Minimal requirements for calcium oscillations driven by the IP₃ receptor

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Introduction

One of the most common and fundamental mechanisms of cell signaling is through changes in the cytosolic free Ca²⁺ concentration ([Ca²⁺]c), which render dynamic regulation of intracellular targets. Imaging of fluorescent Ca²⁺ indicators located within intracellular Ca²⁺ stores was used to monitor IP₃ receptor channel (IP₃R) function and to demonstrate that IP₃-dependent oscillations of Ca²⁺ release and re-uptake can be reproduced in single permeabilized hepatocytes. This system was used to define the minimum essential components of the oscillation mechanism. With IP₃ clamped at a submaximal concentration, coordinated cycles of IP₃R activation and subsequent inactivation were observed in each cell. Cycling between these states was dependent on feedback effects of released Ca²⁺ and the ensuing [Ca²⁺]c increase, but did not require Ca²⁺ re-accumulation. [Ca²⁺]c, can act at distinct stimulatory and inhibitory sites on the IP₃R, but whereas the Ca²⁺ release phase was driven by a Ca²⁺-induced increase in IP₃ sensitivity, Ca²⁺ release could be terminated by intrinsic inactivation after IP₃ bound to the Ca²⁺-sensitized IP₃R without occupation of the inhibitory Ca²⁺-binding site. These findings were confirmed using Sr²⁺, which only interacts with the stimulatory site. Moreover, vasopressin induced Sr²⁺ oscillations in intact cells in which intracellular Ca²⁺ was completely replaced with Sr²⁺. Thus, [Ca²⁺]c, oscillations can be driven by a coupled process of Ca²⁺-induced activation and obligatory intrinsic inactivation of the Ca²⁺-sensitized state of the IP₃R, without a requirement for occupation of the inhibitory Ca²⁺-binding site.

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Thus, it has been proposed that each $[Ca^{2+}]_c$ transient is initiated by a local elevation of trigger $Ca^{2+}$ that activates IP$_3$Rs in the immediate vicinity to yield the rapid rising phase of $Ca^{2+}$ release and propagation of $[Ca^{2+}]_c$ waves. This process is believed to be terminated by a negative feedback effect of the elevated $[Ca^{2+}]_c$, that inactivates the IP$_3$R and allows the $Ca^{2+}$ pumps to re-sequester the released $Ca^{2+}$. Experiments in intact cells have provided evidence for both the positive and negative effects of $[Ca^{2+}]_c$ on the IP$_3$R (Parker and Ivorra, 1990; DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992; Oancea and Meyer, 1996), and regulation by luminal $Ca^{2+}$ has also been proposed (Missiaen et al., 1991, 1992; Nunn and Taylor, 1992; Tanimura and Turner, 1996a). However, it is apparent that recovery from the inhibited state of the IP$_3$R is dependent on factors other than the decline of $[Ca^{2+}]_c$ (Ilyin and Parker, 1994; Oancea and Meyer, 1996).

Previous studies of the mechanisms underlying $[Ca^{2+}]_c$ oscillations have relied on manipulations in intact cells, or have examined the individual components in isolation using subcellular systems. In the present study, we have established a permeabilized cell system in which $Ca^{2+}$ oscillations can be evoked by global application of IP$_3$. Repetitive cycles of $Ca^{2+}$ release and re-uptake were monitored using low affinity fluorescent $Ca^{2+}$ indicators localized within the intracellular $Ca^{2+}$ stores (Hofer and Machen, 1993, 1994), and changes in IP$_3$R permeability were also monitored using the retrograde flux of Mn$^{2+}$ to quench luminal dye (Hajnoczy and Thomas, 1994; Hajnoczy et al., 1994). This approach has allowed us to define the minimum requirements for intracellular $[Ca^{2+}]_c$ oscillations and dissect the mechanism in a single experimental system. IP$_3$-induced $Ca^{2+}$ oscillations were found to depend on fluctuations of $[Ca^{2+}]_c$, but $Ca^{2+}$ re-uptake and control by the luminal $Ca^{2+}$ content of the stores were not essential components of the mechanism. While this manuscript was in preparation, Tanimura and Turner (1996b) reported similar findings in salivary epithelial cells. In addition, we found that the IP$_3$R undergoes an obligate inactivation from the $Ca^{2+}$-sensitized state without the need for occupation of the inhibitory $Ca^{2+}$-binding site. Although this intrinsic inactivation is likely to occur together with $Ca^{2+}$-induced inhibition, experiments utilizing Sr$^{2+}$ in place of $Ca^{2+}$ suggest that the coupled processes of $Ca^{2+}$-dependent activation and subsequent obligatory inactivation of the IP$_3$R is sufficient to generate $[Ca^{2+}]_c$ oscillations in intact cells without utilizing the inhibitory $Ca^{2+}$-binding site of the IP$_3$R.

