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THE RELATIONSHIP OF INTRACELLULAR FREE CALCIUM ACTIVITY TO AMYLASE SECRETION IN SUBSTANCE *P*- AND ISOPRENALINE-STIMULATED RAT PAROTID ACINI

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Abstract—The relationship between intracellular free calcium activity ($[Ca^{2+}]_i$) and stimulated amylase secretion was investigated in rat parotid acini by measuring the effects of substance *P* methyl ester and isoprenaline on quin2 fluorescence and amylase release. Although both of these drugs evoked concentration-dependent increases in $[Ca^{2+}]_i$ and amylase release, the tachykinin had a greater effect on $[Ca^{2+}]_i$ while the catecholamine was the more effective secretagogue.

Whereas isoprenaline exerted equipotent effects on amylase secretion and $[Ca^{2+}]_i$, the dose–response relationship for stimulation of secretion by substance *P* was dissociated by three orders of magnitude to the right of that for elevation of $[Ca^{2+}]_i$ by this peptide.

It is concluded that these data do not support the hypothesis that substance *P*-stimulated amylase secretion is mediated solely through an increase in $[Ca^{2+}]_i$ and that other second-messengers may be involved in mediation of this secretory response.

Secretagogues which stimulate amylase release in the rat parotid salivary gland appear to exert their effects upon the secretory acinar cells through activation of two alternative second-messenger systems [1]. Whereas amylase release in response to muscarinic cholinergic, α-adrenergic or tachykinin receptor stimulation is associated with the generation of inositol polyphosphates [2] and mobilisation of Ca^{2+} from both rapidly exchanging extracellular, and slowly exchanging intracellular, sites [3], the larger enzyme secretory response to β-adrenergic agonists is associated with the generation of cyclic AMP and mobilisation of Ca^{2+} from intracellular sites [4]. Although it is generally accepted that Ca^{2+} mobilisation, which is a common feature of both of these second-messenger pathways, may reflect changes in cellular Ca^{2+} metabolism leading to an increase in $[Ca^{2+}]_i$ in the acinar cell, until recently, support for this hypothesis depended upon data obtained from the use of experimental techniques which could only provide an indirect indication of changes in $[Ca^{2+}]_i$. However, the introduction of Ca^{2+}-sensitive fluorescent dyes such as quin2 has made possible more direct measurement of $[Ca^{2+}]_i$ [5].

In the experiments described in this paper we have utilized the quin2 fluorescence technique to assess the effects of substance *P* methyl ester and of isoprenaline on $[Ca^{2+}]_i$ in isolated rat parotid acini. These data have been compared with the concentration-dependence for stimulation of amylase release by these secretogogues in order to test the hypothesis that this response is mediated by an increase in $[Ca^{2+}]_i$. A preliminary account of some of this work has already been reported [6].

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MATERIALS AND METHODS

Preparation of isolated, quin2-loaded acini. Male Wistar rats, 180–220 g body weight, were killed by cervical dislocation and exsanguinated. In each experiment the ventromedial portions of both parotid glands were removed from three rats and trimmed free of lymph nodes and adipose tissue. The glands were placed in a Petri dish and 4 ml of Krebs–Henseleit buffer (composition: NaCl, 104 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.4 mM; D-glucose, 10 mM; HEPES, 2 mM; adjusted to pH 7.4 with NaOH) containing 100 U/ml collagenase was injected into the interstitium of the glands. The bloated glands were then transferred to a conical flask and finely chopped with scissors. The flask was top-gassed with 100% O_2, stoppered and incubated in a water bath at 37° for 15 min. At the end of this incubation period as much of the supernatant was removed as possible. A further 4 ml of Krebs–Henseleit buffer containing collagenase (100 U/ml) was added and the tissue suspension was passed ten times through a pipette tip (1.2 mm i.d.), then top-gassed with 100% O_2 and incubated at 37° for a further 15 min. At the end of this second incubation period the tissue suspension was passed ten times through a pipette tip (1.2 mm i.d.) and then five times each through syringes fitted with 19 and 21 gauge hypodermic needles respectively. The disrupted tissue was then transferred to a centrifuge tube, diluted to 14 ml with Krebs–Henseleit buffer containing 0.1% (w/v) bovine serum albumin (which was added to all subsequent buffers) and centrifuged at 500 *g* for 3 min. The supernatant was discarded and the pellet resuspended in 14 ml Krebs–Henseleit buffer before re-centrifugation at 500 *g* for a further 3 min. The supernatant was discarded and the pellet was resuspended in 5 ml of the same buffer. The

