Differential regulation of types-1 and -3 inositol trisphosphate receptors by cytosolic Ca^{2+}

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Biphasic regulation of inositol trisphosphate (IP_3)-stimulated Ca^{2+} mobilization by cytosolic Ca^{2+} is believed to contribute to regenerative intracellular Ca^{2+} signals. Since cells typically express several IP_3 receptor isoforms and the effects of cytosolic Ca^{2+} are not mediated by a single mechanism, it is important to resolve the properties of each receptor subtype. Full-length rat types-1 and -3 IP_3 receptors were expressed in insect Sf9 cells at levels 10–40-fold higher than the endogenous receptors. The expressed receptors were glycosylated and assembled into tetramers, and binding of [^{3}H]IP_3 to each subtype was regulated by cytosolic Ca^{2+}. The effects of increased [Ca^{2+}] on native cerebellar and type-1 receptors expressed in Sf9 cells were indistinguishable. A maximally effective increase in [Ca^{2+}] reversibly inhibited [^{3}H]IP_3 binding by approx. 50% by decreasing the number of IP_3-binding sites (B_{max}) without affecting their affinity for IP_3. The effects of Ca^{2+} on type-3 receptors were more complex: increasing [Ca^{2+}] first stimulated [^{3}H]IP_3 binding by increasing B_{max}, and then inhibited it by causing a substantial decrease in the affinity of the receptor for IP_3. The different effects of Ca^{2+} on the receptor subtypes were not a consequence of limitations in the availability of accessory proteins or of artifactual effects of Ca^{2+} on membrane structure. We conclude that Ca^{2+} can inhibit IP_3 binding to types-1 and -3 IP_3 receptors although by different mechanisms, and that IP_3 binding to type-3 receptors is stimulated at intermediate [Ca^{2+}]. A consequence of these differences is that, at resting cytosolic [Ca^{2+}], type-3 receptors are more sensitive than type-1 receptors to IP_3, but the situation reverses at higher cytosolic [Ca^{2+}]. Such differences may be important in generating the spatially and temporally complex changes in cytosolic [Ca^{2+}] evoked by receptors linked to IP_3 formation.

INTRODUCTION

The receptors for inositol 1,4,5-trisphosphate (IP_3) comprise a family of closely related channels that release Ca^{2+} from intracellular stores when they are activated by both IP_3 and cytosolic Ca^{2+} [1]. In mammals, at least three IP_3 receptor subtypes (1–3), which share up to 70% amino acid similarity, are encoded by distinct genes [2–4], and further diversity is generated by alternative splicing of the type-1 IP_3 receptor [5,6]. Each of these proteins is large (2671–2749 residues) and each is believed to assemble into tetrameric complexes of either the same [7] or different [8] subunits to form functional IP_3-gated Ca^{2+} channels. Another feature that appears to be shared by all IP_3 receptors is the presence of three major functional domains. The IP_3-binding domain lies largely within the N-terminal sequence [9,10] and the channel domain is formed by six or more membrane-spanning regions within the C-terminus [7]. An intermediate, and more diverse, modulatory domain includes phosphorylation sites and, according to receptor subtype, sites to which modulators such as ATP, Ca^{2+} and calmodulin can bind [3,10–22].

Limited progress has so far been made towards understanding the functional significance of IP_3 receptor diversity. The receptor subtypes are differentially expressed in different cell types [5,6,13,14], within different subcellular locations [15,16], and at specific developmental stages [6]. They are also susceptible to differential down-regulation during sustained activation of Ca^{2+}-mobilizing receptors [13]. Splice variants of the type-1 receptor vary in their phosphorylation by cAMP-dependent protein kinase [5], and the types-1 and -2, but not the type-3, receptor binds Ca^{2+}/calmodulin [17]. From analyses of fusion proteins encoding the N-terminal IP_3-binding domain [3,18], the three receptor subtypes have been proposed to differ in their affinities for IP_3 ($2 > 1 > 3$). The pattern is, however, less clear when the relative affinities of native IP_3 receptors from cells in which a single subtype predominates are compared [19,20].

The types-1 and -3 receptor have also been proposed to fulfil different roles in Ca^{2+} signalling, with the type-1 receptor perhaps mediating Ca^{2+} mobilization [21] and regenerative Ca^{2+} signals [22], while the type-3 receptor is possibly involved in regulating Ca^{2+} entry across the plasma membrane [22,23]. Type-3 receptors have also been specifically implicated in apoptosis in lymphocytes [24]. Selective disruption of the genes encoding IP_3 receptors has recently challenged suggestions that each subtype has a unique role. In these experiments, the Ca^{2+} entry evoked by depletion of intracellular Ca^{2+} stores persisted in the complete absence of IP_3 receptors, and IP_3-evoked Ca^{2+} mobilization was abolished only when expression of all three IP_3 receptor subtypes was prevented [25].

In most tissues, although perhaps not all, cytosolic Ca^{2+} biphasically regulates IP_3-stimulated Ca^{2+} mobilization, and this regulation is believed to be an important component of the mechanisms responsible for propagation of regenerative intracellular Ca^{2+} waves [1]. However, neither the effects of Ca^{2+} on binding of IP_3 to its receptors [26,27] nor the role of phosphorylation in mediating the effects of Ca^{2+} on IP_3-stimulated Ca^{2+} mobilization [27,28] are conserved between tissues. Both the

Abbreviations used: B_{max}, maximal number of binding sites; $[Ca^{2+}]_m$, medium free [Ca^{2+}]; CLM, cytosol-like medium; ECL, enhanced chemiluminescence; IP_3, inositol 1,4,5-trisphosphate; *h*, Hill coefficient; Sf9/IP_3R-1, Sf9/IP_3R-3, *Spodoptera frugiperda* cells expressing types-1 and -3 IP_3 receptors respectively; ConA, concanavalin A.

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effects of Ca^{2+} on IP_3 receptors and the underlying mechanisms may therefore differ between IP_3 receptor subtypes.

Further progress in understanding the significance of IP_3 receptor diversity requires a more complete characterization of the properties of each subtype, but progress is compromised by considerable practical problems. Cells, even clonal cell lines, almost invariably express a mixture of IP_3 receptor subtypes [13,14,29], which together with the existence of heterotetramers [8], leaves little scope for selecting cells in which to examine the behaviour of only a single receptor subtype. Expression of fragments of IP_3 receptors has been useful in, for example, defining the sites to which IP_3, ATP and Ca^{2+}/calmodulin bind [3,17]. However, such studies are unable to resolve the more complex properties of the receptors, the influence of the modulatory domain on IP_3 binding, for example. Expression of full-length IP_3 receptors in mammalian cells is likely to be severely compromised by the presence of an endogenous complement of receptors and by the complex compensatory changes in cellular properties that appear to follow overexpression of IP_3 receptors in mammalian cells [30].

Baculovirus expression systems allow high levels of expression of large recombinant proteins in insect Sf9 cells under conditions in which post-translational modifications are likely to be similar to those occurring in mammalian cells [31]. In the present study, we have expressed rat types-1 and -3 IP_3 receptors in Sf9 cells and examined the effects of Ca^{2+} on IP_3 binding. The high levels of expression obtained allowed the properties of the recombinant rat IP_3 receptors to be characterized without significant interference from endogenous receptors and in a cellular context that was identical for each receptor subtype. A preliminary abstract of this work has been published [32].

