A novel role for calmodulin: Ca^{2+}-independent inhibition of type-1 inositol trisphosphate receptors

Thomas J. A. CARDY and Colin W. TAYLOR[1]

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

Calmodulin inhibits both inositol 1,4,5-trisphosphate (IP_3) binding to, and IP_3-evoked Ca^{2+} release by, cerebellar IP_3 receptors [Patel, Morris, Adkins, O'Beirne and Taylor (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 11627–11632]. In the present study, full-length rat type-1 and -3 IP_3 receptors were expressed at high levels in insect *Spodoptera frugiperda* 9 cells and the effects of calmodulin were examined. In the absence of Ca^{2+}, calmodulin caused a concentration-dependent and reversible inhibition of [^{3}H]IP_3 binding to type-1 IP_3 receptors by decreasing their apparent affinity for IP_3. The effect was not reproduced by high concentrations of troponin C, parvalbumin or S-100. Increasing the medium free [Ca^{2+}] ($[Ca^{2+}]_m$) inhibited [^{3}H]IP_3 binding to type-1 receptors, but the further inhibition caused by a sub-maximal concentration of calmodulin was similar at each $[Ca^{2+}]_m$. In the absence of Ca^{2+}, ^{125}I-calmodulin bound to a single site on each type-1 receptor subunit and to an additional site in the presence of Ca^{2+}. There was no detectable binding of ^{125}I-calmodulin to type-3 receptors and binding of [^{3}H]IP_3 was insensitive to calmodulin at all $[Ca^{2+}]_m$. Both peptide and conventional Ca^{2+}–calmodulin antagonists affected neither [^{3}H]IP_3 binding directly nor the inhibitory effect of calmodulin in the absence of Ca^{2+}, but each caused a $[Ca^{2+}]_m$-dependent reversal of the inhibition of [^{3}H]IP_3 binding caused by calmodulin. Camstatin, a peptide that binds to calmodulin equally well in the presence or absence of Ca^{2+}, reversed the inhibitory effects of calmodulin on [^{3}H]IP_3 binding at all $[Ca^{2+}]_m$. We conclude that calmodulin specifically inhibits [^{3}H]IP_3 binding to type-1 IP_3 receptors: the first example of a protein regulated by calmodulin in an entirely Ca^{2+}-independent manner. Inhibition of type-1 IP_3 receptors by calmodulin may dynamically regulate their sensitivity to IP_3 in response to the changes in cytosolic free calmodulin concentration thought to accompany stimulation of neurones.

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP_3) receptors belong to a family of intracellular Ca^{2+} channels that release Ca^{2+} from the endoplasmic reticulum in response to the concerted effects of increases in cytosolic Ca^{2+} and IP_3 concentrations. At least three closely related subtypes (1–3) of the IP_3 receptor, which assemble into both homo- and heterotetrameric complexes [1], are expressed in mammalian cells. The subtypes are differentially expressed [1–3], they differ in the rates at which they are degraded during chronic cell stimulation [4], they are differentially regulated by cytosolic Ca^{2+} [5,6] and they differ in their phosphorylation [1]. A recent study, in which expression of each of the three receptor subtypes was abolished, established that each is capable of mediating Ca^{2+} release from intracellular stores and none are required to allow empty Ca^{2+} stores to activate capacitative Ca^{2+} entry [7]. In view of this evidence, it seems likely that IP_3 receptor subtypes may differ rather subtly in their physiological roles.

Calmodulin is a small, acidic and almost perfectly conserved Ca^{2+}-binding protein that is expressed in every eukaryotic cell and mediates many of the effects of increases in cytosolic [Ca^{2+}] on such diverse processes as enzyme and ion-channel activity, motility and gene expression [8]. Calmodulin is particularly abundant in the soluble fractions from brain, where it accounts for about 1 % of all proteins [9]. Binding of Ca^{2+} to the four 'EF-hand' structures of calmodulin, two in each of its two globular domains, causes calmodulin to adopt a more compact structure that exposes hydrophobic residues, which are important in allowing its Ca^{2+}-dependent interactions with both other proteins and several calmodulin antagonists [10,11]. In addition to its roles in allowing Ca^{2+} to regulate cellular activities, calmodulin also directly regulates many Ca^{2+} transport processes. The Ca^{2+} pump of the plasma membrane is stimulated by Ca^{2+}–calmodulin [12], and many Ca^{2+}-permeable channels within the plasma membrane, including cyclic-nucleotide-gated channels [13], those encoded by the *trp* and *trpl* genes [14,15] and *N*-methyl D-aspartate receptors [16], are directly regulated by Ca^{2+}–calmodulin. In addition, Ca^{2+}–calmodulin indirectly regulates many Ca^{2+} channels through its ability to regulate protein kinases, protein phosphatases and the levels of cAMP [17].

Calmodulin also interacts with IP_3 receptors and with their close relatives, ryanodine receptors [18–20]. A short sequence within the modulatory domain of the type-1 IP_3 receptor binds calmodulin only in the presence of Ca^{2+} [21]; a similar sequence is present in the type-2, but not in the type-3, receptor. The functional consequences of Ca^{2+}–calmodulin binding to this site are unknown. Previous studies of the interactions between calmodulin and IP_3 receptors have failed to reveal a consistent pattern of regulation, with calmodulin causing inhibition, stimulation or having no effect on IP_3-stimulated Ca^{2+} mobilization [22]. Furthermore, in the presence of Ca^{2+}, IP_3 receptors from cerebellum ([21,23], but see [24]) and *Xenopus* oocytes [25] adhere to calmodulin columns; but cerebellar IP_3 receptors lose that ability without detectable loss of IP_3 binding during storage [23]. We recently established that in the absence of Ca^{2+}, calmodulin binds to IP_3 receptors purified from rat cerebellum and inhibits both IP_3 binding and Ca^{2+} mobilization [22]. The aim of the present study was to further characterize this interaction between calmodulin and IP_3 receptors using full-length recombinant receptors.

Abbreviations used: B_{max}, maximal number of binding sites; $[Ca^{2+}]_m$, medium free [Ca^{2+}]; CLM, cytosol-like medium; IP_3, inositol 1,4,5-trisphosphate; K_d, equilibrium dissociation constant; *h*, Hill coefficient; Sf9, *Spodoptera frugiperda* 9; Sf9/IP_3R1, Sf9/IP_3R3, *Spodoptera frugiperda* cells expressing IP_3 receptors types-1 and -3, respectively; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

[1] To whom correspondence should be addressed (e-mail cwt1000@cam.ac.uk).

We established previously that a baculovirus system [26] allowed high levels of expression of rat type-1 and -3 IP_3 receptors in insect *Spodoptera frugiperda* 9 (Sf9) cells. The expressed receptors bound IP_3 with high affinity, they were glycosylated appropriately, assembled into tetramers and were regulated by cytosolic Ca^{2+} [6]. In the present study, we have used the same expression system to examine the effects of calmodulin on type-1 and -3 IP_3 receptors.

