Quantal responses to inositol 1,4,5-trisphosphate are not a consequence of Ca^{2+} regulation of inositol 1,4,5-trisphosphate receptors

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Submaximal concentrations of inositol 1,4,5-trisphosphate ($Ins P_3$) rapidly release only a fraction of the $Ins P_3$-sensitive intracellular Ca^{2+} stores, despite the ability of further increases in $Ins P_3$ concentration to evoke further Ca^{2+} release. The mechanisms underlying such quantal Ca^{2+} mobilization are not understood, but have been proposed to involve regulatory effects of cytosolic Ca^{2+} on $Ins P_3$ receptors. By examining complete concentration–effect relationships for $Ins P_3$-stimulated $^{45}Ca^{2+}$ efflux from the intracellular stores of permeabilized hepatocytes, we demonstrate that, at 37 °C, responses to $Ins P_3$ are quantal in Ca^{2+}-free media heavily buffered with either EGTA or BAPTA [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid]. Lower concentrations of $Ins P_3$ were used to examine the kinetics of Ca^{2+} mobilization at 2 °C, because at the lower temperature the stores were more sensitive to $Ins P_3$: the concentration of $Ins P_3$ causing half-maximal Ca^{2+} release (EC_{50}) after a 30 s incubation decreased from 281 ± 37 nM at 37 °C to 68 ± 3 nM at 2 °C. At 2 °C, the EC_{50} for $Ins P_3$-stimulated Ca^{2+} mobilization decreased as the duration of exposure to $Ins P_3$ was increased: the EC_{50} was 68 ± 3 nM after 30 s, and 29 ± 2 nM after 420 s. $Ins P_3$-stimulated Ca^{2+} mobilization is therefore non-quantal at 2 °C: $Ins P_3$ concentration determines the rate, but not the extent, of Ca^{2+} release. By initiating quantal responses to $Ins P_3$ at 37 °C and then simultaneously diluting and chilling cells to 2 °C, we demonstrated that the changes that underlie quantal responses do not rapidly reverse at 2 °C. At both 37 °C and 2 °C, modest increases in cytosolic Ca^{2+} increased the sensitivity of the stores to $Ins P_3$, whereas further increases were inhibitory; both Ca^{2+} effects persisted after prior removal of ATP. We conclude that the effects of Ca^{2+} on $Ins P_3$ receptors are unlikely either to be enzyme-mediated or to underlie the quantal pattern of Ca^{2+} release evoked by $Ins P_3$.

INTRODUCTION

Many extracellular stimuli cause an increase in cytosolic $[Ca^{2+}]$ by stimulating formation of inositol 1,4,5-trisphosphate ($Ins P_3$). $Ins P_3$ then binds to an intracellular receptor through which Ca^{2+} leaks from the lumen of the endoplasmic reticulum [1]. Muallem and his colleagues [2] were the first to report that the kinetics of $Ins P_3$-stimulated Ca^{2+} mobilization are curious in that submaximal concentrations of $Ins P_3$ rapidly release only a fraction of the $Ins P_3$-sensitive Ca^{2+} stores [3]. The inability of low concentrations of $Ins P_3$ to empty the $Ins P_3$-sensitive stores completely, even during prolonged incubations, has been described as quantal Ca^{2+} mobilization. These quantal responses to $Ins P_3$ do not result from classical desensitization, because the total amount of Ca^{2+} mobilized by a submaximal concentration of $Ins P_3$ is similar whether $Ins P_3$ is added as a single dose or as sequential additions of lower concentrations, each triggering quantal Ca^{2+} mobilization [4]. This behaviour, which may allow cells to respond best to changes in stimulus intensity, has now been observed in many cells [5–9] and in functionally reconstituted purified $Ins P_3$ receptors [10]. Quantal responses may also be a characteristic of the related family of intracellular Ca^{2+} channels, the ryanodine receptors [11,12].

The mechanisms underlying quantal responses to $Ins P_3$ are at present unknown. Both all-or-nothing emptying of stores that differ in their sensitivities to $Ins P_3$ [2,7,13] and partial depletion of Ca^{2+} stores, reflecting inactivation of $Ins P_3$ receptors [8,14–16], have been proposed as possible mechanisms. Regulatory effects of increased cytosolic $[Ca^{2+}]$ [15], decreased luminal $[Ca^{2+}]$ [14], or $Ins P_3$ binding itself [16], have all been proposed as possible means of $Ins P_3$-receptor inactivation.

In attempting to understand better the mechanisms underlying quantal Ca^{2+} mobilization, we have examined the effects of temperature on both the kinetics of $Ins P_3$-evoked Ca^{2+} mobilization and the regulatory effects of cytosolic Ca^{2+}.

