Synthetic partial agonists reveal key steps in IP₃ receptor activation

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃R) are ubiquitous intracellular Ca²⁺ channels. IP₃ binding to the IP₃-binding core (IBC) near the N-terminal initiates conformational changes that lead to opening of a pore. The mechanisms are unresolved. We synthesized 2-O-modified IP₃ analogues that are partial agonists of IP₃R. These are like IP₃ in their interactions with the IBC, but they are less effective than IP₃ in rearranging the relationship between the IBC and N-terminal suppressor domain (SD), and they open the channel at slower rates. IP₃R with a mutation in the SD occupying a position similar to the 2-O-substituent of the partial agonists has a reduced open probability that is similar for full and partial agonists. Bulky or charged substituents from either the ligand or SD therefore block obligatory coupling of the IBC and SD. Analysis of ΔG for ligand binding shows that IP₃ is recognised by the IBC and conformational changes then propagate entirely via the SD to the pore.
SD, but it no longer opens the pore4,5. However, the links between IP₃ binding and gating are not understood, and nor do we have a structure of the entire IP₃R at sufficient resolution to provide insight into these gating mechanisms1,6.

Activation of ligand-gated ion channels begins with agonist binding to a stable closed state and proceeds via many short-lived intermediates to a state in which the pore is open7. This activation may proceed entirely through a sequence of incremental changes in the receptor or be dominated by a single concerted transition between two stable conformations9.

Agonists differ in both the strength of their binding (affinity) and in their ability to drive the receptor to its open state (efficacy)10. A ligand with reduced efficacy must occupy more receptors than a full agonist to evoke the same cellular response. Such partial agonists, by occupying receptors, diminish the response to a full agonist11. Partial agonists are particularly useful for exploring the mechanisms of receptor activation because they lie between full agonists and antagonists in their ability to activate receptors7,10. This is true for all receptors, but ligand-gated ion channels are uniquely amenable to such analyses because single-channel recording allows key conformational changes of single receptors to be determined with outstanding temporal resolution7.

For these ligand-gated ion channels, full and partial agonists may differ in either the frequency with which they cause the receptor to visit the fully active open state or they may stabilize different open states that mediate lesser ion fluxes. In both cases, a partial agonist evokes lesser activation. Two subtypes of ionotropic glutamate receptors (iGluR) illustrate the distinction. These receptors, which mediate most excitatory neurotransmission in the brain, are those for which the structural basis of efficacy has been most thoroughly explored12,13. For the AMPA subtype of iGluR, a series of partial agonists, which differ from each other by only a single atom, close the clam-like binding site to varying degrees, but less completely than do full agonists. Partial agonists thereby preferentially open the pore to states with lesser conductance12. For full and partial agonists of the NMDA subtype of iGluR, the conformational changes in the binding site are more subtly different. Both cause similar closure of the clam, and they fully open the pore, but the conformational changes proceed more slowly for partial agonists14,15.

Affinity and efficacy are distinguishable, but the two properties are not independent because energy provided by agonist binding drives the conformational changes that cause channel opening10,16,17. This “binding-gating problem” is a fundamental issue in pharmacology10, but it can also be turned to advantage because the interplay depends upon both the efficacy of the ligand and the presence of the parts of the receptor through which conformational changes must pass. The former because partial agonists divert less binding energy than full agonists into effective conformational changes; and the latter because receptors lacking essential domains are expected to be less able to divert binding energy into conformational changes (Fig. 1b). Analyses of the free energy changes for ligand binding (ΔG = -RTlnK_d, where K_d is the equilibrium dissociation constant) can thus provide insight into the conformational changes evoked by agonist binding. Comparisons of ΔG for full and partial agonists, and for agonist binding to normal and truncated IP₃R, can therefore contribute to defining the links between IP₃ binding and opening of the pore.

Here we synthesize a new series of partial agonists of the IP₃R, and, in defining their properties, we identify a novel form of partial agonism that allows us to define key steps in IP₃R activation.
RESULTS

Synthesis of 2-O-modified analogues of IP$_3$

All high-affinity agonists of all IP$_3$R have structures equivalent to the vicinal 4,5-bisphosphate and 6-hydroxyl of IP$_3$ (Fig. 1c), but the axial 2-hydroxyl is not required. The essential phosphate moieties interact predominantly with opposite sides of the clam-like IBC (P-4 with the $\beta_2$-domain, and P-5 with the ARM domain) (Fig. 1a), suggesting that agonists might close the clam in a manner reminiscent of glutamate binding to iGluR6,13.

In seeking to develop novel high-affinity ligands of IP$_3$R that might differ in efficacy, we focused on the 2-OH group of IP$_3$ because earlier structure-activity analyses had suggested that analogues modified at this position retain activity. The X-ray structure of the IBC with IP$_3$ bound subsequently confirmed that the 2-OH group of IP$_3$ makes no significant contacts with the IBC.

We began by preparing homo-dimers of IP$_3$ with linkers of various lengths (2, 3 and 4 in Fig. 1c), aiming initially to define the separation of IP$_3$-binding sites within a tetrameric IP$_3$R. However, informed by our initial results and cognizant that dimeric cGMP is a partial agonist of a cGMP-gated cation channel, we extended our work to include syntheses of additional 2-O-modified analogues (Fig. 1c) and an assessment of their efficacy.

