Evidence for a non-capacitative Ca^{2+} entry during $[\text{Ca}^{2+}]$ oscillations

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Current models for the agonist-induced activation of Ca^{2+} entry from the extracellular medium in non-excitable cells generally emphasize a capacitative mechanism whereby Ca^{2+} entry is activated simply as a result of the emptying of intracellular Ca^{2+} stores, without any direct involvement of inositol phosphates. To date, the activation and control of Ca^{2+} entry have generally been studied under conditions where the agonist-sensitive stores undergo a profound and sustained depletion. However, responses under more normal physiological conditions typically involve the cyclical release and refilling of the stores associated with oscillations in $[\text{Ca}^{2+}]$, and the nature and control of entry under these conditions has received relatively little attention. In this study, using isolated cells from the exocrine avian nasal gland as

a model system, we show that: (a) the agonist-enhanced rate of Mn^{2+} quench is independent of the cyclical emptying and refilling of the agonist-sensitive Ca^{2+} pool during oscillations; (b) the Ca^{2+} entry pathway is maintained in an activated state for extended periods following inhibition of oscillations under conditions in which agonist-sensitive stores can be shown to be full; (c) no Ca^{2+} entry could be detected in oscillating cells in experiments that followed a definitive protocol for the demonstration of capacitative entry; and (d) on initial exposure to low agonist concentrations, activation of Ca^{2+} entry preceded any detectable release of Ca^{2+} from the stores. We conclude that the essential characteristics of the control of Ca^{2+} entry during oscillations are incompatible with current capacitative models.

INTRODUCTION

In most non-excitable cells, agonist-induced increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are known to result from the combination of a release of Ca^{2+} from intracellular stores, and an entry of Ca^{2+} from the extracellular medium. In recent years, considerable advances have been made in detailing the processes involved, particularly the generation of inositol 1,4,5-trisphosphate (InsP_3) and its role in the release of Ca^{2+} from specific intracellular stores. Far less is known about the Ca^{2+} entry component. Despite some continued controversy, current ideas concerning the activation of this latter component are focused on the so-called capacitative model [1,2] in which the enhanced entry of Ca^{2+} results solely from the emptying of the agonist-sensitive intracellular Ca^{2+} stores, without any direct requirement for inositol phosphates. Evidence in support of the existence of such a mechanism in a wide variety of different cell types is extensive, and direct electrophysiological characterization of a Ca^{2+} -selective current that is activated by depletion of the intracellular Ca^{2+} stores has recently been reported [3–9].

An important point to note is that these studies on Ca^{2+} entry have almost exclusively focused on responses under conditions where there is an extensive and sustained depletion of the agonist-sensitive Ca^{2+} stores (e.g. at high agonist concentrations, or in the presence of SERCA pump inhibitors such as thapsigargin). Under these conditions, the role of the Ca^{2+} entry component in generating the sustained elevation of $[\text{Ca}^{2+}]_i$, typically associated with such conditions, is clear. However, $[\text{Ca}^{2+}]_i$ signals in response to lower, more physiologically relevant, concentrations of agonists are typically far more complex and subtle than the simple elevation of $[\text{Ca}^{2+}]_i$ to a new sustained level. These complex responses include repetitive oscillations or waves of $[\text{Ca}^{2+}]_i$ [10–13]. To date, the nature and role of the Ca^{2+} entry component in these types of signal has received relatively little attention, and it is generally considered that Ca^{2+} entry

plays only a minor role in such responses. For example, in each of the various models for $[\text{Ca}^{2+}]_i$ oscillations that have been proposed [14–18], the basic mechanism underlying the oscillations is essentially intrinsic to the cell and the assumed role of Ca^{2+} entry is simply to replenish intracellular Ca^{2+} lost during the oscillations and to recharge the intracellular stores [19,20]. Accordingly, studies indicating that Ca^{2+} entry was capable of modulating the frequency of such oscillations (for example in hepatocytes [21] and in *Xenopus* oocytes [22]) were explained on the basis that the timing of the repetitive release of Ca^{2+} was determined by the progressive recharging of stores during the interspike interval, and that Ca^{2+} entry influenced the rate at which this occurred [21,23,24].

