Capacitative calcium entry in parotid acinar cells

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The intracellular Ca^{2+} indicator, fura-2, was used to monitor changes in cytosolic $[\text{Ca}^{2+}]$ in parotid acinar cells. When parotid cells were incubated in a medium containing low $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]$ was restored to the physiological range, there was a small increase in cytosolic $[\text{Ca}^{2+}]$. If, however, the cells were first activated by a muscarinic agonist, and receptor activation was terminated before the addition of Ca^{2+} by the addition of a pharmacological excess of the muscarinic-receptor antagonist atropine, the initial increase in cytosolic $[\text{Ca}^{2+}]$ was faster and transiently larger than in the control cells which had not been previously stimulated. This suggested that a stimulation of Ca^{2+} entry occurred owing to the prior emptying of the agonist-regulated intracellular Ca^{2+} pool. This extra Ca^{2+} influx seen in pool-depleted cells persisted even when the interval between the addition of atropine and Ca^{2+} was increased from 1 to 20 min. Also, when the pool was allowed to refill by adding atropine in the presence of extracellular Ca^{2+} , and Ca^{2+} was then sequentially removed and restored, the rise in cytosolic $[\text{Ca}^{2+}]$ after the addition of extracellular Ca^{2+} was not rapid, and resembled the increase seen in unstimulated cells. These results indicate that, when the agonist-sensitive Ca^{2+} pool is emptied by an agonist, Ca^{2+} influx across the plasma membrane is increased. This influx of Ca^{2+} occurs independently of the concentrations of inositol phosphates and probably of any second messengers linked directly to receptor activation. It appears rather to be a consequence of the empty state of the Ca^{2+} pool. Further, we suggest that, whenever the agonist-sensitive Ca^{2+} pool is emptied by agonist activation, the plasma-membrane permeability to Ca^{2+} will be increased, and this may account, at least in part, for the phenomenon of receptor-activated Ca^{2+} entry.

INTRODUCTION

Activation of cell-surface receptors for a variety of hormones, neurotransmitters and growth factors results in an increase in the cytoplasmic Ca^{2+} concentration (Putney, 1987). In most cases, this increase in cytosolic Ca^{2+} can be attributed to the release, or mobilization, of Ca^{2+} from intracellular stores and to the entry of Ca^{2+} from the extracellular space (Putney, 1987; Putney *et al.*, 1981). Stimulation of these same receptors also results in the phospholipase C-catalysed hydrolysis of the minor plasma-membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with the concomitant formation of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] (Berridge, 1983). There is strong evidence that $\text{Ins}(1,4,5)\text{P}_3$ stimulates Ca^{2+} release from intracellular stores (Berridge, 1986); however, the mechanism underlying receptor-regulated Ca^{2+} entry is not well understood. According to one proposal, termed capacitative Ca^{2+} entry, as the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool is depleted, a process for rapidly refilling the pool is activated (Casteels & Droogmans 1981; Putney, 1986). Therefore, in the continued presence of $\text{Ins}(1,4,5)\text{P}_3$, the activated pathway of Ca^{2+} entry into this pool, and subsequently out via the $\text{Ins}(1,4,5)\text{P}_3$ -activated channel, would result in a sustained process of Ca^{2+} entry to the cytoplasm. This implies a mechanism for communication between the Ca^{2+} pool and the plasma membrane such that the permeability of the plasma membrane, perhaps in discrete regions, would be increased when the intracellular pool is empty. Here

we describe experimental results, obtained with the intracellular Ca^{2+} indicator fura-2 (Tsien *et al.*, 1985), which for the first time demonstrate this ability of the agonist-regulated Ca^{2+} pool to enhance Ca^{2+} influx at the level of the plasma membrane.

METHODS

Rat parotid acinar cells were prepared as described previously (Sugiyama *et al.*, 1987) with slight modification. Briefly, minced rat parotid glands were incubated with trypsin, followed by EGTA plus trypsin inhibitor, and then collagenase in Dulbecco's Modified Eagle's Medium containing 0.5% bovine serum albumin. $[\text{Ca}^{2+}]$ was determined with fura-2 essentially as described previously (Hughes *et al.*, 1988; Merritt & Rink, 1987a,b). The cells, suspended in Dulbecco's medium containing 0.5% albumin, were incubated with 2 μM -fura-2/AM for 45 min at 37 °C. The fura-2-loaded cells were washed twice, resuspended in a Hepes-buffered Krebs–Ringer medium (KRH) containing 0.2% albumin and kept at room temperature. KRH consisted of the following (mM): NaCl, 120; KCl, 5.4; MgSO_4 , 0.8; CaCl_2 , 1.0; glucose, 11.1; Hepes, 20 (pH 7.4). Just before use, batches of cells were washed by centrifugation, and resuspended in fresh Ca^{2+} -free KRH containing 0.2 mM-EGTA at 37 °C in a quartz cuvette. In this and all subsequent experiments, final extracellular $[\text{Ca}^{2+}]$ was assumed to be the total concentration, minus 0.2 mM complexed by the EGTA. The fluorescence of fura-2-

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]$, intracellular Ca^{2+} concn.

