Ca^{2+} entry modulates oscillation frequency by triggering Ca^{2+} release

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As in many cells, the frequency of agonist-induced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations in exocrine avian nasal gland cells is dependent on the rate of Ca^{2+} entry. Experiments reveal that the initiation of each oscillatory spike is independent of the relative fullness of the stores and, furthermore, the oscillating pool is normally fully refilled by the end of each $[Ca^{2+}]_i$ spike. Therefore, contrary to current models, the interspike interval (which essentially sets the frequency) does not reflect the time taken to recharge the oscillating stores. Instead, the data show that it is the previously demonstrated role that Ca^{2+} entry plays in triggering the repetitive release of Ca^{2+} from the oscillating stores, rather than the recharging of those stores, that provides the basis for the observed effects of Ca^{2+} entry rate on oscillation frequency.

INTRODUCTION

In exocrine cells, as in many other cells, a key feature of many of the pathways linking activation of receptors on the cell surface with the initiation and control of cell function is the elevation of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$). These increases in $[Ca^{2+}]_i$ are known to comprise two components: (i) a release of Ca^{2+} from intracellular stores, and (ii) an entry of Ca^{2+} from the extracellular medium. In recent years it has become clear that the $[Ca^{2+}]_i$ responses to lower, presumably more physiologically relevant, agonist concentrations are far more complex and subtle than the simple elevation of $[Ca^{2+}]_i$ to a new sustained level. These complex responses include repetitive oscillations of $[Ca^{2+}]_i$, or waves of elevated $[Ca^{2+}]_i$ that spread through the cytosol [1–4], and it has become increasingly clear that the specific spatial and temporal pattern of $[Ca^{2+}]_i$ signalling in many cells has a major impact on the effective control of cell activity. Of particular interest in exocrine cells is the generation of oscillatory $[Ca^{2+}]_i$ signals. Current models for such oscillations include those in which the concentration of agonist-generated inositol 1,4,5-trisphosphate ($Ins P_3$) oscillates [5,6], and those where $Ins P_3$ concentrations are constant. Among the latter are those in which the oscillations derive from a Ca^{2+}-induced Ca^{2+} release (CICR) from $Ins P_3$-insensitive pools [7], and those which involve repetitive Ca^{2+} release and re-uptake from the $Ins P_3$-sensitive stores, a response arising as a result of the bell-shaped dependency of $Ins P_3$-induced release on $[Ca^{2+}]_i$ [8,9]. In each case, the basic mechanism underlying the oscillations is essentially intrinisic to the cell and the principal assumed role of Ca^{2+} entry is simply to recharge intracellular stores and replenish intracellular Ca^{2+} lost during the oscillations [10,11]. Consequently, the role of the Ca^{2+} entry component in these specific types of response has received relatively little attention. In recent studies [12] we have demonstrated a role for Ca^{2+} entry in $[Ca^{2+}]_i$ oscillations that goes far beyond the previously supposed simple recharging of stores and replenishment of Ca^{2+} lost to the cell. We found that, at low agonist concentrations (i.e. under the very conditions where oscillations are most commonly observed), agonist-generated levels of $Ins P_3$ are themselves inadequate to result in a regenerative $[Ca^{2+}]_i$ signal, and that a component of Ca^{2+} entry is essential to drive the generation of such signals. Acute inhibition of Ca^{2+} entry by a variety of means results in the immediate cessation of oscillations, but not because of any depletion of the oscillating stores which remain full under these conditions. The critical role of Ca^{2+} entry therefore appears to involve inducing the repetitive release of Ca^{2+} from internal stores [12]. These findings raise the possibility of previously unsuspected roles for agonist-activated Ca^{2+} entry in the modulation of oscillatory $[Ca^{2+}]_i$ signals, particularly those that involve only $Ins P_3$-sensitive stores. For example, it has been shown, in a variety of different cells, that the rate of Ca^{2+} entry significantly affects oscillation frequency [13–17]. This effect was assumed to result from changes in the rate of refilling of the agonist-sensitive stores [13], and it is a central premise of current versions of both the single-pool and two-pool models for $[Ca^{2+}]_i$ oscillations that the interspike interval is principally set by the rate of refilling of the oscillating stores which, in turn, is dependent on the rate of Ca^{2+} entry [11,18,19] (see [20] for a theoretical analysis). In this report, using cells from the avian nasal gland (which is a model exocrine cell), we examine this assumption. We present evidence to show that oscillation frequency is not related to store refilling, and that the observed effects actually result from the direct action of Ca^{2+} entry in the triggering of Ca^{2+} release.

