Incremental Ca^{2+} mobilization by inositol trisphosphate receptors is unlikely to be mediated by their desensitization or regulation by luminal or cytosolic Ca^{2+}

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The kinetics of Ins(1,4,5)P_3 (InsP_3)-stimulated Ca^{2+} release from intracellular stores are unusual in that submaximal concentrations of InsP_3 rapidly release only a fraction of the InsP_3-sensitive Ca^{2+} stores. By measuring unidirectional $^{45}Ca^{2+}$ efflux from permeabilized rat hepatocytes, we demonstrate that such quantal responses to InsP_3 occur at all temperatures between 2 and 37 °C, but at much lower rates at the lower temperatures. Preincubation with submaximal concentrations of InsP_3, which themselves evoked quantal Ca^{2+} release, had no effect on the sensitivity of the stores to further additions of InsP_3. The final Ca^{2+} content of the stores was the same whether they were stimulated with two submaximal doses of InsP_3 or a single addition of the sum of these doses. Such incremental responses and the persistence of quantal behaviour at 2 °C indicate that InsP_3-evoked receptor inactivation is unlikely to be the cause of quantal Ca^{2+} mobilization. Reducing the Ca^{2+} content of the intracellular stores by up to 45 % did not affect their sensitivity to InsP_3, but substantially reduced the time taken for each submaximal InsP_3 concentration to exert its full effect. These results suggest that neither luminal nor cytosolic Ca^{2+} regulation of InsP_3 receptors are the determinants of quantal behaviour. Our results are not therefore consistent with incremental responses to InsP_3 depending on mechanisms involving attenuation of InsP_3 receptor function by cytosolic or luminal Ca^{2+} or by InsP_3 binding itself. We conclude that incremental activation of Ca^{2+} release results from all-or-nothing emptying of stores with heterogeneous sensitivities to InsP_3. These characteristics allow rapid graded recruitment of InsP_3-sensitive Ca^{2+} stores as the cytosolic InsP_3 concentration increases.

INTRODUCTION

The importance of Ins(1,4,5)P_3 (InsP_3) in mediating the effect of many receptors on the release of intracellular Ca^{2+} stores is well established [1]. The kinetics of InsP_3-stimulated Ca^{2+} release are unusual: even very prolonged incubation with a submaximal concentration of InsP_3 fails to empty the InsP_3-sensitive Ca^{2+} stores completely. This 'quantal' pattern of Ca^{2+} release, which was first observed in pancreatic acinar cells [2] and has since been confirmed in many cell types [3,4], may provide a means whereby cells can rapidly respond to graded changes in InsP_3 concentration without exhausting their finite intracellular Ca^{2+} stores [5]. The mechanisms responsible for quantal Ca^{2+} mobilization, which may also be a characteristic of ryanodine receptors [6], are unknown. Two fundamentally different mechanisms have been proposed: all-or-nothing emptying of discrete Ca^{2+} stores that differ in their sensitivity to InsP_3 [2,7] or a form of InsP_3 receptor desensitization that becomes effective before the stores lose all of their Ca^{2+}. Both the fall in luminal [Ca^{2+}] [8,9] and the rise in cytosolic [Ca^{2+}] [10] that follow InsP_3 receptor activation have been invoked as potential mediators of InsP_3 receptor inactivation, as has a more direct effect of InsP_3 binding [11]. Evidence in favour of each of these mechanisms, which need not be mutually exclusive, has been presented, but none is entirely consistent with the established characteristics of quantal Ca^{2+} release, which include its persistence in the absence of Ca^{2+} fluxes [12] and in purified InsP_3 receptors [13,14].

Analyses of the effects of temperature on quantal responses to InsP_3 have yielded conflicting results. In some reports, responses to InsP_3 become non-quantal at low temperature [15,16], whereas in others quantal responses are observed at all temperatures [17,18]. Some of the problems are consequences of experimental artifacts (see [19]). In the present study, we have re-examined the effects of temperature on the kinetics of InsP_3-evoked Ca^{2+} release. Our results indicate that InsP_3 causes quantal Ca^{2+} release at all temperatures without diminishing the ability of the stores to respond to subsequent additions of submaximal concentrations of InsP_3. The underlying mechanism is unlikely to result from any of the previously proposed forms of InsP_3 receptor desensitization and is most likely to reflect all-or-nothing emptying of Ca^{2+} stores with heterogeneous sensitivities to InsP_3, as first proposed by Muallem and his colleagues [2].