MATERIALS AND METHODS

Expression of IP_3 receptors in Sf9 cells

Full-length cDNAs encoding the rat type-1 (lacking the S1 splice site) [27] and -3 [28] IP_3 receptors were subcloned into the baculovirus transfer vector pBacPAK9 (Clontech, Palo Alto, CA, U.S.A.) as described previously [6]. Recombinant viruses were produced in Sf9 cells by standard techniques [26] from transfer vectors and linearized *Autographa californica* nuclear polyhedrosis viral (AcMNPV) DNA using a linear transfection module (Invitrogen, NV Leek, The Netherlands). Automated sequencing of both strands of our constructs identified minor differences from the original type-1 submission (GenBank/EMBL accession number J05510); the revised sequence is more similar to that of other species and subtypes. The differences (nucleotides numbered according to [27]) are: $^{2180}G \rightarrow A$, codon ^{4443}TTC absent, $^{6020}A \rightarrow G$, $^{7644}C \rightarrow T$ and $^{7663}C \rightarrow T$; in the translated sequence they result in loss of ^{1372}Phe, $^{2439}Pro \rightarrow Ser$ and $^{2445}Ala \rightarrow Val$. Sequencing of our type-3 construct confirmed the original submission [28].

Sf9 cells (2×10^5 cells/ml) were cultured in spinner flasks at 27 °C in serum-free complete TNM-FH insect medium (300 ml) supplemented with fungizone (2.5 μg/ml) [26]. Cells in the logarithmic phase of growth (5×10^5 cells/ml) were infected with recombinant virus stocks at a multiplicity of infection ($\approx$ 2–5) adjusted to ensure similar levels of expression of the two receptor subtypes, as determined by immunoblotting of membrane fractions (see below). Infected cells were harvested 40–42 h after infection by centrifugation (1000 ***g***, 5 min) at 2 °C. The methods used to prepare membranes were described in detail previously [6]. Briefly, the cell pellets were washed twice in PBS, resuspended in Ca^{2+}-free cytosol-like medium (CLM) supplemented with a protease-inhibitor cocktail and homogenized using an Ultra-Turrax T25 homogenizer. The homogenate was centrifuged (3000 ***g***, 10 min) and the membrane pellet resuspended in Ca^{2+}-free CLM (4–6 mg of protein/ml) before rapid freezing in liquid nitrogen and storage at −80 °C.

Equilibrium [^{3}H]IP_3 and ^{125}I-calmodulin binding assays

Membranes (100 μg of protein/ml) from infected Sf9 cells were resuspended in CLM (0.5 ml) with the appropriate medium free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) and containing [^{3}H]IP_3 (0.6 nM) and various concentrations of unlabelled IP_3. Ca^{2+}-free CLM had the following composition: 140 mM KCl/20 mM NaCl/2 mM $MgCl_2$/1 mM EGTA/20 mM Pipes/0.1 mM PMSF/10 μM leupeptin/1 mM benzamidine/0.1 mM soya bean trypsin inhibitor/0.1 mM captopril (pH 7.0). After 5 min at 2 °C, during which equilibrium was attained, the incubations were stopped by centrifugation (20000 ***g***, 5 min at 2 °C) and the supernatants were removed by aspiration. The radioactivity associated with the pellets was measured by resuspending them in 1 ml of Ecoscint-A (National Diagnostics, Aylesbury, Bucks, U.K.) for liquid scintillation counting. Since the effects of calmodulin were similar after preincubation for 5 or 45 min, membranes were routinely preincubated for 10 min with calmodulin (or the other Ca^{2+}-binding proteins) before addition of [^{3}H]IP_3 for a further 5 min. Where appropriate, the calmodulin antagonists or peptides were included during the preincubation.

For ^{125}I-calmodulin binding, membranes (500 μg of protein) were resuspended in Ca^{2+}-free CLM (200 μl) containing [^{3}H]inulin (4 μCi, to correct for trapped volume), ^{125}I-calmodulin (0.4 μCi) and appropriate concentrations of unlabelled calmodulin. After 10 min at 2 °C, during which time equilibrium was attained (results not shown), the incubations were stopped by centrifugation as described above. Non-specific binding was determined in the presence of a high concentration of unlabelled ligand (1 μM IP_3, 10 μM calmodulin) or by extrapolation of the curve fits to infinite ligand concentration (see below) with indistinguishable results. Subtraction of the specific ^{125}I-calmodulin binding to membranes from uninfected cells from that observed with Sf9/IP_3R1 (Sf9 cells expressing IP_3 receptor type-1) membranes allowed specific binding to type-1 IP_3 receptors to be resolved (see the Results section for further details).

Results from equilibrium competition binding experiments were fitted to four-parameter logistic equations using least-squares curve-fitting routines (Kaleidagraph; Synergy Software, Reading, PA, U.S.A.):

$$B = N + \frac{T - N}{1 + \left(\frac{[L]}{IC_{50}}\right)^h}$$

where T is the total amount of radioligand bound in the absence of competing ligand; N is the amount of non-specific binding; B is the total amount of radioligand bound in the presence of a defined concentration of unlabelled ligand, [L]; IC_{50} is the concentration of unlabelled ligand causing half-maximal displacement of specifically bound radioligand and h is equivalent to the Hill coefficient [29]. The IC_{50} values derived from the curve fits were used to calculate the equilibrium dissociation constants (K_d) [30]:

$$K_d = IC_{50} - [L]$$

The maximal number of binding sites (B_{max}) was then calculated from:

$$B_{max} = S\left(\frac{K_d}{[L^*]} + 1\right)$$

where S is the amount of specifically bound radioligand, L*, when its concentration is [L*].

Other methods

Protein concentrations were determined using the Bradford assay [31] with BSA as the standard. The levels of expression of the two IP_3 receptor subtypes were quantified by means of immunoblotting of the membrane fractions using an antiserum (AbC) to a peptide conserved in all IP_3 receptor subtypes and which we demonstrated previously to bind equally well to the type-1 and -3 receptors [6]. The free $[Ca^{2+}]$ of CLM was determined fluorimetrically with Fura 2 using a K_d for Ca^{2+}-Fura 2 of 372 nM at 2 °C.

Materials

Cell-culture materials were from Life Sciences (Paisley, Scotland, U.K.). Fura 2 was from Molecular Probes (Leiden, The Netherlands). [^{3}H]IP_3 (48 Ci/mmol) was from Amersham (Little Chalfont, Bucks, U.K.) and IP_3 was from American Radiolabeled Chemicals Inc. (St Louis, MO, U.S.A.). ^{125}I-Calmodulin ($\approx$ 70 μCi/μg), prepared using Bolton Hunter reagent, was from

NEN (Brussels, Belgium). Bovine brain calmodulin was from either Calbiochem (Nottingham, U.K.) or Upstate Biotechnology (Lake Placid, NY, U.S.A.) and recombinant chicken calmodulin was from Calbiochem. W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide], trifluoperazine and bovine brain S-100 protein were from Calbiochem. Troponin was from Sigma and the concentrations referred to in the text have been corrected to account for the preparation including only 23% troponin C. Peptides were supplied by Research Genetics Inc. (Huntsville, AL, U.S.A.) and their purity (> 80%) was verified by MS. FK506 was a gift from Dr. K. Murato, Fujisawa GmbH (Munich, Germany). All other reagents, including parvalbumin, were from Sigma.

