THE UNIVERSITY OF MANITOBA

SECRETION AND THE SUBMANDIBULAR SALIVARY GLAND OF THE RAT

Studies of K^+-release, Protease Secretion and changes in cyclic GMP Concentrations

By

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DEDICATION

This thesis is dedicated to my wife, Janet Spearman. Without her support, patience, and sacrifices, this work would not have been possible.

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ABSTRACT

Secretion, and the control of secretion, were investigated using rat submandibular gland slices *in vitro*. Kallikrein and two trypsin-like proteases were measured in crude extracts of these glands and selective inhibition of the three activities by various effectors was interpreted as confirmation that the measured activities were due to distinct enzymes. Kallikrein is known to be located in the convoluted granular tubule cells in rat submandibular glands and the convoluted granular tubule cells are reported to be more prevalent in the submandibular glands of the males of the species. The finding that the two trypsin-like proteases were, like kallikrein, present in larger amounts in the submandibular glands of male rats, and, are secreted in parallel to kallikrein, indicate that they are also located in the convoluted granular tubule cells.

The secretion of all three enzymes from these cells was found to be mediated primarily through α-adrenergic stimulation. The increased secretion produced by phenylephrine, an α-adrenergic agonist, and epinephrine, a mixed α- and β-adrenergic agonist, were specifically abolished by preincubation of the tissue with phentolamine, an α-adrenergic antagonist. α-adrenergic agonists were able to increase protease secretion only when Ca^{2+} was present in the tissue medium. However, increasing Ca^{2+} influx into the tissue by exposure of the slices to Ca^{2+} after they had been preincubated with the divalent cationophore A23187 only marginally increased enzyme secretion.

The release of K^{+} from these slices was also studied and was found to be stimulated by epinephrine, phenylephrine,

carbamylcholine, physalaemin, and an eledoisin related peptide. Ouabain also produced K^+ release which was additive to, and therefore independent of, that elicited by epinephrine and carbamylcholine. The effect of epinephrine and phenylephrine could be prevented by preincubation of the tissue with the α-adrenergic antagonist, phentolamine. The β-adrenergic antagonist, propranolol, was without effect. Similarly, the effect of carbamylcholine was prevented by pretreatment with the muscarinic cholinergic antagonist, atropine. The K^+ release produced by physalaemin and the eledoisin-related peptide was not affected by any of these antagonists.

Extracellular Ca^{2+} was required for epinephrine, carbamylcholine, and physalaemin to increase K^+ release. The eledoisin-related peptide produced similar K^+ release in the absence and presence of this ion. Preincubation of tissue with the ionophore A23187 in the absence of Ca^{2+} resulted in K^+ release upon Ca^{2+} addition, in spite of the fact that all normal neurotransmitter receptors were blocked by appropriate antagonists.

The effects of submaximal concentrations of carbamylcholine and epinephrine on K^+ release were potentiated by the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine (IBMX), suggesting a role for cyclic GMP in neurotransmitter induced K^+ release.

Cholinergic stimulation of rat submandibular gland slices resulted in a rapid increase in the level of cyclic GMP. This increase was dependent upon the presence of Ca^{2+} and was potentiated by a phosphodiesterase inhibitor, IBMX. Adrenergic agonists did not produce a statistically significant elevation in the cyclic GMP concentration. The addition of Ca^{2+} to slices preincubated with the

divalent ionophore A23187 caused a rapid rise in the cyclic GMP concentration which was not affected by simultaneous cholinergic stimulation. While these results could support a function for cyclic GMP in cholinergic-mediated K^+ release from these glands, they do not support a role for this nucleotide in α-adrenergic agonist induced K^+ release or protease secretion.

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ABBREVIATIONS

ATP	- adenosine triphosphate
cyclic AMP	- adenosine 3':5'-cyclic-monophosphate
cyclic GMP	- guanosine 3':5'-cyclic-monophosphate
KRB	- Krebs-Ringer bicarbonate medium, pH 7.4
IBMX	- 3-isobutyl-1-methyl xanthine
EGTA	- ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid
BAEE	- α-N-benzoyl-L-arginine ethyl ester
BANA	- α-N-benzoyl-DL-arginine-β-napthylamide.HCl
BAPA	- α-N-benzoyl-L-arginine-p-nitroanilide
CGT	- convoluted granular tubule
TCA	- trichloroacetic acid
RNA	- ribonucleic acid
DNA	- deoxyribonucleic acid

RATIONALE AND EXPERIMENTAL APPROACH

The purpose of this study was to further our knowledge of the mechanisms by which glandular tissues produce and release their secretions. Special emphasis has been placed on the mechanisms by which neural activity regulates these processes. Salivary glands were chosen as model systems in this study as they are innervated by both the sympathetic and the parasympathetic branches of the autonomic nervous system. Thus, the mechanisms by which extracellular α-adrenergic, β-adrenergic, and cholinergic stimulation produce their intracellular effects could be studied in the same experimental system. In addition, a substantial amount of research had already been carried out with these glands and a basic outline of the secretory process had been obtained. This provided a solid foundation on which further studies on the control of this process could be based.

Due to the importance of secretion, its regulation has been the subject of intensive research for several years. By the time this study was initiated, the relationship of the β-adrenergic receptor and intracellular cyclic AMP to amylase release from the parotid gland of the rat had been elucidated. In addition, slices of this gland had been shown to release K^+ upon α-adrenergic and cholinergic stimulation. This K^+ release had been hypothesized to be related to the physiological secretion of fluid. It had also been shown that in many tissues, including the rat submandibular gland, cholinergic stimulation resulted in an increase in the cyclic GMP content of the

tissue. This resulted in the suggestion that this nucleotide might function as an intracellular second messenger. These published results and theories suggested the possibility that cyclic GMP could be an important intermediate in the neural regulation of the secretion of the fluid component of saliva. Experiments were performed to test this hypothesis in the rat submandibular gland.

Two approaches were taken to study the relationship between cyclic GMP and fluid secretion in this gland. The first was to examine K^+ release from slices of this gland and to study the effects on this release of substances known to affect fluid secretion *in vivo*. Substances known to influence the metabolism of cyclic GMP were also examined for an effect on K^+ release. The second approach was to measure the levels of cyclic GMP in slices of this gland and to examine the effects on these levels of substances known to affect K^+ release and/or fluid secretion.

The possibility that cyclic GMP may function as a second messenger in a process unrelated to fluid secretion was also considered. As the most plausable alternate process in which cyclic GMP could conceivably be involved appeared to be exocytosis, the secretion of macromolecules was studied. Three proteases were selected for study and their suitability as monitors of exocytosis was evaluated. The control of their secretion was investigated to determine whether neurotransmitter-induced changes in cyclic GMP levels correlated more closely with exocytotic secretion of macromolecules or with fluid secretion.

LITERATURE REVIEW

Structure and innervation of salivary glands

Salivary glands consist of a specialized collection of cells organized for the purpose of producing and secreting saliva. Saliva is a complex mixture of water, salts and macromolecules, notably mucins and enzymes. The secretion of saliva is not a continuous process, but varies according to the degree of stimulation the glands receive, mainly through the nervous system supplying them. The processes of the formation and secretion of saliva, and especially the aspect commonly called stimulus-secretion coupling, are the subjects of this study. In order to facilitate discussion of control of salivary secretion, a brief description of the morphology of the glands is presented in this section, based mainly on a review by Leeson (70).

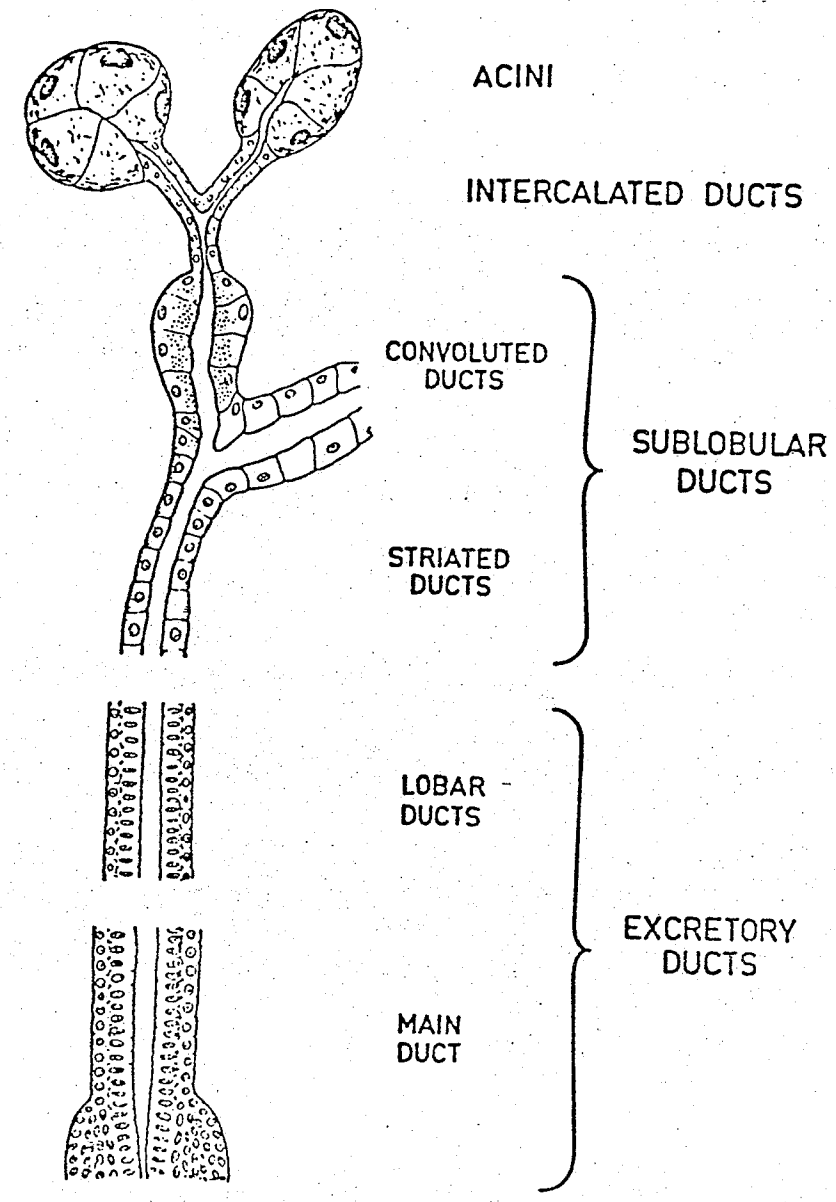
Rats have several distinct salivary glands, the parotid, the submandibular, the sublingual, and minor salivary glands. The major glands are present in pairs and consist of masses of glandular tissue, the parenchyma, and considerable amounts of areolar connective tissue, the stroma. The connective tissue encloses each gland in a fibrous capsule from which numerous septa pass into the interior to divide the gland into lobes, which are further subdivided into lobules. The cells of the lobules are pyramidal in shape and are arranged around a central lumen to form compact units termed "acini". Fine intercellular canals (secretory capillaries) which are continuous with the lumen have been observed at the interface between

adjacent acinar cells (80,96,125). Each acinus is bounded by a distinct basement membrane (80).

The acini are the terminations of a branching system of ducts. Adjacent to the acini are intercalated ducts which are continuous with the striated ducts. These intralobular ducts empty into the extralobular or excretory ducts, which empty into the oral cavity. An important difference in the duct system of the parotid and submandibular glands is the presence in the latter of convoluted granular tubule (CGT) cells. These constitute the proximal portion of the intralobular duct system and consist of tall columnar cells containing secretory granules (32,33,30,125). Thus submandibular glands have two different cell types able to store and secrete protein, the acinar cells and the CGT cells. In parotid gland only the acinar cells can secrete protein.

Secretory acini have been divided into 3 main types; serous, mucous, and mixed. Serous cells secrete a "watery" product and contain no demonstrable polysaccharide in their secretory granules (71). Mucous cell secretions are rich in mucopolysaccharide. Mixed glands contain both serous and mucous acini or have acini containing both serous and mucous cells. Parotid glands contain only serous tubules and are therefore said to be serous glands. Submandibular and subligual glands are of the mixed type.

Each major gland receives nerve fibers from both the parasympathetic and the sympathetic divisions of the autonomic nervous system. Both cholinergic and adrenergic nerve terminals are closely associated with acinar cells in rat parotid (72,73,74,77) and submandibular (73,75,76,77) glands. In fact, Hand (72) reported



The glandular unit of the fully differentiated submandibular gland of the adult rat (as described by Jacoby and Leeson (1959) in J. Anat. 93 201).

finding instances where a single parotid acinar cell was seen to be in close association with both types of terminal. Parasympathetic stimulation causes a copious watery secretion while sympathetic stimulation produces a small volume of viscous, organic-rich, saliva (1).

Development of salivary glands in rodents

Rodent salivary glands are not fully developed at birth (30,111). A substantial amount of research has been done with these glands by investigators using them as a model system for the study of cell differentiation and development. At birth, the glands show lobular organization but large amounts of loose connective tissue are found instead of the mature glandular structure. The rudiments of the duct system are present but acinar cells are not apparent (30,111,156). An unspecialized type of cell, termed terminal tubule cells (30,125,148,198), are found at the terminations of the duct system (30,111,148,198,155). These cells divide and differentiate into typical acinar and intercalated duct cells as the animal matures (30,125,148,198). Another type of cell, termed proacinar cell, has also been distinguished, and may represent an intermediate form in the differentiation of the terminal tubule cells to acinar cells (155-157,160,198).

The typical secreted proteins of adult glands are not present in significant quantity at birth (112,148,149,154) and the increase in cyclic AMP concentration seen on stimulation in adult glands (see below) is also absent (112,153,159). The development of the secreted

enzyme amylase in the parotid gland, and the ability of the gland to respond to neurotransmitter additions with amylase secretion and increased adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations were found to precede full morphological development of this gland (112). Terminal tubule cells were found to have a membrane potential similar to that in mature acinar cells (141). These observations, taken together, suggest a secretory function of the developing terminal tubule or proacinar cells.

The development process has been divided into two phases. The first, occurring primarily from birth until the age of about 25 days, consists largely of cell division (147,148). The second, most rapid at about 25 days and lasting to maturity, consists largely of cellular enlargement (147,148). The rat submandibular gland requires about 3 months for complete maturation with the development of the CGT cells occurring later than the acinar cells (30). Development of the rat parotid appears to be complete after about 2 months (142). Precocious development can be induced by repeated systemic injections of isoproterenol, a potent β-adrenergic agonist (111,135,141,150-152,157). Rats so treated have more mature acinar cells and less terminal tubule cells than control rats of the same age (150-152). Both hyperplasia and hypertrophy are involved (150). Upon the cessation of these treatments growth and differentiation slow until their appearance becomes that of untreated animals of the same age (150). It thus appears that the differentiation and maturation of the glands is influenced by the degree of stimulation they receive.

The influence of hormones on the development process has been

studied. The submandibular gland has been a particularly interesting subject for these studies as a sexual dimorphism has been observed in this tissue in the mouse (32,33,149). The CGT cells are more prominent in the male (33,149) and the development of these cells has been shown to require the presence of androgens (32,33,149). Their development in males can be prevented by castration (33,149) and testosterone injection of females leads to their submandibular glands having male proportions of these cells (149). Sexual dimorphism regarding the size or preponderance of the CGT cells has also been reported in rat submandibular glands by some workers (30,37,39) while others report finding no such differences (34,38). However, a consensus in favor of sexual dimorphism in the rat seems to be developing. Similar to mice, the development of the CGT cells has been shown to require testosterone (37,38) and thyroxine (37) and their development in males can be prevented by castration (37,38).

The size of the glands in the adult is dependent on the amount of functional stimulation they receive. Denervation of the glands leads to their atrophy (119,127,130,134,146) and a decrease in the amount of secretory product they contain (119). Similar decreases in gland size were obtained when normally innervated rats were fed on a liquid diet (118,143,145). Immature rats fed on this diet showed slower gland maturation than normally fed rats (143,145). When rats so fed are returned to solid food the changes are reversed (143,145). In contrast, rats fed on diets containing increasing amounts of non-nutritive cellulose increased their dietary intake in compensation and their gland weights increased (109). Similar increases in gland weight can be obtained by the amputation of the

incisors (126,108,109,131,133,134,186) or by oral or dietary protease administrations (108,109). These experimentally induced changes in gland weight seem to be mainly due to changes in the size and number of the acinar cells (118,119,143), the duct cells seem little changed in these experiments (186). These treatments require intact innervation for their expression (120) and are believed to produce their effects by affecting the level of neural stimulation to the glands (133). When treatments causing glandular enlargement are discontinued, the glands slowly revert to their original state (108,118,126,131,132,137).

Denervation studies have shown that both branches of the autonomic nervous system are involved in the maintenance of gland size (109,119,120,127,130,134,146,169). The sympathetic component of this effect appear to involve only the β-adrenergic receptor, as the increased gland size seen on electrical sympathetic stimulation (158) and oral papain administrations (108) are blocked by propranolol, a β-adrenergic antagonist, but not phentolamine, an α-adrenergic antagonist. Repeated injections of isoproterenol, a potent β-adrenergic agonist, in adult rats leads to an increase in gland weight (131,132,135-137,158,194,206) due to both hypertrophy (an increase in cell size) (129,132,194) and hyperplasia (an increase in the number of cells) (108,128,132,137,139) of the acinar cells. Isoproterenol has also been shown to increase DNA synthesis (110,137,139,140,144,158,206) and thymidine kinase and DNA polymerase activities (110) in rat submandibular gland. Intact innervation is not required for isoproterenol to exert its effects (131) but they can be prevented by simultaneous treatment with a β-adrenergic

antagonist (136,137). The functional relationship between the β-adrenergic receptor and adenylate cyclase has been convincingly demonstrated (64) and cyclic AMP has been postulated to be an intermediate in β-adrenergic agonist induced glandular enlargement (109,216). Enlargement of normally innervated, but not denervated, glands can be induced with repeated injections of the cyclic nucleotide phosphodiesterase inhibitor, theophylline (109,216), supporting the involvement of cyclic AMP in regulating gland size. Parasympathectomy was found to lead to changes in the histology of the striated ducts in rat parotid while sympathectomy did not affect these cells (169). However, little is known of the mechanism by which the parasympathetic system influences gland size.

Ultrastructure of secretory cells

As the principle "form follows function" applies very well in cell biology, different secretory tissues would be expected to have similarities in their ultrastructure. This has indeed been found to be the case. Microscopy of secretory cells (77-80,83,85) has revealed the same gross organization and relative preponderance of intercellular structures in most secretory cells. The most noticeable feature is the presence of a large number of secretory granules (78,85,88,116) which are membrane bounded (79,80,96,98) and appear preferentially in the apical portion of the pyramidal acinar cells (80,83,96). Variation in the electron density of these granules has been noted, those close to the apex have a high electron density while many in the more central locations have lesser density

(78,79,83). The Golgi apparatus is usually well developed and is often associated with numerous peripherally located vesicles which may be derived from it (80,83,96,116). The nuclei appear in a basal position (78,85,116,138) as do many of the mitochondria (80,138). Large amounts of highly developed endoplasmic reticulum are present, which, in the basal portions of the cells, are arranged in flat, parallel cisternae (78,80,83,96). Large numbers of ribosomes, both free and attached to the endoplasmic reticulum, are also present (80,83). Acinar cells make close attachment with each other and have areas of cellular interdigitations, desmosomes and tight junctions effectively isolating the lumen from the intracellular space on the basal side of the cell (80,85,96,113) although these junctions may loosen somewhat upon stimulation (113). Numerous microvilli have been noted on all sides of the cells (78,85). The basal membrane is usually highly folded (116) with invaginations which sometimes appear to give rise to cytoplasmic vesicles in the immediate vicinity (80). Similar basal membrane infoldings have also been noted in the cells of the intercalated and striated ducts (125).

Synthesis and storage of exportable protein

Many different cell types have secretory functions. Digestion, nervous transmission, the immune response, and hormonal regulation of metabolism and homeostasis all involve, at some point, the process of secretion. Fortunately, the process of the synthesis, storage, and secretion of macromolecules appears to possess enough similarities that studies on one cell system (i.e. model) can be related to other

secretory cell types. Much of our understanding of the cell biology of the secretory process is based on experiments on the pancreas. Parotid gland has also been popular as a model system for secretion due to its paucity of proteases and lipases (83,88) and because its secretable enzymes are synthesized in active form (87). This is in contrast to pancreas which contains a great deal of protease and lipase activity and whose secretable enzymes are synthesized and secreted in inactive zymogen forms and require activation prior to assay (121) for reproducible results. In addition, the dual innervation of salivary glands have made them popular models for the study of stimulus-effect coupling. In this section results obtained with salivary glands are quoted whenever possible, results from pancreas are used when necessary (for a review of this topic see Ref. 81,29,87).