MATERIALS AND METHODS

Materials

InsP_3 was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.), and thapsigargin was from Alamone Laboratories (Jerusalem, Israel). All other reagents were from supplies reported previously [20].

Measurement of $^{45}Ca^{2+}$ efflux

Hepatocytes were isolated from the livers of male Wistar rats as previously described [20] and then permeabilized in suspension by incubation with saponin (10 μg/ml; 10 min) in cytosol-like

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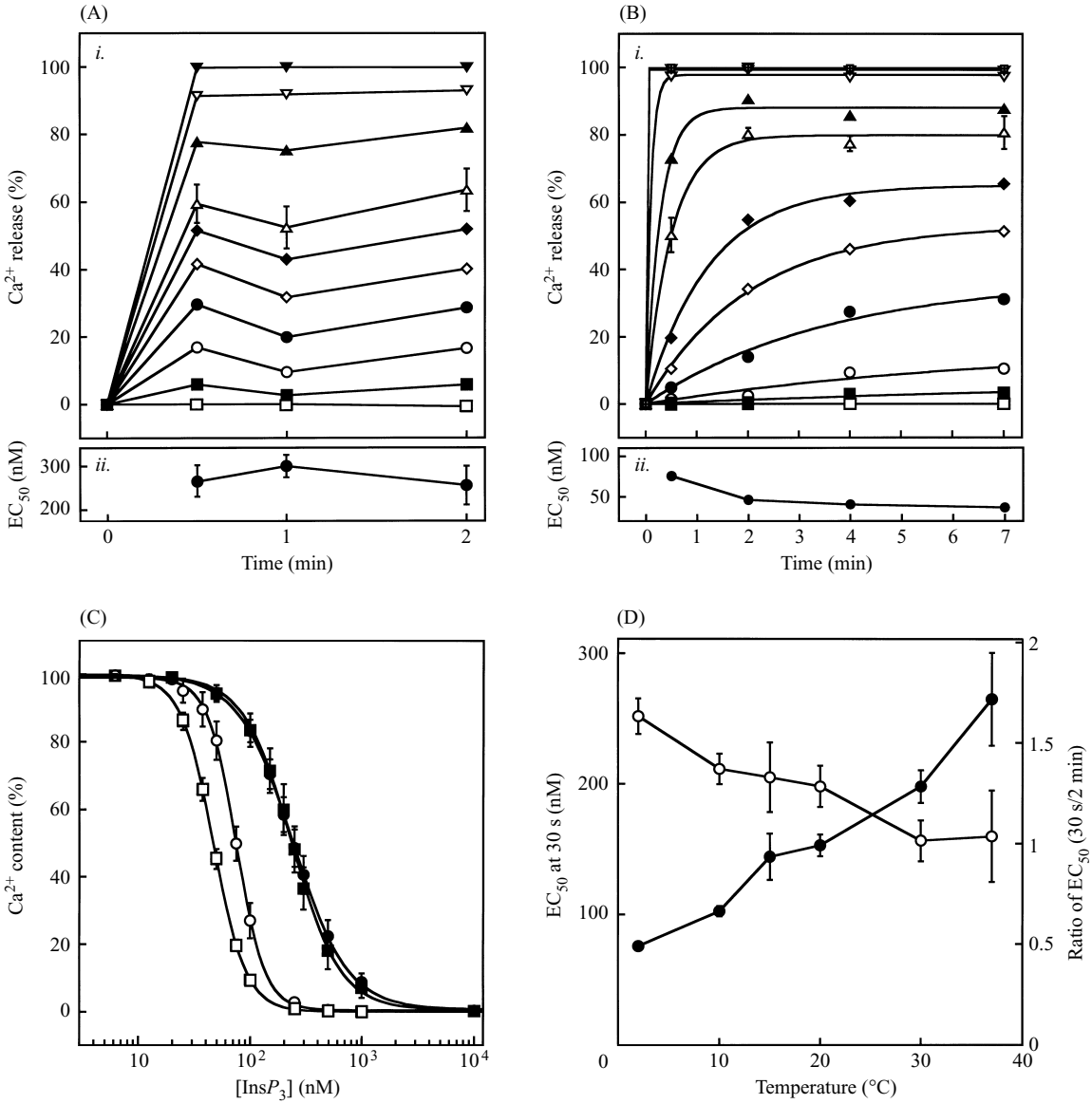