RESULTS

Calmodulin inhibits [^{3}H]IP_3 binding to type-1 IP_3 receptors

In Ca^{2+}-free CLM, the characteristics of IP_3 binding to type-1 and -3 IP_3 receptors expressed in Sf9 cells were indistinguishable from those reported previously (Table 1) [6]. Preincubation of membranes from Sf9/IP_3R1 with 50 μM calmodulin in the absence of added Ca^{2+} ($[Ca^{2+}]_m \approx 2$ nM) reduced specific [^{3}H]IP_3 binding by 49.9 ± 0.5% ($n = 6$) (Figure 1A). The inhibition resulted entirely from a decrease in the apparent affinity of the receptor for IP_3 with no significant change in the B_{max} (Table 1). Calmodulin (50 μM) had no effect on [^{3}H]IP_3 binding to membranes from Sf9 cells expressing type-3 IP_3 receptors (Sf9/IP_3R3) (Figure 1B). We demonstrated previously that the inhibitory effect of calmodulin on IP_3 binding to cerebellar IP_3 receptors was not a consequence of it binding to [^{3}H]IP_3 [22]. That result is confirmed by the different effects of calmodulin on [^{3}H]IP_3 binding to Sf9/IP_3R1 and Sf9/IP_3R3 membranes (Figures 1A and 1B).

The half-maximal inhibitory effect (IC_{50}) of calmodulin on [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes in Ca^{2+}-free CLM occurred when the calmodulin concentration was 811 ± 61 nM ($n = 3$) (Figure 1C). Indistinguishable results were obtained using bovine brain calmodulin ($IC_{50} = 811 \pm 81$ nM) or recombinant chicken calmodulin ($IC_{50} = 675$ nM), and the inhibition of [^{3}H]IP_3 binding caused by a submaximal concentration of calmodulin (1 μM) was similar for bovine brain calmodulin supplied by Calbiochem (37%) or Upstate Biotechnology (39%), or for recombinant chicken calmodulin (35%).

The specificity of the calmodulin effect was confirmed by results with three related Ca^{2+}-binding proteins containing 'EF-hand' structures. Troponin C is the Ca^{2+}-binding subunit of striated muscle fibres, S-100 proteins are almost as abundant in brain as calmodulin and parvalbumin is most abundant in skeletal muscle, but it is also present in brain [32]. Troponin C, bovine brain S-100 and parvalbumin (1 μM) had no effect on [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes, and even when their concentrations were increased to 50 μM they inhibited [^{3}H]IP_3

Table 1 Effects of calmodulin on [^{3}H]IP_3 binding to type-1 and -3 IP_3 receptors in the absence of Ca^{2+}

Experiments similar to those shown in Figure 1 were used to determine the effects of calmodulin (50 μM) on equilibrium binding of IP_3 to Sf9/IP_3R1 and Sf9/IP_3R3 membranes in Ca^{2+}-free CLM. The equilibrium dissociation constants (K_d), maximal numbers of binding sites (B_{max}) and Hill coefficients (h) were derived by fitting logistic equations to equilibrium competition binding curves. Results are means ± S.E.M. of n independent experiments.

Receptor subtype	Calmodulin (μM)	Inhibition of binding (%)	K_d (nM)	B_{max} (pmol/mg)	h	n
Type-1	0	0	13.6 ± 0.6	6.4 ± 0.3	1.03 ± 0.06	6
	50	49.9 ± 0.5	25.7 ± 2.7	7.3 ± 0.75	1.2 ± 0.08	6
Type-3	0	0	3.41 ± 0.89	4.62 ± 0.75	0.98 ± 0.13	3
	50	3.7 ± 0.9	3.5 ± 1.0	4.64 ± 0.1	0.91 ± 0.12	3

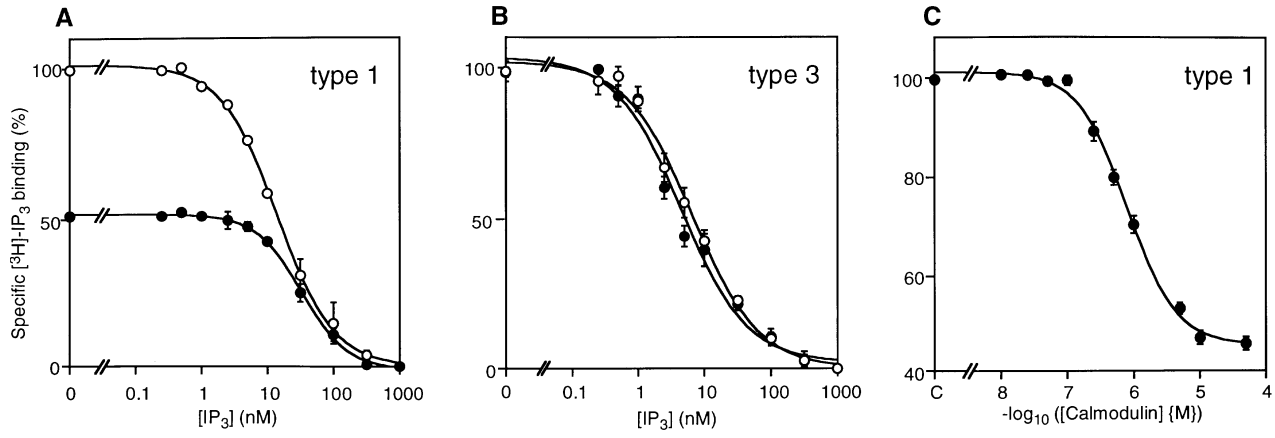

Figure 1 Calmodulin inhibits IP_3 binding to type-1, but not type-3, IP_3 receptors

Specific binding of [^{3}H]IP_3 (0.6 nM) to Sf9/IP_3R1 (**A**) or Sf9/IP_3R3 (**B**) membranes was measured in Ca^{2+}-free CLM with (●) or without (○) calmodulin (50 μM). The effect of varying the calmodulin concentration on [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes is shown in (**C**). Specific [^{3}H]IP_3 binding (typically 3000 dpm) is shown as a percentage of that observed in the absence of both calmodulin and unlabelled IP_3. In these and subsequent Figures, results are means ± S.E.M. of 3 independent experiments, each performed in duplicate (most error bars are smaller than the symbols).

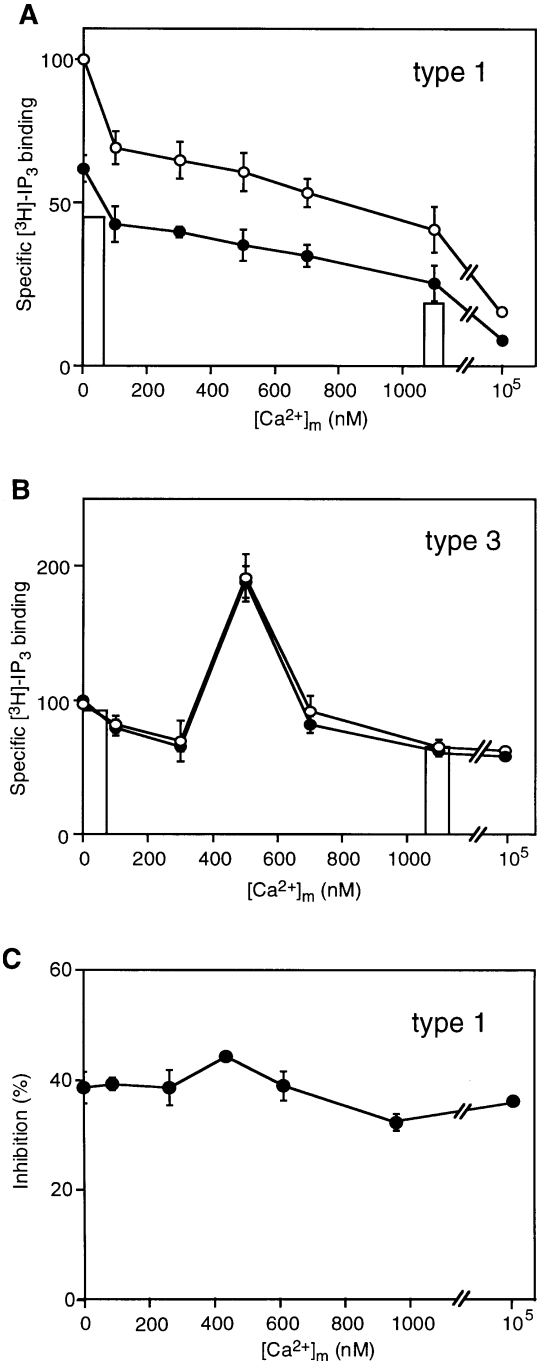

Figure 2 Selective Ca^{2+}-independent inhibition of IP_3 binding to type-1 IP_3 receptors by calmodulin

