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Release of kallikrein-like esterase and tonin from dispersed cells of the rat submandibular gland

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1 Viable dispersed cell preparations of rat submandibular gland were obtained by a tissue-dissociation procedure using collagenase and gentle mechanical force.

2 The cells released kallikrein-like esterase in a time- and calcium-dependent manner in response to noradrenaline (10 μM) at 30°C. The net loss of kallikrein-like esterase content from the dispersed cells corresponded with the increase in kallikrein-like esterase activity in the suspending medium at all concentrations of noradrenaline. These results indicate the viability and functional integrity of this dispersed cell preparation of rat submandibular gland.

3 α-Adrenoceptor agonists such as noradrenaline stimulated kallikrein-like esterase and tonin release in a dose-dependent manner, whereas the β-adrenoceptor agonist isoprenaline and cholinoceptor agonist methacoline were both inactive. Noradrenaline-induced release of kallikrein-like esterase and tonin were completely blocked by prior addition of the α-adrenoceptor antagonist, phenoxybenzamine.

4 It is concluded that the secretion of kallikrein-like esterase and tonin in rat submandibular gland is mediated only via stimulation of α-adrenoceptors.

Introduction

The submandibular glands of several rodent species, including those of the rat, contain two distinctly separate exocrine systems: the acinar secretory component and the convoluted granular tubule (GCT) component (Jacoby & Leeson, 1959; Tamarin & Sreebny, 1965; Dorey & Bhoola, 1972; Cutler & Chaudhry, 1973). Abe & Dawes (1978) reported that in rat submandibular glands the acinar cells secrete protein in response to cholinoceptor, α- and β-adrenoceptor stimulations, with β-adrenoceptor stimuli being most effective, whereas the granular tubules secrete proteins only in response to α-adrenoceptor stimulation.

High concentrations of kallikrein (Bhoola *et al.*, 1973; Brandtzaeg *et al.*, 1976) and tonin (Demassieux *et al.*, 1976; Lis *et al.*, 1977) have been found in rat submandibular gland, although the exact physiological functions of both enzymes have not yet been established. Recent immunohistochemical studies showed the similar localization of kallikrein (Brandtzaeg *et al.*, 1976; Orstavik & Gautvik, 1977) and tonin (Gutkowska *et al.*, 1982) in the GCT segments. However, the secretory mechanism of kallikrein and tonin is different; that is, kallikrein is secreted via the stimulation of α-adrenoceptors (Orstavik & Gautvik, 1977; Spearman & Pritchard, 1980), whereas tonin is via β-adrenoceptors (Garcia *et al.* 1976; 1977; Kondo, 1979; Gutkowska *et al.*, 1982). Such a discrepancy led us to compare the secretory pattern of both enzymes using the dispersed cell preparation of rat submandibular gland which enabled a more detailed analysis of the secretory process at the level of individual cells to be made.

Methods

Preparation of dispersed cells

Male Wistar rats were used in the present studies, unless otherwise described. After decapitation, the submandibular glands together with the adjacent sublingual glands on both sides were removed. The sublingual glands were separated in Ca^{2+}, Mg^{2+}-free Tyrode medium at room temperature. The Tyrode solution had the following composition (mM): NaCl 135, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_2$ 11.9, NaH_2PO_4 0.4, glucose 2.8, sodium pyruvate 4.9, sodium fumarate 2.7, and sodium glutamate 4.9. The glands were finely minced with scissors in the Ca^{2+}, Mg^{2+}-free Tyrode solution and then incubated with 10 ml of Ca^{2+}, Mg^{2+}-free Tyrode

solution containing 0.1% collagenase and 1% bovine serum albumin in an Erlenmyer flask at room temperature. During incubation, the dispersion mixture was stirred moderately for 25 min with magnetic stirrers under an atmosphere of 95% O_2 and 5% CO_2. The dispersed cells were then separated from the solution by centrifugation at 1000 *g* for 3 min. The cells so obtained were washed three times with normal Tyrode solution containing 1% bovine serum albumin and passed through a sieve of 150 mesh (U.S. Standard, Tyler Screen) in order to eliminate undigested pieces of tissue. After a final wash, they were suspended in an appropriate amount of the same solution. The concentration of cells in the medium was adjusted to contain nearly 3.0 mg protein per ml in each tube so as to detect the release of kallikrein-like esterase from the dispersed cells. To each tube, 25 µl of 10 mM Tris-HCl (pH 7.4) buffer with or without the experimental agent, was added. The samples were incubated for 30 min at 30°C in a shaking bath, unless otherwise stated. During incubation, the samples were not gassed, because of the instability of secretagogues such as noradrenaline in the presence of oxygen. After incubation, the dispersed cells were separated from the suspending medium by centrifugation at 1000 *g* for 3 min at 4°C and the sediment was mixed with 1 ml of 50 mM Tris-HCl (pH 8.0) and 50 µl 0.25% Triton X-100. Kallikrein and tonin were measured in cells and/or medium of each sample.

