Type 3 inositol trisphosphate receptors in RINm5F cells are biphasically regulated by cytosolic Ca^{2+} and mediate quantal Ca^{2+} mobilization

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There are three subtypes of mammalian Ins(1,4,5)P_3 (InsP_3) receptor, each of which forms an intracellular Ca^{2+} channel. Biphasic regulation of InsP_3 receptors by cytosolic Ca^{2+} is well documented in cells expressing predominantly type 1 or type 2 InsP_3 receptors and might contribute to the regenerative recruitment of Ca^{2+} release events and to limiting their duration in intact cells. The properties of type 3 receptors are less clear. Bilayer recording from InsP_3 receptors of RIN-5F cells, cells in which the InsP_3 receptors are likely to be largely type 3, recently suggested that the receptors are not inhibited by Ca^{2+} [Hagar, Burgstahler, Nathanson and Ehrlich (1998) Nature (London) **296**, 81–84]. By using antipeptide antisera that either selectively recognized each InsP_3 receptor subtype or interacted equally well with all subtypes, together with membranes from *Spodoptera frugiperda* (*Sf*9) cells expressing only single receptor subtypes to calibrate the immunoblotting, we quantified the relative levels of expression of type 1 (17%) and type 3 (77%) InsP_3 receptors in RINm5F cells. In unidirectional $^{45}Ca^{2+}$ efflux experiments from permeabilized RINm5F cells, submaximal concentrations of InsP_3 released only a fraction of the InsP_3-sensitive Ca^{2+} stores, indicating that responses to InsP_3 are quantal. Increasing the cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) from approx. 4 to 186 nM increased the sensitivity of the Ca^{2+} stores to InsP_3: the EC_{50} decreased from 281 ± 15 to 82 ± 2 nM. Further increases in $[Ca^{2+}]_i$ massively decreased the sensitivity of the stores to InsP_3, by almost 10-fold when $[Ca^{2+}]_i$ was 2.4 μM, and by more than 3000-fold when it was 100 μM. The inhibition caused by 100 μM Ca^{2+} was fully reversed within 60 s of the restoration of $[Ca^{2+}]_i$ to 186 nM. The effect of submaximal InsP_3 concentrations on Ca^{2+} mobilization from permeabilized RINm5F cells is therefore biphasically regulated by cytosolic Ca^{2+}. We conclude that type 3 InsP_3 receptors of RINm5F cells mediate quantal Ca^{2+} release and they are biphasically regulated by cytosolic Ca^{2+}, either because a single type 1 subunit within the tetrameric receptor confers the Ca^{2+} inhibition or because the type 3 subtype is itself directly inhibited by Ca^{2+}.

Key words: InsP_3 receptor subtype, quantal Ca^{2+} release, Ca^{2+} regulation.

INTRODUCTION

There are three closely related subtypes (1–3) of receptors for Ins(1,4,5)P_3 (InsP_3), each of which forms an intracellular Ca^{2+} channel [1] and each of which is expressed to some extent in most cells [2]. Splice variants of the type 1 receptor (and perhaps also of the type 2 receptor) [3], together with assembly of the different subunits into both homotetrameric and heterotetrameric receptor complexes [4,5], add further to the diversity of InsP_3 receptors expressed in mammalian cells. The subtypes are differentially expressed [4,6,7], they differ in their rates of degradation during chronic cell stimulation [8] and in their affinities for InsP_3 [9–11], they are differentially regulated by calmodulin [12] and ATP [13] and they differ in their phosphorylation [3,4].

Biphasic modulation of InsP_3 receptors by cytosolic Ca^{2+}, first reported in smooth muscle [14] but later demonstrated in many cell types [15], has attracted particular attention. The stimulation of InsP_3 receptors by Ca^{2+} is thought to contribute to the regenerative recruitment of InsP_3 receptors in intact cells, whereas the inhibitory effect of Ca^{2+} is proposed to provide the negative feedback that limits the duration of elementary Ca^{2+} release events [16]. The biphasic effect of cytosolic Ca^{2+} on InsP_3-evoked Ca^{2+} mobilization has been most thoroughly examined in cells expressing predominantly type 1 or type 2 InsP_3 receptors [15] and was widely supposed to be a feature of all InsP_3 receptor subtypes. However, Ca^{2+} clearly has different effects on InsP_3 binding to different receptor subtypes [10,11,15]; evidence from intact cells is also consistent with differential effects of Ca^{2+} on InsP_3 receptor subtypes. Type 1 receptors might be specifically implicated in propagating regenerative Ca^{2+} signals [17], whereas type 1 and type 2 receptors, but not type 3 receptors, might be capable of generating oscillatory Ca^{2+} release [13,18]. Bilayer recordings of InsP_3 receptors from RIN-5F cells, which have been suggested (although not quantitatively proved) to express largely type 3 receptors [18], recently suggested that even very high concentrations of Ca^{2+} (100 μM) do not inhibit type 3 InsP_3 receptors. This observation is consistent with the lack of inhibition of type 3 receptors in DT40 cells by more modest increases in cytosolic Ca^{2+} (1 μM) [13] but it is difficult to reconcile with the substantial inhibition of InsP_3-evoked Ca^{2+} mobilization by 10 μM Ca^{2+} in 16HBE14o$^-$ cells, which probably express predominantly type 3 receptors [19].

