Selective recognition of inositol phosphates by subtypes of the inositol trisphosphate receptor

Edmund P. NEROU*, Andrew M. RILEY†, Barry V. L. POTTER† and Colin W. TAYLOR*[1]

*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K., and †Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

Synthetic analogues of inositol trisphosphate (IP_3), all of which included structures equivalent to the 4,5-bisphosphate of (1,4,5)IP_3, were used to probe the recognition properties of rat full-length type 1, 2 and 3 IP_3 receptors expressed in insect *Spodoptera frugiperda* 9 cells. Using equilibrium competition binding with [^{3}H](1,4,5)IP_3 in Ca^{2+}-free cytosol-like medium, the relative affinities of the receptor subtypes for (1,4,5)IP_3 were type 3 ($K_d = 11 \pm 2$ nM) > type 2 ($K_d = 17 \pm 2$ nM) > type 1 ($K_d = 24 \pm 4$ nM). (1,4,5)IP_3 binding was reversibly stimulated by increased pH, but the subtypes differed in their sensitivity to pH (type 1 > type 2 > type 3). For all three subtypes, the equatorial 6-hydroxy group of (1,4,5)IP_3 was essential for high-affinity binding, the equatorial 3-hydroxy group significantly improved affinity, and the axial 2-hydroxy group was insignificant; a 1-phosphate (or in its absence, a 2-phosphate) improved binding affinity. The subtypes differed in the extents to which they tolerated inversion of the 3-hydroxy group of (1,4,5)IP_3 (type 1 > type 2 > type 3), and this probably accounts for the selectivity of (1,4,6)IP_3 for type 1 receptors. They also differed in their tolerance of inversion, removal or substitution (by phosphate) of the 2-hydroxy group (types 2 and 3 > type 1), hence the selectivity of (1,2,4,5)IP_4 for type 2 and 3 receptors. Removal of the 3-hydroxy group or its replacement by fluorine or CH_2OH was best tolerated by type 3 receptors, and accounts for the selectivity of 3-deoxy(1,4,5)IP_3 for type 3 receptors. Our results provide the first systematic analysis of the recognition properties of IP_3 receptor subtypes and have identified the 2- and 3-positions of (1,4,5)IP_3 as key determinants of subtype selectivity.

Key words: Ca^{2+} channel, IP_3 analogues, ligand recognition.

INTRODUCTION

The receptors for inositol trisphosphate (IP_3) are intracellular Ca^{2+} channels that are regulated by cytosolic Ca^{2+} and IP_3; they are thereby responsible for both initiating and propagating cytosolic Ca^{2+} signals evoked by receptors linked to IP_3 formation. Three closely related IP_3 receptor subtypes (1–3), as well as splice variants of the type 1 and 2 receptors, are expressed in mammalian cells, where they assemble into both homo- and hetero-tetrameric complexes [1]. Most cells express a mixture of IP_3 receptor subtypes [1], each of which appears to be capable of forming an IP_3-gated Ca^{2+} channel [2]. However, the subtypes are differentially expressed [1], they differ in their rates of degradation during chronic cell stimulation [3] and in their affinities for IP_3 [4–6], they are differentially regulated by cytosolic Ca^{2+} [4,5,7,8], calmodulin [9], ATP [2], pH and thiol reagents [10], and they differ in their phosphorylation [11].

The physiological significance of this receptor diversity remains to be established. It has been suggested that type 1 and 2, but not type 3, IP_3 receptors may be involved in regenerative Ca^{2+} signals [2,7,12]. Type 1 receptors have been implicated in the induction of long-term depression [13], whereas type 3 receptors may be involved in regulating apoptosis [14]. Type 3 IP_3 receptors were also suggested to be specifically implicated in regulating capacitative Ca^{2+} entry ([12], but see [15]), although more recent evidence suggests that the N-terminal domains of both type 1 and type 3 IP_3 receptors may interact with capacitative Ca^{2+} entry channels [16].

The presence of heterogenous populations of IP_3 receptors in most cells compromises attempts both to establish the characteristics of each receptor subtype and to define the precise roles of each in mediating physiological responses. Ligands selective for the different IP_3 receptor subtypes would greatly benefit such studies. However, analyses of many naturally occurring and synthetic ligands suggest that all agonists of all IP_3 receptors share a structure that resembles the 4,5-vicinal bisphosphate and 6-hydroxy group triad of (1,4,5)IP_3 [17–19]. There are no known agonists lacking a structure analogous to the bisphosphate moiety, and those without a 6-hydroxy group generally bind to IP_3 receptors with very low affinity [20]. The emergence of this critical pharmacophore from analyses of structure–activity relationships in cells expressing different complements of IP_3 receptor subtypes suggests that it is probably an essential requirement for agonist activity at all subtypes. This conclusion is consistent with the high degree of similarity of amino acid sequence within the IP_3-binding domain of each subtype of the rat IP_3 receptor [1]. Nevertheless, published values for the affinities of rat IP_3 receptors for (1,4,5)IP_3 vary enormously, and there is not a clear consensus on the relative affinities of the three subtypes for (1,4,5)IP_3 [4–6,21]. The disparities underscore the need to compare homogeneous populations of receptors under identical conditions and in media that mimic the composition of cytosol.

We recently established a baculovirus/*Spodoptera frugiperda* 9 (*Sf*9) cell system to provide high levels of expression of recombinant mammalian type 1 and 3 IP_3 receptors. The receptors

Abbreviations used: IP_3, inositol trisphosphate; Ab1–3, antisera raised against peptides specific for type 1, type 2 and type 3 IP_3 receptors respectively; AbC, antiserum that equally recognizes all three IP_3 receptor subtypes; CLM, cytosol-like medium; [^{3}H]IP_3, [^{3}H](1,4,5)IP_3; DL-6-CH_2OH-*scyllo*(1,2,4)IP_3, DL-6-deoxy-6-hydroxymethyl-*scyllo*(1,2,4)IP_3, MOI, multiplicity of infection; *Sf*9, *Spodoptera frugiperda* 9; TEM, 50 mM Tris and 1 mM EDTA, pH 8.3.

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

were glycosylated, they were assembled into tetramers and bound (1,4,5)IP_3 with appropriate affinity, and they were regulated in a subtype-selective manner by Ca^{2+} and calmodulin [4,9]. The properties of the full-length recombinant type 2 IP_3 receptor have not yet been examined. In the present study, we use the *Sf*9 expression system to define the recognition properties of type 1, 2 and 3 IP_3 receptors.

MATERIALS AND METHODS

Materials

Cell culture materials, with the exception of TNM-FH medium (Sigma, Poole, Dorset, U.K.), were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). [^{3}H](1,4,5)IP_3 ([^{3}H]IP_3) (30–81Ci/mmol) was purchased from Amersham (Little Chalfont, Bucks., U.K.), ATP was obtained from Roche (Lewes, East Sussex, U.K.), and heparin (molecular mass approx. 3000, from bovine intestinal mucosa) and (4,5)IP_2 were purchased from Sigma. (1,4,5)IP_3 was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). 3-Deoxy-(1,4,5)IP_3, 3-deoxy-3-fluoro-(1,4,5)IP_3, 2,3,6-trideoxy-(1,4,5)IP_3, (3,4,5)IP_3 and (1,3,4,5)IP_4 were purchased from Calbiochem (Nottingham, U.K.). Synthesis of the other inositol phosphates was performed as described previously: L-*scyllo*(1,2,4)IP_3 and *scyllo*(1,2,4,5)IP_4 [22], (1,4,6)IP_3 [23], (1,3,6)IP_3 [24], (2,4,5)IP_3 (S. J. Mills and B. V. L. Potter, unpublished work; details are available from B.V.L.P. on request), (1,2,4,5)IP_4 [25], and DL-6-deoxy-6-hydroxymethyl-*scyllo*(1,2,4)IP_3 [DL-6-CH_2OH-*scyllo*-(1,2,4)IP_3] [26]. The synthesis of pure D-2-deoxy-(1,4,5)IP_3 will be reported elsewhere (details are available from B.V.L.P. on request). *Scyllo*(1,2,4,5)IP_4 is symmetrical and therefore has no enantiomers. DL-6-CH_2OH-*scyllo*(1,2,4)IP_3 is racemic; because only the L-isomer is likely to be active, in comparisons of this analogue with others (e.g., see Figure 5), we refer to it as L-6-CH_2OH-*scyllo*(1,2,4)IP_3 and have assumed its K_d for IP_3 receptors to be half that measured using the racemate (see Table 4). The remaining inositol phosphate analogues, unless otherwise stated, are resolved D enantiomers. The structures are shown in Figure 4 (below). All other reagents were obtained from Sigma.