Preparation of homogenate of rat submandibular gland

The submandibular glands on both sides were removed as described above and were homogenized in the 10 mM Tris-HCl buffer (pH 7.4) and the homogenates were centriguted at 10,000 *g* for 30 min at 4°C. The supernatant was used for the measurement of kallikrein-like esterase and tonin activities.

Kallikrein-like esterase assay

The activity of kallikrein-like esterase was determined as described before (Izumi & Aoki, 1981) by means of a chemical assay method (Brown, 1960). This is a measurement of the esterolytic activity of kallikrein using a synthetic amino acid ester (α-N-benzoyl-arginine ethylester) (Erdos *et al.*, 1968; Orstavik *et al.*, 1977; Spearman & Pritchard, 1980).

Tonin assay

The assay of tonin activity was carried out in a 3.0 ml incubation mixture containing 0.05 M borax-phosphate buffer (pH 7.0). EDTA (1.3 mM), di*iso*propyl-fluorophosphate (0.72 mM, 2% in isopropanol, 2 ml per 300 ml buffer) and dipyridyl (8 mM, 20% in methanol, 2 ml per 300 ml buffer) were added to the buffer solution to inhibit the angiotensin I converting enzyme activity as described by Garcia *et al.* (1977). The reaction was initiated by the addition of the substrate (angiotensin I, 100 µg per tube) and the reaction mixture was incubated at 37°C for 30 min, unless otherwise described. Reactions were terminated by the addition of 0.4 ml of 2 M NaOH. To this reaction mixture, *O*-phthalaldehyde (OPT) reagent (0.1% methanol, prepared freshly daily) (0.1 ml) was added, then mixed with shaking. Four minutes later at room temperature, 3.5 M orthophosphoric acid (0.4 ml) was added to the mixture. The fluorescence was measured at 440 nm with excitation at 360 nm. The concentration of His-Leu released was calculated by comparison of the fluorescence produced with that of standard solutions.

Protein determination

Protein concentration was determined as described by Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

Materials

Collagenase (type 1), (−)-noradrenaline bitartrate, methacholine hydrochloride, atropine sulphate and (−)-isoprenaline hydrochloride were purchased from Sigma Chemical Co. Angiotensin I (human)

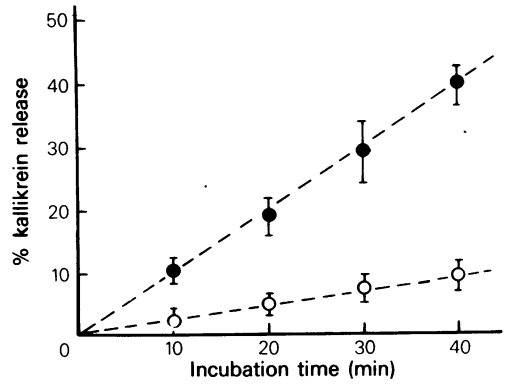

Figure 1 Time-course of noradrenaline-induced release of kallikrein-like esterase from the dispersed cells of rat submandibular gland. Dispersed cells were incubated with (●) or without (○) noradrenaline (10 µM) at 30°C for varying times. Kallikrein release (ordinate scale) is expressed as % of total kallikrein-like esterase activity in the control cells. The intracellular kallikrein-like esterase activity in the absence of noradrenaline without incubation was used as control. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean.