[^{3}H]IP_3 binding to Sf9/IP_3R1 (**A**) or Sf9/IP_3R3 (**B**) membranes was measured in CLM containing the indicated $[Ca^{2+}]_m$ with (●) or without (○) 1 μM calmodulin. Specific [^{3}H]IP_3 binding is shown as a percentage of that observed in Ca^{2+}-free CLM without calmodulin. Open bars denote results from experiments in which the calmodulin concentration was increased to 50 μM. Panel (**C**) shows that although specific [^{3}H]IP_3 binding to type-1 receptors decreases as $[Ca^{2+}]_m$ increases, the inhibition (%) caused by 1 μM calmodulin is similar at all $[Ca^{2+}]_m$.

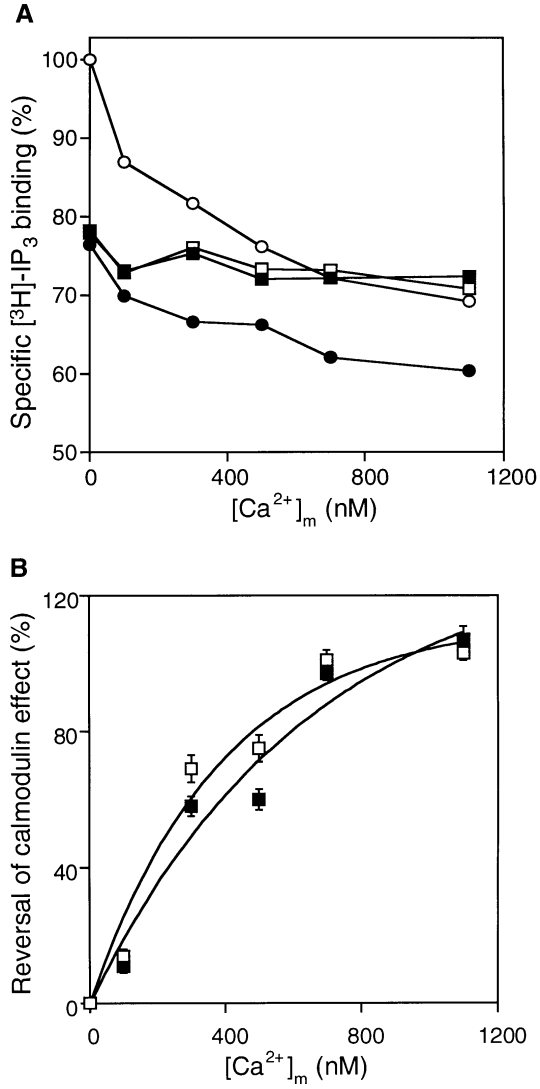

Figure 3 Conventional calmodulin antagonists reverse the effect of calmodulin only in the presence of Ca^{2+}

(**A**) [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes was measured in CLM containing the indicated $[Ca^{2+}]_m$ with (●) or without (○) 1 μM calmodulin. In parallel, W-7 (20 μM, ■) or trifluoperazine (20 μM, □) were added with the calmodulin. Specific [^{3}H]IP_3 binding is shown as a percentage of that observed in Ca^{2+}-free medium without calmodulin. (**B**) Results from (**A**) plotted to illustrate the effects of $[Ca^{2+}]_m$ on the ability of the antagonists to reverse the inhibition caused by calmodulin.

binding to only 16%, 34% and 39% of that caused by a maximal concentration (10 μM) of calmodulin.

The reversibility of the effect of calmodulin was examined by incubating Sf9/IP_3R1 membranes with 1 μM calmodulin and [^{3}H]IP_3 for 10 min before diluting the incubations 10-fold into the same medium, but lacking calmodulin (final calmodulin concentration, 0.1 μM). The results demonstrate that while 1 μM calmodulin inhibited specific [^{3}H]IP_3 binding by 32 ± 2% ($n = 3$), during the subsequent 10 min incubation with 0.1 μM calmodulin the inhibition reversed to 3 ± 1%, which was indistinguishable from that observed with membranes incubated with 0.1 μM calmodulin throughout (5.0 ± 1.5%, $n = 3$).

Ca^{2+}-independent inhibition of IP_3 binding to type-1 IP_3 receptors by calmodulin

In keeping with our previous report [6], increasing $[Ca^{2+}]_m$ from ≈ 2 nM to 1.1 μM inhibited specific [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes by 46 ± 6% ($n = 3$; Figure 2A), but the further

Table 2 Synthetic peptides

The peptides used to examine the effects of calmodulin on IP_3 receptors are shown using single letter amino acid codes; underlined residues are discussed in the text.

	Sequence	Source
Pep-1	KSHNIVQKTALNWRLSARNAAR	Ca^{2+}–calmodulin-binding site of type-1 IP_3 receptor
Pep-2	KSHNIVQKTALNARLSARNAAR	Mutant (inactive) Ca^{2+}–calmodulin-binding site of type-1 IP_3 receptor
Pep-3	LKKFNARRKLKGAILTTMLA	Ca^{2+}–calmodulin binding domain of calmodulin kinase II
Pep-4	APETERAAVAIQAQFRKFQKKKAGS	Camstatin, a Ca^{2+}-independent calmodulin-binding peptide

inhibition (38–42%), caused by addition of a submaximal concentration of calmodulin (1 μM), was similar across the entire range of $[Ca^{2+}]_m$ (Figures 2A and 2C). The effects of $[Ca^{2+}]_m$ on [^{3}H]IP_3 binding to Sf9/IP_3R3 membranes were biphasic [6] and entirely insensitive to 1 μM calmodulin at any $[Ca^{2+}]_m$ (Figure 2B). Even when the calmodulin concentration was increased to 50 μM, it failed to affect [^{3}H]IP_3 binding to Sf9/IP_3R3 membranes in CLM containing either $\approx$ 2 nM or 1.1 μM Ca^{2+} (Figure 2C).

The Ca^{2+}-independence of the effects of calmodulin on [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes was further investigated using two calmodulin antagonists, W-7 and trifluoperazine, which bind only to Ca^{2+}–calmodulin [11,33]. In the absence of calmodulin, neither antagonist (20 μM) had any effect on [^{3}H]IP_3 binding at any $[Ca^{2+}]_m$ ($\approx$ 2 nM–1.1 μM; results not shown), consistent with previous reports indicating that inhibition of IP_3 binding to type-1 receptors by Ca^{2+} is not mediated by calmodulin. In Ca^{2+}-free CLM, neither W-7 nor trifluoperazine (both 20 μM) had any effect on the inhibition of [^{3}H]IP_3 binding caused by calmodulin (1 μM) (Figure 3A). However, as the $[Ca^{2+}]_m$ was increased, both inhibitors antagonized the effect of calmodulin, such that when $[Ca^{2+}]_m$ was $\geqslant$ 700 nM, they fully reversed the inhibition of [^{3}H]IP_3 binding (Figure 3B). Under the conditions used for these experiments, half-maximal reversal of the inhibitory effect of calmodulin by the antagonists occurred when $[Ca^{2+}]_m$ was 304 $\pm$ 30 nM (trifluoperazine) and 358 $\pm$ 12 nM (W-7) (Figure 3B).

