# $Ca^{2+}$-calmodulin inhibits $Ca^{2+}$ release mediated by type-1, -2 and -3 inositol trisphosphate receptors

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$Ins P_3$ binding to type-1, but not type-3, $Ins P_3$ receptors is inhibited by calmodulin in a $Ca^{2+}$-independent fashion [Cardy and Taylor (1998) Biochem. J. **334**, 447–455], and $Ca^{2+}$ mobilization by type-1 $Ins P_3$ receptors of cerebellum is inhibited by calmodulin [Patel, Morris, Adkins, O'Beirne and Taylor (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 11627–11632]. Using cell types expressing predominantly type-1, -2 or -3 $Ins P_3$ receptors, we show that $Ins P_3$-evoked $Ca^{2+}$ mobilization from each is similarly inhibited by calmodulin. In SH-SY5Y cells, which express largely type-1 receptors, calmodulin ($IC_{50} \approx 15\ \mu M$) inhibited $Ins P_3$-evoked $Ca^{2+}$ release only in the presence of $Ca^{2+}$. The inhibition was unaffected by calcineurin inhibitors. The effect of calmodulin did not result from enhanced metabolism of $Ins P_3$ because calmodulin also decreased the sensitivity of the $Ca^{2+}$ stores to adenophostin A, a non-metabolizable $Ins P_3$-receptor agonist. Protein kinase A-catalysed phosphorylation of type-1 $Ins P_3$ receptors was unaffected by $Ca^{2+}$-calmodulin. Using a scintillation proximity assay to measure $^{125}$I-calmodulin binding to glutathione S-transferase-fusion proteins, we identified two regions of the type-1 $Ins P_3$ receptor (cyt1, residues −6 to 159; and cyt11, residues 1499–1649) that bound $^{125}$I-calmodulin. The higher-affinity site (cyt11) was also photoaffinity labelled with *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB)–calmodulin. We speculate that $Ca^{2+}$-independent binding of calmodulin to a site within the first 159 residues of the type-1 $Ins P_3$ receptor inhibits $Ins P_3$ binding and may thereby regulate the kinetics of $Ca^{2+}$ release. $Ca^{2+}$-dependent inhibition of $Ca^{2+}$ release by calmodulin is mediated by a different site: it may reside on an accessory protein that associates with all three receptor subtypes, or $Ca^{2+}$-calmodulin binding to a site lying between residues 1499 and 1649 of the type-1 receptor may inhibit $Ca^{2+}$ release from any tetrameric receptor that includes a type-1 subunit.

Key words: $Ca^{2+}$ regulation, $Ins P_3$-receptor subtype.

## INTRODUCTION

Calmodulin is a small, highly conserved $Ca^{2+}$-binding protein which acts as a $Ca^{2+}$-dependent regulator of many proteins, including enzymes, ion channels and cytoskeletal elements [2]. It is present in micromolar concentrations in all eukaryotic cells [3] and is especially abundant in brain [4]. Calmodulin is not only involved in decoding $Ca^{2+}$ signals, but also in regulating, both directly or via protein kinases and phosphatases, the many $Ca^{2+}$-transporting proteins that control the cytosolic $Ca^{2+}$ concentration. The latter targets include cyclic-nucleotide-gated [5] and voltage-gated [6,7] $Ca^{2+}$ channels, plasma-membrane $Ca^{2+}$ pumps [8], the *Drosophila* $Ca^{2+}$ channels encoded by the *trp* and *trpl* genes [9–11] and *N*-methyl-D-aspartate receptors [12].

Intracellular $Ca^{2+}$ channels are also regulated by calmodulin. Both ryanodine and $Ins P_3$ receptors are phosphorylated by $Ca^{2+}$-calmodulin-dependent protein kinase II and de-phosphorylated by calcineurin [13–18]. In addition, calmodulin, in both the absence and presence of $Ca^{2+}$, binds directly to both ryanodine [19,20] and $Ins P_3$ receptors [21–24]. $Ca^{2+}$-calmodulin binds to a short stretch of residues within the modulatory domain of the type-1 $Ins P_3$ receptor [22] and a similar sequence is present in type-2, but not type-3, $Ins P_3$ receptors. The functional consequences of calmodulin binding to this site are unknown. A second site, which is present in type-1, but appears not to be present in type-3, $Ins P_3$ receptors, binds calmodulin with similar affinity in both the absence and presence of $Ca^{2+}$; occupancy of this site inhibits $Ins P_3$ binding [23,24]. This calmodulin-binding site may be located within the N-terminal region of the type-1 receptor, because calmodulin, in both the presence and absence of $Ca^{2+}$, inhibits $Ins P_3$ binding to a protein that includes only the first 581 residues of the receptor [25]. Again, however, the functional consequences of calmodulin binding to this $Ca^{2+}$-independent site have not been established. We have speculated that it may inhibit $Ins P_3$-evoked $Ca^{2+}$ mobilization and thereby allow changes in free calmodulin concentration to modulate $Ins P_3$-receptor sensitivity [23]. In the present study, we directly test this speculation by examining whether the effects of calmodulin on inhibition of $Ins P_3$ binding ($Ca^{2+}$-independent and specific for type-1 receptors) match the effects of calmodulin on $Ins P_3$-evoked $Ca^{2+}$ mobilization.

## METHODS AND MATERIALS

### Cell culture and isolation of hepatocytes

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Bethesda, MD, U.S.A.; passages 28–35) were grown

Abbreviations used: CLM, cytosol-like medium; cyt1–18, fusion proteins derived from type-1 $Ins P_3$ receptor as defined in [1]; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoate; *Sf*9/$Ins P_3$R1, *Spodoptera frugiperda* cells expressing rat type-1 $Ins P_3$ receptor; SPA, scintillation proximity assay; GST, glutathione S-transferase; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid.

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at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum, 7 mM L-glutamine and 0.9 % non-essential amino acids in an atmosphere of 5 % $CO_2$ and 95 % air. Cells were passaged every 7 days when they were confluent. The same methods were used to culture RINm5F insulinoma cells (from Dr. P. Brown, Manchester University, Manchester, U.K.; passages 78–83), except for changing the medium to RPMI 1640 and reducing the concentration of foetal calf serum to 5 %. Rat hepatocytes were isolated by collagenase digestion and stored for up to 24 h at 4 °C in Eagle's minimal essential medium buffered with 26 mM $NaHCO_3$ [26].