EXPERIMENTAL

Expression of IP_3 receptors in Sf9 cells

Full-length cDNAs for rat type-1 IP_3 receptor (lacking S1 splice site) in pCMVI-9 [2] and rat type-3 IP_3 receptor in pCB6 [4] were gifts from Dr. T. C. Südhof (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) and Dr. G. I. Bell (University of Chicago, IL, U.S.A.) respectively. Each was subcloned into the baculovirus (*Autographa californica*) transfer vector pBacPAK9 (Clontech, Palo Alto, CA, U.S.A.). Recombinant viruses were produced in Sf9 cells by standard techniques [31] from transfer vectors and linearized *Autographa californica* nuclear polyhedrosis virus DNA using a transfection kit (Invitrogen, NV Leek, The Netherlands).

Sf9 cells (2×10^5/cm^2) were cultured in TNM-FH insect medium (Sigma) supplemented with foetal calf serum (10 %), fungizone (2.5 μg/ml) and gentamicin (50 μg/ml), and then infected with recombinant viruses at a multiplicity of infection of 2–6. Infected cells were harvested 40 h after infection by scraping and then centrifugation at 1000 ***g*** for 5 min at 2 °C. Cell pellets were washed twice in PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.76 mM KH_2PO_4, pH 7.0) at 2 °C and resuspended (10^6 cells/ml) in Ca^{2+}-free cytosol-like medium (Ca^{2+}-free CLM: 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). The cells were then homogenized using an Ultra-Turrax T25 homogenizer (10 strokes at 9000 rev./min) and the homogenate was centrifuged (3000 ***g***; 10 min) to sediment a pellet (P1) containing both membranes and nuclei. The resulting supernatant was then further centrifuged (100000 ***g***; 60 min) to sediment the remaining membranes (P2) that lacked nuclei. Each fraction (4–6 mg of protein/ml) was resuspended in Ca^{2+}-free CLM and stored at −80 °C after rapid freezing in liquid nitrogen. Protein concentrations were determined [33] using BSA as a standard.

IP_3 receptors were solubilized from Sf9 membranes (4 mg of protein/ml) by incubation at 2 °C for 2 h in Ca^{2+}-free CLM containing 1 % (w/v) CHAPS. After centrifugation (130000 ***g***; 1 h), the supernatant was rapidly frozen in liquid nitrogen and then stored at −80 °C. For both Sf9/IP_3R-1 and Sf9/IP_3R-3 membranes, approx. 60 % of the [^{3}H]IP_3-binding sites were recovered in the supernatant. [^{3}H]IP_3 binding (1 nM) to the solubilized receptors was then characterized in CLM containing 1 % CHAPS using a poly(ethylene glycol) precipitation method as previously described [34].

Antibody methods

Peptides corresponding to the C-terminal residues (2724–2739) of rat type-1 IP_3 receptor (Pep1: CLLGHPPHMNVNPQQPA) and to N-terminal residues that are similar in all IP_3 receptors (PepC: PMNRYSAQKQFWKAC, residues 62–75 in rat type-1 IP_3 receptor; F is replaced by Y in types-2 and -3 receptors) were used to raise antibodies. The terminal cysteine residue of Pep1, which is not present in the IP_3 receptor, allowed coupling to maleimidobenzoyl-*N*-hydroxysuccinimide-activated keyhole limpet haemocyanin at pH 7 using an ImJect Kit (Pierce and Warriner, Chester, Lancs., U.K.). The terminal cysteine of PepC, which is also absent from the IP_3 receptor, was coupled to maleimidobenzoyl-*N*-hydroxysuccinimide-activated keyhole limpet haemocyanin at pH 6 because it was insoluble at pH 7 [35]. Male New Zealand White rabbits (750 g) were injected subcutaneously with 1 mg of conjugated peptide (0.5 ml) mixed with Freund's complete adjuvant (0.5 ml). At monthly intervals, rabbits were boosted with 1 mg of conjugated peptide (0.5 ml) mixed with Freund's incomplete adjuvant (0.5 ml), until sera of appropriate specificity and titre were obtained [35]. An anti-peptide rabbit antiserum (Ab3) specific for rat type-3 IP_3 receptors (Pep3: LGFVDVQNCMSR, residues 2659–2670) was a gift from Dr. J.-P. Mauger (INSERM 0274, Orsay, France).

For immunoblotting, proteins (10–30 μg) were resuspended in SDS sample buffer (final concentrations 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.2 % Bromophenol Blue and 62.5 mM Tris/HCl, pH 6.8) and then boiled (3 min). After SDS/PAGE (5 % gel), te separated proteins were electrotransferred [35] to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) using a cooled wet-tank transfer system (Bio-Rad, Hemel Hempstead, Herts., U.K.). Blots were probed with primary antisera (Ab1, Ab3, AbC; diluted 1:1000 in PBS), reprobed with goat anti-rabbit antibody coupled to horseradish peroxidase (diluted 1:2000 in PBS; Sigma) [35], and then visualized with the enhanced chemiluminescence (ECL) system using Hyperfilm (Amersham).

The binding of AbC to types-1 and -3 IP_3 receptors was compared by quantitative comparison of immunoblots with protein measurements of the IP_3 receptor bands. Gels loaded with a range (5–40 μg) of membrane protein (P1 fraction) from cells expressing either the type-1 (Sf9/IP_3R-1) or type-3 receptor (Sf9/IP_3R-3) were stained with Coomassie Blue, and the IP_3 receptor bands (250–260 kDa) were quantified by scanning densitometry. Parallel gels were immunoblotted and the reactivity of the corresponding bands with AbC was also quantified by scanning densitometry. For both measurements, there was a linear relationship between absorbance and the amount of membrane protein loaded on to the gel, indicating that the ratio of the two measurements (immunoreactivity/protein) pro-

vides a reliable index of the reaction between the antiserum and the two receptor subtypes.

To establish the oligomeric state of the expressed IP_3 receptors, the IP_3 receptor subunits of membranes from Sf9/IP_3R-1 and Sf9/IP_3R-3 were covalently cross-linked before agarose/SDS/PAGE. Membranes from infected cells were washed twice with sodium phosphate (50 mM, pH 8.0), and then resuspended in the same buffer at a protein concentration of approx. 4 mg/ml. Samples (200 μl) were then incubated with a range of concentrations (0–10 mM) of bis(sulphosuccinimidyl) suberate for 20 min at 20 °C. The incubations were quenched by addition of ammonium acetate (1 M; 40 μl), the mixtures were centrifuged (20000 ***g***; 1 min), and the membranes were then solubilized in SDS/PAGE sample buffer (3 min; 100 °C). The samples were then analysed by SDS (1 %)/agarose (0.5 %)/polyacrylamide (1.75 %) gel electrophoresis [36] and immunoblotted with AbC (see above).

The glycosylation of cerebellar and recombinant IP_3 receptors was compared after SDS/PAGE of membrane protein (5 or 10 μg) followed by quantification of the immunoreactivity (to AbC) of the 250–260 kDa bands. Gels run in parallel were blotted on to nitrocellulose membranes (Millipore), blocked with PBS containing 0.1 % Tween 20, and incubated with peroxidase-labelled concanavalin A (ConA) (1 μg/ml in PBS; Sigma); they were then washed and visualized using ECL and Hyperfilm (see above). Since there was no ConA staining of the 250–260 kDa band from mock-infected Sf9 cells (not shown), the ratio of ConA staining to AbC immunoreactivity (each of which increased linearly with the amount of membrane loaded on to the gel) was used to provide an index of glycosylation.

