Control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+}

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The synergistic action of cytosolic Ca^{2+} and inositol 1,4,5-trisphosphate (InsP_3) in releasing intracellular Ca^{2+} stores has been suggested to be responsible for the complex intracellular Ca^{2+} signals observed during hormonal stimulation of many cell types. However, the ability of cytosolic Ca^{2+} to potentiate Ca^{2+} release has recently been questioned because of the observed inhibitory effects of Ca^{2+} chelators used in previous studies. In the present study, EGTA and BAPTA [1,2-bis-(2-amino-phenoxy)ethane-*NNN'*-tetra-acetic acid] poorly inhibited InsP_3 -induced Ca^{2+} release from permeabilized A7r5 smooth-muscle cells. Additionally, stimulatory effects of cytosolic and luminal Ca^{2+} were observed either in the complete absence of Ca^{2+} chelator or at constant Ca^{2+} -free chelator concentration.

These data suggest that potentiation of InsP_3 -induced Ca^{2+} release by Ca^{2+} in A7r5 cells reflects an interaction between Ca^{2+} and InsP_3 receptors, rather than a decrease in chelator-dependent inhibition. The EC_{50} for activation of InsP_3 -induced Ca^{2+} release by cytosolic Ca^{2+} was unaffected by ATP, or by changing InsP_3 concentration, although InsP_3 -induced Ca^{2+} release became less sensitive to the inhibitory effects of cytosolic Ca^{2+} as the InsP_3 concentration was elevated. Increasing H^+ or Mg^{2+} concentration shifted the Ca^{2+} -activation curve towards higher Ca^{2+} concentrations. These data suggest that, in addition to the InsP_3 -binding site, the affinity of the Ca^{2+} -binding site(s) on InsP_3 receptors can be modulated by intracellular cations.

INTRODUCTION

Hormone-receptor-mediated increases in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{free}}$) often result from both mobilization of Ca^{2+} from intracellular stores and transmembrane Ca^{2+} influx [1,2]. In individual cells, the hormone-stimulated elevation of intracellular $[\text{Ca}^{2+}]$ has a complex temporal and spatial regulation, as the rise in intracellular $[\text{Ca}^{2+}]$ is often observed as a series of repetitive oscillations, or spikes [3–5]. The spatial counterpart of a $[\text{Ca}^{2+}]_{\text{free}}$ spike is a wave, where an initial local $[\text{Ca}^{2+}]_{\text{free}}$ elevation ultimately spreads throughout the cell in a regenerative manner [6–8].

The link between hormone-receptor activation and Ca^{2+} release is the intracellular messenger inositol 1,4,5-trisphosphate (InsP_3), which mobilizes Ca^{2+} by binding to specific intracellular receptors. These receptors also form the channels through which stored Ca^{2+} is released [9,10]. In non-excitable cells the Ca^{2+} spikes and waves, described above, are due to the cyclical and co-ordinated activation of InsP_3 receptors (InsP_3R).

The precise mechanism underlying the cyclical activation of InsP_3R is unclear, although several models have been proposed [1–5,11]. A common feature of these models is the requirement for positive feedback to generate robust regenerative responses. It has been suggested that a likely candidate for providing this feedback is cytosolic Ca^{2+} itself. In this scheme, InsP_3 -released Ca^{2+} diffuses from its site of release to neighbouring InsP_3R , and stimulates additional channel activity [2,11,12]. This model is supported by experimental observations using several different tissues, which indicate that InsP_3 and Ca^{2+} act synergistically to release Ca^{2+} [13–19].

Although it is a generally accepted hypothesis, the concept of cytosolic Ca^{2+} feedback being responsible for Ca^{2+} spiking and wave propagation is not entirely without problems. Firstly, in

some studies, stimulatory effects of cytosolic Ca^{2+} on InsP_3 -mediated Ca^{2+} release were not observed [20,21]. Secondly, under conditions where effects of cytosolic Ca^{2+} have been observed, the extent to which Ca^{2+} potentiated InsP_3 -mediated Ca^{2+} release, as well as the range of $[\text{Ca}^{2+}]$ needed for channel activation, were variable. Additionally, most of the experiments addressing the effects of cytosolic Ca^{2+} on InsP_3R activity have relied on chelators, such as EGTA and BAPTA [1,2-bis-(2-amino-phenoxy)ethane-*NNN'*-tetra-acetic acid], to buffer the $[\text{Ca}^{2+}]_{\text{free}}$. Richardson and Taylor [22] demonstrated that these Ca^{2+} chelators, in their Ca^{2+} -free form, competitively antagonized InsP_3 binding and InsP_3 -stimulated Ca^{2+} release. These studies were recently extended by Combettes and co-workers [23,24], who showed that the activation of InsP_3 -mediated Ca^{2+} release by cytosolic Ca^{2+} in cerebellum was weak when the concentration of Ca^{2+} -free chelator was fixed. These studies suggest that adding increasing amounts of Ca^{2+} to a fixed concentration of chelator, to give different $[\text{Ca}^{2+}]_{\text{free}}$, results in an apparent increase in the sensitivity of receptors, which could be due to a decrease in the concentration of Ca^{2+} -free chelator. Since observations of synergism between InsP_3 and cytosolic Ca^{2+} are central to our understanding of how complex intracellular $[\text{Ca}^{2+}]$ signals are generated, it is important to re-assess whether previously observed cytosolic Ca^{2+} effects were artefactually enhanced by Ca^{2+} -chelator complexation.

