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Introduction of BAPTA into intact rat submandibular acini inhibits mucin secretion in response to cholinergic and β-adrenergic agonists

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Incorporation of the calcium chelator BAPTA into isolated, intact rat submandibular acini by hypotonic swelling, resulted in complete inhibition of carbamylcholine, noradrenaline and isoproterenol stimulation of mucin secretion. No effects of intracellular BAPTA on cell viability or β-adrenergic stimulation of cyclic AMP formation were observed. The data are the first to demonstrate that calcium is necessary for β-adrenergic stimulation of secretion and suggest that Ca^{2+} provides a common link in the triggering of exocytosis.

Submandibular; Mucin secretion; β-Adrenergic; Cholinergic; Calcium chelator

1. INTRODUCTION

Mucin secretion from rat submandibular acinar cells is primarily mediated by the stimulation of β-adrenergic receptors [1–3]. Increased cyclic AMP levels, leading to the activation of protein kinase A and phosphorylation of specific proteins [4] have been suggested as the trigger of exocytosis. However, incorporating excess cyclic AMP phosphodiesterase into intact cells, using a hypotonic swelling method [5], abolished the cyclic AMP rise elicited by a maximal concentration of isoproterenol, without affecting the stimulation of mucin secretion. It was suggested therefore, that mucin release is stimulated by a second messenger other than cyclic AMP. The role of Ca^{2+} is not clear: whereas β-agonist-stimulated mucin secretion was partially inhibited by the removal of extracellular Ca^{2+} and isoproterenol mobilized intracellular Ca^{2+} without increasing $Ins(1,4,5)P_3$ formation [3,6], ionophore A23187, at non-lytic concentrations, increased the intracellular free Ca^{2+} concentration in rat submandibular acini bud did not trigger mucin secretion [7]. It is also not clear whether cholinergic agonists, which act via Ca^{2+} in stimulating enzyme secretion from pancreatic acinar cells [8], increase mucin secretion from submandibular acinar cells [1,2,9].

In order to directly investigate the role of Ca^{2+} in the mucin secretion response to physiological agonists, we have examined the effects, on cholinergic and β-adrenergic stimulation of mucin secretion, of introducing the calcium chelator BAPTA into intact rat submandibular acini by hypotonic swelling.

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy) ethane-*NNN'N'*-tetra-acetic acid; BSA, Bovine serum albumin; KHB, Krebs-Henseleit bicarbonate; TES, *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid

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2. EXPERIMENTAL

Submandibular acini were isolated from overnight-fasted Wistar rats (250–280 g) as described previously [3]. BAPTA was incorporated into isolated acini as previously described [5]. Briefly, to acini suspended in TES-buffered saline (10 mM TES, pH 7.4 containing 143 mM NaCl, 4.7 mM KCl, 1.1 mM $MgCl_2$, 1 mg/ml BSA) was added an equal volume of either 10 mM TES, pH 7.4 (swollen) or TES-buffered saline (unswollen), each containing 5 mM ATP and 10 mM BAPTA where indicated, for 1 min at room temperature, followed by washing and resuspension in KHB buffer (see [5]) containing 20 mg/ml BSA.

Mucin secretion was measured as described previously [3,5], except that acini were labelled with 5 μCi/ml D-[1-^{3}H]glucosamine-HCl (3.92 Ci/mmol from Amersham). After exposure to hypotonic or isotonic medium followed by 15 min incubation at 37°C in KHB buffer, as described above, acini were washed and incubated under experimental conditions at 37°C. ^{3}H-labelled mucins, released into the medium at zero time and after 30 min, were precipitated and their radioactivity measured as previously described [3,5]. Protein content of cell pellets was determined by the method of Lowry et al. [10] and mucin release over 30 min expressed as dpm/mg protein. Owing to the variation in unstimulated mucin release between experiments (range for unswollen acini: 721–2454 dpm/mg protein; for swollen acini: 601–4357 dpm/mg protein), the data in each experiment are presented as % basal (stimulated release/unstimulated release) × 100. Medium collected in the same way but not precipitated was used to assay lactate dehydrogenase release as previously described [5].

