falch, D. & Kjekshus, J. (1994). Magnesium status in healthy freeliving elderly Norwegians. *J. Am. Coll. Nutr.* **13,** 45-50.

Gunther, T. (1990). Functional compartmentation of intracellular magnesium. In: *Metal Ions in Biological Systems: Compendium on Magnesium and its role in Biology, Nutrition and Physiology*. Eds: Sigel H, Sigel A, Marcel Dekker, NY.

Hagishengallis, G.; Nikolova, E. & Russel, M.J. (1992). Inhibition of *Streptococcus mutans* adherence to saliva coated hydroxyapatite by human secretory IgA antibodies to the cell surface antigen I/II: Revearsal by IgA1 protease cleavage. *Infect. Immun.* **60,** 5057-5064.

Hand, A.R.; Pathmanathan, D. & Field, R.B. (1999). Morphological features of the minor salivary glands. *Arch Oral Biol.* **1,** S3- S10.

Hanckock, J.T. (1997). *Cell Signalling.* Longman England.

Hatton, M.N.; Loomis, R.E.; Levine, M.J. & Tabak, L.A. (1985). Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex. *Biochem. J.* **230,** 817-820.

Hay, D.I. (1973). The isolation from human parotid saliva of a tyrosine rich acidic peptide which exibits high affinity for hydroxyapatite surfaces. *Arch. Oral. Biol.* **18,** 1531-1541.

Hay, D.I. (1975). Fractionation of human parotid salivary proteins and the isolation of an histidine-rich peptide which shows high affinity for hydroxyapatite surfaces. *Arch. Oral. Biol.* **18,** 1531-1541.

Hay, D.I. (1995). Salivary factors in caries models. *Adv. Dent. Res.* **9(3),** 239-243.

Hay, D.I. & Gron, P. (1976). Inhibitors of calcium phosphate precipitation in human whole saliva. *In* proceedings, microbial aspects of dental caries, HM Styles, WJ Loesche & TC O' Brien, Eds., Sp. Supp. *Microbiol abst,* Washington: Information retrieval, pp. 143-150.

Heaton, F.W. (1990). Role of magnesium in enzyme systems. In: *Metal Ions in Biological Systems: Compendium on Magnesium and its role in Biology, Nutrition and Physiology.* Eds: Sigel H, Sigel A, Marcel Dekker, NY.

Heaton, F.W. (1993). Distribution and function of magnesium within the cell. In: Magnesium and the Cell. Ed: Birch NJ, Academic Press, London, pp 121-136.

Hellman, J.; Ambudkar, I.S. & Baum, B.J. (1987). Adrenorecptor mobilisation of calcium in rat submandibular gland cells. *Eur. J. Pharmacol.* **143,** 65-72.

Henrotte, G.J. (1993). Genetic regulation of cellular magnesium content. In: *Magnesium and the Cell.* Ed: Birch NJ. Academic Press, London, pp 177-196.

Henskens, Y.M.; Van Den Keijbus, P.A.; Veerman, E.C.; Van Der Weidjen, G.A.; Timmerman, M.F.; Snoek, C.M.; Van Der Velden, U.; Amerongen, A.V. (1996). Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of Cystatins, Albumin, Amylase and IgA. *J. Periodontal. Res.* **31,** 57-65.

Herrera, J.L.; Lyons, M.F. & Johnson, L.F. (1988). Saliva: Its role in health and disease. *J. Clin. Gastroenterol.* **10**(5), 579-588.

Hersey, S.J. & Perez, A. (1990). Permeable cell models in stimulus-secretion coupling. *Ann. Rev. Physiol.* **52,** 345-361.

Hershey, A. & Krause, J.E. (1990). Molecular characterization of functional cDNA encoding the rat substance P receptor. *Science* **247,** 958-962.

Hicks, M. J.; Carter, A.B.; Rossman, S.N.; Demmler G.J.; Simmon, C.L. & Cron, S.G. (1998). Detection of fungal organisms in saliva from HIV-infected children: A preliminary cytologic analysis. *Pediatr. Dent.* 20: 162-168.

Hinkle, M.T. & Soor, S.K. (1992) Stimulation of rat parotid secretion by cAMP analogues that sinergistically activate the type II isoenzyme of cAMP-dependent protein kinase. *Arch. Oral Biol.* **37(2)**, 85-92.

Hiltke, T.R.; Lee T.C. & Bobek L.A. (1999). Structure and function analysis of human cystatin SN and comparision of the cysteine proteinase inhibitory profiles of human cystatins C and SN. *J. Dent. Res.* **78(8),** 1401-1409.

Hiramatsu, Y.; Kagami, H.; Kosaki, K.; Shigetomi, T.; Ueda, M. & Kobayashi, S. (1994). The localization of basic fibroblast growth factor (FGF-2) in rat submandibular glands. *Nagoya J. Med. Sci.* **57,** 143-152.

Holbrook, I.B. & Molan, P.C. (1975). The identification of a peptide in human parotid saliva particulary active in enhancing the glycolytic activity of the salivary micro-organisms. *Biochem. J.* **149:** 489-492.

Hug, M.; Pahl, C. & Novack, I.. (1994). Effect of ATP, Carbachol and other agonists on intracellular calcium activity and mambrane voltage of pancreatic ducts. *Pfluegers. Arch.* **426,** 412-418.

Humphreys-Beyer, M.G.; Macauley, S.P.; Chegini, N.; Van Setten, G.; Purushotham K.; Stuart, C. (1994) Characterization of the synthesis and secretion of transforming growth factor alpha from salivary glands and saliva. *Endocrinology* **134,** 963-970.

Hurley, T.W.; Ryan, M.P. & Shoemaker, D.D. (1994). Mobilisation of Ca^{2+} influx, not stored Ca^{2+}, by extracellular ATP in rat submandibular gland acini. *Arch. Oral Biol.* **39,** 205-212.

Inoue, Y.; Kohei, K.; Kaneko, T.; Yanaihara, N. & Kanno, T. (1983). Intestinal peptide binding to specific receptors on rat parotid acinar cells induces amylase secretion acompained by intracellular acumulation of cyclic adenosine 3' 5',-monophosphate. *Endocrinology.* **116,** 686-697.

Iontcheva, I.; Oppenheim, F.G.; Troxtler, R.F. (1997). Human salivary MG1 selectively forms heterotypic complexes with amylase, proline rich proteins, statherin and histatins. *J. Dent. Res.* **76(3),** 734-743.

Isemura, S.; Saitoh, E.; Ito, S. & Isemura, M. (1984). Cystatin-S: A cysteine proteinase inhibitor of human saliva. *J. Biochem.* **96,** 1311-1314.

Isemura, S.; Saitoh, E. & Sanada, K. (1986). Characterization of new cysteine proteinase inhibitor of human saliva, cystatin-SN, which is immunologically related to cystatin-S. *FEBS. Lett.* **198,** 145-149.

Isemura, S.; Saitoh, E. & Sanada, K. (1987). Characterization and amino acid sequence of a new acidic cystein proteinase inhibitor (Cystatin-AS) structurally closely related to cystatin-S from human whole saliva. *J. Biochem.* **102,** 693-704.

Ishizuka, R.J.; Bold, C. M.; Townsend, Jr. & Thomson, J.C. (1994). *In vitro* relationship between magnesium and insulin secretion. *Magnesium Res.* 7, 17-22 .

Ito S; Isemura S; Saitoh E; Sanada K; Suzuki T & Shibata A. (1983) Imunohistochemical demonstration of salivary proline-rich peptide P-C like imunoreactivity in human pancreatic β cells. *Acta Endocr* **103:** 544-551.

Ito, S.; Isemura, S.; Saitoh, E.; Sanada, K.; Suzuki, T. & Shibata, A. (1984). A study on salivary proline- rich peptide P-C with special reference to the presence of salivary peptide P-C in the human respiratory tract. *Folia. Endocrinol. Jap.* **60,** 16-22.

Iwamoto, Y. & Matsumura, T. (1966). Purification and characterization of the salivary anti-microbial factor (AS factor). *Arch. Oral. Biol.* **12,** 1009-1012.

Iyengar, R. (1993). Molecular and functional diversity of mammalian Gs stimulated adenyly cyclases. *FASEB J.* 7, 768-775.

Jansen, J. W.; Shreurs, V. V. ; Schwartz, H.G. ; Fleuren-Jacobs, A. M. ; de Pont, J. J. & Bonting, S.L. (1980). Role of calcium in exocrine pancreatic secretion. VI. Characteristics of paracellular pathways for divalent cations. *Biochim. Biophys. Acta.* 599, 315-323

Jaskoll, T.; Choy, HÁ & Melnick, M. (1994). Glucocorticoids, TGFβ, and embrionic mouse salivary gland morphogenesis. *J. Craniofac. Genet. Dev. Biol.* **14,** 217-230.

Jenkins JN. (1978) Saliva. In: *The physiology and biochemistry of the mouth.* 4th ed. London: Blackwell pp 285-381.

Jensen, JL.; Lamkin, MS.; Troxler, RF & Oppenheim, FG. (1991). Multiple forms of statherin in human salivary secretions. *Arch. Oral Biol.* **36(7),** 529-534.

Jin, Y. & Hak-Kong, H. (2002). Supragengival calculus: Formation and Control. *Crit.Rev.Oral Biol. & Med.* **13(5),** 426-441.

Jones, R.E. & Ship, J.A. (1995). Major salivary gland flow rates in young and old, generally healthy African American & Whites. *J. Nat. Med. Assoc.* **87**, 131-135.

Juma, L.M.O.; Wisdom, D.M. & Singh, J. (1996). Magnesium homeostasis in the exocrine rat pancreas: Effects of electrical field stimulation and ACh. In: *Current Research In Magnesium.* Eds: Halpern, MJ, Durlach J; John Libbey, London, pp 319-322.

Jung, D.W. & Brierley, G. (1994). Magnesium transport by mitochondria. *J. Bioenergetics Biomembr.* **26** (5), 527-535.