The bulk of our present knowledge about the process of cellular secretion of macromolecules was obtained through the use of two main techniques or approaches to the problem. The first approach is histological (83,85,96,98,116,138); glandular tissue is fixed and the intracellular structure examined by light or electron microscopy. Tissue fixed at various times after the induction of secretion is compared to unstimulated tissue and structural changes accompanying secretion are noted. The second approach can be termed a biochemical one (83,86,89-91,115). Glandular tissue is placed in a media containing amino acids, one of which carries a radioactive label. As secretory cells synthesize exportable protein faster than other, constitutive proteins, the exportable proteins are preferentially labelled (115). By exposing the tissue to the label for a short time,

washing away unincorporated label, and then continuing incubation in unlabelled media, a "pulse" of radioactivity is "chased" through the cells' synthetic, storage, and secretory organelles. The progress of the labelled pulse is monitored through cell fractionation studies or autoradiography. It should be realized that these two approaches are often used together and the wide acceptance the following scheme enjoys is due to the fact that the predictions resulting from this scheme are compatible with results obtained from both approaches.

Secretory cells have the ability to selectively secrete protein. Some proteins are synthesized solely for export, others are synthesized for use within the cell. The means by which cells distinguish between secretable and non-secretable proteins has been the subject of much study (99,100). According to one theory, proteins destined for export are synthesized on membrane bound ribosomes while all other proteins are synthesized on unattached ribosomes (for a review see Ref. 102). Blobel and coworkers (101,103) hypothesize that all mRNA initially binds to free ribosomes but the mRNA coding for exportable protein differs from that coding for constitutive protein in that it posesses a special nucleotide sequence at its 5' terminal. As the mRNA is translated the peptide sequence specified by this special nucleotide sequence (signal piece) begins to emerge from the ribosome. This signal piece is hypothesized to form an attachment to the membrane of the endoplasmic reticulum, binding the ribosome to the endoplasmic reticulum and initiating the transfer of the peptide across the membrane into the cisternal space. Subsequent cleavage of the signal piece, the addition of carbohydrate moieties, the formation of intrachain disulfide bridges, or the folding of the

peptide into its native tertiary conformation would then trap the secretable proteins within this compartment. The transfer process and processing of the nascent polypeptide chain appears to begin before the complete sequence of the protein is translated (103,104). According to this hypothesis, mRNA coding for constitutive proteins lack this special nucleotide sequence. Thus the synthesis of these proteins would be completed on free ribosomes and are confined to the cytosol on separation from the ribosome. It should be emphasised, however, that the signal hypothesis has not received universal acceptance. Ovalbumin is transferred across the endoplasmic reticulum but does not appear to undergo proteolytic processing (245), leading Palmiter and coworkers to conclude that it lacks a signal piece (245).

The secretable proteins are then concentrated and packaged into secretory granules. Little is known about the mechanism by which they are transferred from the cisterns of the rough endoplasmic reticulum to the secretory granules but autoradiographic (83), morphologic (85), and cell fractionation (89) studies have implicated the Golgi apparatus, or its peripheral elements, as being involved. The varying electron density of the secretory granules noted by some investigators may be due to a maturation process in which the granule contents become more concentrated (83,116). The end result of the process is the packaging of the exportable protein in the typical, electron dense secretory granule (90,115) ready for discharge. Protein synthesis is not required for the transport and packaging of previously synthesized exportable protein (91).

The time course of the synthesis and packaging of exportable

protein has been studied in the rabbit parotid by autoradiography (83). One min. after the addition of radioactive leucine the majority of the label is associated with the endoplasmic reticulum. After an 11 min. chase with unlabelled leucine some activity was associated with the Golgi region, where most of the label was found 26 min. post-chase. By 36 min. heavy labelling of the exit side of the Golgi was noted. After 56 min. of incubation the label was found in apparent condensing granules, distinguishable from both the Golgi region and earlier synthesized mature granules. 176 min. post-chase the label appeared in mature granules. Rat pancreas appears to be faster as labelling of the granule fraction in this tissue was seen to be essentially complete within one hour (90).

Exocytosis

Upon appropriate stimulation of the cell the secretory granules move towards the luminal surface of the cell and their membranes fuse with that of the lumen (85,92,96,98,116). Layers of the two apposed membranes are excluded until a single membrane separates the granule contents and the lumen. This finally ruptures, releasing the granule contents into the lumen. After a period of stimulation a decrease in the number of granules is histologically apparent, the length of the luminal membrane and the size of the lumen are increased and the intercellular canals become enlarged (85,88,92,96,116). The process of the fusion of the membranes of the granule and the lumen with the consequent extrusion of the granule contents is known as exocytosis. Serial fusion of granules i.e. a granule fuses with the membrane and

then a second granule fuses with the first, has been noticed when a strong stimulus of secretion is used (85,96,114,116). However, fusion of two granules is only seen when one has already fused with the luminal membrane (88), implying that the fusion of a granule with the luminal membrane results in an alteration in the properties of its membrane.

It should be noted that, according to the above scheme of exportable protein synthesis, storage and secretion, exportable proteins are separated from the cytoplasm by membrane barriers at all times. Many secreted proteins are degradative in function and would wreak havoc with the cellular machinery if released to the cytoplasm in active form. Relatively small amounts of secretable proteins found to be soluble in cell fractionation studies are usually attributed to redistribution from membrane bound vesicles during homogenization. Although this theory of the transport and secretion of macromolecules is currently widely accepted, the experimental evidence in support of it is not conclusive, and other interpretations of the data are possible. Rothman (97) maintains that inhibitors of the exportable enzymes exist to prevent cytoplasmic damage and that, at least under some conditions, enzymes can be secreted directly from the cytoplasm without going into membrane bound structures.

The process of granule lysis has been studied <u>in vitro</u> with secretory granules isolated from a variety of glands. Possible inducers of lysis can be added to these preparations; if lysis occurs the contents of the granules are released and will not sediment in a centrifuge as will the contents of unruptured granules. Such studies have suggested a role for adenosine triphosphate (ATP) and Cl^- in the

release of catecholamines from granules isolated from the adrenal medulla (95). An electrogenic Cl^- pump has been hypothesized to be involved in the release process in this tissue. It is interesting to note that the Cl^- concentration required for release is approximately that of the extracellular fluid but is much higher than that of the intracellular fluid (95). Release may therefore be effected when the granule becomes exposed to the extracellular fluid upon fusion with the luminal membrane.

When granules from rat parotid were incubated with ATP, an ATP regenerating system, cyclic AMP and protein kinase and then fixed for microscopy, the granules were found to have developed pseudopodia (88,94). These were also noted in intact cells, oriented such that the pseudopodia were reaching towards the lumen (85), implying that the pseudopodia formation seen in isolated granule fractions may have physiological significance. Other experiments with granules from this tissue have implicated Mg-ATP, low concentrations of Ca^{2+}, and heat labile cytoplasmic factors in the rupture of the granules (105) and the release of their contents (106-107).

The induction of secretion by the appropriate stimuli is an energy dependent process (82) and requires oxygen (84,87). "Old" exportable protein seems to be secreted before newly synthesized exportable protein (86,114) at low levels of stimulation. Secretory cells which synthesize and secrete more than one protein package the various proteins in the same granules with each granule appearing to contain roughly the same proportions of the proteins as the next (82,84).

During the period of recovery from a strong stimulation of

secretion the length of the luminal membrane and the size of the lumen are decreased (85,96). This may be accomplished by the budding off of excess membrane at the latero-basal (80,122) or luminal (85,113) end of the cell through the formation of intracellular vesicles. Reuse of this membrane material has been suggested (84,93,123). Although membrane does not appear to be reused directly its constituents may be reused after lysosomal degradation (113). The proteins of the granule membrane appear to be resynthesized from amino acid precursors concomitantly with the exportable protein (124). Histochemical alterations of gland cells noted after extensive secretion include an increased prominence of the Golgi apparatus (92,96), the appearance of autophagic vacuoles (92,96,113,122) and an increase in the number of ribosomes bound to the endoplasmic reticulum (92). Resynthesis and repackaging of new secretable protein in rat parotid was apparent 6 hours after the amylase initially present was discharged by injecting the rat with isoproterenol (85). The amylase content of the glands reached 76 % of the initial level in 20 hours (85).

Methodology of the study of salivary function

Several different experimental approaches have been used to study the function of salivary glands. The first technique commonly employed involves the cannulation of the duct of the gland <u>in situ</u> for the collection of saliva, the volume and composition of which can then be determined. Salivary flow can be induced by injecting the animals with neurotransmitters or by the electrical stimulation of

the glands' surgically exposed nerve supply. The glands may be left connected to its normal blood supply or perfused with a buffer of near physiological composition. Experiments of this type are hereafter referred to as in vivo experiments.

A technique developed latter involves the removal of the gland from the animal, the preparation of gland slices or minces, and the suspension of these slices in physiological buffer. As first demonstrated by Hokin (161) with pigeon pancreas slices, under the proper conditions the tissue responds to the addition of neurotransmitter to the buffer with an increase in the rate of release of secretory protein into the media. This technique has since been widely used to study secretion in rat parotid gland as well as secretion in other glands and in other species. A latter refinement of this technique involves controlled enzymatic dissociation of the tissue slices to liberate isolated acini or single cells (183,190,193). Experiments where the gland is removed from the animal before the stimulation of secretion are hereafter referred to as in vitro procedures.

In vitro experiments permit closer control of the experimental conditions; for example, pulse-chase experiments give clearer results in in vitro experiments as the pulse can be administered for shorter time periods with a more effective and faster washout of unincorporated label. Larger numbers of samples can be handled simultaneously with greater ease in in vitro experiments compared to in vivo experiments. Levels of possible intracellular intermediates of stimulus-secretion coupling can be determined at shorter times after the exposure of the gland to secretagogues in in vitro

experiments than are possible in in vivo experiments.

An advantage of dissociated cell over slice preparations is that each cell in such preparations have direct and equal access to the experimental media. In slice systems only the cells on the extreme periphery of the slice are in this situation, the majority of the cells are deep within the slice and considerable diffusion must take place, and, therefore, considerable time must pass, before these cells see added secretagogue. Because a sample of dissociated cells is composed of a larger number of independent units than a slice sample of an equal amount of tissue, better randomization of the samples are possible, leading to more uniform samples. Dissociated cell experiments therefore tend to give faster and sharper kinetics and better agreement among duplicate samples.

A disadvantage of in vitro experiments is that stringent conditions must be met to maintain the viability of the tissue and its ability to respond to stimulation in the normal manner. In in vivo experiments, the normal homeostatic mechanisms of the animal help to maintain these conditions.

Amylase release from rat parotid gland

Considerable differences exists in the enzymic content of corresponding salivary glands from different species. Differences may also exist in the mechanisms regulating secretion in different species. The rat parotid gland has been the most popular salivary gland used to study secretion and correspondingly, more is known about exocytosis in this gland than other salivary glands in the rat

or about the parotid gland in other species. In order to avoid confusion, only results obtained with this gland are quoted in this section, except where clearly indicated.

A substantial portion of the amylase content of rat parotid glands has been shown to be contained in membrane bound secretory granules (163). Rat parotid saliva and the protein contents of isolated parotid secretory granules have been fractionated by ion exchange chromatography (194,217). Protein from both sources were resolved into 6 fractions. The largest peak in the elution profile has been demonstrated to be α-amylase. Two of the other peaks have been identified as RNAse and DNAse. No enzymic activity has yet been associated with the remaining peaks. DNAse has been found to be secreted concomitantly with, and in the same proportions as amylase (164,84), suggesting that both enzymes are found in the same granules. Large amounts of Ca^{2+} have also been found in the granules (173) and are secreted concomitantly with the protein (173). It has been postulated that the Ca^{2+} may have a role in the packing of the proteins within the secretory granules (173).

An important advance in our understanding of the regulation of exocytosis in the parotid gland was made when it was discovered that isolated sliced glands suspended in physiological saline would respond to electric pulses (84), or neurotransmitter additions to the media (162), with an increased rate of amylase release. The most effective neurotransmitter or synthetic neurotransmitter in inducing amylase release was found to be the specific β-adrenergic agonist, isoproterenol (169,185,190), which also causes amylase secretion <u>in vivo</u> (165) and in dissociated cell preparations (183,184,190,193).

The effect of this drug is prevented by pretreatment with the β-adrenergic antagonist, propranolol (184). The physiological neurotransmitters, epinephrine (164,181,167,174,4,184,193) and norepinephrine (7,107,167,4,182), also increase the rate of amylase release. Although epinephrine and norepinephrine bind to both α- and β-adrenergic receptors, their effect on amylase release is blocked by β-adrenergic antagonists, such as propranolol (7,4,182,184), but relatively unaffected by α-adrenergic antagonists, such as phentolamine (7,4,182,184). However, a small increase in amylase release has been reported to be induced by α-adrenergic agonists (67,191,193) which is blocked specifically by α-adrenergic antagonists (191). Acetylcholine or its synthetic analogues, carbamylcholine and pilocarpine, have been reported to cause an increase in the rate of amylase release (162,84,168,7,171,172,174,180,182,191,66,193). Although part of the effect of pilocarpine *in vivo* has been attributed to an indirect stimulation of adrenergic receptors mediated through the superior cervical ganglion and the sympathetic postganglionic fibers (166,169), the muscarinic cholinergic antagonist, atropine, has been reported to block the effect of carbamylcholine *in vitro* (171,180,182,191,66), while propranolol was without effect (191,66), suggesting a direct cholinergic influence on amylase secretion. However, the effect of cholinergic and α-adrenergic agonists is much smaller than the effect of β-adrenergic agonists (7,174,191) and there is no doubt that the β-adrenergic receptor is the physiologically more important inducer of amylase secretion. In addition, the polypeptides, substance P, physaelamin, and eledoisin

have been found to induce amylase release which is not blocked by the normal neurotransmitter antagonists (192). The action of these polypeptides may reflect a hypothalamic influence on salivary secretion (223).

The functional interaction between the β-adrenergic receptor and adenylate cyclase has been clearly demonstrated (for a review see Ref. 64). Although the mechanism is as yet unclear, it is now widely accepted that the binding of an agonist to this receptor activates adenylate cyclase which is situated on the inner surface of the plasma membrane. This activation thus leads to an increase in the intracellular cyclic AMP concentration.

Sutherland (207) listed 4 criteria he believed should be satisfied to assign a second messenger role to cyclic AMP for a given effect in a given tissue:

1) adenylate cyclase in broken cell preparations of the tissue should respond to the agents which cause the effect in the intact tissue
2) the level of cyclic AMP in intact tissue should change appropriately upon stimulation of the effect
3) submaximal stimulation of the effect should be potentiated by phosphodiesterase inhibitors
4) the effect should be mimicked by exogenous cyclic AMP or cyclic AMP analogues

Isoproterenol, epinephrine or norepinephrine additions to the media of rat parotid slices (188,67,191,204) or dissociated cells (183) has been shown to result in rapid cyclic AMP increases which

can be prevented by pretreatment of the tissue with β-adrenergic antagonists (188). Similar increases in cyclic AMP have been noted in glands after they had their sympathetic nervous supply electrically stimulated in situ (205). Plasma membranes isolated from rat parotids contain an adenylate cyclase, which is stimulated by β-adrenergic agonists (188,199). The order of potency of a series of β-adrenergic agonists was found to be the same for the stimulation of adenylate cyclase in isolated membranes, cyclic AMP accumulation in parotid slices, and amylase release from parotid slices (188). Thus, Sutherland's first and second criteria have been satisfied.

Theophylline and caffeine, inhibitors of the cyclic nucleotide phosphodiesterases (the enzymes responsible for cyclic AMP degradation) induce amylase secretion themselves in a slice system (63,167,172) and theophylline potentiates the effects of β-adrenergic agonists on amylase secretion (63,172). Theophylline has been reported to have no effect on unstimulated amylase release in a dissociated cell system (183) but other workers have reported an increase in amylase release induced by the more effective phosphodiesterase inbibitor, IBMX (193). Thus Sutherland's third criterion has also been satisfied.

Cyclic AMP additions to the media in parotid slice systems are without effect (63,167), but this is thought to be due to the poor penetration of the nucleotide into the cell. The dibutyryl derivative, which is more permeable to cellular membranes, is able to evoke amylase release in both slice (63,167,185,188) and dissociated cell preparations (184,193). The effect of dibutyryl-cyclic AMP is not inhibited by propranolol (7,184), showing that this substance

does not induce secretion by liberating endogenous catecholamine. Thus, all of the criteria Sutherland proposed to establish a second messenger role for cyclic AMP have been satisfied in β-adrenergic induced amylase secretion in rat parotid gland.

However, it should be pointed out that experiments using dibutyryl-cyclic AMP must be interpreted with caution, as it has been shown that many effects of this agent in many cell types are due to the butyrate portion rather than the cyclic AMP portion of the molecule (218,219). Additionally, isoproterenol induced secretion and dibutyryl-cyclic AMP induced secretion have been found to differ in respect to their sensitivity to ouabain and extracellular Ca^{2+} (196), suggesting that they may exert their effects through different mechanisms.

It should also be noted that other mechanisms of inducing amylase release, not involving cyclic AMP, may exist. The amylase release induced by α-adrenergic (67) agonists, and substance P and eledoisin (192), is not accompanied by increases in cyclic AMP levels. In fact, cholinergic and α-adrenergic agonists decrease the amount of cyclic AMP which accumulates in response to β-adrenergic agonists (66,67,204). Butcher *et al.* reported that amylase release induced by cholinergic agonists was not accompanied by an increase in cyclic AMP (66) while Harper and Brooker report a very small increase in cyclic AMP levels upon stimulation with carbamylcholine (204). This increase was prevented by atropine pretreatment but not by propranolol (204). Carbamylcholine did not require extracellular Ca^{2+} to increase cyclic AMP levels (204).

Most, if not all of the actions of cyclic AMP in mammalian

systems are thought to be mediated by protein phosphorylation (208). Cyclic AMP has been proposed to induce amylase release in rat parotid gland through such a mechanism (187). High affinity cyclic AMP binding has been found in a microsomal fraction prepared from a rat parotid homogenate (200). Cyclic AMP-stimulated protein kinase activity has been demonstrated in parotid glands from rats (179,203) and other species (201,202). Tolbutamide, an inhibitor of these enzymes, has been shown to inhibit isoproterenol induced amylase secretion from rat parotid slices (179). Rat parotid slices incubated with [^{32}P]-Pi incorporate more of the label into their proteins when stimulated with isoproterenol or dibutyryl-cyclic AMP than in control incubations with no secretagogue (185). These observations all support the hypothesis that cyclic AMP induces amylase release through a cyclic AMP-dependent protein kinase. However, the physiological substrates for these kinases and the function of the phosphorylated proteins are not known at present.

The importance of Ca^{2+} in salivary secretion has been known for some time. Douglas and Poisner (221,222) showed that acetylcholine-evoked salivary secretion from cat submandibular gland was reduced when Ca^{2+} was omitted from the perfusing buffer. Ca^{2+} also appears to play a role in amylase secretion. Amylase secretion induced by cholinergic (107,191,193,196) or α-adrenergic (67,193,196) agonists, substance P and eledoisin (192) and high K^+ levels (107) occurs only when the media contains Ca^{2+}. Prolonged incubations in the presence of the calcium chelator, ethyleneglycol-bis(-aminoethylether)-N,N'-tetraacetic acid (EGTA), are not necessary to demonstrate reduced amylase secretion with these

secretagogues. Introduction of extracellular Ca^{2+} into the cells with the divalent ionophore A23187 results in a small but significant increase in amylase secretion (180,189,193,195). Neurotransmitter antagonists do not block the effect of the ionophore (189). However, the increase in amylase secretion seen with the ionophore, α-adrenergic, and cholinergic agonists is much smaller than that seen with β-adrenergic agonists.