Results and discussion

$Ca^{2+}$ oscillations in intact and permeabilized hepatocytes

Treatment of hepatocytes with vasopressin causes a dose-dependent generation of IP$_3$ (Thomas et al., 1984), which is accompanied by $[Ca^{2+}]_c$ oscillations at submaximal agonist doses and sustained $[Ca^{2+}]_c$ increases with high levels of vasopressin (Figure 1A). The $[Ca^{2+}]_c$ oscillations in hepatocytes represent a very clear example of frequency modulation. The interspike period and initial latency decrease as the agonist dose is increased, but the amplitude and kinetics of the individual $[Ca^{2+}]_c$ spikes remain constant over a broad range of agonist doses (Woods et al., 1986; Rooney et al., 1989). The permeability of the intracellular $Ca^{2+}$ release channels during stimulation with vasopressin was monitored in intact hepatocytes by measuring the Mn$^{2+}$ quench of furap2 compartmentalized within the $Ca^{2+}$ stores (Glennon et al., 1992; Hajnoczy et al., 1993, 1994; Renard-Rooney et al., 1993). Addition of submaximal vasopressin after pre-loading the cytosol with Mn$^{2+}$ resulted in a series of brief steps of rapid quench reflecting the opening of the intracellular channels, and these steps were separated by extended periods of slow quench where channel permeability was low (Figure 1B). The Mn$^{2+}$ quench steps and $[Ca^{2+}]_c$ oscillations showed similar sensitivities to vasopressin dose for latency and frequency, and high levels of vasopressin caused a sustained Mn$^{2+}$ quench that also paralleled the $[Ca^{2+}]_c$ response. Importantly, the rapid phase of Mn$^{2+}$ quench occurred with the same rate throughout the effective vasopressin dose range (2.09 ± 0.18%/s and 2.11 ± 0.09%/s at 50 and 0.5 nM vasopressin, respectively). These data show that submaximal vasopressin doses cause synchronized periodic activation and subsequent deactivation of the entire population of intracellular $Ca^{2+}$ channels that can be activated by saturating levels of vasopressin. Thus, it appears that $[Ca^{2+}]_c$ oscillations in the intact cell are driven by cycling between a fully open and a largely closed state of the IP$_3$R channels.

In order to dissect the mechanisms involved in this process, we established a permeabilized cell system that responds to a fixed level of exogenously added IP$_3$ with autonomous oscillations of $Ca^{2+}$ release and re-uptake at the single cell level. Hepatocytes were permeabilized with digitonin using a protocol that preserves the functional integrity of the endoplasmic reticulum (ER) $Ca^{2+}$ stores (Renard-Rooney et al., 1993; Hajnoczy et al., 1994). Luminal $[Ca^{2+}]_c$ ($[Ca^{2+}]_{ER}$) was measured with compartmentalized low affinity $Ca^{2+}$ indicators (furap2FF or furaptra) (Hofer and Machen, 1993, 1994). Submaximal doses of IP$_3$ evoked oscillations of $[Ca^{2+}]_{ER}$ that were inverted relative to vasopressin-induced $[Ca^{2+}]_c$ spikes in intact hepatocytes (Figure 1C). Thus, each $[Ca^{2+}]_{ER}$ spike consisted of a rapid $Ca^{2+}$ release phase followed by a slower re-accumulation of $Ca^{2+}$. The $[Ca^{2+}]_{ER}$ oscillations occurred in a coordinated manner throughout each cell, but adjacent cells in the imaging field responded asynchronously (Figure 2A). Stepped increases in IP$_3$ concentration increased the oscillation frequency, with little change in the kinetics or amplitude of the individual $[Ca^{2+}]_{ER}$ spikes (Figure 2B). The interspike period varied from 20 to 240 s, which is similar to the range observed for agonist-induced $[Ca^{2+}]_c$, oscillations in intact hepatocytes (Rooney et al., 1989). Maximal doses of IP$_3$ caused a rapid and persistent loss of $[Ca^{2+}]_{ER}$ (Figures 1C and 2B). Mn$^{2+}$ quench of compartmentalized furap2 was also used to monitor changes in IP$_3$R permeability during IP$_3$-induced $[Ca^{2+}]_{ER}$ oscillations in the permeabilized hepatocyte preparation. Consistent with the intact cell data (Figure 1B), submaximal doses of IP$_3$ evoked brief bursts of rapid Mn$^{2+}$ entry into the stores, separated by extended periods where Mn$^{2+}$ permeability returned close to the basal rate, whereas maximal IP$_3$ caused a sustained and complete quench of the luminal dye (Figure 1D). Taken together, these data demonstrate that the entire process responsible for $[Ca^{2+}]_c$ oscillations can be reproduced in
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**Fig. 1.** Oscillations of IP$_3$R permeability during [Ca$^{2+}$]$_{c}$ spikes in intact and permeabilized hepatocytes. (A) [Ca$^{2+}$]$_{c}$ responses to submaximal (0.5 nM) and maximal (50 nM) vasopressin (VP) monitored using cytosolic fura2 in single intact hepatocytes. Agonists were present continuously from the addition arrow. (B) Activation of intracellular Ca$^{2+}$ channels evoked by submaximal and maximal vasopressin monitored by Mn$^{2+}$ quench of compartmentalized fura2 in intact hepatocytes. Addition of 100 µM MnCl$_2$ to fura2-loaded hepatocytes resulted in rapid quench of the cytosolic dye (not shown), followed by slow quench of the compartmentalized dye. Extracellular Mn$^{2+}$ was washed out after complete quench of the cytosolic fura2 (prior to start of traces). The rate of quench reflects the penetration of Mn$^{2+}$ into the fura2-containing intracellular compartment. (C) Effects of submaximal (125 nM) and maximal (7.5 µM) IP$_3$ on [Ca$^{2+}$]$_{ER}$ were monitored using compartmentalized furaptra in single permeabilized hepatocytes. (D) IP$_3$R activation by submaximal and maximal IP$_3$ was monitored as the Mn$^{2+}$ quench (50 µM MnCl$_2$) of compartmentalized fura2 in single permeabilized hepatocytes.