In the present study, we quantitatively determined the relative levels of expression of InsP_3 receptor subtypes in RINm5F cells, established that cytosolic Ca^{2+} exerts typical biphasic effects on InsP_3-evoked Ca^{2+} mobilization in permeabilized RINm5F cells, and demonstrated the quantal nature of the Ca^{2+} release.

MATERIALS AND METHODS

Cell culture

RINm5F cells (from Dr. P. Brown, University of Manchester, Manchester, U.K.) were cultured at 37 °C under air/CO_2 (19:1) in RPMI 1640 medium containing L-glutamine (2 mM), foetal

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CLM, cytosol-like medium; InsP_3, Ins(1,4,5)P_3; *Sf*9/InsP_3R1 and *Sf*9/InsP_3R3, *Spodoptera frugiperda* cells expressing type 1 and type 3 InsP_3 receptors respectively.

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calf serum (5%, v/v), penicillin (50 i.u./ml) and streptomycin (50 μg/ml). The cells were passaged every 3 or 4 days when confluent and were used for experiments when they were confluent. SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Manassas, VA, U.S.A.) were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, 7 mM L-glutamine and 0.9% non-essential amino acids under air/CO_2 (19:1). Cells were passaged every 7 days when they were confluent.

Measurement of unidirectional $^{45}Ca^{2+}$ efflux from permeabilized cells

RINm5F rat insulinoma cells (passage numbers 105–129) were scraped into cold Hepes-buffered saline [HBS: 155 mM NaCl/10 mM Hepes/0.68 mM EDTA, pH 7.4 at 0 °C], washed by centrifugation (650 ***g***, 4 min), and resuspended (5×10^6 cells/ml) 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 7.0 at 37 °C]. The cells were permeabilized by incubation with saponin (10 μg/ml) for 5 min at 37 °C [20] and washed by centrifugation (650 ***g***, 4 min). The permeabilized cells were resuspended in CLM with a free [Ca^{2+}] of 186 nM (total [Ca^{2+}] 300 μM) and containing carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (10 μM) and $^{45}Ca^{2+}$ (10 μCi/ml). ATP (1.5 mM), creatine phosphate (5 mM) and creatine phosphokinase (5 units/ml) were then added to allow the active sequestration of $^{45}Ca^{2+}$ into the intracellular stores. Under these conditions, the cells rapidly ($t_{\frac{1}{2}} \approx 30$ s) accumulated $^{45}Ca^{2+}$ to reach a steady state (26 ± 1 pmol/10^6 cells, mean $\pm$ S.E.M., $n = 3$) within 10 min, which was then maintained for at least 40 min (results not shown). In all experiments, active $^{45}Ca^{2+}$ uptake was defined as that which could be released by ionomycin (10 μM). For experiments examining the reversibility of Ca^{2+} inhibition, cells were loaded under similar conditions but with the total [EGTA] decreased to 200 μM and the total [Ca^{2+}] to 60 μM (free [Ca^{2+}] 186 nM) to allow subsequent sequential changes in free [Ca^{2+}] without increasing the total EGTA concentration excessively.

The effects of InsP_3 on unidirectional $^{45}Ca^{2+}$ release were determined by loading cells with $^{45}Ca^{2+}$ for 15 min and then diluting them 1:1 into CLM containing an appropriate free [Ca^{2+}], InsP_3 and thapsigargin (1 μM) to inhibit the Ca^{2+} pumps of the endoplasmic reticulum. After appropriate intervals at 37 °C (2 min for concentration–effect relationships), the reactions were terminated by the rapid addition of ice-cold sucrose solution (310 mM) with sodium citrate (1 mM); the $^{45}Ca^{2+}$ contents of the stores were determined after filtration through GF/C filters using a Brandel receptor binding harvester [20].

Antibody methods

Full-length rat type 1 (lacking the S1 splice site) and type 3 InsP_3 receptors were expressed in *Sf*9 cells (*Sf*9/InsP_3R1 and *Sf*9/InsP_3R3 respectively), as described previously [10,12]. Infected cells were harvested 40–42 h after infection by centrifugation (1000 ***g*** for 5 min) at 2 °C, cell pellets were washed twice in PBS, resuspended in Ca^{2+}-free CLM supplemented with a protease-inhibitor cocktail (0.1 mM PMSF/10 μM leupeptin/1 mM benzamidine/0.1 mM soya-bean trypsin inhibitor/0.1 mM captopril) and homogenized [12]. The homogenate was centrifuged (3000 ***g*** for 10 min) and the membrane pellet was resuspended in Ca^{2+}-free CLM (4–6 mg/ml protein) before rapid freezing and storage at −80 °C. The same methods were used to prepare membranes from RINm5F cells. Protein concentrations were determined with the Bradford assay [21], with BSA as standard. Immunoblotting was performed as described previously [10] and immunoreactive bands were quantified by densitometric scanning (NIH Image, Bethesda, MD, U.S.A.).

