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Regulation of inositol trisphosphate receptors by luminal Ca^{2+} contributes to quantal Ca^{2+} mobilization

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The quantal behaviour of inositol trisphosphate ($InsP_3$) receptors allows rapid graded release of Ca^{2+} from intracellular stores, but the mechanisms are unknown. In Ca^{2+}-depleted stores loaded with Fura 2, $InsP_3$ caused concentration dependent increases in the rates of fluorescence quench by Mn^{2+} that were unaffected by prior incubation with $InsP_3$, indicating that $InsP_3$ binding did not cause desensitization. When Fura 2 was used to report the luminal free [Ca^{2+}] after inhibition of further Ca^{2+} uptake, submaximal concentrations of $InsP_3$ caused rapid, partial decreases in fluorescence ratios. Subsequent addition of a maximal $InsP_3$ concentration caused the fluorescence to fall to within 5% of that recorded after ionomycin. Addition of all but the lowest concentrations of $InsP_3$ to stores loaded with the lower affinity indicator, Calcium Green-5N, caused almost complete emptying of the stores at rates that increased with $InsP_3$ concentration. The lowest concentration of $InsP_3$ (10 nM) slowly emptied ~80% of the stores, but within 3 min the rate of Ca^{2+} release slowed leaving ~7 μM Ca^{2+} within the stores, which was then rapidly released by a maximal $InsP_3$ concentration. In stores co-loaded with both indicators, $InsP_3$-evoked Ca^{2+} release appeared quantal with Fura 2 and largely non-quantal with Calcium Green-5N; the discrepancy is not, therefore, a direct effect of the indicators. The fall in luminal [Ca^{2+}] after activation of $InsP_3$ receptors may, therefore, cause their inactivation, but only after the Ca^{2+} content of the stores has fallen by ~95% to ≤10 μM.

Keywords: Ca^{2+} channel/desensitization/endoplasmic reticulum/kinetics

Introduction

The ability of inositol 1,4,5-trisphosphate ($InsP_3$) to stimulate Ca^{2+} release from the intracellular stores of innumerable cell types is an essential step in the sequence linking activation of receptors in the plasma membrane to the complex cytosolic Ca^{2+} signals evoked by extracellular stimuli (Berridge, 1993). Activation of $InsP_3$ receptors with consequent opening of their intrinsic Ca^{2+} channels not only causes the initial mobilization of Ca^{2+} stores, but may also underlie the propagation of regenerative Ca^{2+} signals (Leichleiter and Clapham, 1992) and the stimulation of the capacitative Ca^{2+} entry pathway by empty Ca^{2+} stores (Berridge, 1995). The kinetics of $InsP_3$-stimulated Ca^{2+} mobilization are unusual in that submaximal concentrations of $InsP_3$ rapidly deplete only a fraction of the $InsP_3$-sensitive Ca^{2+} stores without affecting their ability to respond to further increases in $InsP_3$ concentration (Muallem *et al.*, 1989; Taylor, 1992). Such quantal responses to $InsP_3$ allow both rapid graded Ca^{2+} mobilization (Meyer and Stryer, 1990; Taylor, 1995) and graded activation of the capacitative Ca^{2+} entry pathway (Berridge, 1995). The mechanisms underlying the quantal behaviour of $InsP_3$ receptors are unknown, although both all-or-nothing emptying of heterogeneous Ca^{2+} stores (Muallem *et al.*, 1989; Hirose and Iino, 1994) and desensitization of $InsP_3$ receptors have been proposed as possible mechanisms. Evidence that such desensitization might be mediated by a decrease in luminal [Ca^{2+}] (Missiaen *et al.*, 1992), an increase in cytosolic [Ca^{2+}] (Iino, 1990; Bezprozvanny *et al.*, 1991; Marshall and Taylor, 1993) or directly by $InsP_3$ binding (Hajnóczky and Thomas, 1994) has been presented, but none of these mechanisms is wholly consistent with the characteristics of quantal Ca^{2+} release.

Cytosolic Ca^{2+} indicators have revealed the complex spatio-temporal organization of the increases in cytosolic [Ca^{2+}] that follow activation of receptors linked to $InsP_3$ formation (Berridge, 1993). The fluorescent Ca^{2+} indicators (e.g. Fura 2) that are widely used to observe the cytosolic free [Ca^{2+}], have more recently been employed to examine the free [Ca^{2+}] within intracellular organelles. Such studies have attempted to identify the intracellular localization of $InsP_3$-sensitive organelles (Hofer and Machen, 1993; Short *et al.*, 1993) to estimate the free [Ca^{2+}] within organelles (Hofer and Machen, 1993), the Ca^{2+} exchanges that occur between different organelles (Hajnóczky *et al.*, 1994), to characterize the mechanisms underlying quantal Ca^{2+} mobilization by $InsP_3$ (Renard-Rooney *et al.*, 1993; Short *et al.*, 1993; Hajnóczky and Thomas, 1994) and to examine the behaviour of $InsP_3$ receptors in intact cells stimulated with physiological stimuli (Chiavaroli *et al.*, 1994). In the present study, we have loaded the intracellular stores of hepatocytes with Fura 2 and Calcium Green-5N and then examined the effects of $InsP_3$ on the luminal free [Ca^{2+}] of the stores of single cells. Our results allow us to estimate the free [Ca^{2+}] within the $InsP_3$-sensitive stores of unstimulated cells and they provide evidence that more than one mechanism contributes to the quantal pattern of $InsP_3$-stimulated Ca^{2+} mobilization.

Results and discussion

$InsP_3$ causes non-quantal Mn^{2+} quenching of Fura 2 trapped within intracellular stores

In order to examine opening of $InsP_3$ receptors in the complete absence of Ca^{2+} fluxes, we recorded the quench-

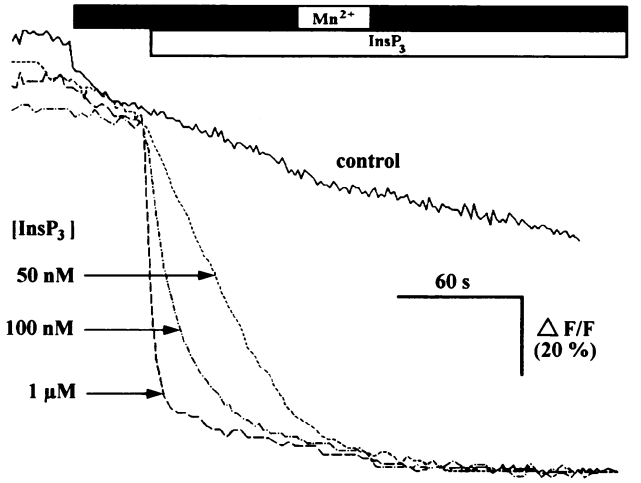

Fig. 1. Non-quantal responses to $InsP_3$ revealed by Mn^{2+} quenching of Fura 2 trapped within the intracellular Ca^{2+} stores of single permeabilized hepatocytes. Intact hepatocytes loaded with Fura 2 were treated with thapsigargin (1 μM) for 4 min before permeabilization and during the subsequent incubation in CLM without ATP (free $[Ca^{2+}]$ ~3 μM) $MnCl_2$ (25 μM) was present for the period shown by the solid bar and $InsP_3$ was present at the concentrations indicated for the period shown by the open bar. Ionomycin (2 μM) was added at the end of each experiment to ensure complete quenching of the trapped Fura 2; the fluorescence changes ($\Delta F/F_{total}$: λ_{ex} = 360 nm; λ_{em} = 510 nm) are expressed as fractions of the ionomycin-sensitive fluorescence signal for each cell. Each of the traces shown is the average of at least three cells from a field, and each is representative of five independent experiments.