**Fig. 2.** Coordination and frequency modulation of IP$_3$-induced [Ca$^{2+}$]$_{ER}$ oscillations in single permeabilized hepatocytes. (A) Images of fura2FF-loaded permeabilized hepatocytes showing sites of [Ca$^{2+}$]$_{ER}$ decrease (red overlay) during IP$_3$-induced [Ca$^{2+}$]$_{ER}$ oscillations. The red overlay was calculated by differentiation of the images through time, using a step value of 6 s. (B) Time courses of [Ca$^{2+}$]$_{ER}$ change for numbered cells of (A) during incubation with 100 nM, 150 nM and 2.5 µM IP$_3$, as indicated.
Mechanisms of permeabilized cell Ca\(^{2+}\) oscillations

Oscillations of [Ca\(^{2+}\)]\(_{ER}\) occurred at a constant level of IP\(_3\) applied in a bath volume >10 000-fold in excess of the original intracellular volume, suggesting that oscillatory changes of [IP\(_3\)] were not required in this system. This is supported by experiments in which [Ca\(^{2+}\)]\(_{ER}\) oscillations were induced by maximal IP\(_3\) in the presence of the competitive IP\(_3\)R blocker heparin (Ghosh et al., 1988). In contrast to the stimulation of IP\(_3\) binding by [Ca\(^{2+}\)]\(_{i}\) (Pietri et al., 1990), heparin affinity is not affected by Ca\(^{2+}\) (Rouxel et al., 1992), making it a good tool to shift the range of IP\(_3\) sensitivity. Heparin addition terminated [Ca\(^{2+}\)]\(_{ER}\) oscillations induced by submaximal IP\(_3\) (Figure 3A). However, in the presence of heparin, [Ca\(^{2+}\)]\(_{ER}\) oscillations could be observed with micromolar IP\(_3\) concentrations that would otherwise cause sustained [Ca\(^{2+}\)]\(_{ER}\) release (Figure 3B). Cellular formation or breakdown of IP\(_3\) is unlikely to contribute significantly under these conditions, because [IP\(_3\)] is effectively clamped at a high level. These findings are consistent with previous reports in which non-metabolizable IP\(_3\) analogs induced [Ca\(^{2+}\)]\(_{i}\) oscillations in intact cells (Wakui et al., 1989; DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992). A potential problem with the interpretation of the intact cell experiments is that there may be a contribution from oscillations of IP\(_3\) formation secondary to the Ca\(^{2+}\) release triggered by the non-metabolizable IP\(_3\) analog. However, in our permeabilized cell studies, the presence of heparin would greatly reduce the efficacy of any endogenous IP\(_3\) formation, which would also be diluted rapidly into the essentially infinite sink of extracellular medium containing high levels of exogenous IP\(_3\).

One potential mechanism by which [Ca\(^{2+}\)] oscillations could occur at a constant IP\(_3\) level is through feedback regulation of the IP\(_3\)R channel by [Ca\(^{2+}\)]\(_{ER}\) (Missiaen et al., 1991, 1992; Nunn and Taylor, 1992; Tanimura and Turner, 1996a). In this model, the cycling between open and closed states is dependent on both Ca\(^{2+}\) release and re-uptake, such that inhibition of the ER Ca\(^{2+}\) pump with thapsigargin would be expected to terminate the oscillations of [Ca\(^{2+}\)]\(_{ER}\). When thapsigargin was added either during IP\(_3\)-induced [Ca\(^{2+}\)]\(_{ER}\) oscillations (Figure 3C) or together with IP\(_3\) (Figure 3D), the re-uptake phase of the [Ca\(^{2+}\)]\(_{ER}\) spikes was completely prevented. However, Ca\(^{2+}\) release still occurred in a periodic manner and, as a result, [Ca\(^{2+}\)]\(_{ER}\) declined in a series of discrete steps. The Mn\(^{2+}\) quench approach (see Figure 1D) also showed the same cycling between the high and low permeability states of the IP\(_3\)R when thapsigargin was added shortly before IP\(_3\) under these conditions (data not shown). Thus, feedback regulation by [Ca\(^{2+}\)]\(_{ER}\) is not an essential component of the Ca\(^{2+}\) oscillation mechanism.

The role of [Ca\(^{2+}\)]\(_{i}\) in the permeabilized hepatocyte system was investigated using 10 mM BAPTA to buffer the medium Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{o}\)). Under these conditions, the bell-shaped dependence on [Ca\(^{2+}\)]\(_{o}\) (Lino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991) was clearly apparent at submaximal IP\(_3\), with properties similar to those described previously for hepatocytes permeabilized in suspension (Marshall and Taylor, 1993). For example, at 250 nM IP\(_3\), the rate of [Ca\(^{2+}\)]\(_{ER}\) release was stimulated 15 ± 6-fold when [Ca\(^{2+}\)]\(_{o}\) was increased from <5 to 500 nM, whereas Ca\(^{2+}\) release rates decreased with higher [Ca\(^{2+}\)]\(_{o}\), and were barely detectable at >5 μM [Ca\(^{2+}\)]\(_{o}\) (not shown). IP\(_3\) released Ca\(^{2+}\) throughout the range of 1–2000 nM [Ca\(^{2+}\)]\(_{o}\), but [Ca\(^{2+}\)]\(_{ER}\) oscillations were never observed in the presence of the Ca\(^{2+}\) buffer (e.g. Figure 4A), indicating that fluctuations in [Ca\(^{2+}\)]\(_{o}\) are necessary for [Ca\(^{2+}\)]\(_{ER}\) oscillations. Although the Ca\(^{2+}\) buffer prevented oscillations, Ca\(^{2+}\) release induced by submaximal IP\(_3\) was still transient at [Ca\(^{2+}\)]\(_{o}\) levels >200 nM (Figure 4A). Since [Ca\(^{2+}\)]\(_{o}\) was highly buffered, Ca\(^{2+}\) re-uptake reflects refilling of the same Ca\(^{2+}\) store at steady-state, which implies an inactivation of the IP\(_3\)-R channel sufficient to allow the ER Ca\(^{2+}\) pump to overcome the IP\(_3\)-activated release pathway. This was demonstrated directly by addition of thapsigargin at steady-state, which revealed a Ca\(^{2+}\) release rate >10-fold slower than the rate when IP\(_3\) was added initially or when IP\(_3\) and thapsigargin were added simultaneously to naive cells (Figure 4A). By contrast, Ca\(^{2+}\) release rates recovered when IP\(_3\) was washed out and then added again (Figure 4B). The high level of BAPTA makes it unlikely that steady-state inactivation of the IP\(_3\)-R was mediated by released Ca\(^{2+}\) during sustained incubation with IP\(_3\). An alternative to Ca\(^{2+}\) feedback inhibition that might contribute to the decline in Ca\(^{2+}\) release under these conditions is the ligand-induced inactivation of the IP\(_3\)-R by IP\(_3\), which occurs in a time-dependent manner at fixed [Ca\(^{2+}\)]\(_{o}\) (Hajnoczy and Thomas, 1994).