Cyclic AMP was measured after 5 min under experimental conditions, in aliquots of acini suspensions (0.5 ml), rapidly frozen in liquid nitrogen, followed by the addition of an equal volume of 20% trichloroacetic acid and thawing on ice with thorough mixing. ATP was measured after 30 min under experimental conditions in acini pellets (following centrifugation for 10 s in an Eppendorf Microfuge) mixed with 0.5 ml 10% trichloroacetic acid and left on ice for 20 min. In both cases, extracts were thoroughly mixed with 2 Vols of 0.5 M tri-*n*-octylamine (Aldrich Chemicals) in 1,1,2-trichlorofluoroethane

(BDH), centrifuged and the aqueous phase removed and stored at −20°C prior to assay. Cyclic AMP was assayed using a radioimmunoassay kit (Amersham); ATP was assayed as previously described [3], using a luminometer (Berthold Biolumat LB9500T).

3. RESULTS

3.1. *Dose–response of carbamylcholine stimulation of mucin release*

Fig. 1 shows that carbamylcholine stimulated mucin release from rat submandibular acini in a concentration-dependent manner, with maximum stimulation at 10 μM. The response was completely blocked by the cholinergic antagonist, atropine. Maximum stimulation of mucin secretion by the β-adrenergic agonist isoproterenol, included in the same experiments, was approx. twice that induced by the cholinergic agonist (Fig. 1).

3.2. *Actions of intracellular BAPTA*

Table I shows that unswollen and swollen submandibular acini have similar secretory responses to isoproterenol, noradrenaline and carbamylcholine. Basal mucin release was unchanged following swelling in the presence or absence of BAPTA (Unswollen: 1152 ± 151, n = 12; Swollen: 1017 ± 64, n = 11; Swollen + BAPTA: 1533 ± 297, n = 12, dpm/mg protein). Isoproterenol and noradrenaline stimulated secretion to the same degree (approx. 3-fold) at a maximally effective concentration, in both swollen and unswollen cells. When directly compared in the same experiments carbamylcholine was less effective (Fig. 1), although the data in Table I are not significantly different. Stimulation was unaffected by incubation of acini for 1 min at room temperature with 10 mM BAPTA in isotonic medium. However, stimulation by all agonists was abolished by intracellular incorporation of the calcium chelator BAPTA, at a concentration (10 mM) during swelling which would give an intracellular concentration of approx. 1 mM [11] and inhibit a stimulated Ca^{2+} rise [12,13].

Table II shows the effects of swelling acini in the presence of BAPTA on two parameters of cell viability, ATP content and lactate dehydrogenase leakage. None of the secretagogues affected either parameter in swollen or unswollen cells (data not shown). It can be seen that ATP concentrations and LDH leakage were not different in unswollen cells and cells swollen in the presence of BAPTA, suggesting that inhibition of mucin release under these conditions is not due to a decrease in cell viability.

The effect of the introduction of BAPTA into submandibular acini, on the maximum stimulation of cyclic AMP levels in response to the three agonists, was also investigated. Table III shows that isoproterenol and noradrenaline, but not carbachol, significantly stimulated cyclic AMP levels by approx. 6-fold, in agreement with previous data [5,14]. Cyclic AMP formation in response to β-adrenergic stimulation was unaffected by the incorporation of BAPTA.

4. DISCUSSION

The hypotonic swelling method, developed using rat pancreatic [11,15] and submandibular [5] acini, has allowed the direct manipulation of concentrations of putative intracellular regulators, by the incorporation of normally impermeant molecules such as calcium chelators and enzymes into intact cells [5,11]. It was previously shown, in rat submandibular acini [5], that preventing the increase in cyclic AMP in response to isoproterenol, did not inhibit stimulation of mucin release, suggesting that other second messengers are involved in the cellular response to β-adrenergic stimulation. In the current study, two lines of evidence suggest that calcium is a primary regulator of mucin secretion: firstly carbamylcholine, an agonist known to act via calcium to stimulate protein secretion in pancreatic and parotid acinar cells (see [8]), also stimulated mucin secretion. Secondly, incorporation of the calcium chelator BAPTA into in-

Table I

Effects on mucin release of BAPTA introduced into rat submandibular acini by hypotonic swelling