The shortest IP$_3$ dimer (2) is a symmetrically substituted $N,N'$-diethyl urea, synthesized by crosslinking of a protected D-2-O-(2-aminoethyl)-IP$_3$ building block using bis(4-nitrophenyl) carbonate. A modification of this synthetic method was used to synthesize hetero-dimers such as 5, 6 and 7, in which the second IP$_3$ moiety is replaced by a different inositol phosphate or by inositol (Scheme 1). We also synthesised an $L$-IP$_3$ homo-dimer (8, the enantiomer of 2) (Scheme 1) and an IP$_3$-adamantane conjugate (9). The syntheses of 2-deoxy-IP$_3$ (10)22 and 2-O-(2-aminoethyl)-IP$_3$ (11) were reported previously. Details of the synthetic procedures and compound characterizations are provided in Supplementary Methods online.

2-O-modified IP$_3$ analogues are high-affinity agonists of IP$_3$R

We used a cell line that expresses only recombinant rat IP$_3$R1 (DT40-IP$_3$R1 cells) to measure Ca$^{2+}$ release from intracellular stores, and IP$_3$R1 purified from rat cerebellum to measure IP$_3$ binding. These analyses show that homo-dimers of IP$_3$ linked through the 2-O-positions of the inositol rings are high-affinity agonists of IP$_3$R. The shortest dimer (2) (Fig. 1c) binds to IP$_3$R1 with greater affinity than IP$_3$ (Table 1 and Supplementary Fig. 1a online) and stimulates Ca$^{2+}$ release from intracellular stores at lower concentrations than does IP$_3$ (Table 1 and Supplementary Fig. 1b online). 2 is the most potent inositol phosphate-based agonist so far identified.

A homo-dimer of L-IP$_3$ (8) is, as expected, inactive because L-IP$_3$ does not bind to the IBC. However, homo-dimers of IP$_3$ with longer linkers (3, 4) also bind to IP$_3$R with greater affinity than IP$_3$, as do hetero-dimers in which IP$_3$ is linked to inositol (inositol-IP$_3$, 7), an unrelated bulky hydrophobic group (adamantane-IP$_3$, 9), or to an inositol phosphate that does not itself bind to the IBC (IP$_3$-IP$_5$, 6; or IP$_{3\alpha}$-IP$_3$, 5) (Table 1). The latter (5-7 and 9) demonstrate that high-affinity binding of IP$_3$ dimers does not result from an interaction with a second specific IP$_3$-binding site, nor does it result from alternating association of the two IP$_3$ moieties with the IBC. Furthermore, the two components of the dimer must be linked, because a high concentration of IP$_5$ (12, 10μM) had no effect on the Ca$^{2+}$ release evoked by IP$_3$ or 2-deoxy-IP$_3$ (10) (Supplementary Fig. 1c online). We conclude that
addition of bulky or charged groups to the 2-O-position of IP$_3$ produces high-affinity agonists of the IP$_3$R.

**A new family of partial agonists of IP$_3$R**

Because a partial agonist less effectively activates its receptor than a full agonist, it must occupy more receptors to evoke the same cellular response. In our Ca$^{2+}$ release assays, where each ligand caused the same maximal Ca$^{2+}$ release as IP$_3$ (Table 1), we can therefore gain some insight into the efficacy of a ligand by comparing the concentration that causes 50% of the maximal response (EC$_{50}$) with that which occupies 50% of the binding sites (K$_d$).

For each ligand, we compared the EC$_{50}$/K$_d$ ratio using DT40-IP$_3$R1 cells for the functional assays and purified IP$_3$R1 to measure IP$_3$ binding (K$_d$). Our results suggest that 2 occupies more IP$_3$R than IP$_3$ to evoke the same Ca$^{2+}$ release (2 has a higher EC$_{50}$/K$_d$ ratio, Fig. 2a and Table 1). This indicates that 2 may be a partial agonist. These characteristics, an increase in both affinity and EC$_{50}$/K$_d$ ratio, are shared by very different 2-O-modified IP$_3$ analogues (Fig. 2a and Table 1). They do not, therefore, depend upon precise structural features: an IP$_3$ moiety that binds to the IBC and a 2-O-substituent larger than adamantane (9) are sufficient to increase both the affinity and EC$_{50}$/K$_d$ ratio.

The EC$_{50}$/K$_d$ ratio for adenophostin A (AdA, 13, Fig. 1c) is similar to that for IP$_3$ (Fig. 2a and Table 1). This is consistent with single channel analyses, where IP$_3$ and AdA cause the IP$_3$R to open to the same maximal single channel open probability ($P_o$, the fraction of time that each channel spends in its open state) (Fig. 2b and Supplementary Table 1 online).26 We conclude that IP$_3$ and AdA are full agonists of the IP$_3$R, whereas 2-7 and 9 appear to be partial agonists.

The results shown in Fig. 2c confirm that 2 must occupy more IP$_3$R than the full agonist, AdA, to evoke the same Ca$^{2+}$ release. DT40-IP$_3$R1 cells were first pre-treated with concentrations of AdA or 2 that caused the same Ca$^{2+}$ release (5.7 ± 1.0% and 5.4 ± 1.3% of the intracellular stores, respectively) and then stimulated with IP$_3$. More IP$_3$ is required to evoke further Ca$^{2+}$ release after treatment with 2 than after AdA (EC$_{50} = 44.4 ± 3.19$ and $4.37 ± 0.18 \text{nM}$, respectively). This confirms that 2 occupies more IP$_3$R than AdA to evoke the same Ca$^{2+}$ release.

Because the nuclear envelope is continuous with the endoplasmic reticulum, we can use patch-clamp recording from the outer nuclear envelope of DT40-IP$_3$R1 cells to resolve the behaviour of single IP$_3$R. To both maximize the amplitude of the currents recorded and to avoid the complexity of feedback regulation of IP$_3$R by Ca$^{2+}$ passing through them, we used K$^+$ as the charge-carrier in these experiments. Both the single channel K$^+$ conductance ($\gamma_K$) and the mean channel open time ($\tau_o$) were the same for all agonists examined (Fig. 2b,d and Supplementary Table 1 online). The open state of the IP$_3$R thus appears to be similar whether it is evoked by binding of a full (AdA, IP$_3$ and 10) or partial agonist (2, 6 and 9).

