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Ca^{2+} influx drives agonist-activated $[Ca^{2+}]_i$ oscillations in an exocrine cell

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Abstract In current models describing agonist-induced oscillations in $[Ca^{2+}]_i$, Ca^{2+} entry is generally assumed to have a simple sustaining role, replenishing Ca^{2+} lost from the cell and recharging intracellular Ca^{2+} stores. In cells from the avian nasal gland, a model exocrine cell, we show that inhibition of Ca^{2+} entry by La^{3+}, SK&F 96365, or by membrane depolarization, rapidly blocks $[Ca^{2+}]_i$ oscillations but does so without detectible depletion of agonist-sensitive Ca^{2+} stores. As the rate of Mn^{2+} quenching during $[Ca^{2+}]_i$ oscillations is constant, Ca^{2+} entry is not directly contributing to the $[Ca^{2+}]_i$ changes and, instead, appears to be involved in inducing the repetitive release of Ca^{2+} from internal stores. Together, these data contradict current models in that (i) at the low agonist concentrations where $[Ca^{2+}]_i$ oscillations are seen, generated levels of $Ins(1,4,5)P_3$ are *themselves* inadequate to result in a regenerative $[Ca^{2+}]_i$ signal, and (ii) Ca^{2+} entry is necessary to actually drive the intrinsic oscillatory mechanism.

Key words: $[Ca^{2+}]_i$ oscillation; Ca^{2+} entry; Exocrine cell; Ca^{2+} release

1. Introduction

Changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting from receptor activation by appropriate agonists are known to comprise two components, namely the release of Ca^{2+} from intracellular stores and the entry of Ca^{2+} from the extracellular medium. The essential role of agonist-activated Ca^{2+} entry in the sustained elevation of $[Ca^{2+}]_i$ seen at high agonist concentrations is clear, but the role of Ca^{2+} entry in the oscillatory $[Ca^{2+}]_i$ signals often seen at lower agonist concentrations is less well-defined. Current models of $[Ca^{2+}]_i$ oscillations are broadly divided into those in which the concentration of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) oscillates [1,2], and those where $InsP_3$ concentrations are constant [3–5]. The latter can be further subdivided into those in which the oscillations derive from the periodic release of Ca^{2+} from an $InsP_3$-insensitive pool (by a process of Ca^{2+}-induced Ca^{2+} release, CICR) [3], and those in which the oscillations reflect repetitive Ca^{2+} release from the $InsP_3$-sensitive stores as a result of a biphasic $[Ca^{2+}]_i$-sensitization and desensitization of the $InsP_3$-induced release mechanism [4,5]. Although, in all these models the basic mechanism underlying agonist-induced $[Ca^{2+}]_i$ oscillations is entirely intrinisic, such oscillations can frequently be sustained only for a limited time in the absence of extracellular Ca^{2+}, although the length of time before decay varies considerably dependent on cell type and on the agonist involved. Such an effect is generally explained by assuming that the role of Ca^{2+} entry is simply to recharge intracellular stores and replenish intracellular Ca^{2+} lost during the $[Ca^{2+}]_i$ oscillations [6,7].

A few studies have shown that modulation of Ca^{2+} influx can markedly affect both the temporal and spatial patterns of $InsP_3$-mediated Ca^{2+} release [8,9], suggesting a more direct role for Ca^{2+} entry in influencing certain types of oscillatory $[Ca^{2+}]_i$ signals. However, the underlying bases for these changes were not examined in detail and were generally assumed to result from effects on store replenishment [8]. In the study reported here, we have investigated the role of Ca^{2+} entry in agonist-induced $[Ca^{2+}]_i$ oscillations in individual isolated cells from the avian nasal gland, a model exocrine cell [10–13], using both microfluorimetric determinations of $[Ca^{2+}]_i$ and perforated patch-clamp measurements of Ca^{2+}-activated membrane currents. Our results indicate that, contrary to current views, agonist-activated Ca^{2+} entry plays an essential and critical role in $[Ca^{2+}]_i$ oscillations apparently by driving the repetitive release of Ca^{2+} from agonist-sensitive stores.