Role of IP\(_3\)-induced inactivation of the IP\(_3\)-R

The time-dependent inactivation of the IP\(_3\)-R by IP\(_3\) was demonstrated originally by measuring IP\(_3\)-R permeability...
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**Fig. 4.** Transient [Ca$^{2+}$]$_{ER}$ release by IP$_3$ in the presence of Ca$^{2+}$. BAPTA buffer. Permeabilized fura2FF-loaded hepatocytes were incubated in intracellular medium supplemented with 10 mM Na-BAPTA (Calbiochem) and 7.5 mM CaCl$_2$, giving a measured [Ca$^{2+}$]$_o$ of ~400 nM calibrated with fura2 (using $K_d = 220$ nM). Ruthenium red was also included to block mitochondrial Ca$^{2+}$ uptake. (A) Cells were first incubated with 250 nM IP$_3$ and then 4 µM thapsigargin (Tg) was added once [Ca$^{2+}$]$_{ER}$ had returned to a steady-state in the continuous presence of IP$_3$ (trace a), or IP$_3$ and thapsigargin were added simultaneously (trace b). Traces are averaged from the entire cell population in the imaging field (30–50 cells). (B) Cells were incubated with 250 nM IP$_3$, followed by washout (three changes of medium) and then readdition of the same level of IP$_3$ where indicated. Using retrograde Mn$^{2+}$ flux through the channel to quench luminal fura2 at various times after addition of IP$_3$ (Hajnoczy and Thomas, 1994). In those experiments, suspensions of permeabilized hepatocytes were pre-incubated with thapsigargin to deplete the Ca$^{2+}$ stores. However, Combettes et al. (1996) suggested recently that there may have been sufficient residual Ca$^{2+}$ within the stores under these conditions to sensitize the IP$_3$R to IP$_3$, and that loss of this [Ca$^{2+}$]$_{ER}$ during the incubation with IP$_3$ could account for the inactivation of the IP$_3$R. Although we observed no Ca$^{2+}$ release and no change in [Ca$^{2+}$]$_{ER}$ measured with luminal fura2 in response to IP$_3$ in thapsigargin-treated cells in our previous studies (Hajnoczy and Thomas, 1994), we have re-examined this question using both low and high affinity Ca$^{2+}$ indicator dyes. In the present experiments, suspensions of hepatocytes were permeabilized in the presence or absence of 2 µM thapsigargin with [Ca$^{2+}$]$_o$ buffered to 400 nM with 15 mM BAPTA. When [Ca$^{2+}$]$_{ER}$ was monitored with luminal fura2FF (Figure 5A), inclusion of thapsigargin from the start of the experiment (lower two traces of Figure 5A) completely blocked Ca$^{2+}$ uptake and depleted the Ca$^{2+}$ stores to the point where IP$_3$ was unable to cause any further loss of [Ca$^{2+}$]$_{ER}$. Thapsigargin was less effective in depleting the Ca$^{2+}$ stores when added after completion of ATP-dependent Ca$^{2+}$ uptake (upper traces of Figure 5A), but treatment with ionomycin caused a rapid decrease of [Ca$^{2+}$]$_{ER}$ to the level measured in cells pre-treated with thapsigargin. Pre-incubation with thapsigargin was also found to eliminate the Ca$^{2+}$ release response to IP$_3$ in experiments using luminal fura2, which has the advantage of being sensitive to [Ca$^{2+}$]$_{ER}$ in the submicromolar range (Figure 5B).

The same fura2-loaded cell preparation examined in Figure 5B was also used for Mn$^{2+}$ quench measurements of IP$_3$R permeability (Figure 5B inset). These experiments demonstrate that the initial fast phase of Mn$^{2+}$ quenching observed when IP$_3$ was added together with the Mn$^{2+}$ was greatly reduced when the cells were pre-incubated with IP$_3$ for 30 s. This reflects the time-dependent inactivation induced by IP$_3$, as reported previously (Hajnoczy and Thomas, 1994). These findings also provide direct evidence that the IP$_3$R remains permeable to Mn$^{2+}$ even when the stores are completely depleted of Ca$^{2+}$, in contrast to the findings of Tanimura and Turner (1996a). Another potential problem with the use of Mn$^{2+}$ to study IP$_3$R permeability is that Mn$^{2+}$ may displace Ca$^{2+}$ from other binding sites, which could then contribute to the inhibition...
of the IP₃R (Combettes et al., 1996). However, the presence of 2 mM Mg-ATP in our experiments provides additional Ca²⁺ and Mn²⁺ buffering capacity, which prevents the substantial Ca²⁺ changes that might otherwise occur on Mn²⁺ addition (calculated [Ca²⁺]), increased from 435 to 515 nM after MnCl₂ addition in Figure 5B). Moreover, this [Ca²⁺], change occurs only at the time of Mn²⁺ addition, and so cannot explain the time-dependent decrease in Mn²⁺ uptake rate during pre-incubation of the cells with IP₃. It should also be noted that Striggow and Ehrlich (1996) have concluded that the free [Mn²⁺] used in these experiments (2 μM) is close to the optimum for measuring IP₃R permeability.

