Interactions of Inositol 1,4,5-Trisphosphate (IP3) Receptors with Synthetic Poly(ethylene glycol)-linked Dimers of IP3 Suggest Close Spacing of the IP3-binding Sites*

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The distances between the inositol 1,4,5-trisphosphate (IP3)-binding sites of tetrameric IP3 receptors were probed using dimers of IP31 linked by poly(ethylene glycol) (PEG) molecules of differing lengths (1–8 nm). Each of the dimers potently stimulated 45Ca2+ release from permeabilized cells expressing predominantly type 1 (SH-SY5Y cells) or type 2 (hepatocytes) IP3 receptors. The shortest dimers, with PEG linkers of an effective length of 1.5 nm or less, were the most potent, being 3–4-fold more potent than IP31. In radioligand binding experiments using cerebellar membranes, the shortest dimers bound with highest affinity, although the longest dimer (8 nm) also bound with almost 4-fold greater affinity than IP31. The affinity of monomeric IP3 is with only the PEG attached was 2-fold weaker than IP3, confirming that the increased affinity of the dimers requires the presence of both IP3 motifs. The increased affinity of the long dimer probably results from the linked IP3 molecules binding to sites on different receptors, because the dimer bound with greater affinity than IP3 to cerebellar membranes, where receptors are densely packed, but with the same affinity as IP3 to purified receptors. IP31 and the IP3 dimers, irrespective of their length, bound with similar affinity to a monomeric IP3-binding domain of the type 1 IP3 receptor expressed in bacteria. Short dimers therefore bind with increased affinity only when the receptor is tetrameric. We conclude that the four IP3-binding sites of an IP3 receptor may be separated by as little as 1.5 nm and are therefore likely to be placed centrally in this large (25 × 25 nm) structure, consistent with previous work indicating a close association between the central pore and the IP3-binding sites of the IP3 receptor.

Inositol 1,4,5-trisphosphate (IP3)1 receptors are intracellular Ca2+ channels that are expressed in many cells and that mediate the release of Ca2+ from intracellular stores evoked by receptors that stimulate IP3 formation. The three subtypes of mammalian IP3 receptor are closely related to each other and to the receptors expressed in birds, Xenopus, crayfish, Drosophila and Caenorhabditis elegans (2). For each of these receptors, the functional IP3-gated Ca2+ channel is thought to be a tetramer, which may either be homomeric or, in those species that express more than one receptor subtype, heteromeric (3). Although the different subtypes of mammalian IP3 receptor differ in their distribution (4), differ modestly in their affinity for IP3 and their ability to recognize different inositol phosphates (4), and may be differentially modulated (5), the physiological significance of this diversity is unclear. More striking than the differences between IP3 receptor subtypes are the similarities: the primary sequences of the subunits are closely related, each assembles to form a tetrameric IP3-gated Ca2+ channel, they recognize similar ligands with broadly similar affinities, and most are biphosphorylated regulated by cytosolic Ca2+ (6).

Analyses of the relationships between structure and function have focused primarily on the mammalian type 1 IP3 receptor, but other subtypes are likely to be similar. Electron microscopy of IP3 receptors in cerebellar Purkinje cells (7) or after purification from cerebellum (8, 9) or smooth muscle (10) suggests that as viewed from the cytosol they exist as either square (7–9) or pinwheel (8, 10) structures. Recent evidence suggests that Ca2+ might regulate the transition between these states (8). In negatively stained images of pure receptors, the sides of the structures are about 25 nm in length (8–10), but they appear smaller (about 15 nm) in single particle reconstructions of purified receptors in vitreous ice (11) and smaller still (about 12 nm) in images of native receptors (7).