In the present study, we used the smooth-muscle cell line A7r5 as a model system to study effects of cytosolic and luminal Ca^{2+} on InsP_3 -mediated Ca^{2+} release. Our data indicate that both cytosolic and luminal Ca^{2+} potentiated InsP_3 -induced Ca^{2+} release, either in the complete absence of Ca^{2+} chelator, or at a constant Ca^{2+} -free chelator concentration. These data support the idea that synergism between InsP_3 and Ca^{2+} is a physiological mechanism controlling the release of Ca^{2+} from intracellular

Abbreviations used: InsP_3 , inositol 1,4,5-trisphosphate; $[\text{X}]_{\text{free}}$, free concentration of X; InsP_3R , InsP_3 receptor; BAPTA, 1,2-bis-(2-amino-phenoxy)ethane-*NNN'*-tetra-acetic acid; SR, sarcoplasmic reticulum.

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stores. Furthermore, we investigated the effect of various experimental conditions on the sensitivity of the InsP_3 -sensitive Ca^{2+} stores in A7r5 cells to cytosolic Ca^{2+} .

MATERIALS AND METHODS

Cell culture

A7r5 cells, which are derived from embryonic-rat aorta, were cultured as previously described [23]. Cells were seeded in 12-well dishes (Costar; 4 cm²) at a density of approx. 10⁴ cells/cm². Experiments were carried out with confluent monolayers of cells (7.5 × 10⁴ cells/cm²) between days 7 and 10 after plating. During all the experimental procedures, the 12-well dishes were maintained on a slowly rotating thermostatically controlled plate at 25 °C.

Cell permeabilization and ⁴⁵Ca²⁺ uptake

At the start of the experiment, culture medium was aspirated from the cells, and 1 ml of permeabilization medium was added, containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl₂, 1 mM ATP, 1 mM EGTA and 20 µg/ml saponin. After a 10 min incubation, the saponin-containing solution was removed, and the cells were washed once with a similar solution not containing saponin. To obtain 'partially loaded' stores (see below), the cells were incubated in this solution for a further 40 min, during which time the intracellular stores became depleted of ⁴⁰Ca²⁺. ⁴⁵Ca²⁺ uptake into the non-mitochondrial Ca²⁺ stores was accomplished by incubation in a loading medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 5 mM MgCl₂, 5 mM ATP, 0.44 mM EGTA and 10 mM NaN₃, supplemented with 5 µCi/ml ⁴⁵Ca²⁺, and CaCl₂ to give the required [Ca²⁺]_{free}. The extent of Ca²⁺-store loading was determined by varying both the duration of incubation with loading medium (2.5 or 40 min) and the [Ca²⁺]_{free} of the loading medium (11 or 241 nM). In the present study, cells incubated for 40 min with a [Ca²⁺]_{free} of 241 nM were designated as 'fully loaded', whereas cells incubated for 2.5 min with a [Ca²⁺]_{free} of 11 nM were designated as 'partially loaded'. These two loading conditions conferred different advantages for subsequent experiments; fully loaded stores are more sensitive to InsP_3 and have a large window of specific InsP_3 -releasable ⁴⁵Ca²⁺, and were therefore used for investigating potential inhibitory effects of Ca²⁺ chelators, whereas partially loaded stores had a smaller InsP_3 -releasable ⁴⁵Ca²⁺ pool, but conferred sensitivity to cytoplasmic Ca²⁺ [25,26]. The proportion of Ca²⁺ stores sensitive to InsP_3 was slightly altered by the particular loading conditions: 94 and 80 % for fully loaded and partially loaded stores respectively (results not shown).

⁴⁵Ca²⁺ efflux

After ⁴⁵Ca²⁺ accumulation, the cells were washed for 2 min in 1 ml of efflux medium A, containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl₂, 1 mM EGTA, 5 mM NaN₃ and 2 µM thapsigargin (calculated [Ca²⁺]_{free} was approx. 10 nM). This incubation with thapsigargin-containing medium irreversibly inhibited any further ATP-dependent Ca²⁺ uptake. Then 1 ml of efflux medium A without thapsigargin was added for the next 2 min, and this solution was replaced every 2 min until 14 min had elapsed. InsP_3 and A23187 were added to the cells for 2 min, i.e. between min 4 and 6 of efflux.

