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Synthesis of Inositol Phosphate-Based Competitive Antagonists of Inositol 1,4,5-Trisphosphate Receptors†

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Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are intracellular Ca$^{2+}$ channels that are widely expressed in animal cells, where they mediate the release of Ca$^{2+}$ from intracellular stores evoked by extracellular stimuli. A diverse array of synthetic agonists of IP$_3$Rs have defined structure-activity relationships, but existing antagonists have severe limitations. We combined analyses of Ca$^{2+}$ release with equilibrium competition binding to IP$_3$R to show that (1,3,4,6)IP$_3$ is a full agonist of IP$_3$R1 with lower affinity than (1,4,5)IP$_3$. Systematic manipulation of this meso-compound via a versatile synthetic scheme provided a family of dimeric analogs of 2-O-butyryl-(1,3,4,6)IP$_3$ and (1,3,4,5,6)IP$_3$ that compete with (1,4,5)IP$_3$ for binding to IP$_3$R without evoking Ca$^{2+}$ release. These novel analogs are the first inositol phosphate-based competitive antagonists of IP$_3$Rs with affinities comparable to that of the only commonly used competitive antagonist, heparin, the utility of which is limited by off-target effects.

Introduction

Inositol 1,4,5-trisphosphate receptors (IP$_3$R$^*$) are intracellular Ca$^{2+}$ channels that are almost ubiquitously expressed in animal cells. 1-3 IP$_3$Rs are essential links between receptors in the plasma membrane that stimulate phospholipase C and release of Ca$^{2+}$ from the endoplasmic reticulum (ER). The resulting cytosolic Ca$^{2+}$ signals regulate many diverse cellular processes. 3 The three subtypes of IP$_3$Rs expressed in vertebrates (IP$_3$R1-3) are closely related proteins and they are each regulated by both (1,4,5)IP$_3$ (1, Fig. 1) and Ca$^{2+}$, but they differ in their sensitivity to other forms of regulation and in their subcellular and tissue distributions. 1

Extensive structure-activity studies, 4-8 reinforced by a high-resolution structure of (1,4,5)IP$_3$ bound to the IP$_3$-binding core of IP$_3$R1 (Fig. 1A), 9 established that the vicinal 4,5-bisphosphate moiety is essential for (1,4,5)IP$_3$ binding and the equatorial 6-hydroxyl and 1-phosphate confer high affinity (Fig. 1B). All high-affinity agonists of IP$_3$R have structures equivalent to these substituents. The only endogenous inositol phosphate likely to bind to IP$_3$Rs under physiological conditions is (1,4,5)IP$_3$, the immediate water-soluble product of phospholipase C-catalyzed hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate. However, synthetic ligands of IP$_3$Rs, including many inositol phosphates 1 and derivatives of adenophostins, 10-12 have provided insight into the structural determinants of IP$_3$R activation. These ligands include analogs of (1,4,5)IP$_3$ that are resistant to degradation, 13-15 fluorescent analogs, 14 partial agonists, 13,16 and synthetic derivatives of adenophostins. 10 There are, however, no ligands of IP$_3$R that distinguish effectively between IP$_3$R subtypes, 5,15,16 and the only available antagonists have severe limitations. 17 The commonly used antagonists are heparin, 2-aminoethoxydiphenyl borate (2-APB), xestospongin and high concentrations of caffeine. The limitations of these antagonists include off-target effects, notably interactions with other Ca$^{2+}$ channels, Ca$^{2+}$ pumps, G proteins and other signalling pathways; membrane-impermeability (heparin) and, for xestospongin, an inconsistent history of effectiveness as discussed recently. 17 This study was undertaken with the aim of developing more effective antagonists of IP$_3$R.

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concentrations that regulate IP$_{1}$Rs. Nevertheless, we chose (1,3,4,6)IP$_{4}$ to attempt development of novel antagonists of IP$_{1}$R because it and analogs in which its free hydroxyls are modified (3, 4) are meso compounds that make synthesis more straightforward, and we had initially supposed that (1,3,4,6)IP$_{4}$ might have reduced efficacy. We previously reported that dimers of inositol phosphates are high-affinity partial agonists of IP$_{1}$Rs. We have now developed a family of antagonists of IP$_{1}$Rs (5-12 in Fig. 1C) from the (1,3,4,6)IP$_{4}$ backbone by modification of its free hydroxyls and dimerization of the modified structures. Diesteric or dietheric (X = O or H) linkages of various sizes (n = 1-3) were chosen for these 5-O-homodimers, which were synthesized by means of a diverse and versatile approach. The most useful of these ligands (8, 10 and 12) bind to IP$_{1}$R1 with an affinity comparable to that of the best available competitive antagonist of IP$_{1}$R4, heparin, the utility of which is limited by its off-target effects.