Recent studies have revealed a role for Ca^{2+} entry in $[\text{Ca}^{2+}]_i$ oscillations that goes far beyond the previously supposed simple recharging of stores and replenishment of Ca^{2+} lost to the cell [25,26]. Contrary to current assumptions [20,23,24], we found that the interspike interval in oscillating cells does not reflect the period required to refill the stores, and that the intrinsic ability of the stores to oscillate is independent of their degree of loading. Furthermore, at the low agonist concentrations where oscillations are most commonly observed, agonist-generated levels of InsP_3 are inadequate, on their own, to effectively release Ca^{2+} from the intracellular stores and generate oscillations in $[\text{Ca}^{2+}]_i$. In order to drive the generation of such signals, an additional component of Ca^{2+} entry is critically required. This action of Ca^{2+} entry is unrelated to the recharging of the internal stores as previously proposed [20] and instead involves the triggering of the repetitive release of Ca^{2+} from the agonist-sensitive stores. Consistent with this model, we showed that, in the continued presence of agonist, acute inhibition of Ca^{2+} entry results in the immediate cessation of the oscillations, but does so without any detectable depletion of the agonist-sensitive stores.

The demonstration of this previously unsuspected but essential role for Ca^{2+} entry in generating $[\text{Ca}^{2+}]_i$ oscillations raises

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; InsP_3 , inositol 1,4,5-trisphosphate.

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questions as to the nature of the activation and control of such entry under conditions when agonist-sensitive stores are undergoing the cyclical emptying and refilling associated with oscillations. In the study presented here we have examined the characteristics of the agonist-enhanced entry of Ca^{2+} during oscillatory $[\text{Ca}^{2+}]_i$ responses. Using a variety of experimental approaches we show that the nature of the Ca^{2+} entry under these conditions demonstrates properties that are incompatible with current versions of the 'capacitative' model.

MATERIALS AND METHODS

Techniques for the isolation of cells from the nasal glands of six 10-day-old domestic ducklings (*Anas platyrhynchos*), their loading with the fluorescent probe indo 1 and subsequent microfluorimetric measurements in single individual cells, were all as previously described [25,27]. Changes in $[\text{Ca}^{2+}]_i$ were determined as the ratio of the emitted fluorescence, measured as photon counts, at 405 nm and 485 nm with excitation at 350 nm, corrected for background and autofluorescence on-line. The simultaneous determination of fluorescence ratio and Mn^{2+} quench in individual cells was essentially as described previously [27]. Briefly, this involves the summing of the two emitted fluorescences which are factored such that the resulting 'adjusted total fluorescence' is independent of $[\text{Ca}^{2+}]$. Determination of the required factor is performed for each individual cell at the beginning of the experiment by observing the effects of induced oscillatory changes in $[\text{Ca}^{2+}]_i$, in the absence of extracellular Mn^{2+} .