Other methods

For each CLM, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined either fluorimetrically as described previously [22], or for CLM with higher $[Ca^{2+}]_i$ by means of a Ca^{2+} electrode calibrated with Ca^{2+} standard solutions from Molecular Probes. Briefly, for the fluorimetric measurements, fura-2 potassium salt (1 μM) was added to the CLM, and the fluorescence ratio determined at wavelengths appropriate for fura-2 and after correction for autofluorescence following addition of $MnCl_2$. $[Ca^{2+}]_i$ was then calculated [23] but with the K_D for Ca^{2+} at 37 °C corrected to take account of the $MgCl_2$ present in CLM (K_D^{Ca} 210 nM). In three separate determinations of $[Ca^{2+}]_i$ of each of the CLM, the S.E.M. for the determinations was less than 3% of the mean. Within the range of $[Ca^{2+}]_i$ in which the sensitivities of the two methods overlapped, the values determined by fura-2 and the Ca^{2+} electrode agreed closely (Table 1).

Analysis

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.), as described previously [20].

Materials

InsP_3 was from American Radiolabeled Chemicals (St Louis, MO, U.S.A.). $^{45}CaCl_2$ was from ICN (Thame, Oxon, U.K.). Ionomycin was from Calbiochem (Nottingham, U.K.) and thapsigargin was from Alamone Labs (Jerusalem, Israel). Cell culture materials, with the exception of serum (Sigma, Poole, Dorset, U.K.), were from Gibco (Paisley, Renfrewshire, U.K.). Fura-2 potassium salt and the Ca^{2+} calibration kits were from Molecular Probes (Leiden, The Netherlands). Other reagents were either from suppliers named in earlier publications [20] or from Sigma.

RESULTS AND DISCUSSION

Expression of InsP_3 receptor subtypes in RINm5F cells

Previous work ([8], but see [5,24]) had suggested that RINm5F cells express 96% type 3 InsP_3 receptors. We first sought to verify this by quantitative immunoblotting. Because type 2 receptors were undetectable in RINm5F cells (results not shown), we quantitatively examined the expression of only type 1 and type 3 InsP_3 receptors. Four parallel gels were loaded with a range of concentrations of membranes from *Sf*9/InsP_3R1, *Sf*9/InsP_3R3 and RINm5F cells and then immunoblotted with antipeptide antisera selective for type 1 (Ab1) or type 3 (Ab3) InsP_3 receptors, or with an antiserum (AbC) that interacted equally well with both subtypes [10] (Figure 1). Within the ranges used for these experiments, there was a linear relationship between the quantity of membranes loaded and the densitometric measurements of InsP_3 receptor bands (approx. 260 kDa) obtained with each antiserum (results not shown). Because infected *Sf*9 membranes express only a single InsP_3 receptor subtype [10], the degree of immunostaining with the subtype-selective antisera (Ab1, Ab3) relative to that obtained under identical conditions with AbC provides a conversion factor from units of staining with specific antiserum to units of AbC staining. The ratio (Ab1 to AbC) was

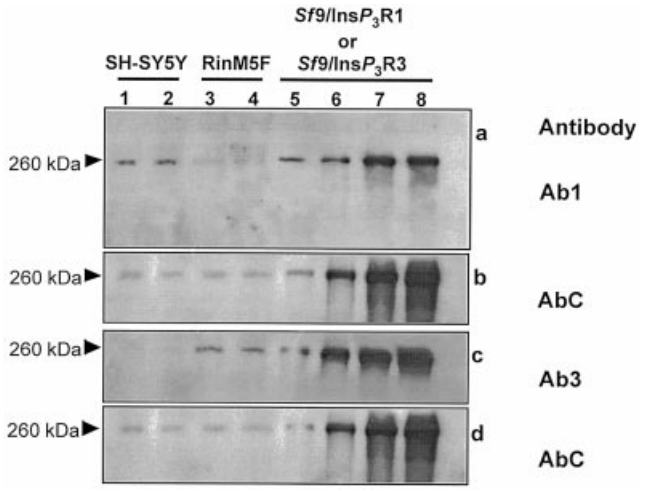

Figure 1 Quantitative analysis of the expression of type 1 and type 3 InsP_3 receptors in SH-SY5Y and RINm5F cells

Lanes were loaded with 300 μg of membranes prepared from SH-SY5Y cells (lanes 1 and 2), RINm5F cells (lanes 3 and 4) or 5, 10, 20 or 30 μg (lanes 5–8) of membranes from *Sf*9 cells expressing either type 1 (**a**, **b**) or type 3 (**c**, **d**) InsP_3 receptors. The blots were probed with each of the three antisera (Ab1, Ab3, AbC).