For the experiments that used skeletal-muscle sarcoplasmic-reticulum (SR) vesicles to decrease [Ca²⁺]_{free}, SR vesicles (44 µg/ml) were added to efflux medium B, which contained

120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl₂, 1 mM ATP, 5 mM NaN₃, 2 mM phosphocreatine and 10 units/ml creatine kinase at 25 °C. The SR vesicles were incubated in this efflux medium for 4 min to obtain a stable [Ca²⁺]_{free}, after which the solution, still containing SR vesicles but with or without InsP_3 , was added to the cells.

At the end of the experiment, the ⁴⁵Ca²⁺ remaining in the cells was released by incubation with 1 ml of 2 % SDS solution for 30 min. The InsP_3 -released Ca²⁺ was calculated as the difference between the control and InsP_3 -treated samples, both of which were corrected for A23187-insensitive ⁴⁵Ca²⁺ uptake.

For experiments where the pH was altered from 6.8 to 7.35 (Figure 6c), the imidazole/HCl buffer was replaced with 30 mM Hepes/KOH. Concentration-response curves were fitted to a logistic equation by using the GraphPAD Inplot curve-fitting program (GraphPAD software, San Diego, CA, U.S.A.) by a non-linear least-squares procedure.

[Ca²⁺]_{free} determinations

The [Ca²⁺]_{free} and [chelator]_{free} in the efflux media used were calculated by using MaxChelator (Dr. C. Patton, Stanford University, CA, U.S.A.) or Cabuf (Dr. G. Droogmans, K.U. Leuven, Belgium). The fluorescence of fluo-3 was calibrated by previously described methods [27], by using a dissociation constant of 400 nM. Changes in [Mg²⁺]_{free} and pH, caused by addition of Ca²⁺ or EGTA/BAPTA, were corrected before starting experiments by addition of MgCl₂ or NaOH respectively.

Measurement of InsP_3 metabolism

A7r5 cells grown on 12-well dishes were permeabilized as described above, and incubated for 2 min at 25 °C in 1 ml of efflux medium A, containing 2 mM, 5 mM or no added Mg²⁺ and supplemented with 4 µM InsP_3 and 0.05 µCi of [³H] InsP_3 (Amersham). After the incubation, the medium was removed from the cells and loaded on to an anion-exchange column (AG 1-X8, formate form, 200–400 mesh, Bio-Rad). InsP_2 and lower inositol phosphates were eluted with 8 ml of 0.5 M ammonium formate/0.1 M formic acid, and InsP_3 was eluted with 8 ml of 1.25 M ammonium formate/0.1 M formic acid. The resolution of InsP_2 from InsP_3 by the anion-exchange columns was confirmed by using [³H]inositol phosphate standards (NEN Research Products).

RESULTS AND DISCUSSION

EGTA and BAPTA are weak inhibitors of InsP_3 -induced Ca²⁺ release in A7r5 cells

Previous studies using A7r5 cells demonstrated a significant (4-fold with supramaximal [InsP_3]) potentiation of InsP_3 -mediated Ca²⁺ release by cytosolic Ca²⁺ over the range 10 nM to 10 µM [25,26]. In these studies, the [Ca²⁺]_{free} was adjusted by adding increasing amounts of Ca²⁺ to a fixed (1 mM) EGTA concentration, giving calculated changes in Ca²⁺-free EGTA from 970 µM to 88 µM, for 10 nM and 10 µM [Ca²⁺]_{free} respectively. Since this is within the range of Ca²⁺-free EGTA concentrations ([EGTA]_{free}) that markedly inhibit InsP_3 -mediated Ca²⁺ release in sheep cerebellar tissues [23,24], we were concerned that the observed potentiation of InsP_3 -mediated Ca²⁺ release by cytosolic Ca²⁺ was an experimental artifact caused by complexation of free chelator.

The ability of Ca²⁺-free EGTA to inhibit InsP_3 -stimulated Ca²⁺ release in A7r5 cells was therefore investigated by incubating permeabilized A7r5 cells with sub-maximal InsP_3 concentrations

Figure 1 Inhibition of InsP_3 -induced Ca^{2+} release from permeabilized A7r5 cells by EGTA and BAPTA

The effect of EGTA and BAPTA on InsP_3 -induced Ca^{2+} release was investigated by incubating fully loaded Ca^{2+} stores (see the Materials and methods section) with either 4 μM ($\square$, $\blacktriangle$) or 0.4 μM ($\circ$) InsP_3 , in solutions containing a fixed (50 nM) $[\text{Ca}^{2+}]_{\text{free}}$ and increasing concentrations of Ca^{2+} -free EGTA ($\square$, $\circ$) or BAPTA ($\blacktriangle$). The data are expressed relative to control experiments, where cells were treated with the EGTA- and BAPTA-containing solutions in the absence of InsP_3 . Values represent means $\pm$ S.E.M. of 3–6 experiments.

(4 and 0.4 μM) in the presence of increasing $[\text{EGTA}]_{\text{free}}$. Cells with 'fully loaded' stores (see the Materials and methods section) were used, since they have a large window of InsP_3 -releasable $^{45}\text{Ca}^{2+}$ (approx. 90%; [25,26]) and an increased sensitivity to InsP_3 , compared with 'partially loaded' stores [26].