Ca^{2+} also appears to be involved in β-adrenergic agonist mediated amylase release. However, this Ca^{2+} involvement is not easily demonstrated as secretion induced by β-adrenergic agonists is unaffected by an absence of Ca^{2+} in the medium (191,193). In fact, slices stimulated with β-adreneric agonists secrete more amylase in a Ca^{2+} free media than they do in a media containing Ca^{2+} (61,196). This may be due to the fact that K^+ release does not occur under these circumstances, thus conserving ATP for exocytosis (61). However if the slices are depleted of Ca^{2+} by lengthy preincubation in Ca^{2+} free media containing the Ca^{2+} chelator EGTA, the ability of the tissue to secrete amylase when stimulated with β-adrenergic agonists (171,195,196), or monobutyryl-cyclic AMP (171) or dibutyryl-cyclic AMP (195,196), is reduced. Butcher demonstrated that isoproterenol-induced amylase secretion was reduced immediately upon Ca^{2+} removal but the response was reaquired within 2-4 min. (196). Prolonged Ca^{2+} depletion resulted in a more long lasting inhibition of secretion (196). However, extracellular Ca^{2+} does not appear to be the main Ca^{2+} source for β-adrenergic induced secretion (181) as isoproterenol increases [$^{45}Ca^{2+}$] efflux rather than influx (195) and inhibitors of transmembrane Ca^{2+} fluxes do not affect

isoproterenol-induced amylase secretion (196). Thus, intracellular Ca^{2+} stores are implicated but it is unclear at this time whether these are mitochondrial, microsomal, or membrane bound Ca^{2+} stores (181). When shorter periods of Ca^{2+} depletion are performed, it has been found that the ability of the slices to secrete protein on stimulation is relatively unimpaired while the ability of the slices to increase cyclic AMP levels is reduced 80 % (177). However, it should be noted that the cyclic AMP levels were still increased 10 fold over basal levels under these conditions (177). Butcher also noted a decreased accumulation of cyclic AMP upon β-adrenergic stimulation in Ca^{2+} free media (196). Thus, Ca^{2+} appears to have a role in the increase in cyclic AMP levels seen on β-adrenergic stimulation.

Experiments demonstrating a decreased cyclic AMP response without a concomitant reduction in amylase secretion appear to weaken the case for cyclic AMP involvement in stimulus-secretion coupling. However, the implicit assumption that the extent of amylase release should be directly proportional to the magnitude of the cyclic AMP concentration increase may be valid only at low levels of the nucleotide. It is possible that at high agonist concentrations cyclic AMP levels are increased well beyond the point where cyclic AMP is the rate-limiting factor. β-adrenergic agonists have been found to measurably increase amylase release at concentrations which do not measurably increase cyclic AMP accumulation (188), leading Butcher and coworkers to suggest that large changes in the level of this nucleotide may not be necessary to induce amylase release (188).

Using slightly different conditions of Ca^{2+} depletion, Putney

(195) found that at 10 uM isoproterenol both cyclic AMP accumulation and amylase secretion were reduced when compared to undepleted slices, but at 1 uM isoproterenol amylase secretion was reduced while cyclic AMP accumulation was unaffected. These results could suggest that another step in the stimulus-secretion coupling process, after the step regulated by cyclic AMP, could be Ca^{2+}-dependent. Some investigators have suggested that Ca^{2+} may be the final mediator of exocytosis in rat parotid gland (193,195) and that the role of cyclic AMP may be to regulate the cytoplasmic ionized Ca^{2+} concentration (181,220).

The secretion of amylase and DNAse has been shown to be an energy dependent process and requires the presence of oxygen (164,84). Epinephrine accelerates oxygen uptake by rat parotid slices (167,172) and oligomycin inhibits both oxygen uptake and epinephrine stimulated amylase secretion (167). Secretion induced with either epinephrine (84,164,167) or dibutyryl-cyclic AMP (167) has been shown to be inhibited by cyanide and dinitrophenol, which are uncouplers of oxidative phosphorylation. Epinephrine (167), pilocarpine (172) and dibutyryl-cyclic AMP (172) have also been shown to be unable to induce amylase release in a nitrogen atmosphere (167). Epinephrine has been demonstrated to reduce ATP levels in rat parotid slices (61,196), but isoproterenol had no effect on these levels (196). β-hydroxybutyrate has been shown to increase the amount of amylase secreted in response to a given dose of epinephrine (167). This effect is thought to be due to the metabolism of this substance helping to maintain intracellular ATP levels (167). Similarly, inosine and adenine have been shown to increase ATP levels in rat

parotid slices (61) and also increase the rate of epinephrine induced amylase secretion (61,175). Ouabain, an inhibitor of (Na^+/K^+)-ATPase, also increases epinephrine-stimulated amylase release (61,175). Although ATP levels were not measured, Selinger *et al.* presume that ouabain increases amylase release by conserving ATP for exocytosis (61,175). Ouabain also potentiates isoproterenol-induced amylase release although ATP levels were unaffected (196), suggesting an effect through a different mechanism. A greater percentage of the amylase content of rat parotid slices is secreted when the β-adrenergic receptor is stimulated without stimulation of the α-adrenergic receptor (61). This is also thought to be due to the conservation of ATP. Amylase release is unaffected by inhibitors of protein synthesis (168,170), indicating that the stimulus-secretion coupling process does not involve the synthesis of new protein. In fact, protein synthesis is reduced during secretion (168,170), although synthesis is increased above basal levels after secretion is terminated (170).

Extracellular K^+ has been shown to be important in amylase secretion. Increasing the K^+ concentration of the medium to over 60 mM resulted in amylase release (193), which was inhibited by dinitrophenol, but was independent of added neurotransmitter (84,164). However amylase release induced by 60 mM K^+ is inhibited by propranolol (7), suggesting that K^+ induces secretion by liberating endogenous catecholamine. Epinephrine induces less amylase secretion in K^+ free media than it does in media containing physiologic concentrations of K^+ (84,164). Ammonium ion can not replace the K^+ requirement for stimulation of secretion (84).

During the process of exocytosis in the rat parotid gland the secretory granules move towards the luminal membrane of the cell. Little is known about the mechanics of this directed motion but microfilaments and microtubules have been suggested to be involved (176,178). When parotid slices were incubated for one hour with cytochalasin B, a drug which disrupts microfilaments, enzyme secretion induced with epinephrine or dibutyryl-cyclic AMP was found to be reduced (176,178). Similar results were obtained with colchicine, a drug which disrupts microtubules, when secretion was induced with epinephrine although a four hour treatment was required to demonstrate the effect (176). However, this drug did not affect secretion induced with dibutyryl-cyclic AMP (176,178).

Enzyme secretion in rat submandibular gland

Much less is known about the secretion of protein in rat submandibular gland than in rat parotid gland. Studies of secretion in this gland are complicated by the fact that it is a mixed mucous-serous type gland and has two types of cells with secretory function; the acinar cells and the CGT cells. As these types of cells are histologically different, it would be expected that they synthesize and secrete different proteins. Splitting the protein secretion between two different types of cells could allow for a more varied repertoire of secretory responses if secretion from the two types of cells is independently regulated. Histological studies have demonstrated exocytosis in rat submandibular gland acinar cells induced by an injection of isoproterenol into the intact animal

(138,231,96) but no changes were noted in the duct cells (138). In contrast, the CGT cells were seen to be degranulated upon pilocarpine stimulation (232,116). It thus appears that secretion from the two different cell types may in fact be independently regulated. However, experiments where pilocarpine is injected into intact animals must be interpreted with caution, as this drug has been found to stimulate adrenergic pathways to the gland at the level of the superior cervical ganglion (166,169).

Tissue slice systems have proven very useful in the study of protein secretion in the rat parotid gland. Rossignol et al. (233) preincubated rat submandibular slices with [^{14}C]-glucose and then studied the release of trichloroacetic acid-precipitable (TCA-precipitable) radioactivity into the incubation media. The release of this material was found to be accelerated by carbamylcholine (233). However this technique is not able to distinguish between secretion from acinar cells and secretion from the CGT cells. An alternate means of studying exocytosis is to measure the release of preformed exportable enzyme into the media by assay of its catalytic activity. If enzymes could be identified which are synthesized and secreted in one cell type but not the other, they could be used to study the secretion of that cell type independently of the other. Amylase (235,236), peroxidase (235,236) and kallikrein (59,60) have been used to monitor secretion from guinea pig submandibular gland slices. Amylase has been reported to be located in acinar cells in this species (239). Peroxidase was initially reported to be present in acinar cells and CGT cells in a light microscopic study (234), but in later studies by the same

investigators using electron microscopy, only an acinar cell location is reported (240,241). The secretion of kallikrein from guinea pig submandibular slices was accompanied by the depletion of secretory granules in the acinar cells; no changes were seen in duct cells (59). However, Schachter et al., using an immunofluorescent technique, report finding kallikrein only in CGT cells in this species (242). The secretion of kallikrein from guinea pig submandibular slices was found to be produced by α-adrenergic (59), β-adrenergic (59), and cholinergic (60) agonists. Cholinergic stimulation has also been reported to cause amylase and peroxidase release from guinea pig submandibular slices (236), but the effect of epinephrine and norepinephrine was found to be inhibited by β-adrenergic antagonists; α-adrenergic antagonists were almost without effect (235). However, species differences in the enzymatic content, and perhaps also in the type of control of secretion, appear to exist. Rat submandibular gland does not synthesize amylase (238,25) and contains much less peroxidase that guinea pig submandibular gland (237). Rat submandibular does contain considerable amounts of kallikrein, however, and has been found by immunofluorescence to be localized to the CGT cells in this species (49,50).

It has been known for some time that rat submandibular gland extracts can degrade proteins (23,24), as can rat submandibular saliva (25-28); indicating exocrine secretion of protease. The submandibular glands of male mice have been found to contain more protease activity than female mice submandibular glands (33) and the development of protease activity in these glands is androgen

dependent (33). As previously discussed, the CGT cells of mice submandibular glands have also been found to be more prevalent in males and are also androgen dependent (32,33,149). The similarities between the sex and androgen dependency of protease activity and the presence of the CGT cells led Junqueira et al. (33) to conclude that these cells are the site of production of protease activity in this particular species. Similar sex-linked differences were observed in rat submandibular gland protease activity by Sreebny (34) and the protease content of male rat submandibular glands was found to be lowered upon castration (121). These observations led Sreebny to conclude that in rats, as in mice, proteases are produced in the CGT cells; although there has been controversy whether or not CGT cells are more prevalent in males in rats (30,34,37,38,39). Histochemical studies support this locale for protease (35,36). In all the above studies the investigators examined only total protease activity although similar results were reported in mice when renin, a trypsin-like protease and a chymotrypsin-like protease were assayed with more specific substrates (149).

Riekkinen and associates (40-47) isolated and characterized several different proteases from rat submandibular gland homogenates. They then examined the age, sex, and androgen dependency of some of these enzymes (48). It was reported that the activity of one enzyme, which they called glandulain, was much higher in males but two other proteases, salivain and cathepsin B, showed no sex-linked differences. If sex-linked differences in activity and androgen dependency indicate a CGT cell localization in the rat, as they do in mice, it can be concluded that glandulain is present in CGT cells but

salivain or cathepsin B are located elsewhere, most probably the acinar cells. In addition, salivain was found to be present in isoproterenol-evoked rat submandibular saliva but glandulain was not found (26). This suggests that the secretion of these two enzymes is independently regulated and supports an acinar cell localization for salivain, as secretion from acinar cells has been observed on isoproterenol stimulation (96,138,231).

Because of the suggestion that different proteases may serve as markers for acinar and CGT cells, a study of the properties, sex-dependency, and secretion of two proteases was undertaken in rat submandibular slices. The protease, kallikrein, whose localization in the CGT cells in rats has been established (49,50), was also included in our study to provide a point of reference.

K^+ release from salivary glands *in vitro*

The secretion of water and electrolytes appears to be different from macromolecule secretion in that the process of exocytosis does not seem to be involved (for a review of salivary secretion of water and electrolytes see Ref. 1). Early ultrastructural studies of exocytosis sometimes revealed the presence of electron-lucent vacuoles in acinar cells upon stimulation (98). At first, attempts were made to relate these vacuoles to the process of exocytosis (98) but later work suggests that these vacuoles are independent of this process. In the rat parotid gland, vacuole formation was found to be associated with α-adrenergic (3,4,5,8) and cholinergic (5) stimulation, whereas exocytotic secretion of amylase is a

predominately β-adrenergic mediated process (4,7,182,184). Through histological observation of the vacuole formation induced by α-adrenergic and cholinergic stimulation and exocytosis induced by β-adrenergic agonists, it has been possible to show that rat parotid acinar cells possess all three classes of receptors (5).

Schneyer and Schneyer (2) demonstrated that rat submandibular gland slices exchanged [$^{42}K^+$] with the medium and that the loss of [$^{42}K^+$] from preloaded slices was accelerated by the cholinergic agonist pilocarpine. Batzri et al. (3) showed that rat parotid gland tissue secreted K^+ into the medium when stimulated with epinephrine, and that this effect was mediated through α-adrenergic receptors (4). Later work by Schramm and Selinger (5) indicated that the synthetic muscarinic cholinergic agonist, carbamylcholine, was also effective in inducing K^+ release in this tissue. In the present study K^+ release by rat submandibular gland slices has been investigated with a wide variety of stimulants and under several different conditions and an hypothesis is presented to explain the mechanism of K^+ release.

Cyclic GMP

The discovery of cyclic AMP and the demonstration of its function as a second messenger was a major advance in understanding cell to cell communication. A number of hormones and neurotransmitters were soon found to increase cyclic AMP levels in their target tissues (207). However a number of other hormones and neurotransmitters were found to either not change, or decrease,

cyclic AMP levels in target tissues (17,18). According to one theory, these agents produce their effects by decreasing cyclic AMP levels (244), but this presently seems unlikely. Rat parotid gland acinar cells have receptors for neurotransmitters of both types (i.e. those thought to act by increasing intracellular cyclic AMP and those theorized to act by decreasing intracellular cyclic AMP), and the effects they mediate can be activated simultaneously (5). Some hormones and neurotransmitters that induce physiological responses without affecting intracellular cyclic AMP levels, produce elevations in the guanosine 3':5'-cyclic-monophosphate (cyclic GMP) content, suggesting that this nucleotide may be a second messenger (209,17,18). This suggestion receives support from the fact that many cells have both cyclic GMP- and cyclic AMP-dependent protein kinases (208). For a review of this topic see Ref. 209.

In rat parotid gland, α-adrenergic and cholinergic stimulation elevates intracellular cyclic GMP (67,66,204,29,31) and induce K^+ release (67,66,8,4,5,211,210); both processes requiring extracellular Ca^{2+} (197,9). Cyclic GMP derivatives have been reported to cause K^+ release when added to parotid slice preparations (67). However, based on their finding that the phosphodiesterase inhibitor, IBMX, did not potentiate the the effect of carbamylcholine (66) or phenylephrine, an α-adrenergic agonist (67), on K^+ release, but did potentiate the cyclic GMP accumulation produced by these agonists, Butcher and coworkers discount the involvement of cyclic GMP in K^+ release from rat parotid. However, Mangos (210), using a dissociated rat parotid acinar cell preparation, found IBMX to increase the amount of K^+ released in response to a submaximal dose of carbamylcholine, and

concluded that cyclic GMP is an intermediate in cholinergic mechanism of K^+ release.

Rat submandibular gland slices, like those from parotid, release K^+ upon appropriate stimulation (56,22,65). The mechanism appears very similar in both tissues as K^+ release is brought about by the same neurotransmitters (8,5,4,211,67,66,56,22,65) and both tissues have similar requirements for extracellular Ca^{2+} (197,9,56,22,65). In contrast to the results of Butcher et al. (66,67) with parotid slices, but in agreement with Mangos (210) with dissociated parotid acinar cells, this laboratory has obtained evidence of a potentiation of K^+ efflux by IBMX (56). Schultz et al. (14) have reported a carbamylcholine induced increase in cyclic GMP in rat submandibular slices incubated with a phosphodiesterase inhibitor which was dependent on external Ca^{2+} (14). However it is not known if α-adrenergic and polypeptide K^+ release inducers (e.g. eledoisin and physalaemin) affect cyclic GMP levels in this gland.

Cyclic GMP has also been implicated in amylase release in mouse (212) and rabbit (16) parotid gland, increased DNA synthesis in mouse parotid gland (213), and kallikrein secretion in guinea pig submandibular gland (60). Considering the present unclear role of cyclic GMP in salivary function, it seemed appropriate to examine the levels of cyclic GMP in rat submandibular gland under various experimental conditions to extend the preliminary observation by Schultz et al. (14). Special emphasis has been placed on the possible relationship of the cyclic GMP response to K^+ release and protease secretion.

MATERIALS

Propranolol, phentolamine, isoproterenol, ethacrynic acid and ionophore A23187 were gifts from Ayerst Labs. (Montreal, Can.), CIBA Pharmaceuticals (Dorval, Can.), Winthrop Labs. (Aurora, Can.), Merck Frosst Labs. (Montreal, Can.) and Lilly Labs. (Indianapolis, U.S.A.), respectively. The phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine (IBMX), was purchased from Aldrich Chemical Co. (Milwaukee, U.S.A.). L-epinephrine bitartrate and phenylmethylsulfonylfluoride were from Calbiochem (San Diego, U.S.A.). [^{125}I]-succinyl-cyclic GMP tyrosine methyl ester, cyclic GMP antibody preconjugated with goat antirabbit antibody, and [^{3}H]-cyclic GMP were purchased from NEN Canada Ltd. (Montreal, Can.) as a cyclic GMP radioimmunoassay kit. [^{3}H]-cyclic GMP was purified before use on Sephadex QAE as described in Methods. The Sephadex QAE A-25 was from Pharmacia (Canada) Ltd. (Dorval, Can.). All other biochemicals were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

METHODS

Buffer

Unless otherwise noted, the media used was Krebs-Ringer saline, buffered with bicarbonate, pH 7.4 (KRB), and continuously gassed with 95% O_2 / 5% CO_2 (247). The buffer was supplemented with 5 mM β-hydroxybutyrate in studies of K^+ release and protease secretion. This substance has been shown to support secretion in rat parotid gland (167). The buffer was maintained at 37°C in a gyrotary water bath (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, N.J.).

Tissue preparation and incubations in slice experiments

Three month old male Long-Evans rats, fed and watered ad libitum, were used unless otherwise noted. Anaesthesia, when used, was acheived with ether. When parotid glands were collected, the anaesthesized rats were killed by heart puncture. At other times rats were killed by decapitation. In early work rats to be decapitated were first anaesthesized, in later work they were decapitated without anaesthetic.

Schramm et al. showed that rat parotid slices had a higher rate of unstimulated amylase release when they had been exposed to low temperatures during collection or preincubation than they had when all steps were carried out at 37°C (69,167). Therefore we placed the glands into prewarmed, pregassed buffer immediately upon removal from

the rats and all tissue manipulations were performed so as to minimize the length of time that the tissues were out of 37°C buffer. After sufficient glands for each experiment had been collected, they were individually removed from the buffer, extraneous tissue and the sublingual glands removed, and the submandibular glands replaced in fresh buffer.

The tissue was sliced by hand with a razor blade for studies of K^+ release and protease secretion. In cyclic GMP experiments, tissues were sliced with a McIlwain tissue chopper. The instrument was set for a 1 mm slice and two passes through the machine were carried out, the second at 90° to the first. The slices were returned to fresh buffer after slicing. After all slicing was completed, the pieces were removed, rinsed twice, blotted carefully, and divided into appoximately equal portions. Each portion was placed in a plastic vial or 14.5 X 96 mm plastic tube containing 3 ml buffer.

After an initial preincubation period of 10 min., the buffer was removed with a Pasteur pipette and replaced with 1 ml of fresh prewarmed and pregassed buffer. Neurotransmitter antagonists, when used, were added at this time. After an additional final preincubation, stimulants were added and the samples incubated. The length of the final preincubation and the incubation itself varied according to the type of experiment performed e.g. K^+ release, protease secretion, etc. These details are described in the sections describing the methodology of the respective projects.

Protease secretion from rat submandibular gland slices

Rats used for these experiments were killed by decapitation without anaesthesia and the submandibular glands removed and prepared as described in "Tissue preparation and incubation in slice experiments". Usually 3 rats were used for each experiment, providing sufficient tissue for 18 to 24 tests. The final preincubation was for 15 min. and the samples were incubated 30 min. with the stimulants unless noted otherwise. The medium was quickly removed with a pipette and the tissue homogenized in 2 ml water with a Potter-Elvehjem tissue grinder. Sample incubation times were staggered so as to allow ample time for processing. Enzyme assays were performed on both the medium and the homogenates the same day.