### $^{45}Ca^{2+}$-flux measurements

SH-SY5Y and RINm5F cells were harvested by scraping into ice-cold medium containing 155 mM NaCl, 10 mM Hepes (pH 7.4) and 0.5 mM EGTA, and pelleted by centrifugation (650 ***g*** for 2 min). Cells were resuspended in $Ca^{2+}$-free medium (100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM Hepes, pH 7, at 37 °C) and permeabilized by incubation with saponin at 37 °C (10 $\mu$g/ml, 5 min). Permeabilized cells were centrifuged (650 ***g***, 2 min) and resuspended in a cytosol-like medium (CLM; 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, 240 $\mu$M EGTA, 64 $\mu$M $CaCl_2$, pH 7, at 20 °C) supplemented with ATP (1.5 mM), phosphocreatine (5 mM), creatine phosphokinase (1 unit/ml), carbonyl cyanide *p*-trifluormethoxyphenylhydrazone (FCCP; 10 $\mu$M) and $^{45}CaCl_2$ (8 $\mu$Ci/ml). The free [$Ca^{2+}$] of CLM, measured using fura 2, was 200 nM. The intracellular stores were allowed to actively accumulate $^{45}Ca^{2+}$ for 15 min at 20 °C before addition of Ins$P_3$ for a further 2 min. The $^{45}Ca^{2+}$ contents of the stores were determined by rapidly terminating the incubations with ice-cold sucrose (310 mM, pH 7) containing trisodium citrate (10 mM) followed by filtration through Whatman GF/C filters [27].

The effects of calmodulin on Ins$P_3$-evoked $Ca^{2+}$ release were first examined by including calmodulin throughout both the loading period and subsequent exposure to Ins$P_3$ (see Figure 1 and Table 1, below). In subsequent experiments examining the $Ca^{2+}$-dependence of the effects of calmodulin (see Table 2, below), permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ were diluted into CLM with or without calmodulin (10 $\mu$M) and with the free [$Ca^{2+}$] buffered by bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA; 5 mM) at either 200 nM or $\approx$ 4 nM (nominally $Ca^{2+}$-free); thapsigargin (1 $\mu$M) was also included to prevent further $Ca^{2+}$ uptake. After 5 min, Ins$P_3$ was added, and 2 min later the $^{45}Ca^{2+}$ contents of the stores were determined.

Permeabilized hepatocytes were actively loaded with $^{45}Ca^{2+}$ (5 min at 37 °C) in medium (140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Pipes, pH 7, 300 $\mu$M $CaCl_2$; free [$Ca^{2+}$] = 200 nM) as described previously [27]. Where indicated, calmodulin was included during this incubation and the effects of Ins$P_3$ were examined by adding it for 1 min before terminating the reactions as described above. The different temperatures for experiments with hepatocytes and cultured cells were required to optimize active $Ca^{2+}$ uptake for each cell type. $^{45}Ca^{2+}$ release from cerebellar microsomes was determined as described previously [23]. In all experiments, active $^{45}Ca^{2+}$ uptake is defined as that which was released by 10 $\mu$M ionomycin.

### Measurements of calmodulin binding

Scintillation proximity assays (SPAs) and photoaffinity labelling were used to identify calmodulin-binding sites within the type-1 Ins$P_3$ receptor.

For SPAs, Protein A-coated SPA beads (3 mg/ml; Amersham, Little Chalfont, Bucks, U.K.) were incubated (1 h, 2 °C) in binding medium (5 mM $KH_2PO_4$, 20 mM Hepes, 10 mg/ml BSA, 0.1 % Surfact-Amps X-100, pH 7.2) with a rabbit anti-glutathione S-transferase (GST) antibody (0.12 mg/ml; Molecular Probes, Leiden, The Netherlands). After centrifugation (6000 ***g***, 2 min at 2 °C), GST-fusion proteins (24 $\mu$g/ml) were coupled to the beads during a further incubation (2 h at 2 °C) in binding medium. The beads, now with GST-fusion protein attached to them via the antibody, were washed (6000 ***g***, 2 min at 2 °C) and resuspended (1.5 mg/ml) in binding medium supplemented with 100 $\mu$M BAPTA, 0.4 nM $^{125}$I-calmodulin and appropriate concentrations of unlabelled calmodulin; $Ca^{2+}$-containing medium also contained 300 $\mu$M $CaCl_2$. After 15 min at 2 °C, equilibrium binding of $^{125}$I-calmodulin was determined by counting vials at 2 °C in a Packard TriCarb 2200CA scintillation counter. The amount of each GST-fusion protein coupled to the SPA beads was quantified by boiling a sample of the beads in SDS sample buffer before SDS/PAGE and quantitative Western-blot analysis using a monoclonal anti-GST antibody (Sigma, Poole, Dorset).

For photoaffinity labelling, *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB)–calmodulin was prepared as described in [28]. Briefly, HSAB (20 $\mu$l, 10 mM in DMSO) was incubated with calmodulin (80 $\mu$l of 3.75 $\mu$M in 200 mM sodium borate, pH 8.5) in darkness for 1 h at room temperature. Excess HSAB was quenched by addition of 50 mM Tris/HCl (500 $\mu$l, pH 7.4) and separated from HSAB–calmodulin on a G-25 Sephadex column (Pharmacia, St Albans, Herts, U.K.). GST-fusion proteins (0.8 $\mu$g) were incubated (10 min on ice) with HSAB–calmodulin (235 nM) in 75 $\mu$l of 50 mM Tris (pH 7.4) containing 0.1 % Surfact-Amps X-100 and either 700 $\mu$M $CaCl_2$ or 7 mM EGTA. The incubations were then irradiated (254 nm, 20 min; Mineralight UVGL-58), and EGTA was added (final concentration, 6.25 mM) to the samples that contained $Ca^{2+}$ (to ensure that calmodulin migrated uniformly in SDS/PAGE). Western blotting was performed using a monoclonal antibody to calmodulin (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and a goat anti-mouse secondary antibody (Sigma); immunoreactive bands were detected using the Pierce Supersignal Ultra system and Hyperfilm (Amersham). The same methods applied to purified calcineurin (Upstate Biotechnology) successfully labelled $\approx$ 20 % of the 60-kDa subunit of the enzyme only when $Ca^{2+}$ was present and only after irradiation (results not shown).