Equilibrium [^{3}H]IP_3-binding assays

Membranes ($\sim$ 100 μg) were incubated at 2 °C in CLM (0.5 ml) containing [^{3}H]IP_3 (0.6 nM) and an appropriate concentrations of unlabelled IP_3. After 5 min, during which equilibrium binding was attained, the incubations were stopped by either centrifugation (20000 ***g***; 5 min) followed by aspiration of the supernatant or addition of 2 ml of cold sucrose (310 mM) containing sodium citrate (10 mM) followed by rapid filtration through Whatman GF/B filters and two washes with 2 ml of the sucrose medium. Similar results were obtained with either method. Equilibrium competition binding results were fitted to a four-parameter logistic equation using least-squares curve-fitting routines (Kaleidagraph; Synergy Software, Reading, PA, U.S.A.) as previously described [37]. Non-specific binding, which was always less than 10 % of total binding, was similar whether determined in the presence of 1 μM IP_3 or by extrapolation of the curve fits to infinite IP_3 concentration.

Membranes were prepared from rat cerebella as previously described [34] and [^{3}H]IP_3 binding was characterized using exactly the same media and methods as were used for membranes from Sf9 cells.

The free [Ca^{2+}] of CLM was determined fluorimetrically with fura 2 using a K_d for Ca^{2+}–Fura 2 of 392 nM at 2 °C as previously described [37]. Each measurement of medium free [Ca^{2+}] ([Ca^{2+}]$_m$) was repeated at least three times with S.E.M.s that were 5 % or less of the mean values reported in the Figures.

Materials

Except where otherwise specified, cell culture materials were from Life Sciences (Paisley, Scotland, U.K.). Peptides were synthesized using the PerSeptire Biosystems 9050 PepSynthesiser (PerSeptive Biosystems, Hertford, Herts., U.K.). Bis(sulphosuccinimidyl) suberate was supplied by Pierce (Rockford, IL, U.S.A.), and fura 2 was from Molecular Probes (Leiden, The Netherlands). Kaleidoscope prestained molecular-mass protein markers were from Bio-Rad (Hercules, CA, U.S.A.). [^{3}H]IP_3 (54 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.). All other reagents were from Sigma.

RESULTS AND DISCUSSION

Expression of types-1 and -3 IP_3 receptors in Sf9 cells

Equilibrium competition binding with [^{3}H]IP_3 revealed that membranes prepared from uninfected Sf9 cells expressed very few IP_3-binding sites ($B_{max} = 0.28 \pm 0.05$ pmol/mg of protein; $K_d = 20 \pm 4.2$ nM; $n = 3$) and the membranes (30 μg) showed no detectable reaction with any of the antibodies (Ab1, Ab3, AbC) (Figure 1). Similar results were obtained with mock-infected Sf9 cells (not shown). In contrast, both the P1 and P2 fractions prepared from Sf9/IP_3R-1 or Sf9/IP_3R-3 cells expressed high levels of IP_3 binding (Table 1) and reacted appropriately with their cognate antibodies (Figure 1). The sizes of the expressed receptors (250–260 kDa) (Figure 1) were as expected from previous analyses of native proteins [18], and the type-3 receptor was consistently about 9 kDa smaller than the type-1 receptor, again consistent with it being 79 residues shorter [2,4]. The P1 fractions of infected cells invariably expressed 2–3-fold higher levels of IP_3 binding than the P2 fractions; the P1 fractions were therefore used for the experiments reported below. There were no significant differences between the P1 and P2 fractions in either the affinity of the expressed receptors for IP_3 or the modulatory effects of Ca^{2+} (not shown).

To provide another means of quantifying levels of IP_3 receptor expression, we raised a polyclonal antiserum to a peptide (PepC) corresponding to a sequence found within the N-terminal of all known IP_3 receptors (see the Experimental section). Quantitative comparison of the protein content of the IP_3 receptor bands on gels with their immunoreactivity with AbC established that the antibody reacted equally well with type-1 IP_3 receptors (immunoreactivity/protein $= 2.9 \pm 0.1$, $n = 3$) and type-3 IP_3 receptors (2.7 ± 0.2, $n = 3$). Densitometric scans of immunoblots after reaction with AbC can therefore be used to quantify the levels of

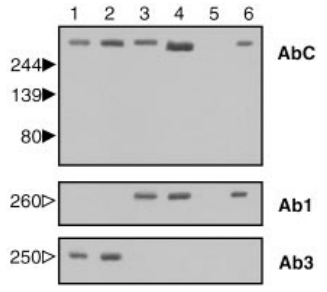

Figure 1 Immunological identification of types-1 and -3 IP_3 receptors expressed in Sf9 cells

Lanes were loaded with 10 μg (lanes 1 and 3) or 20 μg (lanes 2 and 4) of protein from the P1 fractions of Sf9/IP_3R-3 (lanes 1 and 2) or Sf9/IP_3R-1 (lanes 3 and 4) cells. Lane 5 was loaded with 30 μg of protein from uninfected Sf9 cells and lane 6 with 20 μg of protein from rat cerebellar membranes. The blots were probed with each of the three antibodies (Ab1, Ab3 and AbC) as described in the Experimental section and visualized using ECL and Hyperfilm. The results, which are each typical of three independent experiments, show the entire blot probed with AbC and only the relevant area (around 260 kDa) of the blots probed with Ab1 and Ab3. The positions of molecular-mass markers are indicated. The non-specific staining of the blots was similar for each of the antibodies.

Table 1 Effects of $[Ca^{2+}]_m$ on $[^3H]IP_3$ binding to membranes from Sf9 cells expressing types-1 and -3 IP_3 receptors

Equilibrium competition binding curves were performed in CLM containing the indicated $[Ca^{2+}]_m$. B_{max} values are expressed relative to both total membrane protein (pmol/mg of protein) and the amount of immunoreactivity to AbC detected within the 250–260 kDa band after immunoblotting (pmol/AbC). The results are means ± S.E.M. for three to six independent experiments. Most results are those obtained from experiments in which the level of expression (determined using AbC) of type-1 receptor was 134 ± 3% of that of the type-3 receptor. The values in parentheses are from separate experiments in which the levels of expression of both receptor subtypes were higher (573 ± 24 and 274 ± 9% of their levels in the major experiments for types-1 and -3 respectively). nd, not determined.

Receptor subtype	$[Ca^{2+}]_m$ (nM)	K_d (nM)	B_{max} (pmol/mg of protein)	B_{max} (pmol/AbC)	h
1	~ 2	10.8 ± 1.2	6.8 ± 0.2	5.5 ± 0.2	1.09 ± 0.09
		(12.1 ± 1.8)	(65 ± 2)	(9.1 ± 0.3)	(1.0 ± 0.03)
	100	9.8 ± 1.1	6.2 ± 0.9	5.0 ± 0.7	0.91 ± 0.17
	300	10.1 ± 1.3	4.9 ± 0.1	3.9 ± 0.1	0.96 ± 0.13
		(9.0 ± 1.6)	(76 ± 4)	(10.7 ± 0.4)	(0.97 ± 0.1)
	1100	9.6 ± 1.9	3.2 ± 0.13	2.6 ± 0.1	1.0 ± 0.16
		(10.9 ± 1.9)	(32 ± 1)	(4.5 ± 0.1	(1.0 ± 0.04)
3	~ 2	2.05 ± 0.2	4.0 ± 0.2	4.3 ± 0.2	1.08 ± 0.1
		(2.92 ± 1.12)	(10.8 ± 1.44)	(4.3 ± 0.6)	(0.85 ± 0.03)
	100	2.3 ± 0.4	3.1 ± 0.3	3.3 ± 0.3	1.01 ± 0.2
	500	2.0 ± 0.4	7.2 ± 0.3	7.7 ± 0.3	0.93 ± 0.17
		(2.04 ± 0.54)	(21.8 ± 2.2)	(8.6 ± 0.9)	(0.96 ± 0.21)
	1100	14.4 ± 0.9	13.3 ± 0.5	14.3 ± 0.5	0.99 ± 0.05
		(15.7 ± 2.9)	(42.3 ± 4.1)	(16.6 ± 1.6)	(0.96 ± 0.13)
Native Sf9	~ 2	20 ± 4.2	0.28 ± 0.05	nd	0.8 ± 0.1
Cerebellum	~ 2	8.54 ± 0.73	1.42 ± 0.13	12.9 ± 1.2	0.88 ± 0.06
	1100	8.24 ± 0.09	0.89 ± 0.22	nd	1.10 ± 0.07