K^+ release from rat submandibular slices

Two rats were killed by heart puncture and the submandibular glands were removed and prepared as described in "Tissue preparation and incubation in slice experiments". Six samples were prepared from the four glands and the plastic vials were preweighed before use in order to permit calculation of the total volume of the media and tissue at the end of the experiment. Unless noted otherwise the KRB buffer used for these studies had the K^+ concentration reduced from 6 to 4 mM. In order to avoid complications associated with release of endogenous neurotransmitters (2,8) classes of receptors not destined to be stimulated in the experiment were blocked with the appropriate antagonists; phentolamine for α-adrenergic receptors, propranolol for

β-adrenergic receptors, atropine for cholinergic receptors. The final preincubation was for 5 min., after which a 50 ul sample of the media was withdrawn for zero time K^+ determination. Immediately thereafter the stimulant was added and the incubation was continued for 5 min. A sample of the media was then withdrawn for the 5 min. K^+ determination and the tissue was then homogenized in the remaining medium. The K^+ concentration of the homogenate was determined and used to calculate the total K^+ present in the system at zero time. This was accomplished by weighing the vials at the end of the experiment, adding the weight of the sample taken for the determination of the 5 min. K^+ concentration, and subtracting the weight of the empty vials. Assuming that the specific gravity of the medium = 1, this figure then represented the total volume of the system at zero time. Using this figure and the K^+ concentration of an aliquot of the homogenate the total K^+ content of the system was calculated. The K^+ content of an equal volume of the buffer was then subtracted to give the total amount of K^+ in the system which originated from the tissue. The difference in the K^+ concentration of the 5 min. and the zero time samples was multiplied by the appropriate dilution factor to yield the total amount of K^+ released into the media during the final 5 min. incubation. This figure was then divided by the total K^+ content of the tissue and multiplied by 100 to give the % total tissue K^+ secreted into the medium. Ref. 6 should be consulted for experimental details and method of calculation as our procedures were essentially identical to theirs.

Initial experimentation indicated that while variations occurred among different tissue preparations in their response to stimulation,

the patterns were consistant from trial to trial. A complete set of controls was always run with each trial. Usually, three trials were performed each day using freshly prepared tissue and reagents for each trial.

Cyclic GMP levels in rat submandibular slices

Rats used for these experiments were killed by decapitation without anaesthesia and the submandibular glands removed and prepared as described in "Tissue preparation and incubation in slice experiments" except that the Ca^{2+} concentration of the buffer was reduced from 2.5 to 1.0 mM and β-hydroxybutyrate was omitted. One rat was used for each experiment yielding sufficient tissue for 12-15 tests. The final preincubation was for 15 min. and included 500 uM IBMX unless otherwise noted. Final incubations with agonists were for 3 min. except in the experiment described in Fig. 6. The incubation was terminated by the addition of 1 ml of ice cold 10 % w/v TCA and the mixture immediately homogenized for 5-10 seconds with a Polytron PT-10 (Kinematica GMBH, Switzerland) set at maximum speed. Overall experimental time from killing the animal to TCA addition was approximately 45 min. Each experiment consisted of 4 or 5 different treatments repeated 3 times for a total 12 or 15 samples and was performed 3 times on the same day using freshly prepared tissue, buffer and reagents each time. Thus, one day's experimentation resulted in 36 or 45 samples which were purified and assayed together.

Kallikrein

Kallikrein was determined by measuring the amount of α-N-benzoyl- arginine liberated from α-N-benzoyl-L-arginine ethyl ester (BAEE) (51). Diluted medium or homogenate (1:100) was added to 0.5 ml 1 mM BAEE in 50 mM triethanolamine, pH 8.0, maintained at 30° C in cuvettes of a Gilford 2000 Multi-Cell Recording Spectrophotometer (Gilford Instrument Labs. Inc. Oberlin, Ohio) linked to a computer for data processing and readout. Four samples, the medium and homogenates from two tests, were assayed simultaneously. Sequential determinations of the absorbance every 14 sec. were made and converted into a hydrolytic rate by the computer. The volume of sample was chosen such that a constant rate of hydrolysis was maintained for at least 8 determinations, the average of which was used as the measure of kallikrein activity. Because a small, but measurable hydrolysis of BAEE was observed at 253 nm (the absorption maximum) in the absence of enzyme, absorbance was measured at 263 nm. This reduced the sensitivity, necessitating the assay of somewhat larger (or less diluted) aliquots, but eliminated the non-enzymic hydrolysis problem. In spite of this reduction in sensitivity, this assay required less sample than any other assay used in this study. The results are expressed as absorbance change at 263 nm / mg protein / min.

The assay of kallikrein by measuring its hydrolytic effect on BAEE, although commonly employed by many investigators (24,41,51), is not as specific as methods based on the bioassay of kinins (52) released from plasma, i.e. kinogenase activity. Using both methods,

Orstavik and Gautvik (27) calculated that 78 +/- 16 % of total BAEE hydrolysis in rat submandibular gland extracts was due to kallikrein and concluded that variations in BAEE esterase activity clearly reflected quantitative variations in kallikrein activity in rat submandibular gland and its secretions.

pH 8.2 and 9.5 proteases

Both enzymes were estimated by their ability to hydrolyse α-N-benzoyl-DL-arginine-β-napthylamide.HCl (BANA), although at different pH and different buffer compositions. The assay mixture contained 50 mM glycine-NaOH buffer, adjusted to pH 8.2 or pH 9.5 for respective proteases, sample (medium or homogenate) and 0.25 mM BANA in a total volume of 1 ml. The pH 9.5 system also had, in addition, 20 mM $Cacl_2$ and 0.1 mM $CuCl_2$. Incubations were for 30 min at 37°C in a shaking water bath. Reactions were terminated by the addition of 0.5 ml 2.0 N HCl and the liberated β-napthylamine estimated by the method of Martinek et al. (53). Essentially, the free β-napthylamine released by protease action is diazotized with $NaNO_2$ and the product coupled to N-(1-napthyl)ethylenediamine to produce a highly coloured azo dye whose absorbance can be determined at 580 nm. Analysis were done in 16 x 150 mm tubes under reduced lighting as the reaction was found to be sensitive to light (unpublished observation). This procedure was found to be more sensitive than that employing fast Garnet GBC as used by Riekkinen and Niemi (48). The reaction was linear for 30 min provided that the amount of enzyme was selected such that the rate of hydrolysis did not exceed 1.7 nmoles

β-napthylamine released / min. All analysis were performed under these conditions and all determinations were done in duplicate. Activities are expressed as nmoles β-napthylamine released / mg protein / min.

For enzyme secretion studies, the results are expressed as percent of total enzyme activity secreted, thus: % secretion = (total activity of medium / total activity in medium + total activity of homogenate) x 100.

Measurement of K^+

A Perkin-Elmer Atomic Absorption Spectrophotometer Model 503 was used to determine K^+ concentrations. The author wishes to thank Dr. C. Dawes for the use of this instrument.

Cyclic GMP sample purification and assay

To monitor recovery an equal amount (approximately 7000 DPM's) of [^{3}H]-cyclic GMP was added to each sample after which they were centrifuged for 30 min. at 15,000 X g at 4°C. After centrifugation the supernatants were decanted and the pellets were suspended in 0.5 ml ice cold 5% w/v TCA and recentrifuged 15 min. The supernatants were combined. The pellets were dissolved in 2 ml of 0.2 N NaOH and 10 ul aliquots in triplicate were assayed for protein by the procedure of Lowry et al. (54). The combined supernatants were extracted 4 times with 5 ml water-saturated ethyl ether to remove TCA. Residual ether was removed under flowing N_2 at room temperature.

The samples were then taken to dryness in a Vortex-Evaporator (Buchler Instruments, Division of Searle Analytic Inc., Fort Lee, N.J., U.S.A.) at approximately 45°C. The following day the samples were dissolved in 200 ul water and applied to 4 X 0.7 cm columns of Sephadex QAE A-25, Cl^--form. The tubes were then rinsed twice with 0.5 ml water, each rinse being added to the appropriate column. Additional columns received known amounts of a standard cyclic GMP solution (100 - 10,000 fmoles) and approximately 7000 DPM's of [^{3}H]-cyclic GMP. All columns were then eluted with 20 ml 10 mM HCl. This fraction, containing cyclic AMP and IBMX (which interfered with the assay), was discarded. A second elution with 7 ml 50 mM HCl contained the cyclic GMP and was taken to dryness under reduced pressure in the Vortex-Evaporator. The residues were dissolved in 50 mM acetate buffer, pH 6.2, and succinylated according to the procedure of Frandsen and Krishna (246). After dilution to 500 ul with 50 mM acetate buffer pH 6.2, two 25 ul portions were assayed for cyclic GMP in a total volume of 300 ul by radioimmunoassay as described by Steiner et al. (214).

Since EGTA interfered with the radioimmunoassay and eluted from the Sephadex QAE columns with cyclic GMP, samples from experiments in which EGTA was used required additional purification. This was achieved using a modification of the procedure described by Asakawa et al. (215). The ether-extracted, dried samples were dissolved in water and placed on 0.7 cm columns containing 1 g neutral alumina (Sigma, WN-3), which had been washed with 5 ml 50 mM $(NH_4)_2CO_3$, pH 7.5. Elution was with 3 ml 0.2 M $(NH_4)_2CO_3$, pH 7.5. This fraction was collected, evaporated, and then further purified on Sephadex QAE as

described above. Other columns received known amounts of cyclic GMP and [^{3}H]-cyclic GMP and were treated identically.

Calculation of cyclic GMP results

Samples and standards were corrected for losses during purification by measuring the amount of [^{3}H]-cyclic GMP recovered. Results from the cyclic GMP radioimmunoassay were converted to logit % trace binding form (248). A standard curve was constructed by plotting the log of the amount of cyclic GMP standard placed on the columns, adjusted for recovery, versus the corresponding logit % trace binding values. Linear regression analysis of standard curves gave correlation coefficients in excess of 0.99. The amount of cyclic GMP in the experimental samples was calculated from the slope and intercept of the standard curve, adjusted for recovery, and divided by the protein content.

Serial dilutions of purified samples and standards containing equivalent amounts of cyclic GMP were assayed and the results subjected to Hill analysis. This resulted in two essentially parallel lines with Hill coefficients very close to one indicating that samples from this tissue purified by the above procedure do not contain significant amounts of substances interfering with the radioimmunoassay. Treatment of samples prior to assay with phosphodiesterase, calmodulin (supplied by Dr. J.H. Wang, Dept. of Biochemistry) and Ca^{2+} resulted in the destruction of at least 90 % of the immunoreactive material.

Protein determination

Protein determinations throughout this study were by the procedure of Lowry *et al* (54) using bovine serum albumin as standard.

RESULTS

Protease secretion from rat submandibular slices

Riekkinen et al. established that purified glandulain and salivain could degrade a number of synthetic substrates, among them α-N-benzoyl-L-arginine-p-nitroanilide (BAPA), BANA and BAEE (43,47), the last of which is commonly used to assay kallikrein (24,41,51). Salivain and glandulain were found to differ in respect to their pH optima and in the effect of a number of substances on their activity. The activity of purified glandulain was completely inhibited by 0.02 mM Cu^{2+} with 0.5 mM BAPA as substrate in tris-HCl, pH 8.0 (47). Purified salivain was shown to display increased activity in the presence of Ca^{2+} but 0.2 mM Cu^{2+} had no effect on its activity. A 40 % inhibition was noted when the level of Cu^{2+} was 2mM (43). Because of the small difference in the pH at which the enzymes used in the present study are assayed, and because of the partial overlap in substrate specificity of the proteases reported by Riekkinen, it seemed important to examine the characteristics of the assayed activities and to show that 3 different enzymes are in fact being measured.

BANA hydrolysis by rat submandibular gland homogenates was stimulated by the presence of Ca^{2+} at both pH 8.2 and 9.5 (Table I). EGTA abolished hydrolysis at pH 8.2 but had little effect at pH 9.5. Addition of excess Ca^{2+} did not restore the activity at pH 8.2. Kallikrein appeared little affected by EGTA and Ca^{2+}. Cu^{2+}, at concentrations up to 0.5 mM, had little influence on BANA hydrolysis

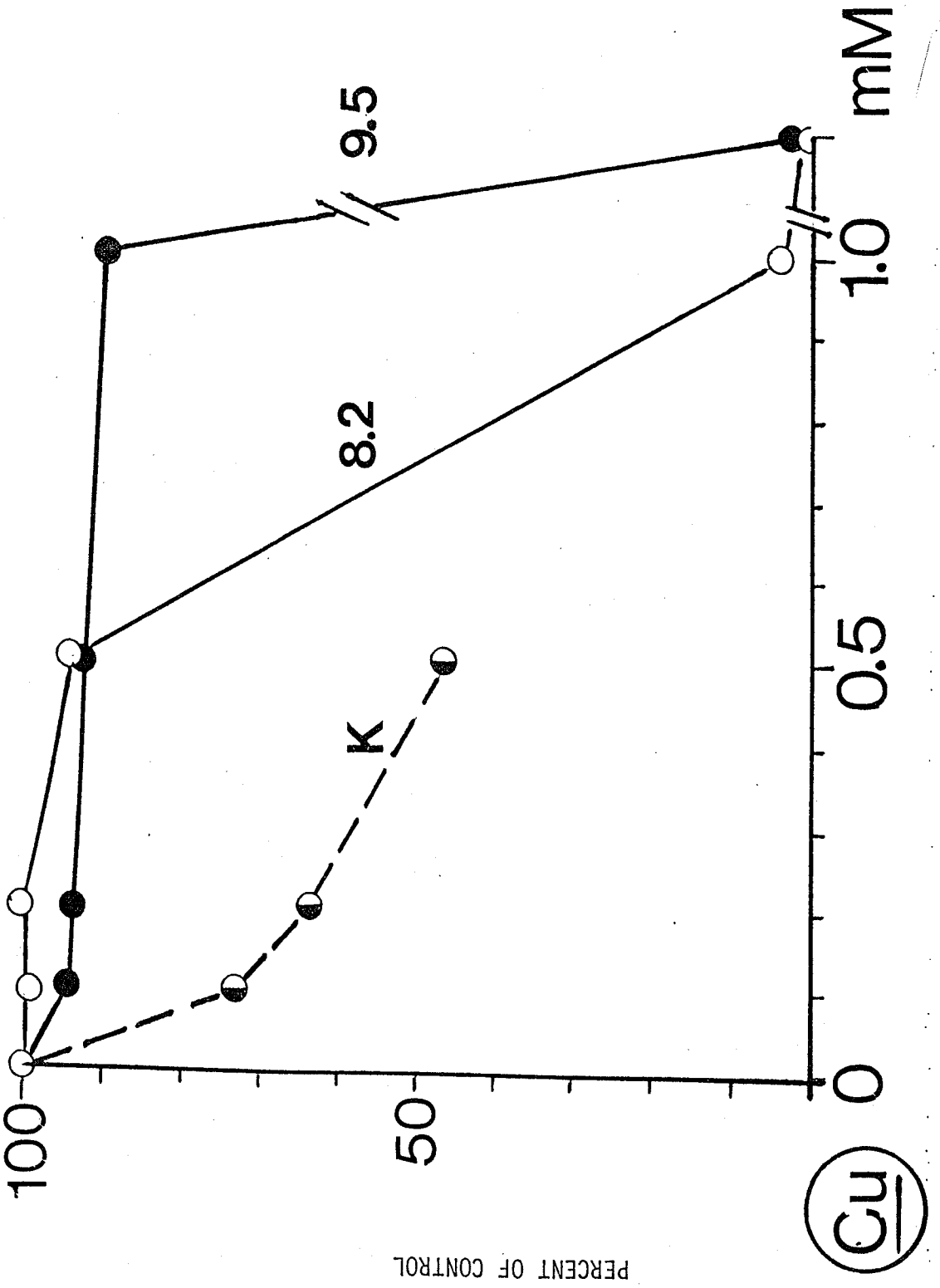
TABLE I. FACTORS INFLUENCING PROTEASE ACTIVITY OF RAT SUBMANDIBULAR GLAND HOMOGENATES

Suitable equal portions of filtered aqueous homogenate was added to tubes containing the appropriate buffer maintained in an ice-bath. and containing the additions shown. After 30 min the tubes for the assay of pH 8.2 and 9.5 proteases were transferred to a water bath at 37°, substrate added, and the incubation continued 30 min. Kallikrein activity was measured at 30° by the procedure noted in methods. All concentrations are final. The results are expressed as [Specific activity of test / specific activity of control] x 100.

Additions	Relative Activity		
	Kallikrein	pH 8.2 Protease	pH 9.5 Protease
Substrate (mM)	BAEE (1.0)	BANA (0.25)	
Nil	100	100	100
EGTA (4 mM)	103	2	101
$CaCl_2$ (20 mM)	93	126	144
$CaCl_2$ (20 mM) + EGTA (4 mM)	87	2	139
$CuCl_2$ (0.1 mM)	73	99	94
(0.2 mM)	64	102	94
(0.5 mM)	46	93	92
(1.0 mM)	-	4	90
(10.0 mM)	-	0	5
Aprotinin (50 units)	21	4	6
Soybean trypsin inhibitor (50 µg)	87	72	64
(250 µg)	66	57	52
Phenylmethylsulfonyl-flouride (50 µg)	84	92	95
(100 µg)	66	26	100

Addendum to Table I. Plot of enzyme activities in the presence of various concentrations of copper chloride.

K - kallikrein
8.2 - pH 8.2 protease
9.5 - pH 9.5 protease



at either pH. In contrast, Riekkinen found purified glandulain to be completely inhibited by 0.02 mM Cu^{2+} (47). However, increasing the Cu^{2+} level to 1 mM caused complete inhibition of the pH 8.2 activity. Kallikrein was more sensitive to Cu^{2+} inhibition than the other two proteases. However, it appeared that all three activities could be completely inhibited by Cu^{2+} if high enough concentrations were used. The concentration of Cu^{2+} that could be tested in the kallikrein assay was limited by the blue color of the $CuCl_2$ interfering with the spectrophotometric determination of BAEE hydrolysis. The activity of all three proteases was decreased by the tryptic-inhibitors aprotinin and soybean trypsin inhibitor (Table I). Aprotinin was much more effective than soybean trypsin inhibitor. Phenylmethylsulfonylfluoride, another protease inhibitor (55), inhibited the activity of kallikrein and the pH 8.2 protease but was without effect on the pH 9.5 activity. Although the activities measured in this study do not appear to have exactly the same characteristics as those reported by Riekkinen for purified salivain and glandulain, the differing effects of EGTA, phenylmethylsulfonylfluoride and 1 mM Cu^{2+} on the three activities strongly suggest that these activities do represent different enzymes.

A comparison of the specific activities of the proteases between male and female 3 month old littermates is shown in Table II. Males had both a higher total activity per gland and a higher specific activity of all three proteases.

Fig. 1 describes the effect of different secretagogue concentrations on the release of protease from rat submandibular

TABLE II. DIFFERENCE IN PROTEASE ACTIVITY BETWEEN SEXES

Three male and three female rats from the same litter were used. Each gland was homogenized individually in 2 ml water resulting in 6 samples for each sex. Assays were done as described in the METHODS using 5 µl directly and 5 µl diluted 1:100 for BANA and BAEE hydrolysis respectively. Each value represents the mean ± S.E. from 6 glands. The difference in enzyme activity between sexes for all 3 enzymes was significant at $P < 0.01$.