Calmodulin-binding peptides confirm the Ca^{2+}-independent effects of calmodulin

A short peptide sequence within the modulatory domain of the mouse type-1 IP_3 receptor, which is conserved in that of the rat, binds calmodulin, but only in the presence of Ca^{2+} [21]. The sequence has some similarity with the consensus structure, a basic amphipathic α helix of about 20 residues, found in other Ca^{2+}–calmodulin-binding proteins [34]. The Ca^{2+}-dependence of calmodulin binding to this site clearly distinguishes it from the Ca^{2+}-independent effects of calmodulin on [^{3}H]IP_3 binding (Figure 2C), but the peptide (Pep-1) is nevertheless a useful tool with which to further examine the effects of calmodulin on [^{3}H]IP_3 binding to type-1 IP_3 receptors. The peptides used are summarized in Table 2.

Inclusion of Pep-1 (10 μM) during the preincubation with calmodulin (1 μM) inhibited its effect on [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes only in the presence of Ca^{2+} (Figure 4). With $[Ca^{2+}]_m \geqslant$ 700 nM (EC_{50} for Ca^{2+} = 351 $\pm$ 23 nM, n = 3), Pep-1 fully restored [^{3}H]IP_3 binding to its control level despite the continued presence of calmodulin (Figure 4B). In the absence of calmodulin, Pep-1 had no effect on [^{3}H]IP_3 binding at any $[Ca^{2+}]_m$ (results not shown). The ability of Pep-1 to bind to Ca^{2+}–calmodulin was previously shown to be abolished when

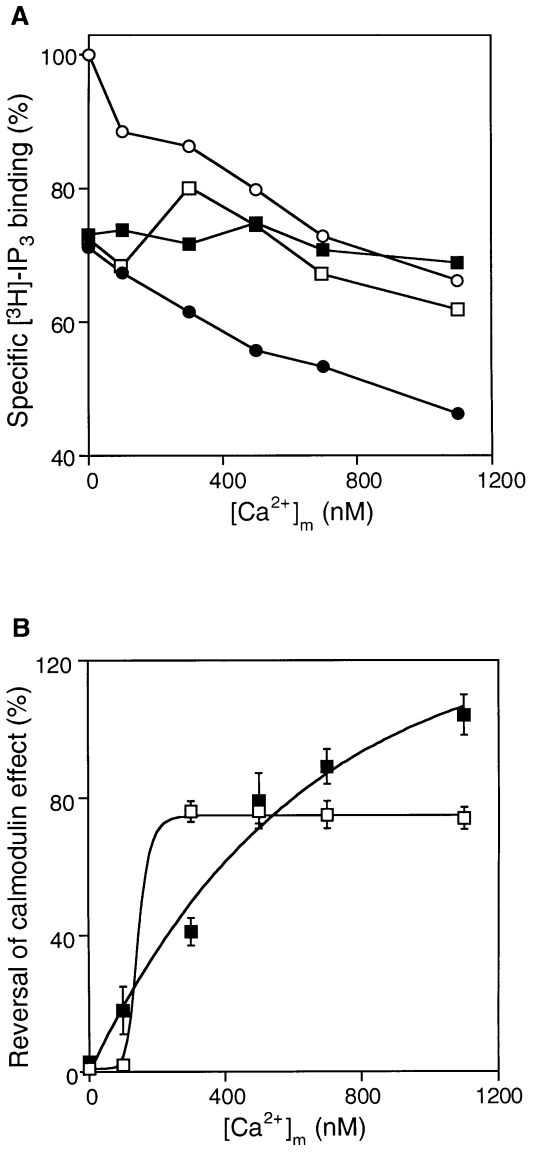

Figure 4 Peptide Ca^{2+}–calmodulin antagonists reverse the effect of calmodulin only in the presence of Ca^{2+}

(**A**) [^{3}H]IP_3 binding to Sf9/IP_3R1 membranes was measured in CLM containing the indicated $[Ca^{2+}]_m$ with (●) or without (○) 1 μM calmodulin. In parallel, Pep-1 (10 μM, ■) or Pep-3 (10 μM, □) were added with the calmodulin. Specific [^{3}H]IP_3 binding is shown as a percentage of that observed in Ca^{2+}-free medium without calmodulin. (**B**) Results from (**A**) plotted to illustrate the effects of $[Ca^{2+}]_m$ on the ability of the peptides to reverse the inhibition caused by calmodulin.

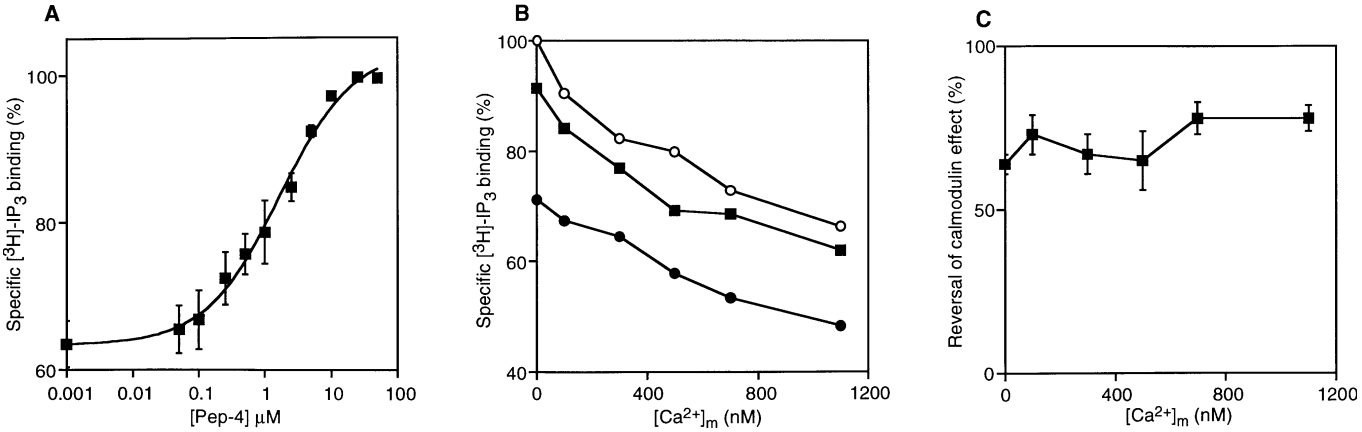

Figure 5 Camstatin reverses the effect of calmodulin irrespective of the $[Ca^{2+}]$

(**A**) The ability of camstatin (Pep-4) to reverse the inhibition of $[^3H]IP_3$ binding to $Sf9/IP_3R1$ membranes caused by 1 μM calmodulin in Ca^{2+}-free CLM is shown. (**B**) $[^3H]IP_3$ binding to $Sf9/IP_3R1$ membranes (percentage of that in Ca^{2+}-free CLM without calmodulin) was measured in CLM containing the indicated $[Ca^{2+}]_m$ in the absence of calmodulin (○) or in the presence of 1 μM calmodulin with (■) or without (●) 10 μM Pep-4. (**C**) Results plotted from (**A**) plotted to illustrate that the ability of Pep-4 to reverse the inhibition caused by calmodulin is independent of $[Ca^{2+}]_m$.