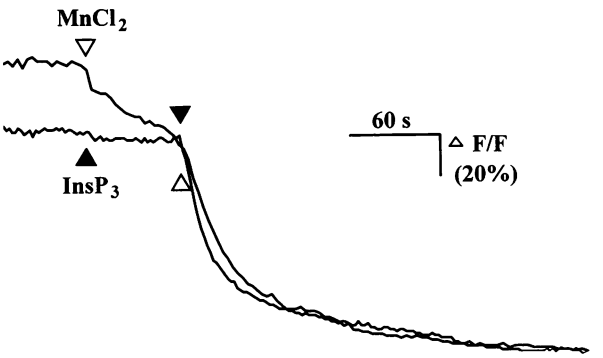

Fig. 2. Prolonged incubation with $InsP_3$ does not cause desensitization of $InsP_3$ receptors. Methods identical to those described in Figure 1 were used to examine $InsP_3$-evoked quenching of trapped Fura 2 by Mn^{2+}. $InsP_3$ (100 nM, solid arrowheads) was either added 60 s before addition of $MnCl_2$ (25 μM, open arrowheads) (lower trace) or 60 s after addition of $MnCl_2$ (upper trace) The traces shown are each the mean of seven cells from a single experiment, and in each the s.e.m. was <3% of the mean. Similar results were obtained in four independent experiments in which 100 nM $InsP_3$ were replaced by 1 μM $InsP_3$.

ing of Fura 2 fluorescence as Mn^{2+} entered empty stores through open $InsP_3$ receptors. Addition of $MnCl_2$ (25 μM) to permeabilized cells caused a slow sustained rate of fluorescence quenching (λ_{ex} = 360 nm) that was substantially increased after addition of $InsP_3$ (Figure 1). Although both maximal and submaximal concentrations of $InsP_3$ increased the rate of quench of almost the entire pool (≥95%) of sequestered Fura 2, the rate of quench increased with increasing concentrations of $InsP_3$ (Figure 1). Similar $InsP_3$ concentration dependent rates of Mn^{2+} quench of the entire pool of compartmentalized Fura 2 have previously been observed in hepatocytes (Renard-Rooney *et al.*, 1993), although our results differ from those in that we found no substantial differences in the sensitivities of individual cells to $InsP_3$ (not shown).

Pre-incubation with $InsP_3$ has been reported to reduce the rate at which Mn^{2+} enters the Fura 2-loaded intracellular stores of permeabilized hepatocytes (Hajnóczky and Thomas, 1994). However, under our experimental conditions, pre-incubation with $InsP_3$ (100 nM or 1 μM) for 1 min did not significantly affect the subsequent rate of Mn^{2+} entry (Figure 2). Since the concerted actions of Ca^{2+} and $InsP_3$ determine the behaviour of $InsP_3$ receptors (Hajnóczky and Thomas, 1994; see Taylor and Traynor, 1995 for review), different patterns of stimulation with the two agonists in our experiments and those of Hajnóczky and Thomas (1994) may underlie the different results. It is impossible to control reliably the free cytosolic $[Ca^{2+}]$ under the conditions needed for Mn^{2+} quench experiments because all practicable Ca^{2+} buffers bind Mn^{2+} with greater affinity than they bind Ca^{2+} (Martell and Smith, 1974). From measurements with both Quin 2 and Fluo 3, the free $[Ca^{2+}]$ of CLM was 2.9 ± 0.5 μM (n = 4) and in our experiments this is unlikely to change significantly after addition of Mn^{2+} because both endogenous and exogenous Ca^{2+} buffers (e.g. Fura 2), from which Ca^{2+} could be displaced by Mn^{2+}, were removed during permeabilization. This situation is very different from that prevailing in the previous study (Hajnóczky and Thomas, 1994), in which Mn^{2+} quench rates were measured without first removing the cytosolic Fura 2 from the incubation medium; addition of Mn^{2+} (40 μM) would therefore inevitably be accompanied by a substantial increase in the free $[Ca^{2+}]$ of the incubation medium ($[Ca^{2+}]_m$). In both our experiments (Figure 2) and a study of purified $InsP_3$ receptors reconstituted in lipid vesicles (Ferris *et al.*, 1992), there was no indication that $InsP_3$ caused desensitization of its receptor. In both cases $[Ca^{2+}]_m$ was relatively high (2.9 μM and ~10 μM, respectively) and unlikely to change significantly during the assay. These results suggest that either elevated $[Ca^{2+}]_m$ alone (≥2.9 μM) can mimic the combined effects of lower $[Ca^{2+}]$ and $InsP_3$ in causing inactivation of $InsP_3$ receptors; our results with Mn^{2+} would then reflect the activity of a desensitized form of the $InsP_3$ receptor. Alternatively the inactivation previously reported in hepatocytes (Hajnóczky and Thomas, 1994) occurred only because there were changes in both $[Ca^{2+}]$ and $InsP_3$.

Both our results and a previous report (Renard-Rooney *et al.*, 1993) suggest that when Mn^{2+} is used to report the opening of $InsP_3$ receptors, the responses of the intracellular Ca^{2+} stores of single hepatocytes to $InsP_3$ is non-quantal. The unusual experimental conditions needed to make such measurements may, however, have influenced the pattern of response because both luminal and cytosolic Ca^{2+} have been reported to influence the behaviour of $InsP_3$ receptors (Taylor and Traynor, 1995). The decrease in luminal $[Ca^{2+}]$ that would normally accompany the activation of $InsP_3$ receptors has been proposed to decrease the sensitivity of $InsP_3$ receptors (Irvine, 1990; Nunn and Taylor, 1992) and to thereby cause quantal Ca^{2+} mobilization (Missiaen *et al.*, 1992). The increase in $[Ca^{2+}]$ at the cytosolic surface of open $InsP_3$ receptors as