resuspended tissue was filtered through nylon bolting cloth (mesh diameter 150 μm) and the filtrand was washed with 9 ml of Krebs–Henseleit buffer. The filtrate was then centrifuged at 500 *g* for 1 min. The supernatant was discarded and the pellet, containing isolated acini, was resuspended in 6 ml Krebs–Henseleit buffer containing 50 μM quin2 acetoxymethyl ester. This acinar suspension was divided into two aliquots which were each incubated in cuvettes with stirring (200 rpm) for 30 min at 37°. At the end of this incubation period the acini were washed by centrifugation at 500 *g* for 1 min and resuspension of the pellet in 3 ml Krebs–Henseleit buffer. This washing procedure was repeated a further four times to remove extracellular quin2 ester and the acini were resuspended to a final volume of 12 ml in Krebs–Henseleit buffer.

Measurement of quin2 fluorescence. Cuvettes containing washed, quin2-loaded acini were placed in a fluorimeter (Perkin–Elmer 3000) and incubated at 37° with stirring (200 rpm). The fluorescent signal of the acinar suspension was continuously recorded using emission and excitation wavelengths of 490 nm and 340 nm respectively. When a stable basal fluorescence had been attained substance *P* methyl ester or isoprenaline were added directly to the incubated tissue as 3 or 10 μl of concentrated stock solutions of 10 nM–1 mM substance *P* or 10 μM–10 mM isoprenaline in Krebs–Henseleit buffer. In control experiments the extracellular fluorescence signal was measured by addition of $MnCl_2$ (20 mM stock) to the cuvettes and Mn^{2+} was subsequently chelated with diethylenetetraminepentaacetic acid (DETPA) (40 mM stock). At the end of each experiment the intracellular and extracellular quin2 fluorescence and autofluorescence were determined by addition of excess $MnCl_2$ followed by chelation of the Mn^{2+} with DETPA, disruption of acini with 10–50 μl of 1% (v/v) Triton X-100 and then addition of excess $MnCl_2$. $[Ca^{2+}]_i$ was calculated as described by Rink and Pozzan [7], assuming a K_d value for quin2–Ca^{2+} of 115 nM and quin2 fluorescence enhancement of 6.2-fold in the presence of Ca^{2+}.

Measurement of amylase release. Quin2-loaded acini were prepared as described above and then divided into 250 μl aliquots which were preincubated for 5 min at 37° in polythene reaction tubes. Substance *P* methyl ester or isoprenaline were then added directly to the incubating tissue as 25 μl of concentrated stock solutions of 1 nM–300 μM substance *P* or 100 nM–300 μM isoprenaline in Krebs–Henseleit buffer. The tissue was then incubated for a further 10 min at 37° prior to sedimentation of the acini by centrifugation at 9700 *g* for 20 sec. One-hundred-and-fifty microlitres of supernatant was removed from each reaction tube and stored at 4° for subsequent determination of secreted amylase activity. Ten microlitres of 0.1% (v/v) Triton X-100 was then added to each reaction tube and the contents vortex-mixed and left to stand for a minimum of 60 min at 4° prior to centrifugation at 9700 *g* for 60 sec and removal of 50 μl aliquots of the supernatant for subsequent determination of tissue amylase content.

The amylase activity in supernatants was determined by Bernfeld's method [8] as previously described [4].

In control experiments quin2-loaded acini which were either only preincubated for 5 min, or preincubated for 5 min and then incubated for a further 10 min after addition of 25 μl of Krebs–Henseleit buffer containing no secretogogues, were centrifuged at 9700 *g* for 15 sec and then treated as described above to determine basal amylase secretion.

