Stimulation of Inositol 1,4,5-Trisphosphate (IP₃) Receptor Subtypes by Adenophostin A and Its Analogues

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃R) are intracellular Ca²⁺ channels that are expressed in almost all animal cells. They allow release of Ca²⁺ from intracellular stores in response to the many stimuli that activate phospholipase C [1,2]. The genomes of vertebrates encode three closely related IP₃R subtypes encoded by vertebrate genomes. Adenophostin A (AdA) is the most potent naturally occurring agonist of IP₃R and it shares with IP₃ the essential features of all IP₃R agonists, namely structures equivalent to the 4,5-bisphosphate and 6-hydroxyl of IP₃. The two essential phosphate groups contribute to closure of the clam-like IP₃-binding core (IBC), and thereby IP₃R activation, by binding to each of its sides (the α- and β-domains). Regulation of the three subtypes of IP₃R by AdA and its analogues has not been examined in cells expressing defined homogenous populations of IP₃R. We measured Ca²⁺ release evoked by synthetic adenophostin A (AdA) and its analogues in permeabilized DT40 cells devoid of native IP₃R and stably expressing single subtypes of mammalian IP₃R. The determinants of high-affinity binding of AdA and its analogues were indistinguishable for each IP₃R subtype. The results are consistent with a cation-π interaction between the adenine of AdA and a conserved arginine within the IBC α-domain contributing to closure of the IBC. The two complementary contacts between AdA and the α-domain (cation-π interaction and 3°-phosphate) allow activation of IP₃R by an analogue of AdA (3°-dephospho-AdA) that lacks a phosphate group equivalent to the essential 5-phosphate of IP₃. These data provide the first structure-activity analyses of key AdA analogues using homogenous populations of all mammalian IP₃R subtypes. They demonstrate that differences in the Ca²⁺ signals evoked by AdA analogues are unlikely to be due to selective regulation of IP₃R subtypes.

Introduction

Inositol 1,4,5-trisphosphate receptors (IP₃R) are intracellular Ca²⁺ channels that are expressed in almost all animal cells. They allow release of Ca²⁺ from intracellular stores in response to the many stimuli that activate phospholipase C [1,2]. The genomes of vertebrates encode three closely related IP₃R subtypes (IP₃R1-3), and most cells from vertebrates express functional IP₃R that are homo- or hetero-tetrameric assemblies of these IP₃R subtypes and their splice variants [3]. The physiological significance of this IP₃R diversity is poorly understood, and nor are there ligands that usefully discriminate between IP₃R subtypes. It is, however, clear that activation of IP₃R is initiated by binding of IP₃ to the conserved IP₃-binding core (IBC, residues 224-604 of IP₃R1) of each IP₃R subunit [4]. Mixed populations of IP₃R in native cells make it difficult to define unambiguously the functional properties of each IP₃R subtype. Stable heterologous expression of mammalian IP₃R in the only vertebrate cell line engineered to lack all endogenous IP₃R (DT40 KO cells) [5] provides an effective means of addressing this difficulty [6]. We previously used DT40 cells expressing homogenous populations of each mammalian IP₃R subtype to define structure-activity relationships for key endogenous and synthetic inositol phosphates [7]. Here, we extend the approach to examine the interactions of each IP₃R subtype with adenophostin A (1, AdA) and its most important analogues [8] (Figure 1A).