However, for IP$_3$R activated by maximal concentrations of AdA, IP$_3$ or 10, $P_o$ was higher than with 2, 6 or 9 (Fig. 2b,e and Supplementary Table 1 online). Increasing the concentration of 2 (from 0.5 to 10$\mu$M, Fig. 2b,e) did not further increase $P_o$, and after stimulation with a mixture of IP$_3$ and 2 (10$\mu$M of each) $P_o$ was significantly less than with IP$_3$ alone (Fig. 2b and Supplementary Table 1 online). These analyses of single IP$_3$R confirm and extend the results obtained with Ca$^{2+}$ release and binding assays (Table 1 and Fig. 2a). Analogues of IP$_3$ with bulky additions to the 2-O-position (2-7 and 9) are high-
affinity partial agonists of IP$_3$R1. Subsequent analyses of the mechanisms underlying these properties of the 2-O-substituted analogues focus on 2 (Fig. 1c).

Our single channel analysis shows that whereas $\tau_o$ is similar for all agonists (Supplementary Table 1 online), mean channel closed times ($\tau_c$) were longer for the partial agonists (2, 6 and 9) than for full agonists (IP$_3$, AdA and 10) (Fig. 2e and Supplementary Table 1 online). The latter, assuming a simplified activation scheme (Fig. 2e, Supplementary Methods online), reveals that the rate constant for channel opening ($\beta = 1/\tau_c$) with partial agonists is less than with full agonists. The 2-O-modified analogues are the first partial agonists of IP$_3$R for which the basis of their reduced efficacy has been established. They open the channel fully (Fig. 2b,d), the channel closes at the same rate whether it has a partial agonist or IP$_3$ bound ($\alpha$ in Fig. 2e), but the rate constant for channel opening ($\beta$) is lower for partial agonists (Fig. 2e and Supplementary Table 1 online). We conclude that these full and partial agonists drive the IP$_3$R into a similar open state, but the partial agonists do so less effectively.

**Full and partial agonists differ in how they rearrange the IBC-SD**

For IP$_3$R, conformational changes evoked by IP$_3$ binding to the IBC near the N-terminal must be transmitted to the pore formed by residues close to the C-terminal (Fig. 1a). Some of the energy provided by IP$_3$ binding is used to drive the opening of the pore. The $K_d$ ($\Delta G = -RT\ln K_d$) measured in a binding assay is therefore determined by both the strength of the contacts between IP$_3$ and the IBC (“intrinsic binding affinity”)16 and the ensuing conformational changes10.

The IBC includes all the amino acid residues that contact IP$_3$ (Fig. 1a,3,28 and each 2-O-modified agonist (2-7 and 9-11) (Fig. 1c) retains the groups within IP$_3$ that interact with the IBC. Furthermore, each of these ligands binds with similar affinity to the IBC alone (Fig. 3a and Table 1). Because the full (IP$_3$) and partial agonists (2-7, 9) are both expected to make the same contacts with the IBC and are also observed to bind to it with similar affinity, we suggest that they do not differ in the binding energy they divert into changing the conformation of the IBC. This contrasts with AMPA receptors, where the clam-like binding site closes more fully with more efficacious agonists12,13. The distinction highlights two fundamentally different ways of reducing efficacy, a defining feature of all ligand-receptor interactions10. A partial agonist may fail to make optimal contacts with the binding site and so less effectively activate the receptor (e.g., AMPA receptors12), or it may impair onward transmission of conformational changes. Subsequent experiments demonstrate that our partial agonists (2-7 and 9) belong to the second category. They are thereby useful in defining the steps that follow IP$_3$ binding.

For all three IP$_3$R subtypes, IP$_3$ binds to the IBC with greater affinity than to either full-length IP$_3$R or the NT (Fig. 3a, Table 1 and Supplementary Table 2 online)28,29. The SD reduces the IP$_3$ binding affinity through its intramolecular interaction with the IBC28 and appears also to mediate communication between the IBC and pore4,5. We therefore examined the contribution of the SD to the conformational changes initiated by IP$_3$ via analysis of $\Delta G$ for ligand binding.

Removal of the SD increases the affinity of the NT for IP$_3$, but it has lesser effects on binding of the partial agonists (Table 1). Efficacy (reported by the EC$_{50}$/K$_d$ ratio) and the difference in $\Delta G$ ($\Delta G = -RT\ln K_d$) for binding to the IBC and NT ($\Delta\Delta G$) are inversely correlated (Fig. 3b). Because we suggest that each agonist contributes similar “intrinsic binding energy”16,17 through the similar interactions that each makes with the IBC (Table 1 and Supplementary Table 2 online), the different $\Delta\Delta G$ for binding of full and partial agonists to the NT must reflect the extent to which each uses binding energy to rearrange the relationship between the IBC and SD16,17,30. We conclude that full and partial agonists
differ minimally in their interactions with the IBC, but radically in how they rearrange its relationship with the SD.

**Conformational changes pass from the IBC entirely via the SD to the pore**

IP$_3$ binds only to a small contiguous sequence within the IP$_3$R, the IBC (Fig. 1a). Truncations of the IP$_3$R might therefore disconnect IP$_3$ binding from downstream conformational changes without directly perturbing the IP$_3$-binding site. These truncated IP$_3$R might then reveal, via analysis of $\Delta$G for ligand binding, the parts of the IP$_3$R through which IP$_3$-evoked conformational changes must pass (Fig. 1b).

