Receptor-activated calcium entry in exocrine cells does not occur via agonist-sensitive intracellular pools

Trevor J. SHUTTLEWORTH
Department of Physiology, Box 642, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A.

Currently, most models describing receptor-activated Ca\(^{2+}\) entry in exocrine cells invoke a pathway for the entry of extracellular Ca\(^{2+}\) directly linking the agonist-sensitive intracellular Ca\(^{2+}\) pools with the plasma membrane. In the avian exocrine tissue, we have found that Ca\(^{2+}\) entry during refilling of the intracellular pools following termination of receptor activation (by atropine) occurs via the cytoplasm and not directly into the empty pools. Under appropriate conditions this can be demonstrated as a transient increase in [Ca\(^{2+}\)]\(_i\) (intracellular Ca\(^{2+}\) concn.) seen on restoration of normal extracellular Ca\(^{2+}\) concentrations after atropine to stimulated cells whose intracellular stores have been prevented from refilling by incubation in a low-extracellular-Ca\(^{2+}\) medium. The magnitude of these [Ca\(^{2+}\)]\(_i\), transients decays with time, but with a time course markedly slower than for the corresponding decrease in intracellular Ins(1,4,5)P\(_3\). Further experiments have revealed that Ca\(^{2+}\) entry into the cytoplasm during the initial stimulation phase is also direct and not via the intracellular pools. Thus the initial rates of increase in [Ca\(^{2+}\)]\(_i\), during stimulation are always faster in conditions where both Ca\(^{2+}\) entry and Ca\(^{2+}\) release occur (i.e., they are additive). These differences could not be explained by any effects of extracellular Ca\(^{2+}\) on the initial increases in intracellular Ins(1,4,5)P\(_3\) after addition of carbachol. These data are therefore inconsistent with the current models in which the rate of Ca\(^{2+}\) entry through the agonist-sensitive pools cannot exceed the rate of Ca\(^{2+}\) release. It appears therefore that Ca\(^{2+}\) entry and Ca\(^{2+}\) release must occur via separate pathways operating in parallel, and not in series as previously predicted.

INTRODUCTION

In most exocrine cells, intracellular signalling pathways for ion and fluid secretion involve increases in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) that comprise two distinct components: a mobilization of intracellular Ca\(^{2+}\) stores, and a sustained entry of Ca\(^{2+}\) from the extracellular medium. Such responses are known to be associated with an increased turnover of membrane phosphoinositides, especially phosphatidylinositol 4,5-bisphosphate, and the subsequent generation of the two second messengers diacylglycerol and Ins(1,4,5)P\(_3\). Considerable evidence obtained from a variety of different cell types has established the critical role of Ins(1,4,5)P\(_3\) in releasing Ca\(^{2+}\) from intracellular stores (Streb et al., 1983; see also Bertridge, 1986), but the limited capacity of these stores means that they are only capable of producing transient increases in [Ca\(^{2+}\)]\(_i\). However, in various exocrine cells, it is clear that the full secretory response is critically dependent on a sustained elevation of [Ca\(^{2+}\)]\(_i\), a response that has been shown to result from an enhanced entry of Ca\(^{2+}\) from the extracellular medium. This receptor-enhanced entry of Ca\(^{2+}\) does not appear to involve the operation of voltage-dependent Ca\(^{2+}\) channels (as found in excitable cells), or to result from a Ca\(^{2+}\)-activated Ca\(^{2+}\) entry process, but, other than this, its nature and control are only poorly understood.

Evidence from a variety of cell types has indicated that the enhanced entry of extracellular Ca\(^{2+}\) is associated, in some way, with the receptor-activated generation of Ins(1,4,5)P\(_3\), but the bulk of such evidence suggests the absence of any direct effect of this inositol phosphate on membrane Ca\(^{2+}\) entry. Instead, most current models of receptor-activated Ca\(^{2+}\) entry in exocrine cells propose increases in membrane Ca\(^{2+}\) permeability that are consequent on the emptying of the agonist-sensitive intracellular stores induced by the action of Ins(1,4,5)P\(_3\). It is envisaged that, during stimulation, the emptied intracellular pools form some kind of physical communication or pathway with the plasma membrane, and entry of extracellular Ca\(^{2+}\) into the cytoplasm then occurs via this pathway and through the open intracellular pools. This has been described as 'capacitative' Ca\(^{2+}\) entry (Putney, 1986).

Initially, the formation of such a direct physical communication between the intracellular pools and the plasma membrane was believed to result simply from the Ins(1,4,5)P\(_3\)-induced emptying of those pools. However, the subsequent demonstration that the metabolism of Ins(1,4,5)P\(_3\) results in the generation of a series of water-soluble inositol phosphates and the finding, at least in certain cells. that one of these metabolites, Ins(1,3,4,5)P\(_4\), appears to be involved with Ins(1,4,5)P\(_3\) in inducing a sustained Ca\(^{2+}\) entry (Irvine & Moor, 1986; Morris et al., 1987), has led to the proposal that Ins(1,3,4,5)P\(_4\) may be involved in creating this physical link between the
emptied agonist-sensitive intracellular pools and the plasma membrane. Several modifications of such a model have been described (Gallacher, 1988; Irvine et al., 1988; Irvine, 1989). However, in each case, the pathway for the receptor-activated entry of Ca\(^{2+}\) is still considered to be through the emptied intracellular pools.