Sex	Gland Weight (mg)	Enzyme Activity / mg Protein		
		Kallikrein (BAEE)	pH 8.2 Protease (BANA)	pH 9.5 Protease (BANA)
Male	244.9 ± 11.4	55.4 ± 5.5	10.0 ± 1.0	14.0 ± 1.2
Female	159.1 ± 3.4	24.9 ± 1.2	4.4 ± 0.2	7.6 ± 0.4
Male / female	1.6	2.2	2.3	2.2

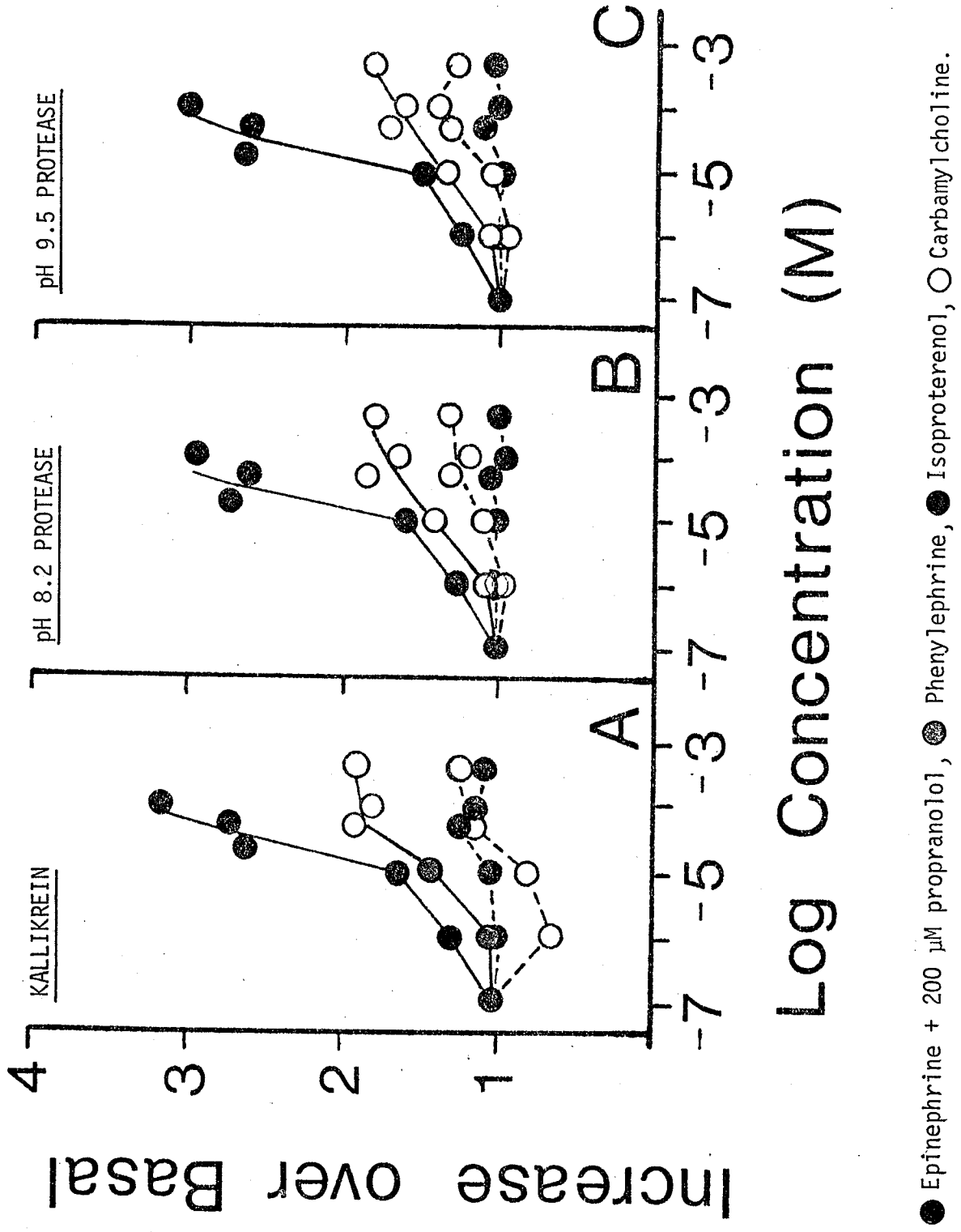


FIGURE I. EFFECT OF SECRETAGOGUE CONCENTRATION ON PROTEASE SECRETION

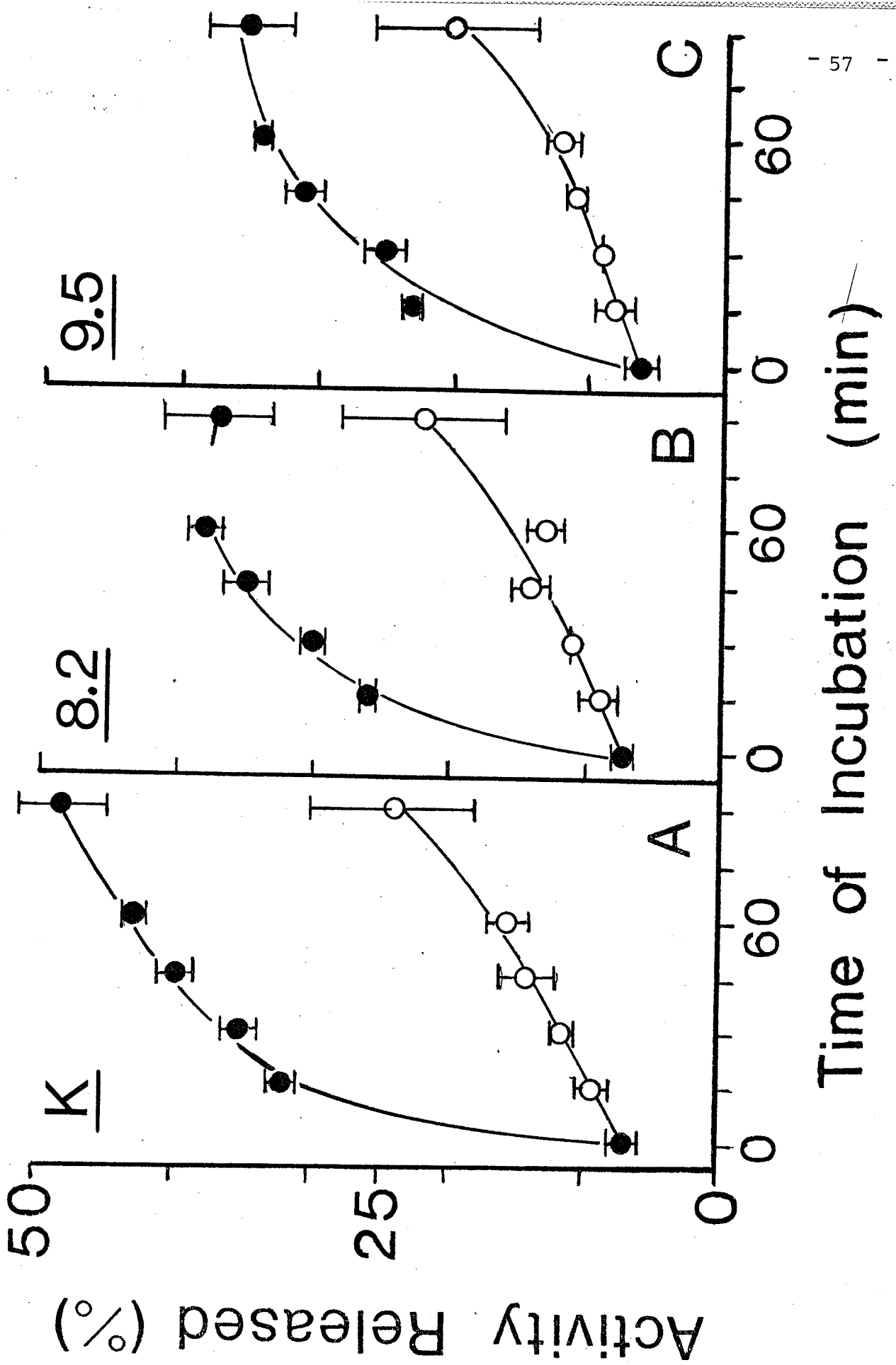


FIGURE 2. CHANGE IN PROTEASE SECRETION RATE DURING INCUBATION

LEGENDS TO FIGURES 1 and 2.

Fig. 1. Effect of secretagogue concentration on protease secretion. Incubations were as noted in text. Slices designated for L-epinephrine treatment had 100 μM propranolol in the buffer 15 min prior to the addition of L-epinephrine. All final incubations were for 30 min with the concentration of secretagogue as noted: epinephrine (●——●), phenylephrine (○——○), isoproterenol (●- - -●) and carbamylcholine (○- - -○). Results, which indicate the activity in the medium only, are expressed as increase over basal activity (no secretagogue) thusly: increase over basal = (test activity / basal activity). Basal and stimulated activities were indistinguishable at 0.1 μM secretagogues. A = kallikrein, B = pH 8.2 protease, C = pH 9.5 protease.

Fig. 2 Change in protease secretion rate during incubation. Each point represents the mean ± S.E. of three individual samples incubated for the times shown after the addition of L-epinephrine (●) or untreated controls (○). Propranolol, 200 μM, was present in buffer 15 min prior to the addition of epinephrine. A, B and C as in Fig. 1.

slices. Phenylephrine and epinephrine (+ propranolol) were used as α-adrenergic agonists with isoproterenol as β-adrenergic and carbamylcholine as muscarinic cholinergic agonists. Epinephrine gave the greatest extent of secretion of all three proteases. Phenylephrine also produced a significant increase in the rate of enzyme secretion. Release was found to be maximal when the concentration of these agonists was about 50-100 uM. Neither isoproterenol nor carbamylcholine caused significant secretion of any of the three proteases. An examination of Fig. 2 shows that the most rapid release of protease occurs within 15 min. of the addition of epinephrine.

Table III presents the results of experiments performed to confirm α-adrenergic stimuli as the main control of protease secretion. As well as the agonists previously noted, α- and β-adrenergic antagonists, phentolamine and propranolol, as well as the cholinergic antagonist, atropine, were employed. It can be seen that only α-adrenergic stimuli, as represented by phenylephrine and epinephrine, gave significant protease release. The polypeptides, eledoisin and physalaemin, which are as effective as phenylephrine in inducing K^+ release (see following section), had no influence on protease secretion.

The secretion of kallikrein and pH 9.5 protease induced by phenylephrine was found to require extracellular Ca^{2+} (Table IV). However, introduction of Ca^{2+} into the cells by means of the ionophore A23187 (Table V) increased protease secretion only slightly; adding epinephrine to these slices resulted in an additional large secretory response. However, epinephrine induced

TABLE III. INFLUENCE OF ADRENERGIC AND CHOLINERGIC AGONISTS AND ANTAGONISTS ON PROTEASE SECRETION

Incubations were as described in text with antagonists (phentolamine, propranolol & atropine) being present in the buffer 15 min prior to the addition of agonists (epinephrine, phenylephrine, isoproterenol & carbamylcholine) after which the incubations continued an additional 30 min. Values underlined were signicantly different from controls at $P < 0.01$. Results are mean ± S.E.

Additions	Percent of Total Activity Secreted (Mean ± S.E.)					
	Kallikrein	(n)	pH 9.5 Protease	(n)	pH 8.2 Protease	(n)
Nil	19.9 ± 1.5	(15)	16.9 ± 1.3	(15)	19.3 ± 2.7	(6)
PHENYLEPHRINE, 50 µM	41.5 ± 1.6	(15)	33.2 ± 1.8	(15)	40.1 ± 1.4	(6)
ISOPROTERENOL, 100 µM	25.9 ± 2.5	(9)	21.1 ± 2.3	(9)	25.1 ± 2.9	(6)
CARBAMYLCHOLINE, 500 µM	21.1 ± 2.4	(6)	16.9 ± 2.1	(6)	21.3 ± 3.5	(3)
PHENYLEPHRINE, 50 µM + PHENTOLAMINE, 100 µM	29.1 ± 5.4	(3)	24.4 ± 4.9	(3)	27.2 ± 5.6	(3)
CARBAMYLCHOLINE, 500 µM + ATROPINE, 1.0 mM	24.4 ± 5.5	(3)	21.5 ± 4.4	(3)	22.8 ± 4.6	(3)
EPINEPHRINE, 50 µM	62.1 ± 2.9	(6)	48.3 ± 2.6	(6)	51.2 ± 2.3	(6)
EPINEPHRINE, 50 µM + PHENTOLAMINE, 200 µM	23.1 ± 1.0	(3)	19.9 ± 0.9	(3)	21.8 ± 0.8	(3)
EPINEPHRINE, 50 µM + PROPRANOLOL, 200 µM	48.4 ± 0.9	(3)	39.2 ± 0.8	(3)	42.7 ± 1.7	(3)
PHENYLEPHRINE, 50 µM + ISOPROTERENOL, 100 µM	53.9 ± 2.1	(3)	43.5 ± 2.0	(3)	46.6 ± 1.2	(3)
ELEDOISIN, 1 µM	22.0 ± 2.7	(3)	16.3 ± 2.7	(3)		
PHYSALAEMIN, 1 µM	24.5 ± 3.2	(3)	19.5 ± 2.0	(3)		
SUBSTANCE P, 1 µM	25.2 ± 3.0	(3)	19.4 ± 3.1	(3)		

TABLE IV. EFFECT OF CALCIUM AND EGTA ON PROTEASE SECRETION

Slices were incubated 10 min in regular buffer, then after aspiration, 1 ml of fresh buffer was added which had either 4 mM EGTA (no calcium) or 2.5 mM calcium chloride as shown. The incubation was continued a further 15 min at which time the secretagogues were added. Final incubation was 30 min.

			Percent of Total Activity Secreted (Mean ± S.E., n = 3)	
Additions	2.5 mM Ca^{2+}	4 mM EGTA	Kallikrein	pH 9.5 Protease
Nil	+	-	18.5 ± 1.9	13.7 ± 0.7
Nil	-	+	18.0 ± 1.9	12.5 ± 1.2
PHENYLEPHRINE, 50 µM	+	-	36.2 ± 2.8 *	27.6 ± 2.3 *
PHENYLEPHRINE, 50 µM	-	+	23.2 ± 4.1	16.3 ± 2.1
CARBAMYLCHOLINE, 500 µM	+	-	18.9 ± 1.1	14.6 ± 0.4
CARBAMYLCHOLINE, 500 µM	-	+	20.1 ± 0.5	15.2 ± 0.9
ISOPROTERENOL, 100 µM	+	-	22.3 ± 1.8	17.0 ± 2.0
ISOPROTERENOL, 100 µM	-	+	18.2 ± 0.9	14.3 ± 0.4

(*) - significantly different from their controls at $P < 0.01$

TABLE V. EFFECT OF IONOPHORE A23187 ON PROTEASE SECRETION

Tissue was prepared Krebs-Ringer bicarbonate buffer, pH 7.4, for A, but the B series contained 0.2 mM EGTA and no calcium (until noted). After an initial 10 min incubation the buffer was discarded and replaced with 1 ml fresh buffer of identical composition. At this time 10 µl methanolic A23187 (10 µg/ml) was added to the B series of samples. After a further 15 min, calcium and epinephrine were added as shown. Final incubation was for 30 min. Results are mean ± S.E. with n as shown.

Additions	Percent of Total Activity Secreted	
	Kallikrein (n)	pH 9.5 Protease (n)
(A) No ionophore		
Control-1 (none)	19.5 ± 1.6 (4)	
Methanol, (10 µl)	20.7 ± 2.5 (4)	
Epinephrine (50 µM)	* 52.8 ± 1.7 (4)	
(B) IONOPHORE A23187		
Control-2 (none)	22.5 ± 1.6 (10)	14.0 ± 1.3 (6)
Ca^{2+} (2.5 mM)	26.2 ± 1.2 (10)	18.4 ± 1.2 (6)
Ca^{2+} (2.5 mM) + Epinephrine (50 µM)	* 39.9 ± 1.4 (4)	

* Indicates significant difference ($P < 0.01$) between values and controls and between each other.

significantly less secretion when added with Ca^{2+} to cells preincubated with the ionophore than it did when added to slices not exposed to the ionophore. The pH 8.2 protease was not measured in these experiments as it is strongly inhibited by EGTA.

K^+ release from rat submandibular slices

In their description of a protocol for the study of K^+ release from rat parotid gland slices, Schramm and Selinger (6) recommended the use of a Krebs-Ringer bicarbonate medium with the K^+ level reduced from 6 to 4 mM. A comparison between the two media was made with submandibular gland slices (Table VI). Carbamylcholine caused the slices to release a similar percentage of their K^+ content regardless of the media. However, the increase in the K^+ concentration of the media was more pronounced in the 4 mM K^+ media and all subsequent studies were done in this medium.

An examination of the rapidity of K^+ release induced by carbamylcholine and epinephrine showed that the slices responded quite quickly. Substantial release was evident at 1 min. and the maximum K^+ concentration was achieved within 5 min. (Fig. 3). A 5 min. incubation period was therefore used for the remainder of the study.

α-adrenergic, β-adrenergic, and muscarinic cholinergic receptors were stimulated separately in order to find the ones associated with the release of K^+ in submandibular gland. Table VII shows that phenylephrine, which specifically stimulates α-adrenergic receptors, induced K^+ release, as did the cholinergic drug carbamylcholine. The

TABLE VI. INFLUENCE OF K^+ LEVELS IN INCUBATION MEDIUM ON K^+- RELEASE

Carbamylcholine added	Percent of tissue K^+ Released		Percent increase in K^+ content of medium	
mM K^+ in medium	4	6	4	6
None	-0.9	3.0	-1.8	5.0
100 µM	25.0 ± 0.3	21.6 ± 2.8	46.8 ± 2.1	30.8 ± 5.5

Tissue samples were incubated in Krebs-Ringer bicarbonate containing 5 mM β-hydroxybutyrate and having either 4 or 6 mM K^+. Both media contained phentolamine and propranolol at 200 µM. Incubation was for 5 min. at 37°. Results are mean ± S.E. where N = 4.

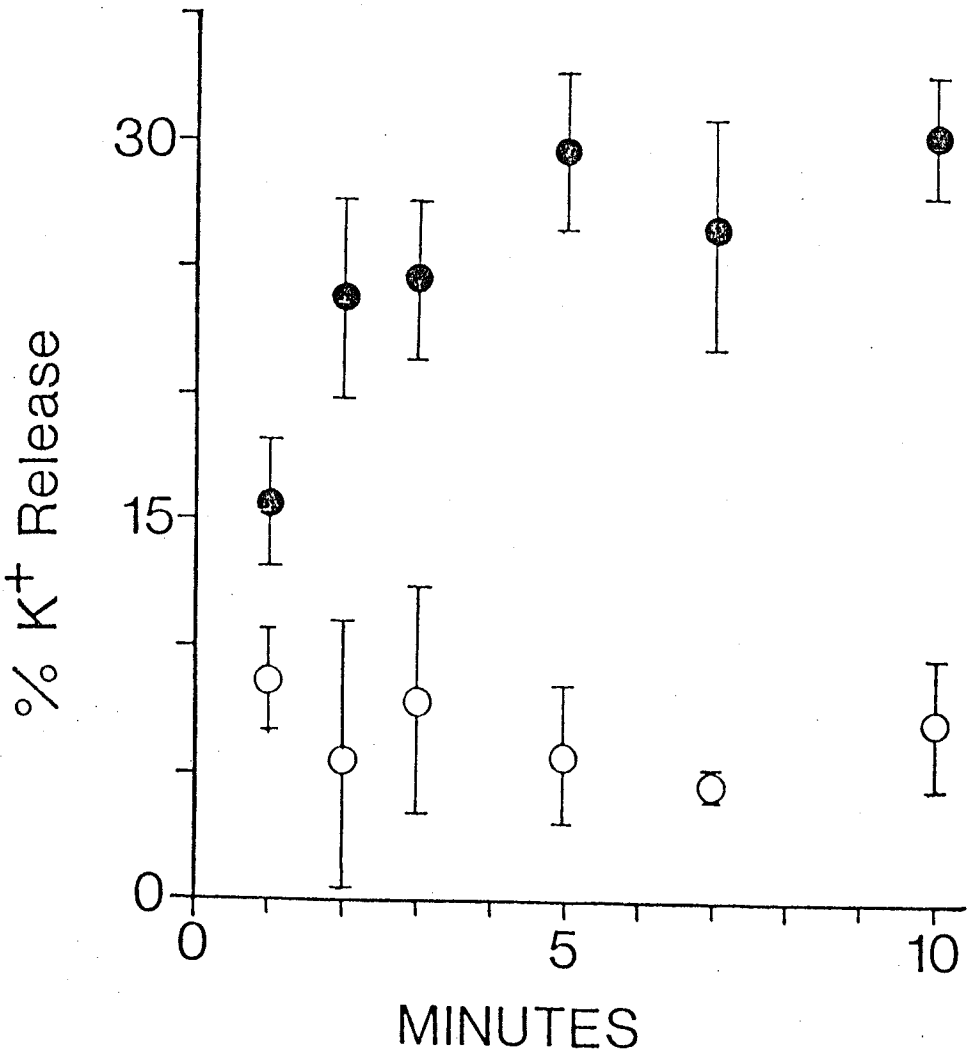


FIGURE 3 Comparison of the effect of incubation time in the presence of carbamylcholine on K^+ release. Buffer contained phentolamine and propranolol at 200 μM during preincubation and incubation periods. Carbamylcholine, when present (●), was added only to the final incubation at 100 μM. Samples were withdrawn for analysis at the times shown. Bars indicate S.E. (n = 6).