Increasing the $[\text{EGTA}]_{\text{free}}$ over the range 90 μM to 9 mM, while keeping cytosolic $[\text{Ca}^{2+}]_{\text{free}}$ constant at 50 nM, decreased the extent of Ca^{2+} release from $73 \pm 2\%$ to $66 \pm 2\%$, and from $31 \pm 2\%$ to $22 \pm 1\%$ for 4 μM and 0.4 μM InsP_3 respectively (Figure 1). These data, indicating that EGTA was a weak inhibitor of Ca^{2+} release from A7r5 cells, are consistent with the study by Richardson and Taylor [22], who observed that EGTA inhibited InsP_3 binding to the rat cerebellar InsP_3R with an $\text{IC}_{50} > 20$ mM.

BAPTA was approx. 60-fold more potent than EGTA in inhibiting InsP_3 binding to the purified rat cerebellar receptor [22]. The effects of changing the $[\text{BAPTA}]_{\text{free}}$ on the Ca^{2+} release induced by 4 μM InsP_3 , in an efflux medium containing 50 nM free Ca^{2+} , is shown in Figure 1. As $[\text{BAPTA}]_{\text{free}}$ was increased from 90 μM to 9 mM the extent of InsP_3 -induced Ca^{2+} release was decreased from $70 \pm 2\%$ to $62 \pm 2\%$. These data suggest that BAPTA had approximately the same weak potency as EGTA in inhibiting the InsP_3Rs expressed in A7r5 cells.

Cytosolic Ca^{2+} potentiates InsP_3 -induced Ca^{2+} release in the absence of EGTA

Since EGTA is a weak inhibitor of InsP_3 -mediated Ca^{2+} release from A7r5 cells, it is unlikely that the changes in $[\text{EGTA}]_{\text{free}}$ that occurred in our previous studies [26,28] complicated the observation that cytoplasmic Ca^{2+} potentiated InsP_3 -induced Ca^{2+} release. However, to demonstrate unambiguously that Ca^{2+} is a co-agonist for the InsP_3R , we examined the ability of Ca^{2+} to synergize with InsP_3 in media without EGTA. In order to decrease $[\text{Ca}^{2+}]_{\text{free}}$ in the test media without adding EGTA, we used SR vesicles from skeletal muscle as an active buffering system. Trace *a* in Figure 2 illustrates that addition of the SR

Figure 2 Ca^{2+} sequestration by SR vesicles

SR vesicles from skeletal muscle (44 $\mu\text{g}/\text{ml}$, labelled 'SR') were added to ATP-free efflux medium B supplemented with 2 μM fluo-3. In trace *b*, efflux medium B was supplemented with 2 μM thapsigargin (TG) to prevent Ca^{2+} uptake by the vesicles. Changes in $[\text{Ca}^{2+}]_{\text{free}}$ were monitored as a function of time after addition of 1 mM ATP. Calibration was performed by adding 2 mM EGTA, followed by 10 mM CaCl_2 , to obtain minimal and maximal fluorescence respectively. The data presented are typical of four experiments.

vesicles (44 $\mu\text{g}/\text{ml}$) decreased the $[\text{Ca}^{2+}]_{\text{free}}$ of the efflux medium to 50 nM in the presence of ATP. The SR vesicles were able to maintain this low $[\text{Ca}^{2+}]_{\text{free}}$ for up to 10 min, which exceeded the duration of their use in a subsequent experiment (see below). In the presence of thapsigargin (2 μM), the SR vesicles were unable to sequester Ca^{2+} (trace *b*), resulting in a $[\text{Ca}^{2+}]_{\text{free}}$ of 5.6 μM .

Exposure of cells with partially loaded Ca^{2+} stores to 4 μM InsP_3 for 2 min in EGTA-free efflux medium B containing active SR vesicles (50 nM $[\text{Ca}^{2+}]_{\text{free}}$) released only $22 \pm 2\%$ of the InsP_3 -releasable Ca^{2+} ($n = 4$, Figure 3a). Addition of 4 μM InsP_3 in EGTA-free efflux medium B containing thapsigargin-inhibited SR vesicles (5.6 μM $[\text{Ca}^{2+}]_{\text{free}}$) released $75 \pm 5\%$ of the InsP_3 -releasable Ca^{2+} ($n = 4$, Figure 3a).

Parallel experiments were performed using efflux media with the same $[\text{Ca}^{2+}]_{\text{free}}$ (50 nM and 5.6 μM) buffered with EGTA (1 mM $[\text{EGTA}]_{\text{total}}$ in each solution). The stimulatory effects of Ca^{2+} were comparable with those obtained by using SR vesicles (Figure 3a), even though in the EGTA-buffered medium there was a change in $[\text{EGTA}]_{\text{free}}$ from 0.9 to 0.09 mM as $[\text{Ca}^{2+}]_{\text{free}}$ was elevated from 50 nM to 5.6 μM . These data are consistent with the results presented above suggesting that EGTA is only a weak inhibitor of InsP_3 -mediated Ca^{2+} release in A7r5 cells, and furthermore they clearly show that the potentiation of Ca^{2+} release by cytosolic Ca^{2+} is not due to complexation of Ca^{2+} -free EGTA.