MATERIALS AND METHODS

Fluorescence measurements were made on single isolated cells obtained from the nasal glands of 6–10-day-old domestic ducklings (*Anas platyrhynchos*) following loading with indo-1 [21,22] using a single-cell photon-counting dual-emission microfluorimetric system (Newcastle Photometrics) mounted on an inverted microscope (Nikon Diaphot) as previously described [22]. 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 in a computer at a rate of 1 Hz. Changes in $[Ca^{2+}]_i$ were determined as the 405/485 ratio. During recording, cells were continuously perfused with saline at a rate of approximately 3.5–4.0 ml/min (chamber volume was approximately 0.5 ml). Brief exposure to external media of changed composition was achieved by perfusion via a separate small pipette positioned within 1–2 mm of the observed cell. All experiments were performed at room temperature (20–22 °C).

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; $Ins P_3$, inositol 1,4,5-trisphosphate.

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RESULTS

As previously shown, $[Ca^{2+}]_i$ oscillations in the avian nasal gland cells are readily induced by low concentrations (0.25–1.0 μM) of the muscarinic agonist carbachol [12,23,24]. They consist of large, regular transients in $[Ca^{2+}]_i$ occurring at a frequency of approximately 2–5 per min, with each transient lasting approximately 8–15 s (Figure 1a). Between each successive oscillation, values of $[Ca^{2+}]_i$ return to resting levels or slightly above resting levels, depending on the oscillation frequency.

The role of agonist-sensitive stores in $[Ca^{2+}]_i$ oscillations

We have previously shown that the carbachol-induced $[Ca^{2+}]_i$ oscillations in these cells involve only InsP_3-sensitive stores and that caffeine-sensitive stores are absent [12,24]. In order to examine the proportion of the total agonist-sensitive stores that are involved in each oscillation, a supramaximal concentration of carbachol (50 μM) was added to an oscillating cell to compare the magnitude of the $[Ca^{2+}]_i$ spike following maximal agonist-dependent release with that occurring with each oscillation. Since the thapsigargin-sensitive and InsP_3-sensitive stores are identical in these cells, and supramaximal agonist addition results in the complete emptying of those stores [25,26], this protocol provides a convenient assay for the Ca^{2+} status of the agonist-sensitive stores. In order to avoid any potential reduction in Ca^{2+} release resulting from inhibition of the InsP_3 receptor by elevated levels of $[Ca^{2+}]_i$, the addition of the supramaximal carbachol concentration was delayed until a point immediately prior to the next anticipated oscillatory $[Ca^{2+}]_i$ spike, or for approximately 15 s after $[Ca^{2+}]_i$ had returned to resting levels. As illustrated in Figure 1(b), the results obtained demonstrate that the magnitude of the $[Ca^{2+}]_i$ spike resulting from the release of Ca^{2+} from the agonist-sensitive stores following application of a supramaximal concentration of carbachol (maximal agonist-dependent release) is not significantly larger than that seen during the regular oscillations. It is concluded that each $[Ca^{2+}]_i$ oscillation therefore reflects the cyclical emptying and refilling of essentially the entire agonist-sensitive Ca^{2+} pool of the cell.