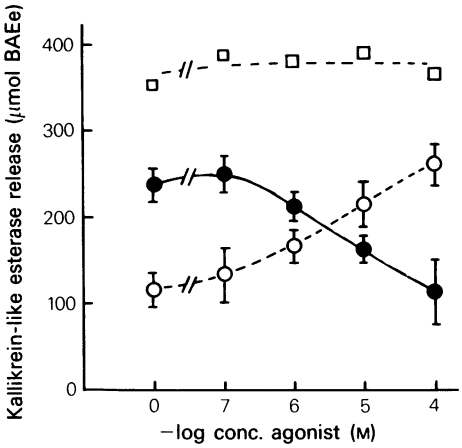

Figure 2 Comparison of net decrease of kallikrein-like esterase activity in the cells (●) and increase in the supernatant (○) by stimulation with noradrenaline (10 μM). Total values (□) are the sum of kallikrein-like esterase activity in the cells and supernatant. Release of kallikrein-like esterase (ordinate scale) is expressed as the amount (μmol) of BAEe hydrolysed in 10 min per tube at 37°C. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean.

was obtained from Protein Research Foundation. Crystalline bovine serum albumin (fraction V) was obtained from Wako Pure Chemical Industries Ltd. Phenoxybenzamine hydrochloride was obtained from Tokyo Kasei Co. Ltd. All other chemicals were reagent grade obtained from general commercial sources.

Results

Preliminary experiments for the dispersed cells

Dispersed cells were incubated with noradrenaline (10 μM) at 30°C for various times (Figure 1). Release of kallikrein-like esterase was observed in response to noradrenaline in a time-dependent fashion at least up to 40 min. Slight spontaneous leakage also occurred. In the following studies, all of the incubations were carried out at 30°C for 30 min. Dispersed cells released kallikrein into the supernatant, and cellular kallikrein-like esterase activity correspondingly decreased (Figure 2). As expected, the algebraic sum of the kallikrein-like esterase activity in the medium and that remaining cells was approximately constant regardless of the concentration of noradrenaline. In the following studies, unless otherwise noted, the percentage decrease of kallikrein-like esterase and tonin activity compared to control (without secretagogue) was used as a measure of kalikrein-like esterase and tonin release from the dispersed cells.

Release of kallikrein-like esterase

Figure 3 represents the effects of noradrenaline, isoprenaline and methacholine on release of kallikrein-like esterase from dispersed cells of rat submandibular glands of both sexes. Dose-related release of kallikrein-like esterase was induced by noradrenaline, whereas no significant release of kallikrein-like esterase occurred in response to isoprenaline or methacholine. There was no difference in

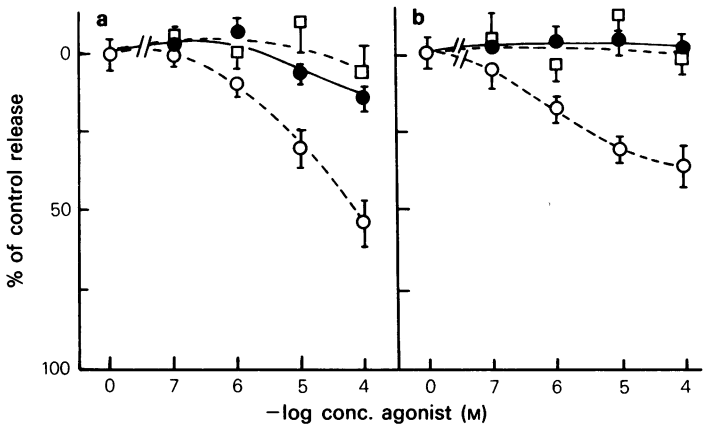

Figure 3 Effects of noradrenaline, isoprenaline and methacholine on release of kallikrein-like esterase from dispersed cells of male (a) and female (b) rat submandibular gland. Dispersed cells were incubated for 30 min at 30°C in the absence (control) and presence of various concentrations of noradrenaline, isoprenaline and methacholine. Kallikrein-like esterase release (ordinate scale) is expressed as % of total kallikrein-like esterase activity in the control cells. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean. Each experiment is the average of duplicate determinations. (○) Noradrenaline; (●) methacholine; (□) isoprenaline.