Figure 1 Temperature affects the time taken for quantal Ca^{2+} release by InsP_3

(**A**) The time course of InsP_3-evoked Ca^{2+} release at 37 °C (% of InsP_3-sensitive Ca^{2+} stores) is shown in (i) for the following final concentrations of InsP_3 (from bottom to top, nM): 0, 50, 100, 150, 200, 250, 300, 500, 1000, 10000. Results are from four to nine independent experiments. For clarity, error bars (S.E.M.) are shown on only one trace, the error bars were smaller on all other traces. (ii) EC_{50} for InsP_3-evoked Ca^{2+} release at each time. (**B**) Results from experiments similar to those shown in (**A**) but at 2 °C and with the following concentrations of InsP_3 (from bottom to top, nM): 0, 6.25, 12.5, 25, 37.5, 50, 75, 100, 250, 500, 1000. Results are from three to eight independent experiments; in (**ii**) the error bars were smaller than the symbols. (**C**) The concentration–effect relationships for InsP_3-evoked Ca^{2+} release after a 30 s (● ○) or 2 min incubation (■ □) with InsP_3 at either 2 °C (○ □) or 37 °C (● ■) are shown. The positively co-operative responses to InsP_3 shown [Hill coefficient (h) = 2.8 ± 0.4; n = 27] in these curves were typical of all experiments. (**D**) From experiments similar to those shown in (**A**), the EC_{50} for Ca^{2+} release after a 30 s exposure to InsP_3 was calculated for each incubation temperature (●). The ratio of the EC_{50} derived from a 30 s exposure to InsP_3 to that derived during a 2 min exposure [EC_{50}(30 s/2 min)] is also shown (○) (n = 3–9).

medium containing 140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Pipes (pH 7.0 at 37 °C). The cells were washed and resuspended (10^7 cells/ml) in cytosol-like medium supplemented with $CaCl_2$ (free [Ca^{2+}] = 200 nM), ATP (7.5 mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM) and $^{45}Ca^{2+}$ (7.5 μCi/ml). After 5 min, the stores had loaded to steady-state with $^{45}Ca^{2+}$ [20]. Unidirectional efflux of $^{45}Ca^{2+}$ was then initiated by diluting the cells 5-fold into Ca^{2+}-free cytosol-like medium at appropriate temperatures (2–37 °C) and supplemented with thapsigargin (1 μM final concentration) and EGTA (8 mM). After 30 s, the cells were added to InsP_3 and at intervals thereafter the $^{45}Ca^{2+}$ contents of the stores were determined by quenching the incubations with cold medium containing sucrose (310 mM) and trisodium citrate (1 mM), and then rapidly filtering them through Whatman GF/C filters [20]. In all experiments, the $^{45}Ca^{2+}$ remaining within the intracellular stores was determined by subtraction of the $^{45}Ca^{2+}$ associated with cells that had been incubated with ionomycin (10 μM).

Analysis of results

In all our analyses of the effects of InsP_3 on unidirectional

$^{45}Ca^{2+}$ efflux, the results were corrected for the passive leak of $^{45}Ca^{2+}$. Concentration–response relationships were fitted to a four-parameter logistic equation using a non-linear curve-fitting program (Kaleidagraph; Abeldeck Software, PA, U.S.A.) as previously described [21]. Computer-assisted curve-fitting (Kaleidagraph) was also used to fit exponential equations. For statistical analyses requiring comparison of ratios of two observations, S.E.M. values were determined as described by Colquhoun [22]. All results are reported as means ± S.E.M.

