Stimulation of Inositol 1,4,5-Trisphosphate (IP$_3$) Receptor Subtypes by Adenophostin A and Its Analogues

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP$_3$R) are intracellular Ca$^{2+}$ channels. Most animal cells express mixtures of the three IP$_3$R subtypes encoded by vertebrate genomes. Adenophostin A (AdA) is the most potent naturally occurring agonist of IP$_3$R and it shares with IP$_3$ the essential features of all IP$_3$R agonists, namely structures equivalent to the 4,5-bisphosphate and 6-hydroxyl of IP$_3$. The two essential phosphate groups contribute to closure of the clam-like IP$_3$-binding core (IBC), and thereby IP$_3$R activation, by binding to each of its sides (the $\alpha$- and $\beta$-domains). Regulation of the three subtypes of IP$_3$R by AdA and its analogues has not been examined in cells expressing defined homogenous populations of IP$_3$R. We measured Ca$^{2+}$ release evoked by synthetic adenophostin A (AdA) and its analogues in permeabilized DT40 cells devoid of native IP$_3$R and stably expressing single subtypes of mammalian IP$_3$R. The determinants of high-affinity binding of AdA and its analogues were indistinguishable for each IP$_3$R subtype. The results are consistent with a cation-$\pi$ interaction between the adenine of AdA and a conserved arginine within the IBC $\alpha$-domain contributing to closure of the IBC. The two complementary contacts between AdA and the $\alpha$-domain (cation-$\pi$ interaction and 3$^\circ$-phosphate) allow activation of IP$_3$R by an analogue of AdA (3$^\circ$-dephospho-AdA) that lacks a phosphate group equivalent to the essential 5-phosphate of IP$_3$. These data provide the first structure-activity analyses of key AdA analogues using homogenous populations of all mammalian IP$_3$R subtypes. They demonstrate that differences in the Ca$^{2+}$ signals evoked by AdA analogues are unlikely to be due to selective regulation of IP$_3$R subtypes.

Introduction

Inositol 1,4,5-trisphosphate receptors (IP$_3$R) are intracellular Ca$^{2+}$ channels that are expressed in almost all animal cells. They allow release of Ca$^{2+}$ from intracellular stores in response to the many stimuli that activate phospholipase C [1,2]. The genomes of vertebrates encode three closely related IP$_3$R subtypes (IP$_3$R1-3), and most cells from vertebrates express functional IP$_3$R that are homo- or hetero-tetrameric assemblies of these IP$_3$R subtypes and their splice variants [3]. The physiological significance of this IP$_3$R diversity is poorly understood, and nor are there ligands that usefully discriminate between IP$_3$R subtypes. It is, however, clear that activation of IP$_3$R is initiated by binding of IP$_3$ to the conserved IP$_3$-binding core (IBC, residues 224-604 of IP$_3$R1) of each IP$_3$R subunit [4]. Mixed populations of IP$_3$R in native cells make it difficult to define unambiguously the functional properties of each IP$_3$R subtype. Stable heterologous expression of mammalian IP$_3$R in the only vertebrate cell line engineered to lack all endogenous IP$_3$R (DT40 KO cells) [5] provides an effective means of addressing this difficulty [6]. We previously used DT40 cells expressing homogeneous populations of each mammalian IP$_3$R 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 IP$_3$R 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 IP$_3$R. It is also resistant to degradation by the enzymes that degrade IP$_3$ via phosphorylation or dephosphorylation [10]. Although AdA is based on a glucose ring, rather than the inositol ring of IP$_3$, its structure retains the key functional groups of IP$_3$ that are known to be essential for IP$_3$ activity at IP$_3$R [12] (Figure 1A). Considerable evidence supports the original suggestion [10] that the essential 4,5-bisphosphate and 6-hydroxyl of IP$_3$ are effectively mimicked by the 4$^\circ$,3$^\circ$-bisphosphate and 2$^\circ$-hydroxyl of AdA (red highlights in Figure 1A). The interactions that allow AdA to bind to IP$_3$R with about 10-fold greater affinity than IP$_3$ have been more difficult to resolve. One view was that the 2$^\circ$-phosphate of AdA is equivalent to the 1-phosphate of IP$_3$ and, like the latter [13] (blue in Figure 1A), contributes to high-affinity binding to the IBC. The suggestion was that the 2$^\circ$-phosphate of AdA forms a stronger interaction with the IBC than does the 1-phosphate of IP$_3$. Our recent analyses have challenged this idea and instead suggest that a cation-$\pi$ interaction between the adenine ring of AdA and a guanidinium side chain of an arginine residue within the $\alpha$-domain of the IBC (R504 in IP$_3$R1) may be a more important determinant of the increased affinity of AdA for IP$_3$R [12].
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 IP3R activation and associated changes in cytosolic Ca2+ signalling [12]. There has, however, been no systematic analysis of the activities of AdA or its analogues with defined populations of homogenous IP3R subtypes. The need for such analyses is particularly important in attempting to explain results in which Ca2+ signals evoked by IP3 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 IP3R have been genetically inactivated [5] to stably express homogenous populations of mammalian IP3R subtypes and thereby define structure-activity relationships for AdA and its key analogues for each IP3R 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. IP3 was from Alexis Biochemicals (Nottingham, UK). AdA [23], imidophostin [24], ribophostin [25], furanophostin [26], manno-AdA and xylo-AdA [27], 3’-dephospho AdA and 4’-dephospho AdA [28], and 2’-dephospho AdA were synthesized, purified and characterized as previously described.

