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Functional characteristics of dispersed rat submandibular cells

(tissue dissociation/secretagogue response/receptors/exocrine cells/mucin synthesis)

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ABSTRACT **Rat submandibular gland cells have been obtained through enzymatic dispersion using chromatographically purified collagenase (EC 3.4.24.3) and hyaluronidase (EC 3.2.1.35) and gentle mechanical force. The recovery of viable cells after the isolation procedure was 59% on the basis of total glandular DNA content. Approximately 60% of the total cell population consisted of acinar cells; less than 8% were immature granular duct cells; and the remainder were intercalated duct, striated duct, and myoepithelial cells. Most of the acinar cells were in acinar-intercalated duct complexes. The integrity of the isolated cells was substantiated by their exclusion of trypan blue, intracellular electrolyte composition, incorporation of [^{14}C]glucosamine into trichloroacetic acid + phosphotungstic acid precipitable material at a linear rate for 1.5 hr, secretory responses to parasympathomimetic and sympathomimetic stimulation, and morphologic integrity as determined by light and electron microscopy. The cholinergic receptors were characterized through investigation of the net transmembrane flux of K^+ in response to carbamoylcholine. The α-adrenergic receptors were characterized by investigating the net transmembrane flux of K^+ in response to norepinephine stimulation and the β-adrenergic receptors were characterized by determining the rate of secretion of ^{14}C-labeled mucin after isoproterenol stimulation. A high degree of sensitivity to both cholinergic and adrenergic secretagogues was observed.**

The acinar cells of the rat submandibular gland are best described as seromucous, histochemical analysis showing that they contain large amounts of neutral glycoproteins and some acid glycoproteins (1). Thus, unlike the rat parotid or pancreatic acinar cells, the rat submandibular acinar cells synthesize, store, and secrete appreciable amounts of high molecular weight mucins. In the past, the secretory processes of submandibular gland function have been studied *in vivo* (2) or *in vitro* in gland slices (3, 4). In both of these experimental approaches the interpretation of the biochemical data is complicated by the presence of neural tissue and possible subsequent secondary release of endogenous neurotransmitters, other extracellular elements that impose diffusional barriers, and existence of pericellular compartments that may alter the secretory response.

In 1972 Amsterdam and Jamieson (5) reported a method for the isolation of pancreatic exocrine cells. The method involved the use of crude collagenase (EC 3.4.24.3) and hyaluronidase (EC 3.2.1.35) digestion, chelation of divalent cations, and mild shearing forces. However, due to the lack of reproducibility of this method, they subsequently modified their tissue dissociation procedure (6, 7) by using chromatographically purified collagenase, hyaluronidase, and α-chymotrypsin (EC 3.4.21.1). While the separated cells appeared to be functional, the net release of amylase and protein material after stimulation was 50% less then previously reported for pancreatic slices. In addition, the concentration of secretagogue required to elicit the secretory response had to be increased 10-fold.

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Similar procedures have been developed for the dissociation of salivary gland cells. Barka *et al.* (8, 9) have recently reported a method for the dissociation of rat parotid and submandibular gland cells. However, after dissociation, the submandibular gland cells were unable to respond to β-adrenergic stimulation. The lack of response to stimulation by isoproterenol was probably due to the destruction or perturbation of the β-adrenergic receptors during the isolation procedure. Of the various methods reported for the dissociation of salivary gland cells (8–14), the best-characterized system is that of Mangos *et al.* (10–12). Although the dispersed rat parotid cells were able to respond to cholinergic, α-adrenergic, and β-adrenergic agonists, the resultant cell population had an abnormally low intracellular K^+ concentration, approximately 90 meq/liter of cellular water, indicating some metabolic alterations may have occurred during the isolation procedure.

In this communication we describe a cell dispersion procedure for rat submandibular cells that was derived from the method of Mangos *et al.* (10–12). The procedure results in a population of cells that demonstrate a high sensitivity to cholinergic and adrenergic agonists and are able to retain a more nearly normal intracellular ionic environment after isolation.