MATERIALS AND METHODS

Hepatocytes were isolated by collagenase perfusion of the livers of male Wistar rats (150–200 g) [17]. The cells (2.5×10^5 cells/ml) were resuspended in Ca^{2+}-free cytosol-like medium (CLM; 140 mM KCl/20 mM NaCl/2 mM $MgCl_2$/1 mM EGTA/20 mM Pipes, pH 7 at 37 °C) and permeabilized by incubation with saponin (10 μg/ml) for 10 min at 37 °C. The non-mitochondrial intracellular Ca^{2+} stores of the cells were loaded with $^{45}Ca^{2+}$ by incubating the cells (10^7 cells/ml) at 37 °C in Ca^{2+}-containing CLM (free $[Ca^{2+}]$ = 200 nM) supplemented with ATP (7.5 mM), $^{45}Ca^{2+}$ (2 μCi/ml) and the mitochondrial inhibitor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 10 μM). Steady-state loading (1–2 nmol of $Ca^{2+}/10^6$ cells) was achieved within 5 min.

In order to investigate the influence of temperature on the effects of $Ins P_3$ on unidirectional $^{45}Ca^{2+}$ efflux from the intra-

Abbreviations used: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; CLM, cytosol-like medium; $[Ca^{2+}]_m$, free Ca^{2+} concentration in the medium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; $Ins P_3$, inositol 1,4,5-trisphosphate.

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cellular stores, permeabilized cells that had loaded to steady state with $^{45}Ca^{2+}$ were diluted 5-fold into CLM containing thapsigargin (1.25 μM), an increased concentration of EGTA or BAPTA [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid] (final concentration 8 mM), and various concentrations of $CaCl_2$ at either 37 °C or 2 °C. This allowed the final free $[Ca^{2+}]$ and temperature of the incubation medium to be changed simultaneously with the prevention of further Ca^{2+} uptake into the stores. For experiments in which the final free $[Ca^{2+}]$ exceeded 1.5 μM, EGTA was replaced with the lower-affinity Ca^{2+} chelator, *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA; final concentration 8 mM). After 30 s, InsP_3 was added, and at intervals thereafter the $^{45}Ca^{2+}$ contents of the intracellular stores were determined by rapid filtration through Whatman GF/C filters [17].

The free $[Ca^{2+}]$ of the incubation media ($[Ca^{2+}]_m$) was determined fluorimetrically at 37 °C as described previously [18], by using Fura-2 (K_d^{Ca} = 224 nM at 37 °C) for media buffered with EGTA and Fluo-3 (K_d^{Ca} = 864 nM at 37 °C) for media buffered with HEDTA. Media used at 2 °C had their pH corrected to allow for the effects of chilling, and the affinity of Fura-2 for Ca^{2+} at 2 °C (K_d^{Ca} = 372 nM) was computed according to the method and enthalpy change (ΔH = 10.3 kJ/mol) reported by Shuttleworth and Thompson [19].

Concentration–response relationships were fitted to a four-parameter logistic equation by using a non-linear curve-fitting program (Kaleidagraph; Synergy Software, Reading PA, U.S.A.):

$$R = S - \frac{S}{1 + \left(\frac{[\text{Ins}P_3]}{EC_{50}}\right)^h} \quad (1)$$

where R is the amount of $^{45}Ca^{2+}$ released after incubation with each concentration of InsP_3 ([InsP_3]), S is the $^{45}Ca^{2+}$ content of the InsP_3-sensitive stores, EC_{50} is the [InsP_3] causing half-maximal $^{45}Ca^{2+}$ mobilization, and h is the Hill coefficient.

A fraction ($\leqslant 25\%$) of the ATP-dependent $^{45}Ca^{2+}$ uptake into the stores of permeabilized hepatocytes is not readily released from them after inhibition of further $^{45}Ca^{2+}$ uptake, although it is released by ionomycin. In previous work [13], we corrected for this slowly released Ca^{2+}, but the correction becomes more significant, and therefore prone to greater error, as the stores lose Ca^{2+}. In order to present the effects of InsP_3 most clearly, unidirectional $^{45}Ca^{2+}$ efflux experiments (Figures 1b, 2b and 4) are therefore shown with the amount of Ca^{2+} released by each concentration of InsP_3 expressed as a percentage of that released by a maximal concentration of InsP_3 (calculated from eqn. 1) over the same interval.