Since the Ca²⁺ dependence for sensitization of the IP₃R to IP₃ and for IP₃-dependent inactivation appeared to be similar in hepatocytes (Marshall and Taylor, 1993; Hajnóczky and Thomas, 1994), we hypothesized that these may be coupled events. Therefore, the Mn²⁺ quench approach was used to compare the effects of [Ca²⁺], on IP₃R sensitization and IP₃-induced inactivation in suspensions of fura2-loaded hepatocytes permeabilized in the presence of thapsigargin. The Mn²⁺ quench evoked by 125 nM IP₃ was taken as a measure of IP₃R sensitization, and the inhibition of Mn²⁺ quench evoked by maximal IP₃ after a 20 s pre-pulse with either 125 nM or 7.5 μM IP₃ was used to measure IP₃-dependent inactivation (Hajnóczky and Thomas, 1994). In addition, the [Ca²⁺],- induced increase in the proportion of high affinity IP₃Rs was measured using a low level of [³H]IP₃ (Pietri et al., 1990; Marshall and Taylor, 1994). There was a very marked increase in IP₃R channel activation by 125 nM IP₃ as [Ca²⁺], was increased in the range 300 nM to 1 μM (Figure 6A), and this was paralleled by a dramatic increase in IP₃-induced inactivation (Figure 6B). The [Ca²⁺], dependence of activation and inactivation at submaximal IP₃ was shifted to higher [Ca²⁺], than the sensitization for IP₃ binding (Figure 6C), which may result from the cooperative nature of channel activation at submaximal IP₃ (Meyer et al., 1990). Although maximal IP₃ caused IP₃R activation at all levels of [Ca²⁺], the extent of inactivation was entirely dependent on [Ca²⁺], and was closely correlated with the [Ca²⁺],- induced increase in high affinity IP₃ binding (Figure 6A–C).

The correlation between IP₃R sensitization and inactivation was investigated further by substituting Sr²⁺ and Ba²⁺ for Ca²⁺. Marshall and Taylor (1994) have shown that the sensitizing effects of Ca²⁺ on IP₃ binding and Ca²⁺ release are mimicked by Sr²⁺ but not by Ba²⁺, and that neither Sr²⁺ nor Ba²⁺ is effective in mimicking the direct inhibitory effect of Ca²⁺. To eliminate the potential for feedback by released Ca²⁺, the effects of Sr²⁺ and Ba²⁺ on IP₃R channel activation were monitored by the IP₃-induced retrograde flux of these ions into fura2-loaded stores in the presence of thapsigargin and EGTA. Sr²⁺ caused a marked sensitization to IP₃ (50 nM) compared with Ba²⁺ (Figure 7A), and this can be explained by the differential effects of these ions on IP₃ binding under these conditions (Figure 7C, and see Marshall and Taylor, 1994). These effects on IP₃ sensitivity were paralleled by IP₃-induced inactivation, such that Sr²⁺ but not Ba²⁺ supported the time-dependent inactivation during IP₃ pre-incubation (Figure 7B). The fact that Ba²⁺ was without effect shows that Sr²⁺ was not acting simply by displacing Ca²⁺ from other binding sites. Thus, the differential effects of Sr²⁺ and Ba²⁺ on IP₃-induced inactivation indicate that the Ca²⁺ dependence of this process reflects binding to the stimulatory Ca²⁺ site of the IP₃R.

Taken together, the data of Figures 6 and 7 provide evidence that only the Ca²⁺ -(or Sr²⁺-) sensitized form of the IP₃R undergoes ligand-induced inactivation. Moreover, the fact that Sr²⁺ is effective in supporting IP₃-induced inactivation indicates that this process does not depend on divalent metal ion binding at the inhibitory site of the IP₃R. Finally, the observation that inactivation at submaximal [IP₃] follows a similar cooperative Ca²⁺ dependence to activation (Figure 6A and B) but does not parallel the non-cooperative Ca²⁺ dependence of IP₃ binding suggests that channel opening is a prerequisite for the inactivation process and hence inactivation may be an obligatory consequence of channel activation in the Ca²⁺-sensitized state.
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Fig. 7. Effects of Sr²⁺ and Ba²⁺ on IP₃R activation and inactivation. (A) Effect of Sr²⁺ and Ba²⁺ on IP₃R activation by submaximal and maximal IP₃, measured as the retrograde flux of each divalent cation from the medium and with compartmentalized furax2. Traces show (a) no IP₃, (b) 50 nM IP₃ and (c) 7.5 µM IP₃. (B) Effect of Sr²⁺ and Ba²⁺ on IP₃R inactivation induced by pre-treatment with IP₃, measured as the Mn²⁺ quench of compartmentalized furax2. Traces show (a) no IP₃, (b) 7.5 µM IP₃ added 900s before Mn²⁺ and (c) 7.5 µM IP₃ added simultaneously with Mn²⁺. (C) Effects of Sr²⁺ and Ba²⁺ on steady-state IP₃ binding; the inset shows the time course of the Sr²⁺ effect (50 nM [³H]IP₃). For these experiments, hepatocytes were permeabilized in ICM without MgATP and supplemented with 40 µM EGTA and 2 µM thapsigargin to prevent Ca²⁺ uptake. In (A) and (C), 200 µM SrCl₂ and 140 µM BaCl₂ were added, giving ~160 µM free Sr²⁺ and ~100 µM free Ba²⁺. For (B), SrCl₂ and BaCl₂ were both added at 100 µM and quenching was initiated with 100 µM MnCl₂. The initial rapid phase in the control traces (a) of (A) and (B) reflects fluorescence responses of cytosolic dye that was released to the medium during permeabilization.