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loaded cells was measured with a Delta-Scan 1 spectrofluorimeter (Photon Technology International, Princeton, NJ, U.S.A.) with excitation at 340 nm and 380 nm and emission at 500 nm. $[Ca^{2+}]_i$ was calculated from the measurement of the ratio of fluorescence intensities (Grynkiewicz *et al.*, 1986). The ratios of maximum and minimum fluorescence were then determined by the addition of 0.09% Triton in the presence of free Ca^{2+} concentration of ≥ 1 mM, and in the presence of a concentration of EGTA at least twice the $[Ca^{2+}]$ (pH > 8.3), respectively. Cell autofluorescence, determined with cells not loaded with fura-2, was subtracted from the traces before $[Ca^{2+}]_i$ was calculated. As previously reported (Hughes *et al.*, 1988), leakage of fura-2 from the cells was minimal within the time frame of the experiments described, as determined by the absence of fura-2 fluorescence in the supernatant after centrifugation of the cells, and the absence of effect of addition of Mn^{2+} to the preparation.

RESULTS AND DISCUSSION

Treatment of parotid acinar cells with a muscarinic-cholinergic agonist results in a transient increase in cytosolic Ca^{2+} , which is independent of extracellular Ca^{2+} and therefore attributed to the release of intracellular Ca^{2+} stores. Subsequently, there is a sustained increase in cytosolic Ca^{2+} , which is dependent on the presence of extracellular Ca^{2+} and is believed to result from an enhanced entry of Ca^{2+} from the extracellular space (Putney *et al.*, 1981). Much of the original evidence for the capacitative model for Ca^{2+} entry was obtained from studies of the refilling of the agonist-sensitive Ca^{2+} pool in parotid acinar cells after removal of an agonist. When this agonist-sensitive pool was full, removal of extracellular Ca^{2+} did not lead to its depletion; however, when empty, the pool could be rapidly refilled from the extracellular space (Aub *et al.*, 1982). In our earlier studies, changes in cytosolic $[Ca^{2+}]$ were monitored by measurements of $^{86}Rb^+$ efflux, an indicator of activation of Ca^{2+} -dependent K^+ channels. Since efflux of $^{86}Rb^+$ was not increased during reloading of the pool, it was concluded that the pathway into the pool from the extracellular space did not traverse the cytoplasmic compartment (Aub *et al.*, 1982).

The intracellular Ca^{2+} indicator fura-2 has been used to monitor changes in cytosolic $[Ca^{2+}]$ in parotid acinar cells (Merritt & Rink, 1987a,b; Hughes *et al.*, 1988). With this more-sensitive indicator, we now detect a brief elevation of cytosolic $[Ca^{2+}]$ during the reloading process, which in turn can be used to infer the influence of pool Ca^{2+} content in plasma-membrane Ca^{2+} fluxes. Thus, when parotid cells were incubated in a medium containing low $[Ca^{2+}]$, the cytoplasmic $[Ca^{2+}]$ decreased from 100 nM to about 50 nM, a phenomenon not detected in earlier studies with $^{86}Rb^+$. Likewise, a corresponding restoration of cytosolic $[Ca^{2+}]$ occurred when extracellular Ca^{2+} was increased to physiological (mM) concentrations (Fig. 1). Fig. 1 also illustrates experiments in which muscarinic-receptor activation was terminated before the addition of Ca^{2+} by the addition of a pharmacological excess of the muscarinic-receptor antagonist atropine. This results in a rapid ($t_1 = 7.6$ s) decline in the cellular concentration of the Ca^{2+} -mobilizing second messenger $Ins(1,4,5)P_3$ (Hughes *et al.*, 1988). However, the absence of extracellular Ca^{2+} prevents refilling of the agonist-sensitive

Fig. 1. Increase in $[Ca^{2+}]_i$ owing to the addition of 3.0 mM- Ca^{2+} to parotid acinar cells incubated in medium containing no added Ca^{2+} and 0.2 mM-EGTA