RESULTS AND DISCUSSION

We first examined whether the rate of entry oscillated along with the intracellular stores by following the quenching of intracellular indo by externally applied Mn^{2+} . Using the technique described above, we were able to simultaneously measure the rate of Mn^{2+} quench (following addition of a low concentration of Mn^{2+} to the external medium) and oscillations in $[\text{Ca}^{2+}]_i$ (determined as the 405/485 fluorescence ratio) in single individual cells. Regular $[\text{Ca}^{2+}]_i$ oscillations similar to those reported elsewhere [25,28,29] were induced by addition of low concentrations (0.25–1.0 μM) of carbachol. We have previously demonstrated that $[\text{Ca}^{2+}]_i$ oscillations in the avian nasal gland cells involve the cyclical emptying and refilling of essentially the entire agonist-releasable pool of Ca^{2+} [30]. Simultaneous determination of Mn^{2+} quenching revealed that these oscillations were associated with a significantly enhanced (3–6-fold) increase in the measured quench rate (Figure 1A). More importantly, the observed agonist-enhanced Mn^{2+} quench rate was constant (i.e. non-oscillatory) during the pronounced oscillations in $[\text{Ca}^{2+}]_i$ (Figure 1B). These results confirm our earlier report of a non-oscillatory enhanced rate of Mn^{2+} quenching during oscillations [25] as well as similar findings in HeLa cells [24] and endothelial cells [31]. In contrast, an oscillatory entry of Ca^{2+} has been demonstrated in lymphocytes [32], but it is clear that the nature of the $[\text{Ca}^{2+}]_i$ oscillations and their relationship to Ca^{2+} entry is very different in these cells compared with the exocrine cells investigated here. For example the oscillations in lymphocytes do not require the generation of InsP_3 , nor do they involve any Ca^{2+} -induced Ca^{2+} release, and are typically broad sigmoidal changes in $[\text{Ca}^{2+}]_i$ each lasting for some 100 s and with a periodicity of between 100 and 200 s [32]. More directly relevant to our findings is the report of an oscillatory Mn^{2+} quench during $[\text{Ca}^{2+}]_i$ oscillations in AR42J cells [33], and in hepatocytes [34]. One possible complication in these studies is that Mn^{2+} quenching was measured using an

Figure 1 Quenching of intracellular indo 1 by Mn^{2+} during $[\text{Ca}^{2+}]_i$ oscillations

(A) A cell was bathed in regular saline to which Mn^{2+} (0.2 mM) had been added and $[\text{Ca}^{2+}]_i$ measured as the 405/485 emission ratio with indo (continuous line), along with the resting Mn^{2+} quench (●) which was measured as the decline in the adjusted total fluorescence 'Ftot', (see the Materials and methods section for details). The mean resting quench rate is represented by the line marked 'a'. Addition of 0.5 μM carbachol (added at arrow) induced an oscillatory $[\text{Ca}^{2+}]_i$ response and an increase in the simultaneously determined rate of Mn^{2+} quench (line 'b'). Trace is representative of five separate experiments. (B) Detail from a trace from a similar experiment to that in (A) showing the rate of Mn^{2+} quench during the $[\text{Ca}^{2+}]_i$ oscillations.

isobestic wavelength technique. A potential problem with this technique is that the spectral properties of the Ca^{2+} probes are known to be significantly affected by various intracellular components. Consequently, it cannot be assumed that a wavelength chosen from measurements made in an artificial solution will be truly isobestic in the intracellular environment of any particular individual cell. Even small deviations from a true isobestic value will result in artifactual oscillations in the apparent rate of quench. Furthermore, it is known that AR42J cells, although derived from pancreatic acinar cells, possess voltage-activated Ca^{2+} channels that are not normally present in exocrine cells. It is conceivable that such channels could act in a reciprocal fashion with Ca^{2+} -activated K^{+} channels to produce oscillatory changes in membrane potential and activation of Ca^{2+} entry via such voltage-activated pathways.

Although we have demonstrated a non-oscillatory Mn^{2+} quench during oscillations in $[\text{Ca}^{2+}]_i$, it is important to note that the precise relationships between Mn^{2+} entry (quenching) and Ca^{2+} entry are often unclear. Nevertheless, the observed increase in quench rate presumably indicates that at least some component of bivalent cation entry is increased during oscillations. We have previously shown that the Mn^{2+} quench of intracellular indo shares many of the known properties of the agonist-activated Ca^{2+} entry pathway (e.g. inhibition by SK&F 96365 and La^{3+} , activation by thapsigargin, etc.) [27]. Furthermore, additional