Table 2 Levels of expression of types-1 and -3 IP_3 receptors in Sf9 cells

Expression levels of recombinant IP_3 receptors were quantified by scanning densitometry of Western blots stained with AbC. The first two lines compare the levels of expression obtained (%) from cells in which the multiplicity of infection was adjusted to achieve high or low levels of expression. The lower lines compare the levels of expression of the type-1 receptor relative to the type-3 receptor (%) at both low and high levels of expression of each. Results are from n independent infections.

	AbC immunoreactivity (%)	n
Sf9/IP_3R-1: high/low level expression	573 ± 24	3
Sf9/IP_3R-3: high/low level expression	274 ± 9	3
Low levels of expression: IP_3R-1/IP_3R-3	134 ± 3	3
High levels of expression: IP_3R-1/IP_3R-3	319 ± 32	5

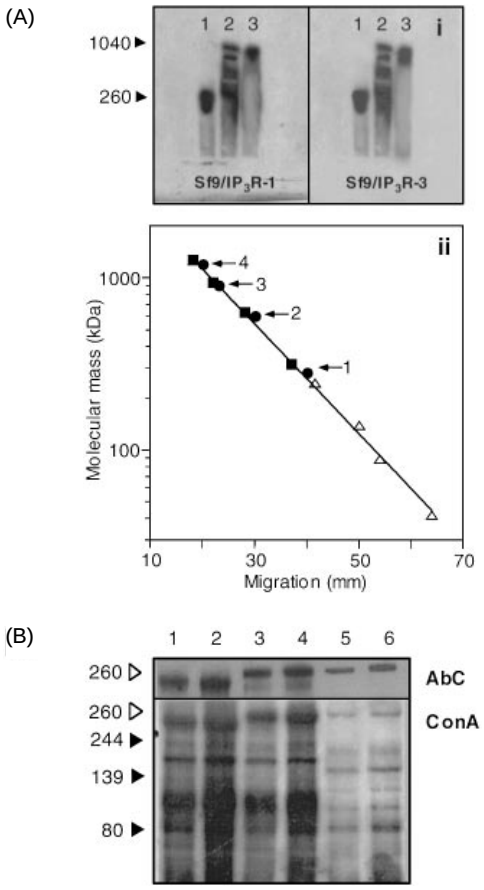

Figure 2 IP_3 receptors expressed in Sf9 cells are tetrameric and glycosylated

(**A**) Membranes from Sf9/IP_3R-1 or Sf9/IP_3R-3 cells were incubated for 20 min (lanes 1 and 2) or 120 min (lane 3) with 0 (lane 1), 10 (lane 2) or 40 (lane 3) mM bis(sulphosuccinimidyl) suberate, the cross-linking agent. The proteins were then separated on SDS/agarose/polyacrylamide gels, and immunoblotted with AbC as described in the Experimental section. Arrowheads indicate the positions of proteins corresponding to a single subunit (260 kDa) and a cross-linked assembly of four subunits (1040 kDa) (i). The lower panel (ii) shows the relative rates of migration of standard protein markers (△) from which the molecular mass of the monomeric IP_3 receptor was estimated by extrapolation of the graph (260 and 250 kDa for types-1 and -3 receptor respectively; labelled 1). The three bands of higher molecular mass (labelled 2, 3 and 4) detected after cross-linking of type-1 (■) and type-3 (●) receptors were assumed to correspond to di-, tri- and tetra-meric cross-linked assemblies, and their molecular masses were therefore plotted as the corresponding multiples of the monomeric masses. The validity of this assumption is confirmed by the demonstration that the mobilities of the cross-linked assemblies correspond closely to the mobilities predicted from their estimated masses and extrapolation of the standard curve. (**B**) Lanes were loaded with either 5 μg (lanes 1, 3 and 5) or 10 μg (lanes 2, 4 and 6) of protein from Sf9/IP_3R-3 (lanes 1 and 2), Sf9/IP_3R-1 (lanes 3 and 4) or cerebellar membranes (lanes 5 and 6). After SDS/PAGE, parallel gels were blotted, probed with either AbC (upper panel) or peroxidase-labelled ConA (major panel), and visualized using ECL. The results, which are typical of three independent experiments, show the entire blot probed with ConA and only the relevant area (250–260 kDa) of that probed with AbC. Filled arrowheads indicate the positions of molecular-mass markers and open arrowheads the positions of the IP_3 receptor bands.

expression of the two IP_3 receptor subtypes. This method established that, under the conditions used for our preliminary experiments, the levels of IP_3 receptor expression were 3.2 ± 0.3-fold ($n = 5$) higher in Sf9/IP_3R-1 cells than in Sf9/IP_3R-3 cells (Table 2). In all comparisons of receptors from these cells (= high level of expression), the amounts of membrane protein added to an incubation were adjusted to ensure that each included the same concentration of IP_3 receptors as determined by their reactivity with AbC. Unless otherwise stated, in all subsequent experiments the multiplicity of infection was adjusted to ensure similar levels of IP_3 receptor expression (measured with AbC) in Sf9/IP_3R-1 and Sf9/IP_3R-3 cells. In these later experiments (= low level of expression), the levels of expression of the type-1 receptor (measured with AbC) was 134 ± 3% of that obtained with the type-3 receptor (Table 2). The levels of expression in infected cells were always at least 10-fold higher than in uninfected cells (Table 1).

Native IP_3 receptors are N-glycosylated [38] and tetrameric [7,8]. Our cross-linking studies with the recombinant receptors established that when expressed in Sf9 cells, both the type-1 and

type-3 receptor subunits also assembled to form homotetrameric structures (Figure 2A). The glycosylation state of cerebellar and recombinant IP_3 receptors was compared by quantifying the amount of peroxidase-labelled ConA bound to each. The ratio of conA binding to AbC immunoreactivity (ConA/AbC) was similar for cerebellar receptors (0.78 ± 0.17; $n = 3$) and for the types-1 (0.72 ± 0.04; $n = 3$) and -3 (0.77 ± 0.07; $n = 3$) recombinant receptors expressed in Sf9 cells (Figure 2B).

These results establish that baculovirus and insect Sf9 cells allow full-length recombinant rat types-1 and -3 IP_3 receptors to be expressed at comparable levels (Tables 1 and 2), in exactly the same cellular context, and at levels that ensure that any contribution from endogenous IP_3 receptors is insignificant. Furthermore, both the assembly of the subunits into tetrameric receptors and the presence of N-linked glycosylation are preserved in Sf9 cells.