Junqueira, L.C. & Carneiro, J. (1990). Glândulas anexas ao tubo digestivo. In: *Histologia Básica.* Guanabara. São Paulo. pp: 240-245.

Kasahara, K.; Tasaka, K.; Masumoto, N.; Nishizaki, T.; Mizuki, J.; Tahara, M.; Miyake, A. & Tanizawa, O. (1993). High magnesium concentration inhibits ligand-stimulated calcium influx and hormone secretion in rat pituitary lactotropes with involvement of intracellular free magnesium. *Biochem. Biophys. Res. Commun.* **197,** 92-99 .

Kauffman, D.L. & Keller, P.J. (1979). The basic proline rich proteins in human parotid saliva from a single subject. *Arch. Oral Biol.* **24,** 249-256.

Kaufman, E. & Lamster, I.B. (2000). Analysis of saliva for periodontal diagnosis: A review. *J. Clin. Periodontol.* **27,** 453-465.

Kerr, M.; Lee, A.; Wang, P.L.; Purushotham, K.; Chegini, N. & Yamamoto, H. (1995). Detection of insulin, insulin like growth factors I and II in saliva and potential synthesis in the salivary glands of mice. Effect of type 2 diabetes mellitus. *Biochem. Pharmacol.* **49,** 1521-1531.

Khan, A.A.; Soloski, M.J.; Sharp, A.H.; Schilling, G.; Sabatini, D.M.; Li, S.H.; *et al.* (1996). Lymphocyte apoptosis: mediation by increased type 3 inostol 1, 4, 5, trisphosphate receptor. *Science.* **273,** 503-507.

Kilian, M. & Russel M.W. (1998). Bacterial evasion of mucosal immune defenses. In: Ogra PL; Mesteky J; Lamm ME; Strober W. *Mucosal Immunity* McGhee JR; Biennenstock J Eds. Ed2 San Diego. Academic Press pp241-251.

Kholer, B. & Bjarnasson, S. (1992). Mutan streptocci, Lactobacilli and caries prevalence in 15-16 year-olds in goteborg. Part II. *Swed. Dent. J.* **16,** 259-259.

Könönen, E. (2000). Development of oral bacterial flora in young children. *Ann. Med.* **32,** 107-112.

Lagerlof, F. (1998). Saliva: Natural protection against caries. *Rev. Belge Med. Dent.* **53(1),** 337-348.

Lamkin M.S.; Arancillo, A.A. & Oppenheim F.G. (1996). Temporal and compositional characteristics of salivary protein adsorption to hydroxyapatite. *J Dent Res* **75(2),** 803-808.

Lazzari, E.P. (1983) Saliva. In: *Handbook of Experimental Aspects of Oral Biochemistry.* CRC press inc. Eds. Boca Raton, Florida pp 285-319.

Lee, M.J.; Xu, X.; Zeng, W.; Diaz, J.; Wojcikiewickz, J.H.; Kuo, T.H. ***et al.*** (1997a). Polarized expression of Ca^{2+} channels in pancreatic and salivary acinar cells: correlation with initiation and propagation of $[Ca^{2+}]_i$ waves. *J. Biol. Chem.* **272,**15765-15770.

Lee, M.J.; Xu, X.; Zeng, W.; Diaz, J.; Kuo, T.H.; Wuytack, F.; ***et al.*** (1997b). Polarized expression of Ca^{2+} channels in pancreatic and salivary acinar cells: role in initiation and propagation of $[Ca^{2+}]_i$ waves. *J. Biol. Chem.* **272,** 15771-15776.

Leeuw, I.D. (2001). Magnesium and diabetes: Is magnesium depletion a risk factor for complications in type 1 diabetic patients. In: *Advances in Magnesium Research Nutrition and Health.* Rayssiguier, Y; Mazur, A & Durlach, J. Eds. John Libbey & Co Ltd. London pp369-374.

Leinonen, J.; Kivela, J.; Parkkila, S.; Parkkila, A.K. & Rajaniemi, H. (1999). Salivary carbonic anhydrase isoenzyme VI is located in human dental pellicle. *Caries Res.* **33(3),** 185-190.

Lennard, R. & Singh, J. (1992). Effects of secretagogues on intracellular free calcium and magnesium concentrations in rat pancreatic acinar cells. *Gen. Pharmacol.* **23,** 903-908.

Levine, M.J.; Reddy, M.S.; Tabak, L.A.; Loomis, R.E.; Bergey, E.J.; Jones, P.C.; Cohen, R.E.; Stinson M.W. & Al-Hashimi, I. (1987). Structural aspects of salivary glycoproteins. *J. Dent. Res.* **66,** 436-441.

Levine, M.J. (1993). Salivary Molecules. Astructure function Synopsis. In: *Saliva as a diagnostic fluid.* Ann NY Acad Sci; 694: 11-15. Eds New York.

Liang, H.; Wang, Y.; Wang, Q. & Ruan, M.S. (1999). Hydrophobic interaction chromatography and capillary zone electrophoresis to explore the correlation between the izoenzymes of salivary alfa amylase and dental caries. *J. Chromatogr. B. Biomed. Sci. Appl.* **724(2),** 381-388.

Linder, M.E.; Ewald, D.A.; Miller, R.J. & Gilman, A.G. (1990). Purification and characterization of $G_{O\alpha}$ after expression in *Escheria coli. J. Biol. Chem.* **265,** 8243-8251.

Liu, B.; Offner, G.D.; Nunes, D.P.; Oppenheim, F.G. & Troxler, R.F. (1998). MUC4 is a major component of salivary mucin MG1 secreted by the human submandibular gland. *Biochem. Biophys. Res. Commun.* **250(3),** 757-761.

Lockwich, T.P.; Liu, X.; Singh, B.B.; Jadlowiec, J.; Weiland, S. & Ambudkar, I.S. (2000). Assembly of Trp1 in a signalling complex associated with caveolin-scaffolding lipid raft domains. *J. Biol. Chem.* **275(16),** 11934-11942.

Loesche, W.J.; Shork, A.; Terpenning, M.S.; Chen, Y.M. & Stoll, J. (1995). Factors which influence levels of selected microorganisms in saliva of older individuals. *J. Clin. Microbiol.* **33,** 2550-2557.

Loomis, R.E.; Prakobphol, A.; Levine M.J.; Redy, M.S. & Jones, P.C. (1987) Biochemical and biophysical comparision of two mucins from human sub-mandibular sub-lingual saliva. *Arch. Biochem. Biophys.* **258 (2),** 452-464.

Lowry, O.H.; Roseborough, N.J.; Forr, A.L. & Randall, R.J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Lundberg, J.M.; Änggard, A.; Famrenkrug, J.; Hökfelt, T. & Mutt, V. (1980). Vasoactive Intestinal Polypeptide in cholinergic neurons of exocrine glands: Functional significance of coexisting transmitters for vasodilatation and secretion. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1651-1655.

Mahay, S.;Winlow, W.; Singh, J.; Pariente, J.; Lajas, A.I. & Adeghate, E. (2002). Effects of ageing on morphology and α-amylase release in the isolated rat parotid gland. *J. Physiol.* **543P**, 15P.

Mandel ID. (1993). Salivary diagnosis: more than a lick and a promise. *J. Am Dent Assoc* **124**, 85-87.

Manganel, M. & Turner, R.J. (1990). Agonist-induced activation of Na'/H* exchange in rat parotid acinar cells is dependent on calcium but not protein kinase. C. *J. Biol. Chem.* **2 (5),** 4284-4289.

Manganel, M. & Turner, R.J. (1991). Rapid secretagogue-induced activation of Na^+/H^+ exchange in rat parotid acinar cells. *J. Biol. Chem.* **266,** 10182-10188.

Markopoulos, A.K. & Belazi, M. (1998). Histopathological and immunohistochemical features of the labial salivary glands in children with type 1 diabetes. *J. Diabetes Complic.* **12**, 39-42.

Marshall, I.C.B. & Taylor, C.W. (1993). Regulation of inositol 1,4,5-trisphosphate receptor. *Biochem. J.* **303,** 591-598.

Martinez, J.R.; Holzgreve, H. & Rick, A. (1966). Micropunture study of submaxillary glands of adult rats. *Pfluggers. Arch.* **190,** 124-133.

Masson, P.L.; Heremans, J.F. & Dive, C. (1966). An iron binding protein common to many external secretions. *Clin. Chim. Acta* **14,** 735.

Mata, A.D.; Marques, D.; Geraldes, M; Rocha, S.; Mesquita, M.F. & Singh, J. (2001). Acetylcholine evoked salivary secretion: Role of extracellular magnesium on acetylcholine evoked secretory responses in the isolated rat submandibular cell glands. In: *Advances in Magnesium Research Nutrition and Health.* Rayssiguier, Y; Mazur, A & Durlach, J. Eds. John Libbey & Co Ltd. London pp 93-96.

Mata, A.D., Marques, D., Mesquita, MF.; Singh, J. (2002) Effect of extracellular magnesium on secretagogue-evoked amylase secretion in the isolated rat parotid gland segments. *Mag. Res.* **(15), 3/4,** 161-165.

Mateos, A.G. (1996). Mecanismos Ionicos de Acoplamiento en la Secrecion Ionica. Tesis Doctoral. Departamento de Fisiologia. Faculdade de Veterinária. Caceres. España.

McKay, B.J.; Pollock, J.J.; Iacono, V.J. & Baum, B.J. (1984). Isolation of miligram quantities of a group of histidine-rich polypeptides from human parotid saliva. *Infect. Immmun.* **44,** 688-694.

McKay, B.J.; Denepitiya, L.; Iacono, V.J.; Krost, S.B. & Pollock, J.J. (1984). Growth inhibitory and bactericidal effects of human parotid salivary histidine rich polypetides on *Streptococcus mutans. Infec. Immun.* **44,** 695-701.

McKay, S.L.D.; Bennet, N.T.; Bland, K.I. & Schultz, G.S. (1992). Growth factors, tumors suppressors and cancer. *Perspect. Gen. Surg.* **3,** 1-25.