AdA, originally isolated from Penicillium brevicompactum [9,10] and later synthesized [11], is a potent agonist of IP₃R. It is also resistant to degradation by the enzymes that degrade IP₃ via phosphorylation or dephosphorylation [10]. Although AdA is based on a glucose ring, rather than the inositol ring of IP₃, its structure retains the key functional groups of IP₃ that are known to be essential for IP₃R activity at IP₃R [12] (Figure 1A). Considerable evidence supports the original suggestion [10] that the essential 4,5-bisphosphate and 6-hydroxyl of IP₃ are effectively mimicked by the 4°,3°-bisphosphate and 2°-hydroxyl of AdA (red highlights in Figure 1A). The interactions that allow AdA to bind to IP₃R with about 10-fold greater affinity than IP₃ have been more difficult to resolve. One view was that the 2°-phosphate of AdA is equivalent to the 1-phosphate of IP₃ and, like the latter [13] (blue in Figure 1A), contributes to high-affinity binding to the IBC. The suggestion was that the 2°-phosphate of AdA forms a stronger interaction with the IBC than does the 1-phosphate of IP₃. Our recent analyses have challenged this idea and instead suggest that a cation-π interaction between the adenine ring of AdA and a guanidinium side chain of an arginine residue within the α-domain of the IBC (R504 in IP₃R1) may be a more important determinant of the increased affinity of AdA for IP₃R [12].

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A

IP₃  

AdA  

Imidophostin  

Rbophostin  

Furanophostin  

Manno-AdA  

Xylo-AdA  

3'-dephospho-AdA  

4'-dephospho-AdA  

2'-dephospho-AdA  

B

C

ATP

IP₃

1 nM

30 nM

100 nM

10 μM

fluorescence (RFU)

time (s)

ATP

AdA

0.1 nM

3 nM

30 nM

1 μM

fluorescence (RFU)

time (s)
The high-affinity and metabolic stability of AdA have generated considerable interest in both the synthesis of AdA analogues and their application to analyses of IP$_3$-R activation and associated changes in cytosolic Ca$^{2+}$ signalling [12]. There has, however, been no systematic analysis of the activities of AdA or its analogues with defined populations of homogenous IP$_3$-R subtypes. The need for such analyses is particularly important in attempting to explain results in which Ca$^{2+}$ signals evoked by IP$_3$ differ from those evoked by AdA [14,15,16,17,18,19,20,21], or where different analogues of AdA evoke different cellular responses [reviewed in 12,22]. Here we use DT40 cells in which all endogenous IP$_3$-R have been genetically inactivated [5] to stably express homogenous populations of mammalian IP$_3$-R subtypes and thereby define structure-activity relationships for AdA and its key analogues for each IP$_3$-R subtype.

### Materials and Methods

#### Materials

Sources of most reagents were provided in a previous publication [7]. The structures of the ligands used and their abbreviations are shown in Figure 1A. IP$_3$ was from Alexis Biochemicals (Nottingham, UK). AdA [23], imidophostin [24], ribophostin [25], furanophostin [26], mannophostin and xylophostin [27], 3'-dephospho AdA and 4'-dephospho AdA [28], and 2'-dephospho AdA were synthesized, purified and characterized as previously described.

#### Measurement Ca$^{2+}$ Release by IP$_3$ Receptors

From quantitative analyses of western blots using antisera that selectively recognize each IP$_3$-R subtype or react equally with all three subtypes, we established that in the DT40 cells used, levels of IP$_3$-R expression (relative to IP$_3$R3) were IP$_3$R1 (71±8%, n = 3), IP$_3$R2 (48±5%) and IP$_3$R3 (100%) [7]. It is impracticable to achieve identical levels of IP$_3$-R expression for each cell line, and differences (albeit modest in our cell lines) may affect both the size of the IP$_3$-sensitive Ca$^{2+}$ pool and its sensitivity to IP$_3$ [29]. The different levels of IP$_3$-R expression do not compromise the analyses reported here, which are entirely concerned with relative potencies of AdA analogues for each IP$_3$-R subtype (see below).