In the original experiments that led to this model, changes in [Ca\(^{2+}\)]\(_i\) were monitored indirectly by measuring the efflux of \(^{86}\)Rb\(^+\), which, it was believed, would reflect changes in Ca\(^{2+}\)-activated K\(^+\) conductance (Aub et al., 1982). These experiments showed that, when full, the Ca\(^{2+}\) content of these pools was essentially unaffected by the removal of extracellular Ca\(^{2+}\). However, once emptied by the action of an appropriate agonist in the absence of extracellular Ca\(^{2+}\), they were rapidly refilled on restoration of extracellular Ca\(^{2+}\) even in the absence of agonist. The critical finding was that this refilling process occurred without any increase in \(^{86}\)Rb\(^+\) efflux and thus, it was concluded, without any elevation of [Ca\(^{2+}\)]\(_i\). This indicated that the pathway for the enhanced Ca\(^{2+}\) entry during this phase did not traverse the cytoplasm, and refilling by extracellular Ca\(^{2+}\) occurred directly into the emptied pools (Poggioli & Putney, 1982; Aub et al., 1982).

More recently, Takemura & Putney (1989) have studied this refilling stage in parotid cells, using fura-2 as a more direct measure of changes in [Ca\(^{2+}\)]\(_i\). In that study, additional evidence for the capacitative nature of the receptor-activated entry of Ca\(^{2+}\) was obtained, and its critical dependence on the Ca\(^{2+}\) content of the intracellular pools (i.e. their 'emptiness'), rather than the direct action of any inositol phosphate, was demonstrated. However, in contrast with the earlier studies, it was revealed that [Ca\(^{2+}\)]\(_i\) markedly increased, at least transiently, during the refilling of the agonist-emptied pools. As mentioned by those authors, this would indicate that the accelerated entry of Ca\(^{2+}\) from the extracellular medium during refilling of the agonist-emptied pools does not occur directly into these pools, as was previously supposed, but occurs first into the cytoplasm and then subsequently into the pools. A similar, although rather smaller, transient increase in [Ca\(^{2+}\)]\(_i\) under the same protocol was also noted in the earlier studies by Pandol et al. (1987) on pancreatic acini.

Of course, such findings relate only to the enhanced entry of Ca\(^{2+}\) during the refilling of the agonist-emptied intracellular pools. However, if confirmed, such a finding would require a significant re-evaluation of current models of the nature of receptor-activated Ca\(^{2+}\) entry, in that it would appear to preclude the presence of a direct physical connection or pathway between the agonist-sensitive pools and the plasma membrane and the use of such a pathway as the route for the enhanced entry of Ca\(^{2+}\) into the cell.

In this paper I have attempted to confirm and extend these findings, using a very different model exocrine cell type, namely the CI–secreting avian nasal gland. Cholinergic (muscarinic) activation of secretion in the isolated cells from this gland involves changes in inositol phosphate generation and [Ca\(^{2+}\)], that are essentially similar to those seen in other exocrine cells (Shuttleworth & Thompson, 1989) and, as in previous studies, the observed increases in [Ca\(^{2+}\)]\(_i\) can be resolved into two distinct components: a transient rise resulting from release of Ca\(^{2+}\) from intracellular stores, and a sustained entry of extracellular Ca\(^{2+}\) across the plasma membrane. In common with other exocrine cells, it is specifically this latter component, i.e. that part entirely dependent on Ca\(^{2+}\) entry from the medium, that is the critical component of the overall signal. These data, together with the relative ease with which viable, essentially homogeneous, isolated cell preparations can be obtained from this tissue, the demonstration of their particular suitability for the use of fluorescent probes such as indo-1, and the large and highly consistent changes in [Ca\(^{2+}\)], observed on muscarinic receptor activation indicated that these cells represented a particularly useful model system for the study of receptor-activated changes in [Ca\(^{2+}\)]\(_i\) and their relationship to the stimulation of exocrine ion and fluid secretion.