RESULTS

Temperature effects of InsP_3-evoked Ca^{2+} release

At 37 °C, quantal responses to all submaximal concentrations of InsP_3 were complete within 30 s of InsP_3 addition (Figure 1A). At 2 °C, responses to InsP_3 were again quantal, but the time taken for each submaximal concentration of InsP_3 to exert its full effect was much longer (Figure 1B). Because the response to a maximally effective concentration of InsP_3 is rapid (Figure 1Bi), prolonging the incubation increases the eventual response evoked by submaximal concentrations of InsP_3 without enhancing that evoked by a maximal concentration. In the interval before the full effect of a submaximal concentration of InsP_3 is achieved, there is therefore a progressive decrease in the concentration of InsP_3 needed to evoke half-maximal release of the InsP_3-sensitive stores (EC_{50}). This phenomenon is illustrated in Figure 1(C). Comparison of EC_{50} values at intervals after InsP_3 addition therefore provides a means of establishing the time course of quantal Ca^{2+} release. The concentration–effect relationship for InsP_3-evoked Ca^{2+} release is the same after a 30 s and a 2 min incubation at 37 °C, whereas at 2 °C the EC_{50} shifts to lower concentrations of InsP_3 as the incubation is prolonged (Figure 1C). The effects of a wider range of temperatures on the ratio of the EC_{50} values for InsP_3-evoked Ca^{2+}-release after a 30 s or 2 min incubation with InsP_3 are shown in Figure 1(D). The results demonstrate that quantal responses are complete within 30 s at 30 °C and 37 °C, but progressively less complete as the temperature is decreased to 2 °C. Figures 1(C) and 1(D) also confirm previous observations [23], by demonstrating that stores are more sensitive to InsP_3 at lower temperatures: after a 30 s incubation with InsP_3, the EC_{50} for Ca^{2+} release was 76 ± 1 nM ($n = 8$) at 2 °C and 265 ± 36 nM ($n = 9$) at 37 °C.

Since the time course of the Ca^{2+} release evoked by submaximal InsP_3 concentrations was both slow and monoexponential at 2 °C (Figure 1B), the time taken to reach half the final response ($t_{1/2}$) provides an estimate of the rate of development of the quantal behaviour. The results demonstrate that quantal responses to low concentrations of InsP_3 take much longer to develop than do those to higher concentrations; a similar pattern was evident from the more limited results obtainable at higher temperatures (Table 1).

Table 1 Effects of InsP_3 concentration on the time taken for completion of quantal Ca^{2+} mobilization

The results show the final extents (% of that released by 1 μM InsP_3) of the Ca^{2+} release evoked by each InsP_3 concentration and the times taken to achieve half of the final response ($t_{1/2}$). Each value is derived from concentration–effect curves similar to those shown in Figures 1(A) and 1(B), each of which was derived from between three and eight independent experiments.

Temperature (°C)	[InsP_3] (nM)	Ca^{2+} release (%)	$t_{1/2}$ (s)
2	25	37 ± 6	150 ± 48
	37.5	53 ± 1	84 ± 6
	50	65 ± 2	51 ± 7
	75	80 ± 1	21 ± 2
	100	88 ± 2	12 ± 1
10	50	32 ± 4	74 ± 28
	75	57 ± 4	38 ± 10
	100	74 ± 2	21 ± 4
20	75	29 ± 2	24 ± 8
	100	42 ± 2	18 ± 4
	150	62 ± 2	13 ± 4

Table 2 Effects of the Ca^{2+} content of the stores on their sensitivity to InsP_3

Intracellular stores were allowed to passively leak Ca^{2+} for the indicated intervals before addition of InsP_3 for a further 2 min. The results show the size of the InsP_3-sensitive Ca^{2+} store (% of that released by 10 μM ionomycin) and the sensitivity of the stores to InsP_3 after the indicated decreases in their initial Ca^{2+} contents.

Duration of passive leak before InsP_3 addition	Ca^{2+} content when InsP_3 added (%)	EC_{50} (nM)	Size of InsP_3-sensitive stores (%)	n
30 s	96 ± 1	46 ± 2	50 ± 4	6
4 min	71 ± 3	48 ± 5	48 ± 1	7
7 min	55 ± 4	57 ± 11	44 ± 3	6

Effects of successive additions of InsP_3

Previous studies of both native [24] and purified InsP_3 receptors [13] have suggested that quantal responses to InsP_3 occur without the receptors losing their ability to respond to further increases in InsP_3 concentration. Such 'incremental' responses to InsP_3 are an important feature of InsP_3 receptors that both distinguishes their behaviour from more conventional forms of receptor desensitization [24] and severely constrains the mechanisms that can be proposed to account for quantal responses [10]. In our experiments, passive leak of $^{45}Ca^{2+}$ from the intracellular stores was too rapid at 37 °C ($t_{1/2} = 77 \pm 10$ s, $n = 4$) to allow the effects of successive additions of InsP_3 to be examined within the temporal resolution of our experiments. At 2 °C, however, the passive leak of $^{45}Ca^{2+}$ was much slower ($t_{1/2} = 496 \pm 48$ s, $n = 3$) and this, together with the occurrence of quantal responses at 2 °C (Figure 1B), allowed us to examine whether a prior quantal response to InsP_3 affected the sensitivity of the stores to subsequent addition of InsP_3. Another requirement of these experiments is that the passive efflux of Ca^{2+} (< 50%) during the relatively prolonged incubations (7 min) should not affect the sensitivity of the stores to InsP_3. That requirement was also satisfied because neither the size of the InsP_3-sensitive Ca^{2+} store nor the sensitivity of the stores to InsP_3 were affected by a prior reduction in their Ca^{2+} content (Table 2, see below).