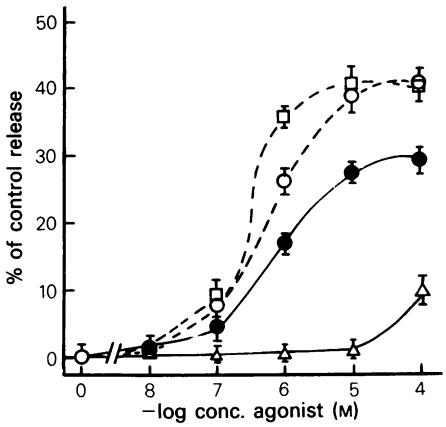

Figure 4 Effects of α-adrenoceptor agonists (noradrenaline, methoxamine and phenylephrine) on release of kallikrein-like esterase and its inhibition by phenoxybenzamine. Dispersed cells were incubated for 30 min at 30°C in the absence (control) and presence of various concentrations of noradrenaline, methoxamine and phenylephrine. Kallikrein-like esterase release (ordinate scale) is expressed as % of total kallikrein-like esterase activity in the control cells. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean. (□) Me thoxamine; (○) noradrenaline; (●) phenylephrine; (△) noradrenaline and phenoxybenzamine (10 μM).

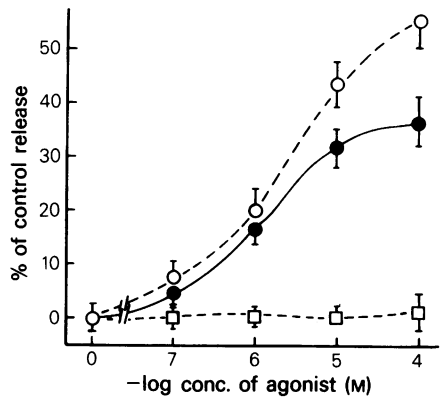

Figure 5 Effects of Ca^{2+} on the release of kallikrein-like esterase produced by various concentrations of noradrenaline. Dispersed cells were incubated in the absence (control) and presence of various concentrations of noradrenaline with or without Ca^{2+}. Kallikrein-like esterase release (ordinate scale) is expressed as % of total kallikrein-like esterase activity in the control cells. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean. (○) Ca^{2+} (9.0 mM) and noradrenaline; (●) Ca^{2+} (1.8 mM) and noradrenaline; (□) noradrenaline without Ca^{2+}.

the secretory pattern between male and female animals. Dopamine, 5-hydroxytryptamine (5-HT) and histamine were almost without effect (data not shown). The ability of three α-adrenoceptor agonists, noradrenaline, methoxamine and phenylephrine to release kallikrein-like esterase was compared (Figure 4). Methoxamine was the most effective and phenylephrine the least (Figure 4). The noradrenaline (0.01 μM–μM)-induced kallikrein-like esterase release was attenuated or completely abolished by prior addition of phenoxybenzamine (10 μM) to the incubation medium (Figure 4).

Effect of Ca-ion on kallikrein-like esterase release

The effects of Ca-ion on the release of kallikrein-like esterase induced by noradrenaline was examined in the dispersed cell preparations (Figure 5). In the absence of Ca-ion, no stimulatory effect of noradrenaline on kallikrein-like esterase release was observed. When Ca (9 mM) was added to the incubation medium, the degree of kallikrein-like esterase release by noradrenaline was slightly higher than those in normal Ca concentration (1.79 mM).

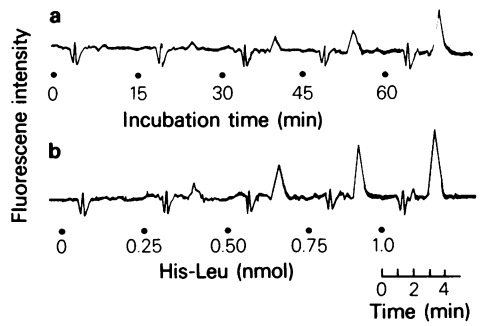

Figure 6 H.p.l.c. chromatograms of the incubation sample of tonin assay (a) and standard solution of His-Leu (b) after OPT reaction. (a) Aliquots of homogenates (2 μg protein) were incubated with angiotensin I (100 μg) for various times as described in Methods and then 100 μl of incubation sample after OPT reaction was supplied into the h.p.l.c. fluorescence analysis system, consisting of a Waters Model 204 Liquid Chromatograph with 6000A Solvent Delivery System, U6K Universal Injector, Radial Pack A column, and Jasco-FP-550A Spectrofluorometer equipped with a flow-cells unit (cell volume; 15 μl). The mobile phase was a mixture of 0.5 M phosphate buffer (pH 4.0) and methanol (40:60). The fluorescence intensity was monitored at Ex. 360/Em.440. (b) After completing OPT reaction with standard solution of His-Leu, aliquots of the reaction mixture were injected into the h.p.l.c. system as described above.