The IP3-binding site (one on each subunit of the receptor) lies within the N-terminal between residues 226 and 576 (12) and is formed by two structurally distinct domains linked by a region that includes the S1 splice site (13). Within this region, conserved lysine and arginine residues are likely to be important for recognition of the phosphate groups of IP3. All four subunits of the tetrameric receptor contribute to the Ca2+ pore, with residues lying close to the C terminus, namely the last two transmembrane regions (TMR-5 and TMR-6) and the intervening loop, forming the Ca2+ channel (14). Although the IP3-binding site and the pore of the channel lie at opposite ends of the primary sequence and are separated by almost 1700 residues (the “modulatory domain”), there is evidence of direct contact between them (15), with the IP3-binding domain of one subunit perhaps interacting directly with the pore region of an adjacent subunit (16). Despite this considerable progress in establishing the structural determinants of IP3 binding and of the pore, the location of the IP3-binding sites within the quaternary structure of the receptor is unknown.

A powerful strategy for the identification of potent and se-
Selective ligands for receptors with multiple binding sites is the use of multivalent ligands (17). Applications of this approach to multi-subunit proteins have generally employed synthetic bivalent constructs in which two molecules of a ligand are linked by a spacer in which the length is customized to span the distance between two binding sites within a protein multimer. Successful examples include the development of selective opioid antagonists such as nornorbinorphine (18), potent muscarinic agonists (19), and inhibitors for β-tryptase (20) and the protease (21). Bivalent ligands may show enhanced affinity and selectivity for multimeric proteins, and they can also be used to investigate the separation of ligand-binding sites. In a recent example, potent bivalent ligands for tetrameric cyclic nucleotide-gated channels of photoreceptors and olfactory neurons were identified from a series of synthetic dimers of the natural ligand (cGMP) linked by PEG chains of various lengths (1). The separation of the binding sites for cGMP in the multimeric channels could then be estimated from the calculated mean separation of the two cGMP molecules in the most active dimers for each type of channel.

Because previous work had established that even bulky additions to the axial 2-oxygen of IP3 only slightly decrease affinity for the IP3 receptor (22), we attached linkers to this position to produce dimers of IP3 (23). In the present work, we use IP3 dimers of varying lengths to examine the distances between IP3-binding sites within a tetrameric IP3 receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—IP3 was from American Radiolabeled Chemicals Inc. (St. Louis, MO) and 3H-IP3 (40 Ci/mmol) was from Amersham Biosciences. Our initial approach to the synthesis of the IP3 dimers employed amide linkages to the PEG (dimers 1 and 3), but a modified strategy employing urethane linkages (dimers 2, 4, 5, and 6 and monomer 8) was later found to be more versatile. Thus, dimers 1 and 3 were synthesized by the reaction of N-2-O-(2-aminooethyl)-3,6-di-O-benzyl-myoinositol 1,4,5-tris-O-(dibenzyl phosphate) (23) with disuccinimidyl esters derived from 3,6,9-trioxaoxadecanedioic acid (Aldrich) and poly(ethylene glycol) 600 diacid (Aldrich), respectively, followed by removal of benzyl-protecting groups by hydrolysis over palladium on carbon. Dimers 2, 4, 5, and 6 were synthesized as described previously (23) by reaction of the same trisphosphate with p-nitrophenyl carbonate-activated poly(ethylene glycol) linkers followed by deprotection. Monomer 8 was synthesized in a similar way by the reaction of the trisphosphate with the p-nitrophenyl carbonate ester derived from monomethoxy-2-(2-aminoethyl)-ethylene glycol (24) followed by deprotection. N-2-O-(2-Aminooethyl)-myoinositol 1,4,5-trisphosphate (7) was synthesized by a two-step deprotection of N-3,6-di-O-benzyl-2-O-[2-(2,2,2-trifluoroacetylamo)-ethyl]-myoinositol 1,4,5-trisphosphate (23) employing hydrolysis over palladium on carbon followed by removal of the trifluoroacetyl protecting group with methanolic ammonia at 60 °C. All polyphosphates were purified by ion-exchange chromatography on Q-Sepharose Fast Flow resin and quantified by total phosphate assay.