Luminal Ca^{2+} potentiates InsP_3 -induced Ca^{2+} release in the absence of EGTA

Several studies have suggested that luminal Ca^{2+} can control the release of Ca^{2+} from intracellular stores, such that stores with a greater Ca^{2+} content are more sensitive to InsP_3 [19,25,26,29–32]. However, we were concerned that the effect of luminal Ca^{2+} may have reflected a decrease in the potential inhibitory effect of EGTA, caused by binding of Ca^{2+} ions to the Ca^{2+} -free EGTA

Figure 3 Cytosolic and luminal Ca^{2+} potentiate InsP_3 -induced Ca^{2+} release in the absence of EGTA

The effect of cytosolic and luminal Ca^{2+} on InsP_3 -induced Ca^{2+} release in the presence and absence of EGTA was investigated as described in the Materials and methods section. The $[\text{Ca}^{2+}]_{\text{free}}$ was adjusted by use of either SR vesicles or EGTA (1 mM total). The concentration of InsP_3 was 4 μM in all the experiments. (a) InsP_3 -induced Ca^{2+} release from partially loaded Ca^{2+} stores at the cytosolic $[\text{Ca}^{2+}]_{\text{free}}$ shown. (b) InsP_3 -induced Ca^{2+} release from cells with partially loaded (denoted as 1% loading) and fully loaded (100% loading) Ca^{2+} stores at a fixed (50 nM) $[\text{Ca}^{2+}]_{\text{free}}$. The data represent means $\pm$ S.E.M. for four independent experiments, each with duplicate samples.

surrounding these stores. In this scheme, cells with more highly loaded intracellular Ca^{2+} stores would appear to be more sensitive to InsP_3 , since the flux of Ca^{2+} , and hence the complexation of Ca^{2+} -free EGTA, would be greatest with these stores.

We therefore tested the ability of InsP_3 to release Ca^{2+} from fully loaded and partially loaded Ca^{2+} stores in the absence and presence of EGTA (1 mM total; 0.93 mM free), at a low cytosolic $[\text{Ca}^{2+}]_{\text{free}}$ (50 nM). To obtain the same cytosolic $[\text{Ca}^{2+}]_{\text{free}}$ in the absence of EGTA, we used SR vesicles as described above. The proportion of the InsP_3 -sensitive Ca^{2+} store released by 4 μM InsP_3 was dramatically increased when the Ca^{2+} content of the stores was elevated, from 1 to 100% (Figure 3b), independent of the presence or absence of EGTA in the efflux medium. These data clearly indicate the dramatic potentiation of InsP_3 -mediated Ca^{2+} release by luminal Ca^{2+} , and also that the effect of luminal Ca^{2+} is not due to the complexation of Ca^{2+} -free EGTA. It is important to note that, since the data in Figure 3 are presented as a proportion of the total InsP_3 -releasable Ca^{2+} , the increase in Ca^{2+} release by 4 μM InsP_3 from fully loaded stores does not reflect selective Ca^{2+} uptake by either the InsP_3 -sensitive or InsP_3 -insensitive stores during the different loading conditions.

The mechanism underlying the ability of luminal Ca^{2+} to potentiate InsP_3 -mediated Ca^{2+} release is at present unknown.

Figure 4 Effect of InsP_3 concentration on the Ca^{2+} -dependent activation of InsP_3 -induced Ca^{2+} release

Cells with partially loaded Ca^{2+} stores were incubated for 2 min with various InsP_3 concentrations at the $[\text{Ca}^{2+}]_{\text{free}}$ shown. Symbols: $\blacklozenge$, 100 μM InsP_3 ; $\square$, 10 μM InsP_3 ; $\bullet$, 3.2 μM InsP_3 . The data points on each curve are expressed relative to control cells that were similarly treated with each $[\text{Ca}^{2+}]_{\text{free}}$ in the absence of InsP_3 . The continuous lines indicate computer-fitted curves over the range of $[\text{Ca}^{2+}]_{\text{free}}$ that activated Ca^{2+} release. The broken line indicates the inhibitory effect of cytosolic Ca^{2+} , and was drawn by hand.