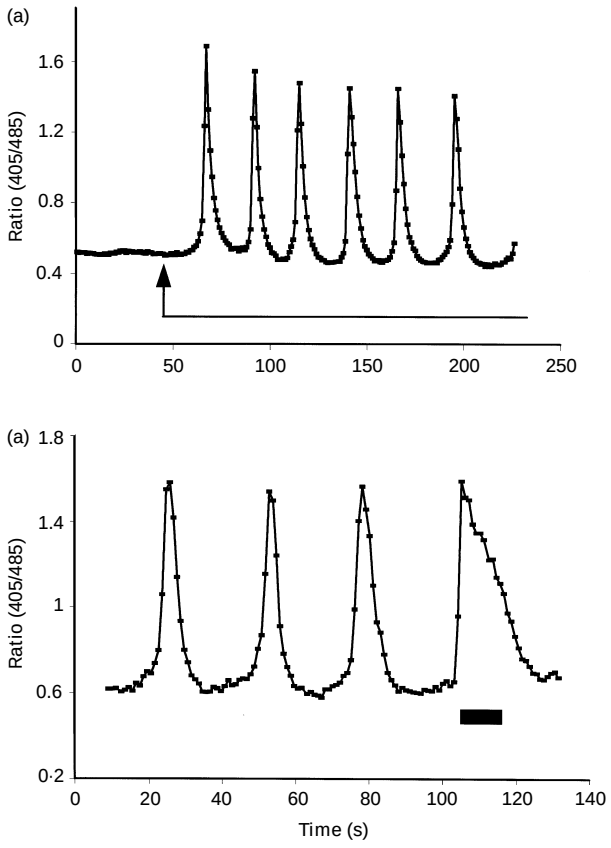

Figure 1 Carbachol-induced $[Ca^{2+}]_i$ oscillations in an avian nasal gland cell

(**a**) The emitted fluorescence ratio (405 nm/485 nm, excitation at 350 nm) from a single cell loaded with indo-1, was recorded as described (see the Materials and methods section) while being continuously perfused with saline. At the point indicated (arrow), the perfusing medium was changed to one containing 0.5 μM carbachol. (**b**) Comparison between maximal agonist-dependent $[Ca^{2+}]_i$ release with that occurring during oscillations. A single indo-1-loaded cell was induced to oscillate by addition of 0.5 μM carbachol. At the point indicated (bar), the carbachol concentration of the perfusion medium was briefly switched to 50 μM, a supramaximal agonist concentration.

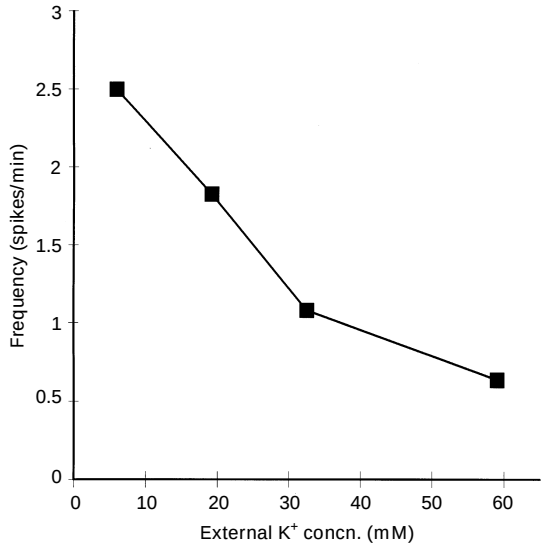

Figure 2 Effect of external K^+ concentration on oscillation frequency

Single indo-1-loaded cells were stimulated to oscillate by addition of 0.5 μM carbachol. They were then perfused with media with various K^+ concentrations (Na^+ removed to maintain isotonicity) and the oscillation frequency [1/peak to peak time (in s) $\times$ 60] recorded. The trace illustrates the data obtained from a single individual cell and is representative of four separate experiments.

The effect of Ca^{2+} entry on oscillation frequency

We have previously shown that the activation of regular $[Ca^{2+}]_i$ oscillations in these cells is associated with an enhanced, constant rate of Mn^{2+} quench of intracellular indo-1, which presumably reflects at least a component of the agonist-activated entry of extracellular Ca^{2+} [12]. In the study reported here, it was first necessary to demonstrate that modulation of this agonist-enhanced rate of Ca^{2+} entry influenced oscillation frequency. Such an effect has previously been reported in a variety of different cell types [13–17].