All full-length IP$_3$R subtypes bind IP$_3$ with only slightly lower affinity than the NT ($\Delta\Delta$G ca. -3kJ/mol)$^{28}$, whereas the NT and IBC differ more substantially in their affinities for IP$_3$ (ca. -6kJ/mol) (Table 1 and Supplementary Table 2 online). This suggests that the most costly conformational changes evoked by IP$_3$ occur within the NT (~6kJ/mol) with downstream events requiring less energy (~3kJ/mol) (Fig. 3c). Removing the SD from full-length IP$_3$R increases its affinity for IP$_3$ by an amount (≤ ca. -9kJ/mol)$^4$ consistent with uncoupling IP$_3$ binding from all the conformational changes downstream of the IBC (Fig. 3c). These analyses suggest that the IBC communicates with the rest of the IP$_3$R entirely via the SD.

A site within the first 340 residues of the IP$_3$R, which includes the SD, appears to interact with a short cytosolic loop linking TMD 4 and 5 (Fig. 3c). This interaction has been proposed to open the pore directly$^{31,32}$. Disruption of this loop increases the affinity of the IP$_3$R for IP$_3$ by an amount (ca. -3kJ/mol)$^{32}$ that matches the estimated cost of all conformational changes downstream of the SD (Fig. 3c).

These analyses corroborate our suggestion that conformational changes pass directly and exclusively from the IBC to the SD, and then perhaps directly to the TMD4-5 loop$^{31,32}$.

**Point mutations within the SD mimic partial agonists**

Removal of the SD and additions to the 2-O-position of IP$_3$ similarly increase binding affinity (Table 1). The latter, we suggest, because the analogues evoke lesser conformational changes in the IP$_3$R. Both modifications also uncouple ligand binding from gating, although removal of the SD does so more completely$^4,5$ than do the 2-O modifications to IP$_3$. We therefore speculated that 2-O-modified analogues partially mimic removal of the SD by disrupting its interaction with the IBC and that this causes both a decrease in efficacy and an increase in affinity.

The SD has a structure reminiscent of a hammer with a large head and short handle (described earlier as an “arm”)$^{33}$ (Fig. 4a). Others$^{33}$ have shown that removing the handle of the SD (residues 67-108) minimally affects IP$_3$ binding to the NT. But mutation of highly conserved residues on the surface of the head domain, most notably within the β2-β3 loop (loop 2)$^{33}$, increases the affinity of the NT for IP$_3$. We therefore tested our hypothesis that 2-O-modified analogues of IP$_3$ disrupt the IBC-SD interface by mutagenesis of residues in the β2-β3 loop and of other residues nearby in the 3D structure of the SD (Fig. 4a). As reported$^{33}$, several mutations increased the affinity of the NT for IP$_3$, with the most effective (V33K) almost mimicking the effect of removing the entire SD. Another mutation (K52E) had no effect (Supplementary Table 3 online)$^{33}$. Furthermore, and consistent with our suggestion that 2-O-substituents of IP$_3$ disrupt the IBC-SD interaction, the effective mutations had lesser effects on binding of 2 to the NT (Fig. 4b and Supplementary Table 3 online). From these non-additive effects, we conclude that binding of 2 displaces the SD in a manner that mimics its removal or displacement by appropriate mutations.
Our results so far establish that the 2-O-substituents of the IP$_3$ analogues and appropriate point mutations within the SD cause similar increases in binding affinity. These effects mimic removal of the SD, leading us to conclude that they result from disrupted communication between the IBC and SD. Given that the 2-O-substituted analogues are partial agonists, and that the SD is required for IP$_3$ to gate the pore$^4,5$, we speculated that the point mutations might further mimic the analogues and give IP$_3$R that even full agonists are unable to activate fully.

In DT40 cells expressing IP$_3$R1 mutated within the SD (Fig. 4a and Supplementary Fig. 2a,b online), IP$_3$ and 2 evoke Ca$^{2+}$ release from permeabilized cells and activate IP$_3$R in nuclear patch-clamp recordings (Fig. 4c-e, Supplementary Fig. 2c online, and Supplementary Table 4 online). The properties of these interactions are consistent with our prediction that disrupting the IBC-SD interaction decreases efficacy and increases agonist affinity by blocking propagation of conformational changes from the IBC. In permeabilized DT40 cells expressing IP$_3$R1 with the V33K mutation (IP$_3$R1$^{V33K}$), IP$_3$ and 2 are equipotent (Supplementary Fig. 2c online), and in single channel recordings each has the same $P_o$ (Fig. 4d,e). This $P_o$ is similar to that observed for normal IP$_3$R stimulated with 2, but lower than the $P_o$ with IP$_3$ (Fig. 2b,e). The less effective mutations have lesser effects (Fig. 4c), consistent with our suggestion that they cause lesser disruption of the IBC-SD interaction.

The structures of the IBC-IP$_3$ and SD are known$^3,33$ (Fig. 1a), but not the relationship between them$^{34}$. We used protein-protein docking to identify a likely relationship between them (Supplementary Methods online). The three IP$_3$R subtypes differ in their affinities for IP$_3$, but their IBC share similar sequences and bind IP$_3$ with the same affinity$^{28}$. A subtype-specific interaction between the IBC and SD determines the different affinities of the three full-length IP$_3$R$^{28}$. Because the residues within the SD that confer these subtype-selective interactions$^{28,33}$ are likely to lie at an IBC-SD interface, this criterion was used to select between possible models of the IBC-SD complex. Our proposed model (Fig. 5a,b and Supplementary Fig. 3 online) is consistent with the radius of the NT-IP$_3$ complex from small-angle X-ray scattering$^{34}$. In this structure, four of the loops (loops 2 and 5, and part of loops 3 and 7)$^{33}$ that link the $\beta$-strands of the SD interact primarily with loops from the $\beta_2$-domain of the IBC (Supplementary Fig. 3 online). Within this IBC-SD structure, the second IP$_3$ moiety of 2 lies close to several point mutations in the SD (V33K, D34R, R36E) that reduce efficacy (Supplementary Table 4 online), each lying on the putative IBC-SD interface (within loop 2). The same interface includes the other effective mutation (K127E, within loop 5), but not the ineffective one (K52E) (Fig. 5a,b and Supplementary Fig. 3c,d online).