Materials. All chemicals were analytical grade. Substance *P* methyl ester was purchased from Cambridge Research Chemicals PLC (Cambridge, U.K.), isoprenaline sulphate from Sigma Chemical Co. (Poole, Dorset, U.K.), collagenase (CLSPA) from Lorne Diagnostics PLC (Twyford, U.K.) and quin2 acetoxymethyl ester from Lancaster Synthesis PLC (Morecambe, U.K.).

Statistical analyses. Significance of difference between means was calculated using Student's *t*-test. The pD_2 values were calculated from regression analysis of the linear portions of dose–response curves and regression and covariance analysis were performed using least-square methods. The level of probability set for rejection of the null hypothesis was $P < 0.05$.

RESULTS

Effects of substance P *on* $[Ca^{2+}]_i$

The calculated $[Ca^{2+}]_i$ in unstimulated acini was 188 ± 6 nM ($N = 168$). Both the total quin2 fluorescence signal and that part of the signal (observed in the presence of manganese) which is believed to be of intracellular origin, remained constant for up to 45 min of incubation. Addition of substance *P* methyl ester (30 pM–1 μM) to the incubated acini stimulated a rapid and concentration-dependent increase in fluorescence which reached a peak value within 30 sec of drug application and then gradually declined towards the basal level (Figs. 1 and 2).

The dose–response relationship for elevation of $[Ca^{2+}]_i$ by substance *P* is illustrated in Figs. 2 and 3. The threshold concentration of the tachykinin was 30 pM or less, and the maximal increase in calculated $[Ca^{2+}]_i$ (201 ± 12% of basal, $N = 14$) was observed when the acini were incubated in the presence of 10 nM substance *P*. The ED_{50} was 575 pM ($pD_2 = 9.2 \pm 0.1$, $N = 68$). At higher concentrations of the tachykinin there was a trend for the stimulated response to decline although this was not confirmed statistically.

In similarity to substance *P*, isoprenaline (10 nM–30 μM) also stimulated a concentration-dependent increase in quin2 fluorescence, but both the time course and the magnitude of this effect were very different to that observed with the tachykinin. Thus isoprenaline stimulated a more gradual increase in fluorescence which took several minutes to develop (Fig. 2) and attained a maximum value, in the presence of 1 μM isoprenaline, which corresponded to only a 24 ± 6% ($N = 14$) increase in calculated $[Ca^{2+}]_i$ above basal (Fig. 3). The ED_{50} for this effect of isoprenaline was 155 nM ($pD_2 = 6.8 \pm 0.1$, $N = 47$).

