Selective determinants of inositol 1,4,5-trisphosphate and adenophostin A interactions with type 1 inositol 1,4,5-trisphosphate receptors

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BACKGROUND AND PURPOSE
Adenophostin A (AdA) is a potent agonist of inositol 1,4,5-trisphosphate receptors (IP3R). AdA shares with IP3 the essential features of all IP3R agonists, namely structures equivalent to the 4,5-bisphosphate and 6-hydroxyl of IP3, but the basis of its increased affinity is unclear. Hitherto, the 2′-phosphate of AdA has been thought to provide a supra-optimal mimic of the 1-phosphate of IP3.

EXPERIMENTAL APPROACH
We examined the structural determinants of AdA binding to type 1 IP3R (IP3R1). Chemical synthesis and mutational analysis of IP3R1 were combined with 3H-IP3 binding to full-length IP3R1 and its N-terminal fragments, and Ca2+ release assays from recombinant IP3R1 expressed in DT40 cells.

KEY RESULTS
Adenophostin A is at least 12-fold more potent than IP3 in functional assays, and the IP3-binding core (IBC, residues 224–604 of IP3R1) is sufficient for this high-affinity binding of AdA. Removal of the 2′-phosphate from AdA (to give 2′-dephospho-AdA) had significantly lesser effects on its affinity for the IBC than did removal of the 1-phosphate from IP3 (to give inositol 4,5-bisphosphate). Mutation of the only residue (R568) that interacts directly with the 1-phosphate of IP3 decreased similarly (~30-fold) the affinity for IP3 and AdA, but mutating R504, which has been proposed to form a cation-π interaction with the adenine of AdA, more profoundly reduced the affinity of IP3R for AdA (353-fold) than for IP3 (13-fold).

CONCLUSIONS AND IMPLICATIONS
The 2′-phosphate of AdA is not a major determinant of its high affinity. R504 in the receptor, most likely via a cation-π interaction, contributes specifically to AdA binding.

Abbreviations
AdA, adenophostin A; CLM, cytosol-like medium; IBC, IP3-binding core; IP2, inositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; Kd, equilibrium dissociation constant; nHill, Hill coefficient; NT, N-terminal; TEM, Tris/EDTA medium

Introduction
Receptors for inositol 1,4,5-trisphosphate (IP3R, nomenclature follows Alexander et al., 2009) are intracellular Ca2+ channels. They are expressed in the membranes of the endoplasmic reticulum of most animal cells (Foskett et al., 2007) and they both initiate and propagate the Ca2+ signals evoked
by receptors that stimulate IP₃ formation (Berridge et al., 2003). In vertebrates, three genes encode closely related subtypes of the IP₃R, which assemble into both homo- and hetero-tetrameric channels (Taylor et al., 1999). The different subtypes share many features (Foskett et al., 2007). Each subunit has a single IP₃-binding site towards the N-terminal, a large cytosolic regulatory domain, and six transmembrane domains, the last pair of which from each subunit, together with the intervening luminal loop, form the pore (Ramos-Franco et al., 1999; Taylor et al., 2004). For all IP₃R, IP₃ binding initiates the conformational changes that lead to opening of the channel. The IP₃-binding core [IBC, residues 224–604 of type 1 IP₃R (IP₃R1)] is entirely responsible for this initial recognition. The two domains (α and β) of the IBC form a clam-like structure lined with the basic residues that coordinate the phosphate groups of IP₃ (Bosanac et al., 2002) (Figure 1A). The 4,5-bisphosphate and 6-hydroxyl groups of IP₃ are important for binding to IP₃R (Potter and Lampe, 1995), and each forms extensive interactions with the IBC. The 4-phosphate forms hydrogen bonds with several residues in the

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**Figure 1**

Structures of the IBC and the ligands used. Structure of the IBC (PDB 1N4K) with the enlarged panel highlighting the residues (RS04, RS68 and KS69) mutated in this study and their interactions with the phosphate groups of IP₃. The red spheres represent water (A). Structures of the ligands used highlighting the 1-phosphate of IP₃ and 2′-phosphate of AdA (B). AdA, adenophostin A; IBC, IP₃-binding core; IP₃, inositol 1,4,5-trisphosphate.
β-domain, the 5-phosphate is hydrogen-bonded to residues predominantly within the α-domain and the 6-hydroxyl interacts indirectly via water with the backbone of KS69 (Figure 1A). The 1-phosphate of IP3 is not essential for binding to IP3R, but it substantially increases the affinity of IP3 (Nerou et al., 2001); it interacts directly only with R568 and indirectly via a water molecule with the backbone of KS69 (Bosanac et al., 2002) (Figure 1A).

