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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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FUFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF ORAL BIOLOGY

WINNIPEG, MANITOBA

March, 1979

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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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.

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. E.T. Pritchard for his guidance, friendship and encouragement during the course of this work. Special thanks are due to Miss J. Cushnie for her excellent technical assistance.

This investigation was supported by a Medical Research Council Studentship and a University of Manitoba Graduate Fellowship.

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).

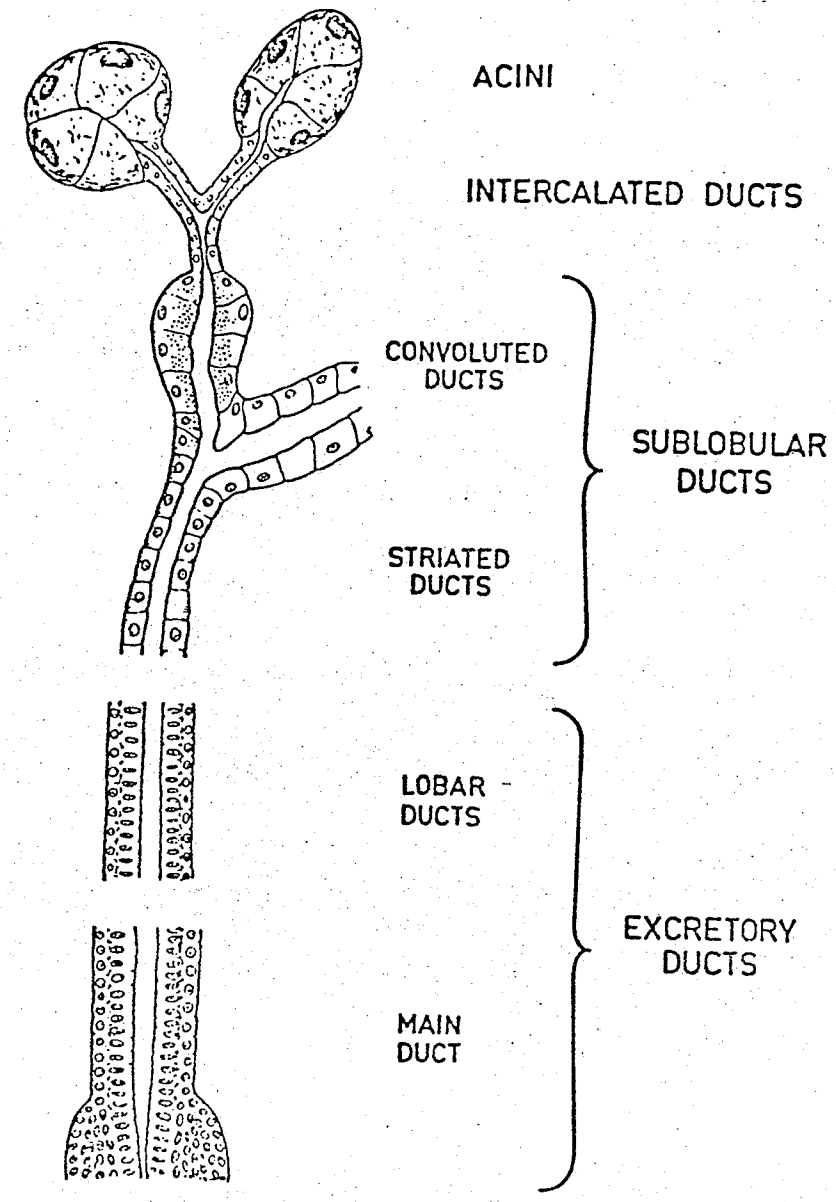
Rats have several distinct salivary glands, the parotid, the submandibular, the sublingual, and minor salivary glands. The major glands are present in pairs and consist of masses of glandular tissue, the parenchyma, and considerable amounts of areolar connective tissue, the stroma. The connective tissue 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