Effects of secretagogues on amylase release

Both substance *P* and isoprenaline stimulated

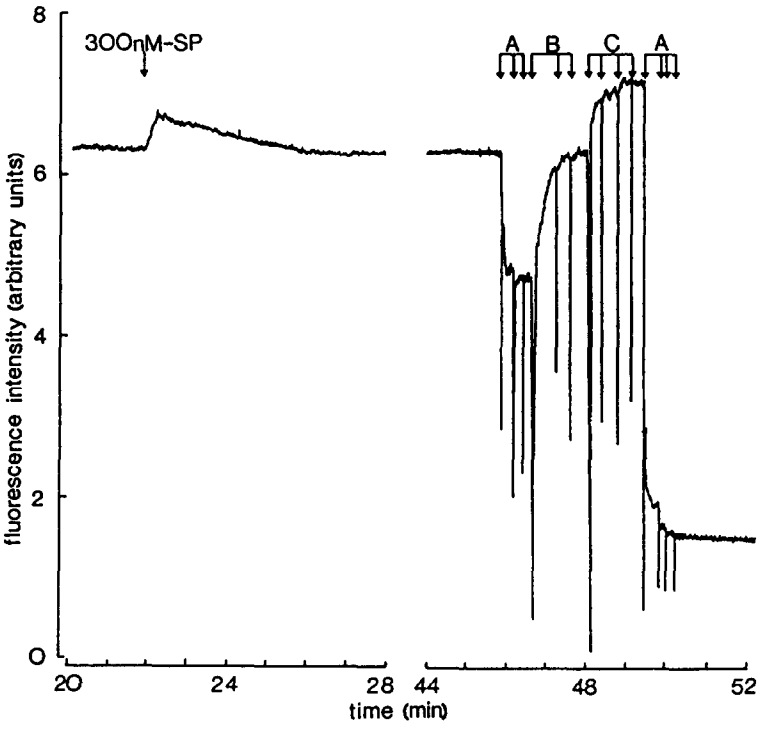

Fig. 1. Representative trace illustrating the time course of substance *P* methyl ester-stimulated quin2 fluorescence in incubated parotid acini and the protocol for calibration of the fluorescence signal. Substance *P* (SP, 300 nM) 20–100 μl of 20 mM stock $MnCl_2$ (A), 20 μl of 40 mM stock DETPA (B) and 20 μl of 1% (v/v) stock Triton X-100 (C) were added to the acinar suspension at times indicated by arrows. The large deflections of the trace observed in the calibration procedure are artefacts introduced during addition of $MnCl_2$, DETPA and Triton X-100 to the acinar suspension.

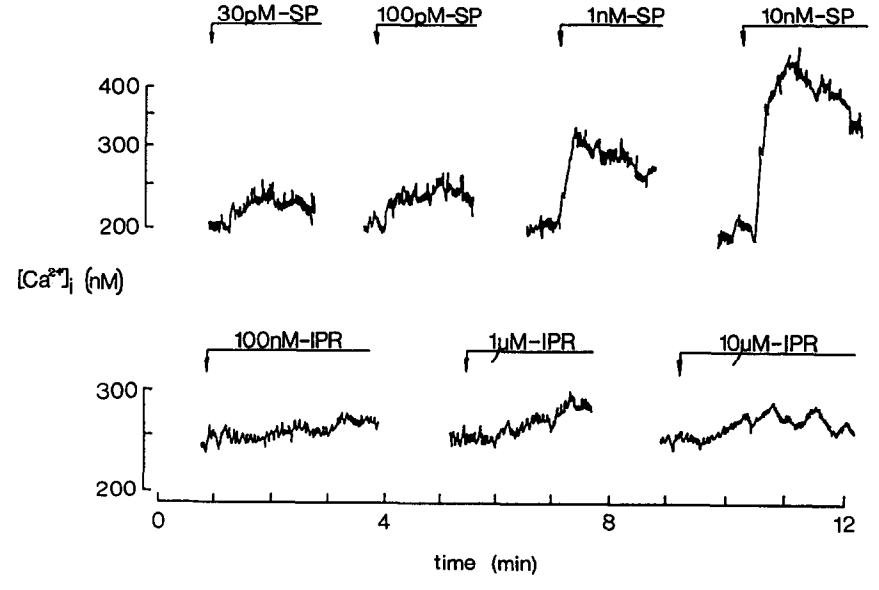

Fig. 2. Individual traces of the effects of substance *P* methyl ester (SP) and isoprenaline (IPR) on quin2 fluorescence in incubated parotid acini. SP and IPR were added to acinar suspensions at the times indicated by arrows and were present for the duration of the horizontal bars. $[Ca^{2+}]_i$ was calculated as described in Methods and is indicated on the ordinate (logarithmic scale). Each trace is representative of between 5 and 24 similar experiments, from which the mean data are illustrated in Fig. 3.