Fig. 8. Sr²⁺ oscillations in single hepatocytes. Fura2-loaded hepatocytes were incubated in Ca²⁺-free ECM supplemented with 0.5 mM Na-EGTA. The cells were first treated with maximal vasopressin (100 nM, VP) and cyclopiazonic acid (200 µM) to deplete intracellular Ca²⁺ stores. These agents were then washed out (4–5 changes of medium) and the cells stimulated a second time with 100 nM vasopressin. After a second washout period, the medium was supplemented with 3 mM SrCl₂, 3 mM BaCl₂ or 2 mM CaCl₂, as indicated, and the cells subsequently challenged again with vasopressin. Fura2 was loaded as fura2/AM for (A), (D) and (E) and microinjected as the free acid form for (B). The downward spikes in the presence of Sr²⁺ in (A) and (D) represent the decrease of [Sr²⁺]ER in cells in which compartmentalized dye predominated. (C) Vasopressin dose–response data for the initial lag time (C) and interspike period (○) for Sr²⁺ oscillations in fura2/AM-loaded hepatocytes. Cells giving sustained Sr²⁺ increases in response to vasopressin were omitted from the frequency analysis, but the proportion of these increased with vasopressin dose: specifically 0, 30, 44 and 57% of cells gave sustained Sr²⁺ responses at 1, 5, 50 and 100 nM vasopressin, respectively.

Agonist-induced oscillations of [Sr²⁺]c in intact hepatocytes

The data presented above and previous studies comparing the effects of divalent metal ions on IP₃R function suggested that Sr²⁺ could be used to distinguish the roles of IP₃-dependent inactivation of the IP₃R and direct feedback inhibition by Ca²⁺ in the generation of [Ca²⁺]c oscillations in intact cells. It has been shown that Sr²⁺ can be accumulated by ATP-dependent intracellular Ca²⁺ stores and subsequently released in response to agonist (Montero et al., 1995). In order to eliminate possible contributions to IP₃R regulation from residual Ca²⁺ in the stores (Morgan and Jacob, 1996), hepatocytes were incubated in the presence of EGTA and treated with high vasopressin and the reversible SERCA Ca²⁺ pump inhibitor cyclopiazonic acid (Figure 8). After washout of these agents, there was no further [Ca²⁺]c response to vasopressin. Following a further washout period, the cells were exposed to SrCl₂, BaCl₂ or CaCl₂, each of which rapidly appeared in the cytosol, presumably as a result of the activated capacitative Ca²⁺ entry pathway. The cells were allowed to re-load with the added divalent cation and, after a steady-state was
achieved, they were challenged again with vasopressin. Vasopressin-induced oscillations of fura2 fluorescence were observed in the cells re-loaded with Sr$^{2+}$ and Ca$^{2+}$, but not in those loaded with Ba$^{2+}$. Although Ba$^{2+}$ did not support oscillations, it did not prevent the induction of oscillations by vasopressin when the medium was supplemented with SrCl$_2$ in the continuing presence of BaCl$_2$.

Loading of hepatocytes with fura2 acetoxymethyl ester (fura2/AM) results in partial compartmentalization of the dye in the ER. This luminal fura2 does not usually contribute to the measured [Ca$^{2+}$], changes in intact cells, because [Ca$^{2+}$]$_{ER}$ remains sufficiently high to saturate the dye unless the cells are treated with agonist in the presence of SERCA pump inhibitors (Glennon et al., 1992). However, the affinity of fura2 for Sr$^{2+}$ is 30-fold lower than for Ca$^{2+}$ and, as a result, the oscillations of fura2 fluorescence recorded in the presence of Sr$^{2+}$ reflect a mixed signal for the cytosolic and ER compartments. This gave rise to a variety of oscillation patterns, ranging from largely cytosolic signals (Figure 8A, cell #2, and D, cell #1) to those cells in which the luminal changes predominate (Figure 8A, cell #3, and D, cell #2). Microinjected fura2 was used to obtain a pure cytosolic signal (Figure 8B). In these experiments, vasopressin gave rise to baseline spikes in the Sr$^{2+}$-loaded cells that propagated throughout the cell and were similar to those observed with Ca$^{2+}$, except the [Sr$^{2+}$], oscillations were 10- to 20-fold smaller in amplitude than the [Ca$^{2+}$], oscillations in the same cells. This can be explained by the lower affinity of fura2 for Sr$^{2+}$, and suggests that the absolute magnitude of [Ca$^{2+}$], and [Sr$^{2+}$], oscillations are similar in hepatocytes. The Sr$^{2+}$ oscillations also demonstrated frequency modulation, such that the initial lag time and the interspike period decreased with increasing vasopressin dose (Figure 8C).