Adenophostins (Figure 1B) were originally isolated from Penicillium brevicompactum (Takahashi et al., 1994a,b,c). They are potent agonists of IP3R (Takahashi et al., 1994b,c; Hirota et al., 1995; Marchant et al., 1997a; Shuto et al., 1998; Correa et al., 2001); they are not metabolized by the enzymes that degrade IP3 and their structures are based on a glucose, rather than a myo-inositol, ring (Figure 1B). Adenophostin A (AdA) has proven a useful tool with which to explore the properties of IP3R (Hirota et al., 1995; Dellls et al., 2006; Marchant and Parker, 1998; Yoshida et al., 1998; Parekh et al., 2002), and it has generated considerable interest in the synthesis of novel AdA analogues (Shuto et al., 1998; Correa et al., 2001; Borissov et al., 2005; Mochizuki et al., 2006).

Inositol 1,4,5-trisphosphate and AdA are each full agonists of the IP3R (Rossi et al., 2009). Both IP3 and AdA bind to the IBC, and despite their structural differences, the 3′,4′-bisphosphate and 2′-hydroxyl groups of AdA evidently mimic the essential 4,5-bisphosphate and 6-hydroxyl of IP3 (Figure 1B) (Takahashi et al., 1994c; Hotoda et al., 1999; Correa et al., 2001; Rosenzweig et al., 2003). These features probably account for the binding of AdA to the IBC (Rosenzweig et al., 2003), but they do not explain the ability of AdA to bind to IP3R with greater affinity than IP3. Hitherto, a favoured suggestion is that the 2′-phosphate of AdA, which is thought to mimic the 1-phosphate of IP3 (Figure 1B), is ‘supra-optimally’ positioned and thereby interacts more strongly with R568 and KS69 than does the 1-phosphate of IP3 (Takahashi et al., 1994c; Wilcox et al., 1995; Hotoda et al., 1999). Alternatively, the adenine of AdA may interact directly with the IP3R (Hotoda et al., 1999; Gloouchankova et al., 2000; Rosenzweig et al., 2003). Such an interaction would need to be rather tolerant of changes to the adenine group because even substantial modifications to it cause only modest decreases in affinity (Correa et al., 2001; Sureshan et al., 2008). Defining the mechanisms responsible for high-affinity binding of AdA would both provide an important step towards rational development of ligands of the IP3R with increased affinity, and contribute to resolving the mechanisms whereby IP3 and AdA can have different effects on Ca2+ signalling (Rossi et al., 2010). Here, we have used synthetic analogues of IP3 and AdA and systematic mutagenesis of the IBC to address the structural basis of the high-affinity binding of AdA to IP3R.

**Methods**

**Stable expression of mutant IP3R1 in DT40 cells**

Cloning of rat IP3R1 (without the S1 splice site) into the pENTR1A vector has been reported previously (Rossi et al., 2009). The QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce point mutations into rat IP3R1 using the primers (5′-3′) forward: TCA CAGCAAGACTACCAGAAACCAAGGATAC, and reverse: GTACTCTGTGTCCTCTGGTATGTGGCTGTGA for R568Q, and forward: TTCTCTAAGCCCAA CCAAGACGGCAGAACCTG, and reverse: CAG CTTCTGCCCCTTGGTGGCTTACAGAA for R504Q. The sequences of all mutant constructs were confirmed by sequencing of the full-length IP3R. Mutated IP3R were subcloned into the expression vector, pcDNA3.2/V5-DEST, by recombination (Invitrogen, Paisley, UK). DT40 cells stably expressing IP3R1 and its mutants were generated by transfection of cells lacking endogenous IP3R (Sugawara et al., 1997). DT40 cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10%), heat-inactivated chicken serum (1%), 2-mercaptoethanol (50 μM) and glutamine (2 mM). Cells were grown in suspension at 37°C in an atmosphere of 95% air and 5% CO2, and passaged or used when they reached a density of ~2 × 10^6 cells·mL^-1 (Tovey et al., 2006). IP3R expression was quantified by immunoblotting using an antiserum (Ab1.5) to a peptide corresponding to residues 2733–2749 of rat IP3R1.