1.46 for *Sf*9/InsP_3R1 and 1.65 for *Sf*9/InsP_3R3 (Ab3 to AbC), allowing the densitometric measurements of type 1 and type 3 InsP_3 receptors (detected with Ab1 and Ab3) from RINm5F membranes to be converted into units of AbC staining. The results indicate that RINm5F cells express $17 \pm 1\%$ ($n = 6$) type 1 receptors and 77% type 3 receptors; parallel experiments with SH-SY5Y neuroblastoma cells (previously suggested to express 99% type 1 receptors [8]) indicate that $89 \pm 6\%$ ($n = 3$) of their InsP_3 receptors are type 1. The total calculated amounts of AbC staining (i.e. the sum of that derived from Ab1 and Ab3 staining) agrees closely with that measured directly after immunoblotting with AbC: 95% of the AbC staining was accounted for by the sum of Ab1 and Ab3 staining in RINm5F cells. We conclude that RINm5F cells express predominantly (77%) type 3 InsP_3 receptors, although the predominance of this subtype is less than previously reported with semi-quantitative methods [8].

Quantal responses to InsP_3 in RINm5F cells

In CLM with $[Ca^{2+}]_i$ buffered at 186 nM, a 2 min incubation with a maximally effective concentration of InsP_3 (10 μM) released $90 \pm 1\%$ of the intracellular Ca^{2+} stores; the EC_{50} was 82 ± 2 nM ($n = 4$) (Table 1). In many cells, the unidirectional $^{45}Ca^{2+}$ efflux evoked by submaximal concentrations of InsP_3 causes only partial emptying of the InsP_3-sensitive stores. Although the mechanisms underlying such quantal responses are not understood and might involve the all-or-nothing emptying of heterogeneous stores [25] or a form of receptor desensitization [26], the behaviour might be important in allowing the graded release of Ca^{2+} stores [27]. The Ca^{2+} signals recorded from intact RINm5F cells were reported to result from an essentially complete emptying of the intracellular stores [18], implying that individual cells were incapable of graded responses to either InsP_3 or agonists that stimulate InsP_3 formation. Because the quantal pattern of Ca^{2+} release might be an element of the mechanisms responsible for graded responses to InsP_3, we examined the kinetics of InsP_3-evoked Ca^{2+} release from permeabilized

Table 1 Effects of $[Ca^{2+}]_i$ on InsP_3-evoked Ca^{2+} release from permeabilized RINm5F cells

Experiments similar to those shown in Figure 3 were used to establish the EC_{50} and Hill coefficient for InsP_3-evoked Ca^{2+} release in CLM containing different $[Ca^{2+}]_i$. The final column shows the percentage of the Ca^{2+} stores released by 10 μM InsP_3 at each $[Ca^{2+}]_i$. Results are means $\pm$ S.E.M. for three to five experiments. The lower $[Ca^{2+}]_i$ were determined by using fura-2; the higher $[Ca^{2+}]_i$ (*) were measured with a Ca^{2+} electrode; where their sensitivity ranges overlapped, the methods yielded similar estimates of $[Ca^{2+}]_i$. Abbreviation: n.d., not determined.

Total $[Ca^{2+}]$ (μM)	$[Ca^{2+}]_i$ (M)	EC_{50} (nM)	Hill coefficient	Maximal Ca^{2+} release (%)
0	$\sim 4 \times 10^{-9}$	281 ± 15	2.33 ± 0.15	79 ± 5
150	72×10^{-9}	100 ± 3	2.26 ± 0.22	88 ± 1
300	186×10^{-9}	82 ± 2	2.33 ± 0.12	90 ± 1
650	869×10^{-9}	128 ± 11	1.75 ± 0.22	88 ± 1
685	1.03×10^{-6}	158 ± 32	1.79 ± 0.24	88 ± 2
	1.17×10^{-6}*			
720	1.27×10^{-6}	195 ± 29	1.49 ± 0.33	88 ± 3
	1.24×10^{-6}*			
750	1.56×10^{-6}	291 ± 33	1.99 ± 0.16	88 ± 2
	1.73×10^{-6}*			
800	2.40×10^{-6}	757 ± 57	1.92 ± 0.59	91 ± 1
	2.03×10^{-6}*			
850	3.0×10^{-6}*	n.d.	n.d.	78 ± 4
900	6.5×10^{-6}*	n.d.	n.d.	58 ± 6
950	15.4×10^{-6}*	n.d.	n.d.	44 ± 5
1100	$\sim 100 \times 10^{-6}$	n.d.	n.d.	22 ± 7
2000	$\sim 10^{-3}$	n.d.	n.d.	16 ± 5

