

Activation of IP₃ receptors by synthetic bisphosphate ligands†

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Ca²⁺ release by D-myo-inositol 1,4,5-trisphosphate receptors (IP₃R) is widely considered to require the vicinal 4,5-bisphosphate motif of IP₃, with P-5 and P-4 engaging the α and β domains of the binding site; using synthesis and mutagenesis we show that the adenine of synthetic glyconucleotides, through an interaction with Arg504, can replace the interaction of either P-1 or P-5 with the α -domain producing, respectively, the most potent bisphosphate agonist yet synthesised and the first agonist of IP₃R without a vicinal bisphosphate motif; this will stimulate new approaches to IP₃R ligand design.

D-myo-Inositol 1,4,5-trisphosphate (IP₃, **1**, Fig. 1) is an intracellular messenger that evokes Ca²⁺ release from the intracellular stores of most animal cells by binding to IP₃ receptors (IP₃R), which are IP₃-gated Ca²⁺ channels.^{1,2} Extensive structure–activity studies suggest that the vicinal 4,5-bisphosphate structure of IP₃ is essential, while the 1-phosphate has an ancillary role that substantially increases affinity.³ For more than 20 years, this enduring conclusion has guided design of new ligands for the IP₃R. Other inositol phosphate regioisomers are active through different binding modes,⁴ but they all have the vicinal bisphosphate structure. The fungal metabolite, adenophostin A (AdA, **2**, Fig. 1),⁵ which is at least ten times more potent than IP₃, provides additional opportunities to explore the mechanisms of IP₃R activation. AdA is a glyconucleotide trisphosphate structurally related to IP₃. It binds to a site that substantially overlaps the IP₃-binding site,⁶ and structure–activity studies with synthetic analogues of AdA have established that the adenine (or another aromatic group) is essential for its enhanced affinity.^{6,7}

The IP₃-binding core (IBC) of the IP₃R forms a clam-like structure with IP₃ held between two domains (α and β) linked by a flexible hinge.⁸ The 4-phosphate group (P-4) of IP₃ interacts mainly with residues in the β -domain, while the 1-phosphate (P-1) and 5-phosphate (P-5) interact predominantly with the α -domain (Fig. 2). We therefore speculated that binding of IP₃ might pull the two domains together in a manner reminiscent of glutamate binding to ionotropic glutamate receptors,⁹ and so cause the IBC to adopt a more constrained structure.¹⁰ Because both P-1 and P-5 interact with the α -domain, it may be possible to bridge the two domains using a ligand with only one of these groups. The

fact that the bisphosphate Ins(4,5)P₂ is a full agonist with low affinity¹¹ supports this suggestion. But the inability¹¹ of Ins(1,4)P₂ to mobilise Ca²⁺ or bind to IP₃R suggests that interaction of P-1 alone with the α -domain, where it directly contacts only Arg568 (R568, Fig. 2), is too weak to stabilise binding. Ins(1,5)P₂ is also inactive¹² presumably because it has insufficient interactions with the β -domain. Hence, the prevailing view is that the vicinal bisphosphate moiety of IP₃ is essential for activity.

Our molecular docking experiments¹³ have suggested that the 2'-AMP motif of AdA interacts with the α -domain of the IBC, perhaps more strongly than P-1 of IP₃. We therefore reasoned that it may be possible for 3''-dephospho-AdA (**3**, Scheme 1) to bridge the domains effectively and release Ca²⁺. Alternatively, if the 2'-AMP interacts with the β -domain, it may allow 4''-dephospho-AdA (**4**) to activate IP₃R. We therefore developed methods to synthesise **3** and **4** (Scheme 1).[‡] Briefly, thioglycosides **5a** and **5b** were used to glycosylate a ribofuranose acceptor leading to disaccharides **6a** and **6b**, which were individually subjected to Vorbrüggen condensations with silylated 6-chloropurine to give **7a** and **7b**, the precursors for **3** and **4**, respectively. Ammonolysis of each installed the N-6 amino group and exposed the required pairs of hydroxyl groups for phosphorylation. Phosphitylation in the presence of imidazolium triflate and *in situ* oxidation followed by removal of benzyl protecting groups by transfer hydrogenolysis gave **3** and **4**, which were purified to homogeneity.