Materials

Fura-2 and Fluo-3 pentapotassium salts were from Molecular Probes (Eugene, OR, U.S.A.), ionomycin was from Calbiochem, thapsigargin was from Alamone Laboratories (Jerusalem, Israel), hexokinase was from Sigma, and all other materials were from the suppliers listed previously [17].

RESULTS

Under the conditions used to examine InsP_3-stimulated unidirectional $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes in Ca^{2+}-free medium at 37 °C, passive leak from the Ca^{2+} stores occurred with a half time of 90±12 s (n = 3). By examining complete concentration–effect relationships for InsP_3-stimulated Ca^{2+}

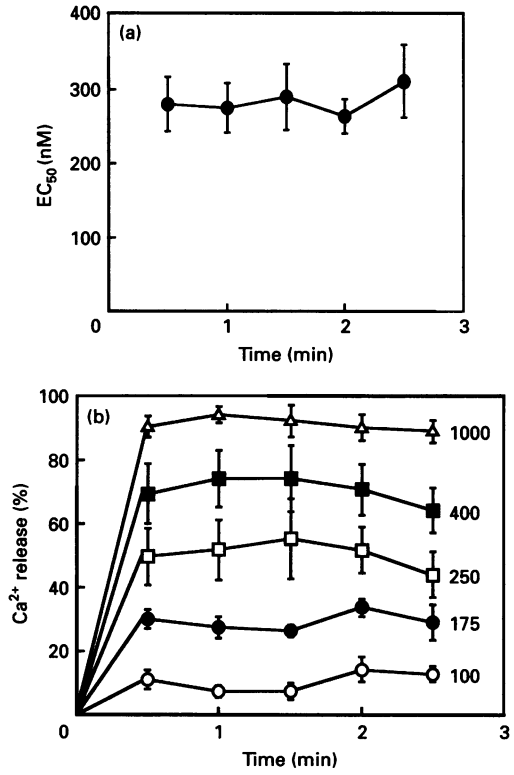

Figure 1 InsP_3-stimulated Ca^{2+} mobilization is quantal in Ca^{2+}-free media at 37 °C

Hepatocytes loaded to steady state with $^{45}Ca^{2+}$ were diluted into Ca^{2+}-free CLM buffered with EGTA at 37 °C. After 30 s, InsP_3 was added, and at intervals thereafter, the $^{45}Ca^{2+}$ contents of the stores were determined. Results are presented as means±S.E.M. of 4 independent experiments. (**a**) The duration of the incubation with InsP_3 does not affect the EC_{50} for InsP_3-stimulated Ca^{2+} mobilization. (**b**) Submaximal concentrations of InsP_3 (shown within the panel, in nM) release a fraction of the stores within 30 s, and thereafter have no further effect. Ca^{2+} release is expressed as a fraction of that released by a maximal concentration of InsP_3.

mobilization at intervals after InsP_3 addition, we demonstrated that, during prolonged incubation at 37 °C, the concentration of InsP_3 needed to cause half-maximal $^{45}Ca^{2+}$ efflux (EC_{50}) is similar after 30 s (EC_{50} = 281±37 nM; n = 4) and after 150 s (EC_{50} = 310±48 nM; n = 4) (Figure 1). These results indicate that submaximal concentrations of InsP_3 release only a fraction of the InsP_3-sensitive Ca^{2+} stores, that the response to InsP_3 is complete within 30 s, and that the fraction of the stores mobilized increases as the InsP_3 concentration is increased (Figure 1b). Similar responses were obtained in Ca^{2+}-free medium containing the faster Ca^{2+} buffer BAPTA (8 mM); after 30 s the EC_{50} for InsP_3-stimulated Ca^{2+} mobilization was 683±113 nM (n = 5) and after 120 s the EC_{50} was 736±114 nM (n = 4). These results therefore establish that InsP_3-evoked Ca^{2+} mobilization is quantal at 37 °C in Ca^{2+}-free medium; similar results were previously reported for cells incubated in medium containing $[Ca^{2+}]$ similar to that found in cytosol [5].