Results and discussion

Chemistry

Synthesis of IP$_{4}$s and IP$_{5}$s. Phosphates 2-4 were all prepared from myo-inositol (13) (Scheme 1). Thus, tetrasodium Ins$_{1}$,(1,3,4,6)P$_{4}$ (2) was synthesized from butanedione-derived acetal 14 following a previously published route. Pentasodium Ins$_{1}$,(1,2,3,4,6)P$_{5}$ (4) was reached via pentol 15a and pentakis phosphate 15b. Modifications on the perphosphorylation and hydrogenolysis protocols of an inositol bicyclohexylidene acetal originated synthetic scheme, were applied in order to solely obtain the pentasodium salt. The preparation of butanoate 3 involved a novel approach. Thus, acetal 14 was initially selectively protected at the C-2 position as the PMB ether to yield 16a. Masking of the remaining C-5 hydroxyl as the benzyl ether gave the fully protected derivative 16b, which was very carefully deprotected upon treatment with aqueous DDQ to reach free alcohol 16c. Introduction of the required butyryl group was performed by esterification with butyric anhydride. The resulting ester (16d) was then exposed to aqueous TFA to cleave both acetics, and the corresponding tetraol (17a) was formed quantitatively. Perphosphorylation of crude 17a was accomplished using a 1H-tetrazole solution in acetonitrile and dibenzyl $N,N$-disopropylphosphoramidate at ambient temperature, followed by direct oxidation of the intermediate phosphate with m-chloroperbenzoic acid at low temperature. Finally, the obtained benzyl tetrakisphosphate 17b was subjected to hydrogenolysis in ethanol/water in the presence of Pd/C and sodium bicarbonate (exactly one equivalent per phosphate group) to yield quantitatively the desired pentasodium salt 3.

Synthesis of dimeric analogs of IP$_{4}$ and IP$_{5}$. For the synthesis of dimers 5-12, we envisioned the retrosynthetic analysis depicted in Scheme 2. Dimers 5-12 could be reached from the corresponding polyols 18 applying sequentially perphosphorylation and global deprotection protocols. The key to obtain all these compounds, differentially substituted...
on C-2, from a common intermediate (19) was to introduce orthogonal protective groups (PG and PG’) at an early stage of the synthesis. In this way, 19 could serve as the sole precursor for both series (2-O-butyrylated and 2-O-phosphorylated derivatives) by selective removal of PG’. Esters and ethers 19 could, in turn, be prepared by dimerization of the corresponding monomers 20 using the appropriate linkers. Since this process involved the relatively hindered secondary alcohols 20, we were keen to explore the feasibility of this approach. Finally, starting from myo-inositol (13) selective introduction of the required protective groups was expected to lead to monomers 20. Monobenzyl ether 21 (Scheme 3) was recognized as a suitable derivative, appropriately functionalized to play the role of 20. Moreover, 21 is easily accessible from myo-inositol through butanedione bisacetal 14. Direct dimerization of this compound was initially investigated using the Steglich esterification approach and employing malonic (n = 1) and succinic acid (n = 2) as linkers (Scheme 3 and Table S1 in ESI). However, these apparently simple couplings were found to be complicated, under various reaction conditions tested, by the formation of acetate 22 (in the first case) and N-acylureas 24a-c (in both cases). Thus, for malonic acid reactions, the presence of DMAP seemed to solely favor the decarboxylation process, regardless of the carbodiimide (DCC or DIC) and the solvent used. We could not securely determine whether this decarboxylation occurred prior to or after the first esterification. However, in other runs we isolated the N-acryleas 24a and 24b, suggesting that acetate 22 is formed from malonic monoester. Although replacing DMAP with DIPEA eliminated this problem, the only product isolated was N-acrylurea 24b, in very low yield, whereas starting material was quantitatively recovered when EDC was used. On the other hand, the reactions performed in the absence of base

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**Scheme 1. Synthesis of phosphates 2-4. Reagents and conditions:** (a) Ref. 25; (b) Ref. 26; (c) Ref. 27; (d) i. (BnO)2P(NiPr)2, 1H-tetrazole, CH3CN, 25 °C, 48 h; ii. m-CPBA, CH2Cl2, –50 to 0 °C, 5 h, for 15b 73%, for 17b 63%; (e) Pd/C, H2 (1 atm), NaHCO3, EtOH/H2O (1:1), 25 °C, 48–96 h, for 3 and 4 100%; (f) i. NaH, DMF, 0 °C, 1 h; ii. PMBCl, 0 to 25 °C, 12 h, 67%; (g) i. NaH, DMF, 0 °C, 1 h; ii. BnBr, 0 to 25 °C, 12 h, 90%; (h) DDQ, CH2Cl2/H2O (10:1), 25 °C, 24 h, 71%; (i) Bt2O, Et3N, DMAP, CH2Cl2, 25 °C, 12 h, 91%; (j) 90% aq. TFA, CH2Cl2, 25 °C, 2 h, 100%

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**Scheme 2. Retrosynthetic analysis for target dimers 5-12.**
were productive, yielding the desired dimer (23a) along with the corresponding N-acrylurea (24a or 24b). The best results were obtained in the case of the DCC-promoted coupling.\(^2\)\(^3\)\(^4\) Surprisingly, applying the same conditions (DCC in Et\(_2\)O) for the coupling of 21 with succinic acid was unsuccessful. In order to reach dimer 23b the presence of DMAP was a crucial factor using either DCC or EDC in CH\(_2\)Cl\(_2\).\(^3\)\(^4\) Again, the reaction with DCC furnished an inseparable mixture of dimer 23b and N-acrylurea 24c, which was subsequently resolved upon hydrogenolysis. In contrast to esters \(23a,b\) the synthesis of dimeric ethers \(25a,b\) was accomplished in a more facile way. Williamson etherifications, through the in situ formed (NaH) sodium alkoxide of 21, were initially attempted in DMF using the required diodo- or dibromo-alkanes, but with poor results. Replacing halo-electrophiles with the more reactive ditosylates \(26\)\(^\text{a}\)\(^\text{, b}\) and \(27\)\(^\text{a}\)\(^\text{, b}\) and applying a protocol\(^5\) which involved KOH as base and a more polar solvent (DMSO) furnished the desired dimers \(25a,b\) in a clean way and in good yields.\(^3\)

With the key intermediate dimers in our hands, we proceeded to the next steps, which installment of the butyryl and phosphate groups. Pd-catalyzed hydrogenolysis of \(23a,b\) and \(25a,b\) led to the corresponding diols \(28\), which were esterified upon exposure to butyric anhydride to give \(29\) in very good yields (Scheme 4).