Effective (r.m.s.) lengths for PEG linkers were calculated according to Kramer and Karpen (1) from previous determinations of PEG lengths (25). Estimates of the extended length of the two shortest dimers (1 and 2) were made using molecular models. Fig. 1 shows the structures of the ligands used.

**Equilibrium 3H-IP3 Binding**—Membranes were prepared from rat cerebellum (26), and tetrameric IP3 receptors were purified from cerebellum using heparin and concanavalin A columns (27). A membrane fraction enriched in IP3-binding sites was prepared from rat liver using a Percoll gradient exactly as reported previously (28). The N-terminal fragment of the rat type 1 IP3 receptor (residues 224–604 and containing the 1 splice site) tagged at the N terminus with hexa-His was expressed in Esherichia coli. The construct was transformed into E. coli strain BL21(DE3), and 1 ml of the culture was grown overnight in Luria-Bertani medium (29) with 50 μg/ml ampicillin at 30 °C. This inoculum was added to 100 ml of Luria-Bertani medium and cultured at 22 °C, and when the A600 had reached 1.0–1.5 (about 7 h), isopropyl-1-thio-β-p-galactopyranoside (0.5 mm) was added. After a further 20 h at 15 °C, cells were harvested by centrifugation (5000 × g, 15 min) and washed in phosphate-buffered saline. The pellet was frozen rapidly in liquid nitrogen and stored at −80 °C. Bacterial lysates were prepared by re-suspending the frozen pellet in 10 ml of Tris/EDTA medium (TEM, 50 mM Tris, 1 mM EDTA, pH 8.3) supplemented with 1 mM β-mercaptoethanol and a protease inhibitor mixture formulated for purification of poly-His-tagged proteins in bacteria (Sigma). The suspension was incubated with lysozyme (100 μg/ml, Sigma) for 30 min on ice followed by five rapid freeze-thaw cycles using liquid nitrogen. The lysate was then sonicated for 20 s (maximal setting on an MSE Soniprep 150), and after centrifugation (30,000 × g, 60 min), aliquots of the supernatant (typically 4 mg protein/ml) were frozen in liquid nitrogen and stored at −80 °C. The major band detected after Western blotting of the final supernatant fraction with a hexa-His antibody had the expected size of 43.5 kDa (Fig. 4A); a very minor band (double arrowheads in Fig. 4A) probably represents dimeric fusion protein. Although we engineered an enterokinase cleavage site into the fusion protein, we were unable to remove the hexa-His tag using enterokinase without causing cleavage of the IP3-binding domain. All experiments with the bacterially expressed IP3-binding domain therefore used the hexa-His-tagged protein.

All equilibrium binding incubations were performed at 4 °C in TEM (final volume 200 μl) containing 3H-IP3 (1–2 nM), membranes (typically 50 μg), bacterial lysate (100 μg) or purified IP3 receptor (8 μg), and appropriate concentrations of competing ligands. After 5 min, reactions were terminated either by centrifugation alone (membranes; 20,000 × g, 5 min) or, for soluble proteins, by the addition of 200 μl of cold TEM containing 30% PEG-8000 and 200 μg of γ-globulin followed by centrifugation. Pellets were solubilized in 1 ml of EcoScint A scintillation mixture, and their activity was determined by liquid scintillation counting. Total 3H-IP3 binding was usually more than 2500 dpm and nonspecific binding was <10% of total binding.

Equilibrium competition binding curves were fitted to logistic equations using nonlinear curve fitting (Kaleidograph, Synergy Software, Reading, PA) from which equilibrium dissociation constants were determined (4).