GST-fusion proteins derived from the mouse type-1 Ins$P_3$ receptor were expressed in *Escherichia coli* as described previously [1]; the same nomenclature is used here to describe the fusion proteins (see Figure 3a, below). Mutagenesis of the cyt11 protein (cyt proteins are fusion proteins derived from type-1 Ins$P_3$ receptor, as defined in [1]) was performed using the Quick Change system according to the manufacturer's instructions (Strategene). The primers used were 5′-GATAACCGCGCGT-TCAGGG-3′ and 5′-CCCTGAACGCGCGGTTATC-3′, and the mutation was confirmed by automated sequencing. Methods for the expression of type-1 Ins$P_3$ receptors in Sf9 cells were described previously [29].

### Phosphorylation of cerebellar Ins$P_3$ receptors

Ins$P_3$ receptors were purified from rat cerebella as described previously [30] except that a protease-inhibitor cocktail [29] was present throughout. The purified protein bound Ins$P_3$ with high affinity ($K_d = 6.6 \pm 0.3$ nM, $n = 3$) and, after SDS/PAGE, silver-staining identified a single band ($\approx$ 260 kDa). For phosphorylation reactions, purified receptor (2.1 $\mu$g) was incubated at

**Table 1 Effects of calmodulin on Ins$P_3$-evoked $Ca^{2+}$ release from cells expressing predominantly type-1, -2 or -3 Ins$P_3$ receptors**

From experiments similar to those shown in Figure 1, the half-maximal effects ($EC_{50}$), Hill coefficients ($h$) and maximal effects of Ins$P_3$ (percentage of actively loaded $Ca^{2+}$ stores) in each of the three cell types were determined in the presence (+Cam) and absence (−Cam) of 10 $\mu$M calmodulin. Results are means ± S.E.M. of three independent experiments. Results from similar experiments with cerebellar microsomes (a single concentration–effect curve established by pooling results from 17 experiments) are shown for comparison. The major receptor subtype expressed in each cell type is shown in parentheses.

| | $EC_{50}$ (nM) | | $h$ | | Maximal response (%) | |
|---|---|---|---|---|---|---|
| | −Cam | +Cam | −Cam | +Cam | −Cam | +Cam |
| Cerebellar microsomes (1) | 44 | 263 | 1.5 | 1.5 | – | – |
| SH-SY5Y cells (1) | 90 ± 6 | 191 ± 6 | 1.7 ± 0.2 | 1.8 ± 0.2 | 66 ± 1 | 68 ± 4 |
| Hepatocytes (2) | 167 ± 1 | 260 ± 15 | 2.3 ± 0.1 | 2.5 ± 0.5 | 57 ± 2 | 55 ± 2 |
| RINm5F cells (3) | 84 ± 4 | 174 ± 27 | 1.9 ± 0.2 | 1.6 ± 0.3 | 80 ± 1 | 67 ± 4 |

20 °C in modified CLM (20 mM NaCl, 140 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM $KH_2PO_4$, 20 mM Hepes, pH 7, 0.1 % Surfact-Amps X-100, with or without 1.5 mM $CaCl_2$) containing catalytic subunit of protein kinase A (200 units/ml; Sigma), ATP (25 $\mu$M) and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci/ml). Reactions were stopped by boiling in SDS sample buffer and, after SDS/PAGE, the bands corresponding to Ins$P_3$ receptors were excised, dissolved in Emulsifier-Safe scintillation cocktail and their $^{32}$P contents determined. Parallel determination of the maximal number of [$^3$H]Ins$P_3$ binding sites ($B_{max}$) was used to define the stoichiometry of the phosphorylation ($^{32}$P/Ins$P_3$-binding site).

## Materials

Ins$P_3$ was from American Radiolabeled Chemicals (St Louis, MO, U.S.A.). $^{45}CaCl_2$ was from ICN (Thame, Oxon, U.K.), $^{125}$I-calmodulin (2280 Ci/mmol) was from Amersham and [$\gamma$-$^{32}$P]ATP (30 Ci/mmol) was from New England Nuclear (Stevenage, Herts, U.K.). ATP, creatine phosphate and creatine phosphokinase were from Boehringer (Lewes, East Sussex, U.K.). Calmodulin was purified from pig brain as described previously [31] and recombinant chicken calmodulin was from Alexis (Nottingham, U.K.): calmodulin from the two sources gave indistinguishable results and was used interchangeably. HSAB and Surfact-Amps X-100 were from Pierce and Warriner (Chester, U.K.). Cell-culture media were from Gibco-BRL (Paisley, Scotland, U.K.). All other reagents, including foetal calf serum, were from Sigma.

## RESULTS

### Inhibition of Ins$P_3$-evoked $Ca^{2+}$ release by calmodulin in cells expressing each of the Ins$P_3$-receptor subtypes

We confirmed previous work [23] by demonstrating that calmodulin (10 $\mu$M) inhibited [$^3$H]Ins$P_3$ (1 nM) binding to cerebellar microsomes by 36 ± 3 % ($n = 4$) and inhibited Ins$P_3$-evoked $Ca^{2+}$ release (Table 1). Subsequent experiments aimed to establish the relationship between these two effects of calmodulin. To allow comparison with other Ins$P_3$-receptor subtypes and assessment of the $Ca^{2+}$-dependence of the calmodulin effect (the rate of passive $^{45}Ca^{2+}$ leak is too fast to allow such studies in cerebellar microsomes), we extended the work to other cells.