Experimental condition	Mucin release (% Control)		
	+10 μM isoproterenol	10 μM noradrenaline	+ 10 μM carbamylcholine
Unswollen	284.4 ± 23.2 (8)	291.1 ± 19.0 (7)	233.3 ± 15.0 (4)
Unswollen + 10 mM BAPTA	245.2 ± 21.1 (4)	243.4 ± 18.4 (3)	211.0 ± 5.8 (4)
Swollen	288.5 ± 18.3 (3)	251.5 ± 28.6 (4)	193.8 ± 18.4 (4)
Swollen + 10 mM BAPTA	98.4 ± 23.2 (4)**	115.8 ± 9.5 (4)*	106.2 ± 2.6 (4)*

After labelling with [^{3}H]glucosamine, isolated acini were incubated for 1 min at room temperature in either isotonic (unswollen) or hypotonic (swollen) buffer ± 10 mM BAPTA. Following washing and incubation for 15 min at 37°C in KHB buffer, acini were placed in the experimental conditions shown above and incubated for a further 30 min at 37°C for measurement of mucin release. Results are mean ± SEM for the number of experiments shown in parentheses (duplicate incubations per experiment). **P<0.001; *P<0.01 for difference from equivalent swollen control.

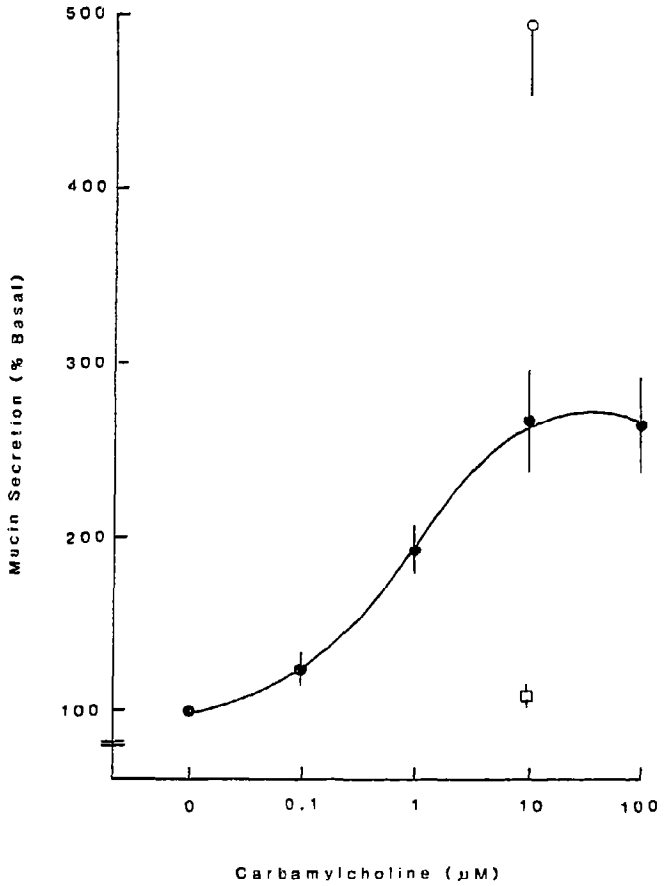

Fig. 1. Effect of carbamylcholine on mucin secretion from rat submandibular acini. Acini were labelled with [^{3}H]glucosamine and incubated for 30 min at 37°C in the experimental conditions, for measurement of mucin release as described in section 2. Results are mean ± SEM for the following numbers of experiments (duplicate incubations per experiment): (●) + carbamylcholine, n = 4–7; (□) + 10 μM carbamylcholine/30 μM atropine, n = 3; (○) + 10 μM isoproterenol, n = 8.

Table II

Effects of the introduction of BAPTA into isolated rat submandibular acini on ATP content and the lactate dehydrogenase (LDH) leakage

Experimental condition	ATP content (nmol/mg protein)	LDH leakage per 30 min (% total cell content)
Unswollen	2.71 ± 0.48 (12)	4.16 ± 0.46 (16)
Unswollen + 10 mM BAPTA	N.D.	3.62 ± 0.44 (16)
Swollen	N.D.	5.16 ± 0.76 (16)
Swollen + 10 mM BAPTA	2.38 ± 0.62 (11)	4.74 ± 0.45 (16)

Isolated acini, incubated for 1 min at room temperature in either isotonic (unswollen) or hypotonic (swollen) buffer ± 10 mM BAPTA, were washed, incubated for 30 min at 37°C in KHB buffer and placed in the experimental conditions shown for a further 30 min at 37°C. LDH release into the medium or cellular ATP content were measured as described in section 2. Results are mean ± SEM for the number of experiments shown in parentheses (duplicate incubations per experiment).

tact, viable cells inhibited stimulation of mucin secretion by physiological neurotransmitters.