MATERIALS AND METHODS

Male Sprague–Dawley rats from Charles River Breeding Laboratories weighing 175–220 g (42–48 days old) were used. The animals were housed under controlled environmental conditions and allowed food and water ad lib. Reagents were obtained from the following sources: chromatographically purified collagenase at 200–400 units/mg, Worthington (listed as EC 3.4.4.19); acetylcholine chloride, atropine sulfate, bovine serum albumin (fraction V), carbamoylcholine (carbamylcholine) chloride, *l*-isoproterenol·HCl, *l*-norepinephrine·HCl, bovine testes hyaluronidase at 300 units/mg, Hepes, D-glucosamine·HCl, Sigma; D-[1-^{14}C]glucosamine·HCl at 59–61 mCi/mmol (1 Ci = 3.7×10^{10} becquerels), Amersham; ^{125}I-labeled albumin, Squibb (New York); basal medium (Eagle) amino acids (100×), GIBCO; *l*-propranolol and *d*-propranolol were the kind gifts of R. Deghenghi, Ayerst Laboratories (New York); phentolamine was a generous gift of C. A. Brownley, Jr., Ciba Pharmaceutical.

Preparation of Cell Suspension. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (6–10 mg/100 g of body weight) and the submandibular–sublingual complexes were removed through a ventral midline incision of the neck. The submandibular glands were separated from the sublingual glands, minced, suspended in the dispersion solution, and incubated in a gyratory shaker for 60 min at 37°C in an atmosphere of 95% O_2/5% CO_2, pH 7.4. The dispersion solution consisted of a Ca^{2+}- and Mg^{2+}-free modified Hanks' salt solution (15 mM Hepes) that contained chromatographically purified collagenase (50–75 units/ml) and hyaluronidase

(1.0 mg/ml). Toward the end of the incubation period, the tissue was repeatedly pipetted and washed twice with a Ca^{2+}- and Mg^{2+}-free modified Hanks' salt solution containing 15 mM Hepes. The cells were passed through a nylon mesh and collected, and the cells were aggregated by centrifugation. The cells were then resuspended in the final incubation medium, a modified Hanks' salt solution containing 15 mM Hepes and 2% bovine serum albumin. The pH of all solutions was adjusted to 7.4 with 0.1 M Na_3PO_4 and the osmolality of all solutions was adjusted to 300 mosmol/kg with 1 M NaCl.

Morphological Studies. The cells and samples of fresh tissue were fixed and embedded in Araldite, and sections were examined by light and electron microscopy as described (15), except that in some instances acrolein was omitted from the primary fixative. The proportions of the total cell population composed of each type of parenchymal cell were estimated for adult and 45-day-old rat submandibular glands and for the pellets by light microscopic surveys of thick (1 μm) Araldite sections. Cells were counted by category to an aggregate total of 1000 cells for each section used. Only clearly recognizable cells with nuclei were counted.

Net K^+ Efflux Studies. The cells were suspended in fresh incubation medium after a 30-min incubation period with a packed cell volume to medium volume of 3.5–5%; 50 μl of cell suspension was removed and centrifuged in an Eppendorf microcentrifuge, model 5412, at 11,000 rpm for 1 min. The supernatant was removed and analyzed for its K^+ content. This sample corresponded to the 0 time sample. After the addition of agonist, antagonist, or both, samples were taken at various time intervals and centrifuged and the supernatants were removed and analyzed for their K^+ content. The total K^+ concentration in the cell suspension system was determined from the supernatant after sonication of the cell suspension. The amount of K^+ released into the medium was expressed as a percentage of the total intracellular K^+ available for release.

Release of ^{14}C-Labeled Material Precipitable by Trichloroacetic Acid and Phosphotungstic Acid ("Acid-Precipitable Material"). The cells were suspended, in the final incubation medium supplemented with basal medium (Eagle) amino acids, incubated for 1 hr in the presence of 1 μCi of D-[1-^{14}C]glucosamine per ml, and then washed four times in incubation medium containing 1 mM glucosamine. After 1 hr in incubation medium containing 1 mM D-glucosamine, the cells were washed three times and resuspended in incubation medium with a packed cell volume to medium volume of 2.5–5%. Ascorbic acid (0.1 mM) was added to the cell suspension to retard the autooxidation of isoproterenol. A 200-μl sample of cell suspension was removed and the amount of acid-precipitable ^{14}C-labeled material was determined. After the addition of agonist, antagonist, or both, samples were removed and the supernatants were analyzed for their acid-precipitable dpm. The amount of acid-precipitable dpm released was expressed as a percentage of the total acid-precipitable dpm in the cell suspension.