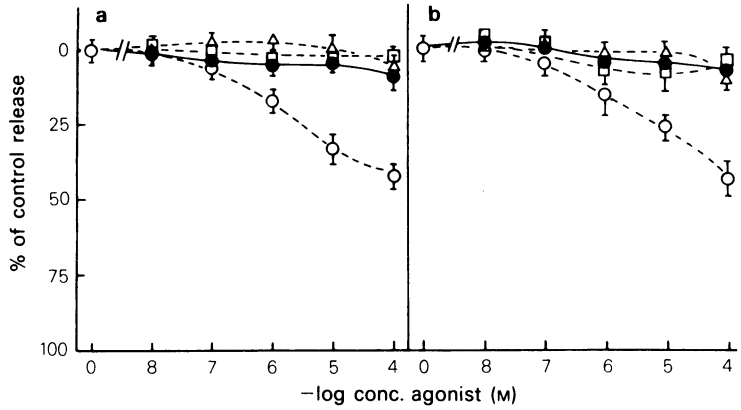

Figure 7 Effects of noradrenaline, isoprenaline and methacholine on tonin release from dispersed cells of male (a) and female (b) rat submandibular glands. Dispersed cells were incubated for 30 min at 30°C in the absence (control) and presence of various concentrations of noradrenaline, isoprenaline and methacholine. Tonin release (ordinate scale) is expressed as % of total tonin activity in the control cells. Results are expressed as mean of 3 experiments; vertical lines show s.e.mean. Each experiment is the average of duplicate determinations. (○) Noradrenaline; (●) methacholine; (□) isoprenaline; (△) noradrenaline and phenoxybenzamine.

Assay of tonin activity

Tonin activity was measured by a fluorometric method with angiotensin I as substrate. In order to examine whether or not the intensity of fluorophore formed after OPT reaction is dependent on the amount of His-Leu released from angiotensin I by tonin, we injected the incubation sample into the high performance liquid chromatography (h.p.l.c.) system. As shown in Figure 6b, the retention time of His-Leu-OPT fluorophore was 3.3 min and this intensity depended on the amount of His-Leu added. Except for this peak, no fluorophore was detected. After incubation with substrate (angiotension I) and homogenates (2.0 μg protein) prepared from rat submandibular glands for various times, the incubation mixtures were treated with OPT. When 100 μl aliquots of these samples were injected into the h.p.l.c. system. Only one peak corresponding to the retention time of His-Leu-OPT fluorophore was observed and the intensity of these peaks increased as a function of incubation time (Figure 6a), indicating that during incubation, only His-Leu was released from angiotensin I by tonin enzyme.

Tonin release

Figure 7 shows the effects of α-, β-adrenoceptor and cholinoceptor agonists on tonin release from dispersed cells of both sexes. The experiments were carried out in the same manner as shown in Figure 3. Only noradrenaline increased the tonin release dose-dependently, and this release was completely abolished by the addition of phenoxybenzamine. There was no significant tonin release by isoprenaline or methacholine. No diffrence of secretory pattern of tonin was observed between cells obtained from male and female rats.

Table 1 Kallikrein-like esterase and tonin activities in the submandibular glands of male and female rats

	Male	*Female*
Kallikrein-like esterase ($\mu mol\ mg^{-1}\ min^{-1}$)	175.39 ± 22.35 (5)	137.81 ± 21.52 (5)
Tonin ($\mu mol\ mg^{-1}\ min^{-1}$)	353.39 ± 89.99 (10)	288.58 ± 19.85 (10)

Assay conditions are described in Methods. Each value represents the mean ± s.e.mean. The number of animals is given in parentheses. Kallikrein-like esterase activity is expressed as μmol BAEe hydrolysed per mg protein and per min. Tonin activity is expressed as μmol His-Leu released per mg protein and per min.

Kallikrein-like esterase and tonin activities in male and female rat submandibular gland

Kallikrein-like esterase and tonin activities in rat submandibular gland were compared between male and female (Table 1). No statistically significant difference in either enzyme was detected between two sexes.