A comprehensive description of the methods used to measure free [Ca$^{2+}$] within the endoplasmic reticulum of permeabilized DT40 cells was provided in preceding publications [7,30]. Briefly, the endoplasmic reticulum of DT40 cells stably expressing each of the three mammalian IP$_3$-R subtypes was loaded with a low-affinity Ca$^{2+}$ indicator (Mag fluo-4) [30]. After permeabilization of the plasma membrane with saponin (10 μg/mL, ∼4 min, 37°C), the permeabilized cells in cytosol-like medium (CLM) were distributed into 96-well plates at 20°C. Addition of MgATP (1.5 mM) then allowed active Ca$^{2+}$ uptake, which was monitored at intervals of ∼1 s using a FlexStation 3 fluorescence plate-reader (MDS Analytical Devices). CLM had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 20 mM Pipes, pH 7, free [Ca$^{2+}$] ∼220 nM (after addition of MgATP), and carbonyl cyanide 4-trifluoromethoxy-phenyl hydrazone (FCCP, 10 μM) to inhibit mitochondrial Ca$^{2+}$ uptake. After 150 s, when the stores had loaded to steady-state with Ca$^{2+}$, IP$_3$, AdA or its analogues was added with thapsigargin (1 μM) to prevent further Ca$^{2+}$ uptake, and after a further 30 s, the response was recorded. Agonist-evoked Ca$^{2+}$ release was expressed as a fraction of that released by ionomycin (1 μM) [30]. All experiments were performed at 20°C.

#### Statistical Analysis

Concentration-effect relationships were fitted to Hill equations using GraphPad Prism (version 5.0) from which Hill coefficients (h), the fraction of the intracellular Ca$^{2+}$ stores released by maximally effective concentrations of agonist, and pEC$_{50}$ values (-log EC$_{50}$) were calculated. For convenience some results are presented as EC$_{50}$ values, but all statistical comparisons use pEC$_{50}$ values. Within each experiment, the pEC$_{50}$ for AdA was determined to allow paired comparisons with values obtained for each AdA analogue. These are reported as ΔpEC$_{50}$, where:

\[
\text{ΔpEC}_{50} = \text{pEC}_{50} \text{AdA} - \text{pEC}_{50} \text{analogue}
\]

We note that Table 1 reports pooled results from experiments collected over a considerable period, whereas ΔpEC$_{50}$ values, like those shown in Table 2, compare only paired values. The latter provide the most robust means of comparing agonist potencies. Results are expressed as means ± SEM from n independent experiments, with each experiment performed in triplicate.

Statistical comparisons used Student’s t-test or ANOVA followed by Bonferroni’s post hoc test, as appropriate, with P<0.05 considered significant. Because not all comparisons of the relative potencies of AdA and IP$_3$ were paired, the SEM of this ΔpEC$_{50}$ value was calculated from:

\[
\text{SEM} = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}
\]

where, $s_p$ is the estimate of the population variance:

\[
s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 - n_2 - 2}}
\]

where, $s_1$ and $s_2$ are the sample standard deviations, and $n_1$ and $n_2$ are the sample sizes [31].

#### Results

##### Quantal Ca$^{2+}$ Release Evoked by AdA and IP$_3$

The kinetics of IP$_3$-evoked Ca$^{2+}$ release from intracellular stores are unexpectedly complex. It is widely observed that under conditions where Ca$^{2+}$ uptake into the endoplasmic reticulum (ER) is inhibited, submaximally effective concentrations of IP$_3$ rapidly release only a fraction of the IP$_3$-sensitive Ca$^{2+}$ stores [32]. Therewith, there is either no, or a massively reduced, effect of IP$_3$ on the rate of Ca$^{2+}$ release. The mechanisms underlying this pattern of response, known as quantal Ca$^{2+}$ release [33], remain

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**Figure 1. Structures of the analogues of AdA used.** (A) Key moieties within IP$_3$ and AdA are highlighted in matching colours to indicate their proposed structural equivalence. (B and C). The Ca$^{2+}$ contents of the intracellular stores of populations of permeabilized DT40-IP$_3$R1 cells are shown after addition of ATP to allow active Ca$^{2+}$ uptake, and then addition of the indicated concentrations of IP$_3$ or AdA with thapsigargin (1 μM) to inhibit further Ca$^{2+}$ uptake. The traces, which are typical of those from all subsequent analyses, show the average response from 2 wells on a single plate. The results demonstrate that both IP$_3$ and AdA evoke quantal Ca$^{2+}$ release.

doi:10.1371/journal.pone.0058027.g001
### Table 1. Effects of AdA analogues on Ca\(^{2+}\) release by subtypes of IP\(_3\) receptor.