McMillan, M.K.; Soltoff, S.P.; Cantley, L.C.; Rudel, R.A. & Talamo, B.R. (1993). Two distinct cytosolic calcium responses to extracellular ATP in rat parotid acinar cells. *Br. J. Pharmacol.* **108,** 453-461.

McMinn, R.M.H.; Hutchings, R.T. & Logan, B.M. (1990). The mouth, palate and pharynx. In: *A Colour Atlas Of Head And Neck Anatomy.* 6th edition. Wolfe Medical Publications. London pp, 136-147.

Meehan, S.M.; Wu, A.J.; Kang, E.C.; Sakai, T. & Ambudkar, I.S. (1997). Interferon γ induces a decrease in the intracellular Ca^{2+} pump in a human salivary gland cell line. *Am. J. Physiol.* **273,** C2030-C2036.

Melvin, J.E.; Koek, L. & Zhang, G.H. (1991). A capacitative Ca^{2+} influx is required for sustained fluid secretion in sub-lingual mucous acini. *Am. J. Physiol.* **261,** G1043-G1050.

Melvin, J.E. & Tamer, R.L. (1992). Cl fluxes related to fluid secretion by the rat parotid: involvement of Cl-HC03 exchange. *Am. J. Physiol.* **262,** 6393-6398.

Merrit, J.E. & Rink, T.J. (1987). Regulation of cytosolic calcium in fura 2 loaded rat parotid acinar cells. *J. Biol. Chem.* **262,** 17362-17369.

Mertz, L.M.; Baum, B.J. & Ambudkar, I.S. (1990a). Ca^{2+} entry in parotid acini: activation by carbachol and aluminium fluoride. *Am. J. Physiol.* **258,** C656-C661.

Mertz, L.M.; Baum, B.J. & Ambudkar, I.S. (1990b). Refill status of the agonist sensitive Ca^{2+} pool regulates Mn^{2+} influx into parotid acini. *J. Biol. Chem.* **265,** 15010-15014.

Mesteky, J.; Lue. C. & Russel, M.W. (1991). Selective transport of IgA:Cellular and molecular aspects. *Clin. North. Am.* **20,** 441-471.

Mesteky, J. & Russel, M.W. (1986). IgA Subclasses. *Monogr. Allergy.* **19,** 277-301.

Métioui, M.; Grosfils, K. & Dehaye, J.P. (1994). Interactions between tapsigargin and ATP^{4-} in the regulation of the intracellular calcium in rat submandibular glands. *J. Cell Physiol.* **161,** 243-248.

Mikoshiba, K. (1993). Inositol 1,4,5-trisphosphate receptor. *Trends. Physiolog. Sci.* **14,** 86-89.

Mitchell, R.H. (1975). Inositol phospholipids and cel surface receptor function. *Biochim. Biophys. Acta.* **415,** 81-147.

Mogami, H.; Tepekin, A.V. & Petersen, O.H. (1998). Termination of the cytosolic Ca^{2+} signals: Ca^{2+} reuptake into intracellular stores is regulated by the Ca^{2+}concentration in the store lumen. *EMBO. J.* **17,** 435-442.

Moller, K.; Benz, D.; Perrin, D. & Solling, H.D. (1996). The role of protein kinase C in carbachol induced and of cAMP-dependent protein kinase in isoproterenol-induced secretion in primary cultured guines pig parotid acinar cells. *Biochem. J.* **314(1),** 181-187.

Montero, M.; Brini, M.; Marsault, R.; Alvarez, R.; Sitia, R.; Pozan, T. *et al.*(1995). Monitoring dinamic changes in free Ca^{2+} concentrations in the endoplasmic reticulum. *EMBO. J.* **14,** 5467-5475.

Moore, P.A.; Guggenheimer, J.; Etzel, K.R.; Weyant, R.J. & Orchard, T. (2001). Type 1 diabetes mellitus, xerostomia, and salivary flow rates. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **92(3)**,281-291.

Mooren, F.C.; Domschke, W.; Stoll, R.; Geada, M.R.; Wisdom, D.M. & Singh, J. (1997). Magnesium homeostasis in exocrine pancreas and its possible therapeutic role in the treatment of pancreatitis. *Adapt. Biol. Med.* **1,** 284-298

Mooren, F.C.; Turi, S.; Günzel, D.; Schlue, W.R.; Domschke, W.; Singh, J. & Lerch, M.M. (2001). Calcium-magnesium interactions in pancreatic acinar cells. *FASEB J.* **15**, 659-672.

Moreno, E.C.; Varughese, K. & Hay, D.I. (1979). Effect of human salivary proteins on the precipitation kinetics of calcium phosphate. *Calcif. Tissue Int.* **28,** 7-16.

Moreno, E.C.; Kresak, M. & Hay, D.I. (1982). Adsorption thermodynamics of acidic proline rich human salivary proteins onto calcium apatites. *J. Biol. Chem.* **257,** 2981-2989.

Morisson, M. & Allen, P.Z. (1963). The identification and isolation of lactoperoxidase from salivary glands. *Biochem. Biophys. Res. Commun.* **13,** 490-494.

Muallem, S.; Shoffield, M.D.; Fimmel, C.J. & Pandol, S.J. (1988). The agonist sensitive calcium pool in the pancreatic acinar cell. *Am. J. Physiol.* **255,** G221-G228.

Muallem, S. (1989). Calcium transport pathways of pancreatic acinar cells. *Ann. Rev. Physiol.* **51,** 83-105.

Mulligan, R. & Sobel, S. (1994). Drugs commonly used by the ederly: a review for dental practice. *J. Calif. Dent. Assoc.* **22,** 35-47.

Murakami, K.; Taniguchi, H. & Baba, S. (1982). Presence of insulin like immunoreactivity and its biosynthesis in rat and human parotid gland. *Diabetologia.* **22,** 358-361.

Murakami, M.; Miyamoto, S. & Imal, Y. (1990). Oxygen consumption for K* uptake during poststimulatory activation of Na', K'-ATPase in perfused rat mandibular gland. *J. Physiol. Lond.* **426,** 127-43.

Murakami, M.; Seo, Y.; Miyamoto, S. et al. (1991). Cation handling by transporters during secretion in the perfused rat mandibular salivary gland. In: Murakami, Seo, Kulwahara and Wataryi, editors. *Ionic basis and energy metabolism of epithelial transport - Hot aspects from exocrine secretion.* National Institute for Physiological Sciences, Japan: Okazaki, pp: 225-8.

Nagler, R.; Marmary, Y.; Fox, P.C.; Baum, B.J.; Har-El, R. &Chevion, M. (1997). Irradiation induced damage to the salivary glands: the role of redox-active iron and copper. *Radiation Res.* **147,** 468-475.

Närhi, T.O. (1994). Prevalence of subjective feelings of dry mouth in the ederly. *J. Dent. Res.* **73(1),** 20-25.

Nathanson, N. (1987). Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10,** 195-236.

Nauntofte, B. (1992). Regulation of electrolyte and fluid secretion in salivary acinar cells. *Am. J. Physiol.* **263,** G823-G837.

Nauntofte, B. Poulse, J.H. (1986). Effects of Ca^{2+} and furosemide on Cl transport and O_2 uptake in rat parotid acini. *Am. J. Physiol.* **2 (1),** 175-185.

Navazesh M. (1993). Methods for collecting saliva. *Ann NY Acad Sci*; 694: 72-82.

Navazesh, M. (1992). Salivary gland hypofunction in ederly patients. *J. Calif. Dent. Assoc.* **22,** 62-68.

Navazesh, M.; Christensen, C. & Brightman, V.J. (1992). Clinical criteria for the diagnosis of salivary gland hypofunction. *J. Dent. Res.* **71**(7), 1363-1369.

Neer, E.J. (1995). Heterotrimeric G proteins: Organizers of transmembrane signals. *Cell* **80,** 249-257.

Nielsen, S.P. & Petersen, O.H. (1972). Transport of calcium in the superfused submandibular gland of the cat. *J. Physiol.* **223,** 685-697.

Nishizuka, Y. (1988). The molecular heterogenity of protein kinase C and its implications for cellular regulation. *Nature* **3334,** 661-665.

Ogra, P.L.; Mesteky, J.; Lamm, M.E.; Strober, W. (1998). *Mucosal Immunity* McGhee JR; Biennenstock J Eds. Ed2 San Diego. Academic Press.

Oppenheim, F.G.; Yang, Y.C.; Diamond, R.D.; Hyslop, D.; Offner, G.D. & Troxler, R.F. (1986). The primary structure and functional characterization of the neutral histidine rich polypeptide from parotid secretion. *J Biol Chem* **261,** 1177-1182.

Oppenheim, F.G.; Xu, T.; McMillian, F.M.; Levitz, S.M.; Diamond, R.D.; Hyslop, D.; Offner, G.D. & Troxler, R.F. (1988). Histatins a novel family of histidine rich proteins in human parotid secretion. *J. Biol. Chem.* **263,** 7472-7477.

Osserman, E.F.; Canfield, R.E. & Beychok, E. (1974). in: *Lysozyme*, Academic Press Eds. New York.

Osterberg, T.; Birkhed, D.; Johansson, C. & Svanborg, A. (1992). Longitudinal study of stimulated whole saliva in an elderly population. *Scand. J. Dent. Res.* **100**, 340-345.

Oxford, G.E.; Nguyen, K.H.; Alford, C.E.; Tanaka, Y. & Humphreys-Beher, M.G. (1998). Elevated salivary EGF levels stimulated by periodontal surgery. *J. Periodontol.* **69(4),** 479-484.

Palade, G.E. (1959). Functional changes in the structure of cell components. In: *Subcellular Particles.* Hiyashi, T. Eds. Ronald Press. New York. pp 64-83.

Page, E. & Polimeni, P. (1972). Magnesium exchange in rat ventricle. *J. Physiol. Lond.* **224**, 121-139.