The properties of the rabbit antisera raised against peptides specific for type 1 (Ab1) and type 3 (Ab3) IP_3 receptors, and to a peptide common to all three IP_3 receptor subtypes (AbC), were described previously [4]. The common peptide sequence (PMNRYSAQKQFWKA; single-letter amino acid notation) is conserved in the *Drosophila* IP_3 receptor, which we assume to be similar to the receptor endogenously expressed in insect *Sf*9 cells. Similar methods were used to generate rabbit antisera specific for type 2 IP_3 receptors (Ab2) using a peptide (CGFLGSNTPHENHHMPPH) corresponding to residues 2685–2701 of the rat type 2 IP_3 receptor (the N-terminal cysteine, which is not present in the IP_3 receptor, was added to allow conjugation with keyhole limpet haemocyanin). The results shown in Figure 1 confirm the specificity of the antisera.

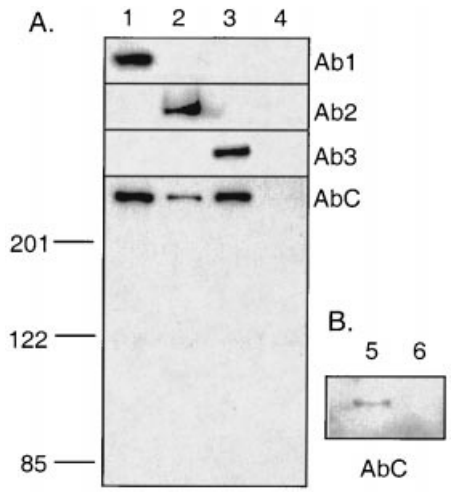

Figure 1 Expression of mammalian IP_3 receptors in *Sf*9 cells

(**A**) Each of four gels was loaded with the following membrane samples: 1, *Sf*9 cells expressing type 1 IP_3 receptors (20 μg); 2, *Sf*9 cells expressing type 2 IP_3 receptors (20 μg); 3, *Sf*9 cells expressing type 3 IP_3 receptors (20 μg); 4, non-infected *Sf*9 cells (100 μg). The gels were then immunoblotted with Ab1, Ab2, Ab3 or AbC as indicated. Only the area of the gel immediately surrounding the IP_3 receptor bands is shown for the subtype-selective antisera; the entire gel is shown for AbC. (**B**) Lane 5 was loaded with membranes (4 μg) from *Sf*9 cells expressing type 2 IP_3 receptors (the subtype expressed at the lowest level in *Sf*9 cells); lane 6 was loaded with 100 μg of membranes from non-infected *Sf*9 cells. The absence of detectable AbC staining in lane 6 establishes that, even for the type 2 receptor, its level of expression exceeded that of the native IP_3 receptors of *Sf*9 cells by at least 25-fold. The gels shown are typical of those from at least three independent experiments.

Expression of mammalian IP_3 receptors in *Sf*9 cells

Full-length rat type 1 (lacking the S1 splice site) [27] and type 3 [28] IP_3 receptors were expressed in *Sf*9 cells essentially as previously described [4,9]. The only significant differences from previous work were that all *Sf*9 cells were grown in suspension in spinner cultures (stirred at 70 rev./min) to allow large-scale production of receptors, and the concentration of foetal calf serum was reduced to 5% (v/v). As before [4], membranes were harvested 40 h after infection; the multiplicity of infection (MOI) was approx. 10.

Although the cDNA sequences of rat and human type 2 IP_3 receptors have been published, they have not been expressed as full-length proteins. The cDNA for the rat type 2 receptor in pCMV5 [29] was provided by Dr G. A. Mignery. Our initial attempts to express the type 2 receptor were unsuccessful, but after sequencing the cDNA we identified a premature stop codon, which we corrected using standard PCR methods. During subsequent sequencing of both cDNA strands of the type 2 receptor, we identified the following minor differences from the originally reported sequence changes to the peptide sequence, when they occur, are shown in parentheses: $A^{603} \rightarrow C$ (Lys → Asn), $A^{1277} \rightarrow G$ (His → Arg), $G^{2468} \rightarrow A$ (Cys → Tyr), $TG^{3074} \rightarrow GT$ (Trp → Val), $T^{4013} \rightarrow C$ (Leu → Pro), $G^{4890} \rightarrow A$ and $G^{4911} \rightarrow A$.

The full-length cDNA of the type 2 receptor was subcloned into a baculovirus transfer vector [4] and recombinant viruses were grown in *Sf*9 cells using a transfection kit (Invitrogen, Groningen, The Netherlands). *Sf*9 cells were grown at 27 °C in spinner cultures in TNM-FH medium with foetal calf serum (5%), gentamicin (50 μg/ml) and fungizone (2.5 μg/ml), infected with recombinant viruses (MOI approx. 20), and the infected cells were harvested 72 h later. The higher MOI and longer incubation period used for *Sf*9 cells expressing type 2 IP_3 receptors were optimized to achieve the highest yield of intact protein.

Membranes from at least six independent preparations of *Sf*9 cells expressing each IP_3 receptor subtype were used for the present study.

Preparation of membranes

Infected *Sf*9 cells were harvested by centrifugation (1000 ***g*** for 5 min) at 2 °C, the cell pellets were washed twice in PBS (140 mM

NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 5 mM KH_2PO_4, pH 7.2) and resuspended in Ca^{2+}-free cytosol-like medium (CLM). The suspension was homogenized using an Ultra-Turrax T25 homogenizer. The homogenate was then centrifuged (3000 ***g*** for 10 min) and the membrane pellet was resuspended in CLM (2–4 mg of protein/ml) before rapid freezing in liquid nitrogen and storage at −80 °C. CLM had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 1 mM EDTA and 20 mM Pipes, pH 7.0. All media were supplemented with a freshly prepared cocktail of protease inhibitors (final concentrations): 250 μM PMSF, 1 μM leupeptin, 1 mM benzamidine, 20 μg/ml soya-bean trypsin inhibitor, 100 μM captopril, 150 nM aprotinin, 1 μM pepstatin and 1 μM bestatin. Membranes were prepared from rat cerebellum and rat liver [30] or RINm5F cells [4] as previously described.

Equilibrium [^{3}H]IP_3 binding assays

Membranes from *Sf*9 cells (10–100 μg of protein) were resuspended in CLM (0.5 ml) containing [^{3}H]IP_3 (1 nM) and various concentrations of unlabelled ligand. To allow effective pH buffering in experiments addressing the effects of pH (see Figure 3 and Table 3), a modified CLM was used that included 10 mM Tris and 10 mM Pipes. After 5 min at 2 °C, during which equilibrium was attained, the incubations were stopped by rapid filtration through GF/B filters (Whatman, Maidstone, Kent, U.K.) using a Brandel receptor binding harvester (Semat Technical, St Albans, Herts., U.K.), followed by rapid washing with ice-cold CLM. Indistinguishable results were obtained when the incubations were terminated by centrifugation (results not shown). The radioactivity associated with the filters was measured by dissolving them in 4 ml of Ecoscint-A (National Diagnostics, Aylesbury, Bucks., U.K.) before liquid-scintillation counting. Total [^{3}H]IP_3 binding was typically 1300 d.p.m., of which approx. 90 % was specific.

To assess the reversibility of the effects of changing pH, membranes expressing type 1 IP_3 receptors were incubated for 10 min in CLM at either pH 7.0 or pH 8.3. The membranes were then centrifuged (20000 ***g*** for 5 min), resuspended in CLM at pH 7.0 or pH 8.3, and specific [^{3}H]IP_3 binding was measured after a further 5 min (see Figure 3C).

To allow detection of [^{3}H]IP_3 binding to membranes prepared from mammalian cells (10–40 μg of protein/500 μl), a different medium (TEM: 50 mM Tris and 1 mM EDTA, pH 8.3) and a higher concentration of [^{3}H]IP_3 (1.5 nM) were used. The methods were otherwise similar to those used for S*f*9 membranes.