<table>
<thead>
<tr>
<th></th>
<th>IP(_3)R1</th>
<th></th>
<th>IP(_3)R2</th>
<th></th>
<th>IP(_3)R3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50})</td>
<td>pEC(_{50})</td>
<td>h</td>
<td>Ca(^{2+})release</td>
<td>n</td>
<td>EC(_{50})</td>
</tr>
<tr>
<td>(1,4,5)IP(_3)</td>
<td>87</td>
<td>7.06 ± 0.05</td>
<td>0.99 ± 0.05</td>
<td>75 ± 1</td>
<td>31</td>
<td>145</td>
</tr>
<tr>
<td>AdA</td>
<td>8.3</td>
<td>8.08 ± 0.09</td>
<td>1.17 ± 0.09</td>
<td>72 ± 3</td>
<td>10</td>
<td>182</td>
</tr>
<tr>
<td>Imidophostin</td>
<td>37</td>
<td>7.43 ± 0.28</td>
<td>1.17 ± 0.21</td>
<td>78 ± 5</td>
<td>3</td>
<td>68</td>
</tr>
<tr>
<td>Ribophostin</td>
<td>40</td>
<td>7.40 ± 0.29</td>
<td>1.34 ± 0.16</td>
<td>77 ± 4</td>
<td>3</td>
<td>102</td>
</tr>
<tr>
<td>Furanophostin</td>
<td>51</td>
<td>7.29 ± 0.25</td>
<td>0.90 ± 0.10</td>
<td>79 ± 6</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>Mann-o-AdA</td>
<td>34</td>
<td>7.47 ± 0.19</td>
<td>1.33 ± 0.30</td>
<td>75 ± 7</td>
<td>3</td>
<td>69</td>
</tr>
<tr>
<td>Xylo-AdA</td>
<td>5.9</td>
<td>8.23 ± 0.17</td>
<td>1.27 ± 0.27</td>
<td>73 ± 7</td>
<td>3</td>
<td>79</td>
</tr>
<tr>
<td>2'-dephospho-AdA</td>
<td>275</td>
<td>6.56 ± 0.13</td>
<td>1.31 ± 0.15</td>
<td>66 ± 7</td>
<td>3</td>
<td>575</td>
</tr>
<tr>
<td>3'-dephospho-AdA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>15 ± 6(^0)</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>4'-dephospho-AdA</td>
<td>Inactive(^a)</td>
<td>Inactive(^a)</td>
<td>Inactive(^a)</td>
<td>ND</td>
<td>6</td>
<td>Inactive(^a)</td>
</tr>
</tbody>
</table>

The EC\(_{50}\) (nM), pEC\(_{50}\) (pM), Hill coefficient (h) and fraction (%) of the intracellular Ca\(^{2+}\) stores released by a maximally effective concentration of each analogue are shown for each IP\(_3\)R subtype. All results (except EC\(_{50}\)) are shown as means ± SEM from n independent experiments.

\(^a\)Inactive at 300 μM.

\(^0\)Ca\(^{2+}\) release evoked by 300 μM 3'-dephospho AdA.

\(^0\)Refer to Table 2 for relative potencies of 3'-dephospho AdA. ND, not determined.

[10.1371/journal.pone.0058027.t001](https://doi.org/10.1371/journal.pone.0058027.t001)
unclear. It may require desensitization of IP₃R as the Ca²⁺ content of the ER declines [34] or heterogeneity among IP₃-sensitive Ca²⁺ stores [35]. The results shown in Figures 1B and C confirm that the Ca²⁺ release evoked by submaximal concentrations of either IP₃ or AdA from permeabilized DT40-IP3R1 cells is quantal. These observations provide the justification for all subsequent experiments in which the concentration-dependent effects of IP₃ or AdA were measured 30 s after their addition (see Methods).