Parekh, A.B. & Penner, R. (1997). Store depletion and calcium influx. *Physiol. Rev.* 77, 901-930.

Pariente, J.A.; Redondo, P.C.; Granados, M.P.; Lajas, A.I. Gonzalez, A.; Rosado, J.A. & Salido, G.M. (2003). Calcium signalling in non-excitable cells. *Mol. Cell Biol.* In press.

Parker, P.J.; Kour, G.; Marais, R.M.; Mitchell, F.; Pears, C.; Shapp, D.; Stabel, S. & Webster, C. (1989). Protein kinase C- a family affair. *Mol. Cell. Endocrinol.* **65,** 1-11.

Parsons, J.T. (1994). Time-dose-volume relation in radiation therapy. *In: Management of Head and Neck Cancer: A Multidisciplinary approach.* Million RR & Cassidy NJ (eds). Lippincott Co:Phyladelphia, pp173-243.

Patel, R.; Singh, J.; Winlow, W.; Adeghate, E.; Martinez-Victoria, E.; Mañas, M.; Yago, M.D. (2002) Morphological changes and secretory responses in streptozotocin-induced diabetic rat pancreas. *J. Physiol.* **543P,** 16 P

Pedersen, A.M.; Bardow, A.; Jensen, S.B. & Nauntofte, B. (2002). Saliva and gastrointestinal functions of taste, mastication, swalloing and digestion. *Oral Dis.* **8(3),** 117-129.

Pellerin, C. & Pellat, B. (1986). La salive. *In: Biochimie Odonto Stomatologique.* Masson. Paris pp 33 – 54.

Peters, E.H. & Azen, E.A. (1977). Isolation and partial characterization of human basic parotid proteins. *Bichem. Genet.* **15,** 947-962.

Petersen, O.H. (1992). Stimulus-secretion coupling: cytoplasmic Ca^{2+} signals and control of ion channels in exocrine acinar cells. *J. Physiol.* **448,** 1-51.

Petersen, O.H; Petersen, C.C.H. & Kasai, H. (1994). Calcium and hormone action. *Ann. Rev. Physiol.* **56,** 297-319.

Petersen, O.H. & Gallacher, D.V. (1988). Electrophysiology of pancreatic and salivary acinar cells. *Ann. Rev. Physiol.* **50,** 65-80.

Petit, J.F. & Jolles, P. (1966). Purification and analysis of human saliva lysozyme. *Nature* **200,** 168-169.

Poggioli, J. & Putney, JW Jr. (1982). Net calcium fluxes in rat parotid acinar cells. *Pflügers. Arch.* **392,** 239-242.

Pollock, J.J.; Denepitiya, L.; McKay, B.J.; Iacono, V.J. (1984). Fungistatic and fungicidal activity of human parotid salivary histidine rich polypeptides on *Candida albicans. Infect. Immun.* **44,** 702-707.

Poulsen, J.H. & Bledsoe, S.W. (1978). Salivary gland K' transport: in vivo studies with K'-specific microelectrodes. *Am. J. Physiol.* **234,** E79-E83.

Poulsen, J.H. & Nauntofte, B. (1990). Role of anion transport in secretion of primary saliva. In: Wong PYD, Young 1A, editors. *Epithelial secretion of water and electrolytes.* Berlin: Springer-Verlag, pp:143-155.

Prakobphol, A.; Levine, M.J.; Tabak, L.A. & Reddy, M.S. (1982). Purification of a low weight mucin type glycoprotein from human submandibular sublingual saliva. *Carbohydr Res.* **108,** 111-122.

Premont, R.T.; Matsuoka, I.; Mattei, M.G.; Pouille,Y.; Defer, N. & Hanoune, J. (1996). Identification and characterization of a widely expressed form of adenylyl cyclase. *J. Biol. Chem.* **271,** 13900-13907.

Purushotham, K.R.; Offenmüller, K.; Bui, A.T.; Zelles, T.; Blazsek, J. & Shultz, G.S. (1995). Absorption of epidermal growth factor occurs though the gastro inestinal tract and oral cavity in adult rats. *Am. J. Physiol.* **269,** G867-G873.

Putney, J.W. Jr. & Bird, G.S.J. (1993a). The inositol-phosphate calcium signalling system in non excitable cells. *Endocrine Rev.* **14,** 610-631.

Putney, JW Jr. (1986a). Identification of cellular activation mechanisms associated with salivary secretion. *Ann. Rev. Physiol.* **30,** 209-245.

Putney, JW Jr. (1986b). A model for receptor-regulated Ca^{2+} entry. *Cell. Calcium.* **7,** 1-12.

Putney, J. (1988). The role of the phosphoinositide metabolism in signal transduction in secretory cells. *J. Exp. Biol.* **139,** 135-158.

Putney, J. (1986). Identification of cellular activation mechanisms associated with salivary secretion. *Ann. Rev. Physiol.* **48,** 75-88.

Quamme, G.A. (1989). Control of magnesium transport in the thick ascending limb. *Am. J. Physiol.* **256,** F197-F210.

Quissell, D.O.; Watson, E. & Dowd, F.J. (1992). Signal transduction mechanisms involved in salivary gland regulated exocytosis. *Crit. Rev. Oral Biol. & Med.* **3** (1/2): 83-107.

Quissel, D.O.; Deisher, L.M. & Barzen, K.A. (1989). Activation and distribution of rat parotid cAMP dependent protein kinase following beta-adernergic receptor stimulation in vitro. *Arch. Oral Biol.* **34(1),** 23-26.

Raj, P.A.; Johnsson, M.; Levine, M.J. & Nancollas, G.H. (1992). Salivary statherin. *J. Biol. Chem.* **267,** 5968-5976.

Rall, L.B.; Scott, J.; Bell, G.I.; Crawford, R.J.; Penschow, J.D. & Niall, H.D. (1985). Mouse prepro epidermal growth facto synthetis by the kydney and other tissues. *Nature* **313,** 228-231.

Randriamampita, C. & Tsien, R.Y. (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature.* **364,** 809-814.

Randriamampita, C. & Tsien, R.Y. (1995). Degradation of a Ca^{2+} influx factor (CIF) can be blocked by phophatase inhibitors or by chelation of Ca^{2+}. *J. Biol. Chem.* **270,** 29-32.

Raymond, J.R.; Hnatowich, M.; Lefkowitz, R.J. &Caron M.J. (1990). Adrenergic receptors, modules for regulation of signal transduction processes. *Hypertension.* **15,** 119-131.

Reinhart, R.A. (1988). Magnesium metabolism. A review with special reference to the relationship between intracellular content and serum levels. *Arch. Intern. Med.* 148, 2415-2420.

Rinderknecht, J. & Marbach, E.P. (1970). A new automated method for the determination of serum α-amylase. *Clinica et Chemica Acta.* **29,** 107-110.

Rodbell, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284,** 17-22.

Roof, S.K. & Maguire, M.E. (1994). Magnesium transport systems: Genetics and protein structure (A Review). *J. Am. Nutri.* **13** (5), 424-428.

Rosenthal, O.; Gottlieb, B.; Gorry, J.D. & Vars, H.M. (1956). Influence of cations on the intracellular distribution of rat liver arginase. *J. Biol. Chem.* **223,** 469-478.

Rouffignac, C.; Mandon, B.; Wittner, M. & Stefano, A. (1993). Hormonal control of renal magnesium handling. *Mineral. Electrolyte Metab.* **19,** 226-231.

Rouvière, H. & Delmas, A. (1990). Apareil Digestif. In: *Anatomie Humaine.* Tome 1. Tête et cou. 12ème edition. Masson. Paris. Pp: 431-477.

Rudich, K. & Butcher, F.R. (1976). Effect of substance P and eledoisin on K^+ efflux, amylase release and cyclic nucleotides levels in slices of rat parotid gland. *Biochim. Biophys. Acta.* **444,** 704-711.

Russel, M.W.; Kilian, M. & Lamm, M.E. (1998). Biological Activities of IgA. In: Ogra PL; Mesteky J; Lamm ME; Strober W. *Mucosal Immunity* McGhee JR; Biennenstock J Eds. Ed2 San Diego. Academic Press, pp225-239.

Russel, M.W.; Hajishengallis, G.; Childers, N.K. & Michalek, S.M. (1999) Secretory immunity in defense against cariogenic mutans streptococci. *Caries Res.* **33,** 4-15.

Ryan, J.; Mantle, T.; McQuaid, S. & Costigan, D.C. (1992). Salivary insulin like growth factors originate from local synthesis. *J. Endocrinol.* **135,** 85-90.

Sakai, T. & Ambudkar, I.S. (1996). Role of protein phophatase in the regulation of calcium influx in parotid gland acinar cells. *Am. J. Physiol.* **271,** C284-C294.

Sakai, T. & Ambudkar, I.S. (1997). Role for protein kinase in Ca^{2+} dependent feed-back modulation of divalent Ca^{2+} store depleted rat parotid gland cells. *Pflügers. Arch.* **433,** 464-471.

Sanderson, M.J. (1996). Intracellular waves of communication. *News. Physiol. Sci.* **11,** 262-269.

Sato, T.P. & Mikami, K. (2002). Recovery of a patient with a recurrent dysgeusia monitored by salivary variables and serum zinc content. *Pathophysiology* **8:(4),** 275-281.

Scannapieco, F.A.; Torres, G.I. & Levine, M.J. (1995). Salivary amylase promotes adhesion of oral Streptococci to hydroxiapatite. *J. Dent. Res.* **74,** 1360-1366.

Schlesinger, D.H. & Hay, D.I. (1977). Complete covalent structure of statherin, a tyrosine rich acidic peptide, which inhibits calcium phosphate precipitation from human parotid saliva. *J. Biol. Chem.* **252,** 1689-1695.

Schneyer, L.H.; Young, J.Á. & Schneyer, C.A. (1972). Salivary secretion of electrolytes. *Physiol. Rev.* **52,** 720-777.

Scott, J. (1987). Structural age changes in salivary glands. *Frontiers of Oral Physiology.* **6,** 40-62.