IP_3 binding to types-1 and -3 IP_3 receptors

In Ca^{2+}-free CLM, [^{3}H]IP_3 bound to a single class of site in both Sf9/IP_3R-1 ($K_d = 10.8 \pm 1.2$ nM; $n = 4$) and Sf9/IP_3R-3 ($K_d = 2.05 \pm 0.20$ nM; $n = 4$) cells, but the type-3 receptor had approx. 5-fold higher affinity for IP_3 (Table 1). Similar results were obtained when the receptors were expressed at higher levels (Table 1). The affinity of Sf9/IP_3R-1 is indistinguishable from that of the native cerebellar type-1 receptor ($K_d = 8.54 \pm 0.73$ nM; $n = 3$) (Table 1) measured under identical conditions. Our results contrast with a similar analysis of human IP_3 receptors in which the type-1 had marginally higher affinity than the type-3 receptor for IP_3 in nominally Ca^{2+}-free medium, and both subtypes had substantially lower affinities ($K_d \sim 100$ nM) than those observed in the present study [39]. The 40-fold discrepancy between the affinities of the type-3 IP_3 receptors in the two studies is particularly striking; it is unlikely to result from differences between species because the human and rat receptors are extremely similar [12]. Nor is the slightly higher pH (7.1 compared with 7.0) used in the former study likely to be significant because when we compared [^{3}H]IP_3 binding to membranes from Sf9/IP_3R-1 ($K_d = 12.1 \pm 1.8$ nM; $n = 4$) and Sf9/IP_3R-3 ($K_d = 2.92 \pm 1.12$ nM; $n = 4$) cells at pH 7.4, there was a similar approx. 4-fold discrepancy in the relative affinities of the two subtypes. A possible explanation is that the approx. 18-fold higher level of expression of the human type-3 receptors relative to the type-1, which was accompanied by increased degradation (Figure 1 of [39]), may have caused the apparent affinity of the type-3 receptor for IP_3 to be reduced [39] (see below).

Previous comparison of the affinities of the bacterially expressed N-terminal IP_3-binding domains of types-1 and -3 IP_3 receptors in Ca^{2+}-free media suggested that the type-1 receptor ($K_d = 6$ nM) bound IP_3 with about 10-fold greater affinity than the type-3 receptor ($K_d = 67$ nM) [18]. Both mutational analyses [9,10] and photoaffinity labelling [40] have established that the N-terminal residues of type-1 IP_3 receptors are major determinants of IP_3 binding. However, in native receptors, this domain may be closely associated with C-terminal residues [41] and these may also influence IP_3 binding [42]. The affinity for IP_3 of native cerebellar IP_3 receptors, which are almost entirely type-1 [29] ($K_d = 8.54 \pm 0.73$ nM), of type-1 IP_3 receptors expressed in Sf9 cells (Table 1; $K_d = 10.8 \pm 1.2$ nM) and of the bacterially expressed N-terminal domain of type-1 IP_3 receptors ($K_d = 6.0 \pm 2.0$ nM) are similar [18]. In contrast, the affinity of IP_3 for full-length type-3 receptors expressed in COS cells ($K_d = 29$ nM) [43] and Sf9 cells ($K_d = 2.05 \pm 0.02$ nM) (Table 1) is higher than that of the N-terminal domain of the type-3 receptor ($K_d = 67$–151 nM) [12,18].

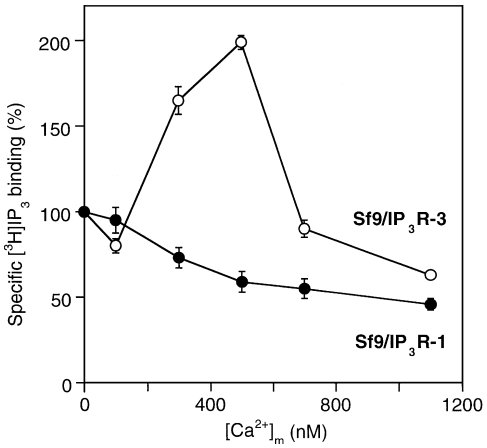

Figure 3 Effects of $[Ca^{2+}]_m$ on [^{3}H]IP_3 binding to Sf9/IP_3R-1 and Sf9/IP_3R-3 membranes

Specific binding of [^{3}H]IP_3 (0.6 nM) to membranes from Sf9/IP_3R-1 (●) and Sf9/IP_3R-3 (○) cells was measured in CLM containing the indicated $[Ca^{2+}]_m$. Specific [^{3}H]IP_3 binding (typically 2500–4000 d.p.m.) is shown as percentages of that observed in CLM containing about 2 nM free [Ca^{2+}]. The results are means ± S.E.M. for three independent experiments, each performed in duplicate (most error bars are smaller than the symbols).

The types-1 and -3 receptors have similar ligand selectivity [12,18], their sequences are very similar within their N-terminal 576 residues (82%) [18], and there is complete conservation of the basic residues shown to be essential for IP_3 binding to type-1 receptors [9]. These observations, together with our results, suggest that although the N-terminal domain includes the major determinants of IP_3 binding for each receptor subtype, regions of the protein beyond these N-terminal residues may enhance IP_3 binding to type-3 receptors. The lower affinity of the type-3 receptors used by Yoneshima et al. [39], which more closely approximates that of its N-terminal domain [12,18], might then be a consequence of having disrupted an association between the N-terminal and more distant residues (see above). Our suggestion would also be consistent with the observation that in Ca^{2+}-free medium, the apparent affinity for IP_3 of receptors in A7r5 cells, which express 73% type-1 and 26% type-3 IP_3 receptor [29], has been reported to be 2-fold higher than that of the type-1 receptors of cerebellum [19]. Our comparison of [^{3}H]IP_3 binding in Ca^{2+}-free CLM at pH 7.4 to membranes from A7r5 cells ($K_d = 6.7 \pm 2.5$ nM; $n = 3$) and cerebellum ($K_d = 10.1 \pm 1.4$ nM; $n = 3$) has confirmed that cells expressing types-1 and -3 IP_3 receptors have a higher apparent affinity for IP_3 than those expressing only type-1 receptors. Assuming that the type-1 receptors of A7r5 cells and cerebellum bind IP_3 with similar affinity [18], these results suggest that in Ca^{2+}-free medium, native type-3 receptors must bind IP_3 with about 3–5-fold higher affinity than native type-1 receptors. This is quantitatively comparable with the results from Sf9 cells in which the full-length type-3 receptor exhibits an approx. 5-fold higher affinity than the type-1 receptor for IP_3 (Table 1).