W^{1576} was mutated to A [21], and in our experiments the mutant peptide (Pep-2, $\leqslant 50\ \mu M$) had no effect on $[^3H]IP_3$ binding, irrespective of the presence of calmodulin or Ca^{2+} (results not shown). In similar experiments, a peptide (Pep-3, 10 μM) corresponding to the Ca^{2+}–calmodulin-binding domain of Ca^{2+}–calmodulin-dependent protein kinase II [35] had no effect on the ability of calmodulin to inhibit $[^3H]IP_3$ binding in the absence of Ca^{2+}, but reversed the effect of calmodulin as $[Ca^{2+}]_m$ was increased (EC_{50} for $Ca^{2+} = 162 \pm 8$ nM) (Figure 4B).

Camstatin is a synthetic peptide (Pep-4, Table 2) [36] modified from a sequence within PEP-19, a neuronal calmodulin-binding protein of unknown function, which includes the very basic IQ motif (commonly IQXXXRGXXXR) found in several proteins that bind calmodulin in the absence of Ca^{2+} [37]. Camstatin binds with similar affinity to calmodulin and Ca^{2+}–calmodulin [36]. In Ca^{2+}-free CLM, Pep-4 caused a concentration-dependent (EC_{50} for Pep-4 $= 2.1 \pm 0.33\ \mu M$, $n = 3$) reversal of the inhibition of $[^3H]IP_3$ binding to $Sf9/IP_3R1$ membranes caused by 1 μM calmodulin (Figure 5A). A submaximal concentration of Pep-4 (10 μM) that reversed the effect of calmodulin by $72 \pm 6\%$ in Ca^{2+}-free medium had the same effect across a range of $[Ca^{2+}]_m$ (Figures 5B and 5C).

Two calmodulin-binding sites on each type-1 IP_3 receptor subunit

In Ca^{2+}-free CLM, ^{125}I-calmodulin bound specifically to $Sf9/IP_3R1$ membranes (Figure 6A). Specific ^{125}I-calmodulin binding to $Sf9/IP_3R3$ membranes and to membranes from uninfected cells was indistinguishable and amounted to only about 14% of that observed for $Sf9/IP_3R1$ membranes (Figure 6A). Non-specific ^{125}I-calmodulin binding (i.e. that persisting in the presence of 10 μM unlabelled calmodulin) was similar for each of the membrane preparations (results not shown). These results indicate that in the absence of Ca^{2+} there was no specific binding of ^{125}I-calmodulin to type-3 IP_3 receptors. The amount of ^{125}I-calmodulin bound specifically to type-1 IP_3 receptors at each point on the equilibrium competition binding curve was calculated by subtracting the small amount of ^{125}I-calmodulin bound specifically to the endogenous calmodulin-binding proteins of membranes from uninfected cells from that bound to $Sf9/IP_3R1$ membranes. The results demonstrate that in the absence of Ca^{2+}, calmodulin binds with high affinity to a site on the type-1 IP_3 receptor ($K_d = 1.02 \pm 0.10\ \mu M$, $B_{max} = 8.8 \pm 0.2$ pmol/mg of protein, $h = 1.18 \pm 0.23$) (Table 3). IP_3 (1 μM) had no effect on this ^{125}I-calmodulin binding to type-1 IP_3 receptors ($96 \pm 1\%$ of control).

As expected, increasing $[Ca^{2+}]_m$ ($EC_{50} \approx 180$ nM) increased ^{125}I-calmodulin binding to $Sf9/IP_3R1$ membranes, consistent with the existence of Ca^{2+}–calmodulin-binding sites [21] (Figures 6A and 6B). Ca^{2+} had no effect on the specific ^{125}I-calmodulin binding to $Sf9/IP_3R3$ membranes or membranes prepared from uninfected Sf9 cells. Specific ^{125}I-calmodulin binding to type-1 IP_3 receptors was again determined by subtraction of the ^{125}I-calmodulin bound specifically to membranes from uninfected cells from that bound to $Sf9/IP_3R1$ membranes (Figure 6B).

The ^{125}I-calmodulin bound to type-1 receptors in the presence of Ca^{2+} is the sum of that bound to the Ca^{2+}-independent and Ca^{2+}-dependent sites. Since calmodulin binds only to the former type of site in the absence of Ca^{2+} and its binding is wholly unaffected by changes in $[Ca^{2+}]_m$ (Figure 2C), the difference between the binding curves in the presence and absence of Ca^{2+} (Figures 6A and 6B) allows the characteristics of the Ca^{2+}–calmodulin-binding site to be isolated (Figure 6C, Table 3). Despite the difficulty of reliably estimating a B_{max} from curves in which only a small fraction of the sites are occupied by ^{125}I-calmodulin, there is reasonable agreement between our estimates of the B_{max} for IP_3-binding sites and for the Ca^{2+}-dependent and Ca^{2+}-independent calmodulin-binding sites (Table 3). Furthermore, our estimated affinity of the single Ca^{2+}–calmodulin-binding site ($K_d = 816 \pm 123$ nM) is similar to that derived from analysis of the peptide (Pep-1) corresponding to the Ca^{2+}–calmodulin-binding domain of the type-1 receptor ($K_d = 700$ nM) [21].

Several lines of evidence, including stoichiometric binding of IP_3 to purified cerebellar IP_3 receptors [24] and identification of the residues responsible for IP_3 binding [38], have unequivocally established that each subunit of the IP_3 receptor has a single IP_3-binding site. The similar estimates of B_{max} for Ca^{2+}–calmodulin-binding and IP_3-binding sites (Table 3) are therefore consistent with earlier evidence suggesting that each subunit also has a single Ca^{2+}–calmodulin-binding site [21]. Our observation that the Hill slope of the calmodulin equilibrium competition binding