We conclude that bulky or charged groups introduced into the IBC-SD interface by either the ligand or the SD disrupt essential communication between the IBC and SD and thereby reduce efficacy.

**DISCUSSION**

We have synthesized and characterized a family of partial agonists of IP$_3$R that differ minimally from full agonists in their interactions with the binding site (IBC), but which have reduced efficacy because they block an obligatory communication between the IBC and SD. These results define two fundamentally different routes to reduced efficacy. A partial agonist may fail to make optimal contacts with the ligand-binding site$^{12,13,35}$. Alternatively, it may, as we have shown for our partial agonists of IP$_3$R, bind normally and then, through additional interactions, block onward transmission of essential conformational changes. These novel properties of our partial agonists allow us to show that the conformational changes initiated at the IBC pass entirely via the SD to the pore (Fig. 5c).
Our activation scheme is consistent with an earlier proposal that IP₃ minimally affects the structures of the three domains of the NT, but rearranges their relationships via flexible linking loops34 (Fig. 5c). We suggest that IP₃ first stabilizes interaction of the β₂ and ARM domains of the IBC by interacting with residues in each3,36. These interactions require the 4- and 5-phosphate groups of IP₃. The IBC then interacts with the SD (=β₁ in Fig. 5c) to give a compact structure34 that allows the SD alone to signal onwards to the pore, probably via its interaction with the TMD4-5 loop (Fig. 5c)32.

IP₃R are close relatives of ryanodine receptors (RyR), sharing most sequence similarity within their N-termini and pores. The likely structural similarities between the SD of IP₃R and the N-terminal of RyR suggests these regions may have similar functions in both families of intracellular Ca²⁺ channels33. Mutations that cause RyR to become dysfunctional in malignant hyperthermia, central core disease (RyR1) and catecholaminergic polymorphic ventricular tachycardia (RyR2) cluster in four regions that include the N-terminal and a region close to the pore37. Furthermore, 3D reconstructions of RyR have shown that activation is associated with major conformational changes within a region that includes the N-terminus38. For RyR1, the same region includes residues that interact with the dihydropyridine receptor, which is the major physiological regulator of RyR1. From structure-based sequence alignment36, it has been suggested that the SD surface opposite to that which we suggest contacts the IBC (Supplementary Fig. 3e,f online) is most conserved between IP₃R and RyR. We speculate that this may be the surface that communicates with the conserved pore region for both IP₃R and RyR.

The SD of an IP₃R activated by a partial agonist fully engages the structures that open the pore because an open IP₃R is the same whether activated by a full or partial agonist (Fig. 2b,d and Supplementary Table 1 online), but it does so less frequently than when activated by a full agonist (Fig. 5c). The many additional proteins that interact with the SD1,33 may exert their effects on IP₃R by targeting this essential link between IP₃ binding and channel opening.

In conclusion, we have synthesized a family of 2-O-modified analogues of IP₃ and shown they are partial agonists of IP₃R. IP₃ and these partial agonists interact similarly with the IBC, but the 2-O-substituents of the analogues block transmission of essential conformational changes from the IBC to the SD. The partial agonists thereby open the channel less effectively. This unusual form of partial agonism allows us to define two means whereby a ligand may have reduced efficacy: it may either fail to make optimal contacts with the binding site, or it may bind like a full agonist but then interfere with subsequent conformational changes. By combining mutagenesis of IP₃R with analyses of the effects of these novel partial agonists, we have shown that the major conformational changes evoked by IP₃ occur within the N-terminal and they pass to the pore entirely via the SD (Fig. 5c).

**METHODS**

**Synthesis of ligands**

Adenophostin A (AdA, 13)39, inositol 1,3,4,5,6-pentakisphosphate (IP₅, 12)40, IP₃ dimers19 2, 3 and 4, D-2-deoxy-IP₃ (10)22, and 2-O-(2-aminoethyl)-IP₃ (11)23 were synthesized as previously reported. Details of the syntheses of compounds 5–9 are given in Supplementary Methods online. IP₃ was from American Radiolabeled Chemicals. [³H]-IP₃ (18-23Ci/mmol) was from Amersham Biosciences.
Stable expression of IP₃R1 in DT40 cells

Rat IP₃R1 were stably expressed in DT40 cells in which the genes for all endogenous IP₃R had been disrupted. The open reading frame of rat IP₃R1 was amplified by PCR using primers P6 and P7 and cloned as an EcoRI fragment into pcDNA3. The CMV promoter was replaced by the chicken β-actin hybrid promoter, excised from the vector pAneo41, to produce the construct pcDNA3-IP₃R1. QuikChange II XL site-directed mutagenesis kit (Stratagene) was used to introduce point mutations in rat IP₃R1, which had been previously cloned into the pENTR1A vector. The primers are listed in Supplementary Table 5 online. Mutated IP₃R1 was subcloned into pcDNA3.2 by recombination (Gateway, Invitrogen). The sequences of all full-length IP₃R constructs were confirmed. DT40 cells stably expressing IP₃R1 and its mutants were generated and cultured as described. Expression of mutant IP₃R in DT40 cell lines was quantified by immunoblotting (Supplementary Fig. 2a,b online).