TABLE VII. EFFECT OF α-, β- AND CHOLINERGIC STIMULATION ON K^+ RELEASE

Stimulant added	Stimulant conc. (μM)	Antagonist present	Percent tissue K^+ released
None	-	all	4.3 ± 1.8
carbamylcholine	100	all	0.8 ± 1.2
carbamylcholine	100	-atropine	19.7 ± 2.4
phenylephrine	100	all	0.9 ± 0.5
phenylephrine	100	-phentolamine	7.0 ± 0.7
epinephrine	50	all	3.9 ± 1.6
epinephrine	50	-phentolamine	19.7 ± 1.6
isoproterenol	100	-propranolol	4.2 ± 1.8

All three antagonists, phentolamine, propranolol and atropine at 200 μM, were present unless otherwise indicated. Incubations were in Krebs-Ringer bicarbonate containing 4 mM K^+. Antagonists were present in the medium during the 5 min. preincubation and 5 min. incubation periods. Stimulants were added at the beginning of the final incubation. Results are mean ± S.E. where N = 5.

effect of L-phenylephrine and epinephrine was abolished by a 15 min preincubation with phentolamine, an α-adrenergic antagonist. Similarly, the effect of carbamylcholine was inhibited by pretreatment of the slices with atropine, a muscarinic cholinergic antagonist. A β-adrenergic agonist, isoproterenol, was not able to effect increased K^+ release and the β-adrenergic antagonist, propranolol, was unable to prevent K^+ release induced by epinephrine, phenylephrine, or carbamylcholine. Batzri et al. (8) reported that epinephrine was more effective than phenylephrine in causing K^+ release from rat parotid gland tissue. Rat submandibular gland tissue is also more responsive to epinephrine (Table VII). In subsequent experiments, when α-adrenergic stimulation was needed, epinephrine in the presence of propranolol and atropine was used.

The effect of increasing concentrations of carbamylcholine and epinephrine on K^+ release is shown in Figs. 4 and 5. Each diagram shows three separate trials to indicate the variation in response by different tissue preparations. The apparent Km for carbamylcholine and epinephrine was approximately 8 uM and 60 uM respectively. These values varied from trial to trial and should be considered in a comparative manner only and not as absolute values. In comparison, Batzri et al. (8) reported 15 uM epinephrine to produce half maximal K^+ release in rat parotid slices.

The effect of ouabain, an inhibitor of (Na^+/K^+)-ATPase, on K^+ release is reported in Table VIII. In both stimulated and unstimulated samples the amount of K^+ lost from the tissues was increased when incubated in the presence of ouabain. It is seen that the effects of ouabain and the neurotransmitters are additive and

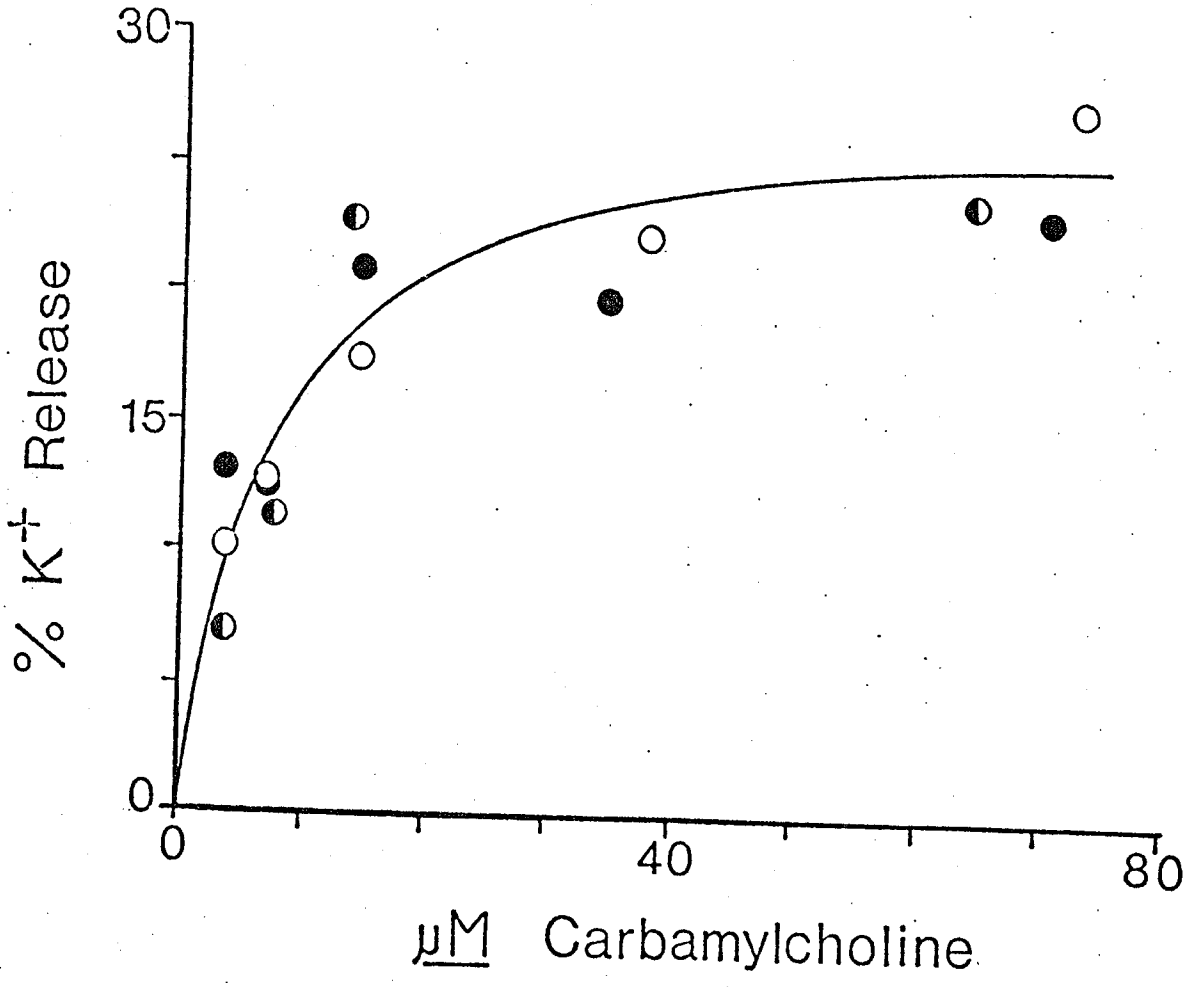


FIGURE 4. Effect of increasing carbamylcholine concentration on K^+ release. The figure shows the results of three trials each represented by a different symbol. Phentolamine and propranolol, at 200 µM, were present during both preincubation (5 min) and incubation (5 min) periods. Carbamylcholine, at the dosages shown, was added at the start of the final incubation. Each point represents 3 samples.

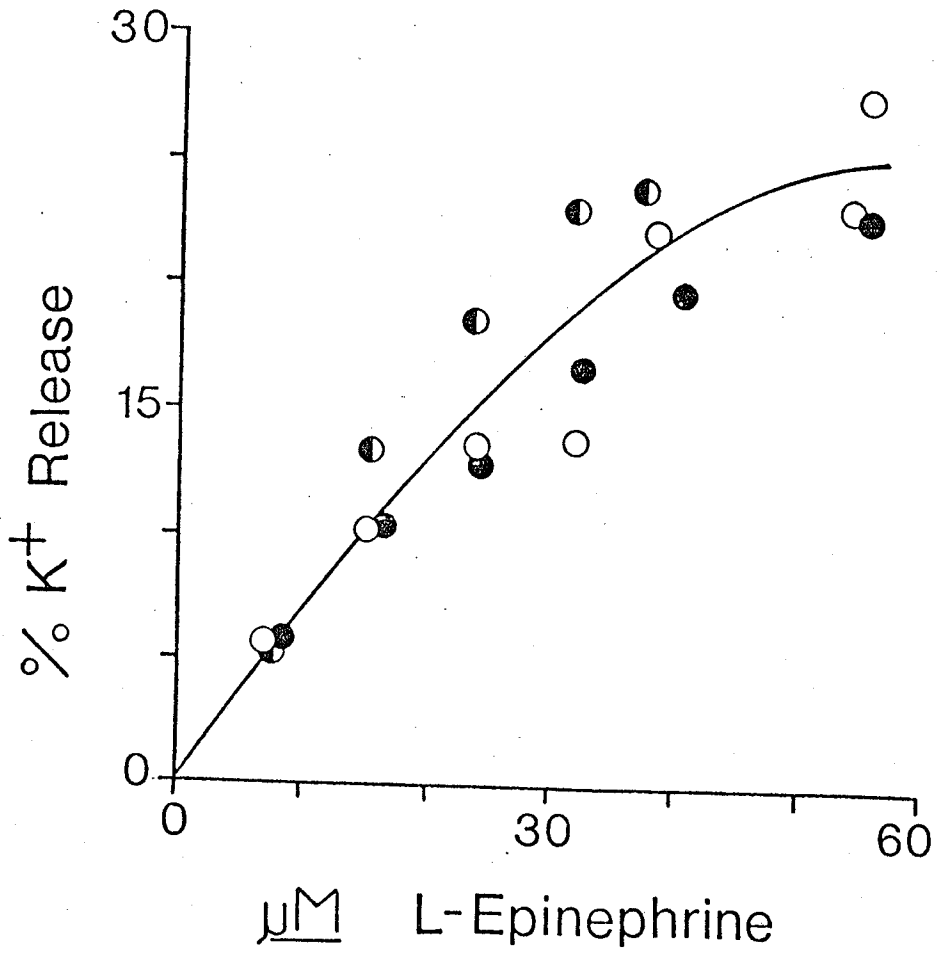


FIGURE 5. Effect of increasing L-epinephrine concentration on K^+ release. Conditions were the same as for Fig. 3. except that the stimulant was L-epinephrine and the antagonists were propranolol and atropine at 200 µM. The figure shows the results of three trials, each represented by a different symbol.

TABLE VIII. EFFECT OF OUABAIN ON K^+- RELEASE

Stimulant added	Antagonist present	Percent tissue K^+ released	
		Without ouabain	With ouabain
None	all	2.8 ± 1.7	14.6 ± 0.3
Carbamylcholine (100 uM)	-atropine	16.3 ± 0.7	37.1 ± 5.3
Epinephrine (50 uM)	-phentolamine	19.5 ± 0.2	40.6 ± 3.7

Conditions were as noted in the legend of Table VII except that 1 mM ouabain was present in the medium during both preincubation and incubation periods. Results are mean ± S.E. where n = 4.

indicate that neither epinephrine nor carbamylcholine induce K^+ release by a mechanism involving the inhibition of the ouabain-sensitive (Na^+/K^+)-ATPase.

Neurotransmitter-induced K^+ release from rat parotid gland slices has been shown to require the presence of extracellular Ca^{2+} (9). The present study indicates that this is also valid for submandibular gland (Table IX). It has also been demonstrated (10) that Ca^{2+} additions to the media could induce K^+ release in rat parotid slices provided that the tissue was preincubated in the absence of this ion with A23187, an ionophore specific for divalent ions. Our results with submandibular gland slices, pretreated with 10 ug/ml A23187, indicate that K^+ release was 5 times greater in the presence of 2.5 mM Ca^{2+} than in its absence (Table IX-A).

Other investigators (11,12) have shown that two polypeptides, eledoisin, found in the salivary glands of two molluscan species, Eledone moschata and Eledone aldrovandi, and physalaemin, found in the skin of a South American toad, Physalaemus fuscumaculatus, were able to evoke in vivo salivary secretion which was unaffected by neurotransmitter blocking agents. The ability of these two peptides to evoke K^+ release from submandibular gland slices is shown in Table X. It is seen that low concentrations of both agents caused substantial K^+ release even when all adrenergic and cholinergic receptors were blocked. Examination of the possible requirement for Ca^{2+} of these two peptides (Table X) indicated that physalaemin required Ca^{2+} but, surprisingly, the action of the eledoisin-related peptide did not.

Employing cat submandibular glands perfused in vivo, Peterson

TABLE IX. DEPENDENCE OF K^+- RELEASE ON THE PRESENCE OF CALCIUM

Stimulant added	Antagonists present	Percent tissue K^+ released	
		With Ca^{2+}	without Ca^{2+}
None	all	4.5 ± 0.9	3.5 ± 1.4
Epinephrine (50 uM)	-phentolamine	19.7 ± 1.6	2.8 ± 0.6
carbamylcholine (100 uM)	-atropine	16.3 ± 0.7	0.4 ± 1.9

Conditions were as noted in the legend of Table VII except that one medium contained 5 mM EGTA in place of Ca^{2+} during both preincubation and incubation periods. Results are mean ± S.E. where n = 5.

TABLE IX-A. EFFECT OF CALCIUM ON K^+ RELEASE IN THE PRESENCE OF THE IONOPHORE A23187

Additions	Percent tissue K^+ released
None	3.8 ± 0.8 (3)
2.5 mM Ca^{++}	18.8 ± 1.5 (6)

Tissue was prepared and preincubated in low K^+ Krebs-Ringer bicarbonate without Ca^{2+} but with 0.2 mM EGTA. After a 10 min. incubation this medium was exchanged for a similar fresh medium containing 10 ug A23187. After 15 min. Ca^{2+} was added as shown. All samples contained phentolamine, propranolol and atropine at 200 µM.
Results are mean ± S.E.

TABLE X. EFFECT OF PHYSALAEMIN AND ELEDOISIN-RELATED PEPTIDE ON K^+ RELEASE

Physalaemin (μM)	Percent tissue K^+ released	
	With Ca^{2+}	Without Ca^{2+}
0	1.3 ± 1.0 (9)	3.5 ± 1.4
0.001	-2.3 ± 1.1	-
0.01	1.3 ± 2.6	-
0.1	20.0 ± 1.2	-
1.0	23.7 ± 2.3 (6)	4.3 ± 2.5
Eledoisin-related Peptide (μM)		
0	4.8 ± 1.6 (9)	3.5 ± 1.4
0.01	2.4 ± 0.3	-
0.1	7.3 ± 0.4	-
1.0	21.7 ± 1.0 (6)	20.1 ± 0.3
10.0	24.0 ± 0.9	-

Phentolamine, propranolol and atropine (all 200 μM) were present in all samples. Polypeptides were added at the beginning of the final 5 min. incubation. Ca^{2+}-free medium (+ EGTA) was as in Table IX. Results are mean ± S.E. where n = 4, except as shown in brackets.

(13) demonstrated that ethacrynic acid inhibited acetylcholine induced salivary secretion. The effect of ethacrynic acid on K^+ release from submandibular gland slices was tested with a concentration of 0.5 mM ethacrynic acid. This level was 5 times greater than Peterson found to be severely inhibitory in vivo. At this level it was found that there was no significant reduction in K^+ secretion evoked by either epinephrine or carbamylcholine (Table XI).

The results of an experiment performed to test the effect of IBMX on K^+ release induced by submaximal epinephrine and carbamylcholine concentrations are shown in Table XII. In the presence of the phosphodiesterase inhibitor at a concentration of 100 uM there was no significant potentiation. However, when the level of inhibitor was raised to 500 uM an increase in K^+ release was observed with both epinephrine and carbamylcholine (Table XII). The increase with carbamylcholine was significant at the 5% level, that with epinephrine at the 10% level.

Mangos (15) reported that 0.01 to 100 uM 8-bromo-cyclic GMP caused a release of K^+ in a preparation of isolated rat parotid cells. With submandibular gland slice preparations no effect could be observed with similar concentrations of this agent either in the presence or absence of a phosphodiesterase inhibitor (data not shown).

This has been a very active area of research and since the completion and publication of the results described in this section (56) a number of related publications have appeared. Martinez and coworkers (22,65,224) also have studied K^+ release from rat submandibular slices and obtained results generally confirming the

TABLE XI. EFFECT OF ETHACRYNIC ACID ON K^+ RELEASE

Stimulant added	Antagonists present	Percent tissue K^+ released	
		ethacrynic acid	
		None	Present
None	all	2.2 ± 2.1	4.8 ± 2.2
epinephrine (50 μM)	-phentolamine	18.9 ± 4.2	17.4 ± 2.0
carbamylcholine (100 μM)	-atropine	16.3 ± 0.7	14.3 ± 2.6

Conditions were similar to those described in the legend to Table VII except that ethacrynic acid (0.5 mM) was added at the same time as the antagonists. Results are mean ± S.E. where n = 6.

TABLE XII. EFFECT OF THE PHOSPHODIESTERASE INHIBITOR, 3-isobutyl-1-methylxanthine, ON K^+ RELEASE INDUCED BY SUBOPTIMAL CONCENTRATIONS OF EPINEPHRINE AND CARBAMYLCHOLINE

Stimulus	Antagonists present (200 μM)			Inhibitor conc. (μM)	Percent tissue K^+ released
	Propranolol	Phentolamine	Atropine		
none	+	+	+	0	0.4 ± 0.1
none	+	+	+	100	2.0 ± 1.1
30 μM epinephrine	+	-	+	0	10.9 ± 0.6
30 μM epinephrine	+	-	+	100	9.1 ± 0.2
10 μM carbamylcholine	+	+	-	0	4.9 ± 0.0
10 μM carbamylcholine	+	+	-	100	5.6 ± 0.4
none	+	+	+	0	2.4 ± 1.0
none	+	+	+	500	2.9 ± 1.3
30 μM epinephrine	+	-	+	0	11.2 ± 2.4
30 μM epinephrine	+	-	+	500	16.6 ± 1.4*
10 μM carbamylcholine	+	+	-	0	2.7 ± 1.2
10 μM carbamylcholine	+	+	-	500	6.7 ± 1.4**

The phosphodiesterase inhibitor and antagonists were present during both preincubation and incubation periods as noted in Table VII. Results are mean ± S.E. where n = 3.

results of the present study. K^+ release was found to be mediated by cholinergic (65) and α-adrenergic (22) stimulation but was not affected by β-adrenergic agonists (22). Release was found to be dependent on extracellular Ca^{2+} (22,65,224) as in the present study. Slices were found to take up secreted K^+ upon the termination of stimulation and this reuptake could be blocked by ouabain (22,65).

Butcher and coworkers confirmed that K^+ release from rat parotid slices is primarily mediated through the cholinergic (66) and α-adrenergic (67) receptor but also reported a small K^+ release to result from specific stimulation of the β-adrenergic receptor (67). K^+ release induced by α-adrenergic or cholinergic stimulation was found to be inhibited by D-600 and tetracaine, agents which block transmembrane Ca^{2+} fluxes (197). In addition these workers confirmed the ability of ionophore A23187-induced Ca^{2+} influx to induce K^+ release in rat parotid slices (197) and found Substance P and eledoisin effective in producing K^+ efflux (192). In contrast to results with submandibular slices reported here (Table X), eledoisin was reported to require extracellular Ca^{2+} to effect K^+ release (192). However, the action of Substance P was found to be uninhibited by D-600 or tetracaine (197). The effect of these substances on eledoisin-induced K^+ release was not tested. These workers also report 2 mM 8-bromo-cyclic GMP to induce K^+ release in parotid slices (67). These are higher concentrations than the ones reported not to induce K^+ release in the present study. These workers report that IBMX did not potentiate the K^+ released by suboptimal phenylephrine (67) or carbamylcholine (66) concentrations in parotid slices, in contrast with this study in submandibular slices, where potentiation

was observed.

Mangos et al. (184,210,225) demonstrated that a dissociated acinar cell preparation from rat parotid glands released K^+ upon α-adrenergic (184) or cholinergic (225) stimulation. IBMX was reported to potentiate the carbamylcholine induced K^+ release in this preparation (210). The kinetics of the release were found to differ from those reported in slice systems (184,225). The K^+ release was found to be very fast; the maximum K^+ concentration of the media is achieved at approximately 30 sec. after stimulation and these levels then fall in spite of the continued presence of secretagogue. By 5 min., the K^+ concentration of the media is almost back to its original level. Subsequent stimulation of the same receptor produces no further K^+ release (225) (the tissue is said to be desensitized).

Lefkowitz and coworkers (211,226) studied the binding by rat parotid dissociated cells and isolated parotid plasma membranes of [^{3}H]-dihydroergocryptine, an α-adrenergic antagonist used to study the binding properties of the α-adrenergic receptor. The ability of agonists to displace this substance from the α-adrenergic receptor was correlated with their ability to produce K^+ release (211). By this method it was found that the desensitization produced by prior stimulation of the receptor is accompanied by a reduction in [^{3}H]-dihydroergocryptine binding (226) and that the desensitization is a result of agonist binding to the receptor rather that the resultant K^+ release. Pretreatment with α-adrenergic agonists did not prevent K^+ on subsequent cholinergic stimulation (226) and desensitization to α-adrenergic stimulation was still observed if the pretreatment with α-adrenergic agonist was performed in the absence

of Ca^{2+} (226), even though α-adrenergic agonists did not cause K^+ release in these circumstances (226).