Shannon, I.L. (1966). Climatological effects on human parotid gland function. *Arch. Oral Biol.* **11**, 451-453.

Sheehan, J.P. (1991). Magnesium deficiency and diabetes mellitus. *Magnesium Trace Elem.* **10**, 215-219.

Ship, J.A.; Pillemer, S.R. & Baum, B.J. (2002). Xerostomia and the geriatric patient. *J. Am. Geriatr. Soc.* **50(3)**, 535-543.

Ship, J.A.; Nolan, N.E. & Puckett, S.A. (1995). Longitudinal analysis of parotid and submandibular salivary flow rates in healthy, different-aged adults. *J. Gerontol.* **50(A)**, M285-M289.

Shomers, J.P.; Tabak, L.A.; Levine, M.J.; Mandel, I.D. & Hay, D.I. (1982). Properties of cysteine containing phosphoproteins from sub-mandibular-sub-lingual saliva. *J. Dent. Res.* **61,** 397-399.

Shomers, J.P.; Tabak, L.A.; Levine, M.J.; Mandel, I.D. & Ellison, S.A. (1982). The isolation of a family of Cystein containing phosphoproteins from sub-mandibular-sub-lingual saliva. *J. Dent. Res.* **61,** 973-977.

Smigel, M.; Katada, T.; Northup, J.K.; Bokoch, G.M.; Ui, M. & Gilamn, A. (1984). Mechanism of guanine nucleotide mediated regulation of adenylate cyclase activity. In: *Advances in Cyclic nucleotid and Protein Phosphorylation Research.* Edited by R. Greengard. **17,** Raven Press. New York, pp:1-18.

Smith, D.J.; Taubman, M.A. & Ebersole, J.L. (1985). Salivary IgA antibody to glucosyltransferase in man. *Exp. Immunol.* **61,** 416-424.

Smith, R.G. (1994). Oral side effects of the most frequently prescribed drugs. *Spec. Care Dentist.* **14,** 96-102.

Soltoff, S.P.; McMillan, M.K.; Cragoe, E.J.; Cantley, L.C. & Talamo, B.R. (1990). Effects of extracellular ATP on ion transport systems and $[Ca^{2+}]_i$ in rat parotid acinar cells. *J. Gen. Physiol.* **95,** 319-346.

Soltoff, S.P.; McMillan, M.K. & Talamo, B.R. (1992). ATP activates a cation permeable pathway in rat parotid acinar cells. *Am. J. Physiol.* **262** *(Cell Physiol.* 31), C934-C940.

Sonju, T. & Rolla, G. (1973). Chemical analysis of the acquired enamel pellicle formed on clean human teeth *in vivo*. Rate of formation and amino acid analysis. *Caries Res.* 7, 30-38.

Spielman, A.I. (1990). Interaction of Saliva and Taste. *J Dent Res* **69(3),** 838-843.

Sreebny, L.M. (1996). *Xerostomia: Diagnosis, Management and Clinical Implications. In:* Saliva and Oral Health, 2nd ed. Edgar, WM, O'Mullane DM (Eds). British Dental Association: London, pp 43-66.

Storch, J. & Schachter, D. (1985). Calcium alters the acyl chain composition end lipid fluidity of rat hepatocyte plama membranes *in vitro. Biochim. Biophys. Acta* **812,** 473-484 .

Streb, H.; Irvine, R.F.; Berridge, M.J. & Shultz, I. (1983). Release of calcium from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5 trisphosphate. *Nature* **306,** 67-68.

Strous, G.J. & Dekker, J. (1992). Mucin type glycoproteins. *Crit Rev Biochem Mol Biol.* **27,** 57-92.

Sueta, C.A.; Clarke, S.W.; Dunlap, S.H.; Jensen, L.; Blauwet, M.B.; Koch, G.; Patterson, J.H. & Adams, K.F. (1994). Effect of acute magnesium administration on the frequency of ventricular arrhythmia in patients with heart failure. *Circulation* **89,** 660-666

Tabak, L.A.; Levine, M.J.; Mandel, I.D. & Ellison, S.A. (1982). Role of salivary mucins in the protection of the oral cavity. *J. Oral Pathol.* **11,** 1-17.

Tabak, L.A. (1995). In defense of the oral cavity: Structure, biosynthesis and function of salivary mucins. *Ann. Rev. Physiol* **57,** 547-564.

Takemura, H. & Putney, JW Jr. (1989). Capacitative calcium entry in rat parotid acinar cells. *Biochem. J.* **258,** 409-412.

Takemura, H.; Hughes, A.R.; Thastrup, O. & Putney, JW Jr. (1989). Activation of calcium entry by tumor promoter tapsigargin in parotid acinar cells: evidence that a intracellular calcium pool and not an inositol phophate regulates calcium fluxes at plasma membrane. *J. Biol. Chem.* **264,** 12266-12271.

Takuma, T. (1990). Evidence for the involvement of cAMP dependent protein kinase in the exocytosis of amylase from parotid acinar cells. *J. Biochem.* **108,** 99-102.

Takuma, T. & Ichida, T. (1994). Catalytic subunit of protein kinase A induces amylase release from strptolysin O-permeabilized parotid acini. *J. Biol. Chem.* **269(35),** 22124-22128.

Tanimura, A. & Turner, R.J. (1996). Ca^{2+} release in HSY cells conforms to a steady-state mechanism involving regulation of the inositol 1,4,5-trisphosphate receptor Ca^{2+} channel by luminal [Ca^{2+}]. *J. cell. Biol.* **132,** 607-616.

Taylor, C.W.; Merrit, J.E.; Putney, J.W. Jr & Rubin, R.P. (1986). A guanine nucleotide dependent regulatory protein couples substance P receptors to phospholipase C in rat parotid gland. *Biochem. Biophys. Res. Commun.* **136,** 362-368.

Taysen, J.H.; Thorn, N.A. & Schwartz, I.L. (1954). Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva. *Am. J. Physiol.* **178,**155-159.

Ten Cate, A.R. (1998). Salivary glands. In: *Oral Histology: Development, Structure And Function.* Mosby St. Louis, Missouri. Pp: 315-344.

Tenovuo, J. (1981). Inhibition of dental plaque acid production by the salivary lactoperoxidase antimicrobial system. *Infect. Immun.* **34,** 208-214.

Tenovuo, J. (1998). Antimicrobial function of human saliva: How important is it for oral health? *Acta Odontol. Scand.* **56(5),** 250-256.

Tenovuo, J. & Kurkijarvi, K. (1981). Immobilised lactoperoxidase as biologically active and stable form of an anti-microbial enzyme. *Arch. Oral Biol.* **26,** 309.

Todd, A.L.; Ng, W.Y.; Lee, Y.S.; Loke, K.Y. & Thai, A.C. (2002). Evidence of autoantibodies to glutamic acid decarboxylase in oral fluid of type 1 diabetic patients. *Diabetes Res. Clin. Pract.* **57(3),** 171-177.

Tojyo, Y.; Tanimura, A. & Matsumoto, Y. (1997). Imaging of intracellular Ca^{2+} waves induced by muscarinic receptor stimulation in rat parotid acinar cells. *Cell Calcium* **22,** 455-462.

Tosiello, L. (1996). Hypomagnesemia and diabetes mellitus. *Arch. Int. Med.* **156,** 1143-1148.

Tsai, H. & Bobek L.A. (2000) Promising anti-fungal therapeutic agents. *Crit Rev Oral Biol Med* **9(4),** 480-496.

Tseng, C.C.; Miyamoto, M.; Ramalingam, K.; Hemavathy, K.C.; Levine, M.J. & Ramasubbu, N. (1999). The roles of histidine residues at the starch binding site in streptococcal –binding activities of human salivary amylase. *Arch. Oral Biol.* **44(2),** 119-127.

Tsien, R.Y.; Pozzan, T. & Rink, T.J. (1982). Calcium Homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescence indicator. *J. Cell Biol.* **94(2),** 325-334.

Turner, R.J. (1993a). Ion transport related to fluid secretion in salivary glands. In: *Biology Of Salivary Glands.* Dobrosielski-Vergona K. Editor. Boca Raton, Florida: CRC press Inc. pp 105-127.

Turner, R.J. (1993b). Mechanisms of fluid secretion by salivary glands. *Ann NY Acad Sci* **694,** 24-35.

Turner, T.J. & Bylund, O. (1987). Characterization of Vasoactive Intestinal Peptide binding assay in membrane preparations. *J. Pharmacol. Exp. Ther.* **242,** 873-881.

Turner, T.J. & Camden, M.J. (1990). The influence of Vasoactive Intestinal Peptide receptors in dispersed acini from rat submandibular gland on cyclic AMP production and amylase release. *Arch. Oral. Biol.* **35,** 103-108.

Twetman, S.; Johansson, I.; Birkhed, D. & Nederfors, T. (2002). Caries incidence in young type 1 diabetes mellitus patients in relation to metabolic control and caries-associated risk factors. *Caries Res.* **36(1),** 31-35.

Tylstrup, A. & Fejerskov, O. (1994). Saliva. In: *Textbook Of Clinical Cariology.* Munksgaard. Copenhagen. pp, 17-42.

Valdez, I.H.; Atkinson, J.C.; Ship, J.A. & Fox, P.C. (1993). Major salivary gland function in patients with reduced xerostomia: flow rates and sialchemistry. *Int. J. Radiation Oncology Biol. Phys.* **25,** 41-47.

Valdez, I.H. & Turner, R.J. (1991). Effects of secretagogues on cytosolic Ca^{2+} levels in rat submandibular granular ducts and acini. *Am. J. Physiol.* **261,** G359-G363.

Van de Put, FHMM & Eliott, AC. (1997). The endoplasmic reticulum can act as functional calcium store in all subcellular regions of the pancreatic acinar cell. *J. Biol. Chem.* **272,** 27764-27770.

Vormann, J. & Gunther, T. (1993). Magnesium transport mechanisms. In: *Magnesium and the Cell.* Ed: Birch NJ, Academic Press, London, pp 137-156.