SH-SY5Y cells, hepatocytes and RINm5F cells express predominantly type-1 (≈ 89 %) [32], type-2 (≈ 80 %) [33,34] and type-3 (≈ 77 %) [32] Ins$P_3$ receptors, respectively. In the absence of mammalian cells that express only a single Ins$P_3$-receptor subtype [34], these cells were chosen to represent the behaviour of each receptor subtype. In all three cell types, Ins$P_3$ caused a steeply (Hill coefficients, $h$, ≈ 2) concentration-dependent release of intracellular $Ca^{2+}$ stores (Table 1 and Figure 1a). Calmodulin (10 $\mu$M), present during both the loading period and the subsequent incubation with Ins$P_3$, significantly reduced the sensitivity of the intracellular stores to Ins$P_3$ in each cell type. In each case, the $EC_{50}$ for Ins$P_3$-evoked $Ca^{2+}$ mobilization increased by ≈ 2-fold, without affecting the response to a maximal concentration of Ins$P_3$ (Table 1). The 2-fold decrease in sensitivity is, however, less than the 6-fold decrease observed in cerebellar microsomes (Table 1) [23] and the ≈ 10-fold shift in A7r5 cells [35]. The apparent decrease in the maximal response to Ins$P_3$ in

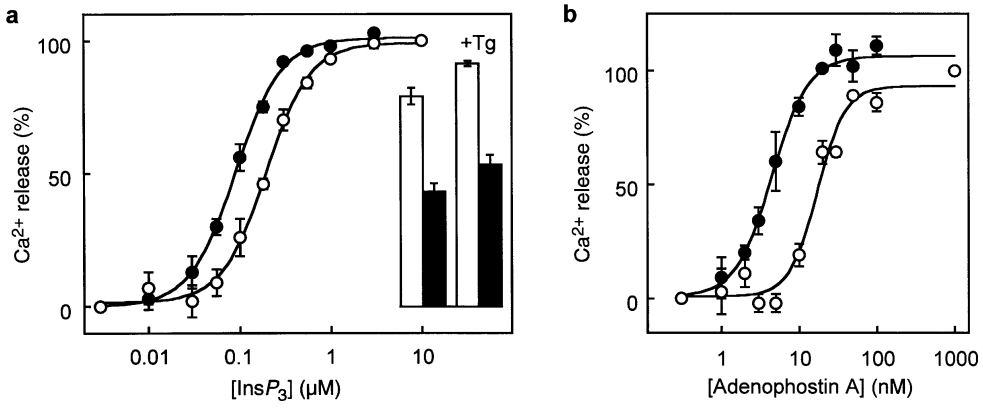


**Figure 1 Calmodulin inhibits Ins$P_3$- and adenophostin A-evoked $Ca^{2+}$ release from permeabilized SH-SY5Y cells**

Permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ at 20 °C in $Ca^{2+}$-containing CLM (free [$Ca^{2+}$], 200 nM) in either the absence (●) or presence (○) of 10 $\mu$M calmodulin, were then stimulated with the indicated concentrations of Ins$P_3$ (**a**) or adenophostin A (**b**) for 2 min. Results (means ± S.E.M. of three experiments) show the $Ca^{2+}$ released as percentages of that released by a maximal concentration (10 $\mu$M) of Ins$P_3$. The inset to (**a**) shows that calmodulin (10 $\mu$M, solid bars; open bars signify no calmodulin present) similarly inhibits the response to 200 nM Ins$P_3$ in the absence and presence of thapisgargin (Tg, 1 $\mu$M).

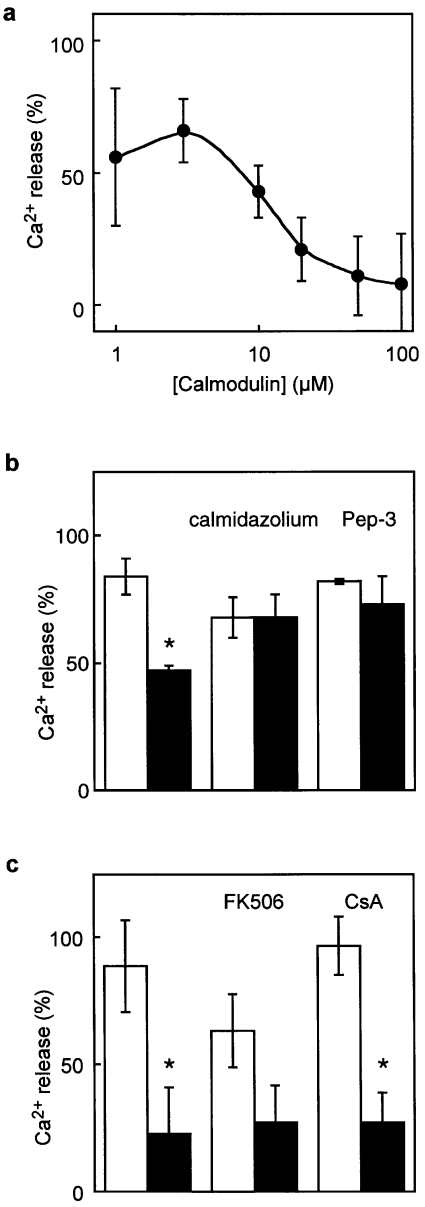


**Figure 2 Inhibition of Ins$P_3$-evoked $Ca^{2+}$ release from permeabilized SH-SY5Y cells by calmodulin**

(**a**) The $Ca^{2+}$ release evoked by a submaximal concentration (200 nM) of Ins$P_3$ is shown in the presence of each of the indicated concentrations of calmodulin in normal CLM (free [$Ca^{2+}$], 200 nM). (**b**) Cells loaded with $^{45}Ca^{2+}$ were added to thapsigargin (1 $\mu$M) with (solid bars) or without (open bars) 10 $\mu$M calmodulin and either calmidazolium (50 $\mu$M) or Pep-3 (20 $\mu$M). After 5 min, 200 nM Ins$P_3$ was added and 2 min later the $Ca^{2+}$ contents of the stores were determined. (**c**) The $Ca^{2+}$ release evoked by a submaximal concentration (200 nM) of Ins$P_3$ is shown in the presence (solid bars) or absence (open bars) of calmodulin (10 $\mu$M) under control conditions or in the presence of 300 nM FK506 or 1 $\mu$M cyclosporin A (CsA). For each panel, results (percentage release of Ins$P_3$-sensitive stores) are means $\pm$ S.E.M. of three experiments. In (**b**) and (**c**), * denotes a significant ($P < 0.05$) effect of calmodulin.