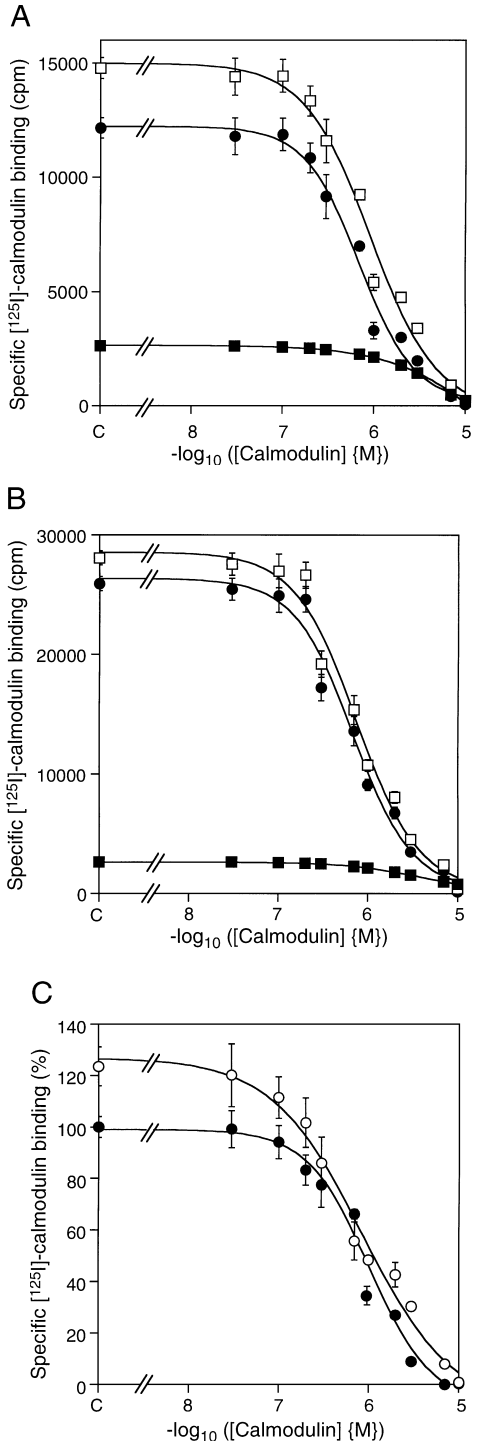

Figure 6 Calmodulin binds to two sites on type-1 IP_3 receptors

(**A**,**B**) Sf9/IP_3R1 membranes (□) or membranes from uninfected Sf9 cells (■) were incubated with ^{125}I-calmodulin (0.4 μCi, 36 nM) and the indicated concentrations of calmodulin for 10 min in either Ca^{2+}-free CLM (**A**) or CLM containing 1.1 μM Ca^{2+} (**B**). Specific ^{125}I-calmodulin binding (cpm) was measured after correction for trapped volume; non-specific binding to both membrane preparations was similar (3939 ± 221 and 4068 ± 53 cpm, for Sf9/IP_3R1 membranes in Ca^{2+}-free and Ca^{2+} containing CLM respectively). (●) Denotes the ^{125}I-calmodulin bound specifically to type-1 IP_3 receptors, calculated by subtraction of the specific binding to uninfected membranes from that to Sf9/IP_3R1 membranes. (**C**) Specific Ca^{2+}-independent calmodulin binding was determined in Ca^{2+}-free CLM (●), and Ca^{2+}-dependent binding (○) by subtraction of the former from that observed in CLM containing 1.1μM CLM (see the Results section). Results (means ± S.E.M. from 3 independent experiments) show specific ^{125}I-calmodulin expressed as percentage of the initial specific ^{125}I-calmodulin binding in Ca^{2+}-free CLM.

Table 3 Characteristics of calmodulin and IP_3 binding to type-1 IP_3 receptors

Sf9/IP_3R1 membranes were used for equilibrium competition binding assays with ^{125}I-calmodulin and calmodulin. The characteristics of the Ca^{2+}-independent calmodulin-binding site were determined in Ca^{2+}-free CLM, and those of the Ca^{2+}-dependent calmodulin-binding site by subtraction of Ca^{2+}-independent calmodulin binding from that measured when $[Ca^{2+}]_m$ was 1.1 μM (see the Results section). Results are means ± S.E.M. from 3 independent experiments. In parallel experiments with the same membranes, IP_3 binding was determined in Ca^{2+}-free CLM as described in Figure 1.

Binding site	B_{max} (pmol/mg)	K_d (nM)	h
IP_3	7.2 ± 0.6	11.4 ± 0.9	0.98 ± 0.09
Ca^{2+}–calmodulin	9.5 ± 1.1	816 ± 123	1.05 ± 0.11
Ca^{2+}-independent calmodulin	8.8 ± 0.2	1021 ± 102	1.18 ± 0.23

curve in the absence of Ca^{2+} is close to unity ($h = 1.18 \pm 0.23$) (Figure 6A, Table 3) is consistent with the existence of a single class of Ca^{2+}-independent calmodulin-binding sites, and because the B_{max} for this site is similar to that for IP_3-binding sites (Table 3), we suggest that each receptor subunit has a single Ca^{2+}-independent calmodulin-binding site. We propose that each subunit of the type-1 IP_3 receptor has a single site for IP_3 binding, a single site for Ca^{2+}-independent calmodulin binding, and a separate single site for Ca^{2+}–calmodulin binding [21].

DISCUSSION

Calmodulin selectively inhibits type-1 IP_3 receptors

In view of the conflicting evidence on interactions between calmodulin and IP_3 receptors (see the Introduction), we wanted both to demonstrate that the effects we observed were attributable to calmodulin, and to provide an explanation for others having failed to detect an effect of calmodulin on IP_3 receptors [23,24]. We demonstrated previously that the high pH of the medium commonly used for [^{3}H]IP_3-binding assays [24] abolished the effect of calmodulin [22]. The unusual Ca^{2+}-independence of the calmodulin effect is also likely to have confused previous analyses because the calmodulin antagonists, W-7 and trifluoperazine, are effective only when $[Ca^{2+}]_m$ is increased (Figure 3). Our observation that calmodulin interacts with type-1 but not type-3 IP_3 receptors (Figures 1, 2 and 6) is likely to have further contributed to the disparate results. Finally, it is noteworthy that the two calmodulin-binding sites of NinaC, an unconventional myosin from *Drosophila*, require different assay conditions for their detection [39].

Type-1 IP_3 receptors are equally sensitive to diverse sources of calmodulin, and much less sensitive to related Ca^{2+}-binding proteins. Furthermore, two conventional Ca^{2+}–calmodulin antagonists (Figure 3) and two peptides (Figure 4), each of which binds only to Ca^{2+}–calmodulin, antagonized the effect of calmodulin, but only in the presence of Ca^{2+}, whereas a control peptide differing from Pep-1 by only a single residue (Pep-2) was ineffective. Another peptide, Camstatin, which is known to bind equally well to calmodulin and Ca^{2+}–calmodulin [36], caused a concentration-dependent reversal of the effect of calmodulin at all free $[Ca^{2+}]_m$ (Figure 5B). This evidence establishes that the inhibition of IP_3 binding must be caused by calmodulin and not by a contaminant of the calmodulin preparations.

The lack of effect of calmodulin on type-3 receptors (Figures 1B and 2B) confirms that calmodulin does not bind to [^{3}H]IP_3. Calmodulin is very acidic and might have exerted its effect by

competing with IP_3 for its binding site on the receptor, but this cannot be the explanation. Firstly, the N-terminal domains within which IP_3 binds are similar for both receptor subtypes [38], yet calmodulin inhibits IP_3 binding only to type-1 receptors (Figures 1 and 2). Secondly, whereas calmodulin does cause an increase in the apparent K_d of type-1 receptors for IP_3 (consistent with competitive antagonism), even a supramaximal calmodulin concentration reduces [^{3}H]IP_3 binding by only $\approx 50\,\%$ (Table 1). Finally, IP_3 had no effect on specific ^{125}I-calmodulin binding to type-1 receptors. We conclude that calmodulin selectively inhibits IP_3 binding to type-1 receptors by binding to a specific site distinct from that to which IP_3 binds

