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

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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-t-aspartate receptors [12].

Intracellular Ca^{2+} channels are also regulated by calmodulin. Both ryanodine and InsP_{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 InsP_{3} receptors [21–24]. Ca^{2+}-calmodulin binds to a short stretch of residues within the modulatory domain of the type-1 InsP_{3} receptor [22] and a similar sequence is present in type-2, but not type-3, InsP_{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, InsP_{3} receptors, binds calmodulin with similar affinity in both the absence and presence of Ca^{2+}; occupancy of this site inhibits InsP_{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 InsP_{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 InsP_{3}-evoked Ca^{2+} mobilization and thereby allow changes in free calmodulin concentration to modulate InsP_{3}-receptor sensitivity [23]. In the present study, we directly test this speculation by examining whether the effects of calmodulin on inhibition of InsP_{3} binding (Ca^{2+}-independent and specific for type-1 receptors) match the effects of calmodulin on InsP_{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...
at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 7 mM t-glutamine and 0.9% non-essential amino acids in an atmosphere of 5% CO₂ 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 (from Dr. P. Brown, Manchester University, Manchester, U.K.; cells were passaged every 7 days when they were confluent. The 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 NaHCO₃ [26].

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 KH₂PO₄, 20 mM Hapes, 10 mg/ml BSA, 0.1% Surfact-Amps X-100, pH 7.2) with a rabbit anti-antithiogulutathione 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 μg/ml) were coupled to the beads for 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 μM BAPTA, 0.4 nM 125I-calmodulin and appropriate concentrations of unlabelled calmodulin; Ca²⁺-containing medium also contained 300 μM CaCl₂. After 15 min at 2 °C, equilibrium binding of 125I-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-azido-benzoate (HSAB)–calmodulin was prepared as described in [28]. Briefly, HSAB (20 μl, 10 mM in DMSO) was incubated with calmodulin (80 μl of 3.75 μM 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 μl, 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 μg) were incubated (10 min on ice) with HSAB–calmodulin (235 nM) in 75 μl of 50 mM Tris (pH 7.4) containing 10% Surfact-Amps X-100 and either 700 μM CaCl₂ or 7 mM EGTA. The incubations were then irradiated (254 nm, 20 min; Merilalight UVGL-58), and EGTA was added (final concentration, 6.25 mM) to the samples that contained Ca²⁺ (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 70% of the 60-kDa subunit of the enzyme only when Ca²⁺ was present and only after irradiation (results not shown).

GST-fusion proteins derived from the mouse type-1 InsP₃ 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 InsP₃ receptor, as defined in [1]) was performed using the Quick Change system according to the manufacturer’s instructions (Strategene). The primers used were 5’-GATAACCGCGCCTGTCAGGG-3’ and 5’-CCCTGAACGCGCGTATC-3’, and the mutation was confirmed by automated sequencing. Methods for the expression of type-1 InsP₃ receptors in SF9 cells were described previously [29].

**Phosphorylation of cerebellar InsP₃ receptors**

InsP₃ receptors were purified from rat cerebella as described previously [30] except that a protease-inhibitor cocktail [29] was present throughout. The purified protein bound InsP₃ with high affinity (Kᵦ = 6.6 ± 0.3 nM, n = 3) and, after SDS/PAGE, silver-staining identified a single band (~260 kDa). For phosphorylation reactions, purified receptor (2.1 μg) was incubated at

**Measurements of calmodulin binding**

Scintillation proximity assays (SPAs) and photoaffinity labelling were used to identify calmodulin-binding sites within the type-1 InsP₃ receptor.

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20 °C in modified CLM (20 mM NaCl, 140 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 5 mM KH₂PO₄, 20 mM Hepes, pH 7.0, 1% v/v Surfact-Amps X-100, with or without 1.5 mM CaCl₂) containing catalytic subunit of protein kinase A (200 units/ml; Sigma), ATP (25 μM) and [32P]ATP (10 μCi/ml). Reactions were started by boiling in SDS sample buffer and, after SDS/PAGE, the bands corresponding to Ins₃P binding sites (B₃₅0) was used to define the stoichiometry of the phosphorylation (32P/Ins₃P-binding site).