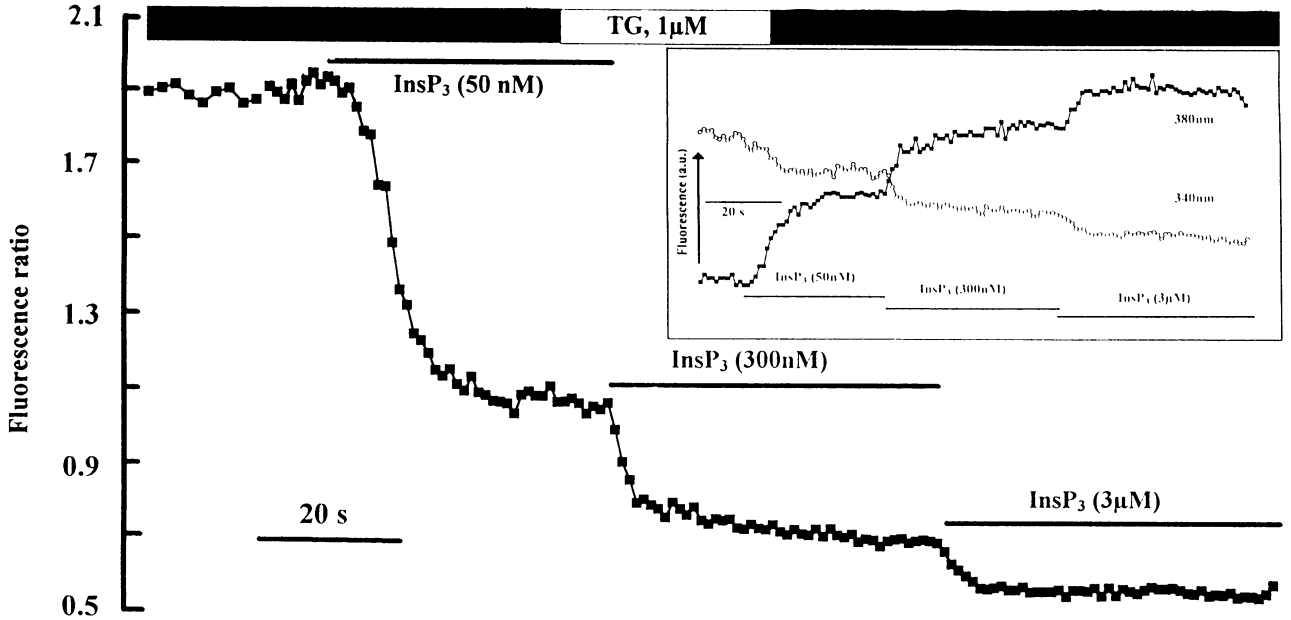

Fig. 3. $InsP_3$ evokes quantal decreases in luminal $[Ca^{2+}]$ from the stores of single permeabilized hepatocytes loaded with Fura 2. Permeabilized cells, in which the intracellular stores were loaded with Fura 2, were incubated with ATP (1 mM) in CLM (free $[Ca^{2+}]$ ~400 nM) for 2–3 min before the beginning of the trace. Thapsigargin (TG, 1 μM) was added at the beginning of the trace to inhibit Ca^{2+} uptake before the indicated stepwise applications of $InsP_3$. The inset shows the changes in Fura 2 fluorescence from a single cell at the two excitation wavelengths, and the main panel shows the fluorescence ratio ($R_{340/380}$) The results, typical of at least seven cells from five independent experiments, suggest that submaximal concentrations of $InsP_3$ rapidly release only a fraction of the $InsP_3$-sensitive Ca^{2+} stores, a quantal pattern of response.

Ca^{2+} leaks through them is known to regulate their behaviour (Iino, 1990; Bezprozvanny *et al.*, 1991; Combettes *et al.*, 1993; Marshall and Taylor, 1993) and it too has been proposed to underlie quantal responses to $InsP_3$ (Swillens *et al.*, 1994). The high free $[Ca^{2+}]$ in the medium, the empty Ca^{2+} stores and the absence of $InsP_3$-evoked Ca^{2+} fluxes in our experiments with Mn^{2+} could therefore have abolished both a normally quantal response to $InsP_3$ and the heterogeneous sensitivity of different stores. We therefore attempted to examine directly the effects of $InsP_3$ on the Ca^{2+} contents of the intracellular stores using trapped fluorescent indicators.

$InsP_3$-stimulated Ca^{2+} mobilization from stores loaded with either Fura 2 or Calcium Green-5N

Fura 2-loaded cells were permeabilized in CLM containing EGTA (60 μM), $CaCl_2$ (free $[Ca^{2+}]$ ~400 nM), ATP and a regenerating system. They were washed twice with the same medium supplemented with thapsigargin (1 μM), but without ATP. Under these conditions, where Ca^{2+} uptake into stores was prevented, sequential additions of sub-maximal concentrations of $InsP_3$ (50 nM and 300 nM) caused rapid, partial decreases in the $R_{340/380}$ fluorescence signals (Figure 3). Addition of 3 μM $InsP_3$, a maximally effective concentration, evoked a further decrease in the $F_{340/380}$ signal causing it to fall to within 5% of the value recorded after addition of ionomycin (2 μM).

In the absence of $InsP_3$, measurements of unidirectional $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes similar to those used for these experiments is approximately monoexponential and occurs with a half-life of ~3 min (Taylor and Potter, 1990), yet for several minutes after inhibition of Ca^{2+} uptake into the Fura 2-loaded stores, there was no detectable change in the Fura 2 fluorescence (not shown). These results establish that Fura 2, because it has such high affinity for Ca^{2+} (K_d^{Ca} = 135 nM), cannot resolve the kinetics of Ca^{2+} efflux from replete stores. Our quantal responses to $InsP_3$ (Figure 3) and those previously reported (Short *et al.*, 1993) therefore reflect the behaviour of only a small fraction of the sequestered Ca^{2+}.

In order to overcome the potential problems with Fura 2, we attempted to load the intracellular stores with fluorescent indicators with lower affinity for Ca^{2+}. Of four indicators tested, Mag Fura 2, Calcium Green-2N, Calcium Green-5N and Fluo 3, the latter two most readily loaded into intracellular stores, and since Calcium Green-5N had the lower affinity for Ca^{2+} and was the least susceptible to photobleaching (not shown), we used it in subsequent experiments. Because the reported affinities of Calcium Green-5N for Ca^{2+} vary substantially (K_d = 10–85 μM) (Suss-Toby *et al.*, 1993; Vergara and Escobar, 1993; Yao and Parker, 1994), we determined the K_d^{Ca} of both compartmentalized and free Calcium Green-5N for Ca^{2+} and obtained an apparent K_d^{Ca} of ~35 μM under each condition (Figure 4).

By measuring the fluorescence of compartmentalized Calcium Green-5N under conditions that have previously been shown to preserve the Ca^{2+} contents of the intracellular stores (Burgess *et al.*, 1983), we estimated that the free $[Ca^{2+}]$ of the intracellular stores in 32 out of 40 cells was 191 ± 15 μM. In the remaining eight cells, the Calcium Green-5N was too close to saturation with Ca^{2+} to allow the luminal free $[Ca^{2+}]$ to be reliably estimated. However, even in the stores with the highest free $[Ca^{2+}]$, removal of ATP together with addition of thapsigargin (1 μM) caused a monoexponential decrease in the fluorescence signal without a detectable delay, indicating that their luminal free $[Ca^{2+}]$ cannot have been substantially higher than ~300 μM (Figure 4).

Loading of intracellular organelles with fluorescent

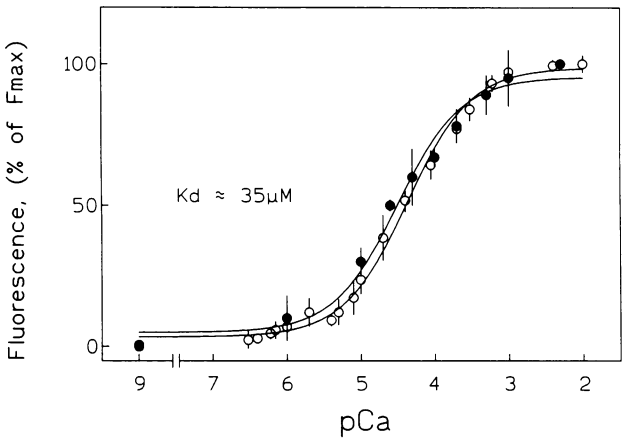

Fig. 4. Ca^{2+} binding to free and trapped Calcium Green-5N. The affinity of trapped Calcium Green-5N (●) was determined by incubating permeabilized hepatocytes, in which the stores had been loaded with the indicator, in CLM containing ionomycin (2 μM) and various free $[Ca^{2+}]$. Fluorescence (λ_{ex} = 480 nm; λ_{em} = 510 nm) was recorded from single cells, and F_{min} and F_{max} were obtained by incubation of the cells in CLM containing 1 mM EGTA or 5 mM $CaCl_2$, respectively. Results are shown as mean ± s.e.m. of 3–4 determinations from three independent experiments. The affinity of the free acid form of Calcium Green-5N (○) was determined using the same media containing 5 μM Calcium Green-5N hexapotassium salt and a Perkin-Elmer LS50B spectrofluorimeter to record fluorescence. The results are the means ± s.e.m. of duplicate determinations from three independent experiments. Calibration media (free $[Ca^{2+}]$ from ≤2 nM to 10 μM) were prepared from mixtures of $CaCl_2$ and EGTA and then confirmed by measurement of Quin 2 or Fluo 3 fluorescence. For higher free $[Ca^{2+}]$ (≥100 μM), EGTA was ommitted and the free $[Ca^{2+}]$ was assumed to equal the total $[Ca^{2+}]$ (the contaminating $[Ca^{2+}]$ was ≤3 μM).