AdA is a Potent Agonist of All Three IP₃ Receptor Subtypes

The results shown in Figure 2 and Tables 1 and 2 demonstrate that AdA is ~10-times more potent than IP₃ at each IP₃R subtype, and for each subtype, maximally effective concentrations of IP₃ and AdA release the same fraction of the intracellular Ca²⁺ stores. This is consistent with many analyses of IP₃ and AdA in a variety of cell types using both functional and binding assays, in which AdA behaves as a full agonist with ~10-fold greater affinity than IP₃ [reviewed in 8]. Our results do, however, provide the first direct demonstration that AdA interacts similarly with all three IP₃R subtypes. Subsequent experiments examine the interactions between key analogues of IP₃ and AdA with each IP₃R subtype.

Trimming the Adenosine Moiety of AdA Reduces its Potency at All IP₃ Receptor Subtypes

Systematic trimming of the adenosine moiety of AdA successively produces imidophostin (which lacks the pyrimidine ring of AdA), ribophostin (in which a methoxy group replaces the adenosine moiety of AdA) and furanophostin (in which only the furanoid ring remains) (Figure 1A). Maximally effective concentrations of each of these analogues released the same fraction of the intracellular Ca²⁺ stores as AdA in cells expressing each of the three IP₃R subtypes, and each analogue was ~5-10-fold less potent than AdA (Figure 3, Tables 1 and 2). These results are consistent with previous analyses of IP₃R in hepatocytes, which express predominantly IP₃R2 [24,36], with analyses of binding of ribophostin and furanophostin to an N-terminal fragment of IP₃R1 [12], and with evidence from other analogues that trimming the adenosine

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**Table 2. Relative potencies of AdA analogues at different IP₃ receptor subtypes.**

<table>
<thead>
<tr>
<th></th>
<th>IP₃R1</th>
<th>IP₃R2</th>
<th>IP₃R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP₃</td>
<td>1.02±0.02</td>
<td>0.9±0.30</td>
<td>1.1±0.30</td>
</tr>
<tr>
<td>Imidophostin</td>
<td>0.78±0.15</td>
<td>0.78±0.08</td>
<td>0.81±0.04</td>
</tr>
<tr>
<td>Ribophostin</td>
<td>0.82±0.18</td>
<td>0.96±0.20</td>
<td>1.06±0.07</td>
</tr>
<tr>
<td>Furanophostin</td>
<td>0.92±0.13</td>
<td>0.83±0.14</td>
<td>1.25±0.05</td>
</tr>
<tr>
<td>Manno-AdA</td>
<td>0.74±0.08</td>
<td>0.79±0.18</td>
<td>0.98±0.08</td>
</tr>
<tr>
<td>Xylo-AdA</td>
<td>−0.01±0.07</td>
<td>−0.3±0.27</td>
<td>0.05±0.08</td>
</tr>
<tr>
<td>2'-dephospho-AdA</td>
<td>1.24±0.33</td>
<td>1.60±0.18</td>
<td>1.68±0.16</td>
</tr>
<tr>
<td>3'-dephospho-AdA</td>
<td>4.03±0.09</td>
<td>4.47±0.30</td>
<td>4.13±0.14</td>
</tr>
</tbody>
</table>

From paired comparisons with AdA, the potency (ΔpEC₅₀) of the analogues relative to AdA is shown for each IP₃R subtype. Results are means ± SEM, with n provided in Table 1. ND, not determined. *Because the very low affinity of 3'-dephospho AdA for IP₃R made it impracticable to stimulate cells with a maximally effective concentration, *ΔpEC₅₀* for 3'-dephospho AdA was estimated by comparing concentrations of it and AdA that evoked the same sub-maximal Ca²⁺ release.