Materials

Ins₃P was from American Radiolabeled Chemicals (St Louis, MO, U.S.A.). 46CaCl₂ was from ICN (Thame, Oxon, U.K.). 125I-calmodulin (2280 Ci/mmol) was from Amersham and [γ-32P]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 Ins₃P-evoked Ca²⁺ release by calmodulin in cells expressing each of the Ins₃P-receptor subtypes

We confirmed previous work [23] by demonstrating that calmodulin (10 μM) inhibited [3H]Ins₃P (1 nM) binding to cerebellar microsomes by 36 ± 3 % (n = 4) and inhibited Ins₃P-evoked Ca²⁺ release (Table 1). Subsequent experiments aimed to establish the relationship between these two effects of calmodulin. To allow comparison with other Ins₃P-receptor subtypes and assessment of the Ca²⁺-dependence of the calmodulin effect (the rate of passive 45Ca²⁺ 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] Ins₃P receptors, respectively. In the absence of mammalian cells that express only a single Ins₃P-receptor subtype [34], these cells were chosen to represent the behaviour of each receptor subtype. In all three cell types, Ins₃P caused a steeply (Hill coefficients, h, ≈ 2) concentration-dependent release of intracellular Ca²⁺ stores (Table 1 and Figure 1a). Calmodulin (10 μM), present during both the loading period and the subsequent incubation with Ins₃P, significantly reduced the sensitivity of the intracellular stores to Ins₃P in each cell type. In each case, the EC₅₀ for Ins₃P-evoked Ca²⁺ mobilization increased by ≈ 2-fold, without affecting the response to a maximal concentration of Ins₃P (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 Ins₃P in

![Figure 1](https://example.com/1.png)

**Figure 1** Calmodulin inhibits Ins₃P₂ and adenophostin A-evoked Ca²⁺ release from permeabilized SH-SY5Y cells

Permeabilized cells loaded to steady state with 45Ca²⁺ at 20 °C in Ca²⁺-containing CLM (free [Ca²⁺]; 200 nM) in either the absence (○) or presence (●) of 10 μM calmodulin, were then stimulated with the indicated concentrations of Ins₃P (a) or adenophostin A (b) for 2 min. Results (means ± S.E.M. of three experiments) show the Ca²⁺ released as percentages of that released by a maximal concentration (10 μM) of Ins₃P. The inset to (a) shows that calmodulin (10 μM, solid bars; open bars signify no calmodulin present) similarly inhibits the response to 200 nM Ins₃P in the absence and presence of thapsigargin (Tg, 1 μM).
RINm5F cells resulted from calmodulin stimulating Ca\(^{2+}\) accumulation into an Ins\(_P_2\)-insensitive pool: the total amount of \(^{45}\)Ca\(^{2+}\) released by 10 \(\mu\)M Ins\(_P_2\) was unaffected by calmodulin; it was 101 ± 16 \%, \((n = 3)\) of that in its absence. Calmodulin also inhibited Ins\(_P_2\)-evoked Ca\(^{2+}\) release when the effects of Ins\(_P_2\) were examined in the presence of thapsigargin (1 \(\mu\)M), indicating that the reduced response was not the result of calmodulin stimulating re-uptake of Ca\(^{2+}\) (Figure 1a, inset).

**Table 2** Ca\(^{2+}\)-dependence of calmodulin inhibition of Ins\(_P_2\)-evoked Ca\(^{2+}\) mobilization

SH-SYSY 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 nM) at either ≈ 4 nM or 200 nM. After 5 min, Ins\(_P_2\) was added and 2 min later the Ca\(^{2+}\) contents of the stores were determined and then expressed (means ± S.E.M. of three independent experiments) as percentages of the response to a maximal concentration (10 \(\mu\)M) of Ins\(_P_2\) in the same CLM without calmodulin. Because the stores are less sensitive to Ins\(_P_2\) in nominally Ca\(^{2+}\)-free medium, the submaximal concentration of Ins\(_P_2\) used when the free [Ca\(^{2+}\)] was ≈ 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.

<table>
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<tr>
<th></th>
<th>Submaximal Ins(_P_2)</th>
<th>Maximal Ins(_P_2)</th>
<th>Submaximal Ins(_P_2)</th>
<th>Maximal Ins(_P_2)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>81 ± 3%</td>
<td>100%</td>
<td>70 ± 4%</td>
<td>100%</td>
</tr>
<tr>
<td>+ Calmodulin</td>
<td>78 ± 2%</td>
<td>104 ± 2%</td>
<td>56 ± 1%*</td>
<td>100 ± 1%</td>
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* Significantly different \((P < 0.05)\) from the results without calmodulin.