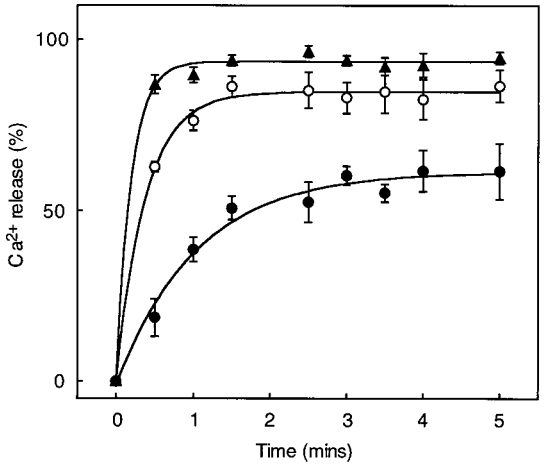

Figure 2 InsP_3 stimulates quantal Ca^{2+} release in RINm5F cells

Permeabilized cells loaded with $^{45}Ca^{2+}$ were diluted 1:1 into CLM ($[Ca^{2+}]_i$ 186 nM) at 37 °C containing thapsigargin (1 μM) and various concentrations of InsP_3: 60 nM (●), 100 nM (○) or 200 nM (▲). The incubations were terminated after the indicated intervals and the $^{45}Ca^{2+}$ released was expressed relative to that released by 10 μM InsP_3, which evoked its maximal effect within 30 s. Results are means $\pm$ S.E.M. for four independent experiments.

RIN5mF cells. After the addition of thapsigargin to cells loaded to steady state with $^{45}Ca^{2+}$, the $^{45}Ca^{2+}$ content of the stores declined approximately mono-exponentially with a $t_{\frac{1}{2}}$ of 266 ± 22 s ($n = 3$). The addition of a maximal concentration of InsP_3 released $85 \pm 3\%$ ($n = 4$) of the Ca^{2+} stores within 30 s. The responses to submaximal concentrations of InsP_3 (60–200 nM)

were slower; however, within 150 s each had exerted its full effect but released only a fraction of the InsP_3-sensitive stores (Figure 2). After 5 min, the fraction of the InsP_3-sensitive stores released by 60, 100 and 200 nM InsP_3 was $62 \pm 6\%$, $82 \pm 5\%$ and $94 \pm 2\%$ ($n = 4$) respectively. We conclude that in RINm5F cells, just as in many other cell types [28], InsP_3 stimulates quantal Ca^{2+} mobilization.

Biphasic effect of $[Ca^{2+}]_i$ on InsP_3-evoked Ca^{2+} release in RINm5F cells

The effects of varying $[Ca^{2+}]_i$ on the concentration–effect relationships for InsP_3 are shown in Figures 3A and 3B. As the $[Ca^{2+}]_i$ was increased from approx. 4 nM (nominally Ca^{2+}-free CLM) to 2.4 μM, the Ca^{2+} release evoked by a submaximal concentration of InsP_3 (200 nM) first increased and then decreased (Figure 3C) to give a characteristic biphasic effect of cytosolic Ca^{2+}. Stimulation by cytosolic Ca^{2+} resulted from a 3.4-fold decrease in the EC_{50} for InsP_3-evoked Ca^{2+} mobilization, as $[Ca^{2+}]_i$ increased from approx. 4 to 186 nM. Further increases in $[Ca^{2+}]_i$ caused an increase in EC_{50} such that at $[Ca^{2+}]_i > 3$ μM even a normally supramaximal concentration of InsP_3 (10 μM) failed to release all the InsP_3-sensitive Ca^{2+} stores (Table 1). Only $22 \pm 7\%$ of the Ca^{2+} stores were released by 10 μM InsP_3 when $[Ca^{2+}]_i$ was 100 μM but the response increased to $34 \pm 4\%$ and $39 \pm 2\%$ release ($n = 3$) when the InsP_3 concentration was increased to 100 and 300 μM respectively. From the $[Ca^{2+}]_i$ ($\leqslant 2.4$ μM) at which it was practicable to determine full concentration–effect relationships for InsP_3, there was no significant effect of Ca^{2+} on the Hill coefficient of the response (Table 1).

Because previous work had suggested that type 3 InsP_3 receptors were not inhibited by Ca^{2+} [18], we examined the inhibitory effect of cytosolic Ca^{2+} on RINm5F cells more closely. In CLM containing a $[Ca^{2+}]_i$ of 100 μM, a normally maximal concentration of InsP_3 (10 μM) stimulated the release of only $10 \pm 2\%$ ($n = 6$) of the intracellular stores; however, when the $[Ca^{2+}]_i$ was then restored to 186 nM for 60 s, the response to 10 μM InsP_3 (release of $77 \pm 3\%$ of the stores) was similar to that of cells treated similarly but bathed in CLM containing a $[Ca^{2+}]_i$ of 186 nM throughout ($86 \pm 2\%$) ($n = 6$). We conclude that the inhibition caused by high $[Ca^{2+}]_i$ is reversible and that it must therefore result from an inhibition of the receptor rather than a loss of Ca^{2+} from the intracellular stores, which (in the presence of thapsigargin) would have been unable to refill during the recovery period.