doi:10.1371/journal.pone.0058027.t002

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**Figure 2. AdA is a potent agonist of all three IP₃ receptor subtypes.** (A) Concentration-dependent effects of AdA on Ca²⁺ release from the intracellular stores of cells expressing IP₃R1, IP₃R2 or IP₃R3. All results are expressed as percentages of the Ca²⁺ release evoked by ionomycin. The same colour codes are used in all subsequent figures. (B) Comparison, for each IP₃R subtype, of the Ca²⁺ release evoked by IP₃ and AdA. Results are means ± SEM from the number of independent experiments given in Table 1. Here, and in many subsequent figures, some error bars are smaller than the symbols.

doi:10.1371/journal.pone.0058027.g002
IP3 Receptors and Adenophostin Analogues

G

IP3R1 492 QDVLEVVSFKPNRERQKLMLRE
IP3R2 491 QDVLDVITKPNRERQKLMLRE
IP3R3 491 QNVLDIMVTKPNRERQKLMLR
mammalian IP3R subtypes (right).

These results are consistent with our earlier conclusion that the 10-fold greater affinity of AdA relative to IP3 requires the adenine moiety of AdA positioned to allow it to form a cation-π interaction with Arg-504 in the α-domain of the IBC [41] and, by interacting with the 3-deoxy-IP3 and analogues with other modifications of the 3-position have reduced affinity for the three IP3R subtypes [40]. With such a massive reduction in potency the lesser importance of 3-deoxy-IP3 and analogues with other modifications of the 3-position of AdA is, however, unlikely to provide the sole explanation for it making no discernible contribution to binding.

Hydroxyl Moieties that are Important for IP3 Binding are Less Important for Binding of AdA

The 3'-CH₂OH and 2'-OH substituents of the glucose ring of AdA are thought to mimic the 3-OH and 6-OH of IP3, respectively (Figure 1A). A structure equivalent to the 6-OH of IP3 is an essential feature of all inositol phosphate analogues that act as IP3R agonists, with Arg-504 in the α-domain of the IBC of IP3R1, a residue that is conserved in all IP3R subtypes [8,12] (Figure 3G). We suggest that this interaction between AdA with IP3R is likely to be similar for all IP3R subtypes.

The 6-OH of IP3 interacts, through a water molecule, with a lysine residue (K569) in the IBC [41] and, by interacting with the adjacent 1-phosphate, it has also been proposed to influence the behaviour of the 4,5-bisphosphate moiety of IP3 [42]. The latter interaction is unlikely to contribute to AdA binding because the structures equivalent to the 6-OH (2''-OH of AdA) and the 1-phosphate of IP3 (2'-phosphate of AdA) are in different rings in AdA (Figure 1A). We suggest that the lesser importance in AdA of the 6-OH is close to the threshold for detecting Ca²⁺ release (Figure 6B). The synthetic route used to prepare 3'-dephospho-AdA (300 μM) failed to evoke Ca²⁺ release via any IP3R subtype (Figure 6A). This is consistent with previous analyses by both functional and binding assays of IP3R1 [28,46]. 3'-dephospho-AdA did, however, cause detectable Ca²⁺ release albeit with much reduced potency (Figure 6B). The synthetic route used to prepare 3'-dephospho-AdA makes it extremely unlikely that the activity could be due to minor contamination with AdA or related structures with a vicinal bisphosphate moiety. Maximal attainable concentrations of 3'-dephospho-AdA (300 μM) failed to release the entire IP3-sensitive Ca²⁺ store, but comparison of the concentrations required to achieve the same submaximal Ca²⁺ release suggests that 3'-dephospho-AdA is ~10,000-fold less potent than AdA at all three IP3R subtypes. With such a massive reduction in potency the lesser sensitivity of DT40-IP3R3 cells to AdA means that even the highest practicable concentration of 3'-dephospho-AdA (300 μM) is close to the threshold for detecting Ca²⁺ release (Figure 6B).