**METHODS**

**Cells**

Isolated cells from the nasal salt gland of ducklings (Anas platyrhynchos) 4–10 days old, and given 1% NaCl in drinking water ad lib, for 48 h immediately before being killed, were obtained as previously described (Shuttleworth & Thompson, 1989). Briefly, the minced tissue was incubated in a medium consisting of 0.25% trypsin (Boehringer) in a 1:1 (v/v) mixture of Dulbecco's Modified Eagle's Medium and Hamm's nutrient F12 medium (Sigma) containing 23 mm-NaHCO\(_3\), gassed with O\(_2\)/CO\(_2\) (19:1), pH 7.4. Dissociation was aided by flushing the tissue fragments through pipettes of decreasing diameter to obtain a cell suspension. The resulting suspension was filtered through nylon mesh to remove any undissected tissue, and the filtrate centrifuged at 60 g for 10 min at room temperature. The supernatant was discarded and the cells were resuspended in 0.2 mg of trypsin inhibitor (Sigma)/ml in Heps-buffered saline gassed with 5% CO\(_2\) in air, pH 7.4, and incubated for 10 min. The saline contained 118.7 mm-NaCl, 4.8 mm-KCl, 1.3 mm-CaCl\(_2\), 23.0 mm-NaHCO\(_3\), 1.2 mm-MgSO\(_4\), 1.2 mm-KH\(_2\)PO\(_4\), 6.0 mm-glucose and 15.0 mm-Hepes, pH 7.4, when gassed with 5% CO\(_2\) in air. The suspension was centrifuged (60 g, 10 min), the supernatant was discarded and the cells were resuspended in Heps-buffered saline. The suspension was filtered again through nylon mesh and then stored in the above Heps-buffered saline at 38 °C and gassed with 5% CO\(_2\) in air until used.

**Determination of [Ca\(^{2+}\)]\(_i\)**

[Ca\(^{2+}\)]\(_i\) was determined in suspensions of dispersed cells as previously described (Shuttleworth & Thompson, 1989) by using the fluorescent probe indo-1 (Gryniewicz et al., 1985). Intracellular loading of indo-1 was done by incubation of the cells for 20 min at 38 °C in Heps-buffered saline containing 5 μM of the acetoxymethyl ester form, indo-1/AM (Calbiochem). The loaded cells were then washed twice by centrifugation at 60 g for 10 min, and the cells were resuspended in air-equilibrated Heps-buffered saline (NaHCO\(_3\) was replaced with the sodium salt of the Hepes buffer with addition of 3.9 mm-Na\(_2\)SO\(_4\)). Cell suspensions were maintained in the dark at 38 °C until use (max. 2 h). Immediately before measurement, each sample of cells was centrifuged at 100 g for 60 s, the supernatant was discarded and the cells were resuspended in air-equilibrated Heps-buffered saline. Measurements were made in a spectrofluorimeter (Perkin–Elmer LS-5B) in a thermostatically controlled
cuvette holder at 38 °C, incorporating a magnetic stirrer. Excitation and emission wavelengths were set at 332 nm and 400 nm respectively. The output from the spectrofluorimeter was recorded on a pen recorder and simultaneously digitized and stored in a microcomputer.

Calibration of the resulting signal and calculation of [Ca\(^{2+}\)]\(_i\) were as previously described (Shuttleworth & Thompson, 1989) and essentially followed the methods of Tsien et al. (1982) and Lückhoff (1986).

**Binding assay for Ins(1,4,5)P\(_3\)**

The cellular content of Ins(1,4,5)P\(_3\) was determined in samples of isolated cells by a specific binding-protein assay (kit from Amersham). Isolated cells, prepared as described above, were washed in air-equilibrated Hepes-buffered saline by centrifugation at 60 g for 10 min and resuspension in fresh saline. After a second centrifugation, cells were resuspended in the appropriate medium (see the Results section) and divided into 250 μl portions. One portion from each medium was centrifuged at 100 g for 60 s, the supernatant was discarded, and the cells were resuspended in distilled water and frozen at −20 °C until analysed for protein content by a Coomassie Blue assay (Pierce). Remaining batches of cells were incubated under the appropriate conditions in large centrifuge tubes (50 ml) in a shaking water bath at 38 °C. Additions were made by rapid injection via a Hamilton syringe, which ensured the rapid mixing necessary for the short time courses studied. Incubations were terminated at the times indicated by the addition of an equal volume of ice-cold 10 % (v/v) HClO\(_4\), and the tubes were left on ice for 15 min. After centrifugation at 15000 g for 2 min, EDTA (pH 7.0, final concn. 2 mM) was added to samples of the supernatant, and the water-soluble components were extracted by the addition of a 1:1 (v/v) mixture of Freon (1,1,2-trichlorotrifluoroethane) and tri-n-octylamine, followed by vortex-mixing and centrifugation at 2000 g for 4 min at 4 °C. Samples of the upper phase (of three) were assayed as described in the assay kit. Briefly, samples are incubated on ice in buffer (pH 9.0) containing 1.0 mM EDTA in the presence of a specific binding protein and tracer amounts of \(^{3}H\)-labelled Ins(1,4,5)P\(_3\). The protein-bound fractions are then separated by centrifugation and the pellets counted for radioactivity in a liquid-scintillation counter after addition of scintillant (Ecoscint A; National Diagnostics). Known Ins(1,4,5)P\(_3\) standards are similarly treated and a calibration curve is constructed. The Ins(1,4,5)P\(_3\) content of the samples is then determined from the calibration curve after appropriate correction for non-specific binding.