The data (Fig. 1 and Table I) showing carbamylcholine stimulation of mucin secretion are in agreement with those of others showing stimulation of sialic acid release from intact tissue fragments and [^{14}C]glucosamine-labelled glycoproteins from isolated acinar cells [1,9] and suggest that dissociated acinar-ductal complex preparations [2] might have lost cholinergic receptors, or the coupled response. The isolated acini preparations used in the present study have low (<5%) ductal contamination and >95% cell viability [3].

Introduction of the calcium chelator BAPTA into isolated submandibular acini not only inhibited stimulation of mucin secretion (Table I) by carbamylcholine, but also both noradrenaline and isoproterenol stimulation of mucin secretion. This is unlikely to be due to a non-specific effect of BAPTA or the swelling procedure, since cell viability was unaffected and manipulations which altered cyclic AMP levels did not affect mucin secretion responses [5]. That intracellular BAPTA did not inhibit the rise in cyclic AMP induced by isoproterenol or noradrenaline (Table III) suggests that the me-

Table III

Effects of the introduction of BAPTA into isolated rat submandibular acini on stimulation of cyclic AMP content

Stimulus	Cyclic AMP content (pmol/mg protein)	
	Unswollen	Swollen + 10 mM BAPTA
None	15.26 ± 1.69	15.03 ± 3.50
10 μM isoproterenol	92.56 ± 7.37	118.92 ± 44.24
10 μM noradrenaline	91.62 ± 8.48	92.75 ± 25.23
10 μM carbamylcholine	22.61 ± 4.43	20.89 ± 4.78

Isolated acini, incubated for 1 min at room temperature in either isotonic (unswollen) or hypotonic (swollen) buffer ± 10 mM BAPTA, were washed, incubated for 15 min at 37°C in KHB buffer and placed in the experimental conditions shown for a further 5 min at 37°C. Cellular cyclic AMP was measured as described in section 2. Results are mean ± SEM for 3 experiments (duplicate incubations per experiment).

chanism by which β-adrenergic agents stimulate secretion in exocrine epithelial cells is not solely through the action of cyclic AMP. This finding has relevance to the abnormalities in exocrine secretion seen in cystic fibrosis [16]. Thus, intracellular BAPTA mimics the defective β-adrenergic stimulation of mucin secretion, with the normal maximal cyclic AMP rise characteristic of CF submandibular and other affected epithelial cells [17,18]. The present data suggest that modulation of Ca^{2+} signalling pathways in CF cells is likely to prove fruitful as a means of correcting the fundamental secretory defect in this disease.

Since an intracellular Ca^{2+} rise induced by ionophore A23187 did not trigger mucin secretion from rat submandibular acini [7], it is likely that physiological neurotransmitters exert their actions by an interaction of Ca^{2+} with other intracellular signals. Isoproterenol stimulated calcium mobilisation [3,19] and increased cytoplasmic free Ca^{2+} concentrations in salivary acinar and canine tracheal cells [13,20–22]. In addition, the introduction of calcium chelators into rat submandibular acinar cells via a patch pipette, inhibited isoproterenol stimulation of whole-cell K^+ and Cl^- currents [23]. Activators of protein kinase C also increased mucin release [24], although β-adrenergic stimulation did not result in the activation of phosphoinositidase C as a route for diacylglycerol production [6].

Thus, the data are a direct demonstration that intracellular Ca^{2+} is essential for triggering mucin secretion by physiological stimuli acting via cholinergic and β-adrenergic receptors. In addition, noradrenaline, the activity of which in stimulating mucin secretion is not identical to that of isoproterenol in that it apparently has a non-α, non-β component to its action [3] was also blocked by chelation of intracellular Ca^{2+}. Although it is likely that secretion can be stimulated independently by the activation of protein kinase A or protein kinase C, the data suggest that during physiological stimulation, calcium acts in a common distal pathway in the stimulation of exocytosis.

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