Ca^{2+} inhibits IP_3 binding to type-1 receptors

Increasing $[Ca^{2+}]_m$ caused a progressive inhibition of [^{3}H]IP_3 binding to membranes prepared from Sf9/IP_3R-1 cells. The inhibition was half-maximal when $[Ca^{2+}]_m$ was about 300 nM and the maximal extent of the inhibition (54 ± 3%), which occurred when $[Ca^{2+}]_m$ was 1.1 μM (Figure 3), could not be

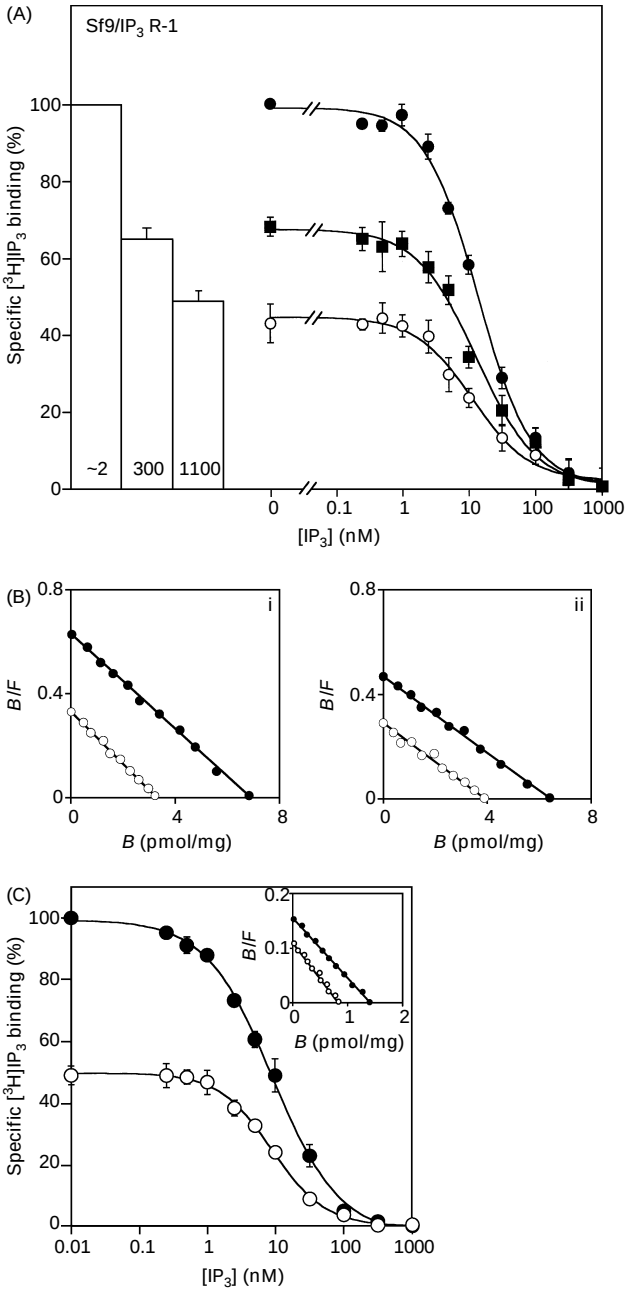

Figure 4 Inhibition of [^{3}H]IP_3 binding to Sf9/IP_3R-1 and cerebellar membranes by increased $[Ca^{2+}]_m$

(**A**) Membranes from Sf9/IP_3R-1 cells were incubated with [^{3}H]IP_3 (0.6 nM) and the indicated concentrations of unlabelled IP_3 in CLM in which $[Ca^{2+}]_m$ was approx. 2 nM (●), 300 nM (■) or 1.1 μM (○). The results (means ± S.E.M. for four independent experiments, each performed in duplicate) are expressed as percentages of the maximal specific binding observed when $[Ca^{2+}]_m$ was about 2 nM. The histograms show the effects of the indicated $[Ca^{2+}]_m$ on specific [^{3}H]IP_3 binding to solubilized receptors from Sf9/IP_3R-1 membranes (means ± S.E.M.; $n = 3$). (**B**) The results obtained from (**A**) (i; $n = 4$) are compared with those obtained from similar experiments in which the [^{3}H]IP_3 concentration was increased to 6 nM (ii; duplicate determinations from a single experiment). Both sets of results are shown as Scatchard plots in which *B* and *F* denote the specifically bound and free [IP_3] respectively, and the symbols are the same as in (**A**). (**C**) Cerebellar membranes were incubated with [^{3}H]IP_3 (0.6 nM) and the indicated concentrations of unlabelled IP_3 in CLM in which $[Ca^{2+}]_m$ was either approx. 2 nM (●) or 1.1 μM (○). The results (means ± S.E.M. for three independent experiments, each performed in duplicate) are expressed as percentages of the maximal specific binding observed when $[Ca^{2+}]_m$ was about 2 nM. The inset shows the corresponding Scatchard plot for cerebellar membranes in which *B* and *F* denote the specifically bound and free [IP_3] respectively.

Table 3 Reversible effects of Ca^{2+} on [^{3}H]IP_3 binding to types-1 and -3 IP_3 receptor

Membranes from Sf9/IP_3R-1 and Sf9/IP_3R-3 cells were incubated for 5 min in CLM with $[Ca^{2+}]_m$ buffered at either about 2 nM or 1.1 μM; the latter reduced specific [^{3}H]IP_3 binding to 49 ± 5% (type 1, $n = 3$) and 66 ± 4% (type 3, $n = 3$) of the level observed when $[Ca^{2+}]_m$ was about 2 nM, confirming the results shown in Figure 3. The membranes were then diluted 2-fold into CLM containing additional EGTA and $CaCl_2$ such that their final concentrations were 45 μM $CaCl_2$ and 5.05 mM EGTA (final $[Ca^{2+}]_m$ about 2 nM) irrespective of the initial incubation conditions. Equilibrium competition binding analyses with [^{3}H]IP_3 were then performed in this medium. The results (means ± S.E.M. for three independent experiments) are from membranes incubated in CLM with $[Ca^{2+}]_m$ about 2 nM throughout, or first with CLM with $[Ca^{2+}]_m$ of 1.1 μM before restoring it to about 2 nM.

	Sf9/IP_3R-1		Sf9/IP_3R-3	
$[Ca^{2+}]_m$ ...	~ 2 nM	1.1 μM to ~ 2 nM	~ 2 nM	1.1 μM to ~ 2 nM
K_d (nM)	10.4 ± 1.1	12.3 ± 0.9	2.4 ± 0.2	2.5 ± 0.3
B_{max} (pmol/mg of protein)	7.1 ± 1.2	7.0 ± 1.1	4.2 ± 0.15	4.4 ± 0.21
h	1.1 ± 0.2	0.94 ± 0.1	1.14 ± 0.2	1.2 ± 0.2

further increased by raising $[Ca^{2+}]_m$ to 1 mM. Over the full range of $[Ca^{2+}]_m$, the Ca^{2+}-mediated inhibition of [^{3}H]IP_3 binding to Sf9/IP_3R-1 membranes was entirely attributable to a progressive decrease in the maximal number of binding sites (B_{max}), with no change in either the Hill coefficient (*h*) or the affinity (K_d) of the sites for IP_3 (Figures 4A and 4B, Table 1). Although these results concur with previous studies in demonstrating that increases in $[Ca^{2+}]_m$ inhibit [^{3}H]IP_3 binding to type-1 IP_3 receptors [26], they differ in that the inhibition has previously been reported to result entirely from a Ca^{2+}-mediated decrease in the apparent affinity of the receptor for IP_3 [39,44,45]. We were concerned at the unexpected decrease in B_{max} caused by increasing $[Ca^{2+}]_m$ and therefore re-examined the effects of $[Ca^{2+}]_m$ on IP_3 binding to Sf9/IP_3R-1 membranes at a 10-fold higher radioligand concentration (6 nM) in order to increase the likelihood of detecting a low-affinity site that might have been undetectable at the lower radioligand concentration (0.6 nM) [46]. The results, derived from only a single experiment because of the substantial cost of the radioligand, confirm that the decrease in [^{3}H]IP_3 binding caused by increasing $[Ca^{2+}]_m$ from about 2 nM to 1.1 μM results entirely from a decrease in B_{max} (Figure 4Bii).