Figure 2 Restoration of $[Ca^{2+}]_i$ oscillations after their inhibition

(A) Effect of restoring Ca^{2+} entry after inhibition of oscillations without depletion of the stores. A cell oscillating in response to $0.5 \mu M$ carbachol was exposed to a high- K^+ medium ($[K^+] = 70$ mM) during the period indicated ('Hi-K'), resulting in the cessation of oscillations. Restoration of normal medium resulted in the rapid resumption of oscillations whose shape and frequency was immediately identical to those seen immediately prior to inhibition [see also (B)]. (B) Superimposition of the last oscillation before depolarization (■) and the first oscillation after repolarization (○) from another experiment similar to that represented in (A). Fluorescence ratio determinations were made at a frequency of 10 Hz. (C) Effect of restoring Ca^{2+} entry after partial depletion of the stores. A cell oscillating in response to $0.5 \mu M$ carbachol was exposed to a modest elevation of medium K^+ concentration ($[K^+] = 30$ mM) during the period indicated by the arrow. This resulted in a partial depletion of the stores, as indicated by the decline in oscillation amplitude. Subsequent restoration of the normal medium produced irregular oscillations of slowly increasing amplitude.

experiments revealed that raising the concentration of Mn^{2+} , relative to that of Ca^{2+} , in the extracellular medium to a higher value (Mn^{2+}/Ca^{2+} ratio > 1) induced a progressive decline in

oscillation amplitude, with the oscillations eventually stopping as the stores became empty (results not shown). This is similar to the response seen on reducing extracellular Ca^{2+} , and suggests that Mn^{2+} and Ca^{2+} are likely to be competing for a common agonist-enhanced entry pathway that is associated with oscillations. Although such findings do not exclude the possibility of the presence of a distinct Ca^{2+} entry pathway that is not recognized by Mn^{2+} , the data do imply that the rate of at least a component of agonist-activated Ca^{2+} entry was unaffected by the cyclical emptying and refilling of the oscillating Ca^{2+} stores. As the oscillations in these cells involve only $InsP_3$ -sensitive stores [29], such data are apparently inconsistent with current capacitative models for Ca^{2+} entry. However, one possible explanation could be that a non-oscillatory rate of entry results from the interpolation of a significant delay in the activation and inactivation of the entry pathway by a signal that is, in fact, generated in an oscillatory manner by the cyclical emptying of the stores. At the present time, we have no way of directly examining such a proposition.

As noted above, we have previously shown that the acute inhibition of Ca^{2+} entry in an oscillating cell results in the immediate cessation of the oscillations, yet this occurs without any detectable depletion of the agonist-sensitive Ca^{2+} stores. We have argued that this results from an absolute requirement for Ca^{2+} entry to trigger the release of Ca^{2+} from the $InsP_3$ -sensitive stores at the low agonist concentrations associated with oscillatory $[Ca^{2+}]_i$ signals [25]. It follows that if, as we have shown, the agonist-sensitive stores are full when the oscillations have been stopped by inhibition of Ca^{2+} entry, then capacitative mechanisms for Ca^{2+} entry should be absent. Holding the cell under these conditions would also allow any lingering capacitative signal to become dissipated. The subsequent restoration of conditions permitting Ca^{2+} entry would then be expected to have no effect, despite the continued presence of agonist, as there would be no Ca^{2+} entry and hence no trigger for the release of intracellular Ca^{2+} . To examine this we initiated oscillations in $[Ca^{2+}]_i$ using a low concentration of carbachol as before. These oscillations were then blocked by depolarizing the cell by exposing it to a medium with an elevated K^+ concentration (70 mM), as reported previously [25]. Calculations indicate that this would depolarize the cell to approx. -10 mV from its resting value of -50 mV [30], and evidence shows that this is sufficient to completely inhibit the sustained $[Ca^{2+}]_i$ signal seen at higher carbachol concentrations and which is known to reflect Ca^{2+} entry (see Figure 3A). Similar data have been reported for various non-excitable cells [35–37]. Following inhibition of $[Ca^{2+}]_i$ oscillations by exposure to the high- K^+ medium, subsequent repolarization of the cell, by restoring the normal medium, resulted in the almost immediate resumption of oscillations (typically within a few seconds of repolarization; see Figure 2A). Furthermore, the essential features of the restored oscillations (frequency, shape, amplitude) are all immediately identical to those seen prior to depolarization (Figure 2B). It is important to note that the response observed is in marked contrast to experiments performed under conditions where oscillations are allowed to run down as a result of a gradual store depletion (for example, by merely reducing Ca^{2+} entry). In these latter experiments, even after only modest depletion of the stores, restoration of Ca^{2+} entry produces a slow increase in $[Ca^{2+}]_i$ followed by gradual resumption of irregular oscillations of progressively increasing frequency (Figure 2C). This clear difference in the response seen on restoring Ca^{2+} entry after even only partial depletion of the stores further confirms that, under conditions of acute inhibition of Ca^{2+} entry, the oscillations must have ceased without any significant depletion of the agonist-sensitive stores.