**RESULTS**

**Effect of external Ca\(^{2+}\) on changes in [Ca\(^{2+}\)]\(_i\) on stimulation**

Fig. 1 shows the changes in [Ca\(^{2+}\)]\(_i\), seen on addition of the muscarinic agonist carbachol (500 μM) to cells incubated in normal medium (1.3 mM) and low-Ca\(^{2+}\) medium (40 μM). As described previously (Shuttleworth & Thompson, 1989), in the normal medium [Ca\(^{2+}\)]\(_i\), rapidly increases from typical resting values of 90–100 nM to approx 450 nM within 5 s. This is followed by a somewhat slower decline before stabilizing at a new elevated value of approx. 300–350 nM, which is sustained for as long as receptor activation is maintained. Addition of the antagonist atropine (100 μM) produces a moderately rapid decline in [Ca\(^{2+}\)]\(_i\), over a period of approx. 30 s, finally stabilizing at a value essentially the same as that seen in unstimulated cells. Suspension of cells in media containing 40 μM-Ca\(^{2+}\) only slightly decreased resting values of [Ca\(^{2+}\)]\(_i\). Addition of carbachol (500 μM) to such cells results in a rapid 4–5-fold increase in [Ca\(^{2+}\)]\(_i\), similar to that seen in the normal medium. However, this increase in [Ca\(^{2+}\)]\(_i\), is only transient and, despite the continued presence of the agonist, [Ca\(^{2+}\)]\(_i\), rapidly returns to a value similar to that seen in unstimulated cells, i.e. there is no sustained elevation of [Ca\(^{2+}\)]\(_i\). In contrast with stimulated cells in the normal medium, addition of atropine (100 μM) to such cells produced only a small decline in [Ca\(^{2+}\)]\(_i\).

**Changes in [Ca\(^{2+}\)]\(_i\) during refilling of agonist-sensitive pools**

In these experiments, a similar protocol to that described in the original studies by Aub et al. (1982) and in the more recent experiments of Takemura & Putney (1989) was employed, except that a low-Ca\(^{2+}\) (40 μM) medium rather than a Ca\(^{2+}\)-free medium was used. Cells were suspended in the low-Ca\(^{2+}\) medium and stimulated with 500 μM-carbachol. When [Ca\(^{2+}\)]\(_i\), had returned to resting values, receptor activation was terminated by the addition of 100 μM-atropine. Fig. 2 illustrates the effect of restoring extracellular Ca\(^{2+}\) (1.3 mM) 1 min after addition of atropine to such cells and compares it with the corresponding effects in unstimulated cells, and in cells in which receptor activation is maintained (i.e. atropine not added). In unstimulated cells, increasing the extracellular [Ca\(^{2+}\)] from 40 μM to 1.3 mM produced only a small increase in [Ca\(^{2+}\)]\(_i\), whereas, in the cells where carbachol stimulation was maintained, restoration of extracellular Ca\(^{2+}\) produced a dramatic, and rapid, increase in [Ca\(^{2+}\)]\(_i\), before stabilizing at approx. 300 nM. Most significantly for this study, restoration of extracellular Ca\(^{2+}\) to the atropine-treated cells resulted in a
pronounced increase in [Ca\(^{2+}\)]\(_i\) to reach a peak of 180 nm before declining to a stable value similar to that seen in the unstimulated cells. Such a marked transient increase in [Ca\(^{2+}\)]\(_i\) suggests that a significant enhanced entry of extracellular Ca\(^{2+}\) still occurs into the cytoplasmic compartment of cells at least 1 min after the termination of receptor activation.

To evaluate how long this enhanced entry of Ca\(^{2+}\) into the cytoplasm persisted after termination of receptor activation, further experiments were performed by using the same protocol as described above except that extracellular Ca\(^{2+}\) was restored to 1.3 mM at different times after the addition of atropine. The results (Fig. 3) show that the size of the transient 'overshoot' in [Ca\(^{2+}\)]\(_i\) declines with time after the termination of receptor activation, such that the response recorded in pre-stimulated cells 3 min after the addition of atropine is virtually indistinguishable from that seen in unstimulated cells. From these data it can be estimated that the 'overshoot' in [Ca\(^{2+}\)]\(_i\) declines after termination of receptor activation, with a \(t_1\) of approx. 40 s.

**Time course of Ins(1,4,5)P\(_3\) metabolism after receptor deactivation**

In order to compare the persistent nature of the enhanced Ca\(^{2+}\) entry into the cytosol after the termination of receptor activation with the corresponding levels of Ins(1,4,5)P\(_3\), experiments were carried out to measure the effect of atropine on the Ins(1,4,5)P\(_3\) content in cells prestimulated with carbachol. In these experiments, a specific binding-protein assay for Ins(1,4,5)P\(_3\) was used (see above). The advantage of this assay over the more conventional techniques involving determination of changes in the incorporation of radiolabelled inositol into inositol phosphates is that it allows absolute quantification of the amount of the inositol phosphate present and avoids possible complications arising from changes in specific radioactivity of different inositol phosphate pools (Challiss et al., 1988; Baird et al., 1989). As shown in Fig. 4, in cells incubated in the low-Ca\(^{2+}\) medium and prestimulated with carbachol (500 \(\mu\)M), addition of 100 \(\mu\)M-atropine resulted in the rapid decline of cellular Ins(1,4,5)P\(_3\) content over the first 15 s before reaching values that remained stable for at least the succeeding 3 min. The stable level attained after atropine (mean of all values obtained between 1 and 3 min after atropine was 12.4 \pm 1.7 pmol/mg of protein; \(n = 9\)) were essentially identical with those seen in unstimulated cells in the low-Ca\(^{2+}\) medium (see below). The data indicate that the decline in cellular Ins(1,4,5)P\(_3\) followed a time course with a \(t_1\) of approx. 3–4 s, a value similar to that previously reported for parotid cells (Hughes et al., 1988). This is some 10 times faster than the observed decline in the enhanced cytoplasmic entry of extracellular Ca\(^{2+}\) noted above.