Immunoblots

Membranes (approx. 20 μg of protein) were resuspended in SDS sample buffer, boiled for 3 min, resolved by SDS/PAGE [5 % (w/v) polyacrylamide gel], and the separated proteins were then electro-transferred on to Immobilon-P (Millipore, Bedford, MA, U.S.A.) using a cooled wet-tank system (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) [4]. Blots were probed with primary antisera (Ab1–3 or AbC; at 1:1000 dilution), re-probed with goat anti-rabbit antibody coupled to horseradish peroxidase (at 1:2000 dilution; AbCam, Cambridge, U.K.) and then visualized with the SuperSignal chemiluminescence system (Pierce and Warriner, Rockford, IL, U.S.A.) using Hyperfilm (Amersham). Immunoblots were quantified by densitometric scanning (NIH Image, Bethesda, MD, U.S.A.). All quantitative analyses were performed on gels where we confirmed the linear relationship between protein loading and scan density.

The levels of expression of the IP_3 receptor subtypes were quantified using an antiserum (AbC) to a peptide conserved in all IP_3 receptor subtypes. We previously demonstrated that AbC recognizes type 1 and 3 IP_3 receptors equally well [4]. Using similar methods we have now confirmed that AbC binds similarly to all three IP_3 receptor subtypes (see below).

Protein concentrations were determined using the Bradford assay with BSA as the standard.

Analysis

Results from equilibrium competition binding experiments were fitted to logistic equations (Kaleidagraph, Synergy Software, Reading, PA, U.S.A.) and IC_{50} values were used to calculate equilibrium dissociation constants (K_d) as previously described [9]. The variance of the ratios of mean values (a and b) were calculated from the variances (var) of each, using the following equation [31]:

$$\mathrm{var}(a/b) = (a/b)^2[(\mathrm{var}(a)/a^2) + (\mathrm{var}(b)/b^2)]$$

RESULTS

Expression of IP_3 receptor subtypes in *Sf*9 cells

Endogenous IP_3 receptors were essentially undetectable in uninfected *Sf*9 cells using antisera selective for each IP_3 receptor

Table 1 Expression of IP_3 receptor subtypes in *Sf*9 cells

Membranes from *Sf*9 cells expressing each of the three rat IP_3 receptor subtypes were used for immunoblotting with AbC ($n = 3$) and for [^{3}H]IP_3 binding, from which the maximal number of IP_3-binding sites (B_{max}) was calculated ($n = 9$–11). To allow quantitative comparisons of results from several gels, AbC immunoreactivity for each receptor subtype (20 μg of protein) is expressed relative to that obtained with 20 μg of membranes expressing the type 1 IP_3 receptor loaded on to the same gel.

Receptor type	B_{max} (pmol/mg)	AbC (%)	AbC/B_{max} (units/pmol)
1	25.3 ± 1.3	100	3.95 ± 0.20
2	2.2 ± 0.2	16.7 ± 5	7.59 ± 2.38
3	13.7 ± 0.7	63 ± 10	4.60 ± 0.77

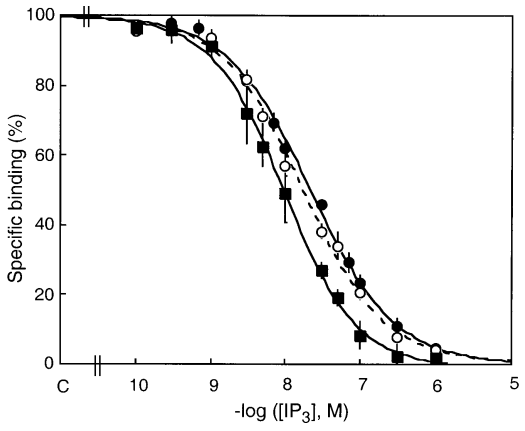

Figure 2 IP_3 binding to IP_3 receptor subtypes expressed in *Sf*9 cells

Specific binding of [^{3}H]IP_3 (1 nM) to type 1 (●), type 2 (○) and type 3 (■) IP_3 receptors expressed in *Sf*9 cells is shown in the presence of the indicated concentrations of IP_3. Results are means ± S.E.M. of four independent experiments.

Table 2 Relative affinities of IP_3 receptor subtypes from native mammalian cells or after expression in *Sf*9 cells

Equilibrium competition binding experiments with [^{3}H]IP_3 were used to determine the K_d values for IP_3 in TEM. Membranes prepared from rat cerebellum (99% type 1) rat liver (approx. 80% type 2) and rat RINm5F insulinoma cells (77% type 3) were used to assess the properties of native IP_3 receptors. Results are means ± S.E.M. from ⩾ three independent experiments.

	K_d (nM)	
Receptor type	Recombinant protein in Sf9 cells	Native protein
1	6.37 ± 0.68	6.37 ± 0.81
2	6.25 ± 0.25	3.05 ± 0.37
3	3.10 ± 0.51	11.3 ± 0.9

Table 3 Effects of pH on IP_3 binding to receptor subtypes

Results (means ± S.E.M., n = 4–11) from experiments similar to those shown in Figure 3(B) are summarized with IP_3 binding measured in Ca^{2+}-free CLM at pH 7.0 and pH 8.3.

	pH 7.0			pH 8.3		
Receptor type	K_d nM	B_{max} (pmol/mg)	h	K_d	B_{max} (pmol/mg)	h
1	24 ± 3	25 ± 1	0.76 ± 0.3	9.8 ± 1.0	53 ± 6	0.79 ± 0.02
2	15 ± 2	2.2 ± 0.2	0.90 ± 0.06	6.20 ± 0.7	2.6 ± 0.2	0.92 ± 0.04
3	11 ± 2	14 ± 1	0.89 ± 0.10	4.6 ± 0.7	36 ± 4	0.89 ± 0.10

subtype (Ab1–3), an antiserum that recognizes all three subtypes (AbC) (Figure 1A) or [^{3}H]IP_3 binding. We [4] and others [5] previously expressed type 1 and 3 IP_3 receptors in *Sf*9 cells; the results shown in Figure 1 confirm that we have now succeeded in expressing full-length type 2 IP_3 receptors. Each of the receptor subtypes migrated as a single band with the expected mobility when resolved by SDS/PAGE; each was only recognized by the appropriate subtype-selective antiserum, and all three subtypes were recognized by the common antiserum (AbC). Because AbC interacts equally well with all three subtypes (see below), it allows the levels of expression of the different subtypes to be reliably compared. The results indicate that under the conditions used for our experiments, the level of expression of the type 1 receptor was approx. 2- and 10-fold greater than that of the type 3 and type 2 receptors respectively (Figure 1A and Table 1). Measure-

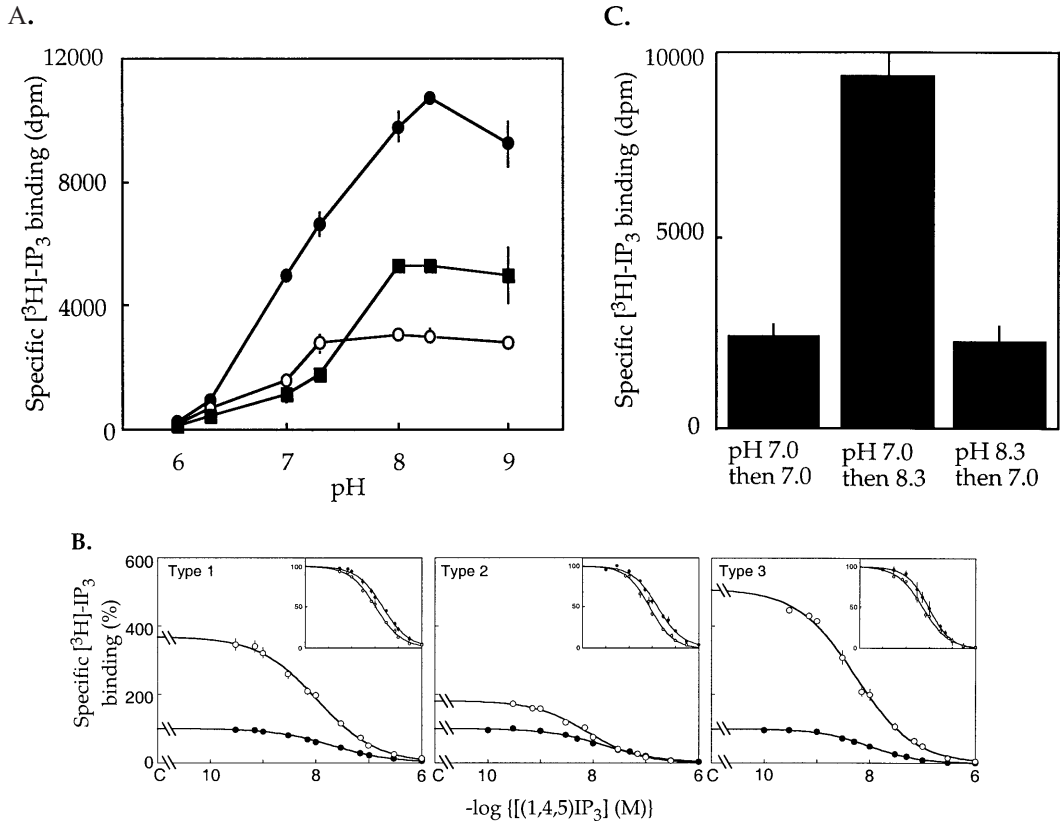

Figure 3 Reversible effects of pH on IP_3 binding to IP_3 receptor subtypes expressed in *Sf*9 cells