Before examining the kinetics of InsP_3-stimulated Ca^{2+} mobilization at 2 °C, we compared the sensitivity of the intracellular stores to InsP_3 at 2 °C and 37 °C to establish the concentration range over which InsP_3 would have submaximal effects at the lower temperature. Permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ at 37 °C were diluted into Ca^{2+}-free CLM at either 2 °C or 37 °C and the response to a 30 s incubation with InsP_3 was determined. The maximal response to InsP_3 was similar at

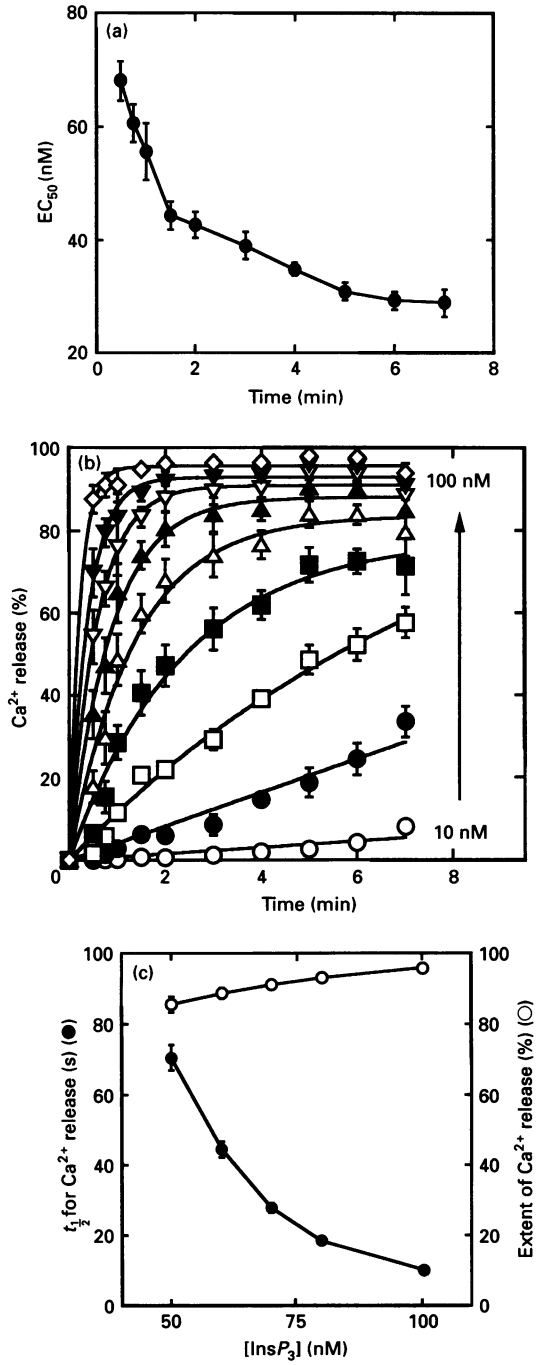

Figure 2 $InsP_3$-stimulated Ca^{2+} mobilization is non-quantal at 2 °C

The results (means ± S.E.M. of 3–7 independent experiments) are from experiments similar to those shown in Figure 1, except that the dilution and subsequent incubation with $InsP_3$ were at 2 °C. (**a**) The EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization decreases as the duration of the incubation with $InsP_3$ is increased. (**b**) Submaximal concentrations of $InsP_3$ (in nM: 10, 20, 30, 40, 50, 60, 70, 80 and 100) eventually release all of the $InsP_3$-sensitive Ca^{2+} stores at rates that increase with increasing $InsP_3$ concentration. The lines shown are fitted to a monoexponential. (**c**) The rate of Ca^{2+} mobilization, plotted as the half-time ($t_{\frac{1}{2}}$) for Ca^{2+} efflux (●), increases as the $InsP_3$ concentration is increased, whereas the extent of the $InsP_3$-stimulated Ca^{2+} mobilization (○) is similar for all $InsP_3$ concentrations. Both the extent and rates were derived from the monoexponential curve fits shown in (**b**).

the two temperatures: $InsP_3$ (10 μM) mobilized 46 ± 4% (n = 9) of the ATP-dependent $^{45}Ca^{2+}$ uptake at 37 °C and 58 ± 4% (n = 9) at 2 °C. However, the sensitivity of the stores to $InsP_3$ was increased by about 4-fold at the lower temperature: at 37 °C, the EC_{50} for $InsP_3$-evoked Ca^{2+} mobilization was 281 ± 37 nM (n = 4) and at 2 °C it was decreased to 68 ± 3 nM (n = 5). Parallel measurements of incubations supplemented with [^{3}H]$InsP_3$ established that degradation of $InsP_3$ never exceeded 10%; lesser degradation of $InsP_3$ at the lower temperature could not therefore account for the greater sensitivity of the stores. These results demonstrate that, at 2 °C, it is necessary to use lower concentrations of $InsP_3$ to examine the kinetics of the responses to submaximal stimulation.