Figure 3 Demonstration of capacitative Ca^{2+} entry in a cell showing a sustained $[\text{Ca}^{2+}]_i$ signal (A), and its absence in a cell showing an oscillating $[\text{Ca}^{2+}]_i$ response (B)

See text for details. Cells were first exposed to either 5 μM carbachol to induce a sustained elevation in $[\text{Ca}^{2+}]_i$ (A), or to 0.5 μM carbachol to induce oscillations in $[\text{Ca}^{2+}]_i$ (B). Subsequently, Ca^{2+} entry was blocked by depolarizing the cells using a high- K^+ medium ($[\text{K}^+] = 70 \text{ mM}$), followed by addition of atropine to displace the carbachol from its receptor (indicated by the block marked 'atro'). Refilling of agonist-depleted stores is prevented by the continued presence of the high- K^+ medium. After the addition of atropine (30 s), the medium was changed to one containing 10 mM Ca^{2+} to reveal any enhanced Ca^{2+} entry remaining. Such entry would correspond to a capacitative entry as it would be dependent only on the depleted state of the agonist-sensitive stores, and not on the sustained activation of the receptor or on the presence of elevated levels of inositol phosphates.

However, as we have seen, restoration of conditions allowing Ca^{2+} entry (i.e. repolarization) resulted in the immediate restoration of normal oscillations. The important point is that, given that we have previously demonstrated that the oscillations are acutely dependent on agonist-activated Ca^{2+} entry, their immediate resumption upon repolarization implies that the Ca^{2+} entry pathway has remained activated during the depolarization. This is despite the fact that the evidence shows that the agonist-sensitive stores are not depleted during this phase. In additional experiments, we have been able to show that the entry pathway can remain activated under these conditions for at least 4 min. This sustained activation of the Ca^{2+} entry pathway under conditions in which the agonist-sensitive stores are not depleted is in direct contradiction to the capacitative model for Ca^{2+} entry. The prolonged activation of the entry pathway for several minutes under such conditions would also tend to rule out the possibility of any sustained capacitative signal being involved.

In an attempt to more directly compare Ca^{2+} entry under oscillating conditions with that seen during a sustained depletion of the agonist-sensitive stores, a protocol was used that essentially

defines the capacitative model. An essential paradigm of this model is that, following depletion of the stores by exposure to an agonist, Ca^{2+} entry remains activated even after removal of the agonist and the decay of elevated levels of inositol phosphates, as long as refilling of the stores is prevented [1,2]. In the protocol we used, cells were stimulated appropriately to produce either an oscillatory $[\text{Ca}^{2+}]_i$ response or a sustained $[\text{Ca}^{2+}]_i$ response (0.5 μM carbachol and 5 μM carbachol respectively). They were then exposed to a high- K^+ medium to inhibit Ca^{2+} entry, followed by atropine to remove the agonist and allow InsP_3 to return to resting levels. Under these conditions, depleted stores are prevented from refilling, despite removal of the agonist. The resulting capacitative entry of Ca^{2+} can then be readily revealed on subsequent repolarization and exposure to a high- Ca^{2+} medium (10 mM). We have previously shown that even at supramaximal carbachol concentrations (500 μM) InsP_3 levels decline to basal values within 15 s of addition of atropine [38]. In the protocol employed here, cells were exposed to atropine for 30 s before restoration of Ca^{2+} entry. As predicted by the capacitative model, in those cells initially showing a sustained elevation in $[\text{Ca}^{2+}]_i$ (treated with 5 μM carbachol), a prolonged rise in $[\text{Ca}^{2+}]_i$ was seen on addition of the high- Ca^{2+} medium, indicative of a pronounced capacitative Ca^{2+} entry (Figure 3A). In marked contrast, absolutely no increase in $[\text{Ca}^{2+}]_i$ could be detected with this protocol in cells showing an oscillatory $[\text{Ca}^{2+}]_i$ signal in response to 0.5 μM carbachol (Figure 3B). These results indicate the absence of any detectable capacitative entry under these circumstances and suggest that Ca^{2+} entry during oscillatory $[\text{Ca}^{2+}]_i$ responses may be more directly dependent on receptor occupation itself.