**Mutagenesis of N-terminal fragments of IP3R1**

N-terminal fragments of IP3R1 (IBC, residues 224–604; NT, residues 1–604) were amplified by PCR from the rat IP3R1 clone lacking the S1 splice site and ligated into pTrcHisA vectors for expression of N-terminally tagged His6 fusion proteins as previously described (Rossi et al., 2009). The IBC included the S1 splice site, but the NT lacked it. The presence of the S1 splice site does not affect the equilibrium dissociation constant (Kd) of the IBC for IP3 (data not shown). All fragments are numbered by reference to the full-length (S1′) rat IP3R1 (GenBank accession number: GQ233032.1). The QuikChange II XL site-directed mutagenesis kit was used to introduce point mutations into the IBC and NT constructs in pTrcHisA.
Expression of fragments of IP₃R1

His₆-tagged IBC and NT fragments were expressed as described previously (Rossi et al., 2009). Briefly, constructs were transformed into E. coli strain BL21(DE3). Cells were grown in Luria-Bertani medium containing ampicillin (100 μg·mL⁻¹) at 22°C until the OD₆₀₀ reached 1.0–1.5. The culture was then induced by addition of isopropyl β-D-thiogalactoside (0.5 mM), and after 20 h at 15°C, cells were harvested and lysates were prepared in Tris/EDTA medium (TEM: 50 mM Tris, 1 mM EDTA, pH 8.3) as described (Rossi et al., 2009). Expression was detected by immunoblotting using an anti-His antibody (Sigma, Poole, Dorset, UK). Proteins were cleaved from the His tags by incubating bacterial lysate (6 h, 4°C) with thrombin (43 units·mg⁻¹) to give a final free [Ca²⁺] of ~220 nM, pH 7.0, followed by centrifugation (20 000 × g, 5 min). Radioactivity was determined by liquid scintillation counting. Non-specific binding, determined by addition of 10 μM IP₃, or by extrapolation of competition curves to infinite IP₃ concentration, was <10% of total binding.

Purification of IP₃R from rat cerebellum

All animal care and experimental procedures complied with UK Home Office policy and with local animal regulations. Adult male Wistar rats were humanely killed by cervical dislocation and cerebella were removed, rapidly frozen in liquid nitrogen and stored at ~80°C. IP₃R was purified from cerebella using heparin-affinity chromatography following a published protocol (Jiang et al., 2002) with some modifications (Rossi et al., 2009). Briefly, cerebella (2 g) were homogenized in homogenization medium (30 mL, HM: 1 M NaCl, 1 mM EDTA, 50 mM Tris, 1 mM benzamidine, Roche protease inhibitor cocktail (1 tablet per 25 mL), pH 8.3), and then centrifuged (100 000 × g, 30 min). The pellet was solubilized in 20 mL of HM without NaCl, but supplemented with CHAPS (1%). After centrifugation (100 000 × g, 1 h), the NaCl concentration of the supernatant was increased to 250 mM, and the supernatant was loaded onto heparin-agarose beads (5 mL). After 30 min, the beads were washed twice in glycerol-containing medium (250 mM NaCl, 50 mM Tris, 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM benzamidine, 1 mM EGTA, 50 mM Tris, 1% CHAPS, pH 8.0). Samples (~100 μg protein per mL) were frozen in liquid nitrogen and stored at ~80°C.