Putney preloaded rat parotid slices with [^{42}K] or [^{86}Rb] and studied the kinetics of the washout of the label (227,229,230). This washout was found to be accelerated by α-adrenergic and cholinergic agonists and the ionophore A23187 but not by β-adrenergic agonists (227). Analysis of neurotransmitter induced washout revealed two components, a transient phase and a sustained phase (227). Only the sustained phase appeared to require extracellular Ca^{2+} (227). The transient phase was suggested to be produced by the mobilization of a bound pool of Ca^{2+} (229). Butcher has obtained evidence in support of this interpretation. Using perifused isolated parotid acinar cells, he also found a transient phase of [^{86}Rb] efflux in Ca^{2+} free buffer. However more extensive Ca^{2+} depletion almost completely eliminated this "Ca^{2+} independent" release (personal communication).

Putney found that stimulation of [^{86}Rb] efflux with either carbamylcholine, phenylephrine, or Substance P caused the tissue to become unresponsive to further additions of not only the original secretagogue, but all the others as well (i.e. cross-receptor inactivation) (229,230). He found that this inactivation could be relieved by Ca^{2+} (229). Based on these results, Putney postulates that the α-adrenergic, cholinergic, and peptide receptors all regulate the same population of Ca^{2+} channels in the membrane (229). By analysis of the washout of [^{45}Ca] from preloaded tissue, Putney has concluded that carbamylcholine increases Ca^{2+} influx in rat parotid gland (228).

Cyclic GMP levels in rat submandibular slices

Increased levels of cyclic GMP were observed on addition of carbamylcholine to submandibular slices incubated in the presence of Ca^{2+} and IBMX (Fig. 6). Significant increases were observed 1 min. after the addition of agonist and this level of cyclic GMP was maintained for at least 10 min., the longest time period employed. The prolonged elevation in cyclic GMP was probably due to the continued presence of phosphodiesterase inhibitor.

Examination of the effect of different concentrations of carbamylcholine on cyclic GMP levels showed that 50-100 uM gave the optimum increase (Fig. 7). These concentrations of agonist are identical to those shown to induce K^+ release (Fig. 4).

The presence of a phosphodiesterase inhibitor has been shown to be necessary to observe secretagogue-induced elevations of cyclic GMP in submandibular slices prepared from both rat (14) and guinea pig (60). Omission of IBMX from the media resulted in low levels of cyclic GMP which were increased by addition of carbamylcholine ($P = 0.02$) (Table XIII). Addition of 500 uM inhibitor markedly elevated both basal and stimulated levels of cyclic GMP and increased the statistical significance of the carbamylcholine effect ($P < 0.01$). All subsequent experiments were performed using 500 uM IBMX.

Ca^{2+} seems to be a requirement for agonist induced increases in cyclic GMP concentrations in many <u>in vitro</u> tissue systems. In the present investigation Ca^{2+} at 1 or 2.5 mM appeared to permit almost identical responses (Table XIV). Omission of Ca^{2+} with the addition of EGTA to the system resulted in significantly reduced basal levels

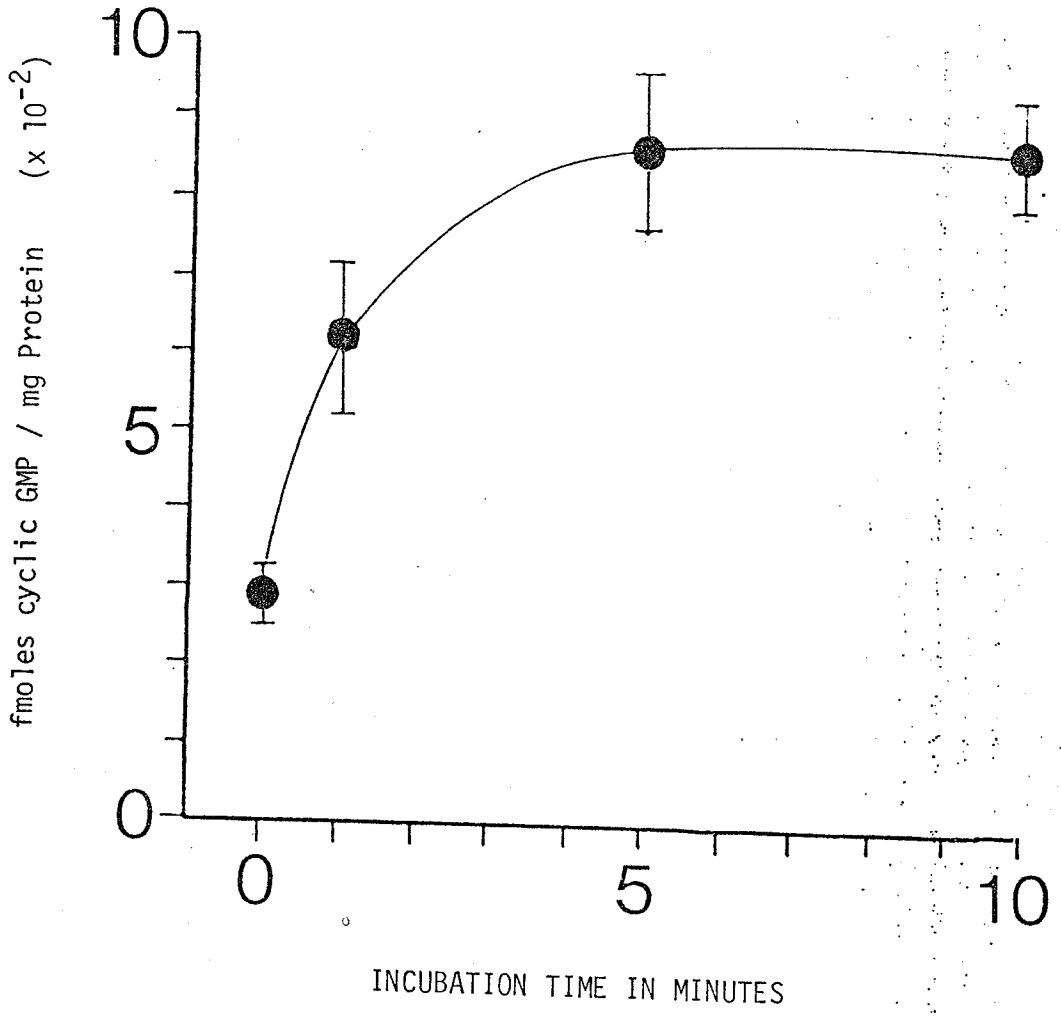


FIGURE 6. EFFECT ON CYCLIC GMP IN SUBMANDIBULAR SLICES OF INCUBATION TIME AFTER THE ADDITION OF CARBAMYLCHOLINE (50 µM)

Experimental details were as described in Methods
Bars represent S.E. of the mean where n = 4.

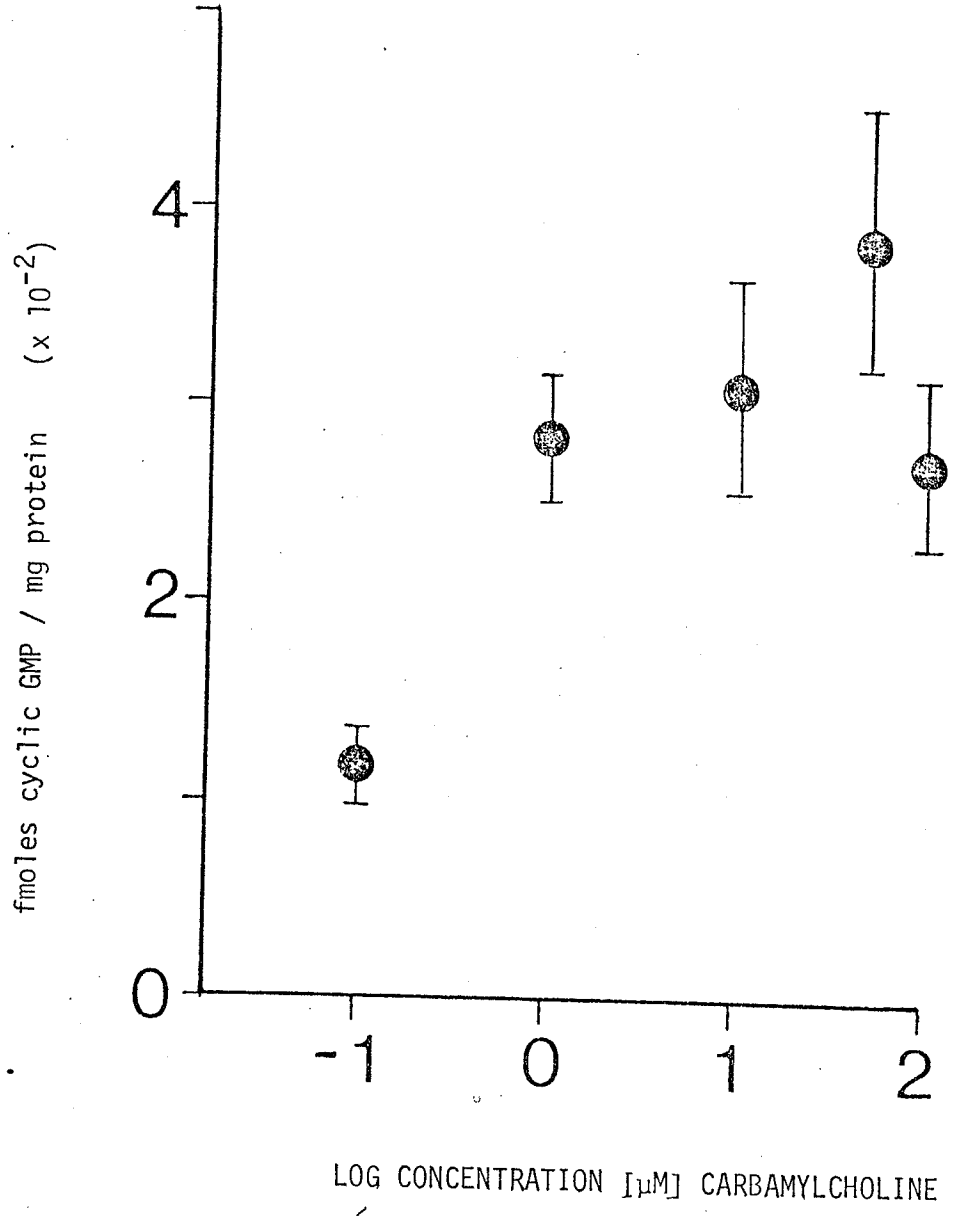


FIGURE 7. CHANGES IN CYCLIC GMP LEVELS 3 MINUTES AFTER THE ADDITION OF INCREASING CONCENTRATIONS OF CARBAMYLCHOLINE

Experimental details were as described in Methods
Bars represent S.E. of the mean where n = 4.

TABLE XIII. INFLUENCE OF 3-ISOBUTYL-1-METHYL XANTHINE (IBMX) ON cyclic GMP LEVELS IN SUBMANDIBULAR GLAND SLICES

Additions			
IBMX (μM)	Carbamylcholine (50 μM)	fmoles cyclic GMP / mg protein	
0	0	42 ± 5	
0	+	154 ± 43	P = 0.02
500	0	104 ± 24	
500	+	702 ± 115	P < 0.01

When present, IBMX was added to buffer 15 min prior to the addition of carbamylcholine. Final incubation with carbamylcholine was for 3 min. Results are mean ± S.E. (n = 9).

TABLE XIV. INFLUENCE OF Ca^{2+} ON cyclic GMP LEVELS

Additions			
Ca^{2+} (mM)	Carbamylcholine (100 µM)	EGTA (4 mM)	fmoles cyclic GMP / mg protein
2.5	0	0	369 ± 67
2.5	+	0	930 ± 171
1.0	0	0	353 ± 67
1.0	+	0	991 ± 139
0.0	0	+	131 ± 12
0.0	+	+	116 ± 19

Experimental details described in Methods. 1 mM Ca^{2+} was present in all buffers until the final 15 min preincubation period. The fresh buffers introduced at this time contained the Ca^{2+} and EGTA as indicated. The final incubation was 3 min with added carbamylcholine when used. Results are mean ± S.E. (n = 9).

of cyclic GMP and abolished the response to carbamylcholine. Preincubation of the tissue with the divalent cation ionophore A23187 resulted in a marked rapid elevation of cyclic GMP levels upon the addition of Ca^{2+}. This increased level could not be augmented by carbamylcholine (Table XV). These results indicate that Ca^{2+} is essential for the carbamylcholine-stimulated increase in cyclic GMP, but it also appears that this result can be produced solely by increasing intracellular Ca^{2+} levels.

As previously demonstrated, cholinergic stimulation gives K^+ release only in the presence of Ca^{2+} (Table IX and 65). As α-adrenergic agonists also bring about K^+ release in a Ca^{2+} dependent manner (Table IX and 22), their effect on cyclic GMP levels was examined (Table XVI). It is evident that neither α-adrenergic or β-adrenergic agonists produce changes equivalent to those produced by carbamylcholine. Combined [α- + β]-adrenergic stimulation (i.e. epinephrine) gave increases of only marginal significance. This lack of adrenergic effect is in contrast to the rat parotid gland, where α-adrenergic agonists have been reported to elevate cyclic GMP to levels comparable to those obtained with cholinergic stimulation (197).

The peptides, eledoisin and physalaemin, which cause K^+ release from rat submandibular slices (Table X), did not affect cyclic GMP levels in this tissue (Table XVII), in agreement with findings using rat parotid slices (192).

TABLE XV. EFFECT OF THE IONOPHORE A23187 ON LEVELS OF TISSUE cyclic GMP

Additions				
A23187 (10 µg/ml)	$CaCl_2$ (1 mM)	Carbamylcholine (50 µM)	fmoles cyclic GMP / mg protein	(n)
0	0	0	51 ± 6	(8)
+	0	0	32 ± 3	(9)
+	+	0	208 ± 24	(9)
+	+	+	207 ± 23	(8)

All buffers were Ca^{2+}-free until final incubation and contained 0.2 mM EGTA throughout the experimental procedures. The ionophore A23187 was present in buffer 15 min prior to the addition of Ca^{2+} or carbamylcholine for the final incubation period (3 min). Results are mean ± S.E. (n = 9).

TABLE XVI. COMPARISON OF ADRENERGIC AND CHOLINERGIC STIMULATION ON CYCLIC GMP LEVELS IN SUBMANDIBULAR GLAND SLICES

Additions	Type of stimulus	fmoles cyclic GMP / mg protein	
Nil	-	193 ± 20	(14)
Phenylephrine	α-adrenergic	281 ± 35	(16)
Isoproterenol	β-adrenergic	306 ± 17	(17)
Carbamylcholine	Cholinergic	828 ± 104	(18)
Nil	-	275 ± 64	(9)
Carbamylcholine	Cholinergic	727 ± 109	(9)
Carbamylcholine + Atropine	Cholinergic antagonist	285 ± 98	(9)
Nil	-	198 ± 33	(16)
Epinephrine	α,β-adrenergic	283 ± 64	(17)
+ propranolol	α-adrenergic	218 ± 43	(17)
+ phentolamine	β-adrenergic	210 ± 45	(18)

Phentolamine, propranolol and atropine, when present, were 200 μM. and were added to incubation 15 min prior to agonists. Final incubations with agonists present were for 3 min. Final concentration of all agonists was 50 μM except for isoproterenol (200 μM). Results are mean ± S.E. (N as shown).

TABLE XVII. FORMATION OF CYCLIC GMP IN THE PRESENCE OF SECRETORY PEPTIDES

Additions (1 μM)	fmoles cyclic GMP / mg protein
nil .	241 ± 47
Eledoisin-related peptide	208 ± 26
Physalaemin	209 ± 50
Substance P	247 ± 61

Peptides were added at the start of the final incubation which was for 3 min. Results are mean ± S.E. (n = 9).

DISCUSSION

Protease secretion from rat submandibular slices

Riekkinen and coworkers isolated and characterized several different proteases from rat submandibular gland homogenates (40-47). The activity of purified glandulain was completely inhibited by 0.02 mM Cu^{2+} with 0.5 mM BAPA as substrate in tris-HCl, pH 8.0 (47). Purified salivain was shown to display increased activity in the presence of Ca^{2+} but 0.2 mM Cu^{2+} had no effect on its activity. A 40 % inhibition was noted when the level of Cu^{2+} was 2mM (43). When they measured these enzymes in unpurified extracts (48), salivain activity was reported as the difference in BANA hydrolysis +/- (0.1 mM $CuCl_2$ + 20 mM $CaCl_2$) at pH 9.5, and glandulain as the difference in BANA hydrolysis +/- 0.1 mM $CuCl_2$ at pH 8.2. They failed to report the actual percentage of the activity inhibited by Cu^{2+} at either pH. In this laboratory the difference in protease activity at pH 8.2 (and 9.5) +/- 0.1 mM $CuCl_2$ was found to be negligible (Table 1). The result was the same whether male or female, Sprague-Dawley or Long-Evans rats were used. Furthermore, homogenizing the tissue in 20 mM $CaCl_2$, reported to stabilize glandulain (47), or storing the homogenates for several days at 4°C to allow possible activation of latent activity, did not change the results with 0.1 mM $CuCl_2$. However, at 1 mM Cu^{2+} complete inhibition of BANA hydrolysis at pH 8.2 was observed (Table 1). Our results, therefore, are somewhat contradictory to those of Riekkinen and associates. Several explanations can be advanced. Either the enzyme named glandulain is

present in our animals in very low concentration and the protease activity at pH 8.2 is really salivain, or the pH 8.2 activity is due to an enzyme distinct from salivain but with a different sensitivity to $CuCl_2$ than that reported for purified glandulain by Riekkinen. It is possible that in crude homogenates a substantial portion of the added Cu^{2+} is bound to protein, thus reducing the concentration of free ionized Cu^{2+}. The ability of protein to bind Cu^{2+} is well known as this is the basis for the Lowry protein assay (54). Although Riekkinen does not report the protein concentration used in their experiments with purified glandulain, it would be expected to be much lower than it would be when crude homogenates are assayed. The observed inhibition by EGTA, phenylmethylsulfonylfluoride and 1 mM $CuCl_2$ of the pH 8.2 but not the pH 9.5 protease activity (Table 1) supports the interpretation that two distinct enzymes have been assayed. However, there appeared to be no justification for routinely assaying the pH 8.2 activity with and without $CuCl_2$ at 0.1 mM, or any other concentration. Similarily, assaying the pH 9.5 activity with and without 0.1 mM $CuCl_2$ + 20 mM $CaCl_2$ and using the difference, did not appear logical considering the properties of the two enzymes (salivain activated by Ca^{2+}, glandulain inhibited by Cu^{2+}). In preliminary secretion experiments, results were calculated this way and compared to results calculated using only the activities obtained in the presence of 0.1 mM $CuCl_2$ + 20 mM $CaCl_2$. Patterns of secretion were found to be similar with either method, but the subtraction method resulted in larger variability. All results reported in this study were calculated by the non-subtraction method. Because of the different Cu^{2+} sensitivity of the enzyme assayed at pH 8.2 in the

present study and that reported for purified glandulain, and because of the different way in which the results have been calculated in this study, the two activities are referred to as pH 8.2 and pH 9.5 proteases rather than glandulain and salivain.

Riekkinen reported that purified glandulain and salivain are able to hydrolyze BAEE, a synthetic protease substrate used in this study to assay kallikrein. It might therefore appear possible that the activity reported as kallikrein in this study might in fact be due to these other proteases. However, the fact that the BAEE hydrolysis was not affected by Ca^{2+} and EGTA as was BANA hydrolysis strongly suggests that this is not the case. As previously noted in methods, it has been concluded that the majority of BAEE hydrolysis by rat submandibular gland homogenates is due to kallikrein and the measurement of kallikrein activity by means of BAEE hydrolysis would appear to be very adequate for rat submandibular gland preparations (27). However, it may not be adequate for preparations from other species (57), hence comparisons of kallikrein, using BAEE, between, and among, species should be done with care.

Erdos et al. (58), using both BAEE and kininogenase assay methods, found high levels of kallikrein in isolated secretory granules obtained from rat submandibular glands. Although kallikrein has been suggested to be localized in acinar cells in mice (149) and guinea pigs (59), immunofluorescent techniques have demonstrated that in rats (49,50) and guinea pigs (242) it is localized in the CGT cells. The present findings that the pH 8.2 and 9.5 proteases are, like kallikrein, present in larger amounts in male glands, and are secreted in parallel to kallikrein, indicate a similar localization.