**45Ca** Release Assays—SH-SY5Y cells were cultured (6) and rat hepatocytes prepared (28) as described previously. Cells were permeabilized by incubation with saponin and loaded with 45Ca (5 μCi/ml) in a cytosol-like medium (KCl 140 mM, NaCl 20 mM, MgCl2 2 mM, EGTA 1 mM, Pipes 20 mM, pH 7.0, ATP 1.5 mM, creatine phosphate 5 mM, creatine phosphokinase 1 unit/ml, carbonyl cyanide p-trifluoromethoxy)phenylhydrazone (FCCP) 10 μM, CaCl2 300 μM to give a free [Ca2+] of 200 nM). Active 45Ca uptake was defined as that which could be released by ionomycin (10 μM). To assess the effects of IP3 or its dimers, they were added with thapsigargin (1 μM to inhibit further Ca2+...
**Spacing of IP₃-binding Sites**

Experiments similar to those shown in Fig. 2A were used to determine the effects of each of the ligands on ⁴⁺/H⁺ release from permeabilized hepatocytes. Results are means ± S.E. of n independent experiments. h, Hill coefficient.

| r.m.s. PEG length² | EC₅₀ | h | Maximal release | n |
|---------------------|------|---|----------------|---|
| nm      | µM  |   | %             |    |
| IP₃  | 141 ± 14 | 1.63 ± 0.12 | 54 ± 3 | 20 |
| 1  | 48 ± 4 | 3.29 ± 0.54 | 48 ± 1 | 6 |
| 2  | 37 ± 5 | 1.86 ± 0.24 | 63 ± 8 | 4 |
| 3  | 75 ± 8 | 2.07 ± 0.33 | 42 ± 4 | 7 |
| 4  | 150 ± 18 | 4.76 ± 1.97 | 72 ± 6 | 4 |
| 5  | 82 ± 17 | 1.42 ± 0.10 | 55 ± 4 | 4 |
| 6  | 98 ± 22 | 1.83 ± 0.4 | 47 ± 4 | 4 |
| 7  | 204 ± 29 | 1.90 ± 0.29 | 35 ± 5 | 3 |

² See footnote 2.

**RESULTS AND DISCUSSION**

**Short IP₃ Dimers Potently Stimulate Ca²⁺ Release from Permeabilized Hepatocytes**—Despite attachment of PEG to the 2-position of IP₃ (Fig. 1), the IP₃ dimers potently stimulated the release of Ca²⁺ from the intracellular stores of permeabilized hepatocytes, with maximally effective concentrations of IP₃ and each of the dimers releasing similar fractions of the intracellular Ca²⁺ stores (Table I, Fig. 2A). To estimate the separation of the two IP₃ structures in each dimer, we followed the approach described by Kramer and Karpen (1) in which the separation is taken as the average (r.m.s.) length of the flexible PEG linker. The r.m.s. lengths can be calculated from previous determinations of the lengths of specific PEGs (25), assuming that the r.m.s. length is proportional to the square root of the number of ethylene glycol monomers. This method predicts r.m.s. lengths of ~1 nm, respectively, for the dimers in the two smallest dimers [1] and [2], increasing to 8 nm for the largest dimer [6]. A comparison of the potencies of the dimers relative to monomeric IP₃ indicates that dimers linked by the shortest linkers (r.m.s. length ≤ 1.5 nm; [1] and [2]) were more than 3-fold more potent than IP₃ (Fig. 2C).

The increased potency of dimeric IP₃ required the presence of both IP₃ motifs, because the monomeric version [7] was less potent than IP₃ (Table I). In equilibrium competition binding analyses using [³²P]IP₃ and membranes prepared from rat liver, a short dimer [2] also bound with higher affinity (Kᵢ = 1.7 ± 0.3 nM, h = 1.20 ± 0.13, n = 5) than either IP₃ (Kᵢ = 3.6 ± 0.9 nM, h = 1.32 ± 0.20, n = 5) or a longer dimer (4, Kᵢ = 8.1 ± 1.1 nM, h = 1.09 ± 0.11, n = 5) (Fig. 2B), where h is the Hill coefficient.