³H-IP₃ binding

Equilibrium-competition binding assays were performed as described (Rossi et al., 2009). Briefly, incubations (500 μL) at 4°C were in either TEM or cytosol-like medium [CLM: 20 mM NaCl, 140 mM KCl, 1 mM EGTA, 20 mM PIPES, 2 mM MgCl₂, 375 μM CaCl₂ (free [Ca²⁺] = 220 nM), pH 7.0] containing ³H-IP₃ (0.75–3 nM), bacterial lysate (~1–10 μg protein) or purified IP₃R (~2.5 μg), and competing ligands. For assays using full-length purified IP₃R, all media also included CHAPS (1%). Reactions were terminated after 5 min by addition of poly(ethylene glycol) 8000 (500 μL, 30%, w/v) and γ-globulin (30 μL, 25 mg·mL⁻¹), followed by centrifugation (20 000 × g, 5 min). Radioactivity was determined by liquid scintillation counting. Non-specific binding, determined by addition of 10 μM IP₃, or by extrapolation of competition curves to infinite IP₃ concentration, was <10% of total binding.

Ca²⁺ release by IP₃R

A low-affinity Ca²⁺ indicator (Mag-fluo-4) was used to monitor the free [Ca²⁺] within the intracellular Ca²⁺ stores of DT40 cells (Laude et al., 2005; Tovey et al., 2006). DT40 cells stably expressing IP₃R or its mutants were centrifuged (650 × g, 2 min) and suspended in medium containing 135 mM NaCl, 5.9 mM KCl, 11.6 mM HEPES, 1.5 mM CaCl₂, 11.5 mM glucose, 1.2 mM MgCl₂, pH 7.3, 1 mg·mL⁻¹ BSA, 0.4 mg·mL⁻¹ Pluronic F127 and 20 μM Mag-fluo-4 AM. After 1 h at 20°C, cells were suspended in Ca²⁺-free CLM supplemented with saponin (10 μg·mL⁻¹) to allow selective permeabilization of the plasma membrane. Permeabilized cells were centrifuged (650 × g, 2 min), re-suspended in CLM without Mg²⁺, but supplemented with 10 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to inhibit mitochondria, and 375 μM CaCl₂ to give a final free [Ca²⁺] of ~220 nM after addition of 1.5 mM MgATP. Cells (~5 × 10⁵ cells per well) were attached to poly-L-lysine-coated, 96-well, black-walled plates (Greiner, Stonehouse, UK). Fluorescence was recorded at 20°C using a FlexStation III plate reader (MDS Analytical Technologies, Woking, Berks, UK) with excitation and emission wavelengths of 485 nm and 520 nm respectively. MgATP (1.5 mM) was added to initiate Ca²⁺ uptake, and when the endoplasmic reticulum had loaded to steady state with Ca²⁺, IP₃, AdA or their analogues were added. Ca²⁺ release is expressed as a fraction of the ATP-dependent uptake (Tovey et al., 2006).
**Data analysis**

Equilibrium binding results and concentration–
effect relationships were fitted to Hill equations
(GraphPad Prism, version 5) from which the Hill
equations \( (n_{\text{Hill}}) \), \(-\log IC_{50}\) \((pIC_{50})\) and \(-\log EC_{50}\)
\((pEC_{50})\) values were obtained. For equilibrium-
competition binding assays, \( pK_d \) values were calcu-
lated using the Cheng and Prusoff equation (Cheng
and Prusoff, 1973). Because \( pEC_{50} \) and \( pK_d \) values are
normally distributed, these results are presented as
means ± SEM from \( n \) independent experiments. For
comparisons of the ratios between mean values
\((EC_{50} \text{ or } K_d)\), statistical analyses compared the differ-
ences between their log values \((\Delta pEC_{50} \text{ or } \Delta pK_d)\)
(Colquhoun, 1971) with the SEM calculated as

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

where, \( s_p \) is the estimate of the population variance:

\[
s_p = \sqrt{s_1^2 + s_2^2}
\]

\[
\frac{n_1 - n_2}{n_1 - n_2 - 2}
\]

where, \( s_1 \) and \( s_2 \) are the sample standard deviations, and \( n_1 \) and \( n_2 \) are the sample sizes.

Although all analyses were performed using log
values, for greater clarity we present some ratios as
the antilogs of the means ± SEM.