(**A**) The effects of varying the pH of a Ca^{2+}-free modified CLM (see the Materials and methods section) on the specific binding of [^{3}H]IP_3 (1 nM) are shown for type 1 (●), type 2 (○) and type 3 (■) IP_3 receptors expressed in *Sf*9 cells (means ± S.E.M., n = 6). (**B**) Equilibrium competition binding curves for IP_3 are shown for each receptor subtype at pH 7.0 (●) and pH 8.3 (○). The results (means ± S.E.M., n = 6) are expressed as percentages of the specific binding observed for that receptor subtype at pH 7.0. Insets show the competition curves standardized to the maximal specific binding detected at each pH (= 100%); the 2-fold increase in affinity with increased pH is clear. (**C**) Specific [^{3}H]IP_3 binding (1 nM) to type 1 IP_3 receptors was measured at pH 7.0 (after preincubation at pH 7.0), at pH 8.3 (after preincubation at pH 7.0) and at pH 7.0 (after preincubation at pH 8.3) (see the Materials and methods section for more details). The results (means ± S.E.M., n = 3) demonstrate that the effects of pH are fully reversible.

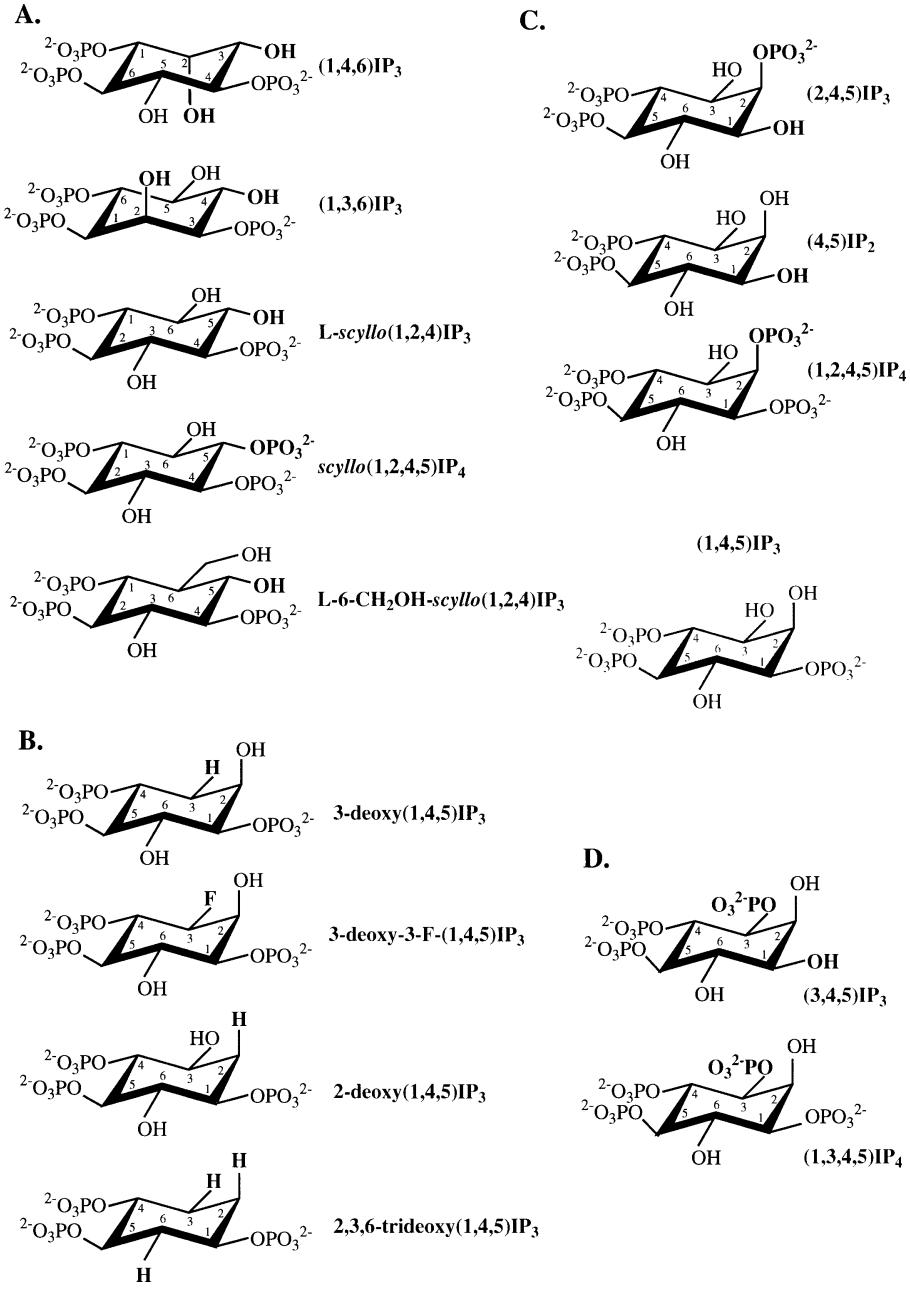

Figure 4 Structures of IP_3 analogues

The analogues used in the present study are shown with the structural differences grouped according to whether the differences from (1,4,5)IP_3 relate to: (**A**) orientation of hydroxy groups; (**B**) number of hydroxy groups; (**C** and **D**) position of phosphate groups.

ments of [^{3}H]IP_3 binding provided similar estimates of the relative levels of expression of the receptor subtypes (Tables 1 and 3). Despite considerable efforts we have not succeeded in increasing levels of expression of the type 2 receptor to those obtained with types 1 and 3. However, comparison of either AbC staining or [^{3}H]IP_3 binding confirm that, even for the type 2 receptor, its level of expression was at least 25-fold higher than that of the endogenous receptors of *Sf*9 cells (Figure 1B).

Our AbC antiserum was previously shown to interact equally well with type 1 and type 3 IP_3 receptors. The results in Table 1 confirm, as expected from the sequence of the peptide antigen, that AbC interacts similarly with type 2 receptors.

IP_3 binding to receptor subtypes

From equilibrium competition binding experiments in Ca^{2+}-free CLM, the affinities (K_d) for (1,4,5)IP_3 of the type 1, 2 and 3 IP_3 receptors expressed in S*f*9 cells were 24 ± 4 nM, 17 ± 2 nM and 11 ± 2 nM respectively (Figure 2, and see Table 4). The density of IP_3-binding sites is too low in most mammalian cells to allow

Table 4 Recognition of IP_3 analogues by IP_3 receptor subtypes

From equilibrium competition binding analysis using [^{3}H]IP_3 and membranes prepared from *Sf*9 cells expressing type 1 2 or 3 IP_3 receptors the affinity (K_d) and Hill coefficients (h) of the IP_3 analogues were determined. All experiments were conducted in Ca^{2+}-free CLM at pH 7.0. Results are means ± S.E.M. of ⩾three determinations [except (3,4,5)IP_3, where $n = 2$]. DL-6-CH_2OH-*scyllo*(1,2,4)IP_3 is the only racemic ligand used in the present study; the results shown in this Table have not been corrected to take account of the inactivity of the D-isomer (see the Materials and methods section).