These results are consistent with short dimers of IP₃ achieving their increased potency because the separation between the pair of IP₃ molecules is sufficient to allow each IP₃ to simultaneously interact with a binding site on a tetrmeric receptor. An obvious prediction would then be that short dimers of IP₃ would not bind with increased affinity to monomeric subunits of the receptor. Unfortunately, the only methods that have succeeded thus far in dissociating native IP₃ receptors into their subunits have also abolished their ability to bind IP₃ (30). The only effective way of examining IP₃ binding to monomeric IP₃-binding sites is, therefore, by expression of recombinant proteins lacking the membrane-spanning regions that mediate oligomerization (30, 31). Type 2 IP₃ receptors are the major subtype (~80%) (32) expressed in hepatocytes, and although we have successfully expressed full-length recombinant type 2 IP₃ receptors (4), we have not succeeded in expressing the type 2 monomeric IP₃-binding domain in bacteria. For subsequent analyses, we therefore used type 1 IP₃ receptors, where it was possible to express monomeric IP₃-binding domains (33, 34).

**Interactions between IP₃ Dimers and Type 1 IP₃ Receptors**—Using SH-SY5Y cells, which express largely (~89%) (6) type 1 IP₃ receptors, we first established that short IP₃ dimers potently stimulate Ca²⁺ release from intracellular stores. In keeping with the results from hepatocytes (Fig. 2), the shortest IP₃ dimer [1] (EC₅₀ = 38 ± 2 nM) was 3-fold more potent than monomeric IP₃ (EC₅₀ = 114 ± 24 nM) in stimulating ⁴⁺/H⁺ release.

Using cerebellar membranes, the richest native source of type 1 IP₃ receptors, we determined the affinities of IP₃ and the IP₃ dimers for type 1 IP₃ receptors using °H/IP₃ in equilibrium competition binding assays (Fig. 3A). The results (Table II) reveal an obvious biphasic effect of the length of the PEG linker on the affinity of a dimer for the type 1 IP₃ receptor (Fig. 3B). The shortest dimer bound with the greatest affinity, and dimers of intermediate length were similar to IP₃, but the longest dimer [6] had significantly greater affinity than IP₃. The high affinity of short IP₃ dimers is consistent with the functional analyses in both hepatocytes (Table I) and SH-SY5Y cells, but the high affinity of the longest dimer was unexpected (see below).

Monomeric versions of a short dimer [2] in which IP₃ was linked to either (CH₂)₄NH₃⁺ (7) or to PEG with one end capped by a methyl group rather than IP₃ (8) (Fig. 1) bound with 4.5- or 1.7-fold lower affinity than IP₃ to cerebellar membranes (Table II). These results established that the high affinity of the dimers for IP₃ receptors requires the presence of both IP₃ motifs; it is not a direct consequence of an interaction with the linking group. Indeed, the reduced affinity of these parent compounds (7, 8) relative to IP₃ suggests that the increased affinity of IP₃ dimers may be rather greater than implied by our comparisons with IP₃ itself. We speculated that the very high density of IP₃ receptors in cerebellar membranes (7) might allow the longest dimer to span IP₃-binding sites between neighboring IP₃ receptors. Subsequent experiments were designed to determine whether the high affinity of the shortest dimers reflects bivalent binding within a tetrmeric receptor and to establish whether the longest dimers might simultaneously bind to sites on adjacent receptors.

**Intra- and Inter-receptor Binding of IP₃ Dimers**—We reasoned that if the high affinity of the long dimer [6] for cerebellar IP₃ receptors (Fig. 3B) resulted from its binding simultaneously to sites on adjacent receptors, then disrupting the close