The inhibition of [^{3}H]IP_3 binding caused by increasing $[Ca^{2+}]_m$ from about 2 nM to 1.1 μM was identical for Sf9/IP_3R-1 membranes (54 ± 3%; $n = 4$) and for solubilized receptors prepared from them (56 ± 3%; $n = 3$) (Figure 4A), confirming that the effects of Ca^{2+} were not an artifactual consequence of Ca^{2+} affecting membrane structure. The effects of increasing $[Ca^{2+}]_m$ were fully reversible (Table 3) in the complete absence of ATP and were not therefore a result of either proteolysis or changes in the phosphorylation of the receptors. The incomplete inhibition of [^{3}H]IP_3 binding in Sf9/IP_3R-1 membranes by increased $[Ca^{2+}]_m$ was unlikely to be a consequence of limiting amounts of an accessory protein, because when the levels of IP_3 receptor expression were increased by about 6-fold (Table 2), the maximal inhibition caused by increased $[Ca^{2+}]_m$ remained constant at 50 ± 9% ($n = 4$). Finally, in parallel comparisons under identical conditions, the effects of increasing $[Ca^{2+}]_m$ from about 2 nM to 1.1 μM on [^{3}H]IP_3 binding were indistinguishable for Sf9/IP_3R-1 and cerebellar membranes (Figure 4C; Table 1). It is noteworthy that increasing $[Ca^{2+}]_m$ was previously reported to abolish [^{3}H]IP_3 binding to cerebellar membranes almost entirely [26,47]. However, these studies were performed at pH 8.3 to optimize IP_3

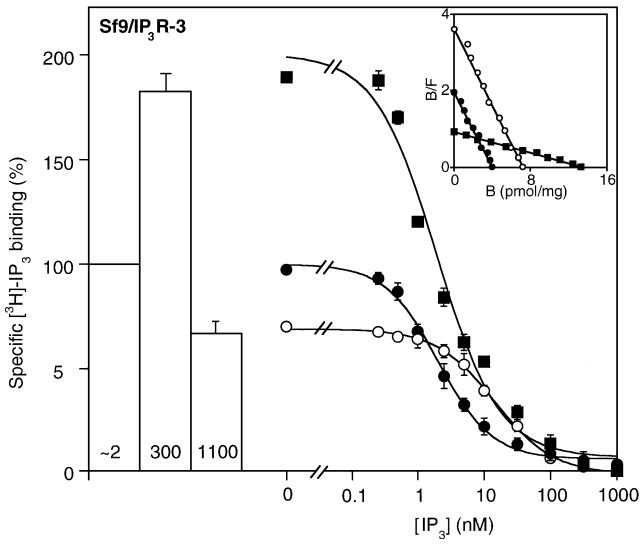

Figure 5 Effects of $[Ca^{2+}]_m$ on $[^3H]IP_3$ binding to Sf9/IP_3R-3 membranes

Membranes from Sf9/IP_3R-3 cells were incubated with $[^3H]IP_3$ (0.6 nM) and the indicated concentrations of unlabelled IP_3 in CLM in which $[Ca^{2+}]_m$ was about 2 nM (●), 500 nM (■) or 1.1 μM (○). The results (means ± S.E.M. for four independent experiments, each performed in duplicate; most error bars are smaller than the symbols) are expressed as percentages of the maximal specific binding observed when $[Ca^{2+}]_m$ was about 2 nM. The histograms show the effects of the indicated $[Ca^{2+}]_m$ on specific $[^3H]IP_3$ binding to solubilized receptors from Sf9/IP_3R-3 membranes (means ± S.E.M., $n = 3$). The inset (symbols as for main panel) shows the corresponding Scatchard plot in which *B* and *F* denote the specifically bound and free $[IP_3]$ respectively.

binding, and at that pH we also found that increasing $[Ca^{2+}]_m$ to 1.1 μM caused 91 ± 2 % ($n = 3$) inhibition of specific $[^3H]IP_3$ binding. The lower inhibition obtained under more physiological conditions (pH 7.0 and appropriate ionic composition) is the same as that obtained under similar conditions by Yoneshima et al. [39], indicating that the maximal inhibitory effect of Ca^{2+} is less profound under physiological conditions. We conclude that under the conditions used for our experiments, recombinant type-1 IP_3 receptors expressed in Sf9 cells and native cerebellar IP_3 receptors are indistinguishable: they have the same affinity for IP_3 and increasing $[Ca^{2+}]_m$ inhibits IP_3 binding to each by causing a reversible decrease in B_{max} (Figure 4, Table 1).

Ca^{2+} both stimulates and inhibits IP_3 binding to type-3 receptors

The effects of $[Ca^{2+}]_m$ on $[^3H]IP_3$ binding to membranes prepared from Sf9/IP_3R-3 cells were more complex. Although increasing $[Ca^{2+}]_m$ from about 2 nM to 100 nM caused a small (20 ± 4 %) decrease in $[^3H]IP_3$ binding, which resulted from a decrease in B_{max} (Table 1), the major effect of modest increases in $[Ca^{2+}]_m$ (to ⩽ 500 nM) was a substantial stimulation (99 ± 4 %) of $[^3H]IP_3$ binding (Figure 3). The enhanced binding resulted entirely from an increase in B_{max} with no significant change in either K_d or h (Table 1). This approx. 2-fold stimulation of $[^3H]IP_3$ binding by submicromolar $[Ca^{2+}]$ is similar to that previously reported by Yoneshima et al. [39]. Our results differ, however, in that further increases in $[Ca^{2+}]_m$ inhibited $[^3H]IP_3$ binding by causing a substantial decrease in the affinity of the receptor for IP_3 (Figures 3 and 5, Table 1). Further increasing $[Ca^{2+}]_m$ to 1 mM had no further effect on $[^3H]IP_3$ binding. The effects of Ca^{2+} on IP_3 binding persisted after solubilization of the receptors (Figure 5) and they were entirely unaffected by increasing the level of expression of the type-3 receptor by about 3-fold (Tables 1 and 2). The discrepancy between our results and those of Yoneshima et al. [39] may result either from them having used about 20-fold higher levels of receptor expression than us and so possibly exhausting a limiting supply of an essential accessory protein, or the higher concentration of $[^3H]IP_3$ used in their studies (4.6 nM compared with 0.6 nM) may have caused the effects of increasing $[Ca^{2+}]_m$ on B_{max} to become more significant than its effect on K_d.

Possible mechanisms of regulation of IP_3 receptors by cytosolic Ca^{2+}

Our results are consistent with those of Yoneshima et al. [39] in showing that the major effect of increased Ca^{2+} on IP_3 binding is inhibitory for type-1 and stimulatory for type-3 receptors. Our results differ in that we have identified an additional inhibitory effect of Ca^{2+} on the type-3 IP_3 receptor (Figures 3 and 5), and we suggest that the mechanisms underlying both the inhibition of $[^3H]IP_3$ binding to type-1 receptors and the stimulation of type-3 receptors by Ca^{2+} result from changes in B_{max} (Figures 4 and 5; Table 1). Both the stimulation and inhibition of IP_3 binding by cytosolic Ca^{2+} were observed in the complete absence of ATP and at 2 °C, indicating that phosphorylation is unlikely to be involved. We previously concluded that the biphasic effects of cytosolic Ca^{2+} on IP_3-stimulated Ca^{2+} release from hepatocytes (81 % type 2, 19 % type 1) [13] were also independent of phosphorylation [27], although in other cell types, phosphorylation has been implicated [28]. Furthermore the effects of cytosolic Ca^{2+} on IP_3 binding were fully reversed after restoration of $[Ca^{2+}]_m$ to about 2 nM (Table 3), indicating that they were not a consequence of protein degradation.