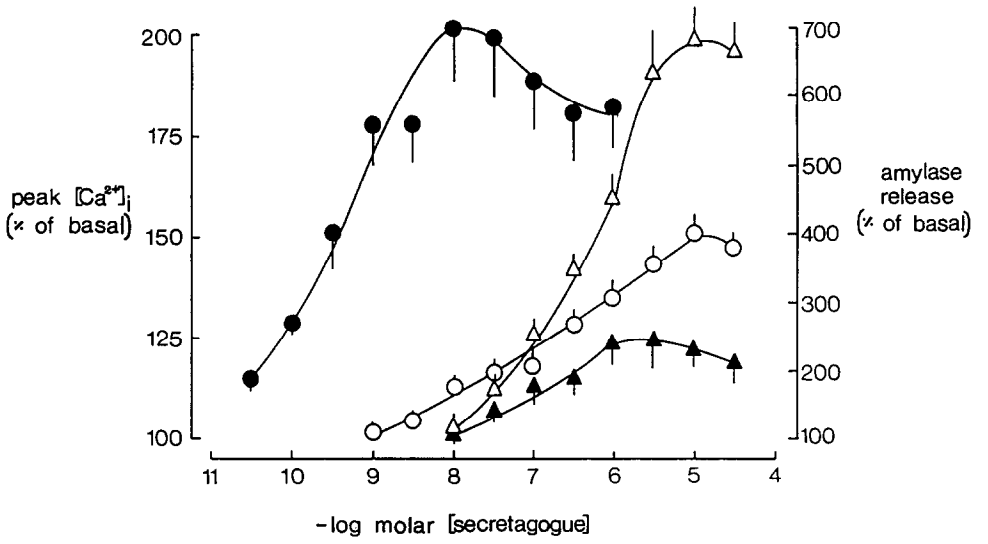

Fig. 3. Dose–response relationships for stimulated increases in $[Ca^{2+}]_i$ and amylase release in parotid acini incubated in the presence of either substance *P* methyl ester or isoprenaline (●, effect of substance *P* on $[Ca^{2+}]_i$; ▲; isoprenaline on $[Ca^{2+}]_i$; ○, substance *P* on amylase release; △, isoprenaline on amylase release). Increase in $[Ca^{2+}]_i$ was calculated from the maximum quin2 fluorescence value observed after addition of secretagogue and is expressed as a percentage of basal $[Ca^{2+}]_i$ calculated from the fluorescence value observed immediately prior to addition of secretagogue. Amylase release is expressed as a percentage of the basal value measured in the absence of secretagogue. Each point is the mean of 5–24 observations and SEM mean is indicated by vertical bars.

amylase release from incubated acini. In contrast to their relative effects on elevation of $[Ca^{2+}]_i$, isoprenaline was a more effective amylase secretagogue than substance *P* (Fig. 3). The concentrations of substance *P* which elicited threshold and maximal stimulation of amylase secretion were 30 nM and 10 μM respectively, the ED_{50} was 138 nM (pD_2 = 6.9 ± 0.1, $N = 72$) and there was significant ($P < 0.001$) dissociation of the dose–response relationships for elevation of $[Ca^{2+}]_i$ and stimulation of amylase secretion by the tachykinin (Fig. 3). Corresponding threshold and maximal values for stimulation of secretion by isoprenaline were 30 nM and 10 μM and the ED_{50} was 347 nM (pD_2 = 6.5 ± 0.1, $N = 56$). There was no significant dissociation of the dose–response curves for the effects of isoprenaline on $[Ca^{2+}]_i$ and amylase release (Fig. 3).

DISCUSSION

The data presented in this paper provide direct demonstration that substance *P* stimulates a concentration-dependent increase in $[Ca^{2+}]_i$ in parotid acini. The basal level of $[Ca^{2+}]_i$ observed here is similar to that previously reported in acinar cells by Takemura [9], and the magnitude of the maximum response to substance *P* (201% of basal $[Ca^{2+}]_i$) is identical to that seen after stimulation of quin2-loaded acini with acetylcholine [9]. The similarity in magnitude of these responses to substance *P* and acetylcholine lends support to the conclusion from other studies that tachykinins and muscarinic cholinergic agonists share common post-receptor mechanisms in the parotid acinar cell [1].