Warner, T.F. & Azen, E.A. (1984). Proline-rich proteins are present in serous cells of sub mucosal glands in the respiratory tract. *Am. Rev. Respir. Dis.* **130,** 115-118.

Watson, E.L.; Abel, P.W.; Di Julio, D.; Zeng, W.; Makoid, M.; Jacobson, K.L.; *et al.* (1996). Identification of muscarinic receptor subtypes in mouse parotis gland. *Am. J. Physiol.* **271,** C905-C913.

Watson, E.L.; Wu, Z.; Jacobson, K.L.; Storm, D.R.; Singh, J.C. & Ott, S.M. (1998). Capacitative calcium entry is involved in cAMP synthesis in mouse parotid acini. *Am. J. Physiol.* **274,** C557-C565.

Weiss, M. & Lasserre, B (1994*).* Should magnesium therapy be considered for the treatment of coronary heart disease? 1. *A* critical appraisal of current facts and hypotheses. *Magnesium Res.* **7,** 135-144.

Williams, R.J.P. (1993). Magnesium: An introduction to its biochemistry. In: *Magnesium And The Cell.* N.J. Birch Eds.Academic press. London, pp. 5-14.

Whittaker, C.J., Klier, C.M. & Kollanbrander, P.E. (1996). Mechanisms of adhesion by oral bacteria. *Ann. Rev. Microbiol.* **50**, 513-552.

Wisdom, D.M., Salido, G.M., Baldwin, L.M. & Singh, J. (1996). The role of magnesium in regulating CCK-8 evoked secretory responses in the exocrine rat pancreas. *Molec. Cell Biochem.* **154(2),**123-132.

Wolfe, P.C.; Chang, E-Y.; Rivera, J. & Frewtrell, C. (1996). Differential effects of the protein kinase C activator phorbol 12- Myristate 13 acetate on calcium responses and secretion in adherent and suspended RBL-2H3 mucosal mast cells. *J. Biol. Chem.* **271** (12), 6658-6665.

Wong, M.M.Y. & Foskett, J.K. (1991). Oscillations of cytosolic sodium during calcium oscillations in exocrine acinar cells. *Science* **254:**1014-6.

Wu, K.D.; Lee, W.S.; Wey, J.; Bungard, D. & Lytton, J. (1995). Localization and quantification of endoplasmic reticulum Ca^{2+} ATPase transcripts. *Am. J. Physiol.* **269,** C775-C784.

Xia, Z. & Storm, D.R. (1996). *Regulatory Properties of the Mammalian Adenylyl Cyclases.* Austin, TX: Landes (Mol. Biol. Intelligence Unit).

Xu, X.; Zeng,W.; Diaz, J.; Lau, K.S.; Gukovskaya, A.C.; Brown, R.J. *et al.* (1997). NNOS and Ca^{2+} influx in rat pancreatic acinar and submandibular salivary gland cells. *Cell Calcium.* **22,** 217-228.

Yago, M.D.; Mañas, M. & Singh, J. (2000). Intracellular free magnesium regulation and transport in epithelial secretory cells. *Frontiers of Biochem.* **5**; 602-619.

Yago, M.D., Mañas, M., Mata, A.D., Mesquita, M.F. & Singh, J. (1999). Effects of extracellular magnesium on basal and on acetylcholine evoked secretory responses in the isolated rat parotid gland. *J. Physiol.* **520P3,** 22P.

Yago, M.D.; Mata, A.D.; Mañas, M. & Singh, J. (2002). Effect of extracellular magnesium on nerve mediated and acetylcholine-evoked secretory responses in the isolated rat parotid gland.*Experimental Physiology* **87.3,** 321-326.

Yao, Y.; Lamkin, M.S.; Oppenheim, F.G. (1999). Pellicle percursor proteins: Acidic proline rich proteins, statherin and histatins and their cross-linking reaction by oral transglutaminase. *J. Dent. Res.* **78 (11),** 1696-1703.

Young, J.A. & Martin, C.J. (1971). The effect of sympatho- and a parasympathomimetic drug on the electrolyte concentrations of primary and final saliva of the rat submaxillary gland. *Pfluegers. Arch.* **327,** 285-302.

Young, J.Á. & Schogel, E. (1966). Micropuncture investigation of sodium and potassium excretion in rat submaxillary saliva. *Pfluggers. Arch.* **291,** 85-98.

Yule, D.I.; Kim, E.T. & Williams, J.Á. (1994). Stimulus-secretion coupling in the pancreatic acinus. In: *Physiology of the Gastrointestinal Tract.* (3 ed.) Edited by Leonard Johnson. Raven Press. New York, pp 1447-1472.

Zahradnick, R.T.; Moreno, E.C. & Burke, E.J. (1976). Effect of salivary pellicle on enamel sub surface demineralization *in vitro. J. Dent. Res.* **55,** 664-670.

Zahradnick, R.T; Propas, D.; Moreno, E.C. (1977). *In Vitro* enamel demineralization by *Strptococcus mutans* in the presence of salivary pellicles. *J. Dent. Res.* **56,** 1107-1110.

Zastrow, M.V. & Castle, J.D. (1987). Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. *J. Cell. Biol.* **105**, 2675-2684.

Zastrow, M.V. & Castle, J.D. (1989). Amonium chloride alters secretory protein sorting within the maturing exocrine storage compartiment. *J. Biol. Chem.* **264,** 6566-6571.

Zelles, T.; Purushotham, K.R.; Macauley, S.P.; Oxford, G.E.; Humphreys-Beher M.G. (1995). Saliva and growth factors the foutain of youth resides in us all. *J. Dent. Res.* **74(12),** 1826-1832.

Zhang, G.H. & Melvin, J.E. (1994). Intracellular magnesium movement during muscarinic stimulation. Magnesium uptake by the intracellular calcium store in rat sublingual mucous acini. *J. Biol. Chem.* **269,** 10352-10356.

Zhang, G.H. & Melvin, J.E. (1995). Regulation by extracellular sodium of cytosolic magnesium concentration in magnesium-loaded rat sublingual acini. *FEBS Lett* **371,** 52-56.

Zhou, J.; Wright, P.S.; Wong, E; Jessen, K; Morand, J.N. & Carlsson, D.M. (1997). Cyclic AMP regulation of mouse proline-rich protein gene expression: isoproterenol induction of AP1 transcription factors in parotid glands. *Arch. Biochem. Biophys.* **338(1),** 97-103.

PUBLICATIONS

Yago, MD; Manas M; **Mata, A.** & Singh, J. (1999). Effects of Extra Cellular Magnesium On Basal and Acetylcholine-Evoked Amylase Secretion and Calcium Mobilisation In The Isolated Rat Parotid gland. *J Physiology* 520P, p 22P.

Mata A, Marques D, Geraldes M, Rocha S, Mesquita MF & Singh J. (2001). Control of acetyl-choline evoked amylase secretion by magnesium in the isolated rat parotid gland. 9th international magnesium symposium. *Magnesium Research*, **14(1/2)**, 91.

Mata A, Marques D, Geraldes M, Rocha S, Mesquita MF & Singh J. (2001). Role of extra-cellular magnesium on acetylcholine-evoked secretory responses in isolated rat sub-mandibular gland. 9th international magnesium symposium. *Magnesium Research,* **14(1/2)**, 139.

Mata, A; Marques, D; Veiga, M; Mesquita, MF & Singh, J Effects of extracellular magnesium on secretagogue evoked secretory responses in the isolated rat submandibular gland. *J. Physiol.* **543P**, 15P.

Mata, A; Marques, D; Lajas, A; Pariente, J; Mesquita, MF & Singh, J. Extracellular magnesium regulates secretagoue-evoked calcium mobilisation in parotid acinar cells. *J. Physiol.* **543P**, 16P.

Mata, A.D., Marques, D., Mesquita, MF.; Singh, J. (2002) Effect of extracellular magnesium on secretagogue-evoked amylase secretion in the isolated rat parotid gland segments. *Mag. Res.* **(15), 3/4,** 161-165.

Yago, M.D.; **Mata, A.D.;** Mañas, M. & Singh, J. (2002). Effect of extracellular magnesium on nerve mediated and acetylcholine-evoked secretory responses in the isolated rat parotid gland.*Experimental Physiology* **87.3,** 321-326.

Mata A, Marques D, Geraldes M, Rocha S, Mesquita MF, Singh J. (2001). Acetylcholine Evoked Salivary Secretion: Role of Extracellular Magnesium On Acetylcholine Evoked Secretory Responses In The Isolated Rat Submandibular Cell Glands. In: *Advances in Magnesium research & Health.* Rassiguier, Y; Mazur,A & Durlach, J. Eds John Libbey & Co. Ltd. pp 93-96.

Forcella, S; **Mata, A**; Mesquita, MF; Spoto, G. (2003). Biochemistry. In: *Basic Sciences For The Dental Student.* Crispian Scully eds. Oxford University Press, Oxford. pp 271-369.

APPENDIX I

SOLUTIONS EMPLOYED IN THE AMYLASE MEASUREMENTS

1) Recipient solution

Composition for 4 L

	MW ($g . mol^{-1}$)	Concentration	G per 4 L
Na Cl	58.44	0.05 M	11.68
Trizma base	121.11	0.1 M	48.44
Triton X-100		0.1 % v/v	4 ml from stock

- A 4 L flask was taken and 3 L of distilled water was added
- The above reagents (NaCl and Trizma base) were added and each was dissolved before adding the other
- Volume was brought to 3.9 L
- The solution was pHed using 1M HCl to pH 7.0
- 4 mL Triton X-100 was added GRADUALLY and stirred THOROUGHLY
- Volume was brought to 2 L with distlled water
- Solution was kept in the fridge

2) Substrate (powder, amylopectin anthranilate).