Discussion

Except for publications dealing with potassium-release (Quissel & Redman, 1979) and morphological changes (Kanamura & Barka, 1975), there are no data in the literature using dispersed cell preparations for the analysis of secretory mechanisms of the rat submandibular gland. The dispersed cells prepared from the rat submandibular gland by the present method released kallikrein-like esterase in response to noradrenaline (10 μM), an α-adrenoceptor agonist, as a function of time at least up to 40 min. The net decrease of kallikrein-like esterase from the cells after stimulation with noradrenaline (10 μM) corresponded to the increase in the incubation medium at all concentrations of noradrenaline used (Figure 2). The other two α-adrenoceptor agonists, methoxamine and phenylephrine, were also effective stimulants for kallikrein-like esterase release. The α-adrenoceptor antagonist phenoxybenzamine suppressed the kallikrein-like esterase release by noradrenaline (Figure 4). These results and the Ca-ion dependence of this response (Figure 5) demonstrate that functional integrity of α-adrenoceptors in the rat submandibular gland was retained after the isolation procedure. In the case of dispersed cell preparations of mouse submandibular gland prepared in the same manner, the functional integrity of cholinoceptors was also retained as well as that of α-adrenoceptors (Izumi *et al.*, 1982; 1983a; Izumi & Aoki, 1984).

It is now believed that proteases such as kallikrein and renin are largely localized within GCT cells of rat (Orstavik & Gautvik, 1977), mouse (Gresik *et al.*, 1978; Simson *et al.*, 1979; Izumi *et al.*, 1982; 1983b), cat (Hojima *et al.*, 1977) and guinea-pig (Schachter *et al.*, 1978) submandibular glands, and that their enzymes are predominantly secreted by α- but not by β-adrenoceptor stimulation (Matthews, 1974; Orstavik & Gautvik, 1977; Abe & Dawes, 1978; Hosoi *et al.*, 1978; Izumi & Aoki, 1981; Izumi *et al.*, 1982). As shown in Figures 3 and 7, the release of kallikrein-like esterase and tonin were elicited only by noradrenaline and were completely inhibited by phenoxybenzamine, suggesting that both kallikrein and tonin secretion in rat submandibular gland are mediated via stimulation of α-adrenoceptors but not by β-adrenoceptors or cholinoceptors. Some caution should be exercised, however, concerning this lack of secretory response by secretagogues, since a reduction or perturbation of receptor activity by proteolytic digestion is known to occur in rat submandibular gland (Kanamura & Barka, 1975) as well as in other hormone receptors (Amsterdam & Jamieson, 1972). Alteration of receptor sensitivity following trypsin digestion has also been reported in rat parotid acinar cells (Mangos *et al.*, 1975). In spite of this possible reservation, it seems unlikely that the lack of secretory response of these isolated cells is due to a destruction or perturbation of β-adrenoceptors or cholinceptors in the plasma membrane by proteolytic enzymes contained in the crude collagenase, since a single injection of isoprenaline or pilocarpine into the rat did not result in a detectable reduction in the activities of total gland esterase (kallikrein) (Orstavik & Gautvik, 1977) or tonin (Garcia *et al.*, 1977).

The secretion of tonin has been reported to be effected by β-adrenoceptor stimulation (Garcia *et al.*, 1976; 1977; Kondo, 1979; Gutkowska *et al.*, 1982). These studies have been carried out *in vivo* (Garcia *et al.*, 1976, 1977; Gutkowska *et al.*, 1982) or *in vitro* using gland slices (Kondo, 1979). In both of these experimental approaches, the interpretation of experimental data by these methods is complicated by the presence of neural tissue and possible subsequent secondary release of endogenous neurotransmitters and the existence of pericellular components that may alter the secretory response, as well as by dilution, metabolic degradation, or biochemical alteration of injected secretagogues in the body fluids.

In the present studies, various secretagogues for kallikrein-like esterase and tonin release in both male and female rats were investigated. A sexual difference in the secretory pattern of renin (Izumi *et al.*, 1982) and esterase (Izumi *et al.*, 1983a; Izumi & Aoki, 1984) in response to methacholine in mouse submandibular gland has previously been reported. As shown in Figures 3 and 7, however, there was no difference in the secretory pattern of kallikrein-like esterase and tonin release between the two sexes in this preparation from rat submandibular gland. Furthermore, no significant difference of kallikrein and tonin activities was observed between male and female (Table 1), suggesting that there is no sexual dimorphism in rat submandibular gland in biochemical as well as in morphological expression (Gresik, 1980).

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