The Ca^{2+} was added either to control cells (not previously stimulated; ·····) or to cells treated with 0.1 mM-methacholine followed by 10 μ M-atropine (—, ———). In the experiment indicated by ———, 0.3 mM- $LaCl_3$ was added with the atropine ($[La^{3+}]$ about 0.1 mM). For clarity, the original traces from a single experiment have been redrawn by hand, in order to superimpose the three different experimental conditions. Examples of original data showing the on-line computer-calculated $[Ca^{2+}]$ data are shown in Fig. 3. The experiments were repeated three to six times; summarized data are shown in Fig. 2.

Ca^{2+} pool (Aub *et al.*, 1982). When extracellular Ca^{2+} is restored under these conditions, i.e. in the absence of receptor activation but when the agonist-sensitive Ca^{2+} pool is depleted, the intracellular pool will rapidly refill (Aub *et al.*, 1982; Merritt & Rink, 1987b; Hughes *et al.*, 1988). As shown in Fig. 1, the initial increase in cytosolic $[Ca^{2+}]$ is transiently larger than in the control cells with the pool intact. The rate of increase of intracellular Ca^{2+} during the first 1 s after introduction of Ca^{2+} was estimated, and this was also slightly increased in the cells whose pool had been depleted ($136 \pm 5\%$ of control). In the experiment in Fig. 1, the basal $[Ca^{2+}]$ appears lower in the cells that had been stimulated than in the control cells, but on average the values were not different in the two groups. The average cytosolic $[Ca^{2+}]$ in the control cells was 55.5 ± 2.4 nM and in the previously stimulated cells it was 55.1 ± 2.2 nM.

Previous studies on Ca^{2+} regulation in the parotid (Merritt & Rink, 1987b) failed to note this additional Ca^{2+} flux, and concluded that the calcium entering the pool did not traverse the cytosol, consistent with the predictions from the studies of $^{86}Rb^+$ flux. However, this additional increase in cytosolic $[Ca^{2+}]$ is apparent on inspection of the data (compare the effect of addition of Ca^{2+} to unstimulated cells shown in Fig. 3(a) with that for previously stimulated cells in Fig. 6(b) of Merritt & Rink (1987b)).

The data of Poggioli *et al.* (1985) indicated that the agonist-activated Ca^{2+} influx has a higher K_m for Ca^{2+} than does the resting leak flux. Thus the reliance of this pool-dependent extra Ca^{2+} flux on the concentration of Ca^{2+} added was examined. Fig. 2 illustrates that the extra influx of Ca^{2+} seen in pool-depleted cells was detectable when extracellular Ca^{2+} was increased to 0.3 or 1.0 mM, and was especially apparent when the concentration was increased to 3.0 or 10.0 mM.

Fig. 2. Effect of addition of various concentrations of Ca^{2+} to extracellular medium containing 0.2 mM-EGTA on $[\text{Ca}^{2+}]_i$ in parotid acinar cells

$[\text{Ca}^{2+}]_i$ from fura-2-loaded cells was measured as described in Fig. 1 legend. The curves in (a), (b), (c) and (d) show results when $[\text{Ca}^{2+}]$ of 0.3, 1, 3.0 and 10 mM respectively was added 1 min after termination of methacholine stimulation by 10 μM -atropine (●) or was added to cells not previously stimulated (control; ○). The experiments with addition of 3 mM- Ca^{2+} were repeated with the interval between the atropine and Ca^{2+} addition increased from 1 to 20 min (panel c: Δ , control; $\blacktriangle$, methacholine + atropine; dotted line). Results are means $\pm$ S.E.M. from three to six separate determinations (S.E.M. values are omitted from the 20 min experiments, for clarity; they were of similar magnitude to those for the 1 min experiments).