Measurement Ca2+ Release by IP3 Receptors

From quantitative analyses of western blots using antisera that selectively recognise each IP3R subtype or react equally with all three subtypes, we established that in the DT40 cells used, levels of IP3R expression (relative to IP3R3) were IP3R1 (71±8%, n = 3), IP3R2 (48±5%) and IP3R3 (100%) [7]. It is impracticable to achieve identical levels of IP3R expression for each cell line, and differences (albeit modest in our cell lines) may affect both the size of the IP3-sensitive Ca2+ pool and its sensitivity to IP3 [29]. The different levels of IP3R expression do not compromise the analyses reported here, which are entirely concerned with relative potencies of AdA analogues for each IP3R subtype (see below).

A comprehensive description of the methods used to measure free [Ca2+]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 IP3R subtypes was loaded with a low-affinity Ca2+ 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 Ca2+ accumulation, 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 [Ca2+]2 ~220 mM (after addition of MgATP), and carbonyl cyanide 4-trifluoromethoxy-phenyl hydrazone (FCCP, 10 μM) to inhibit mitochondrial Ca2+ uptake. After 150 s, when the stores had loaded to steady-state with Ca2+, IP3, AdA or its analogues was added with thapsigargin (1 μM) to prevent further Ca2+ uptake, and after a further 30 s, the response was recorded. Agonist-evoked Ca2+ 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 Ca2+ stores released by maximally effective concentrations of agonist, and pEC50 values (-log EC50) were calculated. For convenience some results are presented as EC50 values, but all statistical comparisons use pEC50 values. Within each experiment, the pEC50 for AdA was determined to allow paired comparisons with values obtained for each AdA analogue. These are reported as ΔpEC50, where:

$$\Delta pEC_{50} = pEC_{AdA} - pEC_{analogue}$$

We note that Table 1 reports pooled results from experiments collected over a considerable period, whereas ΔpEC50 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 IP3 were paired, the SEM of this ΔpEC50 value was calculated from:

$$SEM = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where, s2 is the estimate of the population variance:

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 - n_2 - 2}$$

where, s1 and s2 are the sample standard deviations, and n1 and n2 are the sample sizes [31].

Results

Quantal Ca2+ Release Evoked by AdA and IP3

The kinetics of IP3-evoked Ca2+ release from intracellular stores are unexpectedly complex. It is widely observed that under conditions where Ca2+ uptake into the endoplasmic reticulum (ER) is inhibited, submaximally effective concentrations of IP3 rapidly release only a fraction of the IP3-sensitive Ca2+ stores [32]. Theretofore, there is either no, or a massively reduced, effect of IP3 on the rate of Ca2+ release. The mechanisms underlying this pattern of response, known as quantal Ca2+ release [33], remain
### Table 1. Effects of AdA analogues on Ca^{2+} release by subtypes of IP_{3} receptor.