Functional assay of IP₃R1 in DT40 cells

A low-affinity Ca²⁺-indicator (Magfluo-4) trapped within the intracellular Ca²⁺ stores was used to measure IP₃-evoked Ca²⁺ release.

Cloning and mutagenesis of N-terminal fragments of IP₃R1

Appropriate regions of rat IP₃R1 were amplified by PCR from the full-length receptor clone lacking the S1 splice region (S1⁻). Fragments are numbered by reference to the full-length (S1⁺) rat IP₃R1 (Accession number NM_001007235). PCR used P1 and P2 primers for the fragment including residues 1-604 (NT), and P3 and P2 for residues 224–604 (IBC). Both P1 and P3 insert a thrombin-cleavage site. Fragments were ligated into the pTrcHisA vector at the XhoI/EcoRI sites (Invitrogen) to allow expression of N-terminally tagged His₆ proteins. Insertion of the S1 splice region into the IBC fragment used QuikChange mutagenesis kit with P4 and P5 primers. Mutagenesis of residues within the SD used the same kit. The primers are listed in Supplementary Tables 5 and 6 online. The sequences of all constructs were confirmed by DNA sequencing.

Expression of IP₃R1 fragments in bacteria

Constructs were transformed into E. coli BL21(DE3)43 and 1ml of the culture was grown overnight at 37°C in Luria-Bertani medium (LBM) with 50μg/ml ampicillin. The inoculum was cultured at 22°C in 100ml of LBM until the OD₆₀₀ reached 1.0–1.5, isopropyl β-D-thiogalactoside (0.5mM) was added, and after 20h at 15°C, cells were harvested (5000xg, 5min). The pellet was resuspended in Tris/EDTA medium (TEM: 50mM Tris, 1mM EDTA, pH 8.3) supplemented with 10% PopCulture (Novagen), 1mM 2-mercaptoethanol and protease inhibitor cocktail (Sigma). The suspension was incubated with lysozyme (100 μg/ml) and RNAase (10 μg/ml) for 30min on ice, and the lysate was sonicated for 20s. After centrifugation (30,000xg, 60min), aliquots of supernatant were frozen in liquid nitrogen and stored at -80°C.

For immunoblotting, samples were loaded onto SDS-PAGE gels, transferred to Immobilon membranes (Millipore) and His₆-tagged proteins were identified using an anti-His₆ antibody. Proteins were cleaved from their His₆ tags by incubating bacterial lysates with biotinylated thrombin (Novagen), and thrombin was removed with streptavidin-agarose (Novagen). Cleavage was monitored by immunoblotting using anti-His₆, and Ab142 or Ab1.1 antisera for the NT and IBC fragments, respectively (Supplementary Fig. 4 online and Supplementary Methods online).
Purification of IP$_3$R1 from rat cerebellum

IP$_3$R1 was purified at 4°C from cerebella of adult rats using heparin-affinity chromatography. Frozen cerebella were homogenized in homogenization medium (HM: 1M NaCl, 1mM EDTA, 50mM Tris, 1mM benzamidine, protease inhibitor cocktail tablet (Roche), pH 8.3) and centrifuged (100,000xg, 30min). The pellet was solubilized in HM without NaCl and supplemented with 1.2% CHAPS. After centrifugation (100,000xg, 1h), the NaCl concentration of the supernatant was increased to 250mM before loading onto heparin-agarose beads (Sigma). After 30min, the beads were washed twice in glycerol-containing medium (250mM NaCl, 50mM Tris, 10% glycerol, 1mM 2-mercaptoethanol, 1mM benzamidine, 1mM EGTA, 1% CHAPS, protease inhibitor cocktail, pH 8.0). IP$_3$R were then eluted with elution medium (500mM NaCl, 50mM Tris, 10% glycerol, 1mM 2-mercaptoethanol, 1mM benzamidine, 1mM EGTA, 50mM Tris, 1% CHAPS, pH 8.0), and aliquots frozen in liquid nitrogen before storage at -80°C.

$^3$H-IP$_3$ binding

Equilibrium-competition binding assays were performed at 4°C for 5min in TEM containing $^3$H-IP$_3$ (18-23Ci/mmol, 0.2-1.5nM), bacterial lysate (5-10 μg) or purified IP$_3$R (2.5 μg), and competing ligands. Results were analysed by fitting to a Hill equation (GraphPad Prism) from which the IC$_{50}$, and thereby the K$_d$, were calculated. The variance of the ratios of mean values (a and b) were calculated from the variances (var) of each:

$$\text{var}(a/b) = (a/b)^2\left[\text{var}(a)/a^2 + \text{var}(b)/b^2\right].$$

Single channel recording

Patch-clamp recording from excised nuclear patches of DT40 cells used the methods reported previously. IP$_3$R are relatively non-selective cation channels (P$_{Ba}$/P$_K$ ~6). K$^+$ Ba was therefore used as charge-carrier to increase single channel current amplitudes and avoid feedback regulation of IP$_3$R by permeating Ca$^{2+}$. QuB (http://www.qub.buffalo.edu) was used for analysis of all channel records (Supplementary Methods online).