RINm5F cells resulted from calmodulin stimulating $Ca^{2+}$ accumulation into an Ins$P_3$-insensitive pool: the *total* amount of $^{45}Ca^{2+}$ released by 10 $\mu$M Ins$P_3$ was unaffected by calmodulin; it was $101 \pm 16\%$ ($n = 3$) of that in its absence. Calmodulin also inhibited Ins$P_3$-evoked $Ca^{2+}$ release when the effects of Ins$P_3$ were examined in the presence of thapsigargin (1 $\mu$M), indicating

**Table 2 $Ca^{2+}$-dependence of calmodulin inhibition of Ins$P_3$-evoked $Ca^{2+}$ mobilization**

SH-SY5Y cells were loaded with $^{45}Ca^{2+}$ in normal CLM before dilution into CLM containing thapsigargin (1 $\mu$M), either with or without 10 $\mu$M calmodulin, and in which the free [$Ca^{2+}$] was buffered with BAPTA (5 mM) at either $\approx$ 4 nM or 200 nM. After 5 min, Ins$P_3$ was added and 2 min later the $Ca^{2+}$ contents of the stores were determined and then expressed (means $\pm$ S.E.M. of three independent experiments) as percentages of the response to a maximal concentration (10 $\mu$M) of Ins$P_3$ in the same CLM without calmodulin. Because the stores are less sensitive to Ins$P_3$ in nominally $Ca^{2+}$-free medium, the submaximal concentration of Ins$P_3$ used when the free [$Ca^{2+}$] was $\approx$ 4 nM was higher (1 $\mu$M) than that used (400 nM) when the free [$Ca^{2+}$] was 200 nM. Because the inhibitory effect of calmodulin appears to be slow in onset, the lesser effect of calmodulin in these experiments probably reflects the shorter period of preincubation with calmodulin.

| | $\approx$ 4 nM free [$Ca^{2+}$] | | 200 nM free [$Ca^{2+}$] | |
|---|---|---|---|---|
| | Submaximal Ins$P_3$ | Maximal Ins$P_3$ | Submaximal Ins$P_3$ | Maximal Ins$P_3$ |
| Control | $81 \pm 3\%$ | 100% | $70 \pm 4\%$ | 100% |
| + Calmodulin | $78 \pm 2\%$ | $104 \pm 2\%$ | $56 \pm 1\%$* | $100 \pm 1\%$ |

* Significantly different ($P < 0.05$) from the results without calmodulin.

that the reduced response was not the result of calmodulin stimulating re-uptake of $Ca^{2+}$ (Figure 1a, inset).

## Characteristics of calmodulin inhibition of Ins$P_3$-evoked $Ca^{2+}$ release in SH-SY5Y cells

Previous work established that Ins$P_3$ binding to full-length recombinant type-1, but not type-3, Ins$P_3$ receptors was inhibited by calmodulin in a $Ca^{2+}$-independent fashion [23,24]. Our subsequent work therefore focused on SH-SY5Y cells because they express predominantly type-1 Ins$P_3$ receptors [32]. Figure 2(a) demonstrates that in normal CLM (free [$Ca^{2+}$], 200 nM), half-maximal inhibition of the response to a submaximal concentration of Ins$P_3$ (200 nM) occurred with a calmodulin concentration of $\approx$ 15 $\mu$M. Calmodulin ($\leqslant$ 100 $\mu$M) affected neither $Ca^{2+}$ uptake by SH-SY5Y cells nor their responses to a maximal concentration of Ins$P_3$ (results not shown).

Calmidazolium (50 $\mu$M) almost abolished active $^{45}Ca^{2+}$ uptake and a peptide antagonist (20 $\mu$M) derived from $Ca^{2+}$-calmodulin-dependent protein kinase II (Pep-3 in [24]) substantially reduced it; calmodulin antagonists could not therefore be used during the loading period to establish the specificity of the calmodulin effect. When either antagonist was added with calmodulin (10 $\mu$M) and thapsigargin (1 $\mu$M) to cells that had already been loaded with $^{45}Ca^{2+}$, both antagonists decreased the $Ca^{2+}$ content of the stores (suggesting that endogenous calmodulin may regulate the $Ca^{2+}$ leak), but more importantly the ability of calmodulin to inhibit the $Ca^{2+}$ release evoked by a submaximal concentration of Ins$P_3$ (200 nM) was abolished by the antagonists (Figure 2b). Further evidence that the effect of calmodulin is specific is provided by the indistinguishable inhibition of Ins$P_3$-evoked $Ca^{2+}$ release observed with recombinant chicken calmodulin and calmodulin purified from pig brain, suggesting that the inhibition is unlikely to result from contamination of the calmodulin preparations.

Cyclosporin A (1 $\mu$M), an inhibitor of calcineurin [36], had no effect on the inhibition of Ins$P_3$-evoked $Ca^{2+}$ release caused by 10 $\mu$M calmodulin, and whereas another calcineurin inhibitor, FK-506 (300 nM), itself inhibited Ins$P_3$-evoked $Ca^{2+}$ release, it