|        | IP_{3}R1 |       |       |       | IP_{3}R2 |       |       |       | IP_{3}R3 |       |       |       |
|--------|----------|-------|-------|-------|----------|-------|-------|-------|----------|-------|-------|-------|
|        | EC_{50}  | pEC_{50} | h     | Ca^{2+} release | n | EC_{50}  | pEC_{50} | h     | Ca^{2+} release | n | EC_{50}  | pEC_{50} | h     | Ca^{2+} release | n |
| (1,4,5)IP_{3} | 87 | 7.06 ± 0.05 | 0.99 ± 0.05 | 75 ± 1 | 31 | 145 | 6.84 ± 0.06 | 1.29 ± 0.09 | 61 ± 2 | 34 | 417 | 6.38 ± 0.05 | 1.26 ± 0.07 | 64 ± 2 | 30 |
| AdA | 8.3 | 8.08 ± 0.09 | 1.17 ± 0.09 | 72 ± 3 | 10 | 18.2 | 7.74 ± 0.06 | 1.79 ± 0.21 | 56 ± 2 | 13 | 33 | 7.48 ± 0.09 | 1.13 ± 0.07 | 61 ± 2 | 14 |
| Imidophostin | 37 | 7.43 ± 0.28 | 1.17 ± 0.21 | 78 ± 5 | 31 | 68 | 7.17 ± 0.14 | 1.84 ± 0.50 | 59 ± 3 | 31 | 166 | 6.78 ± 0.16 | 1.73 ± 0.39 | 67 ± 7 | 3 |
| Ribophostin | 40 | 7.40 ± 0.29 | 1.34 ± 0.16 | 77 ± 4 | 31 | 102 | 6.99 ± 0.11 | 1.60 ± 0.50 | 61 ± 2 | 31 | 295 | 6.53 ± 0.21 | 1.42 ± 0.08 | 68 ± 4 | 3 |
| Furaphostin | 51 | 7.29 ± 0.25 | 0.90 ± 0.10 | 79 ± 6 | 31 | 76 | 7.12 ± 0.01 | 1.73 ± 0.20 | 60 ± 3 | 31 | 457 | 6.34 ± 0.18 | 1.27 ± 0.21 | 71 ± 3 | 3 |
| Mannos-AdeA | 34 | 7.47 ± 0.19 | 1.33 ± 0.30 | 75 ± 7 | 31 | 69 | 7.16 ± 0.07 | 1.33 ± 0.22 | 57 ± 3 | 31 | 245 | 6.61 ± 0.23 | 1.23 ± 0.15 | 69 ± 4 | 3 |
| Xylo-AdeA | 4.9 | 8.23 ± 0.17 | 1.27 ± 0.27 | 73 ± 7 | 31 | 79 | 8.10 ± 0.10 | 1.52 ± 0.40 | 52 ± 6 | 31 | 79 | 7.54 ± 0.12 | 1.58 ± 0.29 | 64 ± 9 | 3 |
| 2'-dephospho-AdA | 275 | 6.56 ± 0.13 | 1.31 ± 0.15 | 66 ± 7 | 31 | 575 | 6.24 ± 0.10 | 0.85 ± 0.07 | 63 ± 2 | 31 | 692 | 6.16 ± 0.03 | 1.5 ± 0.22 | 55 ± 7 | 4 |
| 3'-dephospho-AdA | ND | ND | ND | 15 ± 6\(^a\) | 7 | ND | ND | ND | ND | 6 ± 2\(^b\) | 6 | ND | ND | ND | ND | 7 ± 7\(^b\) | 5 |
| 4'-dephospho-AdA | Inactive\(^a\) | Inactive\(^a\) | Inactive\(^a\) | ND | 6 | Inactive\(^a\) | Inactive\(^a\) | Inactive\(^a\) | ND | 6 | Inactive\(^a\) | Inactive\(^a\) | Inactive\(^a\) | ND | 5 |

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.

*Inactive at 300 µM.

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

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

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unclear. It may require desensitization of IP3R as the Ca\(^{2+}\) content of the ER declines [34] or heterogeneity among IP3-sensitive Ca\(^{2+}\) stores [35]. The results shown in Figures 1B and C confirm that the Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 adenine 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 Ca\(^{2+}\) 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