During a 4 min incubation with 25 nM InsP_3 at 2 °C, quantal release of Ca^{2+} was complete, and subsequent addition of a further 25 nM InsP_3 evoked further quantal Ca^{2+} release. The ultimate extent of the quantal Ca^{2+} release evoked by a submaximal concentration of InsP_3 was the same whether the InsP_3 was added as a single addition (50 nM; 54 ± 4%; $n = 6$) or as two successive additions of 25 nM (55 ± 2%; $n = 9$) (Figure 2, top). In a more detailed analysis, cells were first preincubated

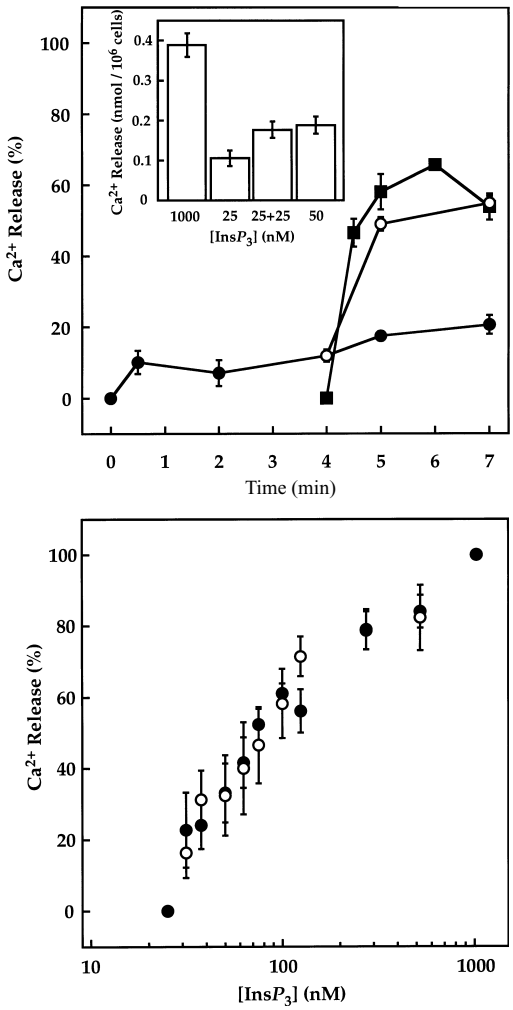

Figure 2 Increment detection at 2 °C

Top, time course of Ca^{2+} release (% $InsP_3$-sensitive stores) from cells that were exposed to 25 nM $InsP_3$ throughout the experiment (●), to 25 nM $InsP_3$ for the first 4 min before addition of a further 25 nM $InsP_3$ (○) or to 50 nM $InsP_3$ alone at 4 min (■). Results are from 5–14 independent experiments (most error bars are smaller than the symbols). The inset shows the results of the same experiments plotted to illustrate the absolute amount of Ca^{2+} (nmol/10^6 cells) released in response to the indicated additions of $InsP_3$. Bottom, cells were incubated for 4 min in efflux medium with (●) or without (○) 25 nM $InsP_3$; additional $InsP_3$ was then added to give the final $InsP_3$ concentrations indicated. After a further 3 min the Ca^{2+} contents of the stores were determined. The results are plotted with Ca^{2+} release shown as a percentage of that released by maximal $InsP_3$ and with the Ca^{2+} release evoked by 25 nM $InsP_3$ set to 0%. Results are from six independent experiments.