Passive efflux of $^{45}Ca^{2+}$ from the intracellular stores is much slower at 2 °C ($t_{\frac{1}{2}}$ = 372 ± 36 s; n = 3) than at 37 °C ($t_{\frac{1}{2}}$ = 90 ± 12 s; n = 3). Nevertheless, the declining Ca^{2+} content of the stores and the proportionately increasing contribution from a very slowly exchanging fraction of the stores (see the Materials and methods section) limits the time-course (to ~ 7 min) over which the effects of low concentrations of $InsP_3$ can be reliably determined. Within this time-scale, the pattern of response to submaximal $InsP_3$ concentrations was very different at 2 °C from that observed at 37 °C. At the lower temperature, the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization progressively decreased as the duration of the incubation with $InsP_3$ was increased (Figure 2a). After a 30 s incubation with $InsP_3$, the EC_{50} for $InsP_3$-stimulated Ca^{2+} efflux was 68 ± 3 nM (n = 5), and it decreased to 29 ± 2 nM (n = 5) after a 420 s incubation (Figure 2a). This change in $InsP_3$ sensitivity with incubation time at 2 °C is a consequence of the ability of submaximal $InsP_3$ concentrations to empty the $InsP_3$-sensitive stores completely at rates that increase with increasing $InsP_3$ concentration (Figure 2b). $InsP_3$-stimulated Ca^{2+} mobilization is therefore non-quantal: the $InsP_3$ concentration determines the rate, but not the final extent, of Ca^{2+} mobilization (Figure 2c).

Since changes in cytosolic [Ca^{2+}] have been proposed as possible mechanisms underlying quantal responses to $InsP_3$ [15,20], we compared the effects of changes in $[Ca^{2+}]_m$ on $InsP_3$-stimulated Ca^{2+} efflux at 37 °C and 2 °C. Increasing $[Ca^{2+}]_m$ from 2 nM to 1.4 μM at 37 °C, caused a 3-fold increase in the sensitivity of the stores to $InsP_3$: the EC_{50} for $InsP_3$-stimulated Ca^{2+} efflux decreased from 281 ± 37 nM (n = 4) to 99 ± 9 nM (n = 6). More substantial increases in $[Ca^{2+}]_m$ inhibited responses to $InsP_3$: when $[Ca^{2+}]_m$ was ~ 30 μM, the response to a normally supramaximal $InsP_3$ concentration (10 μM) was decreased to 9.5 ± 3.5% (n = 9) of that observed when $[Ca^{2+}]_m$ was 50 nM. Half-maximal inhibition was achieved when $[Ca^{2+}]_m$ was 4.4 ± 0.4 μM (n = 5). These results confirm the biphasic effects of cytosolic Ca^{2+} on $InsP_3$-stimulated Ca^{2+} mobilization from hepatocytes [18]. Neither effect of cytosolic Ca^{2+} on $InsP_3$ receptor function required the presence of ATP, because removal of ATP by addition of glucose (10 mM) and hexokinase (10 units/ml) 30 s before changing $[Ca^{2+}]_m$ affected neither sensitization of the $InsP_3$ receptor when $[Ca^{2+}]_m$ was increased to 1.4 μM nor the inhibition when it was increased to ~ 30 μM (Table 1). These results do not conflict with the established stimulatory effect of ATP on $InsP_3$ receptor function, because either ATP or the ADP formed after addition of glucose and hexokinase can fulfill that role [21].

In order to examine the effects of cytosolic Ca^{2+} at 2 °C, cells were pre-chilled for 60 s, then incubated at 2 °C in media buffered at different free [Ca^{2+}] for 30 s, and finally incubated with $InsP_3$ for a further 30 s. Under these conditions, both the stimulatory and inhibitory effects of $[Ca^{2+}]_m$ were similar to those observed at 37 °C. Increasing $[Ca^{2+}]_m$ from 2 nM to 560 nM caused the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization to decrease from 68 ± 3 nM (n = 5) to 36 ± 1 nM (n = 4), and when $[Ca^{2+}]_m$ was further increased to ~ 30 μM, $InsP_3$ (1 μM) released only 12 ± 4% (n = 7) of the $InsP_3$-sensitive stores (Figure 3). These results

Table 1 Biphasic regulation of InsP_3 receptors by cytosolic Ca^{2+} is unaffected by removal of ATP

Hepatocytes were loaded to steady state with $^{45}Ca^{2+}$, and ATP was then removed by incubation with glucose (10 mM) and hexokinase (10 units/ml) for 30 s before dilution into CLM with the free $[Ca^{2+}]$ buffered as indicated. After 30 s, InsP_3 was added, and the Ca^{2+} contents of the stores were determined after a further 30 s. The results (means ± S.E.M. of n independent experiments) show that biphasic regulation of InsP_3 receptors by Ca^{2+} persists in the absence of ATP.