Using methods reported previously,¹⁴ we show that **4** does not evoke Ca²⁺ release *via* recombinant IP₃R1, but **3** is effective, albeit at high concentrations (Fig. 3). These results support the idea that P-3'' and P-4'' of AdA mimic P-5 and P-4 of IP₃, respectively, and that the 2'-AMP motif of AdA is able to bind the α -domain of the IBC more effectively than P-1 of IP₃. This allows bisphosphate **3** to pull the two domains together and activate IP₃R1, even though it lacks a vicinal bisphosphate. Bisphosphate **3**, the AdA equivalent of Ins(1,4)P₂, is the first agonist of IP₃R without a vicinal bisphosphate motif.

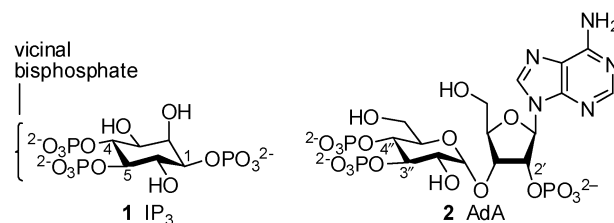


Fig. 1 IP₃ receptor ligands; IP₃ (**1**) and adenophostin A (**2**).

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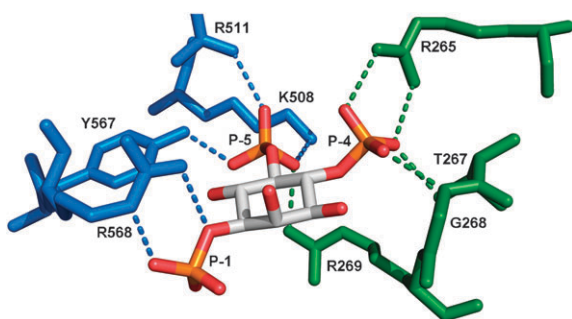
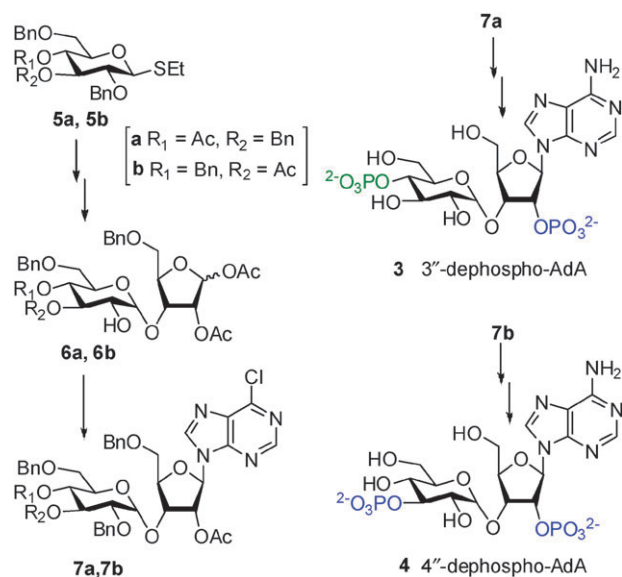


Fig. 2 Interactions of IP₃ with the α (blue) and β (green) domains of the IBC of IP₃R1 (PDB code 1n4k). Water molecules are omitted and only direct interactions between IP₃ and residues in the IBC are shown.



Scheme 1 Structures and syntheses of 3'- and 4'-dephospho-AdA. Phosphate groups are coloured according to putative interactions with the α (blue) or β (green) domains of the IBC. Bn = benzyl.

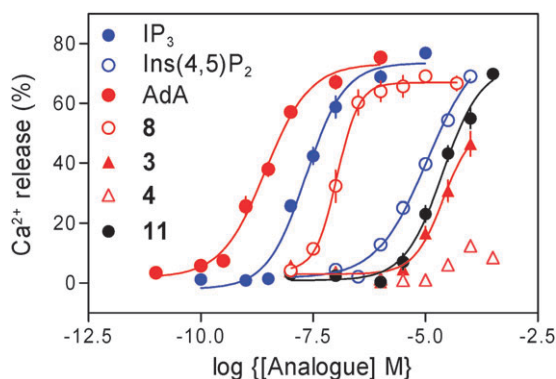
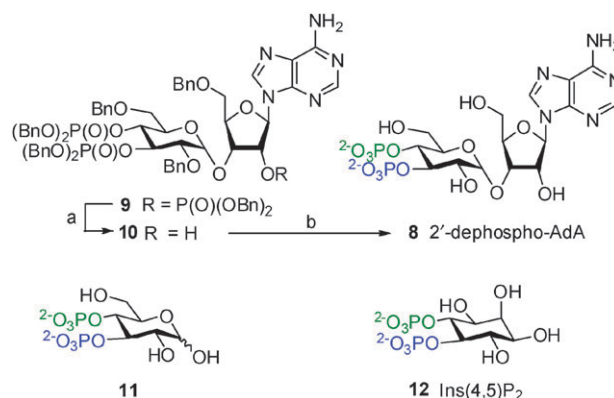