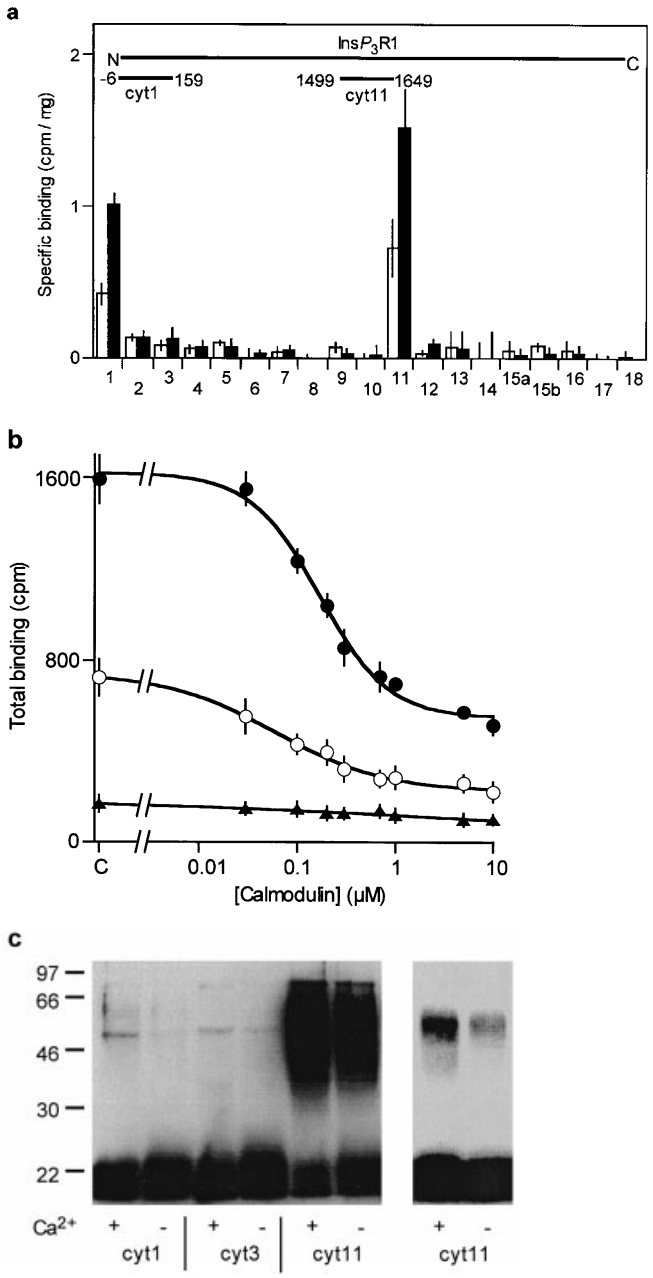


**Figure 3 Calmodulin binding to GST-fusion proteins derived from type-1 Ins$P_3$ receptors**

(**a**) Specific $^{125}$I-calmodulin binding to each of the indicated fusion proteins is shown expressed relative to the amount of GST-fusion protein coupled to the SPA beads (i.e. as c.p.m./mg of GST; see the Methods and materials section). The numbering of the fusion proteins is as reported previously [1], beginning with cyt1 at the extreme N-terminus and ending with cyt18 at the C-terminus. The residue numbers for cyt1 and cyt11 are also shown. (**b**) Equilibrium-competition binding of $^{125}$I-calmodulin to cyt11 is shown in the absence (○, Hill coefficient, $h = 0.81 \pm 0.03$) or presence (●, $h = 1.30 \pm 0.01$) of a free [$Ca^{2+}$] of 200 $\mu$M and the indicated concentrations of calmodulin. GST-syncollin, an 18-kDa secretory granule protein that does not bind calmodulin [42], provides a negative control (▲). Results show total binding (c.p.m.) as means ± S.E.M. of four independent experiments. The higher level of non-specific binding in the presence of $Ca^{2+}$ probably results from the increase in hydrophobicity of calmodulin as it binds $Ca^{2+}$. (**c**) The three fusion proteins (0.8 $\mu$g/lane) shown (each with a molecular mass of ≈ 40 kDa; the molecular-mass markers are shown on the left in kDa) were incubated with 1 $\mu$M HSAB–calmodulin in the presence or absence of $Ca^{2+}$, exposed to UV light, and the cross-linked proteins detected by Western blotting with an anti-calmodulin antibody. The small panel (right-hand side) shows results from another gel that was under-exposed to show more clearly the effect of $Ca^{2+}$ on calmodulin binding to cyt11. The results, indicating that only cyt11 is detectably labelled in either the presence or absence of $Ca^{2+}$, are typical of three experiments.

**Table 3 $^{125}$I-Calmodulin binding to GST-fusion proteins derived from type-1 Ins$P_3$ receptors**

Equilibrium-competition binding experiments similar to those shown in Figure 3(b) were used to define, using SPA, the $IC_{50}$ for $^{125}$I-calmodulin binding to two GST-fusion proteins from the type-1 Ins$P_3$ receptor in the absence and presence of $Ca^{2+}$. Results are means ± S.E.M. of five (cyt1) or seven (cyt11) independent experiments.

| Free [$Ca^{2+}$] | cyt1 | cyt11 |
|---|---|---|
| ≈ 4 nM | 0.76 ± 0.18 $\mu$M | 48 ± 11 nM |
| 200 $\mu$M | 1.42 ± 0.72 $\mu$M | 172 ± 37 nM |

too failed to prevent further inhibition by calmodulin (Figure 2c). The effect of calmodulin did not result from enhanced metabolism of Ins$P_3$, because calmodulin (10 $\mu$M) also reduced the sensitivity of SH-SY5Y cells to adenophostin A, an agonist of Ins$P_3$ receptors that cannot be metabolized [37] (Figure 1b). Indeed, the decrease in sensitivity caused by calmodulin was slightly greater for adenophostin A (3.6-fold; $EC_{50}$ increased from 4.7 ± 3 to 17 ± 2 nM) than for Ins$P_3$ (2.1-fold; $EC_{50}$ increased from 90 ± 6 to 191 ± 6 nM).

The effect of calmodulin was relatively slow in onset. When present during the loading of cerebellar microsomes with $^{45}Ca^{2+}$, calmodulin (10 $\mu$M) inhibited the response to a submaximal concentration of Ins$P_3$ (60 nM) by 91 ± 11 % when included for 5 min, but by only 25 ± 5 % when included for 1 min (results not shown). In the unidirectional $^{45}Ca^{2+}$-efflux experiments required to assess the $Ca^{2+}$-dependence of the calmodulin effect (see the Methods and materials section), it is therefore difficult to both retain sufficient $Ca^{2+}$ within the stores and allow sufficient time for the maximal effect of calmodulin. Nevertheless, Table 2 demonstrates that under these conditions in SH-SY5Y cells, calmodulin inhibited responses to Ins$P_3$, but only in the presence of $Ca^{2+}$.