Analytical Procedures. Cell suspensions, cell pellets, or supernatants were precipitated and washed three times with ice-cold 10% trichloroacetic acid and 0.5% phosphotungstic acid. The precipitates were hydrolyzed in 1.0 ml of 88% (wt/vol) formic acid prior to liquid scintillation spectroscopy. ACS, aqueous counting scintillant, from Amersham was used as the liquid scintillation counting cocktail. Radioactivities of samples were measured in a Beckman 100 LS liquid scintillation spectrophotometer and the values were corrected for quenching by use of an external standard. Counting efficiency was about 80%. Na^+ and K^+ concentrations were determined by flame photometry. DNA content was determined by using the spectrophotometric method of Richards (16).

RESULTS

Dissociation Procedure. The procedure developed for the dissociation of rat submandibular gland cells differs from that of Mangos *et al.* (10–12) in that trypsin or other general proteases were not used and the Hanks' balanced salt solution was supplemented with 15 mM Hepes. When hyaluronidase was omitted the extent of dissociation was not appreciably altered; however, the amount of cells recovered after isolation was markedly decreased. It was also noted that Hanks' balanced salt solution, a bicarbonate buffer, did not provide the necessary buffer capacity during the isolation procedure. Rapid changes in pH were observed and only when 15 mM Hepes was included did the rate of pH change dramatically decrease. However, it still was necessary to monitor the pH and maintain it between 7.4 and 7.2 throughout the entire experimental procedure. Although developed in an empirical fashion, the resultant method produced cells that were viable and functional. In comparison with the original method of Mangos *et al.* (10–12), the total yield of cells and the intracellular electrolyte composition were greatly enhanced by the procedure developed here (Table 1). With this method the proportion of total cells recovered was 59% ± 7.5 (SD), on the basis of total recovered DNA, with a range of 48–70%, whereas the recovery of submandibular cells with the previously described method (10–12) was found to be around 40%, with a range of 34–44%. The *in vivo* intracellular Na^+ and K^+ values were 18 meq/liter of cellular water and 155 meq/liter of cellular water, respectively. Although slightly lower K^+ values and slightly higher Na^+ values were obtained in the cell suspensions (Table 1), the overall intracellular electrolyte values were markedly improved over those obtained with the unmodified cell dispersion procedure.

Morphologic Analysis of Dispersed Cells. The submandibular salivary gland of the adult rat is a tubulo-acinar organ. The acini and intercalated ducts are invested with myoepithelial cells (17, 18). The secretory granules in the granular ducts contain three proteases that are active in alkaline media (19). These ducts differentiate after puberty (about 30 days) and are androgen dependent as well as responsive to nervous stimulation (2, 18, 19). It seemed plausible that the granular duct proteases might be released during the cell dispersion procedure, resulting in damage to cell membranes and secretagogue receptor sites. Therefore, we considered using prepubertal rats. However, submandibular glands from rats younger than about 40 days are complicated, for purposes of studying secretory cells, by the presence of terminal tubule and proacinar cells, in addition to the mature acinar cells (20). Therefore, we selected rats aged 42–48 days in which acinar differentiation is complete while granular duct differentiation is just beginning. In sections of both the intact submandibular gland (Fig. 1 *top*) and the dis-

Table 1. Rat submandibular cellular electrolyte and water composition

Exp.	Cell composition		
	Water, %	Na^+, mM	K^+, mM
1	71.3 ± 0.46	56.6 ± 3.44	121 ± 4.1
2	73.7 ± 0.33	52.0 ± 2.26	119 ± 3.2
3	72.4 ± 0.74	53.3 ± 2.69	129 ± 8.2

Values are mean ± SD for five determinations. Intracellular Na^+ and K^+ were determined after a 30-min incubation of cells after isolation. ^{125}I-Labeled albumin was used as an extracellular space marker. The cells were pelleted by centrifugation and the amount of medium entrapped in the pellets was calculated from the ^{125}I-labeled albumin content. An amount of Na^+ and K^+ equivalent to that amount of medium was subtracted from the total amount in the pellet. The remaining Na^+ and K^+ was assumed to be intracellular.