Luminal Ca^{2+} promotes a conformation of InsP_3R giving an increased affinity for InsP_3 [30,32]. Another possibility is that the greater rate of Ca^{2+} release from more fully loaded Ca^{2+} stores enhances the positive feedback on the cytosolic side of the receptor [17]. Whatever the mechanism, luminal Ca^{2+} clearly regulates InsP_3 -mediated Ca^{2+} release in A7r5 cells, and furthermore our data suggest that luminal Ca^{2+} provides a more effective trigger for Ca^{2+} release than do changes in cytosolic $[\text{Ca}^{2+}]_{\text{free}}$. For example, the relative amount of Ca^{2+} released by InsP_3 from partially loaded stores in the presence of a maximally stimulating cytosolic Ca^{2+} concentration was always less than that released from fully loaded stores (compare the responses of partially loaded stores at 5.6 μM $[\text{Ca}^{2+}]_{\text{free}}$ in Figure 3a with the response of cells loaded to 100% in Figure 3b). Additionally, we have previously shown that, as the Ca^{2+} stores are loaded with Ca^{2+} , or the cytoplasmic $[\text{Ca}^{2+}]_{\text{free}}$ is increased, there is a sensitization of the response to InsP_3 detected by a decrease in the EC_{50} for InsP_3 -mediated Ca^{2+} release. However, the degree of sensitization obtained by elevating cytoplasmic $[\text{Ca}^{2+}]_{\text{free}}$ never matched the effect of loading the stores, even at high $[\text{Ca}^{2+}]_{\text{free}}$ (Figure 2 of [26]). Furthermore, although cytosolic Ca^{2+} both potentiates and inhibits InsP_3 -mediated Ca^{2+} release, luminal Ca^{2+} only appears to enhance Ca^{2+} release [26,32].

Inactivation of InsP_3R by cytosolic Ca^{2+} is less pronounced at high InsP_3 concentrations

In several cell types, InsP_3 -mediated Ca^{2+} release has a biphasic dependence on cytoplasmic $[\text{Ca}^{2+}]_{\text{free}}$, with nanomolar and micromolar $[\text{Ca}^{2+}]_{\text{free}}$ potentiating and inhibiting Ca^{2+} release respectively [13–19,26,28]. We investigated the effect of different InsP_3 concentrations on the activation and inhibition of the InsP_3R by cytosolic Ca^{2+} . Elevating InsP_3 concentration from 3.2 to 100 μM did not cause any apparent shift in the sensitivity of Ca^{2+} release to cytosolic Ca^{2+} in the range of $[\text{Ca}^{2+}]_{\text{free}}$ that potentiated the release (Figure 4). However, with 3.2 μM InsP_3 , there was a significant decrease in the amount of Ca^{2+} released at

Figure 5 Inhibition of InsP_3 -mediated Ca^{2+} release by cytosolic Ca^{2+} is less pronounced at high InsP_3 concentrations

Cells were incubated with either 10 nM (Δ) or 10 μM ($\blacklozenge$) $[\text{Ca}^{2+}]_{\text{free}}$ for 2 min before the addition of various InsP_3 concentrations in the same Ca^{2+} -containing solutions. The data points for each curve are expressed relative to control cells that were similarly treated with 10 nM or 10 μM $[\text{Ca}^{2+}]_{\text{free}}$ in the absence of InsP_3 . Inset: relative inhibition of Ca^{2+} release caused by incubation in 10 μM $[\text{Ca}^{2+}]_{\text{free}}$.

10 μM $[\text{Ca}^{2+}]_{\text{free}}$, which was not apparent with the higher InsP_3 concentrations. These data raised the possibility that Ca^{2+} release at high InsP_3 concentrations was less sensitive to the inhibitory effects of cytoplasmic Ca^{2+} .

However, in these experiments both InsP_3 and Ca^{2+} were added to the permeabilized cells simultaneously, raising the concern that the apparent lower sensitivity of the InsP_3R to cytoplasmic Ca^{2+} at high InsP_3 concentrations was simply due to the ability of these InsP_3 concentrations to bind and activate Ca^{2+} release, before the cytoplasmic Ca^{2+} could exert its inhibitory effect. We therefore adapted the experimental protocol so that the cells were pretreated with 10 μM Ca^{2+} for 2 min before addition of InsP_3 . The data presented in Figure 5 show that preincubation of the cells with 10 μM Ca^{2+} caused a dramatic decrease in the sensitivity of InsP_3 -mediated Ca^{2+} release. This effect, which was much greater than that observed when InsP_3 and 10 μM Ca^{2+} were added together, was consistent with our earlier observations showing that the inhibitory effect of Ca^{2+} is time-dependent [26]. Additionally, Figure 5 shows that the fractional inhibition of the response due to preincubation with 10 μM Ca^{2+} decreased as the InsP_3 concentration was elevated. These data confirm that the inhibitory effects of cytoplasmic Ca^{2+} are less pronounced in the presence of high InsP_3 concentrations. Similar observations were reported for cerebellar membranes [23], where elevating InsP_3 concentration increased the Ca^{2+} concentration required to inhibit InsP_3 -mediated Ca^{2+} release.