Across the entire range of $[Ca^{2+}]_m$ (about 2 nM–1.1 μM), the effect of increasing $[Ca^{2+}]_m$ had opposite effects on the B_{max} of Sf9/IP_3R-1 and Sf9/IP_3R-3 membranes, decreasing that of the former and increasing that of type-3 receptors (Table 1). The effects of $[Ca^{2+}]_m$ on Sf9/IP_3R-3 membranes were unaffected by increasing the level of receptor expression (~ 3-fold; Tables 1 and 2). The opposing effects of $[Ca^{2+}]_m$ on the two receptor subtypes were not therefore a consequence of the modestly different levels of expression achieved in our final analysis (34 ± 3 %; Table 2) causing differential exhaustion of an essential accessory protein. For each receptor subtype, the effect of Ca^{2+} on B_{max} was maximal when $[Ca^{2+}]_m$ was increased to 1.1 μM, although the type-1 receptor was more sensitive to Ca^{2+} (IC_{50} ~ 300 nM) than the type-3 receptor (IC_{50} > 500 nM) (Table 1), consistent with the previous analysis of the relative Ca^{2+} sensitivities of the two receptor subtypes [39]. We propose that for both receptor subtypes, Ca^{2+} regulates the interconversion between two conformations: a state in which the IP_3-binding site is either occluded or of such low affinity as to be undetectable with our radioligand-binding methods, and a second conformation with higher affinity for IP_3 (K_d ~ 10 nM and ~ 2 nM for types-1 and -3 receptors respectively). Increasing $[Ca^{2+}]_m$ favours the high-affinity conformation of the type-3 receptor and the occluded or low-affinity conformation of the type-1 receptor.

Although Ca^{2+} binds to purified IP_3 receptors [48] and a cytosolic Ca^{2+}-binding site has been mapped to 23 residues close to the first membrane-spanning region of the type-1 receptor [49], binding of IP_3 to pure cerebellar IP_3 receptors is entirely insensitive to the changes in $[Ca^{2+}]_m$ that regulate IP_3 binding to native receptors [34,48]. This discrepancy has been attributed to the need for an additional protein, initially thought to be calmedin [48] but see [47], to mediate the effects of cytosolic Ca^{2+}. In other tissues too, accessory proteins have been suggested to be responsible for Ca^{2+} regulation of IP_3-evoked Ca^{2+} release [1,28]. Although our results do not unambiguously establish whether

the effect of Ca^{2+} on [^{3}H]IP_3 binding to Sf9/IP_3R-1 membranes is an intrinsic property of the type-1 IP_3 receptor or mediated by an accessory protein, circumstantial evidence suggests that the latter may be more likely. First, the effects of Ca^{2+} on native cerebellar membranes and type-1 IP_3 receptors expressed at low levels in Sf9 cells were quantitatively comparable, suggesting that the same mechanism is likely to underlie the effects of Ca^{2+} on each; that mechanism is believed to require an accessory protein for cerebellar receptors [34,38]. Secondly, when the type-1 receptor was expressed at high levels, the maximal effect of increasing $[Ca^{2+}]_m$ was unaffected, but lower $[Ca^{2+}]_m$ (300 nM), which caused a decrease in B_{max} at low levels of receptor expression, failed to decrease B_{max} at the higher level of expression (Table 1). This result is consistent with a limiting amount of accessory protein reducing the Ca^{2+} sensitivity of the receptor. Finally, we would expect the ratio of B_{max}/AbC immunoreactivity to be constant at all levels of receptor expression (both measurements should report the number of IP_3 receptors), yet at the higher level of expression of the type-1 receptor, the ratio (B_{max}/AbC) was consistently about 2-fold higher than at the lower level of expression. We therefore detect twice as many IP_3-binding sites per receptor at the higher level of receptor expression, again consistent with inhibition of IP_3 binding (even in the absence of Ca^{2+}) by an accessory protein that can become limiting at very high levels of receptor expression. It is noteworthy that the B_{max}/AbC ratio is similar for Sf9/IP_3R-1 (10.7 ± 0.4; high level of expression; $[Ca^{2+}]_m = 300$ nM), Sf9/IP_3R-3 (16.6 ± 1.6; high level of expression; $[Ca^{2+}]_m = 1.1\ \mu$M) and cerebellar membranes (12.9 ± 1.2; $[Ca^{2+}]_m = \sim 2$ nM) when each is compared under conditions that maximize the B_{max} (Table 1). We conclude that an accessory protein is likely to mediate the inhibitory effect of increased $[Ca^{2+}]_m$ on type-1 IP_3 receptors.

Increasing $[Ca^{2+}]_m$ had an additional effect on type-3 receptors, a decrease in their affinity for IP_3, that became effective at higher $[Ca^{2+}]_m$ (1.1 μM) than were required for the increase in B_{max} (~ 500 nM). The stimulatory and inhibitory effects of Ca^{2+} apparently overlap within this range of $[Ca^{2+}]_m$ (500–1100 nM) because the decreasing affinity of the receptor was accompanied by an increasing B_{max} (Table 1). Our results do not establish whether regulation of type-3 receptors by Ca^{2+} is direct or mediated by additional proteins. Either mechanism would be consistent with our results, although the discrepancy between our results and those of Yoneshima et al. [39] would be most easily explained if the inhibitory effect of Ca^{2+} were mediated by an accessory protein.

Conclusions

We conclude that the baculovirus/Sf9 expression system allows full-length rat IP_3 receptors to be expressed at sufficiently high levels to allow their characterization without significant interference from endogenous receptors. The recombinant receptors are appropriately glycosylated, they assemble into tetramers, and they are regulated by cytosolic Ca^{2+}, with type-1 receptors being more sensitive than type-3 receptors to Ca^{2+}. Whereas the N-terminal domain of type-1 IP_3 receptors is likely to be the major determinant of IP_3 binding, we speculate that additional residues may also be important for IP_3 binding to type-3 receptors. Increases in $[Ca^{2+}]_m$ inhibit IP_3 binding to type-1 receptors by causing up to 50% of them to adopt a conformation with undetectable affinity for IP_3, an effect that seems likely to be mediated by an accessory protein. Increases in $[Ca^{2+}]_m$ unmask latent IP_3-binding sites on the type-3 receptor and, as $[Ca^{2+}]_m$ increases further, there is a substantial decrease in their affinity for IP_3. Our results demonstrate that, although regulation of IP_3 receptors by cytosolic Ca^{2+} is shared by types-1 and -3 receptors, the two subtypes differ in their sensitivity to Ca^{2+}, in its major effects, and in the means whereby Ca^{2+} exerts its effects. One consequence of these differences is that, at resting cytosolic $[Ca^{2+}]$, type-3 receptors are more sensitive to IP_3 ($K_d \sim 2$ nM) and are therefore likely to be activated before type-1 receptors ($K_d \sim 10$ nM). At higher cytosolic $[Ca^{2+}]$, the situation is reversed and type-1 receptors have greater affinity for IP_3 than type-3 receptors. These differences are likely to be important elements of the mechanisms underlying the spatially and temporally complex changes in cytosolic $[Ca^{2+}]$ evoked by receptors linked to IP_3 formation.

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