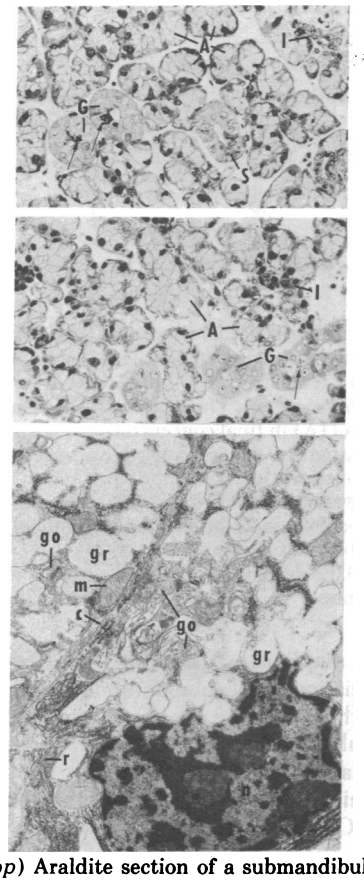

FIG. 1. (*Top*) Araldite section of a submandibular gland of a 45-day-old male rat. The acini (A) and intercalated ducts (I) are fully differentiated; terminal tubule and proacinar cells are not seen. The granular ducts (G) have only a few cells with granules (arrows) and are as numerous as the striated ducts (S), from which they differentiate. (Methylene blue/azure II; ×162.) (*Middle*) Araldite section of a pellet of dispersed cells prepared from the submandibular glands of 42-day-old male rats. Acini (A) generally are intact and frequently remain attached to short segments of intercalated duct (I). In many of the granular duct cells (G), cells with granules have disintegrated (arrow). By this process many segments of granular ducts probably reverted to the appearance of striated ducts. (Methylene blue/azure II; ×162.) (*Bottom*) Electron micrograph of the preparation similar to that in *Middle*. Cell membranes (c), mitochrondria (m), nuclei (n), and rough endoplasmic reticulum (r) generally are in good condition, though an occasional cleft is seen in the rough endoplasmic reticulum (lower left corner). In most cells, the secretory granules (gr) and Golgi apparatus (go) are well preserved, as in the cell on the left. In some, however, they are partly disrupted, as in the cell on the right. (Approximately ×6550.)

persed cell pellets (Fig. 1 *middle*) from 42- to 45-day-old rats, the proportion of acinar cells was about 60%. However, the proportion of granular ducts was decreased (from 10% to 7%) while the striated ducts increased (from 10% to 13%) in the cell dispersions. This appeared to be due to partial degranulation of the granular ducts during the dispersion process, with degranulated segments resembling and being counted as striated ducts (Fig. 1 *middle*). By way of comparison, the proportions of acinar, striated duct, and granular duct cells in intact submandibular glands from mature rats were 55%, 7%, and 22%, respectively.

By both light and electron microscopy (Fig. 1 *middle* and *bottom*), most of the acinar cells emerged from the dispersion process in good condition, with intact cell membranes, minimal loss of secretory granules or leakage of their contents, and usually intact mitochondria and other organelles. This generally healthy appearance was substantiated by the finding that more than 90% of the cells excluded trypan blue for up to 4 hr after dispersion. An important observation was that, after dispersion, most of the acinar cells remained in their natural groupings as acinar–intercalated duct complexes. Ultrastructurally, these complexes were invested by myoepithelial cells, and their lumina were well preserved, including intact junctional complexes (not shown).

Functional Studies. Because rat submandibular acinar cells synthesize, store, and secrete high molecular weight mucous material, the functional integrity of the isolated cells was assessed by measuring the rate of incorporation of radioactively labeled amino sugar into mucins and by determining the cellular responsiveness and sensitivity to cholinergic and adrenergic agonists and antagonists.

The rate of D-[1-^{14}C]glucosamine incorporation into acid-soluble and precipitable fractions is shown in Fig. 2. Within 20 min the intracellular acid-soluble fraction had equilibrated, and, after a short lag period, the synthesis of ^{14}C-labeled acid-precipitable material continued to increase in a linear fashion throughout the rest of the labeling period. Less than 3% of the total acid-precipitable dpm were extractable into chloroform and methanol (2:1 vol/vol). When the acid precipitate was dissolved in 0.1 M Tris·HCl buffer containing 0.1% sodium dodecyl sulfate at pH 9.0 and applied to a Sephadex G-50 column, more than 95% of the total dpm applied to the column were eluted in the void volume; this material thus has a molecular mass of at least 10,000 daltons.