Molecular modelling

We developed a model of the IBC-SD relationship from the coordinate files for the IBC (1N4K) and SD (1XZZ) using protein-protein docking. Coarse-grained models of the complex were first produced using the program Hex5.1 (http://www.csd.abdn.ac.uk/hex/). From these models we selected those in which the linked termini of the SD and IBC were appropriately separated, and then considered only those models in which residues from the SD known to affect binding of IP$_3$ to the IBC were located at an IBC-SD interface. A representative structure was further refined using a local docking search with RosettaDock. Detailed methods are given in Supplementary Methods online. Our predicted structure of the IBC-SD complex (Fig. 5a,b and Supplementary Fig. 3 online) has an inertial radius of gyration (26.1Å), which is compatible with the Guinier radius of gyration (30.7Å) obtained by small angle X-ray scattering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structure of the IP₃R and its ligands. (a) Key domains of IP₃R (numbering from rat IP₃R1, accession code NM_001007235). Pink denotes the SD (residues 1-223), red the IBC (224-604), and black vertical lines represent TMD. The SD (β₁) and IBC (β₂ and armadillo-like repeat, ARM) comprise 3 stably folded domains connected by flexible linkers (L1 and L2). Crystal structures are shown below. (b) Agonist binding (yellow) to a discrete site on the receptor (IBC for IP₃R, red) evokes conformational changes that propagate through the receptor and which then affect (arrows) the binding site. Removal (boxed diagrams) of a domain through which conformational changes must pass prevents this.
energetic interplay between conformational changes and binding. (e) Structures of the ligands used.
Figure 2.
2-**O**-modified IP$_3$ analogues are partial agonists of IP$_3$R. (a) From experiments similar to those shown in Supplementary Fig. 1a,b online, EC$_{50}$/K$_d$ ratios (n ≥ 4) were calculated for each ligand. (b) Traces (each typical of at least 3 similar records) show excised nuclear patch-clamp recordings from DT40-IP$_3$R1 cells with the pipette solution containing ATP (0.5mM), a free [Ca$^{2+}$] of 200nM and the indicated ligands (10μM, except where shown otherwise). The holding potential was +40mV. C denotes the closed state. (c) Cells were treated with IP$_3$ alone, or for 30s with either 0.1nM AdA or 2 and then with the indicated concentrations of IP$_3$. Results (n = 3) show the concentration-dependent release of Ca$^{2+}$ by
IP$_3$. (d) Current-voltage (i-V) relationship for patches stimulated with IP$_3$ or 2 (means ± SEM, n = 3). (e) Summary data showing $P_0$ and mean closed time ($\tau_c$) for IP$_3$R1 stimulated as shown, n = 3-11 (further details in Supplementary Table 1 online). The simplified activation scheme for IP$_3$R shows the transition between closed (C) and open (O) states determined by rate constants, $\beta$ and $\alpha$ (see Supplementary Methods online). All results (a,c-e) are means ± SEM.
Figure 3.
Partial agonists are IP3-like in their interactions with the IBC. (a) Equilibrium-competition binding to IBC (top) and NT (bottom) with 3H-IP3 and either IP3 or 2, n ≥ 17. (b) ΔΔG (ΔG_{IBC} - ΔG_{NT}), reflecting ΔG used to rearrange the IBC-SD relationship, is shown for each ligand, and compared with the efficacy of each (EC_{50}/K_{d}, with K_{d} determined for full-length IP3R1). Results (a,b) are means ± SEM. (c) Estimated ΔG for conformational changes associated with IP3R activation. The affinity (K_{d}) of IP3 for IP3R1 truncated as shown was measured herein (Table 1) or by others: by4 for ΔSD (IP3R1 lacking residues 1-223), and by32 for Δloop (IP3R1 lacking residues 2428-2437); ΔG was then calculated from ΔG = -RTlnK_{d}. The K_{d} for IP3 was not directly measured in32, but under the conditions used the 4-
fold increase in IP₃ binding after deletion of residues 2428-2437 (ie Δloop) is likely to reflect a 4-fold decrease in $K_d$. We assume that deletion of IP₃R fragments through which conformational changes must pass increases IP₃ affinity because less binding energy is diverted into re-arranging the protein (Fig. 1b). Deletions of many other regions (shown in blue) do not increase IP₃ affinity, suggesting that the IP₃-evoked conformational changes do not pass through them. This analysis is consistent with each IP₃ binding event diverting ~9kJ/mol into conformational changes of the IP₃R, of which ~6kJ/mol rearranges the SD-IBC relationship, and ~3kJ/mol is used by the SD to gate the pore.
Figure 4.
Point mutations within the SD mimic partial agonists. (a) The structure of the SD33 is shown highlighting the residues mutated in this study. (b) Relative affinities (Kd) of IP3 and 2 for IBC, NT, and NT with the indicated mutations (Supplementary Table 3 online); n ≥ 5. The dashed line shows KdIP3/Kd2 = 1. (c) Potency (EC50) of IP3 relative to 2 in releasing Ca2+ from permeabilized DT40 cells stably expressing mutant IP3R1 (Supplementary Table 4 online); n ≥ 5. The dashed line shows EC50IP3/EC502 = 1. (d) Typical recordings from excised nuclear patches of DT40-IP3R1V33K cells with 10μM IP3 or 2 in the patch pipette. The holding potential was +40mV. C denotes the closed state. (e) Summary data showing P0
and $\gamma_K$ for IP$_3$R1 and IP$_3$R1$^{V33K}$ stimulated with 10$\mu$M IP$_3$ or 2; $n \geq 3$. Results (b,c,e) are means ± SEM.
Figure 5.
IP$_3$ binding to the IBC activates IP$_3$R entirely via the SD. (a,b) Predicted relationship between the SD (pink) and IBC (red) with 2 bound. Residues within the SD that affect efficacy (V33, D34, R36 and K127) are shown in black (see Fig. 4a and Supplementary Fig. 3 online for details). The ineffective residue K52 is shown in pink. Panel b is an enlargement of the boxed area in panel a, with the IBC-bound IP$_3$ moiety indicated by an arrow. (c) IP$_3$ (yellow) rearranges the 2 domains of the IBC ($\beta_2$ and ARM, red) around its L2 loop causing rearrangement of the SD (=$\beta_1$, pink) around the L1 loop. The SD is then entirely responsible for transmitting conformational changes towards the pore, probably by directly interacting with the TMD4-5 loop of an adjacent subunit.$^{31,32}$ $\Delta$G associated with...
rearranging the SD and its subsequent communication with the pore region is shown. Partial agonists effectively rearrange the IBC, but the inositol 2-\(O\)-substituent (or point mutations in the SD; black circle) disrupt the IBC-SD interface and so block communication with the SD. The latter is now less likely to contact the TMD4-5 loop, but once it makes contact the channel gates normally.
Scheme 1.
Syntheses of hetero-dimers 5, 6 and 7, and L-IP$_3$ dimer 8. Dimers 5 and 8 were synthesized from L-IP$_3$-based building block 15, obtained from diol 148 (see Supplementary Methods online). The N-trifluoroacetyl protecting group was removed, generating an unstable amine which was reacted with 0.5 equivalents of bis(4-nitrophenyl) carbonate, giving protected L-IP$_3$ dimer 16. Hydrogenolytic deprotection of 16 gave L-IP$_3$ dimer 8. When 1 equivalent of bis(4-nitrophenyl) carbonate was used, the product was 4-nitrophenyl N-alkylcarbamate 17, which could be isolated and conjugated with ω-IP$_3$ component 11. The conjugation reaction was carried out in CD$_3$OD and monitored by $^{31}$P NMR spectroscopy. Deprotection followed...
by anion-exchange chromatography then gave IP$_3$–IP$_3$ hetero-dimer 5. Dimers 6 and 7 were synthesized from alcohol 1849. Nitrile 19 was reduced, and the amine product was temporarily protected as the N-trifluoroacetamide (20). Acid-labile protecting groups were then removed, giving pentaol 21, which was converted, via 22, into carbamate 23. Carbamate 23 was then conjugated with 11, and deprotection followed by anion-exchange chromatography gave IP$_3$–IP$_3$ hetero-dimer 6. Alternatively, conjugation of carbamate 24 with 11 gave IP$_3$–Ins dimer 7. Reagents and conditions: (i) LiOH, THF, MeOH, H$_2$O; (ii) bis(4-nitrophenyl) carbonate (0.5 equiv), THF; (iii) bis(4-nitrophenyl) carbonate (1 equiv), THF; (iv) H$_2$, Pd(OH)$_2$/C, MeOH, H$_2$O; (v) 11, CD$_3$OD, Et$_3$N; (vi) NaH, BrCH$_2$CN, CH$_3$CN; (vii) LiAlH$_4$, THF; (viii) EtOC(O)CF$_3$, THF; (ix) TFA, H$_2$O; (x) (BnO)$_2$PN$i$Pr$_2$, 1H-tetrazole, CH$_2$Cl$_2$ then 3-chloroperoxybenzoic acid; (xi) Et$_3$N, H$_2$O, reflux; (xii) bis(4-nitrophenyl) carbonate (1 equiv), DMF, Et$_3$N. Bn, benzyl; PMB, 4-methoxybenzyl. All experimental procedures are described in detail in Supplementary Methods online.
Table 1