Both \(28\) and \(29\) were then used to reach the final targets. Thus, careful treatment of these dimers (especially in the case of \(29\)) with aqueous TFA furnished octaols and decaols \(30\), in nearly quantitative yields (Scheme 4). Phosphorylation of these crude polyols was accomplished as described for \(17b\) (Scheme 1) to obtain the protected polyphosphates \(31\). The latter were debenzylated upon hydrogenolysis in the presence of sodium bicarbonate to yield the octakis and decaakis phosphate salts \(5\)\(-\)\(^\text{12}\).\(^3\)

### Biology
\(1,3,4,6\)\(\text{IP}_4\) is a full agonist of \(\text{IP}_\text{R}\). Both \(1,4,5\)\(\text{IP}_3\) (1) and \(1,3,4,6\)\(\text{IP}_3\) (2) stimulated a concentration-dependent release of \(\text{Ca}^{2+}\) from the intracellular stores of permeabilized DT40-\(\text{IP}_\text{R}1\) cells (Fig. 2A, B). The maximal \(\text{Ca}^{2+}\) release evoked by each ligand was similar, but \(1,3,4,6\)\(\text{IP}_3\) was \(21 \pm 3\) fold less potent that \(1,4,5\)\(\text{IP}_3\) (Table S2 in ESI). Membranes from Sf9 cells expressing rat \(\text{IP}_\text{R}1\) (Sf9-\(\text{IP}_\text{R}1\) cells) were used for equilibrium competition binding studies with \(\text{H}-\)\(1,4,5\)\(\text{IP}_3\) because these membranes express full-length \(\text{IP}_\text{R}1\) at ~20-fold higher levels than cerebellar membranes, the richest source of endogenous \(\text{IP}_\text{R}1\) (Fig. 2C). In these analyses, the equilibrium dissociation constants \((K_d)\) for \(1,4,5\)\(\text{IP}_3\) and \(1,3,4,6\)\(\text{IP}_4\) differed by \(46 \pm 19\) fold (Fig. 2C and Table S2 in ESI).

Because both agonists (1 and 2) released the same amount of \(\text{Ca}^{2+}\) at maximally effective concentrations, a comparison of \(\text{EC}_{50}\) and \(K_d\) values allows the effectiveness with which each promotes opening of the \(\text{IP}_\text{R} \ \text{Ca}^{2+}\) channel to be determined. A partial agonist needs to occupy more receptors to elicit the same response, which is then reflected in a higher \(\text{EC}_{50}\)/\(K_d\) ratio (and a lower value for \(\text{pEC}_{50}\)-\(\text{pK}_d\), where \(p\) denotes the negative log).\(^6\) \(1,3,4,6\)\(\text{IP}_4\) and \(1,4,5\)\(\text{IP}_3\) did not differ significantly in their \(\text{pEC}_{50}\)-\(\text{pK}_d\) values (Table S2 in ESI) suggesting that \(1,4,5\)\(\text{IP}_3\) and \(1,3,4,6\)\(\text{IP}_4\) have similar efficacies. We conclude that \(1,3,4,6\)\(\text{IP}_4\) is a full agonist with lower affinity than \(1,4,5\)\(\text{IP}_3\), in agreement with a previous report,\(^2\) but inconsistent with suggestions that it is a partial agonist.\(^9\)\(^\text{,}^\text{12}\)

\(2\)-\(\text{O-Butyryl-(1,3,4,6)}\)\(\text{IP}_4\) is a partial agonist and \(1,2,3,4,6\)\(\text{IP}_5\) is an antagonist of \(\text{IP}_\text{R}\). We synthesized and assessed the biological activity of two analogs with modifications at the 2-position of \(1,3,4,6\)\(\text{IP}_4\), \(2\)-\(\text{O-Butyryl-(1,3,4,6)}\)\(\text{IP}_4\) (3) and \(1,2,3,4,6\)\(\text{IP}_4\) (4) (Fig. 1B). The analogs retained both the essential pharmacophore (Fig. 1B, blue), and the 5-hydroxyl and 6-phosphate groups (equivalent to the.
6-hydroxyl and 1-phosphate of (1,4,5)IP$_3$ that increase binding affinity (Fig. 1B, green).

A maximally effective concentration of 2-O-butyryl-(1,3,4,6)IP$_3$ released a smaller fraction of the intracellular Ca$^{2+}$ stores than did (1,4,5)IP$_3$ (Fig. 28) and it bound to the IP$_3$R1 with 50 ± 22-fold lower affinity than (1,4,5)IP$_3$ (Fig. 2C). The lesser maximal Ca$^{2+}$ release evoked by 2-O-butyryl-(1,3,4,6)IP$_3$ suggests that it is less efficacious than (1,4,5)IP$_3$. Although 2-O-butyryl-(1,3,4,6)IP$_3$ and (1,3,4,6)IP$_3$ differed in their ability to evoke Ca$^{2+}$ release, they bound to IP$_3$R with similar affinities (Fig. 2C and Table S2 in ESI). Hence, addition of a butyryl moiety to the 2-position of (1,3,4,6)IP$_3$ decreased efficacy without affecting affinity. 2-O-Butyryl-(1,3,4,6)IP$_3$ (3) thus replaced (1,3,4,6)IP$_3$ as a lead compound from which we attempted to develop ligands that bind to IP$_3$R without activating it (i.e. competitive antagonists).