indicators lacks the specificity that can be achieved by using targeted protein indicators, such as aequorin (Kendall *et al.*, 1994; Brini *et al.*, 1995), but Calcium Green-5N appears to be selectively accumulated within the ER because in our experiments, mitochondria were inhibited with FCCP, thapsigargin completely prevented Ca^{2+} uptake into the compartment, and ≥95% of the Ca^{2+} detected by sequestered Calcium Green-5N could be released by $InsP_3$. Although we cannot exclude the possibility that Calcium Green-5N accumulates within only a subset of the $InsP_3$-sensitive ER, the subcellular distribution of compartmentalized Fura 2 and Calcium Green-5N provides no evidence for such heterogeneity. Our estimate of the free $[Ca^{2+}]$ within the ER (200–300 μM) is similar to that previously determined, using compartmentalized Mag Fura 2, for gastric epithelial cells (127 μM) (Hofer and Machen, 1993) or hepatocytes (630 μM) (Chatton *et al.*, 1995), but much higher than the estimate of 21 μM derived by a null-point method for liver microsomes (Dawson *et al.*, 1995) or the estimate of ~0.3–1 μM derived from aequorin targeted to the ER of COS cells (Kendall *et al.*, 1994). Our results are, however, similar to more recent measurements from intact cells expressing targeted aequorin modified to have reduced affinity for Ca^{2+} which estimate the luminal free $[Ca^{2+}]$ to be about 1–2 mM (Montero *et al.*, 1995). From our estimate of the luminal free $[Ca^{2+}]$ of the ER (>200 μM), the time course of its monoexponential decline measured with Calcium Green-5N after removal of ATP and addition of thapsigargin ($t_{1/2}$ = 112 ± 9 s, n = 6) and the high affinity of Fura 2 for Ca^{2+} (K_d = 135 nM), it is scarcely surprising that Fura 2 is incapable of reporting the initial rate of passive Ca^{2+} efflux from replete stores.

In contrast to the apparent partial release of Ca^{2+} observed when Fura 2-loaded stores were stimulated with submaximal concentrations of $InsP_3$ (Figure 3), addition of submaximal concentrations of $InsP_3$ to stores loaded with Calcium Green-5N caused almost complete emptying of the stores (Figure 5). Only the lowest concentration of $InsP_3$ (10 nM) evoked a clearly quantal response: it slowly emptied ~80% of the stores, but within 3 min the rate of decline of the luminal $[Ca^{2+}]$ returned to the unstimulated level, and subsequent addition of a maximal concentration of $InsP_3$ (3 μM) rapidly released the remaining Ca^{2+} (Figure 6). A similar pattern of response was observed in all cells (six out of six cells). These results suggest that incomplete emptying of the stores by $InsP_3$ occurs only at relatively low levels of Ca^{2+} loading; these are near the limit of detection of Calcium Green-5N, but readily detected by Fura 2. Indeed, even after 10 nM $InsP_3$ has had its full effect, the Ca^{2+} remaining within the lumen of the ER has a concentration of ~7 μM, more than enough to saturate Fura 2 but not enought to saturate Calcium Green-5N.

$InsP_3$-stimulated Ca^{2+} mobilization from stores co-loaded with Fura 2 and Calcium Green-5N

The intracellular stores of hepatocytes were co-loaded with Fura 2 and Calcium Green-5N, washed and then permeabilized in CLM containing ATP. After permeabilization, saponin, ATP and the cytosolic indicators were removed by washing the cells in CLM (free $[Ca^{2+}]$ ~400nM) containing thapsigargin (1 μM). The fluorescence (λ_{em} ≥ 520 nM) from Fura 2 (λ_{ex} = 340 and 380 nm) and Calcium Green-5N (λ_{ex} = 480 nm) was then simultaneously recorded from single cells. Under these conditions, a decrease in luminal free $[Ca^{2+}]$ was immediately detected by Calcium Green-5N after inhibition of the ER Ca^{2+}-ATPase, but the decrease was not detected by Fura 2 (Figure 7), confirming previous results with cells loaded with only one indicator (Figure 3 and 5). Addition of a submaximal concentration of $InsP_3$ (50 nM) slowly released most (>90%) of the Ca^{2+} stores when observed with Calcium Green-5N, but over a similar time course appeared to release only ~40% of the $InsP_3$-sensitive stores when observed with Fura 2 (Figure 7). In a single cell, therefore, the Ca^{2+} release evoked by $InsP_3$ appeared to be quantal when observed with Fura 2, and largely non-quantal when observed with Calcium Green-5N.

Conclusions

Why should the apparent behaviour of $InsP_3$ receptors in response to submaximal stimulation depend upon the luminal indicator used to measure the free $[Ca^{2+}]$ within the ER and upon whether Ca^{2+} efflux or retrograde Mn^{2+} fluxes are used to measure receptor acitivity? The discrepancies are clearly not a direct effect of the indicators themselves because the discrepancies persist in cells co-loaded with both indicators (Figure 7). Nor are the results a consequence of increasing Ca^{2+} buffering within the ER (Missiaen *et al.*, 1995) because unidirectional Ca^{2+} efflux measured with luminal Calcium Green-5N, in the