2. Experimental

Single cell fluorescence measurements on isolated cells obtained from the nasal glands of 2- to 10-day-old domestic ducklings (*Anas platyrhynchos*) and loaded with indo-1 [12,14] were made using a single cell photon-counting dual-emission microfluorimetric system (Newcastle Photometrics) mounted on an inverted microscope (Nikon Diaphot) as previously described [14]. Emitted fluorescence from individual cells was determined as photon counts at 405 nm and 485 nm with excitation at 350 nm, corrected for background and autofluorescence on-line and stored on computer at a rate of 1 Hz. Changes in $[Ca^{2+}]_i$ were determined as the 405/485 ratio. Simultaneous measurements of the fluorescence ratio of indo-1 and Mn^{2+} quench rates were obtained using the technique described previously [14].

Whole-cell perforated-patch recordings were achieved by use of the pore-forming antibiotic amphotericin B as previously described [15]. The standard intracellular (pipette) solution contained (in mM) 100 K-glutamate, 40 KCl, 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, pH 7.2. The recording of membrane currents (Axopatch-1C, Axon Instruments), generation of voltage pulses (100 ms with a stepping frequency of 1 Hz), and data collection were controlled by custom software. At the resolution shown in the figures the current traces appear continuous. All experiments were performed at room temperature (20–22°C).

3. Results and discussion

Regular $[Ca^{2+}]_i$ oscillations in isolated avian nasal gland cells were induced by addition of low concentrations (0.25–1.0 μM) of the muscarinic agonist carbachol (CCh). The $[Ca^{2+}]_i$ oscillations observed were similar to those obtained elsewhere [13] appearing as large, regular transients, each lasting 8–15 s, with a frequency of 3–5 per minute. Between each transient, $[Ca^{2+}]_i$ generally returned to values slightly elevated over resting levels. Reduction in the extracellular Ca^{2+} concentration (to < 30 μM) resulted in the decay and eventual loss of the oscillations (Fig. 1) (see also [13]). Once oscillations had ceased, the subsequent addition of a supra-maximal concentration of CCh (10 μM) produced no change in $[Ca^{2+}]_i$, indicating that agonist-sensitive

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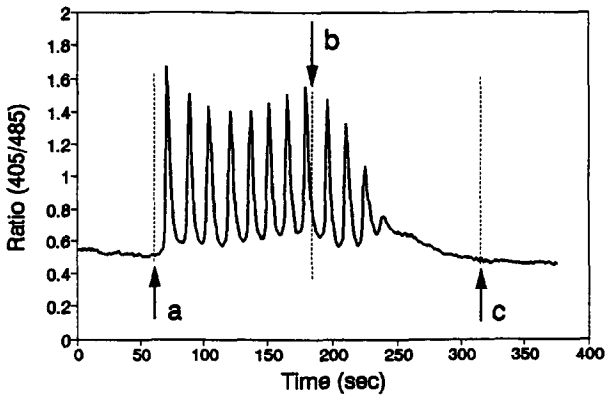

Fig. 1. Effect of reduction in extracellular Ca^{2+} on oscillations in $[Ca^{2+}]_i$ in nasal gland cells. $[Ca^{2+}]_i$ in individual cells loaded with indo-1 was determined as the 405 nm/485 nm fluorescence emission ratio as described in Section 2. Cells were stimulated with a low concentration of CCh (0.5 μM) at arrow 'a'. At arrow 'b' extracellular Ca^{2+} concentration was reduced to < 30 μM followed (at 'c') by addition of a supramaximal concentration of CCh (10 μM).

intracellular Ca^{2+} stores were essentially empty. Such data are consistent with the current view that Ca^{2+} entry is required to replenish Ca^{2+} lost during the oscillations.