Even a very high concentration (100 µM) of (1,2,3,4,6)IP$_3$ (4) failed to release Ca$^{2+}$ (Fig. 2B), but it bound to IP$_3$R1 with a $K_d$ of 22.9 µM (Fig. 2C and Table S2 in ESI). Hence, (1,2,3,4,6)IP$_3$ has 230 ± 100-fold lower affinity than (1,4,5)IP$_3$ for IP$_3$R1 and significantly lower affinity than (1,4,5)IP$_3$ or 2-O-butyryl-(1,3,4,6)IP$_3$ (5.2 ± 0.5 and 4.8 ± 0.3-fold lower, respectively) (Fig. 2C, and Table S2, ESI). (1,2,3,4,6)IP$_3$ retains the essential pharmacophore and moieties known to be crucial for high-affinity binding (Fig. 1B), but it has an axial phosphate at the 2-position [equivalent to the 3-position of (1,4,5)IP$_3$]. Others have reported that an axial phosphate at the 3-position of (1,4,5)IP$_3$ reduced affinity. The important observation is that addition of an axial 2-phosphate to (1,3,4,6)IP$_3$ to give (1,2,3,4,6)IP$_3$ abolishes residual efficacy, albeit with some (5.2 ± 0.5-fold) loss of affinity.

Pre-equilibration of permeabilized DT40-IP$_3$R1 cells with (1,2,3,4,6)IP$_3$ (100 µM, 2 min), shifted the sensitivity of the Ca$^{2+}$ release evoked by (1,4,5)IP$_3$ by 2.4 ± 0.2-fold, without affecting either the maximal Ca$^{2+}$ release or Hill coefficient (Fig. 3 and Table S3, ESI). From the dose ratios (see Experimental Section), this functional analysis suggests that (1,2,3,4,6)IP$_3$ binds to the (1,4,5)IP$_3$-binding site of IP$_3$R1 with a $K_d$ of ~70 µM. Given the non-equilibrium conditions and the different temperatures used for functional (20 °C) and radioligand binding (4 °C) experiments, this measurement is in reasonable agreement with the affinity determined from equilibrium binding to IP$_3$R1 ($K_d$ ~23 µM) (Fig. 2C, 3B and Tables S2, S3 in ESI). These results demonstrate that (1,2,3,4,6)IP$_3$ is a competitive antagonist of IP$_3$R with an affinity of ~20-70 µM.

Dimeric analogs of 2-O-butyryl-(1,3,4,6)IP$_3$ or (1,2,3,4,6)IP$_3$ are antagonists of IP$_3$R1 with reasonable affinity. We reasoned from past precedent that dimeric versions of 2-O-butyryl-(1,3,4,6)IP$_3$ or (1,2,3,4,6)IP$_3$ might improve affinity or [for 2-O-butyryl-(1,3,4,6)IP$_3$] the loss of efficacy. We linked 2-O-butyryl-(1,3,4,6)IP$_3$ and (1,2,3,4,6)IP$_3$ through the 5-O-position [analogous to the 6-hydroxyl of (1,4,5)IP$_3$] to provide homo-dimeric ligands (5-12, Fig. 1C).

The activities of (1,2,3,4,6)IP$_3$ (4) and the dimer 6 are directly compared in Fig. 3. Neither 4 nor 6 (100 µM) evoked Ca$^{2+}$ release, but they reduced the sensitivity of the Ca$^{2+}$ release evoked by (1,4,5)IP$_3$ by 2.4 ± 0.2 and 20.9 ± 0.7-fold, respectively, without affecting the maximal response or Hill coefficient. Hence the dimer 6, like the monomer 4, is a competitive antagonist, but 6 has an apparent affinity that is 8.8 ± 1.0-fold greater than 4 (Table S3 in ESI).
Fig. 2. 2-O-Butyryl-(1,3,4,6)IP, (3) is a partial agonist and (1,2,3,4,6)IP, (4) is an antagonist of IP3R1. (A) Typical experiment showing Ca^{2+} uptake into the ER of permeabilized DT40-IP3R1 cells after addition of MgATP (1.5 mM), recorded with a luminal Ca^{2+} indicator (mag-fluo-4). Addition of (1,4,5)IP3 (concentrations in nM), with cyclopiazonic acid (CPA, 10 µM) to inhibit the Ca^{2+} pump, reveals the concentration-dependent effect of (1,4,5)IP3 on Ca^{2+} release. Results show fluorescence (relative fluorescence units, RFU) as means from triplicate determinations in a single experiment. (B) Summary results show effects of the indicated analogs on Ca^{2+} release (% of Ca^{2+} content of intracellular stores). (C) Equilibrium competition binding with 3H-(1,4,5)IP3 and the indicated analogs using membranes from Sf9-IP3R1 cells in CLM containing 1.5 mM MgATP. Results in B and C are means ± s.e.m., n = 3. The inset shows a representative Western blot (n = 2) demonstrating expression of IP3R1 in membranes from rat cerebellum (5 µg protein) and Sf9-IP3R1 cells (0.3 µg). Data summarized in ESI in Table S2.

The results with 6, suggesting that a dimer of (1,3,4,5,6)IP5 retained the lack of efficacy of (1,3,4,5,6)IP3, while displaying improved affinity, prompted analysis of seven additional dimeric analogs of 2-O-butyryl-(1,3,4,6)IP3 and (1,2,3,4,6)IP3 (Fig. 1C). None of the dimers (5-12, 100 µM) evoked Ca^{2+} release, and they all significantly decreased the sensitivity to (1,4,5)IP3 without affecting the maximal Ca^{2+} release or Hill coefficient (Fig. 4 and Table 1). All of the dimers (5-12) are therefore competitive antagonists.