- 200 mL of distilled water was heated to 35°C and temperature was checked using a thermometer.
- The following compounds were added:

- Maize starch 100g
- NaCl 1.8 g
- Na_2CO_3 2 g
- Isatoic anhydride 2g
- The solution was maintained at 37°C with continuous stirring for 4 hours
- After the 4 hours-period, a 400 mL mixture absolute ethanol: methanol (1:1 v/v) was made
- The solution was filtered through a Watman N°1 filter using a side arm Buchner funnel and a vacuum pump
- The remaining solid was resuspended in 200 mL of distilled water and filtered again, this process was repeated twice
- The solid was resuspended in 200 mL mixture ethanol : methanol, filtered, and the process repeated one more time
- The resultant white solid was dried over silica gel in a dessicator before use

The resultant white solid was dried over silica gel in a dessicator before use

The solid was stored in a dessicator under vacuum at 0-5° C.
Under these conditions the solid remained viable without loss of activity for 3 months

3) Substrate (colloidal solution)

- 2.0 L recipient solution was placed into a 4L beaker and heated to 95°C (a thermometer was used to check temperature)
- 10 g amylopectine anthranilate was weighed and dissolved in 100 mL of recipient solution (final concentration was 0,5% w/v)
- When temperature (95°C) of the recipient was reached, the solution of amylopectin was added gradually
- Solution was heated and stirred for another 5-10 minutes
- Heating was switched off and solution was cooled at room temperature with continuous stirring
- Substrate was finally filtered using nylon mesh before use

The solution was stored at 0-4°C for 2 days while still retaining optimum sensitivity. When diluting substrate was necessary, recipient solution was used.

RESULT TABLES

CHAPTER 3

Figure 3.1

$[Mg^{2+}]_0$	Basal	SEM
0	213.69	6.7
1.1	250.31	11.03
5	164.21	7.35
10	185.16	10.44

Figure 3.2

$[Mg^{2+}]_0$	Control	SEM	ACh 10^{-5} M	SEM
0	165.65	11.07	203.78	10.53
1.1	338.63	8.05	303.43	22.77
5	179.01	6.59	205.00	9.78
10	148.61	15.31	209.00	30.78

Figure 3.3

$[Mg^{2+}]_0$	Control	SEM	NA 10^{-5} M	SEM
0	236.56	13.72	320.89	11.57
1.1	291.82	19.77	491.76	31.72
5	124.32	18.43	216.75	9.14
10	222.09	9.46	253.21	16.35

Figure 3.4

$[Mg^{2+}]_0$	Control	SEM	Phe 10^{-5} M	SEM
0	226.12	10.45	254.92	8.95
1.1	277.96	11.98	319.93	12.91
5	161.11	8.7	202.77	10.09
10	152.72	9.2	213.19	15.49

CHAPTER 4

Figure 4.1 Basal Amylase Secretion

mM $[Mg^{2+}]_0$	Mean Basal Amylase Output	SEM
0	3.36	0.19
1.1	5.43	0.44
5	3.51	0.16
10	2.98	0.17

Figure 4.3 EFS 10 Hz

mM $[Mg^{2+}]_0$	Mean EFS 10 Hz	SEM
0	20.78	4.28
1.1	24.59	5.05
5	21.49	3.69
10	20.33	2.70

Figure 4.4 NA 10^{-5} M

mM $[Mg^{2+}]_0$	Mean NA 10^{-5} M	SEM
0	9.12	0.91
1.1	20.60	2.02
5	16.35	0.61
10	10.21	1.65

Figure 4.5 Phe 10^{-5} M

mM $[Mg^{2+}]_0$	Mean Phe 10^{-5} M	SEM
0	9.85	0.85
1.1	14.04	0.79
5	12.80	3.96
10	11.18	0.92

Figure 4.6 ISO 10^{-5} M

mM $[Mg^{2+}]_0$	Mean ISO 10^{-5} M	SEM
0	10.21	1.94
1.1	14.54	1.53
5	16.27	1.72
10	19.08	0.88

Figure 4.7 ACh 10^{-5} M

mM $[Mg^{2+}]_0$	Mean ACh 10^{-5} M	SEM
0	3.67	0.32
1.1	7.11	0.72
5	6.56	0.55
10	4.25	0.42

Figure 4.8 ACh 10^{-8} to 10^{-5} M

	0	1.1	5	10	mM $[Mg^{2+}]_0$
ACh					
10-8 M	1.610 ± 0.460	2.120 ±0.180	1.120 ±0.0880	0.8480 ±0.230	
10-7 M	2.130 ± 0.223	5.360 ±0.530	2.560 ±0.4840	2.6200 ±0.565	
10-6 M	3.376 ± 0.262	5.928 ±0.612	4.666 ±0.2400	3.1200 ±0.427	
10-5 M	3.890 ± 0.370	7.110 ±0.720	6.560 ±0.5500	4.2500 ±0.420	

Figure 4.9 NA & Phe 10^{-6} M

$[Mg^{2+}]_0$	0 mM	SEM	1.1 mM	SEM	5 mM	SEM
NA	4.10	±1.050	12.490	±2.310	3.060	±0.670
Phe	3.59	±1.050	5.560	±1.510	2.650	±0.710

Figure 4.10 Amylase in Zero amd 2.56 mM $[Ca^{2+}]_0$

mM $[Mg^{2+}]_0$	0	SEM	1.1	SEM	5	SEM	10	SEM
BASAL2,56 mM [Ca2+]0	4.950	±0.580	5.330	±0.520	3.670	±0.170	2.810	±0.280
BASAL 0 mM [Ca2+]0	3.680	±0.420	2.520	±0.250	2.200	±0.190	1.810	±0.240
ACh 10-5 mM	4.510	±1.260	2.640	±0.480	2.290	±0.330	2.230	±0.510
GRADIENT 2,56 mM [Ca2+]0	5.450	±1.090	1.980	±0.200	1.800	±0.080	1.000	±0.340

Figure 4.11 Basal $[Ca^{2+}]_i$

mM $[Mg^{2+}]_0$	0	SEM	1.1	SEM	5	SEM	10	SEM
1.8 mM $[Ca^{2+}]_i$	0.2590	±0.00610	0.28940	±0.00790	0.24510	±0.00940	0.22190	±0.01170
0 mM $[Ca^{2+}]_i$	0.2499	±0.00578	0.20590	±0.00250	0.19390	±0.00935	0.19490	± 0.00698

Figure 4.14 Peak & Plateau $[Ca^{2+}]_i$ in 1.8 mM $[Ca^{2+}]_0$

mM $[Mg^{2+}]_0$	0	SEM	1.1	SEM	5	SEM	10	SEM
peak	0.67460	±0.01920	0.8000	±0.06680	0.42430	±0.02140	0.370640	±0.01840
plateau	0.44560	±0.01500	0.5348	±0.03800	0.38420	±0.01580	0.369800	±0.01780

Figure 4.15 Peak & Plateau $[Ca^{2+}]_i$ in 0 mM $[Ca^{2+}]_0$

mM $[Mg^{2+}]_0$	0	SEM	1.1	SEM	5	SEM	10	SEM
peak	0.562220	±0.01630	0.510590	±0.01570	0.30971	±0 0.01760	0.26970	±0.01470
plateau	0.027289	±0.00763	0.023864	±0.01370	0.02042	±0.01380	0.020396	±0.01060

Figure 4.17 CCE activation times

mM$[Mg^{2+}]_0$	0	SEM	1.1	SEM	5	SEM	10	SEM
Time (Sec)	225.0	±6.0	35.0	±5.0	245.0	±3.0	171.0	±5.0

Figure 4.18 CCE Values

Time	0	SEM	1.1	SEM	5	SEM	10	SEM	mM $[Mg^{2+}]_0$
100.0	0.450	±0.0120	0.350	±0.0098	0.290	±0.01550	0.280	±0.00931	
200.0	0.440	±0.0150	0.310	±0.0050	0.270	±0.01100	0.280	±0.01100	
300.0	0.450	±0.0160	0.290	±0.0060	0.260	±0.01300	0.250	±0.01220	

Figure 4.19 Basal $[Mg^{2+}]_i$ & ACh 10^{-5} M-evoked $[Mg^{2+}]_i$ decrease

	BASAL	SEM	ACh 10-5 M	SEM
0.0	0.26310	±0.01160	0.24110	±0.00470
1.1	0.30380	±0.01010	0.27740	±0.00907
5.0	0.42480	±0.03170	0.40300	±0.03110
10.0	0.49540	±0.01610	0.45340	±0.01100

Figure 4.20 Continuous increase in basal $[Mg^{2+}]_i$ & ACh 10^{-5} M-evoked $[Mg^{2+}]_i$ decrease in 10 mM $[Mg^{2+}]_0$

	BASAL	SEM	ACh 10-5 M	SEM
0.0	0.26310	±0.01160	0.24110	±0.00470
1.1	0.30380	±0.01010	0.27740	±0.00907
5.0	0.42480	±0.03170	0.40300	±0.03110
10.0	0.49540	±0.01610	0.45340	±0.01100

Figure 4.21 Effect of transport inhibitors on basal $[Mg^{2+}]_i$

Inhibitors	Cytosolic Magnesium Ratio (Mean)	SEM
Basal	0.304	±0.010
Lidocaine 10^{-3} M	0.402	±0.022
Amiloride 10^{-3} M	0.509	±0.004
NMDG	0.563	±0.033
Quinidine 10^{-3} M	0.662	±0.015
DNP 10^{-4} M	0.788	±0.073
Bumetanide 10^{-3} M	0.930	±0.058

Figure 4.22 Effect of transport inhibitors on basal and ACh 10^{-5} M $[Mg^{2+}]_i$

A)

Inhibitors Basal	Cytosolic Magnesium Ratio (Mean)	SEM	Inhibitors plateau	Cytosolic Magnesium Ratio (Mean)	SEM
Basal alone	0.304	±0.010	ACh 10^{-5} M alone	0.284000	±0.0206
Lidocaine 10^{-3} M	0.402	±0.022	Lidocaine 10^{-3} M	0.404900	±0.0296
Amiloride 10^{-3} M	0.509	±0.004	Amiloride 10^{-3} M	0.482416	±0.0036
NMDG	0.563	±0.033	NMDG	0.461190	±0.0291
Quinidine 10^{-3} M	0.662	±0.015	Quinidine 10^{-3} M	0.600490	±0.0133
DNP 10^{-4} M	0.788	±0.073	DNP 10^{-4} M	0.6967142	±0.0584
Bumetanide 10^{-3} M	0.930	±0.058	Bumetanide 10^{-3} M	0.792525	±0.0422