An effective and readily reversible method of modulating Ca^{2+} entry that has been widely used in such studies is by depolarizing the cell membrane potential thereby reducing Ca^{2+} entry, presumably as a consequence of the resulting reduction in the electrochemical driving force on the Ca^{2+} ion (voltage-operated Ca^{2+} channels are absent in these cells). Such a depolarization can be simply achieved by exposing the cell to elevated medium K^+ concentrations. Figure 2 shows the effect of a series of such

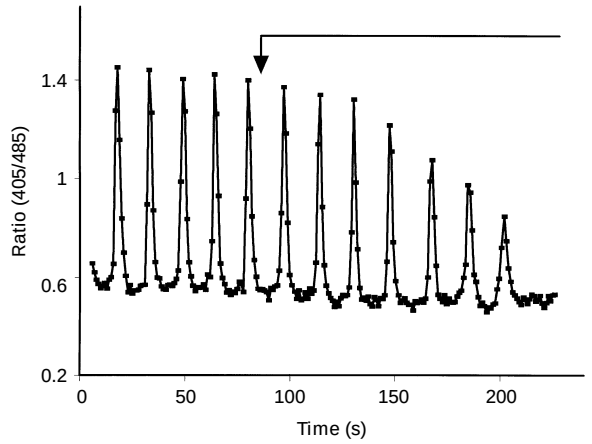

Figure 3 $[Ca^{2+}]_i$ oscillations during progressive store depletion

A single indo-1-loaded cell was stimulated to oscillate by addition of 0.5 μM carbachol, and at the point indicated (arrow), the Ca^{2+} concentration in the perfusing medium was reduced from 1.3 mM to 0.65 mM to induce a slow, progressive depletion of the agonist-sensitive stores. The trace is representative of five separate experiments.

increases of medium K^+ concentrations on oscillation frequency. The data demonstrate a fairly direct relationship between oscillation frequency and the external K^+ concentration. For example, increasing external K^+ concentration to 60 mM (which is predicted to result in a membrane depolarization from a resting value of approximately -50 mV [24] to close to -15 mV) produced a 4-fold change in oscillation frequency. This is similar to the effects seen in other cells where, for example, 3-fold decreases in oscillation frequency were induced by raising the extracellular K^+ concentration from 6 mM to 71 mM in hepatocytes [13], and by membrane depolarization from -50 mV to 0 mV in *Xenopus* oocytes [14]. As noted, where such effects have been observed, they are generally assumed to result from changes in the rate of refilling of the agonist-sensitive stores [13]. Clearly, whatever its precise basis, modulation of Ca^{2+} entry has a significant influence on oscillation frequency.

$[Ca^{2+}]_i$ oscillations during store depletion

As discussed above, current models explaining $[Ca^{2+}]_i$ oscillations under conditions of constant $InsP_3$ concentration propose that the interspike interval is set by the time required to recharge the internal stores sufficiently to induce the next spike [11,18,19,27]. A direct implication of such an interpretation is that it is necessary for the stores to refill to a particular level before they can release their stored Ca^{2+}. There are several ways such a requirement may arise, including possible effects of cytosolic or luminal Ca^{2+} on the relevant Ca^{2+}-release receptor/channel ($InsP_3$ receptor or ryanodine-sensitive receptor) in the internal stores. To examine whether successive oscillations are dependent on the relevent stores reaching a particular level of fullness, oscillating cells were exposed to a modest reduction in extracellular Ca^{2+} sufficient to induce a slow progessive depletion of the agonist-sensitive intracellular stores. Such depletion is confirmed by the subsequent failure to release any further Ca^{2+} on addition of a supramaximal agonist concentration [12]. As can be seen in Figure 3, the oscillatory release of Ca^{2+} continues throughout this process of progressive depletion of the stores, even though the amount of Ca^{2+} available for release becomes successively smaller and smaller. Clearly, the progressive depletion of the stores has little effect on their intrinsic ability to oscillate, and refilling of the store to some particular level is not a prerequisite for the initiation of a $[Ca^{2+}]_i$ spike. Essentially identical data were