The media used for bilayer analysis of InsP_3 receptors from RIN-5F cells included ruthenium red [18] to inhibit ryanodine receptors, but ruthenium red binds to many Ca^{2+}-binding sites, including those on fusion proteins derived from InsP_3 receptors [29]. However, although ruthenium red (10 μM) caused a modest inhibition of responses to InsP_3, it did so across a range of $[Ca^{2+}]_i$ and did not prevent the inhibitory effect of high $[Ca^{2+}]_i$ (Table 2). The inclusion of ruthenium red (2 μM) in bilayer analyses is therefore unlikely to have caused the inhibitory effect of Ca^{2+} to be lost.

We conclude that the InsP_3 receptors of RINm5F cells are biphasically regulated by cytosolic Ca^{2+}: modest increases in $[Ca^{2+}]_i$ increase the sensitivity of the stores to InsP_3 (optimal at $[Ca^{2+}]_i \approx 200$ nM), whereas further increases, although still within a physiologically appropriate range, cause a massive and reversible decrease in sensitivity to InsP_3. Indeed, the biphasic effects of cytosolic Ca^{2+} on the responses of RINm5F and SH-SY5Y cells (which express predominantly type 1 InsP_3 receptors) to InsP_3 are barely distinguishable (Figure 3C, inset).

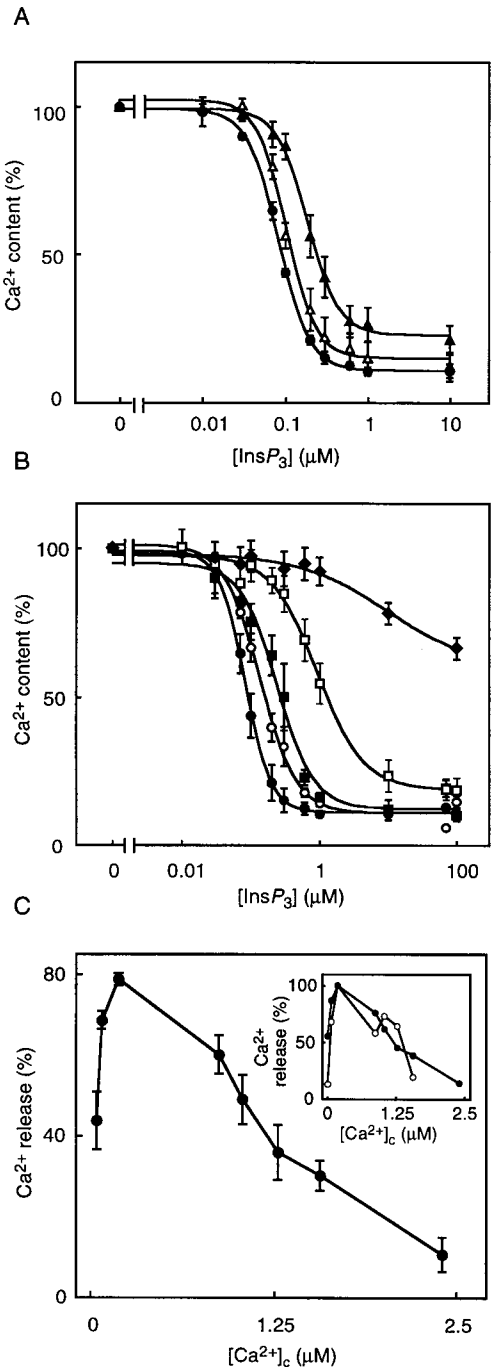

Figure 3 Biphasic regulation by $[Ca^{2+}]_i$ of InsP_3-evoked Ca^{2+} release from permeabilized RINm5F cells

(**A**, **B**) To establish InsP_3-concentration–effect relationships, permeabilized cells were incubated for 2 min with the indicated concentrations of InsP_3 in CLM with $[Ca^{2+}]_i$ of approx. 4 nM (▲), 72 nM (△), 186 nM (●), 869 nM (○), 1.27 μM (■), 2.4 μM (□) or 100 μM (◆). The results (means ± S.E.M., $n = 3$–5) show the Ca^{2+} remaining within the intracellular stores expressed as a percentage of the total actively accumulated $^{45}Ca^{2+}$ content of the stores of cells that had not been exposed to InsP_3. For clarity, responses to the lower $[Ca^{2+}]_i$ (**A**) are plotted separately from those to higher $[Ca^{2+}]_i$ (**B**) and the response in CLM containing 186 nM Ca^{2+} (●) is shown in both panels. (**C**) The effects of varying $[Ca^{2+}]_i$ ($[Ca^{2+}]_c$) on the amount of Ca^{2+} released (percentage of total intracellular Ca^{2+} stores) by a submaximal concentration of InsP_3 (200 nM) are shown as means ± S.E.M. for three to five independent experiments. The inset (from which error bars have been omitted for clarity) shows responses of SH-SY5Y cells (○) and RINm5F cells (●) to submaximal concentrations of InsP_3 (70 and 200 nM respectively for the two cell types) at each of the indicated $[Ca^{2+}]_i$. Responses (means for three to five experiments) show the Ca^{2+} released by the submaximal InsP_3 concentration expressed as percentages of the response to the same concentration of InsP_3 when $[Ca^{2+}]_i$ was 186 nM.