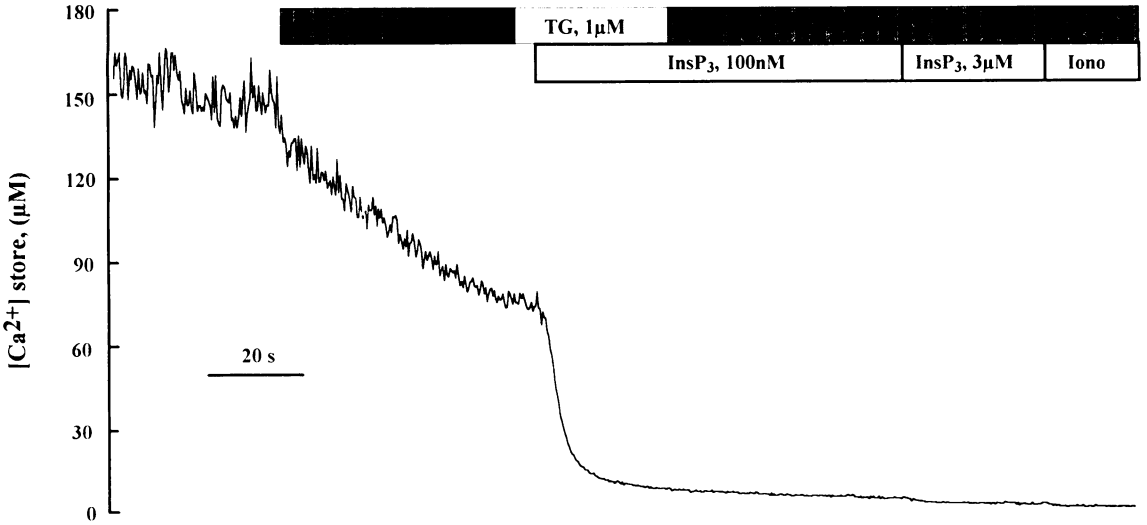

Fig. 5. $InsP_3$ evokes non-quantal decreases in luminal $[Ca^{2+}]$ from the stores of single permeabilized hepatocytes loaded with Calcium Green-5N. Permeabilized hepatocytes, in which the stores had been loaded with Calcium Green-5N, were incubated in CLM (free $[Ca^{2+}]$ ~400 nM) containing ATP (1 mM) for 1 min before the beginning of the recording. ATP was then removed (and TG added), followed by $InsP_3$ addition; unidirectional Ca^{2+} efflux was recorded by measuring Calcium Green-5N fluorescence (λ_{ex} = 480 nm; λ_{em} = 520 nm). At the end of the experiment, cells were perfused with CLM containing ionomycin (2 μM) and first EGTA (1 mM) and then $CaCl_2$ (5 mM) to determine F_{min} and F_{max}, respectively. The trace is shown is representative of at least six cells in the field; similar results were obtained in four independent experiments.

absence of $InsP_3$, occurs at a similar rate to that previously observed using $^{45}Ca^{2+}$ (Taylor and Potter, 1990).

Mn^{2+} entry to stores that had been completely emptied of Ca^{2+} was invariably non-quantal: all concentrations of $InsP_3$ caused almost complete quenching of the compartmentalized indicator at rates that increased with $InsP_3$ concentration (Figure 1). Similar results were previously reported from permeabilized hepatocytes (Renard-Rooney *et al.*, 1993) and they are extended by our results demonstrating that all but the lowest concentrations of $InsP_3$ cause non-quantal release of Ca^{2+} from stores loaded with Calcium Green-5N (Figures 5 and 6). In previous studies of both Mn^{2+} entry to stores (Renard-Rooney *et al.*, 1993) and Ca^{2+} release from them (Taylor and Potter, 1990), responses to $InsP_3$ were quantal after fragmentation of the stores. The quantal behaviour of $InsP_3$ receptors is therefore overt only when each pool of Ca^{2+} is accessible to relatively few receptors. These results are consistent with the suggestion (Muallem *et al.*, 1989; Oldershaw *et al.*, 1991) that the behaviour of the $InsP_3$ receptor is such as to allow essentially all-or-nothing emptying of stores that differ in their sensitivity to $InsP_3$. Such behaviour demands highly cooperative responses to $InsP_3$ and receptors with a range of affinities for $InsP_3$ (Meyer and Stryer, 1990). The extent to which these properties of $InsP_3$ receptors contribute to quantal Ca^{2+} mobilization in intact cells (Bootman *et al.*, 1994) will depend upon both the extent of the luminal communication between stores and the degree to which receptors of different sensitivity are segregated between stores. When the luminal free $[Ca^{2+}]$ was recorded with Fura 2, the response to all submaximal concentrations of $InsP_3$ (50–300 nM) was quantal (Figure 3), whereas only the very lowest (10 nM) concentration of $InsP_3$ caused quantal Ca^{2+} mobilization when responses were monitored with trapped Calcium Green-5N. How can these seemingly discrepant observations be reconciled?

Previous atttempts to explain quantal responses to $InsP_3$ have attempted to distinguish between all-or-nothing

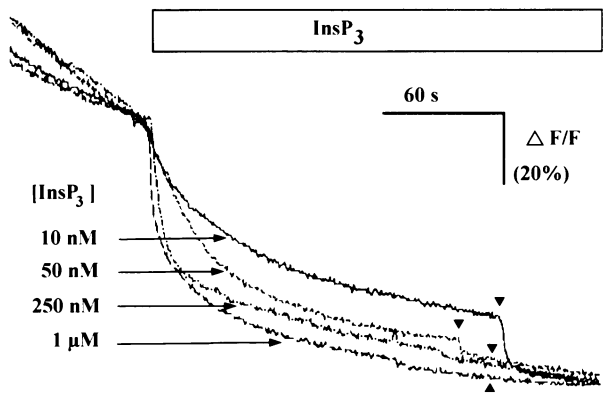

Fig. 6. Concentration-dependence of the $InsP_3$-evoked decrease in luminal $[Ca^{2+}]$ from the stores of single permeabilized hepatocytes loaded with Calcium Green-5N. Methods identical to those described in Figure 5 were used to examine the effects of different concentrations of $InsP_3$ on the luminal $[Ca^{2+}]$ of the stores. Fluorescence changes ($\Delta F/F_{total}$; λ_{ex} = 480 nm, λ_{em} = 520 nm) are each the average of seven cells from a field and each is representative of three independent experiments. The submaximal concentrations of InsP were applied for the times shown by the open bar and the $InsP_3$ concentration was increased to 3 μM at the times shown by arrowheads.

emptying of heterogeneous stores and a form of $InsP_3$ receptor inactivation that leaves Ca^{2+} trapped within the ER after a submaximal concentration of $InsP_3$ has had its full effect. Our results suggest that these mechanisms may not be exclusive and that, according to the Ca^{2+} content of the stores, both may contribute to the kinetics of $InsP_3$-stimulated Ca^{2+} mobilization.

The stimulatory influence of luminal Ca^{2+} on the sensitivity of $InsP_3$ receptors to $InsP_3$ saturates at modest levels of Ca^{2+} loading (Marshall and Taylor, 1993; Missiaen *et al.*, 1994), a characteristic that may account for the failure of several investigators to detect the effect (Shuttleworth, 1992; Combettes *et al.*, 1993; van de Put *et al.*, 1994). Our results suggest that Fura 2, with its high