We have reported (3, 4) that rat submandibular glands when incubated *in vitro* in a gland slice system release K^+ into the medium after stimulation with cholinergic and α-adrenergic secretagogues. The secretory response of the isolated cells to cholinergic secretagogue stimulation is shown in Fig. 3. The net efflux of K^+ was 40% within 30 sec and 37% 1 min after stimulation. Because phentolamine, an α-adrenergic antagonist, could not prevent the net efflux of K^+, and atropine sulfate, a muscarinic cholinergic antagonist, could antagonize the efflux of K^+ after cholinergic stimulation, the net efflux of K^+ in these cells after carbamoylcholine addition appeared to be a muscarinic cholinergic receptor-mediated response. These data also indicate that the cholinergic receptors remained intact and were functional after the isolation procedure.

The effect of various adrenergic agonists and antagonists on the net K^+ efflux was also investigated. As shown in Fig. 4 *upper*, the net efflux of K^+ was 44% at 30 sec after the addition of norepinephrine to the cell suspension and 39% at 1 min. The addition of isoproterenol, a β-adrenergic agonist, resulted in

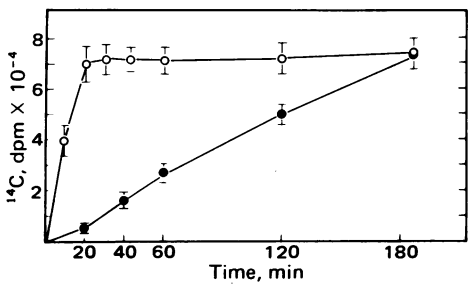

FIG. 2. Incorporation rate of D-[1-^{14}C]glucosamine into the cellular trichloroacetic acid/phosphotungstic acid soluble (○) and precipitable (●) fractions per ml of cell suspension. The submandibular cells were incubated in the presence of 1 μCi of D-[1-^{14}C]glucosamine per ml. At various time intervals a portion of the cell suspension was removed and the cells were washed four times in incubation medium containing 4 mM glucosamine. The washed cells were precipitated and washed three times with ice-cold 10% trichloroacetic acid and 0.5% phosphotungstic acid containing 4 mM glucosamine. Each value represents the mean and SD from four experiments.

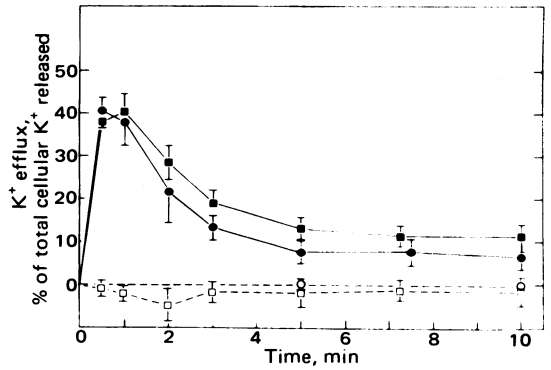

FIG. 3. Net K^+ efflux from isolated submandibular cells as a percentage of total cellular K^+ content after parasympathomimetic stimulation. Carbamoylcholine in concentration of 10 μM (●) induced a rapid K^+ efflux within 30 sec. Prior addition of atropine to the incubation medium in final concentration of 10 μM (□) inhibited the cellular response to the addition of carbamoylcholine (10 μM), whereas the prior addition of phentolamine (■) to the incubation medium in final concentration of 10 μM had no effect on the cellular response to the addition of carbamoylcholine (10 μM). No net K^+ efflux was observed in the absence of secretagogue (○). Each value represents the mean and SD from at least eight experiments.

no net efflux of K^+ from the isolated cells. The efflux of K^+ after norepinephrine stimulation appeared to be an α-adrenergic receptor-mediated response because atropine sulfate or *l*-propranolol did not prevent the net efflux of K^+. However, the prior addition of phentolamine, an α-adrenergic antagonist, did antagonize the cellular response to norepinephrine stimulation (Fig. 4 *lower*). These data demonstrate that the functional integrity of the α-adrenergic receptors was retained after the isolation procedure.