	Type 1		Type 2		Type 3	
IP_3	K_d (nM)	h	K_d (nM)	h	K_d (nM)	h
(1,4,5)IP_3	24 ± 4	0.76 ± 0.04	17 ± 2	0.81 ± 0.02	11 ± 2	0.89 ± 0.1
2-Deoxy(1,4,5)IP_3	41 ± 6	0.69 ± 0.08	9.7 ± 3.5	0.66 ± 0.09	9.7 ± 0.5	0.82 ± 0.02
3-Deoxy(1,4,5)IP_3	320 ± 3	0.86 ± 0.07	333 ± 35	0.8 ± 0	73 ± 2	0.77 ± 0.06
3-Deoxy-3-F-(1,4,5)IP_3	180 ± 10	0.79 ± 0.03	303 ± 40	0.88 ± 0.07	79 ± 4	0.87 ± 0.08
2,3,6-Trideoxy(1,4,5)IP_3	8327 ± 1843	0.87 ± 0.03	3993 ± 631	0.74 ± 0.02	4464 ± 353	0.82 ± 0.05
(1,4,6)IP_3	1825 ± 254	0.85 ± 0.03	3231 ± 330	0.68 ± 0.02	4953 ± 1010	0.72 ± 0.06
(1,3,6)IP_3	8527 ± 1570	0.82 ± 0.03	2893 ± 546	0.87 ± 0.04	1345 ± 122	0.9 ± 0.1
(3,4,5)IP_3	31706 ± 13451	0.96 ± 0.11	3019 ± 472	1.02 ± 0.12	11145 ± 2064	0.7 ± 0.04
(1,3,4,5)IP_4	1394 ± 64	0.86 ± 0.01	483 ± 117	0.85 ± 0.01	401 ± 81	0.75 ± 0.03
L-*scyllo*(1,2,4)IP_3	38 ± 10	0.90 ± 0.12	12.8 ± 2.8	0.81 ± 0.09	10.8 ± 2.8	0.75 ± 0.04
scyllo(1,2,4,5)IP_4	77 ± 6	0.81 ± 0.05	25 ± 3	0.75 ± 0.02	23 ± 8	0.7 ± 0.06
DL-6-CH_2OH-*scyllo*(1,2,4)IP_3	164 ± 11	0.89 ± 0.07	183 ± 4	0.81 ± 0.06	73 ± 7	0.79 ± 0.04
(4,5)IP_2	7069 ± 895	0.92 ± 0.05	1142 ± 105	1.0 ± 0.13	1436 ± 243	0.77 ± 0.03
(2,4,5)IP_3	615 ± 76	0.8 ± 0.07	194 ± 36	0.52 ± 0.04	131 ± 20	0.63 ± 0.07
(1,2,4,5)IP_4	92 ± 7	0.76 ± 0.03	20 ± 3	0.86 ± 0.07	18 ± 0	0.80 ± 0.01

reliable measurements of [^{3}H]IP_3 binding in CLM. We therefore performed equilibrium competition binding experiments in a different medium at higher pH (TEM; see the Materials and methods section) and compared the affinities of the recombinant receptors for (1,4,5)IP_3 with those of receptors from mammalian cells in which a single IP_3 receptor subtype predominates. The results again suggest only modest differences in the affinities (< 4-fold) of the different IP_3 receptor subtypes for (1,4,5)IP_3, although with liver (80% type 2) having the highest affinity and RINm5F cells (77% type 3) having the lowest affinity for (1,4,5)IP_3 (Table 2). When assayed in the same medium (TEM), the relative affinities of the recombinant IP_3 receptor subtypes again differed only modestly in their affinities for (1,4,5)IP_3 (type 3 > type 2 ≈ type 1). It is noteworthy that the affinity for (1,4,5)IP_3 of the only native source of homotetrameric IP_3 receptors (type 1 in cerebellum) was indistinguishable from that of the recombinant type 1 receptor (Table 2). The other sources of native receptors (liver and RINm5F cells) express heterotetrameric IP_3 receptors, albeit with one subtype predominating; their affinities for (1,4,5)IP_3 are slightly, though significantly, different from the appropriate recombinant subtype.

Differential regulation of IP_3 receptor subtypes by pH

Increasing pH stimulated specific [^{3}H]IP_3 binding to each of the IP_3 receptor subtypes and for each the effect was maximal at pH 8.3 (Figure 3A). However, the subtypes differed in both their sensitivities to pH and the magnitude of its effect (Figure 3B). Type 1 receptors were stimulated the most by increased pH and type 2 receptors the least, and the half-maximal effect of pH occurred at lower pH values for type 1 and 2 (approx. pH 7.0), than for type 3 (approx. pH 7.5), receptors. For each subtype, increasing the pH from 7.0 to 8.3 caused the affinity of the receptor for IP_3 to increase by approx. 2-fold (Figure 3B), and for the type 1 and 3 receptors there was, in addition, an approximately 2-fold increase in B_{max} (Table 3). Because the effect of increasing pH was most pronounced for type 1 receptors (Figure 3A) and they are the most widely expressed subtype [1], we chose type 1 receptors to examine the reversibility of the effects of pH. The results (Figure 3C) demonstrate that the (3.8 ± 0.5)-fold increase in specific [^{3}H]IP_3 binding as the pH is increased from 7.0 to 8.3 is fully reversible.

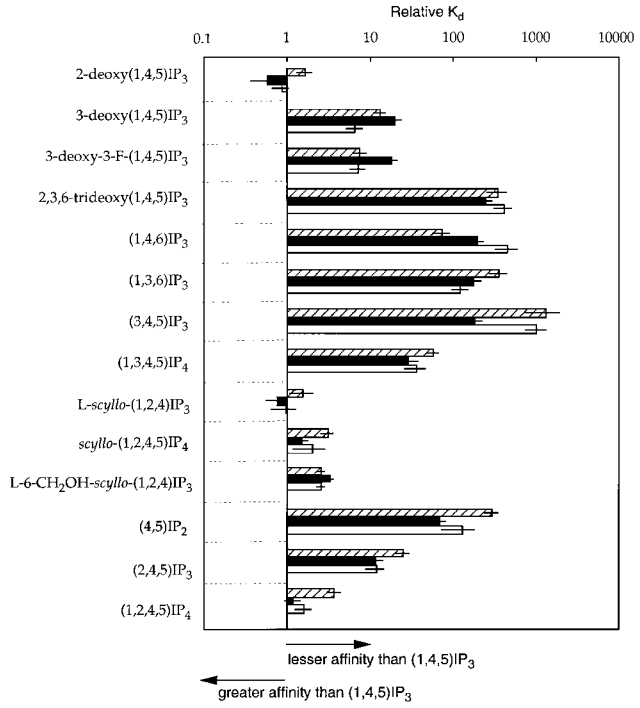

Figure 5 Relative affinities of inositol phosphates for IP_3 receptor subtypes

The data shown in Table 4 have been analysed to show the affinities of each IP_3 receptor subtype for each of the indicated ligands expressed relative to the affinity of the same subtype for (1,4,5)IP_3 [i.e. $K_d^{ligand}/K_d^{(1,4,5)IP_3}$]. Type 1, hatched bars; type 2, solid bars; type 3, open bars. Results (plotted on a log scale) are means ± S.E.M. (n as in Table 4), with error bars for ratios calculated as described in the Materials and methods section. In these comparisons, only the L-isomer of DL-6-CH_2OH-*scyllo*-(1,2,4)IP_3 is assumed to be active (see the Materials and methods section).