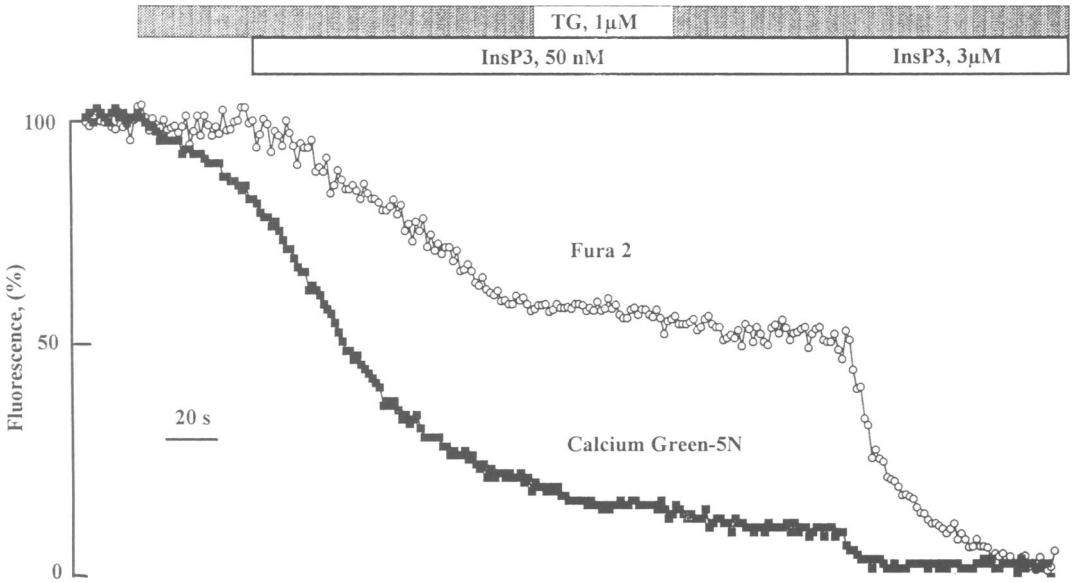

Fig. 7. Quantal or non-quantal appearance of the effect of $InsP_3$ on intracellular stores of single permeabilized hepatocytes co-loaded with Calcium Green-5N and Fura 2. Hepatocytes were first co-loaded with Fura 2 and Calcium Green-5N and then permeabilized in CLM (free $[Ca^{2+}]$ ~400 nM) containing ATP (1 mM). After simultaneous addition of thapsigargin (TG, 1 μM) and removal of ATP, the effects of $InsP_3$ on unidirectional Ca^{2+} efflux were examined by measuring both Fura 2 ($R_{340/380}$) and Calcium Green-5N (λ_{ex} = 480 nm) fluorescence. Because the amount of Ca^{2+} released by ionomycin would be overestimated by Fura 2, the results, from a single hepatocyte, are expressed as fractions of the total $InsP_3$-sensitive fluorescence signals. Traces, each representative of the 5–11 cells examined in each field, are shown. Similar results were obtained in four independent experiments.

affinity for Ca^{2+}, is capable of detecting the residual Ca^{2+} trapped within the stores after stimulation with all submaximal concentrations of $InsP_3$ (Figure 3), whereas Calcium Green-5N, with its lower affinity for Ca^{2+}(K_d ~35 μM), is near the limit of its sensitivity and detects the Ca^{2+} trapped (~7 μM) after the effects of only the very lowest concentrations of $InsP_3$ (Figure 6). The choice of luminal Ca^{2+} indicator is therefore crucial, because an indicator with high affinity for Ca^{2+}(e.g. Fura 2) will overestimate the extent to which $InsP_3$-stimulated Ca^{2+} mobilization is quantal. We conclude that in hepatocytes, the decrease in luminal $[Ca^{2+}]$ that follows activation of $InsP_3$ receptors may limit their opening, but the effect is likely to operate only as the free $[Ca^{2+}]$ within the stores falls to ≤10 μM, corresponding to ~5% of their steady-state loading in our experiments. This effect of luminal Ca^{2+} cannot, therefore, adequately explain the results of $^{45}Ca^{2+}$ flux experiments in which appropriate submaximal concentrations of $InsP_3$ cause quantal responses despite only modest decreases in the Ca^{2+} content of the stores (Muallem *et al.*, 1989; Taylor and Potter, 1990). Our results underscore both the need to deplete completely intracellular stores of Ca^{2+} if the regulatory effects of luminal Ca^{2+} on $InsP_3$ receptor function are to be excluded and the difficulty of effecting such complete emptying. Since in the previous study of $InsP_3$ receptor inactivation (Hajnóczky and Thomas, 1994), the stores were not totally depleted of Ca^{2+} before the first challenge with $InsP_3$, the decreased rate of Mn^{2+} quenching after pre-incubation with $InsP_3$ may be a consequence of the fall in luminal Ca^{2+} rather than to a direct effect of $InsP_3$ binding.

Why, if luminal Ca^{2+} is essential to allow opening of $InsP_3$ receptors by $InsP_3$, should $InsP_3$ stimulate Mn^{2+} entry into stores that are completely depleted of Ca^{2+} (Figure 1)? The most likely explanation is that $InsP_3$ is, in the complete absence of luminal Ca^{2+}, capable of activating its receptor, but far less effectively than in the presence of luminal Ca^{2+}. Our results with Mn^{2+}, and those reported by others (Hajnóczky and Thomas, 1994), would then reflect the behaviour of the $InsP_3$ receptor in a state which mediates undetectable Ca^{2+} efflux. Unfortunately, it is impracticable to compare quantitatively $InsP_3$-evoked Mn^{2+} entry to full and empty stores because competition between the Mn^{2+} and Ca^{2+} for the luminal indicator would obscure the analysis.

From our estimates of the luminal $[Ca^{2+}]$ within the ER of hepatocytes (200–300 μM) and the effects of $InsP_3$ on these stores after co-loading with Fura 2 and Calcium Green-5N, we suggest that luminal Ca^{2+} stimulates $InsP_3$ receptors, but the effect saturates as $[Ca^{2+}]$ within the stores exceeds 10 μM. Our observation that luminal Ca^{2+} influences $InsP_3$ receptors only when the Ca^{2+} content of the stores is very low may both explain the apparent absence of such an effect in some cells (Shuttleworth, 1992; Combettes *et al.*, 1993; van de Put *et al.*, 1994) and contribute to our understanding of quantal Ca^{2+} mobilization. We conclude that near complete emptying of stores that differ in their sensitivity to $InsP_3$ is likely to be a major component of the mechanisms responsible for the quantal Ca^{2+} mobilization observed in many permeabilized cell preparations (Muallem *et al.*, 1989; Oldershaw *et al.*, 1991). In addition, inhibition of $InsP_3$ receptors by the fall in luminal $[Ca^{2+}]$ that follows their activation is likely to trap some Ca^{2+} within stores responding to submaximal concentrations of $InsP_3$. Finally, our evidence suggests that desensitization of $InsP_3$ receptors by $InsP_3$ binding is unlikely to contribute significantly to quantal Ca^{2+} mobilization.

Materials and methods

Materials

Fluorescent indicators were obtained from Molecular Probes Inc. (Eugene, OR, USA), cell culture materials were from GIBCO BRL (Paisley, Scotland), ionomycin was from Calbiochem, collagenase was from Worthington, thapsigargin was from Alamone Laboratories (Jerusalem, Israel), collagen was from Seromed (Berlin, Germany) and $InsP_3$ was from Dr R.F.Irvine (Babraham, UK). All other reagents were purchased from the suppliers listed previously (Nunn and Taylor, 1992).

Loading of intracellular stores with fluorescent indicators

Hepatocytes were prepared by collagenase perfusion of livers from fed male Wistar rats (200–250 g) (Combettes *et al.*, 1986) and stored (2×10^6 cells/ml) at 4°C in Eagle's medium supplemented with $NaHCO_3$ (25 mM), bovine serum albumin (BSA, 15 mg/ml) and glucose (1 mM) and gassed with 95% O_2/5% CO_2 (pH 7.4). Cell viability, estimated by Trypan Blue exclusion, always exceeded 90% and remained stable for 4–5 h. Before loading cells with fluorescent indicators, they were washed twice with Eagle's medium (20°C), and resuspended (2.5×10^5 cells/ml) in loading medium comprising William's medium E supplemented with bovine serum albumin (10 mg/ml), penicillin (20 U/ml), streptomycin (10 μg/ml) and the acetoxymethyl ester (AM) form of the appropriate fluorescent indicator (5 μM). In order to maximize intracellular compartmentalization of the fluorescent indicators, cells (5×10^5 cells/35 mm Petri dish) were plated onto round collagen-coated glass coverslips (No. 2, 22 mm diameter) and then incubated for between 1 and 2 h at 37°C under an atmosphere of 5% CO_2. At the end of the loading period, the coverslip was mounted in a perfusion chamber and the cells were washed twice by perfusion with extracellular medium (EM: NaCl, 116 mM; KCl, 5.4 mM; $MgCl_2$, 0.8 mM; NaH_2PO_4, 0.96 mM; $NaHCO_3$, 5 mM; glucose, 5.6 mM; HEPES, 10 mM; pH 7.4). Medium was continuously aspirated from the chamber, and by allowing the volume of medium to decrease to 500 μl before manually adding 2 ml of replacement medium, medium changes could be effected within 3 s.