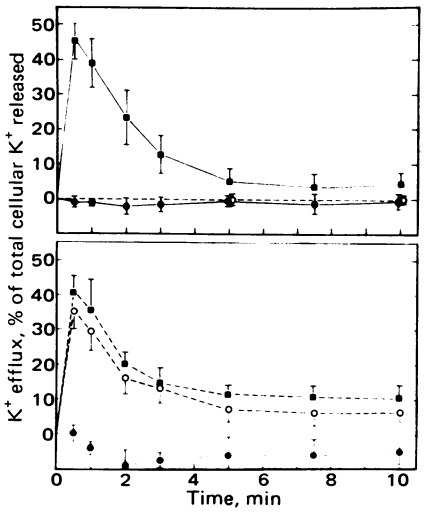

FIG. 4. Net K^+ efflux from isolated submandibular cells as percentage of total cellular K^+ content after sympathomimetic stimulation. As shown in *Upper*, norepinephrine in final concentration of 10 μM (■) induced a rapid efflux of K^+, whereas the addition of isoproterenol in final concentration of 10 μM (●) did not produce a net efflux of K^+. No net K^+ efflux was observed in the absence of secretagogue (○). As shown in *Lower*, the prior addition of phentolamine (●) to the incubation medium in a final concentration of 10 μM inhibited the cellular response to the addition of norepinephrine (10 μM), whereas the prior addition of atropine sulfate (■) or *l*-propranolol (○) to the incubation medium in a final concentration of 10 μM did not inhibit the cellular response to the addition of norepinephrine. Each value represents the mean and SD from at least eight experiments.

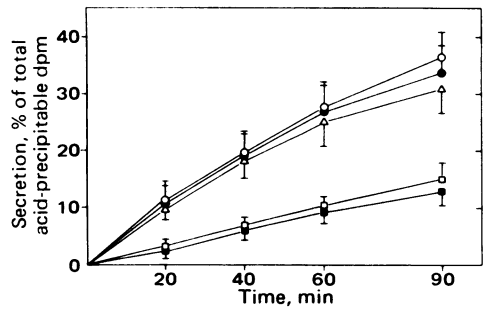

FIG. 5. Secretion of [^{14}C]glucosamine-labeled mucin in response to a sympathomimetic agonist and selected antagonists. *l*-Isoproterenol in final concentration of 10 μM (○) induced a rapid release of acid-precipitable material. The prior addition of phentolamine (●) or *d*-propranolol (△) in final concentration of 10 μM did not antagonize the stimulatory release caused by isoproterenol (10 μM), whereas the prior addition of *l*-propranolol (□) in a final concentration of 10 μM was able to antagonize the release of acid-precipitable material caused by the addition of isoproterenol. A slight loss of acid-precipitable material was observed in the absence of agonist or antagonists (■). Each value represents the mean and SD from at least four experiments.

Kanamura and Barka (9) have recently described a method for the dissociation of rat submandibular cells. However, after dissociation the cells were unable to respond to β-adrenergic agonist stimulation. To assess the secretory response of the rat submandibular cells after isolation by the method described in this communication, the effects of isoproterenol on the rate of release of ^{14}C-labeled acid-precipitable material was determined. After the cells had been labeled for 1 hr with D-[1-^{14}C]glucosamine, they were incubated for 1 hr in medium containing 1 mM glucosamine. The receptor-mediated specificity for the release of acid-precipitable material by isoproterenol was then evaluated. The results of these experiments are summarized in Fig. 5. The prior addition of phentolamine or *d*-propranolol did not antagonize the stimulatory release of acid-precipitable material caused by isoproterenol addition, but *l*-propranolol added to the cell suspension medium prior to stimulation by isoproterenol was able to antagonize this release. These data demonstrate that the β-adrenergic receptors remained intact and were functional after this isolation procedure.

Previous cell dispersion procedures have resulted in a viable cell suspension system, but the sensitivity of the cells to various stimuli was markedly decreased (5, 9). To evaluate the sensitivity of these submandibular cells to various agonists, the dose–response relationships for carbamoylcholine, norepinephrine, and isoproterenol were investigated. The results indicate that the dose–responses for all three agonists were quite similar (Fig. 6) with median effective doses (ED_{50}) at 0.71, 0.56, and 0.25 μM for carbamoylcholine, norepinephrine, and isoproterenol, respectively.