**Characteristics of calmodulin inhibition of Ins\(_P_3\)-evoked Ca\(^{2+}\) release in SH-SYSY 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-SYSY 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), halffmaximal inhibition of the response to a submaximal concentration of Ins\(_P_3\) (200 nM) occurred with a calmodulin concentration of ≈ 15 \(\mu\)M. Calmodulin \((< 100 \mu\)M) affected neither Ca\(^{2+}\) uptake by SH-SYSY 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 porcine 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...
Table 3 ¹²⁵I-Calmodulin binding to GST-fusion proteins derived from type-1 Ins₃ᵣ receptors

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specific binding (cpm/mg)</th>
</tr>
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<tbody>
<tr>
<td>Free [Ca²⁺]</td>
<td>cytl</td>
</tr>
<tr>
<td>≈ 4 nM</td>
<td>0.76 ± 0.18 μM</td>
</tr>
<tr>
<td>200 μM</td>
<td>1.42 ± 0.72 μM</td>
</tr>
<tr>
<td>cytl1</td>
<td>48 ± 11 nM</td>
</tr>
<tr>
<td>cytl11</td>
<td>172 ± 37 nM</td>
</tr>
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Figure 3  Calmodulin binding to GST-fusion proteins derived from type-1 Ins₃ᵣ receptors

(a) Specific ¹²⁵I-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 ¹²⁵I-calmodulin to cyt11 is shown in the absence (○, Hill coefficient, h = 0.81 ± 0.03) or presence (●, h = 1.30 ± 0.01) of a free [Ca²⁺] of 200 μM 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. (c) The three fusion proteins (0.8 μg/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 μM HSAB-calmodulin in the presence or absence of Ca²⁺, 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 underexposed to show more clearly the effect of Ca²⁺ on calmodulin binding to cyt11. The results, indicating that only cyt11 is detectably labelled in either the presence or absence of Ca²⁺, are typical of three experiments.

too failed to prevent further inhibition by calmodulin (Figure 2c). The effect of calmodulin did not result from enhanced metabolism of Ins₃ᵣ, because calmodulin (10 μM) also reduced the sensitivity of SH-SY5Y cells to adenophostin A, an agonist of Ins₃ᵣ 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; EC₅₀ increased from 4.7 ± 3 to 17 ± 2 nM) than for Ins₃ (2.1-fold; EC₅₀ 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 ⁴⁵Ca²⁺, calmodulin (10 μM) inhibited the response to a submaximal concentration of Ins₃ (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 ⁴⁵Ca²⁺-efflux experiments required to assess the Ca²⁺-dependence of the calmodulin effect (see the Methods and materials section), it is therefore difficult to both retain sufficient Ca²⁺ 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 Ins₃, but only in the presence of Ca²⁺.

Binding of calmodulin to type-1 Ins₃ᵣ receptors

Our previous work [24] established that ¹²⁵I-calmodulin bound to two distinct low-affinity sites on membranes prepared from Si9 cells expressing type-1 Ins₃ᵣ receptors: a Ca²⁺-dependent site and a Ca²⁺-independent site. Attempts to identify these sites by photoaffinity-labelling membranes of Spodoptera frugiperda cells expressing rat type-1 Ins₃ᵣ receptor (Si9/Ins₃ᵣ,R1) with HSAB–calmodulin (10 μM) were unsuccessful because there was too much non-specific binding (results not shown). However, using a SPA to measure specific ¹²⁵I-calmodulin binding to GST-fusion proteins (cyt1–18) representing the full length of the type-1 Ins₃ᵣ receptor, we identified specific ¹²⁵I-calmodulin binding to two fusion proteins (Figure 3a). Both cyt1 (residues 6–159) and cyt11 (residues 1499–1649) bound ¹²⁵I-calmodulin in the absence of Ca²⁺, and for both fusion proteins the specific binding was increased in the presence of 200 μM free [Ca²⁺]. From equilibrium-competition binding studies (Figure 3b), the affinities of the calmodulin-binding sites in cyt1 were significantly lower (Kₐ ≈ 1 μM) than those in the cyt11 fragment (Kₐ ≈ 100 nM; Table 3); the former more closely approximate the affinities of the calmodulin-binding sites previously detected by ¹²⁵I-calmodulin binding to Si9/Ins₃ᵣ,R1 membranes (Kₐ ≈ 1 μM) [24].

Photoaffinity labelling of the same GST-fusion proteins clearly identified HSAB–calmodulin binding to cyt11 in both the absence
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 analogous mutation (Trp\(^{1576}\) to Ala) in cyt11 reduced specific \(^{18}\)I-calmodulin-binding by 63 ± 9 % in the presence of Ca\(^{2+}\), it had a similar effect in the absence of Ca\(^{2+}\) (a reduction of 67 ± 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\(^{1569}\)) 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\(^{1568}\)) 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.
Ca\(^{2+}\)-calmodulin inhibition of Ins\(_P_2\) receptors


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