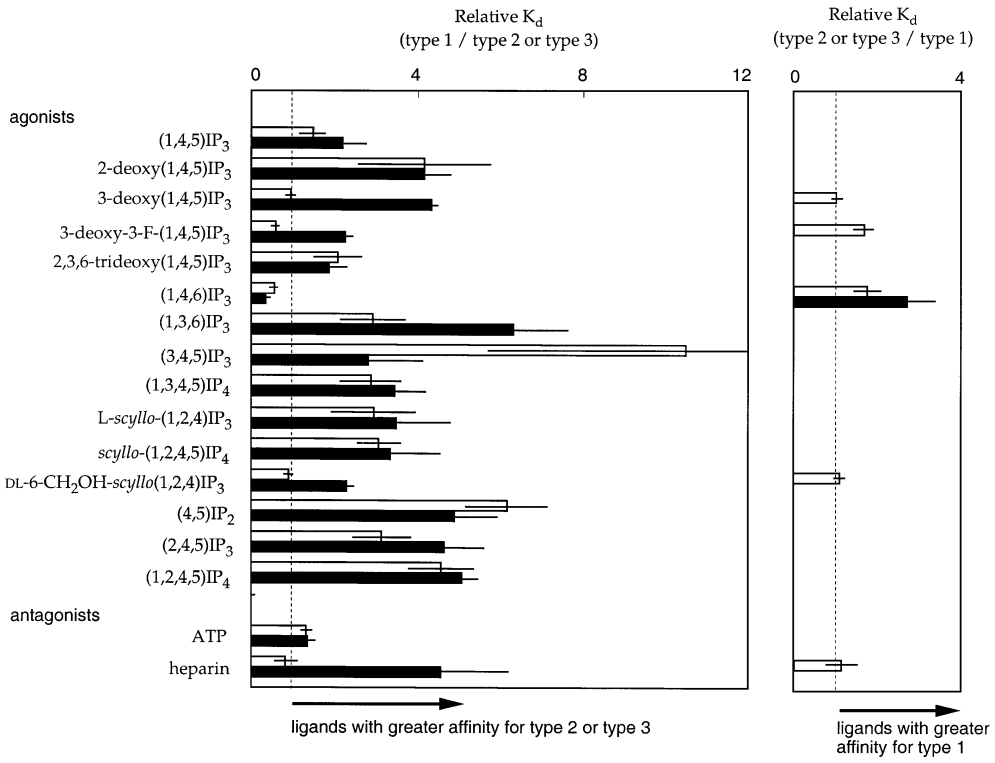

Figure 6 Selective recognition of ligands by IP_3 receptor subtypes

The data shown in Table 4 have been analysed to show the affinities of each ligand for type 2 and type 3 receptors expressed relative to the affinity of the type 1 receptor for the same ligand. In the left panel, a relative K_d [$K_d^{type\ 1}/(K_d^{type\ 2}$ or $K_d^{type\ 3})$] greater than unity (shown by the broken line) denotes a ligand with greater affinity for the type 2 or type 3 IP_3 receptor. The few ligands with greater affinity for type 1 IP_3 receptors are plotted in the right panel [$(K_d^{type\ 2}$ or $K_d^{type\ 3})/K_d^{type\ 1}$]. In both panels, type 2 receptors are shown by open bars, and type 3 receptors are shown by filled bars. Results are means ± S.E.M.

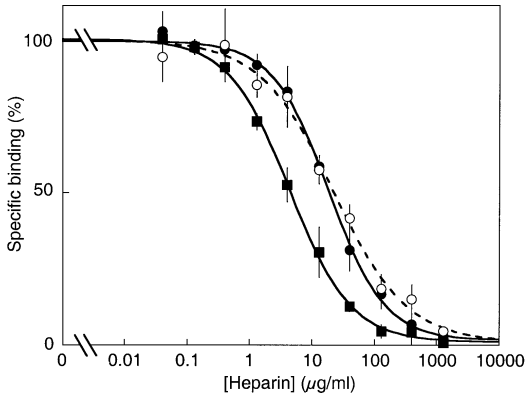

Figure 7 Heparin binding to IP_3 receptor subtypes expressed in *Sf*9 cells

Specific binding of [^{3}H]IP_3 (1 nM) to type 1 (●), type 2 (○) and type 3 (■) IP_3 receptors expressed in *Sf*9 cells is shown in the presence of the indicated concentrations of heparin. Results are means ± S.E.M. of three independent experiments.

Table 5 Antagonist binding to IP_3 receptor subtypes

Methods similar to those shown in Table 4 were used to establish the affinity (K_d) and Hill coefficients (h) of IP_3 receptor subtypes for ATP and heparin. Results are means ± S.E.M. of three determinations.

	ATP		Heparin	
Receptor type	K_d (μM)	h	K_d (μg/ml)	h
1	1041 ± 75	1.00 ± 0	22 ± 6	0.94 ± 0.12
2	767 ± 44	1.04 ± 0.08	24 ± 5	0.71 + 0.07
3	745 ± 71	1.08 ± 0.08	4.6 ± 1.1	0.90 ± 0.10

Recognition of inositol phosphates by IP_3 receptor subtypes

Each of the analogues shown in Figure 4 completely displaced specific [^{3}H]IP_3 binding from each of the three IP_3 receptor subtypes. Table 4 compares the affinities of the three IP_3 receptor subtypes for each of the ligands used. In Figure 5, the affinity of each ligand for each receptor subtype has been expressed relative to the affinity of (1,4,5)IP_3 for the same subtype. Figure 6 shows the same data, but with the affinities of type 2 and type 3 receptors for each ligand expressed relative to the affinity of the type 1 receptor for the same ligand. The results show that most inositol phosphates bind with the highest affinity to type 3 receptors, with lesser affinity to type 2 receptors, and with lowest affinity to type 1 receptors.

There are, however, exceptions to these general patterns. (1,4,6)IP_3 is the only ligand that binds with greater affinity to type 1 receptors than to the other subtypes. There are significant differences in the affinities of 3-deoxy-(1,4,5)IP_3, 3-F-3-deoxy-(1,4,5)IP_3 and DL-6-CH_2OH-*scyllo*(1,2,4)IP_3 for type 2 and

type 3 receptors (Figure 6). Of the ligands examined, (1,4,6)IP_3 had the greatest selectivity for type 1 receptors, (3,4,5)IP_3 had the greatest selectivity for type 2 receptors, and 3-deoxy(1,4,5)IP_3 had the greatest selectivity for type 3 receptors. Finally, both 2-deoxy(1,4,5)IP_3 and L-*scyllo*(1,2,4)IP_3 had slightly greater affinity than the endogenous ligand, (1,4,5)IP_3, for type 2 and 3 IP_3 receptors (Figure 5). The significance of these observations is discussed below.

Antagonist binding to IP_3 receptor subtypes

Heparin and ATP are competitive antagonists of IP_3 receptors and, as expected, each can completely displace specific [^{3}H]IP_3 binding from each of the three IP_3 receptor subtypes (Figure 7). ATP, unlike the agonists of IP_3 receptors (Table 4), binds with similar affinity to all three IP_3 receptor subtypes (Table 5). Heparin, however, has significantly higher affinity for type 3 receptors than for either type 1 or type 2 receptors (Figure 7).

DISCUSSION

Recognition of inositol phosphates by type 1 IP_3 receptors: the roles of hydroxy groups

Most analyses of ligand recognition by IP_3 receptors have used either mammalian tissues, in which type 1 IP_3 receptors predominate [17,19,32,33], or *Xenopus* oocytes [18], in which the only IP_3 receptor isoform is similar to the mammalian type 1 receptor. These studies have established that all high-affinity ligands of type 1 IP_3 receptors share structures analogous to the vicinal 4,5-bisphosphate and 6-hydroxy group of (1,4,5)IP_3. The same 4,5-bisphophate/6-hydroxy group triad is probably essential for high affinity interactions with type 2 and 3 IP_3 receptors (see the Introduction section). The 1-phosphate of (1,4,5)IP_3 increases binding affinity [17]. Each of the inositol phosphates used in the present study has a structure equivalent to the vicinal 4,5-bisphosphate of (1,4,5)IP_3, but they differ in the orientations (see Figure 4A) and numbers of hydroxy groups (see Figure 4B), in whether they have a phosphate equivalent to the 1-phosphate of (1,4,5)IP_3 (see Figure 4C) or phosphates at other loci (see Figures 4A, 4C and 4D).

To simplify subsequent discussion, the analogues are all drawn to allow their vicinal bisphosphates to be superimposed on the 4,5-bisphosphates of (1,4,5)IP_3 (see Figure 4), and in the text we describe structural modifications to the analogues by reference to the analogous positions in (1,4,5)IP_3. For example, when (1,3,6)IP_3 is drawn to allow its 1,6-bisphosphate to mimic the 4,5-bisphosphate of (1,4,5)IP_3, its 3-phosphate then mimics the 1-phosphate of (1,4,5)IP_3 (see Figure 4A); we subsequently refer to this as a 1-phosphate. Our results with recombinant type 1 receptors are consistent with earlier work in confirming that the 6-hydroxy group of (1,4,5)IP_3 is much more important than either the 2- or 3-hydroxy groups, and that the 3-hydroxy group is more important than the 2-hydroxy group [19,20,32,34,35].

Removal of the 6-hydroxy group of (1,4,5)IP_3 [35] or its replacement with a methoxy group [36] is known to massively decrease binding affinity. The very low affinity of 2,3,6-trideoxy(1,4,5)IP_3 for type 1 receptors (see Table 4) is consistent with this established importance of the 6-hydroxy group [20]. Because (1,3,6)IP_3 differs from L-*scyllo*(1,2,4)IP_3 only in the orientation of the hydroxy group equivalent to the 6-hydroxy group of (1,4,5)IP_3, and yet has an affinity similar to 2,3,6-trideoxy(1,4,5)IP_3, the orientation of the 6-hydroxy group (equatorial rather than axial) is clearly essential for high-affinity binding to type 1 receptors. Hirata et al. [34] reached a similar conclusion by comparing the activities of (1,3,6)IP_3 and (1,4,6)IP_3, which differ only in the orientations of the hydroxy groups equivalent to the 3- and 6-hydroxy groups of (1,4,5)IP_3. Our results concur with theirs, with (1,4,6)IP_3 having a (4.6 ± 1.1)-fold higher affinity than (1,3,6)IP_3 (see Table 4).