DISCUSSION

The method described in this communication utilizes the combined enzymatic digestion by collagenase and hyaluronidase coupled with mild shearing forces for the dispersion of functionally intact cells from the rat submandibular gland. The use of other proteolytic enzymes and the use of media with rather low buffer capacity resulted in a population of cells having a low total intracellular K^+ concentration. Gentle pipetting of the cells and slow addition of Na_3PO_4 for pH adjustment was also found to be essential for good recovery and viability.

The fact that this method does not completely dissociate the

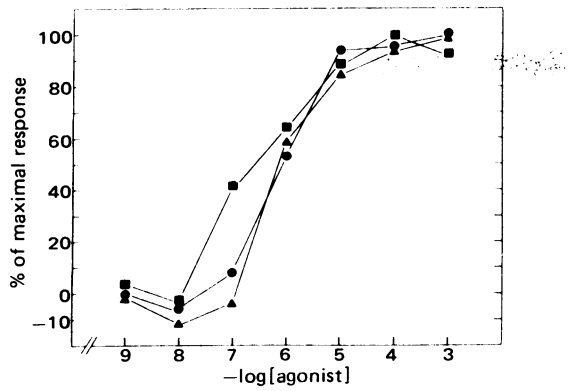

FIG. 6. Dose response of isolated cells to carbamoylcholine (●), norepinephrine (▲), and isoproterenol (■). The secretory response to carbamoylcholine and norepinephrine represents the percent of the maximal net K^+ efflux at 45 sec after stimulation and the secretory response to isoproterenol represents the percent of the maximal amount of acid-precipitable material released in 40 min after stimulation. Each value represents the mean of at least eight experiments.

cells but rather leaves them in secretory complexes may be more relevant to what actually occurs *in vivo*. Recently, Hammer and Sheridan (21) have reported that rat submandibular acinar cells are electrically coupled. Cell-to-cell communication via these low-resistance junctions appears to be important during the secretory response. Presumably the coupling is through the gap junctions between the cells as described by Hand (22). Therefore, it may be necessary to preserve this form of cell-to-cell communication when studying the secretory response of these cells *in vitro*.

When the secretory response to cholinergic and adrenergic agonists was investigated in submandibular cells obtained by using the method of Mangos *et al.* (10–12), the results were qualitatively the same as shown in Figs. 3 and 4 but quantitatively different. That is, the amount of K^+ released at maximal stimulation was significantly ($P < 0.01$) higher (44–48%) with the new method than that obtained by using the method of Mangos *et al.* (10–12) (32–36%). Therefore, not only did the submandibular cells contain higher intracellular levels of K^+ with the new method but also the cells released a greater percentage of their total cellular K^+ during cholinergic and α-adrenergic stimulation. These data suggest that the use of trypsin and the lack of sufficient buffer capacity in the original Hanks' medium may result in a population of cells having an abnormally high flux of ions and subsequently a lower intracellular K^+ concentration. Or a mixed population of cells may be present, some very leaky with very low levels of intracellular K^+ and some not as leaky with near normal intracellular K^+ levels. The difference between the two methods in the resultant cell population may be very dramatic when one begins to examine the stimulus–secretion coupling mechanism in these cells. Abnormally high flux rate of ions or a mixed cell population could conceivably result in an overall abnormal metabolic response.

The fact that the cells obtained by using this new method were able to secrete up to 50% of their total intracellular K^+ and then were able to pump back a large portion of the released K^+ within 5 min also indicates the maintenance of their intracellular metabolism and the functional integrity of their plasma membranes. Glycosyltransferase activity of the isolated cells also appeared to be functional in that a linear incorporation of glucosamine into acid-precipitable material was observed over a 1.5-hr period. The present method has greatly improved the conditions necessary for the isolation of rat submandibular gland cells, although the method and procedures may not be ideal. The method results in the isolation of the principal secretory cells in their natural functional units (acinar-intercalated duct complexes), which demonstrate a high degree of sensitivity to a variety of secretagogues.

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