Thus, any of these three enzymes appear suitable for use as tools to study the regulation of secretion from rat submandibular CGT cells. Kallikrein seems to be best suited to this purpose, due to the ease and reproducibility of its assay. In contrast to results reported here, Riekkinen et al. (48) reported substantial trypsin-like activity (salivain) to be present in equal amounts per unit weight in male and female rats, which suggests an acinar cell localization. In addition these authors state that salivain, but not glandulain, is present in isoproterenol-evoked saliva (26). In the present study no non-secretable protease was found. It is tempting to speculate that these different results may be due to the differences in the methods of calculation between their study (48) and the present one.

The present study clearly demonstrates that α-adrenergic stimulation is responsible for submandibular gland secretion of protease in vitro. This supports in vivo studies. Electrical stimulation of nerves and neurotransmitter agonist and antagonist perfusion of rat submandibular glands in situ have shown that α-adrenergic stimuli induces kallikrein (27) and total casein hydrolyzing activity (28) secretion into saliva. The secretion of male specific components of mouse submandibular glands, assayed by an immunological method, was also found to be induced by α-adrenergic stimulation (117). Control of kallikrein secretion from guinea pig submandibular gland appears to be different from that of rat and the mouse as Bhoola and coworkers (59,60) have reported that α- and β-adrenergic and cholinergic agonists all increase kallikrein secretion from submandibular slices prepared from this species.

In the rat parotid gland, enzyme secretion is mediated by

β-adrenergic receptors (61). Muscarinic cholinergic receptors elicit secretion of rat pancreatic zymogens (62). Thus, although the process of secretion has many similarities among tissues and species, the controls may be dissimilar. This has important implications for investigations on the biochemistry of secretion. Cyclic AMP has been proposed as a controlling factor in rat parotid enzyme secretion (63) via β-adrenergic activation of adenylate cyclase (64). In rat submandibular gland, however, protease secretion is mediated by α-adrenergic receptors, and a different chemical mediator would be expected to be involved. Comparison of tissues where exocytosis is mediated through different receptors should be useful in elucidating both the mechanism of secretion and the way in which different receptors mediate their effects.

A comparison of protease secretion to K^+ release in rat submandibular gland slice preparations shows that cholinergic (Table VII,22), -adrenergic (Table VII,65), and peptide (Table X) agonists cause K^+ release but only α-adrenergic agonists induce protease secretion. The fact that both cholinergic and α-adrenergic agonists cause K^+ release, and both require the presence of extracellular Ca^{2+} to do so (Table IX), suggests that the two receptors work through very similar mechanisms. If K^+ and protease are both released from the same cells, and both types of stimulation releases K^+, then both types of stimulation would be expected to cause protease secretion. Most probably protease secretion and K^+ efflux occur from different cells. Rat parotid gland slices release K^+ in a process bearing great resemblance to that of submandibular slices (8,9,66,67), and histological investigation has demonstrated that this release is

accompanied by vacuolization of the acinar cells (5). The site of K^+ release in submandibular gland is most probably the acinar cells, as in parotid. These cells probably have α-adrenergic and cholinergic receptors which mediate K^+ release and thus the secretion of water, and β-adrenergic receptors, which mediate glycoprotein secretion, while the CGT cells, containing proteases, would have only α-adrenergic receptors. Abe and Dawes (68) reached a similar conclusion regarding the types of receptors possessed by the two types of secretory cells in this gland. They studied the electrophoretic patterns of salivary proteins secreted in response to different types of stimulation. It was found that α-adrenergic stimulation resulted in saliva containing bands of protein in addition to those also appearing in β-adrenergic and cholinergic evoked saliva. They postulate that these extra bands originate in the CGT cells which have only α-adrenergic receptors while the other bands originate in the acinar cells which have α-adrenergic, β-adrenergic, and cholinergic receptors.

In vivo β-adrenergic stimulation has been reported to cause the secretion of rat submandibular saliva with a higher protein content than does α-adrenergic stimulation (1,243). If secretion from acinar cells is mediated primarily through the β-adrenergic receptor, these results do not contradict the present findings that protease secretion is primarily mediated through α-adrenergic receptors. Acinar cells make up approximately 65 % of the volume of the gland (239). Duct cells make up about 35 %, but only a fraction of these are CGT cells. Thus, β-adrenergic stimulation of the more prevalent acinar cells would lead to a higher protein content of saliva than

would α-adrenergic stimulation of fewer CGT cells.

Extracellular Ca^{2+} is required for neurotransmitter-evoked secretion of K^+ (Table IX,22,65) and proteases (Table 4) from rat submandibular gland. The observation that the ionophore A23187 could completely mimic the effects of α-adrenergic induced K^+ release (Table IX-A) suggests that a major function of the α-adrenergic receptor is to regulate a Ca^{2+} gate in the basal plasma membrane. The present studies have shown that the ionophore is ineffective in inducing protease secretion, thus suggesting that α-adrenergic receptors in CGT cells may, in addition to regulating a Ca^{2+} gate, have other functions or influence the production of other, as yet unknown, intracellular second messengers.

K^+ release from rat submandibular slices

The results of this study indicate that rat submandibular gland slices release K^+ by a process very similar to that of rat parotid gland. In both glands K^+ secretion is associated with α-adrenergic and cholinergic receptor stimulation but not with β-adrenergic receptor stimulation. Ca^{2+} appears to play an essential role in neurotransmitter-induced K^+ release and can, by itself, induce secretion when tissues are preincubated with the ionophore A23187.

According to current theories of salivary secretion (1), the acinar cells are responsible for the initial salivary fluid production. As this fluid is moved through the duct system the ionic composition is altered but the volume remains essentially the same. Micropuncture studies (19) have shown that the primary fluid does not

resemble intracellular fluid in ionic composition but its ionic composition does resemble that of blood plasma, suggesting that this could be the source of the fluid component of saliva. Burgen (20) noted a marked drop in the intracellular content of K^+ shortly after the onset of secretion, which implies that a portion of K^+ in initially-secreted saliva may be of intracellular origin. However, the cells do not contain enough Na^+ to support the production of an appreciable amount of saliva of the observed ionic composition. The only possible source of Na^+ is plasma, thus the formation of primary saliva must involve events at both the basal and luminal membranes of the cell i.e. fluid and electrolytes enter the cell at the basal end, move through the cell, and are secreted at the luminal end.

The following sequence of events is proposed as a possible mechanism for the salivary secretion of water and electrolytes. Cholinergic and α-adrenergic agonists interact with their receptors on the basal membrane leading to an increased permeability to Ca^{2+} which would flow into the cells. This Ca^{2+} influx, by an unknown mechanism, leads to an increased permeability of both basal and luminal membranes to Na^+ and K^+. The latter ion would then flow out of the cell into the lumen and into the blood. This idea is supported by the observation that immediately after stimulation <u>in vivo</u>, there is a transitory increase of K^+ in both saliva and in the venous drainage of dog and cat parotid glands (20). Na^+ would, according to its electrochemical gradient, flow into the gland from the blood plasma.

Petersen (13) proposed the presence of two distinct Na^+ pumps in salivary gland, one inhibited by ethacrynic acid, and the other by

ouabain. His postulate is that the ethacrynic acid-inhibited pump is the one responsible for saliva formation and does so by pumping Na^+, Cl^-, and water into the lumen or intercellular spaces contiguous with the lumen. This is consistent with his observation that ethacrynic acid inhibited saliva formation and our observation that K^+ release is unaffected by this agent (Table 6). This pump, on the luminal membrane of the cell, could be activated by high cellular Na^+ levels resulting from neurotransmitter- induced basal membrane permeability increases.

Selinger et al. (9) showed that rat parotid slices were able to reabsorb previously-secreted K^+ on cessation of stimulation, and that this reuptake was prevented by ouabain. Petersen (13) reported that cat submandibular glands, perfused in situ, took up K^+ from the perfusing medium following a period of stimulation and that this uptake was inhibited with ouabain. Therefore, the probable function of the ouabain-sensitive pump is to prevent the occurence of excessively low intracellular K^+ levels. Activation could be effected through lowered K^+ levels within the cells. Our findings that ouabain increased both stimulated and unstimulated K^+ release (Table 2) supports this theory and indicates that submandibular membranes are at least partially permeable to this ion at all times. (Na^+/K^+)-ATPase has been histochemically demonstrated on both the luminal and basal membranes (96).

The fact that a phosphodiesterase inhibitor potentiates K^+ release induced with sub-optimal doses of stimulants (Table 7) tends to support the idea that cyclic GMP is involved in this process. The inability of 8-bromo-cyclic GMP to cause K^+ release may be due to the

slow permeability of this compound.

As the action of physalaemin and eledoisin are apparently unaffected by neurotransmitter blocking agents (Table 5), they probably act either on distinct polypeptide receptors or act on the mechanism of K^+ secretion at some stage distant from the normal neurotransmitter binding. The finding that the two polypeptides have differing requirements for Ca^{2+} to increase K^+ release may suggest that the do not affect the same step of the process.

Cyclic GMP levels in rat submandibular slices

The present investigation has shown that muscarinic cholinergic stimulation of rat submandibular gland in vitro results in a significant increase in cyclic GMP levels. The presence of Ca^{2+} was essential for this effect. The phosphodiesterase inhibitor IBMX increased basal levels of cyclic GMP, and resulted in larger and more significant increase upon carbamylcholine stimulation. Neither α- nor β-adrenergic agonists could elicit a increase comparable to that obtained on cholinergic stimulation. It would, therefore, appear that cyclic GMP levels in rat submandibular gland are primarily regulated by a cholinergic mediated process.

Previous investigation has demonstrated that cholinergic stimulation of submandibular gland slices cause a release of K^+ (Table VII,65), which, like the cyclic GMP increases reported here, requires extracellular Ca^{2+}. Increasing Ca^{2+} influx into the tissue with the ionophore A23187 produces both an increase in cyclic GMP content and an efflux of K^+. It would thus appear that occupation of

the cholinergic receptor by suitable agonists results in a Ca^{2+} influx and this influx then mediates both the increase in cyclic GMP and the release of K^+.

If the cholinergic K^+ release and cyclic GMP increase are causally related, two possibilities exist. The release of K^+ may stimulate cyclic GMP accumulation or the increase in cyclic GMP levels may stimulate the release of K^+. If the release of K^+ stimulates cyclic GMP accumulation, any treatment causing a similar extent of K^+ release should produce a similar elevation of cyclic GMP. As eledoisin, physalaemin and α-adrenergic agonists cause K^+ release without affecting cyclic GMP, it is apparent that the cyclic GMP increases noted with cholinergic stimulation are not the result of K^+ release. If the K^+ release is a result of the increase in cyclic GMP levels, a phosphodiesterase inhibitor should increase the amount of K^+ secreted with suboptimal doses of carbamylcholine by inhibiting the degradation of cyclic GMP and thus potentiating the increase in its level. Results reported in Table XII showed this to be true.

K^+ release can also be produced by other types of agonists, e.g. α-adrenergic agonists and 2 polypeptides, eledoisin and physalaemin. It might be expected that these agonists, because they give rise to the same effect, would do so through the same mechanism. There appear to be, in fact, similarities in the mechanisms of action of agonists producing K^+ release. α-adrenergic agonists and physalaemin, like cholinergic agonists, do not induce K^+ release in the absence of extracellular Ca^{2+}. However physalaemin and α-adrenergic agonists do not significantly elevate cyclic GMP, and because it is not

unreasonable to assume that all agonists effect K^+ release through the same mechanism, it can be argued that the increase in cyclic GMP given by carbamylcholine is not an intermediate in the K^+ release induced by this agonist. However, the assumption that all agonists producing K^+ release do so through the same mechanism may not be valid. In support of this, the K^+ release caused by eledoisin was found to be independent of extracellular Ca^{2+}. In addition, results have been obtained in this laboratory which may indicate that α-adrenergic and cholinergic agonists produce their effects through different mechanisms. As shown in Table III, increasing Ca^{2+} influx with the ionophore A23187 results in a fold increase in cyclic GMP levels comparable to that produced by carbamylcholine, even though both basal and stimulated levels are lower due to the lack of Ca^{2+} during preincubation necessary in this experiment. Adding carbamylcholine to tissue stimulated in this manner does not further increase cyclic GMP levels. In contrast, the secretion of protease from these slices, an α-adrenergic mediated process also dependent on extracellular Ca^{2+}, is only slightly stimulated by an ionophore-induced Ca^{2+} influx, and the addition of an α-adrenergic agonist to slices so treated results in an additional large secretory response. It thus appears possible that α-adrenergic and cholinergic agonists exert their effects through somewhat different mechanisms and it may therefore be that cholinergic agonists induce K^+ release through regulating cyclic GMP levels while α-adrenergic and polypeptide secretagogues produce K^+ release through a mechanism not involving cyclic GMP. Final proof must await further experimentation.

In the foregoing discussion, it was assumed that the release of

K^+ and the increased synthesis of cyclic GMP took place in the same cell type. However, no attempt to definitively identify the cell type or types involved in the cyclic GMP response was made in this study and, therefore, the possibility that the increases in the cyclic GMP levels noted in the present study are due to a small population of some other type of cell can not be conclusively ruled out.

Putney has postulated that cholinergic, α-adrenergic, and peptide receptors all regulate the same population of Ca^{2+} influx sites in rat parotid gland (229). This would not appear to be true in rat submandibular gland, however, as increasing the Ca^{2+} influx with the ionphore increases cyclic GMP levels, as does cholinergic stimulation. Under certain assay conditions, Ca^{2+} has been found to stimulate guanylate cyclase directly (21). If α-adrenergic and polypeptide receptors regulated the same Ca^{2+} influx sites as do the cholinergic receptor, stimulation of these receptors should increase cyclic GMP levels to a similar extent. However, stimulation of these receptors does not significantly increase cyclic GMP accumulation.

Albano et al. (60) have shown that acetylcholine and norepinephrine induce both an increase in levels of cyclic GMP and kallikrein secretion in guinea pig submandibular slices. They also reported that dibutyryl-cyclic GMP caused a release of kallikrein. These results led them to postulate a role for cyclic GMP in the stimulus-secretion of this protease in this tissue. However, as kallikrein secretion in rat submandibular is mediated primarily through the α-adrenergic receptor (Table III), and as α-adrenergic agonists do not significantly increase cyclic GMP levels in this tissue (Tables XVI,XVII), it appears unlikely that cyclic GMP has a

role in the regulation of kallikrein secretion in the rat submandibular gland. This conclusion is supported by the finding that the introduction of extracellular Ca^{2+} into the tissue with the ionophore effectively increasess cyclic GMP (Table III) but only slightly increase kallikrein and protease secretion (Table V).

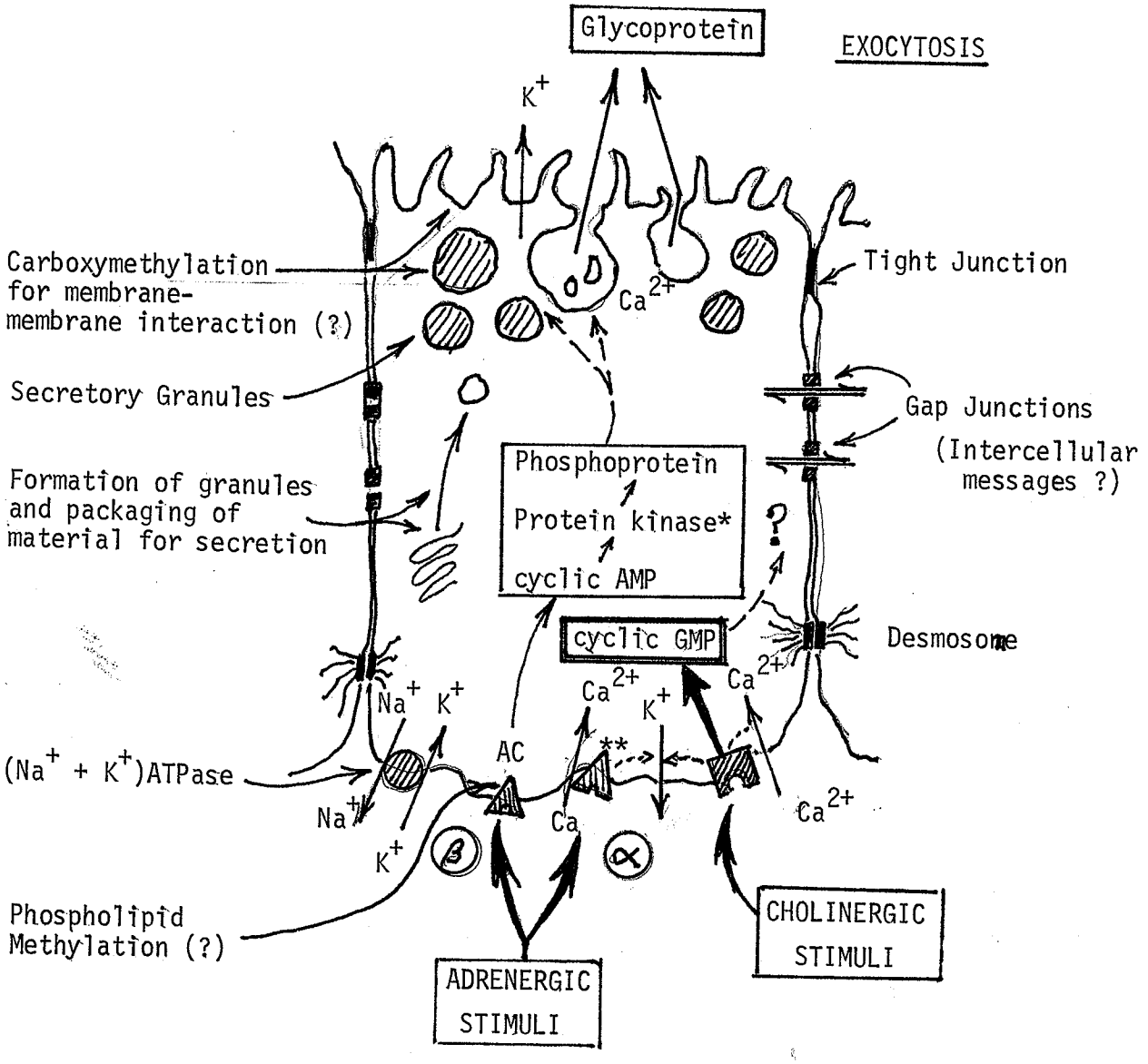
Selective denervation of the two branches of the autonomic nervous system to the salivary glands has demonstrated the importance of both branches in the maintenance of gland size (169). Cyclic AMP appears to be an intermediate in the sympathetic pathway regulating gland size but some other substance must mediate this effect in the parasympathetic pathway as cholinergic agonists do not increase the levels of this nucleotide. In mouse parotid gland, a number of isoproterenol derivatives increased DNA synthesis without increasing cyclic AMP levels (213). As these derivatives were found to increase cyclic GMP levels, a role in cell growth has been proposed for this nucleotide in that gland (213). Cyclic GMP may have a similar function in rat submandibular gland. This does not rule out other possible roles however since cyclic AMP has a dual function in rat parotid, stimulation of growth and induction of exocytosis.

TABLE XVIII. EFFECTORS, MESSENGERS AND RESPONSES IN RAT PAROTID AND SUBMANDIBULAR GLANDS

Stimulus	Secretion		Ca^{2+} required	Phosphodiesterase inhibitor added	cyclic GMP increased	Ref.
	K^+	Protease				
PAROTID						
α-adrenergic	yes	n.d.	yes	no	n.d.	187,229
muscarinic	"	"	"	"	"	187,229
A23187	"	"	"	"	"	10
Substance P	"	"	"	"	"	229
α-adrenergic	yes	n.d	yes	yes	yes	197,67
muscarinic	"	"	"	"	"	66
A23187	"	"	"	"	"	189
Substance P	"	"	"	"	No	192
Eledoisin	"	"	"	"	No	192
SUBMANDIBULAR						
α-adrenergic	yes	yes	yes	yes	No	this study
muscarinic	"	no	yes	yes	yes	"
A23187	"	"	"	"	"	"
Substance P	n.d.	"	n.d.	"	no	"
Eledoisin	yes	"	yes	"	no	"
Physalaemin	"	"	yes	"	no	"

n.d. = not done

FIGURE 8. Schematic representation of exocrine cell



AC = adenylate cyclase

* These processes require Ca^{2+} and calmodulin. Phosphodiesterase(s) probably required with de-activation sequence.

** In CGT cells the alpha adrenergic response causes the secretion of protease via exocytosis.

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