To examine this further, the effect of adding 1 mM La^{3+} to cells induced to oscillate with CCh was investigated. At this concentration, La^{3+} blocks both Ca^{2+} entry and Ca^{2+} efflux via the plasma membrane Ca^{2+} ATPase [16,17] which, in the absence of a detectible Na^+/Ca^{2+} exchange in our cells, is the principal route of Ca^{2+} efflux. This was confirmed in preliminary experiments showing that the normal decline to resting $[Ca^{2+}]_i$ seen in CCh-stimulated (10 μM) cells in a nominally Ca^{2+}-free medium was converted into a sustained $[Ca^{2+}]_i$ signal in the presence of 1 mM La^{3+} (data not shown). It was anticipated that addition of 1 mM La^{3+} to oscillating cells would maintain CCh-induced $[Ca^{2+}]_i$ oscillations despite blocking Ca^{2+} entry because the simultaneous inhibition of Ca^{2+} efflux should prevent loss of Ca^{2+} from the cell. However, rather than sustaining $[Ca^{2+}]_i$ oscillations, addition of 1 mM La^{3+} resulted in their rapid termination (Fig. 2A). Significantly, the subsequent addition of a high concentration of CCh (10 μM) resulted in a large Ca^{2+} release indistinguishable from that seen in cells initially stimulated at this concentration, indicating that the oscillations had ceased without measurable depletion of the agonist-sensitive Ca^{2+} stores. This effect did not result from the La^{3+}-induced blockage of Ca^{2+} efflux mechanisms as essentially identical results were obtained in experiments using SK&F 96365 (Fig. 2B), a drug that has been shown to be an effective inhibitor of receptor-activated Ca^{2+} entry pathways in these and other cells [14,18]. To confirm that the above effects did not result from changes in the cytosolic Ca^{2+} buffering a result of loading of the cells with indo-1, parallel experiments were performed using the perforated patch-clamp technique in unloaded cells. In this technique, the integrity of the normal intracellular Ca^{2+} buffering systems are maintained and $[Ca^{2+}]_i$ can be monitored as changes in Ca^{2+}-activated membrane currents. As previously reported [15] low concentrations of CCh produced oscillatory increases in Ca^{2+}-activated Cl^- currents that were reversibly dependent on the presence of extracellular Ca^{2+}. As was seen in the indo-1 experiments, addition of 1 mM La^{3+} resulted in the rapid inhibition of the oscillations in the CCh-induced Ca^{2+}-dependent current (Fig. 2C).

As a non-pharmacological approach to this question, the effect of reducing the driving force on Ca^{2+} entry by depolarizing the membrane was examined. In perforated-patch experiments, CCh-induced oscillations in membrane currents monitored by pulsing to +30 mV from a holding potential of −60 mV were rapidly terminated on clamping the cell at +30 mV (Fig. 3A). Parallel experiments were performed using indo-1