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

| IP3R Type  | IP3R1 Potency | IP3R2 Potency | IP3R3 Potency |
|------------|---------------|---------------|---------------|
| IP3        | 1.02±0.02     | 0.9±0.30      | 1.1±0.30      |
| Imidophostin| 0.78±0.15     | 0.78±0.08     | 0.81±0.04     |
| Ribophostin | 0.82±0.18     | 0.96±0.20     | 1.06±0.07     |
| Furaphostin | 0.92±0.13     | 0.83±0.14     | 1.25±0.05     |
| Manno-AdA   | 0.74±0.08     | 0.79±0.18     | 0.98±0.08     |
| Xylo-AdA    | −0.01±0.07    | −0.3±0.27     | 0.05±0.08     |
| 2'-dephospho-AdA | 1.24±0.33 | 1.60±0.18 | 1.68±0.16 |
| 3'-dephospho-AdA | 4.03±0.09 | 4.47±0.30 | 4.13±0.14 |

From paired comparisons with AdA, the potency (\(\Delta\text{pEC}_{50}\)) 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, \(\Delta\text{pEC}_{50}\) for 3'-dephospho AdA was estimated by comparing concentrations of it and AdA that evoked the same sub-maximal Ca\(^{2+}\) release.

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**Figure 2.** AdA is a potent agonist of all three IP3 receptor subtypes. (A) Concentration-dependent effects of AdA on Ca\(^{2+}\) release from the intracellular stores of cells expressing IP3R1, IP3R2 or IP3R3. All results are expressed as percentages of the Ca\(^{2+}\) release evoked by ionomycin. The same colour codes are used in all subsequent figures. (B) Comparison, for each IP3R subtype, of the Ca\(^{2+}\) 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.

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IP³ Receptors and Adenophostin Analogues

(A) IP₃R1, IP₃R2, IP₃R3

(B) AdA, Imidophostin, IP₃R1

(C) IP₃R2, Ribophostin, IP₃R1

(D) IP₃R3, Furanophostin, IP₃R1

(E) IP₃R3, Ribophostin, IP₃R3

(F) IP₃R3, Furanophostin, IP₃R3

(G) IP₃R1 492 QDVLEVVFSKPNNRERQKLMRE
IP₃R2 491 QDVLDVVITKPNRERQKLMRE
IP₃R3 491 QNVLDIMVTKPNNRERQKLMRD
moiety decreases affinity for cerebellar IP$_3$R, which are largely IP$_3$R1 [37]. These results are consistent with our earlier conclusion that the 10-fold greater affinity of AdA relative to IP$_3$ requires the adenine moiety of AdA positioned to allow it to form a cation-$\pi$ interaction with Arg-504 in the $\alpha$-domain of the IBC of IP$_3$R1, a residue that is conserved in all IP$_3$R subtypes [8,12] (Figure 3G). We suggest that this interaction of AdA with IP$_3$R is likely to be similar for all IP$_3$R subtypes.

Hydroxyl Moieties that are Important for IP$_3$ Binding are Less Important for Binding of AdA

The 3'-CH$_2$OH and 2'-OH substituents of the glucose ring of AdA are thought to mimic the 3-OH and 6-OH of IP$_3$, respectively (Figure 1A). A structure equivalent to the 6-OH of IP$_3$ is an essential feature of all inositol phosphate analogues that bind to IP$_3$R [13,39,39] and inversion of its orientation from equatorial to axial reduces affinity by more than 100-fold at all IP$_3$R subtypes [40]. It is therefore surprising, but consistent with previous analyses of native hepatic IP$_3$R [36], that manno-AdA, which differs from AdA only in the orientation of its 2'-OH, should be only 5- to 10-fold less potent than AdA at each IP$_3$R subtype (Figures 4A and B, Tables 1 and 2). Why, when the 6-OH of IP$_3$ and 2'-OH of AdA seem to be analogous in the ligand structures, should these moieties make such different contributions to the interactions of IP$_3$ and AdA with IP$_3$R?

The 6-OH of IP$_3$ 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 IP$_3$ [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 IP$_3$ (2'-phosphate of AdA) are in different rings in AdA (Figure 1A). We suggest that the lesser importance in AdA of a structure equivalent to the essential 6-OH of IP$_3$ comes from this hydroxyl mediating a relatively minor interaction with K569 in AdA, whereas for IP$_3$ it contributes also to appropriately orienting the critical 4,5-bisphosphate moiety.