Fig. 3 Concentration-dependent effects of ligands on Ca²⁺ release via IP₃R1 stably expressed in DT40 cells. Results show means $\pm$ SEM; $n = 8$.

The 2'-AMP motif of AdA may allow P-2' to bind more effectively than P-1 of IP₃, or the adenosine itself may have



Scheme 2 Synthesis of 2'-dephospho-AdA and structures of glucose 3,4-bisphosphate and Ins(4,5)P₂: (a) K₂CO₃, BnOH, 70 °C, 93%; (b) Pd(OH)₂/C, cyclohexene, MeOH, H₂O, 80 °C, 92%. Bn = benzyl. Phosphate groups are coloured according to putative interactions with the α (blue) or β (green) domains of the IBC.

interactions with the IBC. To distinguish between these two possible roles, we synthesised 2'-dephospho-AdA, the adenosine equivalent of Ins(4,5)P₂ (**8**, Scheme 2). Interestingly, treatment of a benzyl-protected version of AdA (**9**)¹⁵ with sodium benzoxide in BnOH led to removal of the 2'-dibenzylphosphate group giving bisphosphate **10** as the major product with minor amounts of other by-products. Use of the milder base potassium carbonate resulted in clean and highly selective conversion into **10**. Deprotection and purification as before gave **8** in excellent yield. The 2'-phosphate triester in **9** is comparatively less crowded and hence more prone to nucleophilic attack than those in the 3',4' vicinal positions and this is likely to be the reason for this interesting high selectivity. We also synthesised the related glucose 3,4-bisphosphate (**11**, Scheme 2). Because **11** can be viewed as AdA without the 2'-AMP motif, any difference in the activities of **8** and **11** illustrates the role of the adenosine moiety. In functional assays, **8** was 100 times more potent than **11** (Fig. 3), confirming that the adenosine motif directly contributes to AdA binding even when it lacks the 2'-P group. Compound **8** is the first potent synthetic bisphosphate agonist of the IP₃R; it is >30-fold more potent than Ins(4,5)P₂ (Fig. 3, Table 1).

Our molecular modelling suggests a key role for arginine 504 (R504) in recognising the adenine of AdA, perhaps *via* a cation- π interaction.¹³ Using targeted mutagenesis, we established a cell line expressing only IP₃R1 with R504 mutated to glutamine, to determine whether R504 selectively contributes to the activity of AdA analogues. For the mutant IP₃R, the EC₅₀ for IP₃ and Ins(4,5)P₂ was reduced by 57- and 8.8-fold, respectively. For AdA and **8**, the reductions were 434- and

Table 1 Ca²⁺ release *via* IP₃R1. Results (means $\pm$ SEM, $n = 3-8$) show the EC₅₀ values for each agonist in DT40 cells stably expressing IP₃R1

Agonist	EC ₅₀
IP ₃	35 $\pm$ 5 nM
Ins(4,5)P ₂	7.59 $\pm$ 2.63 μ M
AdA	2.9 $\pm$ 0.9 nM
8	240 $\pm$ 5 nM

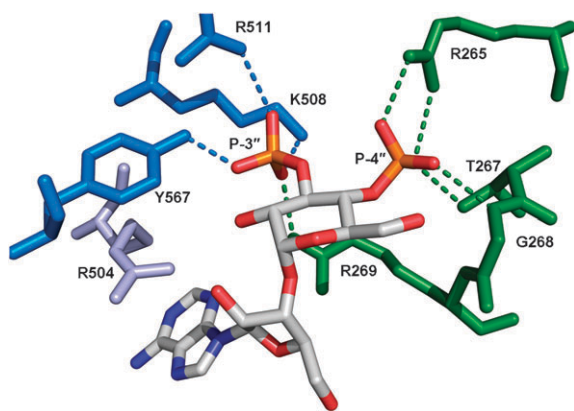


Fig. 4 Suggested interactions of **8** with the IBC of IP₃R1, based on molecular docking experiments using AdA.¹³ The side chain of R504 is shown in pale blue.