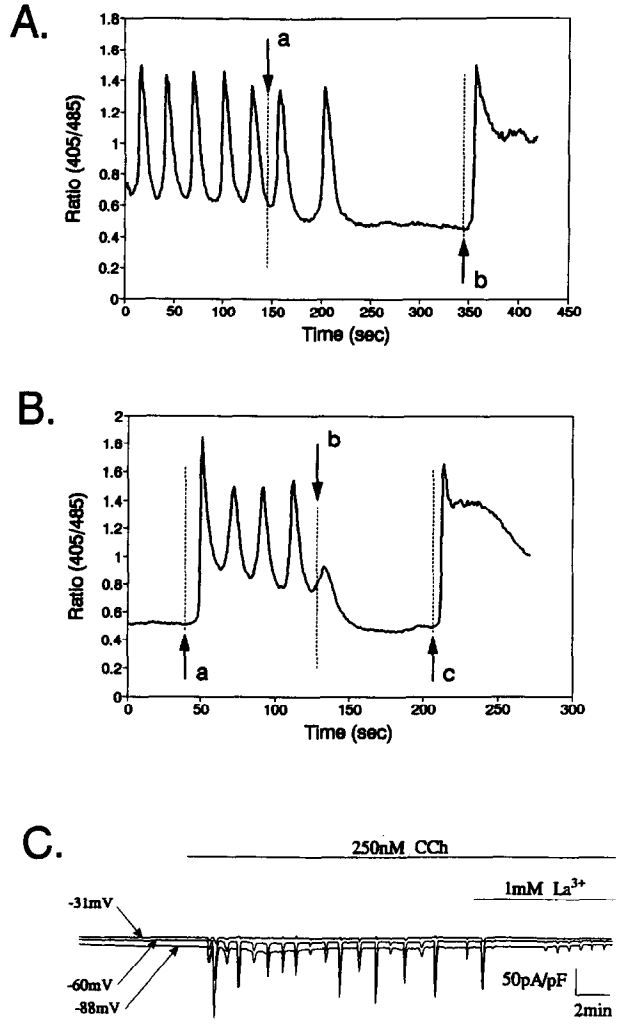

Fig. 2. Effect of La^{3+} and SK&F 96365 on $[Ca^{2+}]_i$ oscillations in nasal gland cells. $[Ca^{2+}]_i$ was measured either as the 405/485 fluorescence ratio in individual cells loaded with indo-1 (A and B) or as Ca^{2+}-activated Cl^- current (C) as described in section 2. In all experiments involving La^{3+} addition, phosphate and sulfate in the standard saline was replaced with chloride to prevent precipitation. (A) A cell oscillating in response to 1 μM CCh was selected and 1 mM La^{3+} added (at arrow 'a'), followed by 10 μM CCh (at arrow 'b'). (B) $[Ca^{2+}]_i$ oscillations were induced in a cell by addition of 0.5 μM CCh (arrow 'a'). SK&F 96365 (50 μM) was added at arrow 'b' followed (at arrow 'c') by a high concentration of CCh (10 μM). All traces are representative of at least 4–6 similar ones. (C) Membrane currents in a cell recorded under perforated patch-clamp conditions. Membrane potential was held −60 mV and pulsed to −88 mV and −31 mV for 100 ms every second to record Ca^{2+}-activated Cl^- currents [11]. At the resolution shown, the indivdual current traces (at −88 mV, −60 mV and −31 mV) appear continuous. Oscillations in this current were induced in a cell by addition of CCh (0.25 μM) and were rapidly terminated on addition of 1 mM La^{3+}.

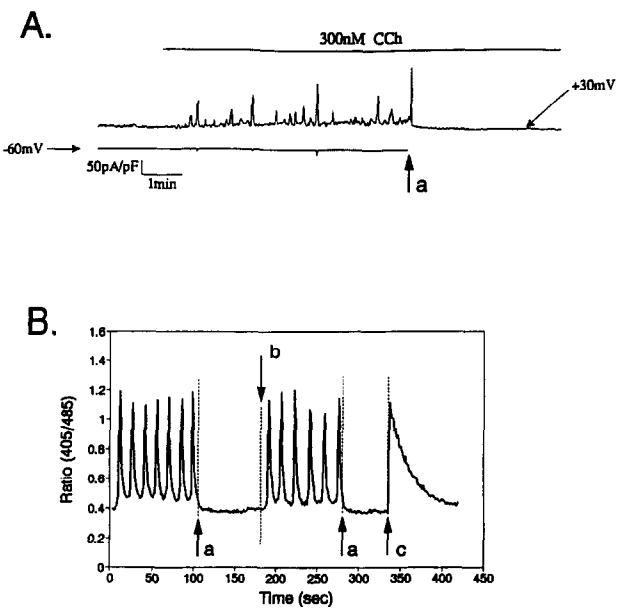

Fig. 3. Effect of membrane depolarization on CCh-induced $[Ca^{2+}]_i$ oscillations. (A) Membrane currents measured in a cell under perforated patch-clamp conditions. Addition of CCh (0.3 μM) induced oscillations in the membrane currents recorded in a cell by pulsing to +30 mV for 100 ms every second from a holding potential of −60 mV. At the point indicated (arrow 'a'), membrane potential was clamped at +30 mV. (B) Effect of high-K^+ medium on $[Ca^{2+}]_i$ oscillations measured as the fluorescence ratio in cells loaded with indo-1 as in Fig. 1. A cell oscillating in response to 0.5 μM CCh was selected. At the points indicated (arrows 'a') the the normal medium was changed to a high-K^+ medium, and returned to normal medium at arrow 'b'. Finally CCh (10 μM) was added (at arrow 'c') to assess the status of the agonist-sensitive stores. Calculations indicate that the membrane would be depolarized to approximately −17 mV by the change to the high-K^+ medium.