The 3-OH group, although less important than the 6-OH, is another feature of IP$_3$ that contributes to high-affinity binding [43]. Our recent analyses of the functional effects of 3-deoxy-IP$_3$ established that it was $\sim$40-fold less potent than IP$_3$ at all three IP$_3$R subtypes [7]. This is consistent with earlier work showing that 3-deoxy-IP$_3$ and analogues with other modifications of the 3-position have reduced affinity for the three IP$_3$R subtypes [40]. However, the equivalent modification of AdA, removal of its 5'-CH$_2$OH to give xylo-AdA (Figure 1A), had no significant effect on its potency at any IP$_3$R subtype (Figures 4C and D, Tables 1 and 2). This is consistent with a previous functional analysis of hepatic IP$_3$R, where xylo-AdA was only marginally less potent than AdA ($\Delta$EC$_{50}$ = -0.28) [36]. Our results suggest that despite the apparent structural similarity between the 3-OH of IP$_3$ and the 5'-CH$_2$OH of AdA (Figure 1A), the two hydroxyl groups do not contribute similarly to ligand binding. Previous analyses of IP$_3$ analogues suggested that replacing the 3-OH with the larger CH$_2$OH moiety caused the affinity to decrease by no more than 7-fold [40]. A partial explanation for the lack of effect of removing the 5'-CH$_2$OH of AdA may therefore be that this moiety is less readily accommodated than a hydroxyl group in the IBC. This would suggest that an analogue of AdA in which the 5'-CH$_2$OH is replaced by 3'-OH might bind with increased affinity. We are unaware of such an analogue having been synthesized. The larger substituent at the 5'-position of AdA is, however, unlikely to provide the sole explanation for it making no discernible contribution to binding.

The 2'-phosphate of AdA is not a Super-Optimal Mimic of the 1-phosphate of IP$_3$

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 IP$_3$ [44,45]. However, our recent study combining structure-activity analyses with mutagenesis of the binding site suggest that the 1-phosphate of IP$_3$ is more important for binding than is the 2'-phosphate of AdA [12]. Removal of the 1-phosphate from IP$_3$ (to give (4,5)IP$_3$) caused its potency and affinity for IP$_3$R1 to decrease by $\sim$100-fold [12], whereas removal of the 2'-phosphate from AdA (2'-dephospho AdA) causes a decrease in potency of $\sim$17-fold in IP$_3$R1 (Figure 5) and $\sim$40-fold decreases in potency were obtained with 2'-dephospho AdA and IP$_3$R2 and IP$_3$R3 (Figure 5, Table 1 and 2). These results establish that for all three IP$_3$R subtypes, the enhanced affinity of AdA is not due to its 2'-phosphate interacting more effectively than the 1-phosphate of IP$_3$ with the IBC.

A Bisphosphate Moiety is not Essential for Activation of IP$_3$ Receptors by AdA

All known active analogues of IP$_3$ have structures equivalent to its 4,5-bisphosphate moiety [13]. Structures of the IBC with and without IP$_3$ bound provide a rationale for this requirement by revealing that these two phosphate groups contact opposite sides (the $\alpha$- and $\beta$-domains) of the clam-like IBC, closure of which initiates IP$_3$R activation [4,41]. Substantial evidence suggests that the 4,5-bisphosphate moiety of AdA mimics the critical 4,5-bisphosphate of IP$_3$ [8] (Figure 1A).

4'-dephospho-AdA at concentrations up to 300 $\mu$M failed to evoke Ca$^{2+}$ release via any IP$_3$R subtype (Figure 6A). This is consistent with previous analyses by both functional and binding assays of IP$_3$R1 [28,46]. 3'-dephospho-AdA did, however, cause detectable Ca$^{2+}$ 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 $\mu$M) failed to release the entire IP$_3$-sensitive Ca$^{2+}$ store, but comparison of the concentrations required to achieve the same submaximal Ca$^{2+}$ release suggests that 3'-dephospho-AdA is $\sim$10,000-fold less potent than AdA at all three IP$_3$R subtypes. With such a massive reduction in potency the lesser sensitivity of DT10-IP$_3$R3 cells to AdA means that even the highest practicable concentration of 3'-dephospho-AdA (300 $\mu$M) is close to the threshold for detecting Ca$^{2+}$ release (Figure 6B).
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.

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The inability of high concentrations of 3'-dephospho-AdA to release the entire IP3-sensitive Ca2+ 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 Ca2+ release via IP3R1 (~21 ± 5%) had no effect on the sensitivity of the Ca2+ release evoked by a subsequent addition of IP3. The pEC50 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 homogenous 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_3R subtypes, suggest that different physiological effects of 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_3R subtypes.

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

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