Statistical analysis used ANOVA followed by Bon-
ferroni test for selected pairs, or unpaired Student’s
t-tests (GraphPad Prism, version 5). \( P < 0.05 \) was
considered significant.

**Materials**

Protease inhibitor cocktail was from Roche
(Burgess Hill, Sussex, UK). Heparin-agarose beads
and sera were from Sigma (Poole, Dorset, UK). Thrombin was from GE Healthcare (Little Chal-
font, Bucks, UK). CHAPS (3-[3-(cholamidopropyl)
dimethylammonio]-1-propane-sulphonate) was
from Helford Laboratories (Suffolk, UK). RPMI 1640
medium, Pluronic F127 and Mag-fluo-4 AM were
from Invitrogen (Paisley, Scotland). \( ^3 \)H-IP3 (681
GBq·mmol\(^{-1} \)) was from PerkinElmer (Bucks, UK). IP3
was from Alexis Biochemicals (Nottingham, UK).
AdA (Borissow et al., 2005), 2′-d(3H-IP3 (681
GBq·mmol\(^{-1} \)) was from PerkinElmer (Bucks, UK). IP3
was from Alexis Biochemicals (Nottingham, UK).
AdA (Borissow et al., 2005), 2′-diphospho-AdA
(Sureshan et al., 2009), furanophostin (Marwood
et al., 1999) and ribophostin (Jenkins et al., 1997)
were synthesized as previously described. Inositol
4,5-bisphosphate (IP\(_3\)) was synthesized by hydro-
genolytic deprotection of 1b-2,3,6-tri-O-benzyl-4,5-
bis(dibenzoyloxyphosphoryl) myo-inositol (Desai
et al., 1994). All ligands were purified by ion-
exchange chromatography, fully characterized by
the usual spectroscopic methods and accurately
quantified by total phosphate assay. The structures
of the ligands used are shown in Figure 1B. Sources
of other reagents either are specified elsewhere in
the methods or were previously reported (Rossi
et al., 2009).

**Results**

**Stimulation of IP\(_3\)R1 by AdA**

We used full-length IP\(_3\)R1 purified from rat cerebel-
um for binding assays, and DT40 cells expressing
only recombinant IP\(_3\)R1 to measure \( Ca^{2+} \) release
from intracellular stores. Most published analyses of
\( ^3 \)H-IP3 binding use media similar to TEM because its
high pH and/or low ionic strength reduce the \( K_d \) of
IP\(_3\)R for IP3, thereby increasing the specific binding
determined with low concentrations of \( ^3 \)H-IP3. At
the densities that recombinant full-length IP\(_3\)R are
expressed, it is impracticable to measure \( ^3 \)H-IP3
binding in CLM, although it is feasible with the
bacterially expressed fragments of IP\(_3\)R. To allow
comparison with published work (Hirota et al.,
1995; Hotoda et al., 1999; Glouchankova et al.,
2000; Rossi et al., 2009) and to provide a direct com-
parison with our analyses of binding to IP\(_3\)R frag-
ments, we first examined IP3 and AdA binding to
IP\(_3\)R1 in TEM.

In both binding (in TEM) and functional analy-
ses (in CLM), AdA was \(-12\)- to 19-fold more potent
than IP3 \((\Delta pK_d = 1.27 \pm 0.09 \text{ and } \Delta pEC_{50} 1.09 \pm
0.05) (Figure 2A and B, Table 1, Table S1). These
results are consistent with many previous studies
(Hirota et al., 1995; Shuto et al., 1998; Correa
et al., 2001; Morris et al., 2002). We note, however, that in
one series of studies (Takahashi et al., 1994a,b,c), the
\( K_d \) values for IP3 and AdA were incorrectly calculated
from the IC\(_{50}\). The correct \( K_d \) for IP3 and AdA calcu-
lated from the data provided are 13 nM and
0.73 nM, respectively, suggesting that in these
studies too, AdA bound with about 18-fold greater
affinity than IP3, rather than the stated 100-fold
difference.