	EC_{50} for Ca^{2+} mobilization		Maximal Ca^{2+} release
$[Ca^{2+}]_m$ ...	< 2 nM	1.4 μM	~ 30 μM
+ATP	281 ± 37 nM (n = 4)	99 ± 9 nM (n = 6)	9.5 ± 3.5% (n = 9)
−ATP	289 ± 51 nM (n = 4)	94 ± 10 nM (n = 4)	16.3 ± 8.8% (n = 4)

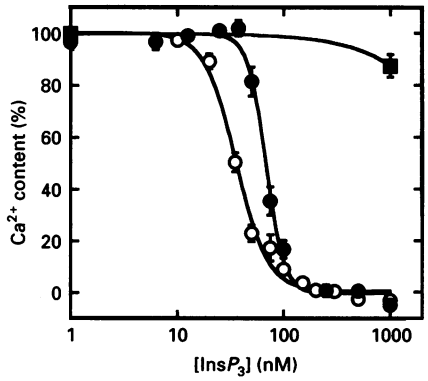

Figure 3 Biphasic regulation of InsP_3-stimulated Ca^{2+} mobilization by cytosolic $[Ca^{2+}]$ persists at 2 °C

Hepatocytes were first loaded with $^{45}Ca^{2+}$ at 37 °C in CLM ($[Ca^{2+}]_m$ = 200 nM) and then incubated at 2 °C for 60 s before dilution into CLM at 2 °C with the free $[Ca^{2+}]$ buffered at 2 nM (●), 560 nM (○) or ~ 30 μM (■). After 30 s, InsP_3 was added, and after a further 30 s the Ca^{2+} contents of the stores were determined. The results (means ± S.E.M. of 4–7 independent experiments) show that, even at 2 °C, modest increases in $[Ca^{2+}]_m$ sensitize InsP_3-stimulated Ca^{2+} mobilization, and further increases are inhibitory.

demonstrate that both the stimulatory and inhibitory effects of cytosolic Ca^{2+} can be activated at 2 °C.

Since responses to InsP_3 are quantal at 37 °C (Figure 1) and non-quantal at 2 °C (Figure 2), we next examined whether quantal responses initiated at 37 °C reversed to give non-quantal responses when the cells were cooled to 2 °C. After incubation of cells with a submaximal concentration of InsP_3 (200 nM) for 30 s at 37 °C, the cells were diluted 4-fold to 2 °C (final InsP_3 concentration = 50 nM). Prolonged further incubation (7 min) at 2 °C with InsP_3 then caused no further Ca^{2+} mobilization (Figure 4). In contrast with these results, cells that were not pre-treated with InsP_3 at 37 °C released progressively more of the Ca^{2+} stores during prolonged incubation with InsP_3 at 2 °C (Figure 4). Under these conditions, 50 nM InsP_3 caused 18 ± 4% (n = 5) mobilization of the InsP_3-sensitive stores after 30 s, and 80 ± 6% (n = 6) after 7 min. These results demonstrate that quantal responses initiated at 37 °C do not rapidly reverse to non-quantal responses at 2 °C.

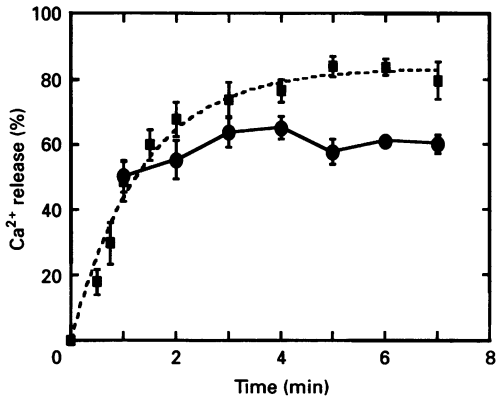

Figure 4 Quantal responses initiated at 37 °C are not reversed at 2 °C

Cells loaded with $^{45}Ca^{2+}$ were diluted into Ca^{2+}-free CLM and then incubated with a submaximal concentration of InsP_3 (200 nM) at 37 °C. After 30 s, they were diluted 4-fold into Ca^{2+}-free CLM at 2 °C, allowing simultaneous lowering of the temperature to 2 °C and of the InsP_3 concentration to 50 nM (●). In parallel experiments, cells were subjected to a similar dilution protocol, but InsP_3 (50 nM) was added 30 s after chilling to 2 °C (■). The results (means ± S.E.M., n = 3–7), shown as the fraction of the InsP_3-sensitive Ca^{2+} stores released at intervals after the onset of the incubation with 50 nM InsP_3 (t = 0), demonstrate that, once initiated at 37 °C, the mechanism underlying quantal Ca^{2+} release does not readily reverse at 2 °C.