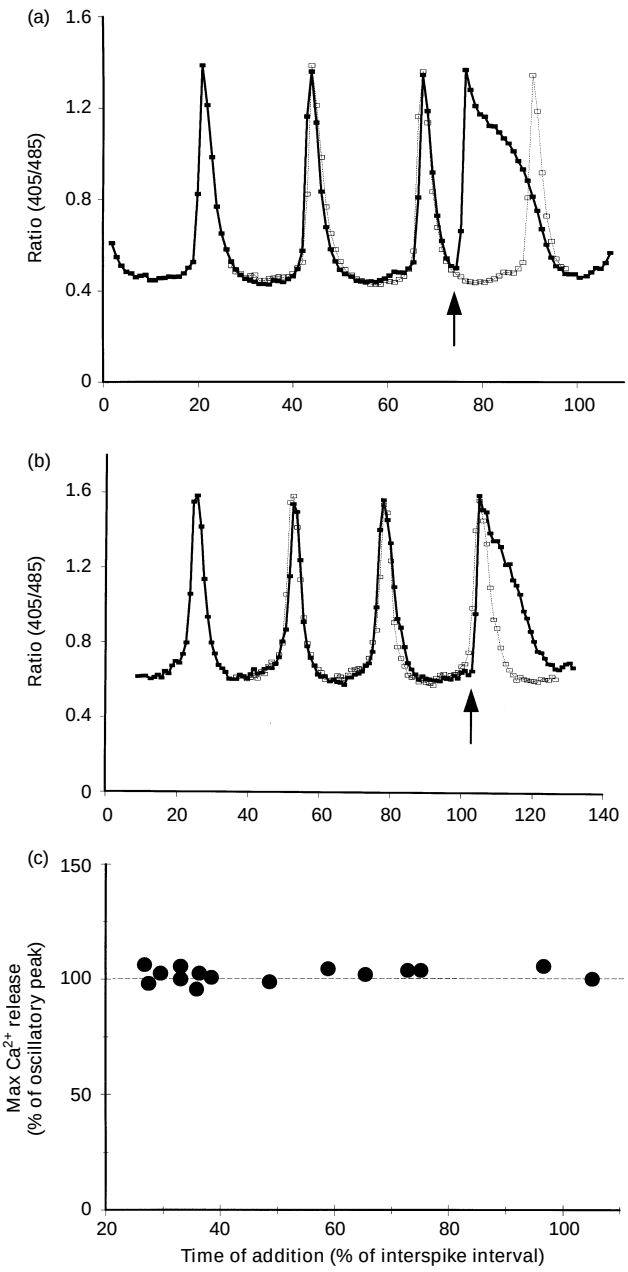

Figure 4 Magnitude of the agonist-releasable Ca^{2+} pool during the interspike interval

Cells induced to oscillate by addition of a low concentration of carbachol (0.5 μM) were briefly exposed to a supramaximal concentration of carbachol (50 μM) at various points (arrow) during the interspike interval to evaluate the size of the agonist-releasable Ca^{2+} pool. Illustrated are representative traces showing such determinations (**a**) at a point immediately after a regular oscillation, and (**b**) at a point immediately before the next anticipated oscillation. Also shown is the $[Ca^{2+}]_i$ trace for the same cell but advanced by one oscillation cycle (□), thereby indicating the predicted pattern of $[Ca^{2+}]_i$ changes in the absence of supramaximal stimulation. In (**c**) the results of several such experiments, each on different individual cells, are presented. The peak $[Ca^{2+}]_i$ value following supramaximal agonist addition (maximal agonist-induced Ca^{2+} release) is expressed as a percentage of the average peak $[Ca^{2+}]_i$ value from the immediately preceding three oscillations. This is plotted against the time of addition, expressed as a percentage of the average interspike interval of the same preceding three oscillations.