When considered in isolation, our data concerning the effects of substance *P* on $[Ca^{2+}]_i$ are consistent with the idea that an increase in $[Ca^{2+}]_i$ is involved in the coupling of this peptidergic stimulus to amylase secretion in parotid acinar cells. However, it should be noted that the dose–response relationship for substance *P*-stimulated increases in $[Ca^{2+}]_i$ lies substantially to the left of that for stimulated amylase secretion. Therefore, although our data are in agreement with the hypothesis that stimulation of amylase secretion by low (< 10 nM) concentrations of substance *P* is mediated through increased $[Ca^{2+}]_i$, we interpret the lack of effect of higher concentrations (> 10 nM) of the tachykinin (which stimulate a further increase in amylase release) on $[Ca^{2+}]_i$ to indicate that this simple model of stimulus–secretion coupling cannot account for the effects of a wide range of substance *P* concentrations on Ca^{2+} metabolism and amylase secretion in the parotid acinar cell.

Possible explanations of this dissociation of the effects of substance *P* on $[Ca^{2+}]_i$ and amylase secretion include variation in the distribution of Ca^{2+} within the intracellular compartment such that our measurements of changes in $[Ca^{2+}]_i$ do not accurately reflect changes in a smaller pool more closely associated with the exocytotic process, and also the involvement of other second-messengers in the secretory response. At present, these suggestions are largely speculative, but Ca^{2+} has been shown to occupy a very restricted domain within the cytosol of other cell types [10, 11] and the results of experiments performed in our laboratory suggest the possibility that diacylglycerol, generated as a consequence of an increase in membrane phosphatidylinositol turnover, may act as an additional second-messenger in the substance *P*-stimulated parotid gland [12].

The dose–response relationship of substance *P*-

stimulated diacylglycerol generation is unknown. However, Dreux *et al.* [13] have recently demonstrated a biphasic relationship between substance *P* stimulation and [^{3}H]-protein secretion with pD_2 values of 9.2 and 6.2 (although no such biphasic response was detected in either the present work or other studies [14] which employed measurements of amylase release to assess secretory activity) and have suggested that the two phases of secretion may be related to inositol trisphosphate-dependent Ca^{2+} mobilisation and diacylglycerol accumulation respectively. Our observed pD_2 of 9.2 for elevation of $[Ca^{2+}]_i$ is consistent with this model.

To provide further examination of the relationship between substance *P*-peptidergic stimulation and secretion, it is of interest to compare dose–response relationships for elevation of $[Ca^{2+}]_i$ and stimulation of amylase release, reported here, with the relationships, as reported previously, for other steps in the series of events which may link this peptidergic stimulation to secretion. The pK_d for substance *P* binding in the parotid gland is reported as 8.4 [15, 16], a value that is very similar to the pD_2 of 8.5 for the substance *P*-stimulated phosphatidylinositol response [17]. The approximation of these values to the pD_2 of 9.2 for elevation of $[Ca^{2+}]_i$ leads us to the conclusion that there may be a close association between receptor binding, the phosphatidylinositol response and elevation of $[Ca^{2+}]_i$ with little spare capacity between these three events in the signal transduction pathway.

Our data concerning the effects of isoprenaline on $[Ca^{2+}]_i$ are in agreement with those presented by Takemura [9] who also reported that the β-adrenergic agonist increases $[Ca^{2+}]_i$ to a maximum of 24% above basal. A comparison of the dose–response relationship for isoprenaline-stimulated increase in $[Ca^{2+}]_i$ with that for stimulation of amylase release reveals that the catecholamine exerts approximately equipotent effects on these two parameters. Thus, in contrast to substance *P*-stimulated acini, an increase in $[Ca^{2+}]_i$ is closely associated with stimulation of amylase secretion by isoprenaline over a wide range of secretagogue concentrations. Although these data may be interpreted as providing indirect support for the view that Ca^{2+} may act as a second messenger during β-adrenergic stimulation of amylase secretion, they do not exclude the possibilities that the stimulated increase in $[Ca^{2+}]_i$ may be unrelated to exocytotic secretion or may serve a modulatory, rather than mediatory, function, and that other second-messengers may play a more direct role in this stimulus–secretion coupling mechanism.

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