The 2'-phosphate of AdA is not a Super-optimal Mimic of the 1-phosphate of IP3

It has been suggested that the 2'-phosphate of AdA interacts with the IBC in a manner that allows it to behave as a super-optimal mimic of the 1-phosphate of IP3 [44,45]. However, our recent study combining structure-activity analyses with mutagenesis of the binding site suggest that the 1-phosphate of IP3 is more important for binding than is the 2'-phosphate of AdA [12]. Removal of the 1-phosphate from IP3 (to give (4,5)IP2) caused its potency and affinity for IP3R1 to decrease by ~100-fold [12], whereas removal of the 2'-phosphate from AdA (2'-dephospho AdA) causes a decrease in potency of ~17-fold in IP3R1 (Figure 5) and ~40-fold decreases in potency were obtained with 2'-dephospho AdA and IP3R2 and IP3R3 (Figure 5, Table 1 and 2). These results establish that for all three IP3R subtypes, the enhanced affinity of AdA is not due to its 2'-phosphate interacting more effectively than the 1-phosphate of IP3 with the IBC.

A Bisphosphate Moiety is not Essential for Activation of IP3 Receptors by AdA

All known active analogues of IP3 have structures equivalent to its 4,5-bisphosphate moiety [13]. Structures of the IBC with and without IP3 bound provide a rationale for this requirement by revealing that these two phosphate groups contact opposite sides (the α- and β-domains) of the clam-like IBC, closure of which initiates IP3R activation [4,41]. Substantial evidence suggests that the 4',5'-bisphosphate moiety of AdA mimics the critical 4,5-bisphosphate of IP3 [8] (Figure 1A).

4'-dephospho-AdA at concentrations up to 300 μM failed to evoke Ca²⁺ release via any IP3R subtype (Figure 6A). This is consistent with previous analyses by both functional and binding assays of IP3R1 [28,46]. 3'-dephospho-AdA did, however, cause detectable Ca²⁺ release albeit with much reduced potency (Figure 6B). The synthetic route used to prepare 3'-dephospho-AdA makes it extremely unlikely that the activity could be due to minor contamination with AdA or related structures with a vicinal bisphosphate moiety. Maximal attainable concentrations of 3'-dephospho-AdA (300 μM) failed to release the entire IP3-sensitive Ca²⁺ store, but comparison of the concentrations required to achieve the same submaximal Ca²⁺ release suggests that 3'-dephospho-AdA is ~10,000-fold less potent than AdA at all three IP3R subtypes. With such a massive reduction in potency the lesser sensitivity of DT40-IP3R3 cells to AdA means that even the highest practicable concentration of 3'-dephospho-AdA (300 μM) is close to the threshold for detecting Ca²⁺ release (Figure 6B).

**Figure 3. Trimming the adenosine moiety of AdA reduces potency.** (A–F) Effects of imidophostin (A), ribophostin (C) and furanophostin (E) on Ca²⁺ release via each of the three IP3R subtypes, and the same analogues compared with AdA (B, D and F). Results are means ± S.E.M. from 3 independent experiments. (G) A cation-π interaction between the adenine of AdA and RS04 within the α-domain of the IBC is proposed to stabilize AdA binding (left). Closure of the clam-like IBC is proposed to be mediated by interactions between the 3'-phosphate of AdA and the α-domain of the IBC (blue ribbon), and between the 4'-phosphate and the β-domain of the IBC (green ribbon). In 3'-dephospho AdA, a cation-π interaction between AdA and the IBC α-domain is proposed to be sufficient to allow some effective closure of the clam. RS04 is conserved in all three mammalian IP3R subtypes (right). doi:10.1371/journal.pone.0058027.g003
Figure 4. Hydroxyl groups within the glucose ring of AdA are unimportant. (A–D) Effects of manno-AdA (A) and xylo-AdA (C) on Ca$^{2+}$ release via each IP$_3$R subtype, and the same analogues compared with AdA (B and D). Results are means ± S.E.M. from 3 independent experiments. doi:10.1371/journal.pone.0058027.g004
The inability of high concentrations of 3'-dephospho-AdA to release the entire IP3-sensitive Ca\(^{2+}\) store is likely to be due solely to its reduced affinity rather than reduced efficacy. A concentration of 3'-dephospho-AdA (30 μM) that caused detectable Ca\(^{2+}\) release via IP3R1 (−21 ± 5%) had no effect on the sensitivity of the Ca\(^{2+}\) release evoked by a subsequent addition of IP3. The pEC\(_{50}\) was 7.00 ± 0.02 and 7.04 ± 0.06 (n = 3) for (1,4,5)IP3 alone and in the presence of 3'-dephospho-AdA, respectively (Figure 6C). A partial agonist would be expected to shift the sensitivity to higher concentrations of IP3. These results suggest that 3'-dephospho-AdA is a low-affinity full agonist of IP3R.