Although 2-O-butyryl-(1,3,4,6)IP4 is a partial agonist with a K_d of 4.8 µM (Fig. 2B and Table S2 in ESI), its dimeric analogs are competitive antagonists with slightly reduced apparent affinities (K_d = 15-44 µM) (Table 1). The decreased affinity is consistent with evidence from analogs of (1,4,5)IP3, where substitution of the 6-hydroxyl (equivalent to the 5-hydroxyl of 3, through which the dimers are linked) reduced affinity.41,42 The 6-hydroxylation of (1,4,5)IP3 is thought to stabilize interactions of the IP3-binding core.43 However, the reduction in affinity between 3 and its dimers is modest by comparison with the 70 to 100-fold decrease for 6-deoxy-(1,4,5)IP3 and 6-methoxy-(1,4,5)IP3 relative to (1,4,5)IP3.41,43 Hence, dimerization of 2-O-butyryl-(1,3,4,6)IP3, to give 5, 7, 9 and 11, successfully reduced efficacy, without improving affinity (Table 1).

The antagonist, 12, is one of three dimers of (1,2,3,4,6)IP5 (4) with equally high affinity and it shifted the EC_{50} for (1,4,5)IP3 by 19.4 ± 6.5-fold, suggesting an apparent K_d of ~7 µM (Fig. 4B). Given the similar affinities of the dimers 8, 10 and 12 (K_d 7-8 µM) in functional assays (Table 1), we examined only 12 in equilibrium competition binding experiments. The K_d value for 12 determined in these experiments (7.7 µM) concurs with the results from functional analyses (Table 1).
These results establish that 8, 10 and 12 are competitive antagonists of IP₃R with low-micromolar affinity. Although modifications of the 6-hydroxyl of (1,4,5)IP₃ reduced affinity, dimerization through the analogous 5-hydroxyls of 3 and 4 caused more modest decreases or increases in affinity, respectively (Table 1). That pattern is similar across the four different linkers used (Fig. 1C). For each linker, dimers of 4 had 2 to 3-fold greater affinity than dimers of 3, even though monomeric 4 has significantly lower affinity than monomeric 3 (Fig. 4, Table 1 and Table S2 in ESI). For dimers of both 3 and 4, the shortest linker (n = 1, Fig. 1C) less effectively increased affinity than did the longer linkers (n = 2-3) (Table 1).

Conclusions

There is a need for selective antagonists of IP₃R so far reported. The affinity of these antagonists for IP₃ varies from low-micromolar affinity to much lower affinity for IP₃, 5-carboxymethyl-(1,4)IP₃ was reported to partially inhibit IP₃-evoked Ca²⁺ release, but only at an extremely high concentration (5 mM). 5-Carboxymethyl-(1,4)IP₃ was the only other inositol phosphate previously shown to be a competitive antagonist, but it bound to IP₃R with lower affinity (Kᵦ ~40 µM) than (1,2,3,4,6)IP₃ (4, Kᵦ ~23 µM) and with substantially lower affinity than the dimers of 4. These comparisons are consistent with our observation that 10 µM (1,2,4,5,6)IP₃ had no detectable effect on (1,4,5)IP₃-evoked Ca²⁺ release, whereas the same concentration of 12 caused a 2.8-fold decrease in (1,4,5)IP₃-sensitivity (not shown). A dimeric benzene with six attached phosphate groups (biphenyl 2,2',4,4',5,5'-hexakisphosphate) was recently reported to be a rather high-affinity (Kᵦ ~200 nM) antagonist of IP₃R, but it inhibited IP₃ 5-phosphatase with very similar potency. Compounds 8, 10 and 12 are the most potent inositol phosphate-based antagonists of IP₃R so far reported. The affinity of these antagonists for IP₃R (Kᵦ ~4 µM), but the new dimeric antagonists are smaller than heparin (Mₐ ~1200 and ~5000, respectively), and less likely to interact with as many additional intracellular targets. None of these antagonists is membrane-permeant, but based on the versatility of our synthetic approach, it may be feasible to esterify the

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**Table 1.** Dimers of 2-O-butyryl-(1,3,4,6)IP₃ and (1,2,3,4,6)IP₃ are competitive antagonists of IP₃R.

<table>
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<th>Ca²⁺ release</th>
<th>pEC₅₀ /M</th>
<th>ΔpEC₅₀ /M</th>
<th>EC₅₀ nM</th>
<th>Maximal release %</th>
<th>nₛ</th>
<th>Kᵦ μM</th>
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<td>(1,4,5)IP₃</td>
<td>7.03 ± 0.02</td>
<td>-</td>
<td>94</td>
<td>70 ± 1</td>
<td>1.31 ± 0.18</td>
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<td>+ 9</td>
<td>6.24 ± 0.07*</td>
<td>0.79 ± 0.09</td>
<td>571</td>
<td>64 ± 4</td>
<td>1.63 ± 0.42</td>
<td>20</td>
</tr>
<tr>
<td>+ 10</td>
<td>5.86 ± 0.06*</td>
<td>1.17 ± 0.05</td>
<td>1393</td>
<td>64 ± 3</td>
<td>1.58 ± 0.17</td>
<td>7</td>
</tr>
<tr>
<td>+ 11</td>
<td>6.27 ± 0.13*</td>
<td>0.76 ± 0.15</td>
<td>537</td>
<td>59 ± 4</td>
<td>1.08 ± 0.17</td>
<td>21</td>
</tr>
<tr>
<td>+ 12</td>
<td>5.84 ± 0.16*</td>
<td>1.19 ± 0.18</td>
<td>1449</td>
<td>60 ± 5</td>
<td>2.13 ± 0.55</td>
<td>7</td>
</tr>
</tbody>
</table>

