Activation of IP3 receptors by synthetic bisphosphate ligands†

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Ca2+ release by d-myo-inositol 1,4,5-trisphosphate receptors (IP3Rs) 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, 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 active through different binding modes,4 but they all have the vicinal bisphosphate structure. The fungal metabolite, adenophysin A (AdA, 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 glycosylated triphosphate 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 (RS68, 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 imidazolinium 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 adenophysin A (2).
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 interactions with the IBC. To distinguish between these two possible roles, we synthesised 2′-dephospho-AdA, the adenosinephostin equivalent of Ins(4,5)P₂ (8, Scheme 2). Interestingly, treatment of a benzyl-protected version of AdA (9) 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 434-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. 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 IP₃R₁, 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 240-fold, respectively.

Table 1 Ca²⁺ release via IP₃,R₁. Results (means ± SEM, n = 3–8) show the EC₅₀ values for each agonist in DT40 cells stably expressing IP₃,R₁.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>EC₅₀</th>
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<tr>
<td>IP₃</td>
<td>35 ± 5 nM</td>
</tr>
<tr>
<td>Ins(4,5)P₂</td>
<td>7.59 ± 2.63 μM</td>
</tr>
<tr>
<td>AdA</td>
<td>2.9 ± 0.9 nM</td>
</tr>
<tr>
<td>8</td>
<td>240 ± 5 nM</td>
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for agonists of IP₃R, these results illustrate the potential to long-standing dogma that a vicinal bisphosphate is essential with only two phosphate groups. Aside from challenging the 8 AdA and R504 in the α-domain. The same interaction can also compensate for a cation–π interaction between adenine 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 by a suitable spacer.

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-6A, H-6B, H-6C). 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, 3.8 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'), 71.60 (d, 3.83 Hz, C-5'), 72.83 (C-4', C-5' 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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