obtained using low concentrations of thapsigargin (20–50 nM), an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, to gradually deplete the stores in oscillating cells (results not shown). It should be noted that reducing extracellular Ca^{2+} might be expected to affect oscillation frequency, yet, in Figure 3, little change in frequency is seen. However, it must be remembered that the interpretation of any predicted changes in frequency under such conditions is significantly complicated by the presumed increase in capacitative Ca^{2+} entry as the stores progressively become depleted. The key finding in these experiments is that, contrary to the predictions of current models, the intrinsic ability of the stores to oscillate is essentially independent of their Ca^{2+} status.

Ca^{2+} store status during the interspike interval

A second, easily testable, implication of the assumption that the interspike interval is the period during which the oscillating Ca^{2+} pool refills, is that the size of the pool (i.e. the amount of Ca^{2+} releasable from the oscillating pool) should increase during the interspike interval. We have shown above that the carbachol-induced $[Ca^{2+}]_i$ oscillations in the avian nasal gland cells involve cyclical release and re-uptake of essentially the entire agonist-sensitive Ca^{2+} pool. It is therefore relatively easy to obtain an estimate of the magnitude of the oscillating pool at any point in the oscillation cycle in these cells simply by adding a supramaximal concentration of the agonist carbachol. Examples of such experiments are illustrated in Figure 4. It can be seen that the application of a supramaximal concentration of carbachol at an early point (Figure 4a) or a late point (Figure 4b) in the interspike interval results in the release of an essentially identical amount of Ca^{2+} relative to that released by the regular oscillations. The results from several such experiments are summarized in Figure 4(c) where the 'test' spike height obtained by adding the supramaximal agonist concentration (expressed as a percentage of the normal oscillating spike height) is plotted against the time of addition (expressed as a percentage of the normal interspike interval). The results obtained reveal that the Ca^{2+} released by such a supramaximal agonist application is the same throughout the interspike interval. The significance of this is that if the interspike interval truely reflected the period during which the oscillating stores progressively refill, then application of a supramaximal agonist concentration early in the interspike interval should result in a significantly smaller $[Ca^{2+}]_i$ spike than that seen later in the interval. In contrast, our data demonstrate that the oscillating stores are essentially full immediately at the end of each $[Ca^{2+}]_i$ spike. Clearly then, the duration of the interspike interval cannot reflect the period of refilling of the oscillating stores, which is essentially complete by the time $[Ca^{2+}]_i$ has returned to resting levels at the end of the spike. Interestingly, similar data were briefly reported in a study on histamine-induced $[Ca^{2+}]_i$ oscillations in human endothelial cells [28], although the implications were not discussed. Clearly, the findings we have obtained may be applicable to oscillatory $[Ca^{2+}]_i$ responses in a wide variety of different cell types.

DISCUSSION

As discussed, we have previously demonstrated a new and critical role for Ca^{2+} entry in $[Ca^{2+}]_i$ oscillations in these model exocrine cells, namely the triggering of repetitive Ca^{2+} release from the InsP_3-sensitive stores thereby driving the oscillations [12]. Such an action is unlikely to result from a cyclical activation of entry just prior to the initiation of each spike as we have previously shown that Mn^{2+} influx is constant during $[Ca^{2+}]_i$ oscillations [12]. Similar data, and a similar identification of the action of Ca^{2+} entry in triggering Ca^{2+} release, were subsequently reported by Thorn [27] in studies on HeLa cells. Despite the demonstration of this role for Ca^{2+} entry during oscillations, Thorn [27] concluded that the interspike interval represented a period during which the intracellular Ca^{2+} stores are progressively refilling and that Ca^{2+} entry 'only realizes a role in spike initiation at a point when intracellular Ca^{2+} stores are repleted' [27]. However, such an interpretation is inconsistent with our demonstration that (a) the initiation of a $[Ca^{2+}]_i$ spike is not dependent on the Ca^{2+} status of the stores (Figure 3), and (b) the stores are essentially replete immediately at the end of a $[Ca^{2+}]_i$ spike (Figure 4). Clearly, the duration of the interspike interval cannot reflect the rate of refilling, which is complete by the time $[Ca^{2+}]_i$ has returned to resting levels at the end of the spike. Nor is refilling of the store a prerequisite for the initiation of a $[Ca^{2+}]_i$ spike, as progressively depleting stores continue to oscillate. Consequently, any effect of Ca^{2+} entry on the rate of store replenishment is unlikely to be the basis for influences on oscillation frequency. It should also be noted that the data obtained directly contradict a primary feature of both the two-pool and single-pool models for $[Ca^{2+}]_i$ oscillations, namely that the interspike interval is determined by the rate of recharging of the oscillating pool.