Finally, it is inherent in the capacitative model that the agonist-induced release of Ca^{2+} from the stores must precede the activation of Ca^{2+} entry. It follows from this that inhibition of agonist-activated Ca^{2+} entry should be without effect on the initial release of Ca^{2+} from the stores. In initial experiments examining this, we compared the time to the first $[\text{Ca}^{2+}]_i$ spike following addition of agonist (latent period) in normal medium to that seen in a high- K^+ medium where Ca^{2+} entry is reduced as a consequence of a partial depolarization of the cell. We first showed that duplicate stimulation of a single individual cell with a 5 min interval between stimulations resulted in no significant change in latent period. The mean latent period for a second control stimulus equalled $102.8 \pm 4.5\%$ of the first latent period (mean $\pm$ S.E.M., $n = 14$). As an extra precaution we randomized the order of treatment (normal versus depolarized) in the different experimental repeats. In a series of such experiments in individual cells, we found that the latent period varied considerably from cell to cell, ranging from 5 to 30 s. However, in each individual cell, depolarization significantly increased the observed latent period by an average of $50.7 \pm 8.4\%$ over control values (mean $\pm$ S.E.M., $n = 21$). In three additional cases, the cells failed to respond at all when depolarized, yet did so normally on repolarization. Current models indicate that latency at low agonist concentrations depends primarily on the rate of generation of InsP_3 and the associated increase in the permeability of the agonist-sensitive stores [39,40]. While this may be modulated by such factors as the extent of cytosolic Ca^{2+} buffering and the rate of Ca^{2+} pumping, etc. [39–41], Ca^{2+} entry itself should have no effect on latency. Our observation that reducing Ca^{2+} entry by depolarizing the cell affects the latency of the initial response to agonist implies an activation of Ca^{2+} entry at low agonist concentrations that precedes any measurable discharge of the intracellular stores. Even more dramatic effects were seen in experiments where inhibition of Ca^{2+} entry was obtained by brief pre-exposure of the cells to SK&F 96365, a drug that is

Figure 4 Effect of SK&F 96365 on the initiation of an oscillatory $[Ca^{2+}]_i$ response

A cell was exposed to SK&F 96365 (30 μ M) for 20 s prior to the addition of carbachol (0.5 μ M). After failing to respond to the carbachol within approx. 140 s, the SK&F 96365 was washed off, whereupon the cell began to oscillate in response to carbachol.

known to effectively inhibit agonist-activated Ca^{2+} entry in a variety of cell types [27,42]. In these, a pre-exposure with 30 μ M SK&F 96365 for only 20 s was sufficient to completely block the initiation of an oscillatory response on addition of agonist. Oscillations rapidly began, however, on subsequent washout of the SK&F 96365 (Figure 4). This effect of SK&F 96365 could not be explained by any inhibition of the carbachol-induced increase in $InsP_3$ levels as these were unaffected by exposure to SK&F 96365. Values obtained from a binding assay (pmol of $InsP_3$ /mg of protein) were: control, 7.9 ± 2.3 ; carbachol-stimulated (5 μ M), 16.9 ± 1.8 ; carbachol-stimulated in the presence of SK&F 96365 (30 μ M), 18.2 ± 0.6 (mean $\pm$ S.E.M., $n = 3$).