### Binding of calmodulin to type-1 Ins$P_3$ receptors

Our previous work [24] established that $^{125}$I-calmodulin bound to two distinct low-affinity sites on membranes prepared from Sf9 cells expressing type-1 Ins$P_3$ receptors: a $Ca^{2+}$-dependent site and a $Ca^{2+}$-independent site. Attempts to identify these sites by photoaffinity-labelling membranes of *Spodoptera frugiperda* cells expressing rat type-1 Ins$P_3$ receptor (Sf9/Ins$P_3$R1) with HSAB–calmodulin (10 $\mu$M) were unsuccessful because there was too much non-specific binding (results not shown). However, using a SPA to measure specific $^{125}$I-calmodulin binding to GST-fusion proteins (cyt1–18) representing the full length of the type-1 Ins$P_3$ receptor [1], we identified specific $^{125}$I-calmodulin binding to two fusion proteins (Figure 3a). Both cyt1 (residues −6 to 159) and cyt11 (residues 1499–1649) bound $^{125}$I-calmodulin in the absence of $Ca^{2+}$, and for both fusion proteins the specific binding was increased in the presence of 200 $\mu$M free [$Ca^{2+}$]. From equilibrium-competition binding studies (Figure 3b), the affinities of the calmodulin-binding sites in cyt1 were significantly lower ($K_d$ ≈ 1 $\mu$M) than those in the cyt11 fragment ($K_d$ ≈ 100 nM; Table 3); the former more closely approximate the affinities of the calmodulin-binding sites previously detected by $^{125}$I-calmodulin binding to Sf9/Ins$P_3$R1 membranes ($K_d$ ≈ 1 $\mu$M) [24].

Photoaffinity labelling of the same GST-fusion proteins clearly identified HSAB–calmodulin binding to cyt11 in both the absence

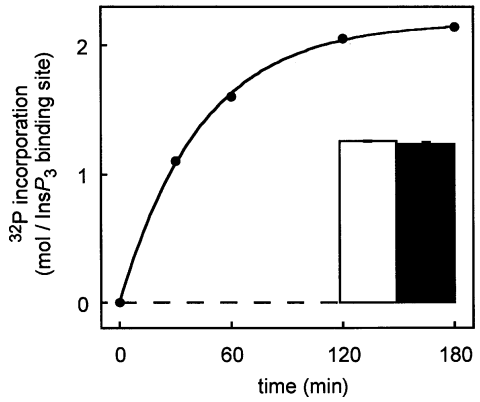


**Figure 4 Calmodulin does not affect protein kinase A-catalysed phosphorylation of type-1 Ins$P_3$ receptors**

Purified cerebellar Ins$P_3$ receptors were incubated with the catalytic subunit of protein kinase A and [$\gamma$-$^{32}$P]ATP for the indicated periods in $Ca^{2+}$-free CLM. The results (means of duplicate determinations from experiments repeated twice with similar results) show the stoichiometry of the $^{32}$P/Ins$P_3$-binding site (mol/mol). The histogram (means $\pm$ S.E.M., $n = 3$; the error bars are barely visible) shows the results obtained after incubation for 30 min in either the presence (open bar) or absence (solid bar) of 10 $\mu$M calmodulin with 500 $\mu$M free $Ca^{2+}$ present throughout.

and presence of $Ca^{2+}$, but failed to detect binding to the lower-affinity sites in cyt1 (Figure 3c). Cyt11 includes the sequence (1564–1585) to which Yamada et al. [22] attributed all $Ca^{2+}$-calmodulin binding in the type-1 Ins$P_3$ receptor, and mutation of $Trp^{1576}$ to Ala within this sequence abolished $Ca^{2+}$-calmodulin binding [22]. We have also confirmed that a peptide corresponding to residues 1564–1585 of the type-1 receptor binds $Ca^{2+}$-calmodulin, but loses that ability after mutation of $Trp^{1576}$ [24]. However, while the analagous mutation ($Trp^{1576}$ to Ala) in cyt11 reduced specific $^{125}$I-calmodulin binding by $63 \pm 9\,\%$ in the presence of $Ca^{2+}$, it had a similar effect in the absence of $Ca^{2+}$ (a reduction of $67 \pm 18\,\%$).

### Calmodulin and phosphorylation of type-1 Ins$P_3$ receptors by protein kinase A

The $Ca^{2+}$-calmodulin-binding site within the type-1 Ins$P_3$ receptor [22] is only a few residues away from a residue ($Ser^{1588}$) that is phosphorylated by protein kinase A. We therefore examined whether calmodulin affected phosphorylation of type-1 Ins$P_3$ receptors. In our experiments, half-maximal phosphorylation of purified cerebellar Ins$P_3$ receptors by protein kinase A occurred after 30 min (Figure 4). Calmodulin (10 $\mu$M) in the presence of a free [$Ca^{2+}$] of 500 $\mu$M (to ensure saturation of the calmodulin with $Ca^{2+}$) had no effect on the protein kinase A-catalysed phosphorylation.

## DISCUSSION

$Ca^{2+}$-independent binding of calmodulin to both purified and native cerebellar Ins$P_3$ receptors (type 1) and to full-length recombinant type-1, but not type-3, Ins$P_3$ receptors was shown previously to inhibit Ins$P_3$ binding [23,24]. Because calmodulin also inhibits Ins$P_3$-evoked $Ca^{2+}$ mobilization from cerebellar microsomes [23] (Table 1), we suggested previously that $Ca^{2+}$-independent calmodulin binding might inhibit $Ca^{2+}$ release by inhibiting Ins$P_3$ binding. The present results suggest that the interactions between calmodulin and Ins$P_3$ receptors are more complex.

First, in cells expressing predominantly type-1 (SH-SY5Y cells), type-2 (hepatocytes) or type-3 (RINm5F cells) Ins$P_3$ receptors, Ins$P_3$-evoked $Ca^{2+}$ release was equally susceptible to inhibition by calmodulin (Table 1), although for each, the extent of the inhibition was less than that observed with cerebellar microsomes (Table 1) or A7r5 cells [35]. Secondly, in SH-SY5Y cells, calmodulin inhibited Ins$P_3$-evoked $Ca^{2+}$ release only in the presence of $Ca^{2+}$ (Table 2). The latter is consistent with a recent study of A7r5 cells, which express Ins$P_3$ receptors types 1 and 3, in which calmodulin inhibited Ins$P_3$-evoked $Ca^{2+}$ release only when the free [$Ca^{2+}$] exceeded 300 nM [35]. We conclude that the $Ca^{2+}$-independent binding of calmodulin that inhibits equilibrium binding of Ins$P_3$ to type-1 receptors [24,25] does not, as we previously supposed [23], underlie the inhibitory effect of calmodulin on Ins$P_3$-evoked $Ca^{2+}$ mobilization.