**Pathways of Ca\(^{2+}\) entry during the initial stimulation phase**

In these experiments, the rates of increase in [Ca\(^{2+}\)]\(_i\), over the first few seconds of stimulation were compared in cells suspended in normal-Ca\(^{2+}\) and low-Ca\(^{2+}\) media. The output from the spectrofluorimeter was sampled by computer every 200 ms and converted into values of [Ca\(^{2+}\)]\(_i\). Only the initial rates of increase in [Ca\(^{2+}\)]\(_i\) were compared, and no attempt was made to quantify any delay or lag times before the onset of the increase.

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**Fig. 2. Effect of restoration of normal extracellular [Ca\(^{2+}\)] (1.3 mM) in cells incubated in low-Ca\(^{2+}\) saline**

Isolated cells were prepared and loaded with indo-1 as before, and resuspended in saline containing 40 \(\mu\)M-Ca\(^{2+}\). Ca\(^{2+}\) was added to the extracellular medium at the point indicated to reach a final concentration of 1.3 mM. Before this, cells either remained unstimulated (— — — —), or had been prestimulated with carbachol (500 \(\mu\)M) for 3–4 min, after which [Ca\(^{2+}\)] had fallen to close to resting levels (curve A), or were similarly prestimulated with carbachol for 3 min, followed by atropine (100 \(\mu\)M) for 1 min before the addition of extracellular Ca\(^{2+}\) (curve B). Representative traces are shown superimposed for comparison.

**Fig. 3. Effect of extracellular Ca\(^{2+}\) restoration on [Ca\(^{2+}\)]\(_i\) in stimulated cells after atropine**

Isolated cells were prepared and loaded with indo-1 as before, resuspended in low-Ca\(^{2+}\) saline ([Ca\(^{2+}\)] = 40 \(\mu\)M), and stimulated with carbachol (500 \(\mu\)M); 3 min later, after which time [Ca\(^{2+}\)] had returned to approximately resting levels, 100 \(\mu\)M-atropine was added. Addition of Ca\(^{2+}\) to the extracellular medium (final concn. = 1.3 mM) was made at the point indicated after 1 min (curve A), 2 min (curve B) or 3 min (curve C) of atropine treatment. Traces are computer averages of four separate experiments in each case, carried out on different cell preparations. The broken line (— — — —) represents the typical trace recorded in unstimulated cells (see Fig. 2) for comparison.
Fig. 4. Effect of atropine on intracellular \( \text{Ins(1,4,5)P}_3 \) levels in carbachol-stimulated cells

Samples of isolated cells were suspended in low-Ca\(^{2+}\) saline ([Ca\(^{2+}\)] = 40 \( \mu \)M) and stimulated with 500 \( \mu \)M-carbachol, for 3 min, after which (zero time) atropine (100 \( \mu \)M) was added. At different times after the addition of atropine, the cells were taken and their intracellular \( \text{Ins(1,4,5)P}_3 \) contents analysed as described. Data represent means ± S.E.M. of three separate cell preparations.

Fig. 5. Effect of extracellular Ca\(^{2+}\) concentration on the initial rate of increase in [Ca\(^{2+}\)], in cells stimulated with carbachol

Cells, isolated and loaded with indo-1 as described, were suspended in either normal-Ca\(^{2+}\) medium ([Ca\(^{2+}\)] = 1.3 mM) (○) or low-Ca\(^{2+}\) (40 \( \mu \)M) medium (■). The initial rate of increase in [Ca\(^{2+}\)], after addition of carbachol (500 \( \mu \)M) was monitored by using a data-sampling rate of 5/s. The data represent typical traces with their time bases shifted and superimposed to permit direct comparison.

Typical traces are shown in Fig. 5 and reveal that, over the first 2 s, the rate of increase in [Ca\(^{2+}\)], is markedly faster in the normal-Ca\(^{2+}\) medium than in the low-Ca\(^{2+}\) medium. Data obtained from a series of such experiments indicated that the initial rate of increase in [Ca\(^{2+}\)], averaged 161.2 ± 18.8 nm/s (n = 8) in the normal-Ca\(^{2+}\) medium, compared with 126.3 ± 17.2 nm/s (n = 6) in the low-Ca\(^{2+}\) medium, a difference that was statistically highly significant (\( P < 0.01 \)).