fluorescence to monitor $[Ca^{2+}]_i$. In these, depolarization of the membrane was achieved by replacing the normal medium with a high-K^+ medium (extracellular Na^+ replaced with K^+ to give a $[K^+]$ = 70 mM). In preliminary experiments, exposure to this medium markedly reduced the sustained elevation in $[Ca^{2+}]_i$ seen in cells stimulated with 10 μM CCh (data not shown). In CCh-stimulated cells showing a oscillatory $[Ca^{2+}]_i$ signal, switching to the high-K^+ medium resulted in an immediate cessation of the oscillations (Fig. 3B). This effect was fully reversible. Addition of a high concentration of CCh (10 μM) to a cell in which $[Ca^{2+}]_i$ oscillations had been blocked by exposure to the high-K^+ medium resulted in a large $[Ca^{2+}]_i$ transient whose magnitude was indistinguishable from the normal response. Once again, this indicates that the termination of $[Ca^{2+}]_i$ oscillations had occurred without detectible depletion of the agonist-sensitive stores.

The key finding in these experiments is that inhibition of Ca^{2+} entry with either La^{3+}, SK&F 96365 or depolarization rapidly blocks $[Ca^{2+}]_i$ oscillations, but does so with the agonist-sensitive stores remaining essentially full despite the continued presence of agonist. Clearly, at these low agonist concentrations, the levels of $InsP_3$ produced are *in themselves* inadequate to effectively release Ca^{2+} from the intracellular stores and to generate oscillations in $[Ca^{2+}]_i$.

To determine whether Ca^{2+} entry was directly contributing to the rise in $[Ca^{2+}]_i$ associated with the agonist-induced oscillations, simultaneous measurements of $[Ca^{2+}]_i$ and Ca^{2+} entry (determined as the Mn^{2+} quench of intracellular indo-1) during oscillations induced by low concentrations of CCh were performed. As demonstrated previously [14], CCh activates a Mn^{2+}-permeable divalent cation entry pathway resulting in the quenching of fluorescence of intracellular indo-1 [14]. Simultaneous measurements of $[Ca^{2+}]_i$ and Mn^{2+} quench in oscillating CCh-stimulated cells showed that the oscillations are associated with a constant, sustained rate of Mn^{2+} quenching (Fig. 4). Previous studies of Mn^{2+} quenching during $[Ca^{2+}]_i$ oscillations have produced contradictory results [19,20]. An oscillatory mechanism involving only $InsP_3$-insensitive (caffeine-sensitive) stores which do not influence Ca^{2+} entry [21] cannot explain our findings as we have previously shown that such stores are not present in nasal gland cells [15]. One possibility is that, at low agonist concentrations, Ca^{2+} entry is controlled primarily by the sustained depeletion of a small subset of the overall agonist-sensitive stores. If so, such stores must be uniquely sensitive to $InsP_3$ and their depletion cannot contribute significantly to the magnitude of overall Ca^{2+} release. Alternatively, we have recently demonstrated the presence of a significant temporal lag in the activation of the Ca^{2+} entry pathway by store emptying [14]. Such a lag could result in the signal derived from the repetitive transient emptying and filling of the stores becoming smoothed into a constant submaximal signal. Whatever the underlying basis for our observations, they indicate that Ca^{2+} entry is constant during agonist-induced oscillations. Ca^{2+} entry cannot, therefore, be making any significant direct contribution to the cyclical changes in $[Ca^{2+}]_i$ observed. Instead, a sustained agonist-activated Ca^{2+} entry appears to be involved in actually driving the $[Ca^{2+}]_i$ oscillations.