B)

Inhibitors	Cytosolic Magnesium Decrease (Mean)	SEM
ACh 10^{-5} M alone	0.026	±0.003
Lidocaine 10^{-3} M	0.011	±0.003
Amiloride 10^{-3} M	0.022	± 0.003
NMDG	0.063	±0.010
Quinidine 10^{-3} M	0.063	±0.008
DNP 10^{-4} M	0.082	±0.019
Bumetanide 10^{-3} M	0.148	±0.019

Chapter 5

Figure 5.1

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	0.7927	0.108400	3.1368	0.294300
35-44	0.4610	0.061250	2.1142	0.218400
45-54	0.3069	0.019420	1.4538	0.114500
55-64	0.2969	0.040360	1.2678	0.129100
65-75	0.2083	0.042560	1.0967	0.185000

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	2.08	0.21	1.52	0.17	1.14	0.10	0.96	0.12	0.88	0.15

Figure 5.2

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	0.59700	0.0820	2.44270	0.17170
35-44	0.34020	0.05748	1.51180	0.16660
45-54	0.40160	0.0403	1.66310	0.12730
55-54	0.20070	0.02766	1.28060	0.23460
65-75	0.22950	0.02581	1.37490	0.11950

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	1.85	0.12	1.17	0.15	1.26	0.11	0.87	0.12	1.07	0.08

Figure 5.3

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	1.34530	0.11350	0.98330	0.09630
35-44	1.62190	0.18730	0.91100	0.11580
45-54	1.66650	0.20310	0.91070	0.11560
55-64	2.58660	0.54380	1.03940	0.10450
65-75	2.76780	0.53710	1.28670	0.17280

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-0.59	0.18	-0.69	0.14	-0.75	0.13	-1.54	0.48	-1.48	0.42

Figure 5.4

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	1.47640	0.1218	0.99110	0.08140
35-44	1.66000	0.1403	0.87230	0.06000
45-54	1.68000	0.2370	0.83940	0.09000
55-54	2.21000	0.2813	0.93660	0.10240
65-75	2.35000	0.1880	1.14960	0.10550

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-0.50	0.13	-0.79	0.11	-0.81	0.18	-1.28	0.25	-1.18	0.22

Figure 5.5

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	39.6722	3.1746	52.5056	6.5017
35-44	34.5842	2.4217	46.6947	5.1161
45-54	33.35	2.1664	65.0591	3.763
55-64	50.56	11.5313	70.52	7.8358
65-75	52.5429	5.6283	90.0857	11.3943

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	13.83	5.78	12.14	4.87	31.70	3.4	19.96	13.02	37.54	14.26

Figure 5.6

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	37.99000	2.9700	53.48000	4.64000
35-44	28.90000	3.7500	48.68000	2.13000
45-54	32.90000	3.7500	52.14000	2.17000
55-54	38.30000	2.8600	75.22000	11.93000
65-75	45.89000	6.0000	95.41000	7.88000

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	15.50	3.94	19.78	3.36	19.42	2.57	36.92	10.49	49.51	10.33

Figure 5.7

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	4.52220	0.40120	3.31670	0.42830
35-44	5.32630	0.50180	3.12110	0.47750
45-54	6.70450	0.87920	4.42730	0.62170
55-64	8.68000	2.70910	5.09000	0.96050
65-75	9.51430	2.62730	8.91430	2.01570

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-1.25	0.31	-2.20	0.61	-2.27	0.78	-3.59	0.74	-0.60	0.47

Figure 5.8

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	5.78600	0.5060	4.05000	0.35000
35-44	5.79000	0.5500	3.79000	0.48000
45-54	4.31000	0.4700	2.65000	0.52000
55-54	6.91000	0.8900	4.86000	0.82000
65-75	5.81000	1.0800	5.74000	0.86000

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-1.74	0.48	-2.00	0.59	-1.66	0.65	-2.05	0.71	-0.07	1.17

Figure 5.9

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	7.27780	0.75750	8.01670	0.81430
35-44	1.78950	0.14370	2.42110	0.21990
45-54	0.91000	0.12640	1.55590	0.14990
55-64	1.54000	0.38130	1.97900	0.56790
65-75	0.98570	0.03401	1.00290	0.16930

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	0.73	0.12	0.63	0.19	0.69	0.11	0.39	0.29	0.017	0.14

Figure 5.10

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	3.50000	0.6330	3.64000	0.70670
35-44	0.76000	0.2900	1.06670	0.21920
45-54	0.49000	0.09999	1.09310	0.14850
55-54	0.25000	0.0824	0.50910	0.09570
65-75	0.52700	0.0328	0.69090	0.05300

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	0.13	0.25	0.30	0.27	0.60	0.17	0.25	0.072	0.16	0.04

Figure 5.11

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	17.27780	1.13700	16.55560	0.89740
35-44	17.82350	0.91650	15.05260	0.81820
45-54	22.40910	1.37170	14.40900	0.70760
55-64	28.66700	5.97220	15.00000	1.37440
65-75	29.14290	4.89130	16.42860	1.70100

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-7.22	1.43	-3.58	1.24	-8.00	1.30	-13.00	5.51	-12.71	4.31

Figure 5.12

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	18.36000	1.4030	15.13640	0.76250
35-44	22.20000	1.5668	16.73330	0.76510
45-54	19.16000	1.2901	15.22220	0.76480
55-54	22.36000	1.1460	15.36360	1.06410
65-75	19.54000	0.8780	12.72730	0.63380

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-3.22	1.20	-5.47	1.51	-4.10	1.37	-7.00	1.16	-6.81	0.99

Figure 5.13

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	195.5560	15.66940	350.9444	37.96110
35-44	108.5789	13.27560	249.8947	44.81670
45-54	157.8636	26.64850	154.5	17.80590
55-64	221.0000	63.84870	190.0	30.28460
65-75	182.8571	49.46740	145.4286	28.26900

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	155.38	46.32	141.31	46.93	2.09	26.67	-31.00	58.34	-37.42	55.04

Figure 5.14

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	182.68000	19.1100	208.18000	19.35000
35-44	154.86000	17.5700	196.46000	31.61000
45-54	111.41000	12.5300	125.24000	12.60000
55-54	119.27000	16.2400	119.63000	26.70000
65-75	66.18000	15.2400	53.18000	10.24000

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	25.50	23.86	41.66	34.12	3.03	24.90	0.36	32.73	-31.18	24.36

Figure 5.15

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	794.50	84.52800	687.000	86.97220
35-44	796.7895	66.83220	766.2032	60.34030
45-54	673.4545	46.10820	481.6818	37.56670
55-64	763.00	93.77400	441.300	42.96800
65-75	688.1429	59.74530	451.5714	45.88120

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	-107.50	68.51	-29.47	99.83	-191.77	51.91	-321.70	90.62	-236.51	42.13

Figure 5.16

A)

AGE	RESTING	SEM	STIMULATED	SEM
20-34	755.36000	45.7600	765.00000	51.75000
35-44	722.60000	45.1700	762.73000	82.89000
45-54	673.17000	46.2800	613.82000	38.90000
55-54	695.09000	68.6600	435.18000	39.82000
65-75	791.81000	48.4600	529.45000	30.93000

B)

AGE	20-34	SEM	35-44	SEM	45-54	SEM	55-64	SEM	65-75	SEM
	5.09	48.26	-53.20	46.96	-59.34	41.10	-259.91	70.39	-262.36	57.17

Figure 5.17

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	0.6897	0.06800	2.760	0.17150
TYPE I	0.222	0.03250	1.230	0.15300
CONTROL	0.3263	0.01710	1.5325	0.06390
TYPE II	0.252	0.02400	1.1791	0.10330

Figure 5.18

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	1.4174	0.08380	0.9881	0.06063
DIABETES	2.602	0.46170	1.3597	0.19250
CONTROL	1.8425	0.10200	0.9501	0.04070
DIABETES	2.3487	0.27840	1.4996	0.11770

Figure 5.19

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	38.745	2.14800	53.495	3.83000
DIABETES	48.570	6.81000	75.540	7.41000
CONTROL	37.1554	1.58640	63.730	2.30000
DIABETES	77.660	4.85000	100.046	5.22000

Figure 5.20

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	5.2175	0.34280	3.720	0.27510
DIABETES	13.228	2.97000	6.0143	0.97840
CONTROL	6.124	0.38150	4.1876	0.29040
DIABETES	9.5885	1.69000	4.715	0.61300

Figure 5.21

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	5.250	0.55490	5.750	0.59800
DIABETES	1.257	0.18180	1.2857	0.14100
CONTROL	0.9382	0.06930	1.344	0.08900
DIABETES	0.7538	0.11190	0.8769	0.17800

Figure 5.22

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	772.975	45.07000	729.900	48.11000
DIABETES	1741.920	178.07000	1327.210	144.22340
CONTROL	711.600	20.88600	577.420	21.06000
DIABETES	1107.580	81.81000	762.500	40.21490

Figure 5.23

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	188.480	12.55000	287.430	29.15000
DIABETES	148.710	21.85000	226.930	20.48000
CONTROL	137.280	10.00000	160.370	12.31000
DIABETES	131.350	27.04000	147.390	17.06000

Figure 5.24

A)

	RESTING	SEM	STIMULATED	SEM
CONTROL	17.875	0.91930	15.775	0.58560
DIABETES	25.780	2.87000	18.1429	0.81780
CONTROL	21.7356	0.75820	15.090	0.33350
DIABETES	23.700	1.43000	16.880	0.82530

Figure 5.17 to 5.24 B)