These data show that inhibition of Ca^{2+} entry can dramatically affect the initial agonist-dependent release of Ca^{2+} from the stores, with the clear implication that the agonist-induced activation of Ca^{2+} entry is occurring prior to Ca^{2+} release. We have previously reported a similar conclusion based on measurements on cell suspensions using fluoroaluminate to slowly initiate the $[Ca^{2+}]_i$ response [43]. Removing external Ca^{2+} has been reported to significantly lengthen the latent period to the initial response to agonists in hepatocytes [44] although its implications were not discussed. Similarly, Berridge [23] recently reported that reducing extracellular Ca^{2+} significantly lengthened the latent period to low 5-hydroxytryptamine concentrations in blowfly salivary glands. These findings were interpreted as indicating a need for a Ca^{2+} entry-dependent recharging of the agonist-sensitive stores prior to their initial discharge. The activation of this Ca^{2+} entry was believed to result from a 'covert' activity of $InsP_3$ on stores close to the plasma membrane resulting in a small, undetectable, capacitative entry [23]. Unfortunately, there is no direct evidence for such a hidden activity, nor for the need to recharge stores before they can be stimulated to release. Indeed, we have previously shown that the ability of the stores to discharge their Ca^{2+} is unrelated to their degree of loading. Furthermore, it is unclear why the stores should be partially empty in the resting cell at all. An alternative explanation for these results is that Ca^{2+} entry is activated independently of store emptying, with the clear implication that the entry of Ca^{2+} under these conditions cannot be gated by any mechanism dependent on depletion of the stores. Hence, by definition, such entry cannot be via any capacitative mechanism.

Conclusions

We conclude that several of the features of Ca^{2+} entry observed during oscillatory $[Ca^{2+}]_i$ responses are inconsistent with the capacitative model, at least as presently understood. In particular, (a) the agonist-enhanced rate of Mn^{2+} quench is independent of the cyclical emptying and refilling of the agonist-sensitive Ca^{2+} pool during $[Ca^{2+}]_i$ oscillations; (b) the Ca^{2+} entry pathway appears to be maintained in an activated state for extended periods following inhibition of oscillations under conditions in which agonist-sensitive stores can be shown to be full; (c) no Ca^{2+} entry could be detected in oscillating cells in experiments that followed a definitive protocol for the demonstration of capacitative entry; and (d) on initial exposure to low agonist concentrations, activation of Ca^{2+} entry preceded any detectable release of Ca^{2+} from the stores.

It has become increasingly clear that the specific spatial and temporal patterns of $[Ca^{2+}]_i$ signalling in exocrine, as well as other, cells have a major impact on the effective control of cell activity. Current models of these phenomena generally emphasize the special properties and modifications of $InsP_3$ -induced Ca^{2+} release, with Ca^{2+} entry playing only a minor sustaining role. We have previously shown that, contrary to such models, agonist-enhanced Ca^{2+} entry has a critical role in the initiation and modulation of such responses, a role that involves the Ca^{2+} entry-induced triggering of $InsP_3$ -dependent intracellular Ca^{2+} release. In the study reported here, using a variety of different experimental protocols, we have demonstrated certain unique features in the control of Ca^{2+} entry at the low agonist concentrations where these types of response are typically produced. Importantly, these features are uniformly inconsistent with the proposal that agonist-activated Ca^{2+} entry during oscillations is occurring via a capacitative mechanism. It would seem that, although a capacitative mechanism undoubtedly exists in many cells (and can be clearly demonstrated in our cells under conditions of a sustained depletion of agonist-sensitive intracellular stores), such a mechanism may not be the primary one controlling Ca^{2+} entry during oscillating $[Ca^{2+}]_i$ responses. Possible alternative mechanisms for controlling Ca^{2+} entry under these circumstances fall into two broad groups: either entry is being controlled by some component of the phospholipase C/inositol phosphate pathway (such as via an action of $InsP_3$ and/or $InsP_4$ at the plasma membrane), or the agonist is activating a distinct signalling pathway that is independently responsible for the control of Ca^{2+} entry. Resolution of these alternatives must await further studies.

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