Conclusions

In keeping with results obtained from bilayer analyses of InsP_3 receptors from RIN-5F cells [18] and from type 3 InsP_3 receptors in DT40 cells [13], modest increases in $[Ca^{2+}]_i$ enhanced the ability of submaximal InsP_3 concentrations to cause Ca^{2+} mobilization from permeabilized RINm5F cells (Figure 3). The results are also consistent with those from recombinant type 3 InsP_3 receptors expressed in *Sf*9 cells, in which modest increases in $[Ca^{2+}]_i$ stimulate [^{3}H]InsP_3 binding [10,11], although the link between the effects of Ca^{2+} on [^{3}H]InsP_3 binding and channel function has yet to be established. However, whereas increasing $[Ca^{2+}]_i$ to 100 μM failed to inhibit RIN-5F InsP_3 receptors in bilayers, the receptors in permeabilized cells were most sensitive to InsP_3 when $[Ca^{2+}]_i$ was 186 nM and were then progressively less sensitive as the $[Ca^{2+}]$ was increased (Table 1). Increasing $[Ca^{2+}]_i$ to only 2.4 μM decreased the sensitivity of the receptors by almost 10-fold, and with 100 μM Ca^{2+} the decrease was at least 3000-fold. A preliminary report has also suggested that recombinant type 3 receptors expressed in the nuclear envelope of *Xenopus* oocytes are regulated biphasically by cytosolic Ca^{2+} and that inhibition occurs over a similar range of $[Ca^{2+}]_i$ for type 1 and type 3 InsP_3 receptors [30]. The disparity between our results and those obtained from receptors in bilayers [18] is not a consequence of the ruthenium red used in the latter experiments blocking a Ca^{2+}-inhibitory site (Table 2). A similar disparity exists for type 2 InsP_3 receptors: in several cell types expressing predominantly type 2 receptors, Ca^{2+} biphasically regulates InsP_3-evoked Ca^{2+} release [15], yet in bilayers cardiac (type 2) InsP_3 receptors are stimulated, but not inhibited, by cytosolic Ca^{2+} [31]. One possibility is therefore that an accessory protein mediates the inhibitory effects of cytosolic Ca^{2+} on type 2 and type 3 InsP_3 receptors and that it is lost when preparing receptors for bilayer recording. This interpretation would be consistent with recent work that suggests that the effects of Ca^{2+} on InsP_3 binding to type 2 InsP_3 receptors are mediated by an accessory protein [32].

Unfortunately, the relative levels of expression of type 1 and type 3 InsP_3 receptors in the RIN-5F cells used for bilayer recording were not quantified [18]. Our results suggest that 77% of the InsP_3 receptors expressed in RINm5 cells were type 3, the remainder being type 1 (Figure 1); it is therefore possible that most tetrameric receptors in RINm5F cells include a type 1 subunit. Indeed, a previous analysis of RINm5F cells failed to detect homomeric type 1 receptors but detected both homomeric and heteromeric type 3 receptors [5]. Therefore another possibility, which would be consistent with the rather similar effects of Ca^{2+} on RINm5F and SH-SY5Y cells (Figure 3C, inset), is that, within a heteromeric channel, the inhibitory effect of Ca^{2+} on a single type 1 receptor subunit dominates, even if the remaining three type 3 subunits are not directly inhibited by Ca^{2+}. The same explanation was proposed to account for the modest inhibition of InsP_3-induced Ca^{2+} release by Ca^{2+} in DT40 cells expressing type 1 and type 3 InsP_3 receptors [13]. However, in those experiments, the inhibition was substantially greater for type 1 receptors alone than for the co-expressed type 1 and type 3 receptors; it therefore seems equally likely that the effects of Ca^{2+} were simply the sum of their independent effects on the two receptor subtypes.

We conclude that in RINm5F cells, which express predominantly type 3 InsP_3 receptors, InsP_3 evokes quantal Ca^{2+} mobilization and that the effects of InsP_3 are regulated biphasically by cytosolic Ca^{2+}. We cannot yet resolve whether the inhibition by Ca^{2+} is a property of the type 3 subtype itself, or whether the inhibition of a single type 1 subunit within the tetrameric InsP_3 receptor is sufficient to ensure the inhibition of the entire receptor. It seems likely that, in most cells, InsP_3 receptors exist largely as heteromeric channels; either of the mechanisms proposed to mediate Ca^{2+} inhibition of type 3 receptors is therefore likely to ensure that in intact cells they, like other InsP_3 receptor subtypes, are biphasically regulated by cytosolic Ca^{2+}.