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² Two sources of error (1) contribute to uncertainty in estimating the effective lengths of PEG linkers: PEGs are flexible and thus each molecule can span a range of lengths; and the PEGs used to synthesize the larger dimers [3 to 6] are polydisperse, consisting of a range of molecules distributed around a mean value of molecular weight.
packing of IP3 receptors in native membranes (7) would reduce its affinity. Whereas dimer 6 bound with 3.4 ± 0.6-fold greater affinity than IP3 to receptors in cerebellar membranes (Fig. 3B), its affinity for purified cerebellar IP3 receptors (Kd = 2.3 ± 0.5 nM) was not significantly different from that of IP3 (Kd = 3.5 ± 0.6 nM). We conclude that the high affinity of 6 for IP3 receptors in cerebellar membranes probably results from an inter-receptor interaction (Fig. 4C, b). This interpretation is consistent with both the reported spacing of IP3 receptors in native membranes (~2–4 nm) (7) and with our observation that the relative potency of 6 is greater in the cerebellum, which has a very high density of IP3 receptors, than in hepatocytes (Fig. 2B), where IP3 receptors are present at lower density. Our results with 6 are therefore consistent with IP3 receptors in the cerebellar membranes being close together, but they do not allow a precise estimate of their spacing. Although the estimated r.m.s. length of the linker in 6 is 8 nm, this dimer was synthesized from a polydisperse PEG containing a range of polymer lengths distributed about a mean molecular weight. Thus, the longer dimers present in 6, particularly in more extended conformations, will be capable of spanning larger distances.

We expressed a near minimal IP3-binding domain of the type 1 IP3 receptor (residues 224–604 with the S1 splice region residues 224–604) in bacteria (Fig. 4A) and used it to establish whether the high affinity of IP3 dimers for native receptors required a tetrameric receptor structure. In keeping with previous results suggesting that residues toward the N terminus may inhibit IP3 binding (12, 34), residues 224–604 bound IP3 with about 10-fold greater affinity than did the full-length receptor (Tables II and III). Previous work established that full-length IP3 receptor or N-terminal fragments (residues 1–604) whether expressed alone or with a hexa-His tag had indistinguishable affinities for IP3 (33), confirming that the tag does not affect IP3 binding. Our results with residues 224–604 (Table III, Fig. 4B), in contrast to those obtained with tetrameric receptors (Figs. 2 and 3), demonstrate that dimers of IP3, whether linked by long [6] or short linkers [1, 2], bind with slightly lower affinity than IP3 to monomeric IP3-binding domains. These results establish that short IP3 dimers bind with high affinity only when the receptor exists in its tetrameric state. Their increased affinity must therefore result from simultaneous binding of the two linked IP3 molecules to binding sites within a tetrameric receptor.

**Conclusions**—In both functional and radioligand binding assays using type 1 and type 2 IP3 receptors, dimers of IP3 linked by short spacers (~1.5 nm) bind to tetrameric IP3 receptors with significantly greater affinity than monomeric IP3. Molecular models show that even in the most extended conformations of the shortest dimer [1] the separation of the two IP3 molecules does not exceed 2 nm. We conclude that the IP3-binding sites of the receptor are likely to lie within 2 nm of each other and must therefore be near the center of the large (12–25 nm) (7–10) tetrameric IP3 receptor structure. Such a location...
would place the IP$_3$-binding sites close to the central pore of the channel, consistent with evidence suggesting a close association between the N terminus and channel region of the receptor (15, 16). In a recent study of purified type 1 IP$_3$ receptors, heparin (a competitive antagonist of IP$_3$ binding) conjugated to gold via albumin was used to locate IP$_3$-binding sites by electron microscopy (8), and the results suggested that the sites might lie toward the periphery of the receptor (i.e. ≥10 nm apart). However, because the albumin (66 kDa) and gold (>5 nm) attached to the heparin are themselves large, they may exaggerate the distance between IP$_3$-binding sites. Alternatively, the binding sites may be paired such that the spacing between sites within a pair is less (≤2 nm) than the spacing between pairs (≥10 nm).}

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receptor. Having established the utility of this approach, future studies with more rigid linkers should provide opportunities to develop very high affinity agonists and antagonists, also allowing more precise estimates of the spacing of IP₃-binding sites within a tetramer and of IP₃ receptors in native membranes. Conversely, as the structure of the IP₃ receptor is resolved at higher resolution, it will become possible to tailor the spacing of bivalent ligands more precisely to match the spacing of the higher resolution, it will become possible to tailor the spacing of IP₃-binding sites and perhaps thereby to produce very high affinity agonists and antagonists of IP₃ receptors.

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