How then does the $Ca^{2+}$-independent effect of calmodulin on equilibrium binding of Ins$P_3$ manifest itself on Ins$P_3$-evoked $Ca^{2+}$ mobilization, and how is the $Ca^{2+}$-dependent inhibition of $Ca^{2+}$ mobilization by calmodulin exercised?

A single $Ca^{2+}$-independent calmodulin-binding site [24] located within the first 581 residues of the type-1 Ins$P_3$ receptor appears to mediate the effects of calmodulin on Ins$P_3$ binding [25]. The present work suggests that this site probably lies within the first 159 residues (i.e. cyt1; Figure 3a), which is just outside the Ins$P_3$-binding core (residues 226–576) [38,39] and is therefore consistent with previous work demonstrating that $Ca^{2+}$-independent calmodulin binding is not prevented by Ins$P_3$ [23]. We speculated previously [23] that $Ca^{2+}$-independent calmodulin binding might inhibit Ins$P_3$-evoked $Ca^{2+}$ mobilization, but both the present work (Table 2) and earlier work with A7r5 cells [35] found no effect of calmodulin on the extent of the $Ca^{2+}$ mobilization evoked by Ins$P_3$ in the absence of cytosolic $Ca^{2+}$. While our work was under review, Michikawa et al. [40] also reported that cerebellar Ins$P_3$ receptors were inhibited by calmodulin only in the presence of $Ca^{2+}$. An alternative possibility is suggested by the observation that Ins$P_3$ binding to its receptor is followed rapidly by a change in the receptor to a state that binds Ins$P_3$ with increased affinity but which is less capable of mediating $Ca^{2+}$ release; a partially inactivated, high-affinity state [41]. $Ca^{2+}$-independent calmodulin binding might therefore decrease Ins$P_3$ binding by favouring the low-affinity *active* conformation and would thereby *increase* the initial rate of $Ca^{2+}$ release, possibly without appreciably affecting its final extent. High-resolution measurements of the kinetics of Ins$P_3$-evoked $Ca^{2+}$ release will be required to test this speculation directly.

We can be less certain of the site through which $Ca^{2+}$-calmodulin inhibits Ins$P_3$-receptor function (Figure 1): it may reside within the receptor itself or on an accessory protein. ATP is not required for calmodulin inhibition of Ins$P_3$-evoked $Ca^{2+}$ release [35], calcineurin is not involved (Figure 2b), and nor does calmodulin influence phosphorylation of the type-1 receptor by protein kinase A (Figure 4). It seems unlikely, therefore, that calmodulin mediates its effects via either phosphorylation or dephosphorylation of the Ins$P_3$ receptor. An attractive possibility is that the $Ca^{2+}$-dependent calmodulin-binding site within cyt11 (Figure 3) mediates the functional effects of calmodulin, but this explanation is not easily reconciled with all the experimental evidence. First, the short sequence ($Lys^{1564}$–$His^{1585}$) within cyt11 to which Yamada et al. [22] first mapped the $Ca^{2+}$-calmodulin binding site is present in receptor types 1 and 2, but absent from type-3 receptors, yet calmodulin inhibits Ins$P_3$-evoked $Ca^{2+}$ release from cells expressing predominantly type-1, -2 or -3 Ins$P_3$

receptors (Table 1). Whereas the peptide corresponding to the site identified by Yamada et al. plainly binds $Ca^{2+}$-calmodulin [22,24], our results suggest that these residues may not provide the entire site to which $Ca^{2+}$-calmodulin binds in type-1 receptors, because the mutation ($Trp^{1576}$ to Ala) which abolished calmodulin binding to the peptide [22] only partially reduced $^{125}$I-calmodulin binding to cyt11. It seems possible that the methods used in the original study (adherence to a calmodulin–Sepharose column) may have detected only high-affinity calmodulin-binding sites and may therefore have attributed a substantial decrease in calmodulin binding (as occurred in our mutant cyt11 protein) to a complete loss of the site. In summary, it remains possible that the determinants of $Ca^{2+}$-calmodulin binding, while including the region identified by Yamada et al. [22], may also involve additional residues within cyt11 that may be expressed in other subtypes. Even that explanation, however, is difficult to reconcile with our inability to detect specific $^{125}$I-calmodulin binding to recombinant full-length type-3 $InsP_3$ receptors in either the presence or absence of $Ca^{2+}$ [24]. An alternative possibility is that because the cells we used express only $\approx 80\,\%$ of the predominant receptor subtype (the remainder being largely type 1), each tetrameric receptor could conceivably include a type-1 subunit and if its binding of $Ca^{2+}$-calmodulin were to cause inhibition of the entire channel complex then the functional effects of $Ca^{2+}$-calmodulin might be mediated entirely via binding to a site within cyt11, but present only in type-1 (and possibly type-2) $InsP_3$ receptors.

We suggest that $Ca^{2+}$-independent binding of calmodulin to a site within the first 159 residues of the type-1 $InsP_3$ receptor inhibits $InsP_3$ binding and may thereby regulate the kinetics of $Ca^{2+}$ release; it is not, however, the means whereby calmodulin inhibits $InsP_3$-evoked $Ca^{2+}$ release. Only $Ca^{2+}$-calmodulin [35,40] (Table 2) has so far been shown to inhibit $InsP_3$-evoked $Ca^{2+}$ release. The site to which $Ca^{2+}$-calmodulin binds to cause inhibition may reside on an accessory protein that associates with all three receptor subtypes, or $Ca^{2+}$-calmodulin binding to a site between residues 1499 and 1649 of the type-1 receptor may inhibit $Ca^{2+}$ release from any tetrameric receptor that includes a type-1 subunit.

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