Understanding the effects of modifying the 3-position of (1,4,5)IP_3 is important, because this position is phosphorylated *in vivo* by (1,4,5)IP_3-3-kinase, and there is a possibility that 3-phosphorylated lipids might also interact with IP_3 receptors. 3-Deoxy(1,4,5)IP_3 binds with (13 ± 2)-fold lower affinity than (1,4,5)IP_3; this is slightly less than the 4-fold difference reported for native IP_3 receptors [20]. Previous work has shown that L-*chiro*(2,3,5)IP_3 [which differs from (1,4,5)IP_3 only in the axial orientation of the 3-hydroxy group] bound with approx. 10-fold less affinity than (1,4,5)IP_3 [37]. Our results are consistent with the idea that inverting the orientation of the 3-hydroxy group causes the affinity to decrease by at least 10-fold: (1,4,6)IP_3, for example, binds with (48 ± 14)-fold less affinity than L-*scyllo*(1,2,4)IP_3, and differs from it only in the orientation of the 3-hydroxy group (see Figure 4A).

Replacement of the equatorial 3-hydroxy group is tolerated only if the substituted group is relatively small [38]. Its replacement with a hydroxymethyl group [L-*scyllo*(1,2,4)IP_3 to L-6-CH_2OH-*scyllo*(1,2,4)IP_3] (see Figure 4A) reduced affinity by only (2.2 ± 0.6)-fold, whereas replacement with a phosphate group [(1,4,5)IP_3 to (1,3,4,5)IP_4] caused the affinity to decrease by (58 ± 10)-fold. Because L-6-CH_2OH-*scyllo*(1,2,4)IP_3 binds with greater affinity [(3.91 ± 0.54)-fold] than 3-deoxy(1,4,5)IP_3, it is clear that for both recombinant and native [38] type 1 IP_3 receptors, the 3-hydroxy group is recognized by a site capable of accommodating groups at least as large as a hydroxymethyl. 3-Deoxy-3-fluoro(1,4,5)IP_3, where the 3-hydroxy group is replaced by a fluorine, which is both smaller than the hydroxy group and capable of accepting, but not donating, a hydrogen bond, also bound with significantly greater affinity [(1.8 ± 0.1)-fold] than 3-deoxy(1,4,5)IP_3. The limited ability of the 3-position to tolerate bulky substitutions and the ability of fluorine to partially substitute for the 3-hydroxy group suggest that the equatorial 3-hydroxy group binds within a spatially restricted site on the receptor and that its modest effect on affinity at least partly results from it accepting a hydrogen bond.

The 2-hydroxy group appears to be relatively unimportant: 2-deoxy(1,4,5)IP_3 has only (1.7 ± 0.4)-fold lower affinity than (1,4,5)IP_3, and even very bulky additions to the axial 2-position of (1,4,5)IP_3 only slightly decrease binding to cerebellar IP_3 receptors [39]. The high affinity of L-*scyllo*(1,2,4)IP_3 [similar to that of 2-deoxy(1,4,5)IP_3], which differs from (1,4,5)IP_3 only in the orientation of the 2-hydroxy group (see Figure 4A), is further evidence that the axial 2-hydroxy group does not contribute significantly to (1,4,5)IP_3 binding to type 1 receptors [33]. *scyllo*(1,2,4,5)IP_4 differs from L-*scyllo*(1,2,4)IP_3 only in the presence of a moiety equivalent to an equatorial 2-phosphate and they differ by (2.0 ± 0.5)-fold in their affinities, whereas (1,2,4,5)IP_4 and (1,4,5)IP_3 differ only in the presence of an axial 2-phosphate and they differ by (3.8 ± 0.7)-fold in their affinities. The similarity suggests that the modest decrease in affinity after attachment of a phosphate to the 2-position is similar whether its orientation is axial or equatorial. We conclude that removal of the axial 2-hydroxy group from IP_3 [2-deoxy(1,4,5)IP_3], inversion of its orientation [L-*scyllo*(1,2,4)IP_3] or even its replacement by a phosphate [(1,2,4,5)IP_4] only modestly affect recognition by type 1 IP_3 receptors. These results suggest that the 2-hydroxy group is recognized by a more open binding site than that to which the 3-hydroxy group binds.

Recognition of inositol phosphates by type 1 IP_3 receptors: role of the 1-phosphate

The substantial increases in affinity observed after addition of a 1-phosphate to (4,5)IP_2 [to give (1,4,5)IP_3], to (2,4,5)IP_3 [to give (1,2,4,5)IP_4], or to (3,4,5)IP_3 [to give (1,3,4,5)IP_4] (see Figure 5) confirms that the 1-phosphate makes an important contribution to high-affinity binding. Clearly, the effect is greatest with (1,4,5)IP_3. This conclusion is consistent with earlier studies of mammalian IP_3 receptors [17,33]; in *Xenopus* oocytes the role of the 1-phosphate is less clear [18]. Although addition of a 2-phosphate to (1,4,5)IP_3 decreases affinity [by (3.8 ± 0.7)-fold], presumably because of electrostatic repulsion between the neighbouring phosphates, the same addition to (4,5)IP_3 substantially increases affinity [by (11 ± 2)-fold]. These results suggest that in the absence of a 1-phosphate, a 2-phosphate can substitute for it and provide a substantial increase in affinity.

Essential features of recognition of inositol phosphates by type 1 IP_3 receptors

We conclude that the minor contribution of the axial 2-hydroxy group to (1,4,5)IP_3 binding is via an interaction with a relatively open site on the type 1 IP_3 receptor. The equatorial 3-hydroxy group makes a greater contribution probably by accepting a hydrogen bond within a more enclosed binding site. The equatorial 6-hydroxy group is essential. A phosphate in either the equatorial 1-position or axial 2-position substantially increases binding affinity; repulsion between adjacent phosphates probably accounts for the decrease in affinity when both phosphates are present.

Our results establish that ligand recognition by recombinant type 1 receptors expressed in *Sf*9 cells is similar to that of native type 1 receptors, and that recombinant receptors can therefore be used legitimately to define the recognition properties of IP_3 receptor subtypes.

Receptor subtypes differ only modestly in their affinities for (1,4,5)IP_3

Several approaches have been used to examine the relative affinities of IP_3 receptor subtypes for (1,4,5)IP_3, including expression of N-terminal IP_3-binding domains [40], examination of cells expressing predominantly one receptor subtype, or selective immunoprecipitation [6] or knockout of receptor subtypes [2], and expression of type 1 and 3 receptors in *Sf*9 cells [4,5]. The results provide both hugely variable estimates of the absolute affinities of each receptor subtype ($>$ 100-fold for type 1 receptors), and different rank orders for the affinities of the subtypes for (1,4,5)IP_3. Differences in the affinities of the subtypes are often rather small ($<$ 3-fold) [5,29,40], but there are again disparities, with some reports suggesting the subtypes may differ by as much as 50-fold in their relative affinities for (1,4,5)IP_3 [6]. The different media and conditions used have undoubtedly contributed to the disparities [4,5,41]. Our results allow the first comparison of all three receptor subtypes expressed as full-length proteins in exactly the same cellular context and free of contamination by endogenous IP_3 receptors (see Figure 1).

In Ca^{2+}-free CLM, the recombinant receptor subtypes differ by no more than approx. 2-fold in their affinities for IP_3, with type 3 receptors having the highest affinity and type 1 receptors the lowest (see Table 4). These results are consistent with those from rat tissues or cell lines expressing a predominant receptor subtype insofar as the affinities of the subtypes for (1,4,5)IP_3 differ only modestly, but in the tissues expressing heterotetrameric IP_3 receptors, the affinities of native and recombinant IP_3 receptors are slightly different. The differences may result either from the different cellular contexts or from interactions between subunits within heterotetrameric receptors. The important point is that for both native and recombinant IP_3 receptors, the subtypes differ only modestly in their affinities for (1,4,5)IP_3.