**Binding**

<table>
<thead>
<tr>
<th>pKᵦ /M</th>
<th>Kᵦ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.90 ± 0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>4.79 ± 0.05</td>
<td>16.4</td>
</tr>
<tr>
<td>5.11 ± 0.08</td>
<td>7.7</td>
</tr>
</tbody>
</table>

*Summary results from Fig. 4 show the effects of (1,4,5)IP₃ alone or in the presence of 100 µM of each analog. Results show pEC₅₀, ΔpEC₅₀, pEC₅₀ - pEC₅₀ and Hill coefficients (nₛ) (means ± s.e.m.) and EC₅₀ for (1,4,5)IP₃-evoked Ca²⁺ release (n = 3). Kᵦ is shown calculated from functional assays and from equilibrium competition binding experiments (n = 3). Statistical differences were determined by one-way ANOVA and Tukey’s post hoc test, and refer to the results with (1,4,5)IP₃ alone. *P < 0.05.
Experimental

Chemistry

Materials and methods. All commercially available reagent-grade chemicals and solvents were used without further purification. Dry solvents were prepared by literature methods and stored over molecular sieves. Whenever possible, reactions were monitored using commercially available precoated TLC plates (layer thickness 0.25 mm) of Kieselgel 60F254. Compounds were visualized by use of a UV lamp and/or phosphomolybdic acid (PMA) or Seebach’s stains upon warming. Column chromatography was performed in the usual way using Merck 60 (40–60 mm) silica gel using as eluents the solvents indicated in each case. Yields are reported for isolated compounds with >96% purity, as established by NMR spectroscopy. FTIR spectra were obtained in a Nicolet 6700 spectrometer. NMR spectra were recorded with a 300 MHz Bruker Avance II spectrometer (1H: 300 MHz, 13C: 75 MHz, 13P: 121 MHz) or an Agilent 500/54 spectrometer (1H: 500 MHz, 13C: 126 MHz, 13P: 202 MHz) using the deuterated solvent indicated. Chemical shifts are given in parts per million and J values in Hertz using solvent or TMS as an internal reference. Assignments of protons were confirmed based on 2D NMR experiments (1H,1H COSY, HSQC, and HMBC, recorded using a standard pulse-program library). High resolution mass spectra (HRMS) were recorded on micrOTOF GC–MS QP 5050 Shimadzu single-quadrupole mass spectrometer. For each known compound 1H and/or 13C NMR spectra along with HRMS spectra were used to establish identity.

Esterification of malonic acid with 21. Malonic acid (280 mg, 2.69 mmol), and DCC (4.43 g, 21.5 mmol) were successively added to a solution of alcohol 21 (2.69 g, 5.39 mmol) in dry Et2O (50 mL). The resulting slurry was vigorously stirred under an Ar atmosphere at room temperature for 24 h, while the reaction progress was monitored by TLC. Upon completion, the solvent was removed in vacuo. The residue was triturated with Et2O and filtered. The solid was further washed with Et2O (25 mL) and the filtrates were concentrated in vacuo and the residue was purified with flash column chromatography (hexanes/ EtOAc 1:1) to give diesters 23a and 510 mg (12%) of ureido derivative 24b.

Esterification of succinic acid with 21. Succinic acid (160 mg, 1.36 mmol), DMAP (133 mg, 1.1 mmol), and DCC (1.69 g, 8.2 mmol) were successively added to a solution of alcohol 21 (1.36 g, 2.72 mmol) in dry CH2Cl2 (25 mL). The resulting slurry was vigorously stirred under an Ar atmosphere at room temperature for 96 h, while the reaction progress was monitored by TLC. Upon completion, the reaction mixture was washed with H2O (25 mL) and saturated brine (25 mL). The combined aqueous phases were back-extracted with CH2Cl2 (4×50 mL), the combined organic phases were dried over Na2SO4, and the solvents were removed in vacuo. The residue was purified with flash column chromatography (hexanes/EtOAc 3:1 to 1:1) to give diesters 23b and 260 mg (12%) of ureido derivative 24c.

General Procedure A: Preparation of 5,5'-ethers 25a,b. Alcohol 21 (1 mmol) was dissolved in a 4:1 mixture of toluene and DMSO (2.5 mL), powdered KOH (140 mg, 2.5 mmol) was added and the mixture was warmed up to 55 °C. Then, 26 or 27 (0.5 mmol) was added in one portion and the resulting slurry was heated at the same temperature for 120 h, while the progress of the reaction was monitored by TLC. Upon completion, the mixture was neutralized with the addition of a saturated aqueous NH4Cl solution. Then, water was added to dissolve all solids and the clear solution was extracted with toluene (50 mL) and CH2Cl2 (2×50 mL). The combined organic phases were dried over Na2SO4, and concentrated in vacuo. The residue was purified with flash column chromatography (hexanes/EtOAc 5:1 to 1:1) to give ethers 25a,b.

General Procedure B: Preparation of diols 28. 10% Pd/C (200 mg) was added to a solution of dibenzyl ether 23 or 25 (1 mmol) in MeOH (60 mL). This mixture was vigorously stirred under H2 (1 atm) at room temperature for 24 h. Then, it was filtered through a pad of Celite®, which was further washed with MeOH (20 mL), CH2Cl2 (20 mL), and MeOH (20 mL). Diols 28 were found to be sufficiently pure and used in the next steps without any further purification.