Ca^{2+}-independent inhibition of type-1 IP_3 receptors by calmodulin

Whereas calmodulin binds to many proteins in the absence of Ca^{2+} [40–43], its ability to *regulate* the activities of its targets has so far proven to be entirely Ca^{2+}-dependent [17]. Even those proteins, notably the unconventional myosins, to which apo-calmodulin binds appear to change their activity only after Ca^{2+} binding [43]. The only possible exception is an unusual extracellular adenylyl cyclase from bacteria [44], but since calmodulin is not expressed in bacteria the significance is unclear. However, whereas calmodulin is essential for survival of yeast, it can complete its entire life cycle using calmodulin from which all Ca^{2+}-binding sites have been removed [45]. The targets through which calmodulin fulfills these Ca^{2+}-independent, but essential, roles have not been identified. The type-1 IP_3 receptor provides the first example of a protein whose activity is regulated by calmodulin irrespective of the free [Ca^{2+}]. Our conclusion is substantiated by several independent lines of evidence. Firstly, the effect of a submaximal calmodulin concentration on IP_3 binding is indistinguishable across a wide range of cytosolic [Ca^{2+}] (Figure 2C). This observation is similar to that reported previously for purified cerebellar receptors [22]. However, whereas pure cerebellar IP_3 receptors are insensitive to Ca^{2+} [46], probably because they have lost an accessory protein that mediates the Ca^{2+} effect, type-1 IP_3 receptors expressed in Sf9 membranes retain their Ca^{2+} sensitivity [6] (Figure 2A). These results establish that neither the effects of Ca^{2+} on calmodulin nor the effects of Ca^{2+} on the IP_3 receptor influence the ability of calmodulin to inhibit IP_3 binding. We conclude that in intact cells, just as with pure cerebellar IP_3 receptors [22], calmodulin-mediated inhibition of IP_3 binding to type-1 receptors is likely to be Ca^{2+}-independent. Secondly, both conventional and peptide antagonists of Ca^{2+}–calmodulin reversed the inhibitory effect of calmodulin only when $[Ca^{2+}]_m$ was substantially increased (Figures 3 and 4), consistent with the established ability of these inhibitors to bind only to Ca^{2+}–calmodulin. Finally, in contrast to the results with the Ca^{2+}–calmodulin antagonists, Camstatin, which binds equally well to calmodulin and Ca^{2+}–calmodulin [36], potently inhibited the effects of calmodulin and was similarly effective at all $[Ca^{2+}]_m$ (Figure 5).

Binding of Ca^{2+} to calmodulin is influenced by interactions between its four Ca^{2+}-binding sites, by interactions with protein targets, and by ionic strength; it is therefore difficult to precisely predict the range of $[Ca^{2+}]_m$ over which calmodulin will become saturated with Ca^{2+} [47]. It could be argued that in our experiments, where $[Ca^{2+}]_m$ was generally $\leqslant 1.1\,\mu$M (Figures 2–5), the increase in $[Ca^{2+}]_m$ was insufficient to reveal a Ca^{2+}-dependent effect of calmodulin. However, the ability of both conventional (Figure 3) and peptide (Figure 4) antagonists to fully reverse the calmodulin-mediated inhibition of IP_3 binding as $[Ca^{2+}]_m$ was increased to $\leqslant 1.1\,\mu$M confirms that the calmodulin had bound sufficient Ca^{2+} to bind to its Ca^{2+}-dependent targets. Furthermore, when $[Ca^{2+}]_m$ was increased to $\approx 100\,\mu$M, the inhibition of [^{3}H]IP_3 binding caused by a submaximal concentration of calmodulin was indistinguishable from that observed at all other $[Ca^{2+}]_m$ (Figure 2A). We conclude that the effect of calmodulin on type-1 IP_3 receptors is unusual in that it is entirely Ca^{2+}-independent.

Mechanism of calmodulin action

We reported previously that calmodulin inhibited IP_3 binding to purified cerebellar receptors [22], but it is now clear that such preparations may retain tightly associated accessory proteins, notably calcineurin and FKBP12 [48]. We therefore considered the possibility that calmodulin might exert its effects through tightly bound calcineurin, which is activated by calmodulin. Such an explanation is unlikely. Firstly, the effect of calcineurin on IP_3 receptors results from its protein phosphatase activity [48], yet the effects of calmodulin (which might then be expected to result from dephosphorylation) on IP_3 binding were fully reversed in the absence of ATP. Secondly, calcineurin is activated by Ca^{2+}–calmodulin, whereas the effects of calmodulin on IP_3 receptors are Ca^{2+}-independent (Figure 2C). Thirdly, the Sf9 membranes used in our experiments were prepared in Ca^{2+}-free medium, which has been reported to cause dissociation of calcineurin from cerebellar IP_3 receptors [48]. Finally, FK506 causes the FKBP12 that anchors calcineurin to the cerebellar IP_3 receptor to dissociate [48], but pretreatment of Sf9/IP_3R1 membranes with FK506 (1 μM, 5 min) had no effect on the ability of 1 μM calmodulin to inhibit IP_3 binding (results not shown). Stimulation of calcineurin is plainly not the means whereby calmodulin inhibits IP_3 binding. We conclude that the effect is likely to be mediated by direct binding of calmodulin to type-1 IP_3 receptors.

The only calmodulin-binding site so far unequivocally identified in the type-1 IP_3 receptor lies within its modulatory domain, but since it binds only Ca^{2+}–calmodulin [21], it cannot be the site through which the Ca^{2+}-independent effects of calmodulin on IP_3 binding are mediated. Whereas a consensus sequence to which apo-calmodulin binds has not been clearly defined [36,40], the IQ motif mediates calmodulin binding to several proteins in the absence of Ca^{2+} [40]. In most cases, however, the interaction of calmodulin with these proteins is not Ca^{2+}-*independent*, but occurs preferentially in the absence of Ca^{2+} [37,43]. It is perhaps not surprising, therefore, that there is no sequence conforming to an IQ motif within the type-1 IP_3 receptor, and nor is there any similarity with the apo-calmodulin-binding sites of other proteins that lack IQ motifs. We speculate from our results with ^{125}I-calmodulin binding (Table 3) that each subunit of the type-1 receptor is likely to provide a Ca^{2+}-independent calmodulin-binding site, but its location has yet to be established.

Physiological consequences

Type-1 IP_3 receptors are prevalent in brain [49], and their inhibition by calmodulin in a Ca^{2+}-independent fashion is likely to have important implications because it endows the receptor with a unique ability to reliably respond to changes in free calmodulin concentration irrespective of changes in cytosolic [Ca^{2+}]. Several abundant neuronal proteins (e.g. neurogranin and neuromodulin) bind apo-calmodulin with greater affinity than Ca^{2+}–calmodulin [41], and they share with other neuronal proteins (e.g. MARCKS, myristolyated alanine-rich C kinase substrate) [50] an ability to release calmodulin after phosphorylation by protein kinase C. It has been speculated that these proteins, and many others that bind only Ca^{2+}–calmodulin, allow

different intracellular messenger pathways to regulate the free cytosolic calmodulin concentration [41]. We suggest that such dynamic changes in calmodulin concentration are likely to profoundly influence the activity of type-1 IP_3 receptors and provide a means both of feedback regulation (via Ca^{2+} and protein kinase C) and of integrating the inputs from other signalling pathways. There is an interesting parallel with the role of calmodulin in light adaptation of *Drosophila* photoreceptors [39,51], where calmodulin-mediated inhibition of ryanodine receptors [52] and trpl channels [53] has been implicated, with the latter perhaps involving two calmodulin-binding sites, one of which binds calmodulin in the absence of Ca^{2+} [42].

Conclusions

The ability of calmodulin to inhibit type-1 IP_3 receptors is unusual in being entirely Ca^{2+}-independent and in thereby endowing the receptors with an ability to sense the free calmodulin concentration irrespective of the cytosolic [Ca^{2+}]. We speculate that this means of regulation, which is not shared with type-3 IP_3 receptors, allows the IP_3 sensitivity of type-1 receptors to be dynamically regulated by changes in free calmodulin concentration during stimulation of neurones.

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