There are two plausible mechanisms that could account for these observations. Firstly, high InsP_3 concentrations may promote an InsP_3R conformation where Ca^{2+} cannot bind to the site that mediates inhibition. Secondly, at high cytosolic $[\text{Ca}^{2+}]_{\text{free}}$, InsP_3R will be in equilibrium between inactive and active states, as Ca^{2+} binds to, and then dissociates from, the site that mediates the Ca^{2+} -dependent inhibition. Under these conditions, elevating InsP_3 concentration will increase the probability of InsP_3 binding and opening the Ca^{2+} channel, during the period when the InsP_3Rs are in an activatable conformation.

Ca^{2+} -dependent inactivation of InsP_3R may be important in controlling $[\text{Ca}^{2+}]_{\text{free}}$ during hormonal stimulation, for example by terminating Ca^{2+} release during a Ca^{2+} spike. However, it has

been suggested that, at its site of production, InsP_3 concentration may be much higher than in the bulk cytosol [33]. These data, along with the observations that Ca^{2+} -dependent inactivation of InsP_3R is less pronounced at high InsP_3 concentrations, presented above, raise the possibility that in discrete areas with high InsP_3 concentrations the InsP_3R may be continually activated, despite the global changes in $[\text{Ca}^{2+}]_{\text{free}}$. Such discrete areas are likely to be located close to the InsP_3 -generating enzymes near the plasma membrane. In this location, the InsP_3R may also be able to generate a signal for the entry of Ca^{2+} across the plasma membrane, and could therefore be responsible for the continuous entry of Ca^{2+} that is required to sustain Ca^{2+} spiking.

Effects of ATP, Mg^{2+} and pH on the activation of InsP_3R by cytosolic Ca^{2+}

We investigated the effects of ATP, Mg^{2+} and pH on the activation of InsP_3 -mediated Ca^{2+} release by cytosolic Ca^{2+} . To ensure that our results reflected real effects of these agents on InsP_3 -mediated Ca^{2+} release, rather than an effect on the concentration of Ca^{2+} -free EGTA, the $[\text{EGTA}]_{\text{free}}$ was maintained at 0.25 mM in all the experiments described below. This ensured that the apparent changes in sensitivity to cytosolic Ca^{2+} described below were not complicated by the small inhibitory effect of Ca^{2+} -free EGTA shown in Figure 1. Additionally, Ca^{2+} -dependent activation of phospholipase C did not contribute to the data presented below, since addition of solutions containing elevated $[\text{Ca}^{2+}]_{\text{free}}$ in the absence of InsP_3 released only a fraction of the $^{45}\text{Ca}^{2+}$ that was released in the presence of InsP_3 . For example, a 2 min incubation of permeabilized cells in efflux medium A with $[\text{Ca}^{2+}]_{\text{free}}$ set at 2 μM (conditions which gave nearly maximal stimulation of InsP_3 -induced Ca^{2+} release) decreased the $^{45}\text{Ca}^{2+}$ labelling of the cells by 25%. The loss of $^{45}\text{Ca}^{2+}$ caused by solutions containing elevated $[\text{Ca}^{2+}]_{\text{free}}$ was due to the activation of a $^{45}\text{Ca}^{2+}$ - $^{40}\text{Ca}^{2+}$ exchange previously demonstrated in these cells [28], and therefore represents a decrease in the specific radioactivity of the accumulated $^{45}\text{Ca}^{2+}$ rather than a release of Ca^{2+} . If unaccounted for, the decrease in specific radioactivity caused by various solutions would have led to an overestimation of the Ca^{2+} released at each $[\text{Ca}^{2+}]_{\text{free}}$. To avoid this problem, we expressed the data relative to controls in which cells were treated with the various $[\text{Ca}^{2+}]_{\text{free}}$ solutions in the absence of InsP_3 .

In a Mg^{2+} - and ATP-free medium, activation of InsP_3 -mediated Ca^{2+} release by cytosolic Ca^{2+} occurred with an EC_{50} of 534 nM Ca^{2+} (Figure 6a). Increasing the ATP concentration to 1 mM did not affect the EC_{50} for activation of Ca^{2+} release, but increased the extent of Ca^{2+} release at all $[\text{Ca}^{2+}]_{\text{free}}$ tested. These data are consistent with previous studies of InsP_3R activity in lipid bilayers, where ATP increased the probability of InsP_3R opening and in some cases stabilized a higher conductance state [14,34,35]. The effects of ATP on Ca^{2+} release were not due to InsP_3R phosphorylation, since ATP was added in the absence of Mg^{2+} .