Differential regulation of IP_3 receptor subtypes by pH

The effects of pH on (1,4,5)IP_3 binding to its receptors are significant, both because physiological changes in cytosolic pH may influence IP_3 receptor behaviour, and to allow comparison with the many studies in which IP_3 binding has been examined at high pH values in order to maximize levels of specific [^{3}H]IP_3 binding. A previous study of membranes from cells expressing predominantly type 1, type 2 or type 3 IP_3 receptors concluded that [^{3}H]IP_3 binding to type 2 receptors was most sensitive to pH, and that type 3 receptors were almost insensitive [10]. In functional analyses, however, type 3 receptors were most sensitive to pH [41].

Our methods allow the effects of pH to be examined in CLM using pure populations of each IP_3 receptor subtype expressed in the same cellular context. Increasing pH within a probable physiological range stimulated [^{3}H]IP_3 binding to all three receptor subtypes, although they differed modestly in their sensitivity to pH (types 1 and 2 were more sensitive than type 3) and in the maximal effect of increased pH (type 1 $>$ type 3 $>$ type 2) (see Figure 3). The effect of pH on [^{3}H]IP_3 binding was due to an increase in apparent affinity, together with an increase in the B_{max} values for type 1 and 3 receptors (see Table 2). The effect of pH is unlikely to result from the loss of an accessory factor occluding IP_3-binding sites, because the stimulatory effect of alkalinization was fully reversible under conditions where any dissociated factor would have been removed by extensive washing (see Figure 3C).

Because the effect of pH on the affinity of the three IP_3 receptor subtypes is so similar and changes in protonation of the phosphate groups of (1,4,5)IP_3 occur across a similar pH range (pH 6–8) [42], it is plausible that the major affect of pH on [^{3}H]IP_3 binding results from changes in protonation of (1,4,5)IP_3. This explanation could readily account for the increase in apparent affinity of all three receptor subtypes, but the additional effect of pH on the B_{max} values of type 1 and 3 IP_3 receptors suggests that they, at least, are directly and reversibly regulated by pH (see Figure 3).

Subtype-selective recognition of an IP_3 receptor antagonist, heparin

Heparin is often used as an antagonist of IP_3 receptors and, ahead of molecular studies, it provided the first suggestion that there may be receptor subtypes [43]. Our results suggest that heparin binds with greater affinity to type 3 receptors than to type 1 or type 2 receptors (see Figure 7 and Table 5). Because heparin (molecular mass approx. 3000 Da) is the largest ligand used, we cannot exclude the possibility that, even in isolated membranes, the different affinities may reflect differences in accessibility of the ligand-binding sites of the receptor subtypes. Heparin, a large poly-sulphated chain, is not amenable to further systematic analysis of the structural determinants of its selectivity.

Selective recognition of inositol phosphates by IP_3 receptor subtypes

The essential features of ligand recognition are similar for type 1, 2 and 3 IP_3 receptors. An equatorial 6-hydroxy group is essential

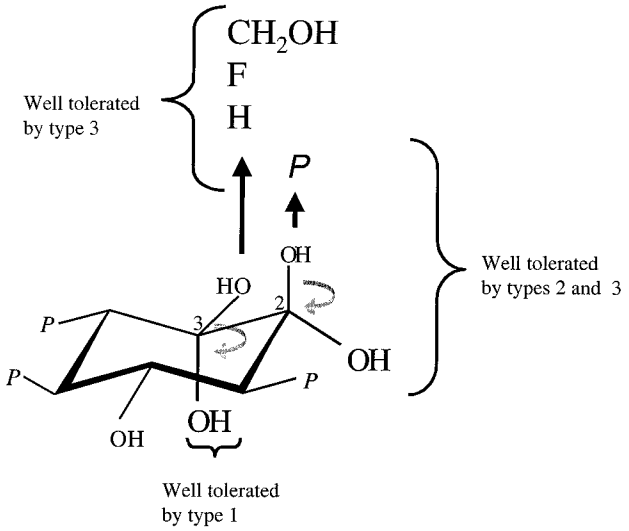

Figure 8 Key determinants of selective recognition of inositol phosphates by IP_3 receptor subtypes

Changes to the structure of (1,4,5)IP_3 that selectively affect binding to the different IP_3 receptors subtypes are shown.

for high-affinity binding: 2,3,6-trideoxy(1,4,5)IP_3 binds with similar low affinity to each receptor subtype, and inversion of its orientation [L-*scyllo*(1,2,4)IP_3 compared with (1,3,6)IP_3] massively decreases affinity. The equatorial 3-hydroxy group makes a significant contribution: 3-deoxy(1,4,5)IP_3 binds with lower affinity than (1,4,5)IP_3 to all three subtypes, and adding a larger group to the 3-position [L-6-CH_2OH-*scyllo*(1,2,4)IP_3 compared with L-*scyllo*(1,2,4)IP_3] reduces affinity. For each subtype, the 2-hydroxy group is relatively unimportant, and a 1-phosphate (or in its absence, a 2-phosphate) increases affinity.

From the analogues examined, (1,4,6)IP_3 was the only inositol phosphate to bind with greatest affinity to type 1 receptors (see Figure 6). Two previous studies are consistent with selective recognition of (1,4,6)IP_3 by type 1 receptors [34,44]. The results were, however, inconclusive, because the selectivity was evident in only one of two cell types expressing predominantly type 1 receptors [44]. Again the disparities may reflect complex effects of heterotetrameric receptor assemblies on ligand binding (see above). (1,4,6)IP_3 differs from (1,4,5)IP_3 only in the orientations of the 2- and 3-hydroxy groups (see Figure 4). However, removal of the 2-hydroxy group [2-deoxy(1,4,5)IP_3] or inversion of its orientation [L-*scyllo*(1,2,4)IP_3] decreases affinity for type 1 receptors, while increasing the affinity for type 2 and 3 receptors. Addition of a phosphate group to the 2-position [(2,4,5)IP_3 and (1,2,4,5)IP_4] likewise disproportionately decreases binding to type 1 receptors. We suggest, therefore, that the inverted orientation of the hydroxy group equivalent to the 3-hydroxy group of (1,4,5)IP_3 underlies the selective binding of (1,4,6)IP_3 to type 1 receptors. Comparison of the affinities of L-*scyllo*(1,2,4)IP_3 with (1,4,6)IP_3, which differ only in the orientation of the 3-hydroxy group (see Figure 4A), substantiates our conclusion. For the three receptor subtypes, this effect of inverting the 3-hydroxy group caused the affinities to decrease by (48 ± 14)-fold (type 1), (252 ± 61)-fold (type 2) and (459 ± 151)-fold (type 3) (Figure 8). We conclude that the type 1 receptor is more tolerant of inversion of the orientation of the 3-hydroxy group and less tolerant of inversion of the 2-hydroxy group, and that the former accounts for the selectivity of (1,4,6)IP_3 for type 1 IP_3 receptors.

Although modifications of the 2-hydroxy group [2-deoxy(1,4,5)IP_3, (2,4,5)IP_3, (1,2,4,5)IP_4 and L-*scyllo*-(1,2,4)IP_3] are better tolerated by type 2 and 3 receptors than by type 1, the effects are similar for type 2 and 3 receptors. Changes to the 3-position, however, differentially affect type 2 and type 3 receptors (Figure 8). Removal of the 3-hydroxy group or its replacement by fluorine or hydroxymethyl [L-6-CH_2OH-*scyllo*(1,2,4)IP_3] more substantially decreases binding to type 2 receptors. We suggest that replacement of the 3-hydroxy group by groups small enough to be accommodated within the restricted binding pocket is better tolerated by type 3, than by type 2, receptors.

Conclusions

Using a *Sf*9/baculovirus expression system to provide homogeneous sources of recombinant mammalian IP_3 receptor subtypes, we have established that the three subtypes are differentially regulated by pH, and differ modestly in their affinities for (1,4,5)IP_3. The receptor subtypes share many elements of ligand recognition, but the type 1 receptor is most tolerant of inversion of the 3-hydroxy group of (1,4,5)IP_3 [accounting for the selectivity of (1,4,6)IP_3 for type 1 receptors] and least tolerant of changes at the 2-position. By contrast, binding to type 2 and 3 IP_3 receptors is improved by removal or inversion of the 2-hydroxy group, and phosphorylation of the 2-position reduces affinity by less than for type 1 receptors, hence the selectivity of (1,2,4,5)IP_4 for type 2 and 3 receptors. Finally, type 2 receptors are less tolerant than type 3 receptors of small modifications to the 3-hydroxy group, accounting for the selectivity of 3-deoxy(1,4,5)IP_3 for type 3 receptors.

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