Fig. 6. Effect of carbachol on intracellular levels of \( \text{Ins(1,4,5)P}_3 \) at normal and low extracellular Ca\(^{2+}\) concentrations

Samples of cells, isolated as described, were resuspended in normal medium ([Ca\(^{2+}\)] = 1.3 mM) (○), or low-Ca\(^{2+}\) (40 \( \mu \)M) medium (■). Carbachol (final concn. 500 \( \mu \)M) was added at zero time, and subsequently, at different times, the cells were taken and their intracellular \( \text{Ins(1,4,5)P}_3 \) contents analysed as described. Data represent means ± S.E.M. of four or five experiments on separate cell preparations.

To determine whether exposure of the isolated cells to the low-Ca\(^{2+}\) medium affected the generation of the critical second messenger \( \text{Ins(1,4,5)P}_3 \), the cellular contents of this inositol phosphate, and the changes induced by addition of carbachol (500 \( \mu \)M), were determined in cells in low-Ca\(^{2+}\) and normal-Ca\(^{2+}\) media. As shown in Fig. 6, resting levels of \( \text{Ins(1,4,5)P}_3 \) in both media within 5 s to reach levels of approx. 35–40 pmol/mg of protein. These levels were sustained, or even slowly increased, over the proceeding 55 s, eventually reaching approx. 40–50 pmol/mg of protein after 5 min (results not shown). Comparison of the responses shows that both the rate and the overall magnitude of the increases in \( \text{Ins(1,4,5)P}_3 \) seen on addition of carbachol were essentially identical in the two media. It should also be noted that, although very few measurements of the actual cellular content of \( \text{Ins(1,4,5)P}_3 \) have been reported for any cell type to date, the values obtained here for avian nasal-gland cells were almost identical with those found in resting and carbachol-stimulated cerebral-cortex slices by Challiss et al. (1988), and broadly similar to those reported by Horstman et al. (1988) for AR42J pancreaticoma cells.

DISCUSSION

Most previous studies on receptor-activated Ca\(^{2+}\) entry in exocrine and other cells have relied largely on the use of Ca\(^{2+}\)-free media to discriminate between Ca\(^{2+}\) mobilization from intracellular stores and Ca\(^{2+}\) entry. A problem with such studies is that this procedure either may lead to the depletion of an essential component of the total intracellular Ca\(^{2+}\) pool, thereby affecting the [Ca\(^{2+}\)]
responses subsequently recorded, or may affect steps in the overall signalling process, such as phospholipase C activity etc., resulting in decreased levels of critical second-messenger moieties. In addition, we have previously demonstrated that exposure of the isolated cells from the avian nasal gland to Ca\(^{2+}\)-free media, even for only a few minutes, appears to result in an irreversible increase in membrane Ca\(^{2+}\) permeability (Shuttleworth & Thompson, 1989). Similar findings have been reported by Joseph et al. (1985) in hepatocytes and by Williams & Fay (1986) in smooth-muscle cells. As an alternative procedure, some studies have used extracellular Mn\(^{2+}\) to mimic Ca\(^{2+}\), and the quenching of the intracellular fluorescence signal of the Ca\(^{2+}\) probe fura-2 as the Mn\(^{2+}\) enters the cytoplasm was determined (Merritt & Hallam, 1988; Merritt et al., 1989; Sage et al., 1989). However, preliminary experiments showed that this technique was inappropriate for the avian cells, as the Ca\(^{2+}\)-entry pathway activated in these cells by carbachol did not appear to permit the passage of Mn\(^{2+}\). Other reports suggest that this is true for receptor-activated Ca\(^{2+}\)-entry pathways of exocrine cells in general (Merritt & Hallam, 1988). In the experiments described in the present study, an alternative procedure of using a low-Ca\(^{2+}\) medium (40 \(\mu\)M) rather than a Ca\(^{2+}\)-free medium was employed. In this low-Ca\(^{2+}\) medium, [Ca\(^{2+}\)] in the stable and only slightly decreased (approx. 10\%) compared with that seen in normal-Ca\(^{2+}\) media, and restoration of normal extracellular [Ca\(^{2+}\)] produced only a small, relatively slow, increase in [Ca\(^{2+}\)] (Fig. 2). Furthermore, the size of the initial peak of [Ca\(^{2+}\)] on stimulation with carbachol was, again, only slightly decreased compared with that seen in normal-Ca\(^{2+}\) media (Fig. 1), and such differences can readily be explained by the absence of a significant Ca\(^{2+}\)-entry component during this initial phase in the low-Ca\(^{2+}\) medium. It appears therefore unlikely that the limited exposure to low-Ca\(^{2+}\) media (40 \(\mu\)M) in these experiments significantly affected intracellular pools of Ca\(^{2+}\) or the discharge of those pools after activation of muscarinic receptors. This is further supported by the finding that incubation in the low-Ca\(^{2+}\) media had no significant effect on the increases in cellular Ins(1,4,5)\(P_3\) content induced by carbachol (Fig. 6).