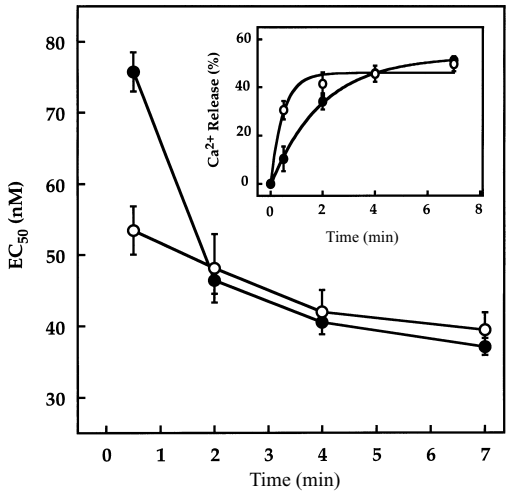

Figure 3 The Ca^{2+} content of the stores affects the time taken for quantal responses to $InsP_3$

Cells at 2 °C were exposed to various concentrations of $InsP_3$ after the stores had been allowed to passively leak Ca^{2+} for 30 s (●) or 4 min (○). Results (means ± S.E.M. for three to eight independent experiments) show the EC_{50} for $InsP_3$-evoked Ca^{2+} release determined at each of the indicated intervals after $InsP_3$ addition. The inset shows the time course (means ± S.E.M. for three to eight independent experiments) of Ca^{2+} release evoked by 37.5 nM $InsP_3$ from cells that had been allowed to leak Ca^{2+} for 30 s (●) or 4 min (○).

Table 3 $InsP_3$-evoked Ca^{2+} release from replete and partially emptied Ca^{2+} stores

Intracellular stores were allowed to passively leak Ca^{2+} for 30 s or 4 min, during which their Ca^{2+} contents fell to 96 ± 1 and 71 ± 3% of their steady-state levels respectively. $InsP_3$ was then added for a further 2 min. Both the final extent of the Ca^{2+} release evoked by each concentration of $InsP_3$ (% $InsP_3$-sensitive stores) and the half-time ($t_{1/2}$) of the Ca^{2+} mobilization response are shown. Results are means ± S.E.M. from six to seven independent experiments.

	Ca^{2+} content when $InsP_3$ added			
	96 ± 1%		71 ± 3%	
[$InsP_3$] (nM)	$t_{1/2}$ (s)	Ca^{2+} release (%)	$t_{1/2}$ (s)	Ca^{2+} release (%)
25	150 ± 48	37 ± 6	37 ± 13	32 ± 3
37.5	84 ± 6	53 ± 1	20 ± 5	46 ± 2
50	51 ± 7	51 ± 7	12 ± 3	57 ± 2

with 25 nM $InsP_3$ for long enough (4 min) to ensure an essentially complete quantal response. A range of $InsP_3$ concentrations was then added and the concentration–effect relationship established after a further 3 min incubation. The results establish that, after a quantal response to 25 nM $InsP_3$, there was no significant difference in the sensitivity of the stores to $InsP_3$ relative to naive cells (Figure 2, bottom). Similar results were obtained when cells were preincubated with 37.5 nM $InsP_3$, which caused quantal release of 46 ± 1% ($n = 4$) of the stores (not shown).

Effects of store Ca^{2+} content on quantal Ca^{2+} release

At 2 °C, the sensitivity of the Ca^{2+} stores to a 2 min exposure to $InsP_3$ was similar whether they were first allowed to passively leak Ca^{2+} for 30 s, 4 min or 7 min, which caused the Ca^{2+} content of the stores to fall by 4, 29 and 45% respectively (Table 2). These results, where the Ca^{2+} content of the stores never fell to below 55% of its steady-state level, are consistent with previous work suggesting that the Ca^{2+} content of the intracellular Ca^{2+} stores fails to affect their sensitivity to $InsP_3$ until it has fallen to below approx. 30% of its steady-state level [17,25]. The Ca^{2+} content of the stores did, however, affect the time taken for quantal Ca^{2+} release to be completed. Stores that were depleted of Ca^{2+} took less time for each submaximal concentration of $InsP_3$ to exert its full effect (Figure 3, Table 3).

Despite the different rates at which quantal Ca^{2+} release occurs from full and partially emptied stores, the eventual Ca^{2+} content of the stores after each $InsP_3$ concentration has exerted its full effect was the same whether $InsP_3$ was added to cells with replete

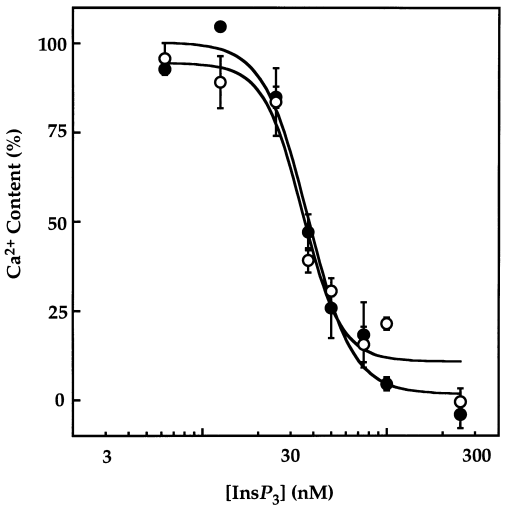

Figure 4 The Ca^{2+} content of the stores does not affect their sensitivity to InsP_3