Fluorescence measurements

The chamber containing the coverslip and cells loaded with either Fura 2 or Calcium Green-5N was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. The cells were then permeabilized by incubation with saponin (50 μg/ml) at 23°C in a cytosol-like medium (CLM: NaCl, 20 mM; KCl, 100 mM; $MgCl_2$, 1 mM; NaH_2PO_4, 1 mM, HEPES 20 mM; pH 7.15) containing ATP (1 mM), creatine phosphate (5 mM), creatine phosphokinase (5 U/ml) and carbonyl cyanide *m*-chlorophenylhydrazone (FCCP, 5 μM). Since the organization of both the cytoskeleton and the endoplasmic reticulum can be disrupted by excessive increases in cytosolic [Ca^{2+}] (Nicotera *et al.*, 1992), CLM was buffered with a mixture of Ca^{2+} (30 μM) and EGTA (60 μM). Assuming that CLM is contaminated with about 3 μM Ca^{2+} (see below), the final free [Ca^{2+}] was ~400 nM (Combettes *et al.*, 1993).

Permeabilization was monitored both by phase-contrast microscopy and by the rapid decrease in the fluorescence intensity of the cells as cytosolic dye leaked through the permeabilized plasma membrane. Permeabilization of most cells (≥90%) was usually achieved within 4 min, whereupon the cells were immediately washed with saponin-free CLM. The decrease in Fura 2 fluorescence (λ_{ex} = 360 nm) that accompanied permeabilization established that with our loading protocol, between 20 and 60% of the intracellular Fura 2 was sequestered within internal stores. The distribution of the compartmentalized Fura 2 (λ_{ex} = 360) was relatively uniform with the exception of the nucleus, which excluded the dye; similar results have previously been reported by others (Hofer and Machen, 1993; Short *et al.*, 1993). Similar results were obtained with Calcium Green-5N. Subsequent experiments (see Results and discussion) demonstrated that the Ca^{2+} content of this compartment is sensitive to both thapsigargin and $InsP_3$; the compartment is presumably, therefore, the endoplasmic reticulum.

By buffering the free [Ca^{2+}] of the incubation medium at about 400 nM during permeabilization, we minimized the formation of plasma membrane blebs which are proposed to reflect disruption of the cytoskeleton (Nicotera *et al.*, 1992): >70% of cells developed only 1–2 blebs and ~10% had none. Only those cells that had such minimal blebbing were used for fluorescence measurements. Further evidence that permeabilization was not accompanied by effects of cytoskeletal disruption on the behaviour of the endoplasmic reticulum (ER) is provided by our observation that $InsP_3$ released ≥90% of the Ca^{2+} stores (see below). Previous studies had suggested that fragmentation of the ER after cytoskeletal disruption significantly reduced the fraction of the stores that were $InsP_3$-sensitive (Renard-Rooney *et al.*, 1993).

Excitation light of different wavelengths (λ_{ex}) was provided by a high pressure xenon arc lamp (100 W) mounted behind a computer-controlled rotating filter wheel containing narrow band (10 nm) interference filters. A computer-controlled shutter was used to minimize photobleaching by limiting exposure of the cells to excitation light to the periods of data collection. The excitation wavelengths used were 340 nm and 380 nm for Fura 2-loaded cells, and 480 nm for cells loaded with Calcium Green-5N. Rapid switching (800 ms/cycle) between all three excitation wavelengths was used for cells co-loaded with Fura 2 and Calcium Green-5N. The more intense fluorescence signal from Calcium Green-5N when the free [Ca^{2+}] was high, required the use of neutral density filters (OD 0.5–2) to attenuate the 480 nm excitation light selectively. Under these conditions, there was no overlap of the fluorescence spectra of the two indicators in either their Ca^{2+}-free or Ca^{2+}-bound forms.

Mn^{2+} quenching measurements

Prolonged incubation (4 min) with thapsigargin (1 μM) in Ca^{2+}-free EM prior to permeabilization, followed by further incubation (~6 min) with thapsigargin in the absence of ATP in CLM during and after permeabilization was essential to empty the intracellular stores of Ca^{2+} completely. After this treatment, there was no detectable change in Fura 2 fluorescence ($R_{340/380}$) after addition of $InsP_3$ (3 μM) or ionomycin (2 μM), indicating that the intracellular Ca^{2+} stores had been completely emptied (not shown). In order to measure Mn^{2+} entry into these empty Fura 2-loaded intracellular stores, the permeabilized cells were washed twice in CLM and then incubated with CLM containing $MnCl_2$ (25 μM). Quenching of the Fura 2 fluorescence was then recorded by measuring fluorescence while rapidly switching between λ_{ex} = 360 nm (the isoemissive wavelength for Ca^{2+}) and 340 and 380 nm (to confirm that there were no Ca^{2+} fluxes).

Emitted fluorescence (λ_{em}) was collected by the objective (×20 or ×40), passed through a dichroic mirror (510 nm) and then a high-pass barrier filter (520 nM) before detection by an intensified CCD camera (Photonic Science). Video images were digitized and stored in the memory of either an Applied Imaging Magiscan on Improvision image analyser. Subtraction of background fluorescence and cellular autofluorescence, and analysis of the corrected cell images using either Applied Imaging TARDIS or Improvision IonVision software was performed as previously described (Hargreaves *et al.*, 1994). Up to 10 cells were analysed in each field and each trace shown is representative of the field; similar results were obtained in at least three independent experiments.

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References

Berridge,M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

Berridge,M.J. (1995) Capacitative calcium entry. *Biochem. J.*, **312**, 1–11.

Bezprozvanny,I., Watras,J. and Ehrlich,B.E. (1991) Bell-shaped calcium-response curves for Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature*, **351**, 751–754.

Bootman,M.D., Cheek,T.R., Moreton,R.B., Bennett,D.L. and Berridge, M.J. (1994) Smoothly graded Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. *J. Biol. Chem.*, **269**, 24783–24791.

Brini,M., Marsault,R., Bastianutto,C., Alvarez,J., Pozzan,T. and Rizzuto,R. (1995) Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ([Ca^{2+}]$_c$). *J. Biol. Chem.*, **270**, 9896–9903.

Burgess,G.M., McKinney,J.S., Fabiato,A., Leslie,B.A. and Putney,J.W.,Jr (1983) Calcium pools in saponin-permeabilized guinea pig hepatocytes. *J. Biol. Chem.*, **258**, 15336–15345.

Chatton,J-Y., Liu,H. and Stucki,J.W. (1995) Simultaneous measurements of Ca^{2+} in the intracellular stores and the cytosol of hepatocytes during hormone-induced Ca^{2+} oscillations. *FEBS Lett.*, **368**, 165–168.