Table 2 Ruthenium red does not prevent the inhibitory effect of $[Ca^{2+}]_i$

The release of the intracellular Ca^{2+} stores (%) evoked by submaximal (200 nM) or maximal (10 μM) concentrations of InsP_3 was determined at the indicated $[Ca^{2+}]_i$ in either the absence or the presence of 10 μM Ruthenium red. Results are means ± S.E.M. for three independent experiments.

		Release of intracellular Ca^{2+} (%)	
$[Ca^{2+}]_i$ (μM)	InsP_3 concentration (μM)	Control	Ruthenium red
0.186	0.2	71 ± 2	57 ± 2
0.869	0.2	44 ± 5	27 ± 2
1.27	0.2	25 ± 10	21 ± 8
2.4	0.2	–1 ± 2	–1 ± 8
100	0.2	5 ± 3	4 ± 4
100	10	15 ± 2	12 ± 4

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REFERENCES

1 Sugawara, H., Kurosaki, M., Takata, M. and Kurosaki, T. (1997) EMBO J. **16**, 3078–3088
2 De Smedt, H., Missiaen, L., Parys, J. B., Henning, R. H., Sienaert, I., Vanlingen, S., Gijsens, A., Himpens, B. and Casteels, R. (1997) Biochem. J. **322**, 575–583
3 Patel, S., Joseph, S. K. and Thomas, A. P. (1999) Cell Calcium **25**, 247–264
4 Mikoshiba, K. (1997) Curr. Opin. Neurobiol. **7**, 339–345
5 Nucifora, F. C., Sharp, A. H., Milgram, S. L. and Ross, C. A. (1996) Mol. Biol. Cell **7**, 949–960
6 Nakagawa, T., Okano, H., Furuichi, T., Aruga, J. and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6244–6248
7 Joseph, S. K. (1996) Cell Signalling **8**, 1–7
8 Wojcikiewicz, R. J. H. (1995) J. Biol. Chem. **270**, 11678–11683
9 Wojcikiewicz, R. J. H. and Luo, S. G. (1998) Mol. Pharmacol. **53**, 656–662
10 Cardy, T. J. A., Traynor, D. and Taylor, C. W. (1997) Biochem. J. **328**, 785–793
11 Yoneshima, H., Miyawaki, A., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1997) Biochem. J. **322**, 591–596
12 Cardy, T. J. A. and Taylor, C. W. (1998) Biochem. J. **334**, 447–455
13 Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T. and Iino, M. (1999) EMBO J. **18**, 1303–1308
14 Iino, M. (1990) J. Gen. Physiol. **95**, 1103–1122
15 Taylor, C. W. (1998) Biochim. Biophys. Acta **1436**, 19–33
16 Berridge, M. J. (1997) J. Physiol. (London) **499.2**, 291–306
17 DeLisle, S., Blondel, O., Longo, F. J., Schnabel, W. E., Bell, G. I. and Welsh, M. J. (1996) Am. J. Physiol. **270**, C1255–C1261
18 Hagar, R. E., Burgstahler, A. D., Nathanson, M. H. and Ehrlich, B. E. (1998) Nature (London) **296**, 81–84
19 Missiaen, L., Parys, J. B., Sienaert, I., Maes, K., Kunzelmann, K., Takahashi, M., Tanzawa, K. and De Smet, H. (1998) J. Biol. Chem. **273**, 8983–8986
20 Marchant, J. S., Beecroft, M. D., Riley, A. M., Jenkins, D. J., Marwood, R. D., Taylor, C. W. and Potter, B. V. L. (1997) Biochemistry **36**, 12780–12790
21 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
22 Marshall, I. C. B. and Taylor, C. W. (1993) J. Biol. Chem. **268**, 13214–13220
23 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
24 De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van den Bosch, L. and Casteels, R. (1994) J. Biol. Chem. **269**, 21691–21698

25 Beecroft, M. D. and Taylor, C. W. (1997) Biochem. J. **326**, 215–220
26 Missiaen, L., Sipma, H., Parys, J. B., De Smet, P., Callewaert, G., Hill, E., McCarthy, T. V. and De Smedt, H. (1999) Pflügers Arch. **437**, 691–694
27 Taylor, C. W. (1995) Biochem. Soc. Trans. **23**, 637–642
28 Bootman, M. D. (1994) Curr. Biol. **4**, 169–172
29 Sienaert, I., Missiaen, L., De Smedt, H., Parys, J. B., Sipma, H. and Casteels, R. (1997) J. Biol. Chem. **272**, 25899–25906
30 Mak, D.-O., McBride, S., Yue, Y. and Foskett, J. K. (1999) Biophys. J. **76**, A375
31 Ramos-Franco, J., Fill, M. and Mignery, G. A. (1998) Biophys. J. **75**, 834–839
32 Picard, L., Coquil, J.-F. and Mauger, J.-P. (1998) Cell Calcium **23**, 339–348

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