DISCUSSION

Previous studies of permeabilized hepatocytes [16] and cerebellar microsomes [15] suggested that quantal responses resulted, at least in part, from a form of inactivation of the InsP_3 receptor that depended on the presence of both cytosolic Ca^{2+} and InsP_3. Quantal responses were abolished in media containing low $[Ca^{2+}]$ (≤ 30 nM) [15,16], and responses to InsP_3 that had inactivated in the presence of cytosolic Ca^{2+} were re-activated by removal of Ca^{2+} [16]. By contrast, in A7r5 cells quantal responses occurred in Ca^{2+}-free medium, and restoration of cytosolic Ca^{2+} failed to re-activate the response to InsP_3 unless the intracellular stores were allowed to reload with Ca^{2+} [8]. By examining complete concentration–effect relationships for InsP_3-stimulated Ca^{2+} mobilization at intervals after InsP_3 addition, we established that in Ca^{2+}-free medium ($[Ca^{2+}]_m$ < 2 nM) responses to InsP_3 were quantal at 37 °C (Figure 1). Similar quantal responses were obtained in media buffered with either EGTA or the faster Ca^{2+} buffer, BAPTA. The possibility that even the fastest Ca^{2+} buffers may fail to prevent substantial local increases in free $[Ca^{2+}]$ near the mouths of open InsP_3 receptors [20,22] needs to be considered when comparing our results (Figure 1) with those suggesting that quantal responses are abolished in Ca^{2+}-free media [15,16]. We cannot exclude the possibility that, in our experiments, even BAPTA failed to prevent the $[Ca^{2+}]$ near open channels from transiently reaching concentrations capable of inactivating InsP_3 receptors. However, inhibition by increased cytosolic $[Ca^{2+}]$ reverses within seconds when $[Ca^{2+}]$ is returned to its resting level [15–18], yet in our experiments InsP_3-evoked Ca^{2+} mobilization was complete within 30 s, after which there was no further Ca^{2+} mobilization during a subsequent 2 min incubation (Figure 1). Even if a local, ineffectively buffered, increase in $[Ca^{2+}]$ near open InsP_3-receptor channels caused their inactivation, we would therefore expect the inactivation to reverse rapidly as the Ca^{2+} gradient dissipated after channel closure. The InsP_3 receptor would then be capable of again responding to InsP_3, allowing sustained, non-quantal, Ca^{2+} mobilization. We conclude that

inhibition of $InsP_3$ receptors by increased cytosolic [Ca^{2+}] is not essential for quantal responses to $InsP_3$.

Biphasic effects of cytosolic Ca^{2+} on $InsP_3$ receptors are a common, and perhaps ubiquitous, feature that is both shared with ryanodine receptors [23–25] and likely to be important in mediating regenerative intracellular Ca^{2+} signals [1,26]. The mechanisms underlying the regulatory effects of Ca^{2+} on $InsP_3$ receptors are not, however, understood: in hepatocytes, they appear to be mediated by two distinct Ca^{2+}-binding sites [27], but it has not yet been resolved whether they reside on the $InsP_3$ receptor or accessory proteins [28]. A previous study of $InsP_3$ receptors in fibroblasts suggested that the stimulatory effects of cytosolic Ca^{2+} were mediated by calmodulin-dependent protein kinase II and the inhibitory effects by protein phosphatase 2B [29]. We previously reported that calmodulin inhibitors failed to abolish the effects of Ca^{2+} on liver $InsP_3$ receptors [18], and our present results demonstrate that both effects of cytosolic Ca^{2+} on $InsP_3$ receptors persist at 2 °C and neither is affected by complete removal of ATP (Table 1). We conclude that, in hepatocytes, the biphasic affects of Ca^{2+} on $InsP_3$ receptors are not mediated by phosphorylation and are unlikely to involve enzyme-catalysed modifications of $InsP_3$ receptors.