107-fold, and the response to **3** (≤ 3 mM) was abolished. The greater effect of this mutation on the potency of AdA and **8** is consistent with the proposed interaction between adenine and R504 (Fig. 4). Indeed, the potency of AdA and **8** at IP₃R1 both depend largely on R504 because, at the mutated receptor, AdA is only equipotent with IP₃, and **8** is less than 3-fold more potent than Ins(4,5)P₂.

We conclude that, while the three phosphate groups and adenine of AdA most likely make incremental contributions to IP₃R binding, a vicinal bisphosphate moiety is not essential for IP₃R activation. P-4 of IP₃, which contacts the β -domain of the IBC, is required, but P-5, which contacts the α -domain, can (as in **3**) make an interaction that can be substantially compensated by a cation– π interaction between the adenine of AdA and R504 in the α -domain. The same interaction can also serve to mitigate the loss of P-1 to provide a potent agonist (**8**) with only two phosphate groups. Aside from challenging the long-standing dogma that a vicinal bisphosphate is essential for agonists of IP₃R, these results illustrate the potential to design less polar IP₃R ligands. At their simplest, these might comprise two motifs that interact with the IBC domains linked by a suitable spacer.

Since inositol polyphosphates often bind to sites rich in Arg and Lys residues, replacing interactions with a polar phosphate by cation– π interactions should have more general applications in the chemical biology of inositol phosphate signalling and probably elsewhere.

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Notes and references

‡ All new compounds were thoroughly characterised and exhibited satisfactory parameters using standard spectroscopic techniques. A typical example follows.

Synthesis of 3'-O-(α -D-glucopyranosyl)-adenosine-3'',4''-bisphosphate (**8**).

A suspension of bisphosphate **10** (45 mg, 0.037 mmol) and 20% Pd(OH)₂ on carbon (120 mg) in a mixture of cyclohexene (1.6 mL), MeOH (3 mL) and H₂O (0.22 mL) was heated at 80 °C overnight. The mixture was filtered through a membrane filter and solvents evaporated *in vacuo*. Purification on an AG ion-exchange column (0–100% gradient elution, with 150 mM TFA and water) afforded pure bisphosphate **8** (20 mg, 92%). ¹H NMR (400 MHz, D₂O): 3.74–3.90 (m, 6H, H-2'', H-5'', H-5A', H-5B' H-6A'', H-6B''), 4.10 (ddd, like a q, 1H, 9.66 Hz, 9.66 Hz, 9.66 Hz, H-4''), 4.42 (dd, 1H, 7.24 Hz, 3.86 Hz, H-4'), 4.47–4.55 (m, 2H, H-3', H-3''), 4.87 (dd, like a t, 1H, 5.80 Hz, 5.31 Hz, H-2'), 5.19 (d, 1H, 3.38 Hz, H-1''), 6.19 (d, 1H, 5.80 Hz, H-1'), 8.40 (s, 1H, H-2), 8.50 (s, 1H, H-8). ¹³C NMR (100 MHz, D₂O): 60.08 (C-6''), 60.79 (C-5'), 70.48 (C-2'', ³¹P coupled), 71.60 (d, 3.83 Hz, C-5'', ³¹P coupled), 72.83 (C-4'', ³¹P coupled), 73.48 (C-2'), 76.37 (C-3'), 77.71 (C-3'', ³¹P coupled), 83.95 (C-4'), 88.23 (C-1'), 99.02 (C-1''), 118.91 (C-5), 142.81 (C-8), 144.43 (C-2), 148.16 (C-4), 149.91 (C-6). ³¹P NMR (161.94 MHz, D₂O with excess of TEA): 4.50, 3.62. *m/z* (ES+) = 590.2 [(M + H)⁺, 100%]; 612.1 [(M + Na)⁺, 100%]; *m/z* (ES–) 588.2 [(M – H)⁺, 100%]; HRMS: mass calcd for C₁₆H₂₆O₁₅N₅P₂ [(M + H)⁺, 590.0895; found, 590.0895.

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