There are several ways in which this could occur. The direct recharging of agonist-sensitive stores by Ca^{2+} entry, as previously proposed [7,21,22], can be excluded in our experiments as inhibition of Ca^{2+} entry blocked $[Ca^{2+}]_i$ oscillations without a corresponding measurable depletion of the stores. Clearly, the key role of Ca^{2+} entry is linked to the emptying of agonist-sensitive stores not their refilling. One way in which Ca^{2+} entry

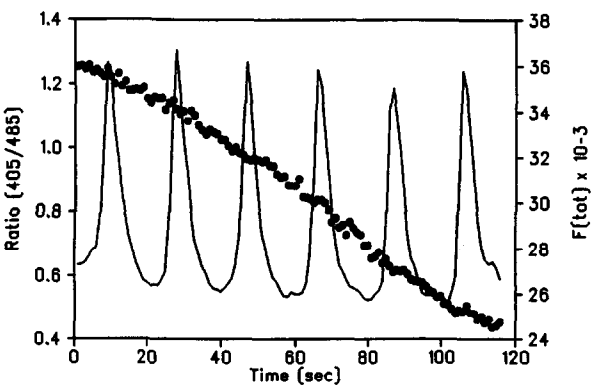

Fig. 4. Rate of Mn^{2+} quench during $[Ca^{2+}]_i$ oscillations. An indo-1 loaded cell oscillating in response to CCh (0.5 μM) was selected and Mn^{2+} (1 mM) added to obtain a measure of Ca^{2+} entry rate from the measured rate of quenching of indo-1. $[Ca^{2+}]_i$ (as fluorescence ratio, continuous line) and Mn^{2+} quench (●) were measured as described in section 2. The observed rate of Mn^{2+} quench is typical of agonist-activated cells and is some 3–5 times higher than that seen in non-stimulated cells. The trace is representative of at least 5 others.

could be involved in store refilling and yet have a critical role in triggering Ca^{2+} release would be through an effect on $InsP_3$-induced Ca^{2+} release via a luminal Ca^{2+} modulatory site [23–25]. We were unable to detect any evidence for such sites in our cells [26], and the presence and/or physiological relevance of such sites is currently controversial (see, for example [27]).

We conclude that a strictly cytosolic site for the Ca^{2+} entry effect on Ca^{2+} oscillations, is indicated. Direct actions on phospholipase C (PLC) influencing the generation of $InsP_3$ [28] are unlikely as physiological levels of $[Ca^{2+}]_i$ appear to have minimal effects on PLC activity in these [29] and many other cells [30], and raising $[Ca^{2+}]_i$ with A23187 never results in oscillations [15]. Effects involving feedback on PLC activities also seem unlikely as these usually involve a PKC-mediated negative feedback. If Ca^{2+} entry was involved in the cyclical activation of PKC then the inhibition of Ca^{2+} entry would remove this negative feedback and inhibit oscillations with the stores empty, not full as we have observed.