Elevating $[\text{Mg}^{2+}]_{\text{free}}$ to 2 mM induced a rightward shift in the activation curve for Ca^{2+} , resulting in an EC_{50} for Ca^{2+} -dependent activation of 1.7 μM (Figure 6b). With 5 mM $[\text{Mg}^{2+}]_{\text{free}}$, there was also an apparent rightward shift in the Ca^{2+} -activation curve, and a decrease in the extent of Ca^{2+} release. The inhibition of Ca^{2+} release by 5 mM Mg^{2+} was completely reversible (results not shown). The effects of Mg^{2+} were not due to activation of the Mg^{2+} -dependent InsP_3 phosphatase for several reasons: firstly, changes in InsP_3 concentration do not cause a shift in the sensitivity to cytosolic Ca^{2+} (Figure 4); secondly, 2 mM Mg^{2+} had a similar inhibitory effect on Ca^{2+} release induced by 10 μM $\text{Ins}(2,4,5)\text{P}_3$, which is a poor substrate for the InsP_3 phosphatase

Figure 6 Effect of ATP, Mg^{2+} and pH on the activation of $InsP_3$ -induced Ca^{2+} release by cytosolic Ca^{2+}

This Figure describes the effect of various experimental conditions on the Ca^{2+} release induced by $4 \mu M$ $InsP_3$ from cells with partially loaded Ca^{2+} stores. The Ca^{2+} -free [EGTA] was fixed at 0.25 mM in all conditions. (a) Effect of adding ATP. The symbols represent: ●, Ca^{2+} release after incubation of cells with $InsP_3$ in efflux medium A without $MgCl_2$; △, Ca^{2+} release after incubation of cells in efflux medium A without $MgCl_2$, but with 1 mM ATP. (b) Effect of increasing $[Mg^{2+}]_{free}$ concentration. The symbols represent: ●, no added Mg^{2+} ; □, 2 mM Mg^{2+} ; ◆, 5 mM Mg^{2+} . (c) Effect of pH. The symbols represent: ●, Ca^{2+} release after incubation in efflux medium A without $MgCl_2$, at pH 6.8; ○, Ca^{2+} release after incubation in efflux medium A without $MgCl_2$, at pH 7.35. The data represent means $\pm$ S.E.M. for at least three independent experiments, each with duplicate samples. Error bars are shown when larger than the symbol. The continuous lines indicate computer-fitted curves, from which the EC_{50} for Ca^{2+} -dependent activation of Ca^{2+} release was estimated. The broken lines indicate the inhibitory effect of cytosolic Ca^{2+} , and were drawn by hand.

[36–38]; results not shown), thirdly, measurement of $[^3H]InsP_3$ metabolism under our experimental conditions indicated that only 6 or 7% of the $InsP_3$ was metabolized after incubation with solutions containing 2 or 5 mM Mg^{2+} respectively.

Previous studies using dog cerebellar membranes have shown that Mg^{2+} inhibits $InsP_3$ -induced Ca^{2+} release in a concentration-dependent manner [39]. The inhibition of Ca^{2+} release by Mg^{2+} is probably due to the integrated effect of several independent mechanisms. It has been demonstrated that the binding of $InsP_3$

to its receptor is inhibited by Mg^{2+} in a concentration-dependent non-competitive manner [39–41]. Additionally, millimolar levels of Mg^{2+} will bind to $InsP_3$ itself, possibly decreasing the effective $InsP_3$ concentration [40]. These effects may account for the decreased extent of Ca^{2+} release that we observed in the presence of 5 mM Mg^{2+} (Figure 6b). However, decreasing the effective concentration of $InsP_3$ cannot account for the shift in sensitivity to cytosolic Ca^{2+} , since different $InsP_3$ concentrations have similar Ca^{2+} -dependencies (Figure 4). We therefore suggest that Mg^{2+} also inhibits the binding of Ca^{2+} to an activatory site on the $InsP_3R$. The effects of Mg^{2+} on $InsP_3$ -mediated Ca^{2+} release are clearly complex, with several potential binding sites.

Cytoplasmic alkalinization has been found to increase the sensitivity of $InsP_3$ -mediated Ca^{2+} release [42–44], an effect that is commonly ascribed to an increase in the affinity of $InsP_3R$ for $InsP_3$ [40,43,45,46]. However, the data presented in Figure 6(c) show that alkalinization sensitized $InsP_3$ -mediated Ca^{2+} release to cytosolic Ca^{2+} , suggesting that there is an interaction between H^+ and the Ca^{2+} -binding site on $InsP_3R$. A similar pH-dependent sensitization of $InsP_3$ -mediated Ca^{2+} release to cytosolic Ca^{2+} was reported by Tsukioka and colleagues [44]. However, in their study, alkalinization only affected the activatory part of the response; there was no apparent shift in the $[Ca^{2+}]_{free}$ required for maximal Ca^{2+} release. These observations differ from the results shown in Figure 6(c), where alkalinization decreased both the EC_{50} for Ca^{2+} -dependent activation (from 534 nM to 299 nM) and the $[Ca^{2+}]_{free}$ required for maximal Ca^{2+} release (from 5.6 μM to 1 μM).

The data presented above confirm that the regulation of $InsP_3$ -mediated Ca^{2+} release is complex. In addition to their previously reported effects on $InsP_3$ binding, various cytosolic cations also appear to compete for the Ca^{2+} -binding site(s) on the $InsP_3R$. Changes in the cytoplasmic concentration of these regulatory ions, e.g. the change in pH which can occur during stimulation of cells with growth factors [47], will alter the activity of the intracellular Ca^{2+} -signalling machinery.

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