In the experiments shown in Figs. 1 and 2, the addition of Ca^{2+} occurred 1 min after the addition of atropine. Thus it is unlikely that the additional flux can be attributed to persisting $\text{Ins}(1,4,5)\text{P}_3$, which decreases with a half-time of 7.6 s (Hughes *et al.*, 1988). However, other inositol phosphates formed in acinar cells decrease with half-times of the order of minutes (Hughes *et al.*, 1988), and some of these have been suggested to have Ca^{2+} -mobilizing or regulating actions, e.g. inositol cyclic 1:2,4,5-trisphosphate (Wilson *et al.*, 1985), inositol 1,3,4-trisphosphate (Irvine *et al.*, 1986), inositol 1,3,4,5-tetrakisphosphate (Morris *et al.*, 1987). Two experimental protocols were utilized to demonstrate the independence of the pool-dependent Ca^{2+} influx on the presence or metabolism of inositol phosphates or other agonist-dependent messengers. First, increasing the time interval between the addition of atropine and Ca^{2+} from 1 to 20 min had no effect on the additional Ca^{2+} influx observed in the pool-depleted cells (Fig. 2c). In a second strategy, the pool was allowed to refill by adding atropine in the presence of extracellular Ca^{2+} . Extracellular Ca^{2+} was subsequently removed, and the rise in cytosolic $[\text{Ca}^{2+}]_i$ after application of 3 mM extracellular Ca^{2+} was determined. Under these conditions, the rise in cytosolic $[\text{Ca}^{2+}]_i$ after the addition of extracellular Ca^{2+} was not rapid, and resembled the increase seen in unstimulated cells (Fig. 3). That is, the additional Ca^{2+} influx could be

observed as late as 20 min after the blockade of the muscarinic receptor as long as refilling of the agonist-sensitive pool during the intervening period was prevented by the absence of extracellular Ca^{2+} . Conversely, the extra influx was not observed 3 min after atropine treatment if the intracellular pool was first allowed to refill.

Taken together, these results demonstrate that, when the agonist-sensitive Ca^{2+} pool is emptied, Ca^{2+} influx across the plasma membrane is increased. This influx of Ca^{2+} occurs independently of the increased concentrations of inositol phosphates caused by receptor activation, and probably of any second messengers linked directly to receptor activation. Rather, it appears to be a consequence of the empty state of the Ca^{2+} pool. Since the influx is exaggerated relative to the basal flux at high $[\text{Ca}^{2+}]_i$, the extra flux would seem to have a higher K_m for Ca^{2+} than the basal flux, in agreement with the findings by Poggioli *et al.* (1985). Therefore it follows that, whenever the agonist-sensitive Ca^{2+} pool is emptied by agonist activation, the plasma-membrane permeability to Ca^{2+} will be increased. In these experiments, this effect is short-lived because the test application of Ca^{2+} serves to restore the pool. However, in the continued presence of an agonist, $\text{Ins}(1,4,5)\text{P}_3$ would prevent the refilling of the pool, and a sustained influx of Ca^{2+} would result.

Fig. 3. Methacholine followed by atropine does not induce an augmented Ca^{2+} influx if the intracellular Ca^{2+} pool is filled

In (a), atropine and, 3 min later, 3.0 mM- Ca^{2+} were added to parotid acinar cells incubated in a medium lacking extracellular Ca^{2+} and containing 0.2 mM-EGTA. In (b), 0.1 mM-methacholine was added to cells in the presence of 1.0 mM- Ca^{2+} , and then atropine was added 3 min later. This was followed 1 min later by excess EGTA (3.0 mM), and then, after 1 min more, with an additional 5.0 mM- CaCl_2 ($[\text{Ca}^{2+}] \approx 3.0$ mM). Previous studies (Hughes *et al.*, 1988) have shown that, after the addition of atropine in the presence of extracellular Ca^{2+} , the agonist-sensitive Ca^{2+} pool is completely refilled within 1 min. A single experiment is shown; two others produced similar results.

These data provide strong evidence in favour of the capacitative model for Ca^{2+} entry in the parotid acinar cell, and may well apply for a number of other cell types in which Ca^{2+} fluxes are under primary control of inositol-lipid metabolism. However, it is also quite possible that other mechanisms may regulate transmembrane Ca^{2+} fluxes in other systems, or that under some circumstances this mechanism of Ca^{2+} entry can be regulated or modified

by other second messengers, such as inositol 1,3,4,5-tetrakisphosphate (Morris *et al.*, 1987).

How information concerning the state of the intracellular agonist-sensitive Ca^{2+} pool is conveyed to the plasma membrane is not directly addressed by these experiments. The rapidity of Ca^{2+} entry into the pool from the extracellular space has suggested proximity between these organelles (Putney, 1986), and subcellular-fractionation results support this idea (Guillemette *et al.*, 1988). Thus there may be substantial compartmentation of the cytosolic compartment through which this flux occurs, and the buffering action of fura-2 may serve to short-circuit some of this flux, making it more readily detectable as a rise in total cytosolic $[\text{Ca}^{2+}]$. The mechanism of interaction or communication between these important Ca^{2+} -regulating cellular components will be a challenging topic for future investigations.

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