An effect of Ca^{2+} entry on Ca^{2+} release is therefore indicated. As nasal gland cells do not possess $InsP_3$-insensitive CICR stores [15], only models of $[Ca^{2+}]_i$ oscillations involving $InsP_3$-sensitive stores need be considered. Such models are based on evidence that $InsP_3$-induced Ca^{2+} release is modulated by $[Ca^{2+}]_i$ in a biphasic manner, being facilitated by moderate increases in $[Ca^{2+}]_i$ but inhibited, on a slower time-scale, by higher values of $[Ca^{2+}]_i$ [31,32]. The basic oscillatory mechanism is therefore entirely intrinisic to the stores and the process is, theoretically, self-sustaining. However, we have shown that a component of Ca^{2+} entry appears to play a critical role in driving or priming this oscillatory mechanism. Two possible explanations for our data appear most likely. First, it has recently been proposed in pancreatic acinar cells [33] that saturation of cytosolic Ca^{2+} buffers may be necessary before Ca^{2+} release can be initiated. It is possible that low concentrations of $InsP_3$ could partially deplete the stores but such release, in itself, is inadequate to saturate cytosolic Ca^{2+} buffers sufficiently to raise $[Ca^{2+}]_i$ to the level required to initiate a generalized $InsP_3$-sensitive Ca^{2+} release. Simultaneous activation of Ca^{2+} entry by the initial partial depletion of the stores could serve to saturate these buffers such that this release was now sufficient to raise $[Ca^{2+}]_i$ above threshold. Alternatively, low concentrations of $InsP_3$ could selectively empty a small proportion of the total intracellular stores that are preferentially coupled to the Ca^{2+} entry mechanism (see above). The induced Ca^{2+} entry could then raise $[Ca^{2+}]_i$ sufficiently to render the remaining stores sensitive to the low $InsP_3$ concentration resulting in a $InsP_3$-sensitive Ca^{2+}-induced release. The presence of subsets of agonist-sensitive stores with differing sensitivity to $InsP_3$ has already been demonstrated in various cells [34,35]. Either of the above mechanisms would be dependent on elevated levels of both $InsP_3$ and Ca^{2+} entry and, as found, the latter would not oscillate but would be constant.

Interestingly, a version of the single-pool model has proposed that, if maintained $InsP_3$ gradients exist in cells, then oscillations could be initiated by the release of Ca^{2+} from pools closest to the region of highest $InsP_3$ concentration, with the released Ca^{2+} then sensitizing the $InsP_3$ receptors on the remaining pools [7]. Whether such gradients in $InsP_3$ exist is unknown. However if, as we suggest, the triggering event was a local elevation of $[Ca^{2+}]_i$ resulting from Ca^{2+} entry then this would indeed give rise to an analogous situation that is consistent with our data. In our model, $InsP_3$ concentrations are constant *and* subthreshold throughout the cell and Ca^{2+} entry has the essential role of driving the $[Ca^{2+}]_i$ oscillations by initiating Ca^{2+} release. Inhibition of Ca^{2+} entry would cause the oscillations to cease with the stores full despite continued agonist action and $InsP_3$ generation, exactly as we have observed. As such, Ca^{2+} entry provides 'primer' Ca^{2+} much as previously proposed [7], but with the critical difference that its role is not to recharge the internal stores [7] but to initiate their release. That Ca^{2+} entry can indeed drive Ca^{2+} release in the presence of subthreshold levels of $InsP_3$ was demonstrated in a recent study in *Xenopus* oocytes [36], although it was considered that such effects would only be of significance in cells possessing voltage-activated Ca^{2+} channels and undergoing large fluctuations in membrane potential, conditions that do not apply in our cells.

Although the above arguments are based on an effect on $InsP_3$-sensitive stores only, there is no reason why a similar effect may not apply to those cells where $[Ca^{2+}]_i$ oscillations involve $InsP_3$-insensitive stores and a CICR mechanism. Presumably, however, the effects we have described would not apply to those agonist-induced oscillations that are resistant to removal of extracellular Ca^{2+} and/or inhibition of Ca^{2+} entry, such as those induced by CCK in pancreatic cells [37]. However, it is currently unclear whether these differences reflect the involvement of a fundamentally different mechanism of oscillation or not. For example, transitions between different types of oscillation (sinusoidal versus transient) with corresponding differences in sensitivity to extracellular Ca^{2+} removal can be induced by the same agonist in the same cell simply by modifying cytosolic Ca^{2+} buffering [38], or resting $[Ca^{2+}]_i$ [39].

In conclusion, we have shown that the role of Ca^{2+} entry in certain agonist-induced $[Ca^{2+}]_i$ oscillations goes far beyond the previously supposed simple recharging of intracellular stores and replenishment of Ca^{2+} lost to the medium. Significantly, at low agonist concentrations where oscillations are most commonly observed, generated levels of $InsP_3$ are *themselves* inadequate to result in a regenerative $[Ca^{2+}]_i$ signal. A component of Ca^{2+} entry is essential to drive the generation of such oscillatory signals, apparently by inducing the repetitive release of Ca^{2+} from internal stores.

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