The data presented show that muscarinic-receptor activation in these cells results in an enhanced entry of Ca\(^{2+}\) across the plasma membrane. This can be seen in the sustained elevation of [Ca\(^{2+}\)], in cells stimulated in normal saline, and the dependence of this elevated [Ca\(^{2+}\)], on continued receptor activation and on the presence of adequate levels of Ca\(^{2+}\) in the extracellular medium. This is also supported by the large, rapid and sustained increase in [Ca\(^{2+}\)], seen in stimulated cells induced by raising extracellular Ca\(^{2+}\) concentration from 40 \(\mu\)M to 1.3 mM, whereas only a marginal increase in [Ca\(^{2+}\)] is produced in unstimulated cells. The data further showed that, in cells whose intracellular Ca\(^{2+}\) pools have been emptied by appropriate prestimulation in low-Ca\(^{2+}\) media, followed by termination of receptor activation with atropine, restoration of normal concentrations of extracellular Ca\(^{2+}\) is associated with a marked, transient, increase in [Ca\(^{2+}\)]. In the low-Ca\(^{2+}\) medium, the magnitude of this transient ‘overshoot’ in [Ca\(^{2+}\)] decreases with time, indicating that it is not merely an artefact resulting from the sudden re-exposure of the cells to normal extracellular Ca\(^{2+}\) concentrations. Evidence showed that the time course for this decline in the magnitude of the transient overshoot is far slower than the corresponding removal of the Ca\(^{2+}\)-mobilizing second messenger Ins(1,4,5)\(P_3\), suggesting that it is unlikely to be associated with any persistent inositol phosphate action. A similar conclusion was reached in the recent studies by Takemura & Putney (1989), where a transient increase in [Ca\(^{2+}\)], representing an accelerated rate of Ca\(^{2+}\) entry into the cytosol, could be observed as much as 20 min after addition of atropine so long as pool refilling was prevented by maintenance of zero extracellular [Ca\(^{2+}\)]. Consequently, it seems most probable that the decay in the magnitude of the [Ca\(^{2+}\)] overshoot seen in the present study reflects the slow refilling of the agonist-sensitive Ca\(^{2+}\) pools in the low-Ca\(^{2+}\) medium used. Whatever their precise origin, such increases in [Ca\(^{2+}\)], are clearly inconsistent with a direct refilling of the agonist-sensitive pools from the extracellular medium and, instead, show that the enhanced entry of extracellular Ca\(^{2+}\) during refilling occurs initially into the general cytosolic compartment.

It was also found that, on stimulation with carbachol, [Ca\(^{2+}\)], increased at a faster rate in the presence of normal extracellular Ca\(^{2+}\) than in low-Ca\(^{2+}\) media. Such an effect could not be ascribed to any differences in the rate or magnitude of the corresponding increases in Ins(1,4,5)\(P_3\) in the two media, which were essentially identical. As has been shown in many different cell types, the initial increase in [Ca\(^{2+}\)], in low-Ca\(^{2+}\) media principally reflects the rate of release of Ca\(^{2+}\) from the agonist-sensitive intracellular stores, which should therefore be independent of extracellular Ca\(^{2+}\) concentration. In the low-Ca\(^{2+}\) medium used, we have already shown that the contribution of receptor-enhanced Ca\(^{2+}\) entry from the extracellular medium is minimal (Fig. 1). The accelerated rise in [Ca\(^{2+}\)], seen in normal-Ca\(^{2+}\) medium therefore presumably represents the additional contribution of receptor-activated Ca\(^{2+}\) entry to the overall increase in [Ca\(^{2+}\)]. However, the finding that receptor-activated Ca\(^{2+}\) entry and Ca\(^{2+}\) release are additive is not compatible with models where receptor-activated Ca\(^{2+}\) entry occurs through the agonist-sensitive intracellular Ca\(^{2+}\) pools via a pathway that is critically controlled by the Ca\(^{2+}\) levels in those pools. In such models, if the rate of Ca\(^{2+}\) entry from the medium exceeded the rate of Ca\(^{2+}\) release, this would result in the pools becoming full, and the pathway would close. Hence, the fact that Ca\(^{2+}\) release and Ca\(^{2+}\) entry are in series in such models means that the maximum rate of addition of Ca\(^{2+}\) to the cytosol can never exceed the rate of Ca\(^{2+}\) release from the agonist-sensitive pools. The data presented here clearly contradict such a model and suggest that, after stimulation, Ca\(^{2+}\) release and Ca\(^{2+}\) entry operate in parallel, i.e. via separate pathways.

As noted above, in most of the current models describing receptor-activated Ca\(^{2+}\) entry in exocrine cells, the pathway for such entry is considered to be via the intracellular Ca\(^{2+}\) pools emptied by the action of Ins(1,4,5)\(P_3\). Most of the evidence for this derives from studies of the refilling of the agonist-sensitive pools after termination of receptor activation, showing that this occurs without any significant change in [Ca\(^{2+}\)]. However, in pancreatic acini, Pandol et al. (1987) reported that [Ca\(^{2+}\)], increased on restoration of extracellular Ca\(^{2+}\) after addition of atropine to cells first depleted of intracellular Ca\(^{2+}\) by stimulation with carbachol in Ca\(^{2+}\)-free media. The increases in [Ca\(^{2+}\)], seen were only small.
Receptor-activated Ca\textsuperscript{2+} entry