Cells at 2 °C were exposed to the indicated concentrations of InsP_3 after the stores had been allowed to passively leak Ca^{2+} for 30 s (●) or 4 min (○). Both sets of incubations were then terminated at the same time, 7 min after initiation of passive Ca^{2+} efflux. The concentration–effect relationship for cells to which InsP_3 was added after 30 s was computed directly (see the Materials and methods section) from the $^{45}Ca^{2+}$ activities detected on the filters. The $^{45}Ca^{2+}$ contents (c.p.m.) of these unstimulated cells (set to 100%) and cells maximally stimulated with InsP_3 (set to 0%) were then derived from the fitted curves. In order to allow direct comparison of the absolute Ca^{2+} contents of the stores under the two conditions, the same absolute maximal and minimal $^{45}Ca^{2+}$ activities (c.p.m.) were then used to calculate the percentage Ca^{2+} content of the stores stimulated with InsP_3 after 4 min of passive leak. The results (means ± S.E.M. for three independent experiments) demonstrate that each concentration of InsP_3 caused the Ca^{2+} content of the stores to fall to the same level at 7 min, irrespective of whether the InsP_3 was added after 30 s or 4 min of passive leak.

stores or to cells that had first been allowed to passively leak Ca^{2+} for 4 min (Figure 4).

DISCUSSION

Quantal Ca^{2+} release at 2 °C

Our results establish that the quantal pattern of Ca^{2+} release typically evoked by InsP_3 at physiological temperatures [2,3,26] also occurs at 2 °C (Figure 1). In these unidirectional efflux experiments, each submaximal concentration of InsP_3 emptied only a fraction of the InsP_3-sensitive Ca^{2+} stores. We have already discussed some of the experimental problems likely to have confused previous analyses of InsP_3-evoked Ca^{2+} release at low temperatures [19]. These include effects of temperature on Ca^{2+} buffering and the sensitivity of the Ca^{2+} indicator used, and inadequate allowance for the substantial increase in the sensitivity of the stores to InsP_3 at reduced temperature (Figures 1C and 1D). The discrepancy with our previous report, in which we concluded that responses to InsP_3 were not quantal at 2 °C [19], is a consequence of both the very slow time course of the quantal response at 2 °C (Figure 1B) and of restricting our original analyses to the effects of only the higher concentrations (> 50 nM) of InsP_3. Although many cellular functions are modified by reducing the temperature to 2 °C, the preservation of the quantal pattern of InsP_3-evoked Ca^{2+} mobilization at this temperature allowed the underlying mechanisms to be addressed under more experimentally tractable conditions.

Desensitization of InsP_3 receptors is not the cause of quantal Ca^{2+} release

Binding of InsP_3 has been reported to inactivate directly hepatic InsP_3 receptors ([11], but see [27]), and to thereby generate quantal Ca^{2+} mobilization. Our results demonstrate that, whatever role such inactivation may play in regulating InsP_3 receptor behaviour, it is not essential for quantal Ca^{2+} release. Responses to InsP_3 were quantal at 2 °C (Figure 1B), whereas receptor inactivation has been reported not to occur at 4 °C [11], and in our experiments, responses to submaximal InsP_3 concentrations at 2 °C were unaffected by prior exposure to InsP_3 (Figure 2).