Recently, Petersen et al. [29] presented data from experiments investigating the effects on oscillation frequency of partial inhibition of the store pump by low concentrations of thapsigargin. Although not directly discussed by the authors, the data obtained also contradict models proposing that the rate of store refilling determines oscillation frequency because they report that inhibition of the pumps on the stores led to an increase, not a decrease, in frequency. The authors instead proposed that the interspike interval was determined by the rate of loading of cytosolic buffers with Ca^{2+} released from the stores as a result of the sustained action of the low agonist-induced levels of InsP_3. They speculated that the loading of these buffers would be influenced by the rate of Ca^{2+} removal from the cytosol, either by sequestration into stores or pumping into the extracellular medium, as well as by the rate of Ca^{2+} entry. However, for the rate of Ca^{2+} entry to influence oscillation frequency, it is implicit in the model presented by Petersen et al. [29] that the cytosolic buffers be relatively full (loaded) immediately prior to each Ca^{2+} oscillation, and relatively empty (depleted) immediately following each oscillation. In other words, such buffers would have to discharge their bound Ca^{2+} during some stage of each $[Ca^{2+}]_i$ spike in order to be available for recharging during each interspike interval. Only in this way could Ca^{2+} entry affect oscillation frequency, by influencing the rate at which such cytosolic buffers become loaded. However, it is difficult to envisage exactly how such buffers could unload or discharge their bound Ca^{2+} during an oscillation when cytosolic levels of Ca^{2+} are clearly elevated above resting values, and yet recharge during the interspike interval when $[Ca^{2+}]_i$ is at or close to resting values. We believe that a simpler explanation for the data reported by Petersen et al. [29] is that the application of thapsigargin results in the progressive partial depletion of the stores with a concomitant increase in capacitative Ca^{2+} entry. The observed increase in oscillation frequency results simply from this enhanced Ca^{2+} entry.

We conclude that neither an action on the rate of store replenishment [11,18,19,27], nor one on the rate of loading of cytosolic buffers [29] can adequately explain the observed influence of Ca^{2+} entry rate on $[Ca^{2+}]_i$ oscillation frequency. Instead, the results are consistent with the demonstrated action of Ca^{2+} entry in driving oscillatory $[Ca^{2+}]_i$ responses by triggering

the repetitive release of Ca^{2+} from agonist-sensitive stores. Although not directly demonstrated, this action presumably results from the Ca^{2+}-induced sensitization of the InsP_3 receptors on at least a portion of the stores to the low agonist-enhanced levels of InsP_3.

Finally, the fact that the stores in our cells normally refill so rapidly (i.e. during the period of the down-stroke of a single oscillation, or within approx. 5 s) suggests that little Ca^{2+} is lost to the medium during each oscillation. Given this finding, it might be expected that the maintenance of $[Ca^{2+}]_i$ oscillations would be largely independent of reductions in Ca^{2+} entry (for example by removing extracellular Ca^{2+}). Yet, as we have previously shown, oscillations are immediately terminated by the acute inhibition of Ca^{2+} entry [12]. Significantly, this apparent contradiction is immediately resolved with the realization that, as we have shown, the key role of Ca^{2+} entry during $[Ca^{2+}]_i$ oscillations lies not in replenishing the stores, but rather in triggering their emptying.

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