probably reflecting the fact that, in the cell preparation used, receptor-activated Ca\textsuperscript{2+} entry appeared to make only a minor contribution to the overall Ca\textsuperscript{2+} signal (e.g. no sustained elevation in [Ca\textsuperscript{2+}]) was seen in stimulated cells in normal Ca\textsuperscript{2+}-containing extracellular media. Furthermore, the values of [Ca\textsuperscript{2+}] in the Ca\textsuperscript{2+}-depleted cells before addition of extracellular Ca\textsuperscript{2+} were significantly depressed below normal resting levels, so it is possible that the increases in [Ca\textsuperscript{2+}], seen reflected a simple restoration of normal Ca\textsuperscript{2+} balance in previously Ca\textsuperscript{2+}-depleted cells, rather than any sustained receptor-activated pathway. In the exocrine cell type which has been most extensively studied to date, the parotid, the original studies by Poggioli & Putney (1982) and Aub et al. (1982), using \textsuperscript{86}Rb\textsuperscript{+} to identify changes in [Ca\textsuperscript{2+}], failed to detect any changes in [Ca\textsuperscript{2+}], during refilling of the agonist-emptied intracellular Ca\textsuperscript{2+} stores. This, and other evidence showing the slow exchange of \textsuperscript{45}Ca\textsuperscript{2+} between loaded pools and the extracellular medium compared with the rapid exchange when the pools were depleted (Poggioli & Putney, 1982), were instrumental in the development of the ‘capacitative’ model for receptor-regulated Ca\textsuperscript{2+} entry (Putney, 1986). Similarly, in studies using the fluorescent Ca\textsuperscript{2+} probe fura-2, Merritt & Rink (1987b) reported that reloading of the agonist-sensitive intracellular pools by a brief application of external Ca\textsuperscript{2+} occurred without a substantial elevation of [Ca\textsuperscript{2+}], leading the authors to propose that receptor-enhanced Ca\textsuperscript{2+} entry involved a direct pathway for external Ca\textsuperscript{2+} connecting the plasma membrane and the agonist-sensitive intracellular pools. The essential property required of this proposed pathway was its critical dependence on the Ca\textsuperscript{2+} concentration within the intracellular Ca\textsuperscript{2+} pools. Thus, under resting conditions the pathway would be closed by the high [Ca\textsuperscript{2+}] within the pools, but would open when the pools became depleted of Ca\textsuperscript{2+} after receptor activation. However, the recent studies by Takemura & Putney (1989) also using fura-2, clearly showed that refilling of the agonist-sensitive pools in parotid cells is associated with marked transient changes in [Ca\textsuperscript{2+}]. Although somewhat larger, the responses seen in those studies reported here are essentially identical to those described by Takemura & Putney (1989) and, as noted by those authors, are inconsistent with models in which enhanced Ca\textsuperscript{2+} entry during refilling of the pools occurs directly into those pools.

We have also reported evidence, based on an analysis of the initial rate of increase in [Ca\textsuperscript{2+}] after receptor activation, that indicates an independent direct entry of extracellular Ca\textsuperscript{2+} into the cytosol during the initial phase of stimulation operating in addition to, and separate from, the release of Ca\textsuperscript{2+} from agonist-sensitive intracellular pools. Merritt & Rink (1987a) considered such a direct entry of extracellular Ca\textsuperscript{2+} into the cytosol during the initial phase of stimulation as unlikely because of the presence of a finite lag time before the onset of a rise in [Ca\textsuperscript{2+}], after stimulation, and the absence of any effect of removal of external Ca\textsuperscript{2+} on this lag time. However, Merritt & Rink (1987a) did find that the initial rate of increase in fluorescence after stimulation was more rapid in the presence of extracellular Ca\textsuperscript{2+} than in its absence, and they pointed out that such data are hard to rationalize with models where Ca\textsuperscript{2+} entry is dependent on, and subsequent to, release of Ca\textsuperscript{2+} from intracellular stores.

It is concluded that, contrary to the generally accepted current models, the receptor-enhanced entry of extracellular Ca\textsuperscript{2+} in exocrine cells occurs directly into the cytosol, and not via the agonist-sensitive intracellular pools. Such a direct entry can be shown to operate during both the initial phases of stimulation and the refilling of agonist-depleted intracellular pools. A significant consequence of these findings is the removal of the requirement for a close physical association of the agonist-sensitive intracellular pools with the plasma membrane. This, itself, raises an important problem. Thus, other workers have suggested that the enhanced entry of Ca\textsuperscript{2+} across the plasma membrane in exocrine cells is determined by the level of Ca\textsuperscript{2+} in the agonist-sensitive pools, yet it has been shown above that the pathway for this enhanced Ca\textsuperscript{2+} entry is separate from, and operates in parallel to, the release of Ca\textsuperscript{2+} from those pools. The physical separation of these two components of the overall Ca\textsuperscript{2+} signal in such cells clearly raises the problem of precisely how depletion of the agonist-sensitive Ca\textsuperscript{2+} pools is able to activate a Ca\textsuperscript{2+}-entry pathway in the plasma membrane.

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