Responses to IP3 analogues. The effects of each analogue on Ca\(^{2+}\) release from the intracellular stores of permeabilized DT40-IP3R1 cells and on \(^3H\)-IP3 binding to full-length purified IP3R1 (FL), its N-terminal (NT, residues 1-604) or the IP3-binding core (IBC, residues 224-604) are summarized (n ≥ 4). For dimers, the estimated separation of the two moieties is shown calculated as in\(^50\). Results are means ± SEM. *No detectable Ca\(^{2+}\) release with 30μM 8. ND, not determined.

|  | EC\(_{50}\) (nM) | Release % | FL | NT | IBC |
|---|---|---|---|---|---|
| 1 | IP3 | 20±2 | 77±5 | 12.5±1.06 | 1.6±0.2 | 2.8±0.26 | 0.21±0.03 |
| 2 | (IP3)\(_{2}\) 0.8nm | 1.5±0.1 | 75±6 | 1.26±0.11 | 1.2±0.01 | ND | ND |
| 3 | (IP3)\(_{2}\) 1.5nm | 4.9±0.3 | 74±6 | 0.86±0.19 | 5.7±1.3 | 0.47±0.09 | 0.18±0.01 |
| 4 | (IP3)\(_{2}\) 8nm | 13±1 | 73±7 | 1.39±0.23 | 9.3±1.6 | 1.37±0.20 | 0.48±0.02 |
| 5 | IP3-IP3 0.8nm | 5±1 | 70±7 | 0.90±0.12 | 5.6±1.3 | 0.42±0.02 | 0.14±0.02 |
| 6 | IP3-IP3 0.8nm | 5±0 | 72±5 | 0.89±0.11 | 5.6±0.7 | 0.36±0.03 | 0.20±0.01 |
| 7 | IP3-Ins 0.8nm | 21±1 | 76±4 | 7.82±1.73 | 2.7±0.6 | 2.8±0.22 | 0.33±0.01 |
| 8 | (-IP3)\(_{2}\) 0.8nm | Inactive* | ND | ND | ND | ND | ND |
| 9 | 2-adamantane-IP3 | 30±3 | 71±1 | 7.62±0.51 | 3.9±0.5 | 1.63±0.022 | 0.20±0.02 |
| 10 | 2-deoxy-IP3 | 32±7 | 65±3 | 17.1±1.1 | 1.9±0.4 | 4.01±0.38 | 0.22±0.03 |
| 11 | 2-aminoethyl-IP3 | 42±5 | 69±6 | 43.6±4.6 | 0.95±0.02 | 13.5±2.0 | 0.52±0.04 |