General Procedure C: Preparation of butyrates 29. Dry Et3N (0.56 mL, 4 mmol) and DMAP (50 mg, 0.4 mmol) were added to a solution of diol 28 (1 mmol) in dry CH2Cl2 (10 mL) under an Ar atmosphere at room temperature. Butyric anhydride (0.50 mL, 3 mmol) was added and the mixture was stirred at room temperature until the full consumption of starting material (TLC monitoring, about 24 h). The reaction mixture was diluted with CH2Cl2 (20 mL) and successively washed with saturated aqueous sodium bicarbonate solution (3×10 mL) and saturated brine (10 mL). The aqueous phase was back-extracted with CH2Cl2 (10 mL) and the combined organic phases were dried over Na2SO4, and concentrated in vacuo. The residue was purified with flash column chromatography (hexanes/ EtOAc 7:1 to 1:1) to give butyrates 29.

General Procedure D: Removal of acetal protecting groups. A 90% aqueous solution of TFA (10 mL) was added dropwise to a solution of starting acetal (16d or 28 or 29, 1 mmol) in CH2Cl2 (10 mL) at room temperature. The resulting mixture was stirred at the same temperature for 2 h. Then, the volatiles were removed under reduced pressure (40 °C). The residue was successively treated with toluene (10 mL) and absolute Et2O (3×10 mL) and each time the solvent was removed under reduced pressure. The resulting polyl was found to be sufficiently pure by NMR and used in the next step without any further purification.

General Procedure E: Phosphorylation of polyls. A 0.45M solution of 1H-tetrazole in CH3CN (3 equiv. per OH) was added to a flask containing neat starting polyl (15a or 17a or 30, 1 mmol) under an Ar atmosphere at room temperature. Then, dibenzyl N,N-diisopropylphosphoramidite (1.6 equiv. per OH)
was added dropwise over a period of 30 min. The resulting mixture was stirred for 24 h at room temperature, and an additional amount of the phosphorylating agent was added (0.3 equiv. per OH). After 24 h the reaction mixture was diluted with CH₂Cl₂ (10 mL) and cooled to –50 °C. A solution of 70% m-CPBA (2.4 equiv. per OH) in CH₂Cl₂ (1.6 mL/mmol m-CPBA) was added dropwise and the mixture was left to vigorously stir for 5 h at 0 °C. The reaction mixture was further diluted with CH₂Cl₂ (120 mL) and successively washed with a 10% aqueous solution of sodium sulfite (2×150 mL), a saturated aqueous solution of NaHCO₃ (2×120 mL), and H₂O (120 mL). The combined aqueous phases were back-extracted with CH₂Cl₂ (100 mL). The combined organic phases were washed with saturated brine (120 mL), and dried over Na₂SO₄. The solvents were removed under reduced pressure and the residue was purified with flash column chromatography (initially hexanes/ EtOAc 2:1 to 1:2 and then 2-5% CH₂OH in EtOAc).

**General Procedure F: Final deprotection.** The starting benzyl phosphate (15b or 17b or 31, 1 mmol) was dissolved in EtOH (50–70 mL). Deionized H₂O (50–70 mL) and NaHCO₃ (1 equiv. per phosphate group) were added. Then, 10% Pd/C (1 g) was added to the resulting emulsion and the mixture was vigorously stirred under H₂ (1 atm) at room temperature for the indicating period of time. The reaction progress was monitored by 1H NMR. Upon completion the catalyst was removed by filtration through an LCR/PTFE hydrophilic membrane (0.5 mm); the membrane was washed with a 1:1 mixture of EtOH and deionized H₂O (3×30 mL). The combined filtrates were evaporated under reduced pressure (55 °C), and the resulting residue was dried under high vacuum for 24 h to yield the desired phosphate salt.

**Biology**

**Ca²⁺ release from permeabilized DT40-IP₃R₁ cells.** DT40 cells with disrupted endogenous IP₃R genes, and stably expressing rat IP₃R₁ (DT40-IP₃R₁ cells) were cultured as described. For measurements of free [Ca²⁺] within the lumen of the ER, cells were incubated with mag-fluo4/AM (20 µM, Life Technologies, Paisley, UK) under conditions that favor sequestration of the indicator into the ER lumen. Cells were washed, permeabilized using saponin, resuspended in cytosol-like medium (CLM) supplemented with FCCP (10 µM, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) to inhibit mitochondria, and distributed into black half-area 96-well plates (Greiner Bio-One). CLM had the following composition: 2 mM NaCl, 140 mM KCl, 1 mM EGTA, 20 mM PIPES, 375 µM CaCl₂ (free [Ca²⁺] ~230 nM), pH 7.0. Fluorescence (excitation at 485 nm, emission at 525 nm) was recorded at 1.44-s intervals at 20 °C using a FlexStation 3 plate-reader (MDS Analytical Devices, Berkshire, UK). Ca²⁺ uptake into the ER was initiated by addition of 1.5 mM MgATP, and after 2 min (1,4,5)IP₃ or an analog was added with cyclopiazic acid (CPA, 10 µM, R&D Systems Europe, Oxford, UK) to inhibit further Ca²⁺ uptake. Ca²⁺ release was recorded after a further 10-20 s and reported as a fraction of the Ca²⁺ uptake evoked by ATP. Antagonists were added 2 min before (1,4,5)IP₃.