Chiavaroli,C., Bird,G.St.J. and Putney,J.W.,Jr (1994) Delayed 'all-or-

none' activation of inositol 1,4,5-trisphosphate-dependent calcium signaling in single rat hepatocytes. *J. Biol. Chem.*, **269**, 25570–25575.
Combettes,L., Berthon,B., Binet,A. and Claret,M. (1986) Glucagon and vasopressin interactions on calcium movements in isolated hepatocytes. *Biochem. J.*, **237**, 675–683.
Combettes,L., Claret,M. and Champeil,P. (1993) Calcium control of $InsP_3$-induced discharge of calcium from permeabilized hepatocyte pools. *Cell Calcium*, **14**, 279–292.
Dawson,A.P., Rich,G.T. and Loomis-Hesselbee,J.W. (1995) Estimation of the free [Ca^{2+}] gradient across endoplasmic reticulum membranes by a null-point method. *Biochem. J.*, **310**, 371–374.
Ferris,C.D., Cameron,A.M., Huganir,R.L. and Snyder,S.H. (1992) Quantal calcium release by purified reconstituted inositol 1,4,5-trisphosphate receptors. *Nature*, **356**, 350–352.
Hajnóczky,G. and Thomas,A.P. (1994) The inositol trisphosphate calcium channel is inactivated by inositol trisphosphate. *Nature*, **370**, 474–477.
Hajnóczky,G., Lin,C. and Thomas,A.P. (1994) Luminal communication between intracellular calcium stores modulated by GTP and the cytoskeleton. *J. Biol. Chem.*, **269**, 10280–10287.
Hargreaves,A.C., Lummis,S.C.R. and Taylor,C.W. (1994) Ca^{2+} permeability of cloned and native 5-hydroxytrytamine type 3 receptors. *Mol. Pharmacol.*, **46**, 1120–1128.
Hirose,K. and Iino,M. (1994) Heterogeneity of channel density in inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores. *Nature*, **372**, 791–794.
Hofer,A.M. and Machen,T.E. (1993) Technique for *in situ* measurement of calcium in intracellular inositol 1,4,5-trisphosphate-sensitive stores using the fluorescent indicator mag-fura-2. *Proc. Natl Acad. Sci. USA*, **90**, 2598–2602.
Iino,M. (1990) Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. *J. Gen. Physiol.*, **95**, 1103–1122.
Irvine,R.F. (1990) Quantal Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates—a posible mechanism. *Febs Lett.*, **263**, 5–9.
Kendall,J.M., Badminton,M.N., Dormer,R.L. and Campbell,A.K. (1994) Changes in free calcium in the endoplasmic reticulum of living cells detected using targeted aequorin. *Anal. Biochem.*, **221**, 173–181.
Leichleiter,J.D. and Clapham,D.E. (1992) Molecular mechanisms of intracellular calcium excitability in *X. laevis* oocytes. *Cell*, **64**, 283–294.
Marshall,I.C.B. and Taylor,C.W. (1993) Biphasic effects of cytosolic calcium on Ins(1,4,5)P_3-stimulated Ca^{2+} mobilization in hepatocytes. *J. Biol. Chem.*, **268**, 13214–13220.
Martell,A.E. and Smith,R.M. (1974) *Critical Stability Constants. Volume 1*. Plenum Press, New York, pp. 204–269.
Meyer,T. and Stryer,L. (1990) Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate. *Proc. Natl Acad. Sci. USA*, **87**, 3841–3845.
Missiaen,L., De Smedt,H., Droogmans,G. and Casteels,R. (1992) Ca^{2+} release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca^{2+} in permeabilized cells. *Nature*, **357**, 599–602.
Missiaen,L., De Smedt,H., Parys,J.B. and Casteels,R. (1994) Co-activation of inositol trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+} is loading-dependent. *J. Biol. Chem.*, **269**, 7238–7242.
Missiaen,L., Parys,J.B., De Smedt,H., Sienaert,I., Henning,R.H., Casteels,R., Mezna,M., Michelangeli,F., Hirose,K. and Iino,M. (1995) Opening up Ca^{2+} stores with $InsP_3$. *Nature*, **376**, 299–301.
Montero,M., Brini,M., Marsault,R., Alvarez,J., Sitia,R., Pozzan,T. and Rizzuto,R. (1991) Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. *EMBO J.*, **14**, 5467–5475.
Muallem,S., Pandol,S.J. and Beeker,T.G. (1989) Hormone-evoked calcium release from intracellular stores is a quantal process. *J. Biol. Chem.*, **264**, 205–212.
Nicotera,P., Bellomo,G. and Orrenius,S. (1992) Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 449–470.
Nunn,D.L. and Taylor,C.W. (1992) Luminal Ca^{2+} increases the sensitivity of Ca^{2+} stores to inositol 1,4,5-trisphosphate. *Mol. Pharmacol.*, **41**, 115–119.
Oldershaw,K.A., Nunn,D.L. and Taylor,C.W. (1991) Quantal Ca^{2+} mobilization stimulated by inositol 1,4,5-trisphosphate in permeabilized hepatocytes. *Biochem. J.*, **278**, 705–708.
Renard-Rooney,D.C., Hajnóczky,G., Seitz,M.B., Schneider,T.G. and Thomas,A.P. (1993) Imaging of inositol 1,4,5-trisphosphate-induced Ca^{2+} fluxes in single permeabilized hepatocytes: demonstration of both quantal and nonquantal patterns of Ca^{2+} release. *J. Biol. Chem.*, **268**, 23601–23610.
Short,A.D., Klein,M.G., Schneider,M.F. and Gill,D.L. (1993) Inositol 1,4,5-trisphosphate-mediated quantal Ca^{2+} release measured by high resolution imaging of Ca^{2+} within organelles. *J. Biol. Chem.*, **268**, 25887–25893.
Shuttleworth,T.J. (1992) Ca^{2+} release from inositol trisphosphate-sensitive stores is not modulated by intraluminal [Ca^{2+}]. *J. Biol. Chem.*, **267**, 3573–3576.
Suss-Toby,E., Rom-Glas,A., Payne,R. and Minke,B. (1993) The light response of *Drosophila* photoreceptors is accompanied by an increase in intracellular calcium. *Biophys. J.*, **64**, A218.
Swillens,S., Combettes,L. and Champeil,P. (1994) Transient inositol 1,4,5-trisphosphate-induced Ca^{2+} release: a model based on regulatory Ca^{2+} binding sites along the permeation pathway. *Proc. Natl Acad. Sci. USA*, **91**, 10074–10078.
Taylor,C.W. (1992) Kinetics of inositol 1,4,5-trisphosphate-stimulated Ca^{2+} mobilization. *Adv. Second Messenger Phos. Res.*, **26**, 109–142.
Taylor,C.W. (1995) Why do hormones stimulate Ca^{2+} mobilization? *Biochem. Soc. Trans.*, **23**, 637–642.
Taylor,C.W. and Potter,B.V.L. (1990) The size of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores depends on inositol 1,4,5-trisphosphate concentration. *Biochem. J.*, **266**, 189–194.
Taylor,C.W. and Traynor,D. (1995) Calcium and inositol trisphosphate receptors. *J. Membr. Biol.*, **145**, 109–118.
van de Put,F.H.M.M., De Pont,J.J.H.H.M. and Willems,P.H.G.M. (1994) Heterogeneity between intracellular Ca^{2+} stores as the underlying principle of quantal Ca^{2+} release by inositol 1,4,5-trisphosphate in permeabilized pancreatic acinar cells. *J. Biol. Chem.*, **269**, 12438–12443.
Vergara,J. and Escobar,A. (1993) Detection of Ca^{2+} transients in skeletal muscle fibers using the low affinity dye calcium-green-5N. *Biophys. J.*, **64**, A37.
Yao,Y. and Parker,I. (1994) Ca^{2+} influx modulation of temporal and spatial patterns of inositol trisphosphate-mediated Ca^{2+} liberation in *Xenopus* oocytes. *J. Physiol.*, **476**, 17–28.

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