Several reports of the effects of temperature on quantal responses to $InsP_3$ have appeared, but the results are inconsistent. In rat basophilic leukaemia (RBL) cells, responses to $InsP_3$ were quantal at 37 °C [4], but at 11 °C similar concentrations of $InsP_3$, which caused submaximal initial rates of Ca^{2+} mobilization, were capable of emptying the entire $InsP_3$-sensitive Ca^{2+} store [30]. Initial rates of Ca^{2+} mobilization are, however, both expected [21] and demonstrated [15,31] (but see [32]) to be more sensitive to $InsP_3$ than are the eventual extents of store depletion. Irrespective of the effects of temperature, concentrations of $InsP_3$ that evoke submaximal initial rates of Ca^{2+} mobilization need not therefore cause submaximal extents of Ca^{2+} mobilization. The conclusion, that quantal responses are lost at 11 °C in RBL cells, may therefore be compromised by both the greater sensitivity of the stores to $InsP_3$ at lower temperatures (the present results) [33] and by examining the extent of store depletion with only the higher $InsP_3$ concentrations (> 25 nM) [4]. The same problems may compromise interpretation of similar results in platelets [34]. In contrast with these findings, quantal responses to $InsP_3$ have been reported both at 37 °C and at lower temperatures (4–12 °C) in A7r5 cells [35], bovine adrenal cortex microsomes [36], and our previous results with hepatocytes [13]. The reasons for these apparent discrepancies are unclear: they may include both the effects of temperature on $InsP_3$ sensitivity and the substantial changes in $[Ca^{2+}]_m$ that occurred in some analyses [30,36], but probably not in others [13,35].

In an attempt to resolve these conflicting reports, we examined complete time courses of the concentration-dependence of $InsP_3$-evoked Ca^{2+} mobilization in Ca^{2+}-free medium at 2 °C. Our results demonstrate that, under these conditions, responses to $InsP_3$ are not quantal: all concentrations of $InsP_3$ cause complete emptying of the $InsP_3$-sensitive stores at rates that increase with $InsP_3$ concentration (Figure 2). We conclude that responses to $InsP_3$ are not quantal at 2 °C. This conclusion conflicts with our previous study [13] in which hepatocytes were incubated with $InsP_3$ at 2 °C in medium containing Ca^{2+} (free [Ca^{2+}] ~ 200 nM). The discrepancy is not, however, a consequence of this difference in $[Ca^{2+}]_m$, because, when we examined complete concentration–effect relationships for $InsP_3$-stimulated Ca^{2+} mobilization at 2 °C in media containing ~ 200 nM free [Ca^{2+}], we again found a progressive decrease in the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization (from 37 nM at 30 s to 14 nM at 180 s) (results not shown). Although we cannot readily explain the discrepancy between our previous [13] and present results, the method used in the present study, i.e. complete $InsP_3$ concentration–effect relationships for each incubation period, is more robust than that used previously [13] and circumvents the need to correct for the slowly exchanging fraction of the Ca^{2+} stores (see the Materials and methods section).

Our observation that the biphasic effects of cytosolic Ca^{2+} are unaffected by low temperature (Figure 3), whereas quantal responses to $InsP_3$ are abolished (Figure 2), provides further circumstantial evidence in favour of our conclusion that changes in cytosolic [Ca^{2+}] are not responsible for quantal responses.

By initiating quantal responses to $InsP_3$ at 37 °C and then simultaneously chilling the cells and diluting them to ensure that the $InsP_3$ concentration was decreased while preserving both the pH and free [Ca^{2+}] of the medium, we established that the mechanism that leads to quantal behaviour does not rapidly reverse at 2 °C (Figure 4). This result conflicts with the conclusion of a previous study of RBL cells in which Ca^{2+} release was monitored by including Fluo-3 in the incubation medium [37]. However, interpretation of that work is compromised because chilling increases the sensitivity of the $InsP_3$ receptor, allowing concentrations of $InsP_3$ that were submaximal at 37 °C to become maximal at 2 °C, the pH and thereby free [Ca^{2+}] of the medium will change during the chilling protocol used, and no correction was made to accommodate changes in either the rate of passive efflux from the stores or the K_d of the Fluo-3 for Ca^{2+} after chilling.

We have demonstrated that the biphasic regulation of $InsP_3$ receptors by cytosolic Ca^{2+} is unaffected by either low temperature or removal of ATP, indicating that neither effect requires phosphorylation and neither is likely to be enzyme-mediated. Our results suggest that these regulatory effects of cytosolic Ca^{2+} are not an essential feature of the mechanisms underlying quantal responses to $InsP_3$. Quantal responses persist during prolonged incubations in heavily buffered Ca^{2+}-free media, and whereas the regulatory effects of cytosolic Ca^{2+} persist at 2 °C, the quantal pattern of Ca^{2+} mobilization is abolished. The mechanism underlying quantal Ca^{2+} mobilization remains unclear, but we suggest that both its initiation and reversal include temperature-sensitive processes that may either be conformational changes in the receptor or reflect the involvement of enzyme-catalysed steps.

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