These results extend our previous analyses of IP3R1 by demonstrating that for all IP3R subtypes, the 4'-phosphate group of AdA is essential for activity, whereas the 3'-phosphate is important but not essential. 3'-dephospho-AdA is the only known agonist of IP3R to lack a structure equivalent to the 4,5-bisphosphate moiety of IP3.

**Discussion**

AdA is a high-affinity full agonist of IP3R that has been extensively used to explore the behaviour of IP3R [reviewed in8]. The activity of AdA has been confirmed in many cell types, but hitherto there has been no assessment of its activity in homogeneous populations of IP3R subtypes. We have demonstrated that AdA is ~10-fold more potent than IP3R at each IP3R subtype (Figure 2, Tables 1 and 2), and the structural determinants of its high-affinity interaction with IP3R are similar for all three IP3R subtypes. Contrary to an earlier suggestion that the 2'-phosphate of AdA mediates its enhanced affinity by forming a stronger interaction with the IBC than the analogous 1-phosphate of IP3, we find that the 1-phosphate makes a greater contribution to IP3 binding than does the 2'-phosphate of AdA (Figure 5) [12]. A more likely explanation for the enhanced affinity of AdA is a cation-π interaction between its adenine moiety and R504 within the α-subunit of the IBC (Figure 3G) [28]. That explanation is supported by results for each IP3R subtype showing that truncation of the adenosine moiety of AdA brings the potency of the resulting analogues (imidophostin, ribophostin and furanophostin) close to that of IP3 (Figure 3).

A key step in the initial activation of IP3R by IP3 appears to be closure of its clam-like IBC as the 4-phosphate of IP3 contacts one side of the clam (its β-domain) and the 5-phosphate contacts the other side (α-domain) [4]. That mechanism provides a satisfying explanation for the long-standing observation that all inositol phosphates that activate IP3R share this essential 4,5-bisphosphate moiety. AdA is different in that its 4'-phosphate (analogous to the 4-phosphate of IP3, Figure 1A) is essential, but 3'-dephospho-AdA retains activity at all three IP3R subtypes, albeit with very low affinity (Figure 6). We suggest that for AdA, the need for the bisphosphate moiety to cause closure of the IBC can be partially replaced for all IP3R subtypes by having an interaction between the adenine of AdA and the α-domain substitute for the interaction between the 3'-phosphate (analogous to the 5-phosphate of IP3) and the α-domain [28]. Finally, whereas the 6-OH and, to a lesser extent, the 3-OH of IP3 are important for IP3 binding, the equivalent structures within AdA play lesser roles.
Both store-operated Ca\(^{2+}\) entry, which is triggered by depletion of IP\(_3\)-sensitive Ca\(^{2+}\) stores [47], and the spatial organization of subcellular Ca\(^{2+}\) signals have been reported to be differentially affected by IP\(_3\), AdA or its analogues [14,16,17,19,21,22]. Our present results, which demonstrate that AdA structure-activity relationships are similar for all IP\(_3\), AdA or its analogues are more likely to result from differences in their affinities, kinetics or rates of degradation than from selective interactions with different IP\(_3\)-R subtypes.

Author Contributions
Conceived and designed the experiments: CWT SCT BVLP AMR. Performed the experiments: HS AMR. Analyzed the data: CWT HS SCT. Contributed reagents/materials/analysis tools: BVLP AMR. Wrote the paper: CWT.

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