Since both the stimulatory and inhibitory effects of cytosolic Ca^{2+} on InsP_3-stimulated Ca^{2+} mobilization occur at 2 °C [19], the persistence of quantal Ca^{2+} release at 2 °C need not exclude those models that invoke Ca^{2+} inhibition of InsP_3 receptors as the underlying mechanism [10]. Several other lines of evidence, however, suggest that such mechanisms are unlikely to comprise an essential element of the quantal mechanism. We previously observed quantal responses to InsP_3 in media heavily buffered with bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) [19], and others have reported quantal opening of InsP_3 receptors in the apparent absence of Ca^{2+} fluxes [12,13]. In the present study, global increases in the free [Ca^{2+}] would be effectively buffered by EGTA, but local increases in free [Ca^{2+}] near open InsP_3 receptors might regulate their behaviour [10]. Our results provide further evidence that such regulation by cytosolic Ca^{2+} is not likely to underlie the incomplete emptying of InsP_3-sensitive Ca^{2+} stores by submaximal concentrations of InsP_3. Our observation that it takes longer for a submaximal InsP_3 concentration to exert its full effect when added to replete Ca^{2+} stores (Figure 3) cannot easily be reconciled with feedback inhibition by cytosolic Ca^{2+} as the mechanism responsible for preventing complete Ca^{2+} release. The converse would be expected if inhibition by an increase in cytosolic [Ca^{2+}] were the mechanism: Ca^{2+} leaking from replete stores would be expected to cause a more rapid inactivation of InsP_3 receptors than the lesser Ca^{2+} flux from more depleted stores.

The third mechanism proposed to cause closure of InsP_3 receptors before they can completely drain the stores of Ca^{2+} arises from the suggestion, which remains controversial [28], that the receptors are co-regulated by InsP_3 and luminal Ca^{2+} [8,9]. In this model, submaximal concentrations of InsP_3 are assumed to only partially empty the stores because the luminal [Ca^{2+}] falls below the level needed to sustain channel opening; Ca^{2+} is thereby trapped within the stores. This model is, however, impossible to reconcile with our present results. The luminal Ca^{2+} model predicts that, for each concentration of InsP_3, there is a critical Ca^{2+} content of the stores below which InsP_3 will fail to cause channel opening. Since the fraction of the Ca^{2+} stores released by a maximal concentration of InsP_3 is the same whether it is added to replete stores or those that have leaked 45% of their Ca^{2+} (Table 2), the rates of passive Ca^{2+} efflux must be similar from InsP_3-sensitive and insensitive stores. The total Ca^{2+} content of the stores therefore provides an accurate measure of the Ca^{2+} content of the InsP_3-sensitive stores. Addition of 25 nM InsP_3 to replete stores evoked quantal release of 37 ± 6% of the InsP_3-sensitive stores (Figure 1B, Table 1) and it released 32 ± 3% of the remaining InsP_3-sensitive stores when added after 29 ± 3% of their Ca^{2+} had already leaked (Table 3). Partial emptying of the InsP_3-sensitive stores by allowing Ca^{2+} to passively leak from them does not therefore mimic the effect of a submaximal concentration of InsP_3. These results, and similar results with a range of submaximal concentrations of InsP_3 (Figure 4), demonstrate that allowing the average Ca^{2+} contents

of the stores to passively fall to levels similar to those prevailing after quantal responses to InsP_3, has no significant effect on the subsequent response to a submaximal InsP_3 concentration. These observations, where reducing the luminal [Ca^{2+}] failed to mimic the effects of InsP_3, are not consistent with luminal Ca^{2+} as a significant determinant of quantal behaviour.

Our results are entirely consistent with the model first proposed by Muallem and his colleagues [2] in which quantal responses were proposed to arise from all-or-nothing emptying of stores (quanta) with heterogeneous sensitivities to InsP_3. In this model, depletion of the Ca^{2+} stores would not be expected to affect their sensitivity to InsP_3, although partially depleted stores would, in agreement with our results (Table 3), be expected to take less time to respond fully to each InsP_3 concentration (they each have less Ca^{2+} to lose). The half-times for completion of quantal responses would be expected to be shorter for higher InsP_3 concentrations (Table 1), because the time courses simply reflect the rates of InsP_3-evoked Ca^{2+} leak, rather than the more complex regulatory phenomena required of other models. Finally, the all-or-nothing model readily accommodates the incremental pattern of responses to InsP_3 [13,24] (Figure 3) because each addition of InsP_3 is assumed to evoke release from only those stores with appropriate sensitivity irrespective of their previous exposure to InsP_3.

We conclude that in hepatocytes, quantal responses to InsP_3 do not require desensitization of InsP_3 receptors of their regulation by luminal or cytosolic Ca^{2+}, but result entirely from all-or-nothing emptying of stores that must be both heterogeneous in their sensitivity to InsP_3 and respond to its co-operativity. These characteristics ensure that intracellular stores can respond rapidly to both InsP_3 and graded changes in its intracellular concentration [5] by progressive recruitment of InsP_3-sensitive stores.

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