The fact that the cells were incubated in the presence of EGTA and depleted of Ca$^{2+}$ to the point where there was no detectable Ca$^{2+}$ release to vasopressin should ensure that the oscillations in Sr$^{2+}$-loaded cells are due predominantly to Sr$^{2+}$ fluxes. A number of other lines of evidence support the conclusion that these are Sr$^{2+}$ oscillations and that they are driven directly by Sr$^{2+}$ feedback effects rather than as a secondary consequence of residual Ca$^{2+}$ fluxes. The observation of oscillatory decreases in [Sr$^{2+}$]$_{ER}$ in fura2/AM-loaded cells indicates that [Ca$^{2+}$]$_{ER}$ must have been reduced to the submicromolar range where it was no longer able to saturate the luminal fura2. Furthermore, Sr$^{2+}$-dependent oscillations continued for >30 min, often with little change in amplitude through many cycles. This repetitive cycling would be expected to chase out any residual Ca$^{2+}$ that could play a role in feedback regulation at the IP$_3$R. Finally, the small amplitudes of the oscillations measured with microinjected cytosolic fura2 are consistent with [Sr$^{2+}$]$_{c}$ spikes, whereas if these reflected [Ca$^{2+}$]$_{c}$ spikes they would be inadequate to elicit the feedback activation of the IP$_3$R necessary to propagate the release throughout the cell. The small amplitude of these spikes also shows that [Sr$^{2+}$] does not achieve the near millimolar concentrations where it might act at the inhibitory Ca$^{2+}$ site of the IP$_3$R (Marshall and Taylor, 1994). We were unable to determine whether Sr$^{2+}$ alone could support IP$_3$-induced oscillations in our permeabilized cell system, because we cannot use chelators in this preparation, and the contaminating Ca$^{2+}$ and effectively infinite volume of the incubation medium are sufficient to allow substantial Ca$^{2+}$ loading of the intracellular stores. Nevertheless, the observation of [Sr$^{2+}$] oscillations in intact cell experiments and the demonstration that Sr$^{2+}$ mimics the effects of Ca$^{2+}$ in sensitizing the IP$_3$R and supporting IP$_3$-dependent inactivation in permeabilized cells provides strong evidence that Sr$^{2+}$ is able to substitute effectively for Ca$^{2+}$ in driving the basic oscillation mechanism. Moreover, the inability of Sr$^{2+}$ to substitute for Ca$^{2+}$ at the inhibitory binding site of the IP$_3$R suggests that this form of negative feedback control by Ca$^{2+}$ is not essential to obtain [Ca$^{2+}$]$_{c}$ oscillations.

**Conclusions**

Our findings with permeabilized hepatocytes demonstrate that feedback regulation of the IP$_3$R by [Ca$^{2+}$]$_{c}$ at a constant level of IP$_3$ represents the minimum requirement for oscillatory Ca$^{2+}$ release. In addition, it appears that the stimulatory Ca$^{2+}$-binding site of the IP$_3$R can effect both activation and termination of Ca$^{2+}$ release, with the latter process occurring through the intrinsic slow inactivation that follows IP$_3$-induced activation of the Ca$^{2+}$-sensitized state of the IP$_3$R. This does not exclude an additional contribution from direct negative feedback by released Ca$^{2+}$ at the inhibitory Ca$^{2+}$-binding site. It is also difficult to formally exclude the possibility that IP$_3$ binding to the Ca$^{2+}$-sensitized IP$_3$R leads to a change in the properties of the inhibitory Ca$^{2+}$-binding site that increases its affinity for Ca$^{2+}$, and perhaps Sr$^{2+}$. It is possible that other regulatory mechanisms may also contribute to [Ca$^{2+}$]$_{c}$ oscillations in intact cells, including regulation of the IP$_3$R by [Ca$^{2+}$]$_{ER}$, [Ca$^{2+}$]$_{cyt}$ stimulation of IP$_3$ formation and enhanced plasma membrane Ca$^{2+}$ entry. However, our findings in the permeabilized cell preparation demonstrate that these are not essential components of the [Ca$^{2+}$]$_{c}$ oscillator.

Our studies suggest a basic mechanism of [Ca$^{2+}$]$_{c}$ oscillations that depends on [Ca$^{2+}$]$_{c}$-dependent interconversion between two modes of IP$_3$R channel activation, a Ca$^{2+}$-free basal state that requires high levels of IP$_3$ for activation and a Ca$^{2+}$-sensitized state that can be activated at much lower levels of IP$_3$ but undergoes an intrinsic slow inactivation when IP$_3$ is bound. The Ca$^{2+}$- (and Sr$^{2+}$-) dependent interconversions of the IP$_3$R are shown in Figure 9, with the postulated predominant pathway underlying a [Ca$^{2+}$]$_{c}$ spike shown by the thick blue arrows. At the resting [Ca$^{2+}$] between [Ca$^{2+}$]$_{c}$ spikes, IP$_3$ affinity is low and the IP$_3$R channel does not inactivate, so that submaximal levels of IP$_3$ cause continuous low level Ca$^{2+}$ release. As [Ca$^{2+}$]$_{c}$ rises, IP$_3$Rs convert to a conformation with high affinity for IP$_3$, which accelerates Ca$^{2+}$ release by these channels resulting in positive feedback by [Ca$^{2+}$]$_{c}$ that effectively recruits all available IP$_3$Rs to the high affinity activated conformation. A key observation of the present study is that there is an obligatory coupling between channel opening and inactivation in the Ca$^{2+}$-sensitized state of the IP$_3$R. Thus, in the high affinity conformation, IP$_3$R activation occurs in a phasic manner, whereby channel opening is followed by a time-dependent inactivation that does not require further Ca$^{2+}$ binding. An obligatory linkage between the activation and inactiv-
Calcium oscillations driven by the IP₃ receptor

Fig. 9. Scheme showing coupled Ca²⁺-dependent activation and inactivation of the IP₃R. Each subunit of the tetrameric IP₃R is depicted with an IP₃-binding site (yellow), a site for stimulation by Ca²⁺ (green) that increases IP₃ affinity and an inhibitory Ca²⁺ site (red) that inactivates the channel independently of IP₃. The channel pore is shown in gray for closed and inactivated conformations, and as a clear white diamond where channel opening can occur. The scheme is arranged in three columns, the Ca²⁺-unbound state at low [Ca²⁺]c (left), the IP₃-sensitized state induced by submicromolar [Ca²⁺]c levels (middle) and the inactivated state elicited by higher [Ca²⁺]c (right). The predominant pathway of coupled IP₃R activation and inactivation proposed here for the generation of a [Ca²⁺]c spike is shown by the blue arrows.