**Equilibrium binding of [³H]-1H-(1,4,5)IP₃ and competing ligands to IP₃R₁.** These assays were performed at 4 °C in 500 µL of CLM containing 1.5 mM MgATP, membranes (~20 µg protein) prepared from Sf9 cells expressing rat IP₃R₁ (Sf9-IP₃R₁ cells), [³H]-1H-(1,4,5)IP₃ (1.5 nM, 19.3 Ci/mmol, Perkin Elmer, Waltham, MA, USA) and appropriate concentrations of competing ligand. Non-specific binding was determined by addition of 10 µM (1,4,5)IP₃ (Enzo Life Sciences, Exeter, UK). Reactions were terminated after 5 min by centrifugation (20,000 xg, 5 min, 4 °C). The pellet was washed with 700 µL of CLM, resuspended in 200 µL of CLM, and radioactivity was determined by liquid scintillation counting. Culture of Sf9 cells, infection with baculovirus encoding rat IP₃R₁, and preparation of membranes were as described previously. Quantification of IP₃R₁ expression by Western blotting, using an anti-peptide antiserum to IP₃R₁ was performed as described.

**Analysis.** For each individual experiment, concentration-effect relationships were fitted to a Hill equation using non-linear curve-fitting (GraphPad Prism, version 5). From each experiment, pEC₅₀ or pIC₅₀ (log of the half-maximally effective (ECₕ₅₀) or inhibitory (IC₅₀) concentration in M), Hill coefficient (nₕ), and the maximal response were obtained and then used for statistical analyses. All reported comparisons of ligand potencies rely on comparisons within experiments because EC₅₀ values for (1,4,5)IP₃-evoked Ca²⁺ release can vary between passages of cells. For convenience, figures illustrating concentration-effect relations show average results from several experiments, but the values (pEC₅₀, etc) determined from fitting curves to individual experiments were used for statistical analyses. Most statistical comparisons were paired, and used Student’s t-test or one-way ANOVA with Tukey’s post hoc test as appropriate. P < 0.05 is considered significant.

The dose ratio (DR = EC₅₀/IP₃ₙₜ, where EC₅₀ and IC₅₀ are the EC₂₀ values for (1,4,5)IP₃-evoked Ca²⁺ release determined in the presence and absence of antagonist, respectively) was used to calculate the apparent affinity (Kₐ) of IP₃R₁ for antagonists from functional assays:

\[ K_d = \frac{[\text{Antagonist}]}{(DR - 1)} \]

From equilibrium competition binding experiments, the Kₐ of competing ligands was calculated from the concentration (IC₅₀) required to cause 50% displacement of the specifically bound [³H]-1H-(1,4,5)IP₃:

\[ K_d = \frac{I_{C_{50}}}{1 + \left(\frac{[\text{³H}-(1,4,5)\text{IP}_3]}{K_{d}^{[\text{³H}-(1,4,5)\text{IP}_3]}}\right)} \]

The [³H]-1H-(1,4,5)IP₃ was 1.5 nM, and Kₐ [³H-(1,4,5)IP₃] (127 nM) (ESI Table S2). pKₐ values were then used for statistical analyses. For comparisons of differences between pEC₅₀ and pKₐ values (pEC₅₀-pKₐ), the standard deviation of the difference (σₚEC₅₀-Kₐ) was calculated from the individual variances (σₚEC₅₀ and σₚKₐ)².
\[ \sigma_{pEC_{50}-K_d} = \sqrt{\sigma_{pEC_{50}}^2 - \sigma_{K_d}^2} \]

Acknowledgements

Supported by a Senior Investigator Award from the Wellcome Trust 101844 (to C.W.T.), Biotechnology and Biological Sciences Research Council UK and the German Academic Exchange Service (to V.K.). A.E.K. thanks the Research Committee of AUTH for financial support. C.W.T. and V.K. thank Dr S. B. Shears (N.I.E.H.S, U.S.A.) for his helpful advice.

Notes and references

† Abbreviations used: Bt, butyryl; CLM, cytosol-like medium; m-CPBA, m-chloro-perbenzoic acid; DCC, N,N'-dicyclohexylcarbodiimide; DDQ, 2,3-dichloro-5,6-dicyano-1,4-dimethylaminopyridine; EC50 ([I]50), half-maximal effective (inhibitory) concentration; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ER, endoplasmic reticulum; IP3, inositol trisphosphate; IP6, inositol hexakisphosphate; IP10, inositol pentakisphosphate (structures of the analogs and their codes are shown in Fig. 1); Kd, equilibrium dissociation constant; pEC50 (pKd), -logEC50 (Kd); PMB, p-methoxybenzyl; TFA, trifluoroacetic acid.

2 C. W. Taylor and S. C. Tovey, Cold Spring Harb. Persp. Biol., 2010, 2, a004010.
8 The reaction was closely monitored in order to avoid the oxidative removal of the benzyl group.
12 We have practically recovered the rest of unreacted starting material [21].
14 For large scale runs we found it more convenient to subject the mixture of 23 and 24 to hydrogenolysis (see Scheme 4), since the debenzylated derivatives were more easily separable.
18 Only traces of the corresponding 5-O-but-3-enyl- and 5-O-pent-4-enyl-derivatives (elimination products) were detected in the reaction mixture.
For compounds 5 and 6 a rapid H-D exchange of malonic protons in the NMR solvent (D₂O) occurs. Therefore, these protons disappear in the ¹H NMR spectrum, whereas a quintet is observed in the ¹³C NMR spectrum for the CD₂ group (around 40 ppm). The complete insolubility of these compounds in non-protic solvents did not allow us to run other NMR experiments. The provided data (NMR and HRMS) are consisted with the given structures.


S. C. Tovey, Y. Sun and C. W. Taylor, Nat. Prot., 2006, 1, 259.