The ryanodine receptor by depolarization in skeletal muscle has also been reported (Pizarro et al., 1996), suggesting that this may be a common property of intracellular Ca²⁺ release channels. This process is ideally suited to generate a stable transient increment of Ca²⁺ release during each [Ca²⁺]c spike. In the final phase of the [Ca²⁺]c oscillation cycle, the intrinsic inactivation, perhaps in combination with direct feedback inhibition by [Ca²⁺]c, allows a return to basal [Ca²⁺]c through the action of Ca²⁺ pumps. Recovery of the IP₃R from IP₃-dependent inactivation can occur either by dissociation of Ca²⁺ from the stimulatory site or by removal of IP₃ (Hajnoczky and Thomas, 1994) but, since Ca²⁺ regulates IP₃ affinity, both Ca²⁺ and IP₃ are expected to dissociate from the IP₃R during the recovery phase. Although IP₃R inactivation reverses more slowly than the direct inhibitory effect of Ca²⁺ (Finch et al., 1991; Ilyin and Parker, 1994; but cf. Oancea and Meyer, 1996), it is not slow enough to account for the long interspike periods. Therefore, other factors may be involved in resetting the system prior to the next [Ca²⁺]c spike or, alternatively, the primary determinant of oscillation frequency may be the time required to generate a sufficient Ca²⁺ trigger signal to initiate the next Ca²⁺ release spike. Overall, the coupled feedback regulation of the IP₃R by Ca²⁺ and IP₃ is likely to play a key role in ensuring that the amplitude and duration of each [Ca²⁺]c spike is constant over a range of IP₃ and agonist doses, perhaps in combination with direct feedback inhibition by [Ca²⁺]c, allowing a return to basal [Ca²⁺]c through the action of Ca²⁺ pumps. Recovery of the IP₃R from IP₃-dependent inactivation can occur either by dissociation of Ca²⁺ from the stimulatory site or by removal of IP₃ (Hajnoczky and Thomas, 1994) but, since Ca²⁺ regulates IP₃ affinity, both Ca²⁺ and IP₃ are expected to dissociate from the IP₃R during the recovery phase. Although IP₃R inactivation reverses more slowly than the direct inhibitory effect of Ca²⁺ (Finch et al., 1991; Ilyin and Parker, 1994; but cf. Oancea and Meyer, 1996), it is not slow enough to account for the long interspike periods. Therefore, other factors may be involved in resetting the system prior to the next [Ca²⁺]c spike or, alternatively, the primary determinant of oscillation frequency may be the time required to generate a sufficient Ca²⁺ trigger signal to initiate the next Ca²⁺ release spike. Overall, the coupled feedback regulation of the IP₃R by Ca²⁺ and IP₃ is likely to play a key role in ensuring that the amplitude and duration of each [Ca²⁺]c spike is constant over a range of IP₃ and agonist doses, to yield an essentially pure frequency-modulated [Ca²⁺]c signal.

Materials and methods

Imaging measurements in intact and permeabilized hepatocytes

Hepatocytes were isolated from the livers of Sprague–Dawley rats by collagenase perfusion and maintained in primary culture for 3–24 h in Williams E medium, as described previously (Rooney et al., 1989; Hajnoczky et al., 1993), except that dexamethasone was omitted.

For measurements of [Ca²⁺], [Sr²⁺] and [Ba²⁺] in intact hepatocytes,
the cells were loaded with 5 µM fura2/AM for 15 min in the presence of 100 µM sulfonpyrazone or were microinjected with fura2 free acid as described previously (Rooney et al., 1989; Lin et al., 1994). Measurements of Mn$^{2+}$ quench of compartmentalized fura2 utilized cells loaded with 5 µM fura2/AM for 45–60 min (Renard-Rooney et al., 1993; Hajno´czky et al., 1994). Fura2 and other Ca$^{2+}$ indicators were loaded into intact hepatocytes incubated at 37°C in intracellular medium (ECM) composed of 121 mM NaCl, 5 mM NaHCO$_3$, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 10 mM glucose and 2% bovine serum albumin (BSA), pH 7.4. Intact cell experiments were carried out in the same buffers with BSA reduced to 0.25%, and CaCl$_2$ was omitted for measurements of Mn$^{2+}$ quench and intracellular [Sr$^{2+}$] and [Ba$^{2+}$]. The free [Ca$^{2+}$] of this Ca$^{2+}$-free ECM was 400 nM measured using fura2 free acid (1.5 µM).

Measurements of [Ca$^{2+}$]$_{i}$ in compartmentalized hepatocytes were carried out by first loading the intact cells for 60–120 min with 6 µM fura2/AM or 6 µM fura2FF/AM. These Ca$^{2+}$ indicators have Ka values of 53 µM for furaptra (Hofer and Machen, 1994) and 35 µM for fura2FF (A.Minta, TEF LABS), making them suitable for measuring changes in [Ca$^{2+}$]$_{i}$. Dye-loaded hepatocytes were washed with Ca$^{2+}$-free buffer and permeabilized by incubation for 6 min with 15 µg/ml digitonin in intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH$_2$PO$_4$, 20 mM Tris-HEPES at pH 7.2 with 2 mM MgATP and 1 µg/ml each of antipain, leupeptin and pepstatin. To increase [Ca$^{2+}$]$_{i}$, the ICM was passed through a Chelex column prior to addition of EGTA (5–23 µM) and CaCl$_2$ (0–45 µM) or in the presence of 160 µM SrCl$_2$ or BaCl$_2$. The bound and free fractions were separated by filtration (GF/B filters, transit time 2–3 s). Non-specific binding determined in the presence of 10 µM unlabeled IP$_3$ was <5% of total binding. Specific binding was normalized to maximum binding attained in the presence of Ca$^{2+}$ (14.8 ± 1.5 fmol/mg cell protein). The time course of increased IP$_3$ binding induced by Sr$^{2+}$ was measured after 4 min pre-incubation with 50 nM [${}^{1}$H]IP$_3$.

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