Inositol 1,4,5-trisphosphate binding is entirely
mediated by residues in the IBC (Bosancic et al.,
2002) (Figure 1A). We therefore compared IP3 and AdA
binding to the isolated NT, initially in TEM. The
results, consistent with a previous analysis of a
slightly shorter NT fragment of IP\(_3\)R1 (residues
1–580) (Glouchankova et al., 2000), establish that
the NT alone binds AdA with about 15-fold greater
affinity \((\Delta pK_d = 1.18 \pm 0.12) \) than IP3 (Figure 2C,
Table 2 and Table S1). To allow more direct
comparisons with functional assays, we compared IP$_3$ and AdA binding to the isolated IBC and NT in CLM. These results confirm the expected substantial decrease in affinity for IP$_3$ in CLM (~20-fold relative to TEM). More importantly, they establish that in CLM the relative affinities for AdA and IP$_3$ are not significantly different for the IBC and NT ($\Delta pK_d = 1.37 \pm 0.07$ for the IBC, and $1.14 \pm 0.21$ for the NT).

Figure 2
Interactions of AdA, IP$_3$, and their dephospho analogues with IP$_3$R and its N-terminal (NT) fragments. Equilibrium-competition binding to purified IP$_3$R1 using $^3$H-IP$_3$ (1.5 nM) and the indicated ligands in TEM (A). Ca$^{2+}$ release from permeabilized DT40-IP$_3$R1 cells evoked by the indicated ligands (B). Equilibrium-competition binding to the NT using $^3$H-IP$_3$ (1.5 nM) and the indicated ligands in TEM (C). Equilibrium-competition binding to the IBC using $^3$H-IP$_3$ (0.75 nM) and the indicated ligands in CLM (D). Equilibrium-competition binding to the NT using $^3$H-IP$_3$ (1.5 nM) and the indicated ligands in CLM (E). The key to the symbols shown in panel A applies to all five panels (A–E). For each analysis (A–E) the $K_d$ (from binding) or EC$_{50}$ (from functional assays) is shown as a ratio for IP$_3$ versus AdA (F). For each analysis (A–E), the $K_d$ or EC$_{50}$ is shown as a ratio for the dephospho analogue relative to IP$_3$ or AdA (G). Results are means ± SEM, n = 4. DT40-IP$_3$R1 cells, DT40 cells stably expressing rat type 1 IP$_3$R. AdA, adenophostin A; CLM, cytosol-like medium; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; $K_d$, equilibrium dissociation constant; TEM, Tris/EDTA medium.
Indeed in all our assays, the relative affinities of IP3 and AdA for binding to the full-length receptor and its fragments, and their relative potencies in functional assays are not significantly different (Figure 2F, Table S1). It is noteworthy that the \( n_H \) for the interactions of AdA with IP3R consistently tend to be greater than unity (see Tables 1–4), even when the interactions are with monomeric IBC or NT (Tables 2 and 3). We and others have reported similar observations previously (reviewed in Rossi et al., 2010), although the underlying mechanism is unresolved.

These results, which establish that the IBC includes the structural determinants for high-affinity binding of AdA, provide our justification for using the IBC in subsequent experiments to explore the structural determinants of AdA binding. With the exception of Gly-268, the residues within the IBC that coordinate IP3 are conserved between all three IP3R subtypes (Bosanac et al., 2002), and the IBC from each subtype binds IP3 with the same affinity (Iwai et al., 2006). This, together with evidence that AdA is more potent than IP3 in cells that predominantly express each IP3 receptor subtype (Rossi et al., 2010), suggests that the results obtained from our subsequent analysis of IP3R1 and the IBC from IP3R1 are probably applicable also to IP3R2 and IP3R3.

**The 2’-phosphate is not responsible for high-affinity binding of AdA**

The suggestion that the 2’-phosphate of AdA provides a supra-optimal mimic of the 1-phosphate of IP3 (Takahashi et al., 1994c; Wilcox et al., 1995; Hotoda et al., 1999) predicts that removal of each phosphate moiety should more profoundly reduce the affinity of AdA for IP3R relative to IP3. We tested this prediction using synthetic IP2 and 2’-dephospho-AdA (Figure 1B). In equilibrium-competition binding analyses with full-length IP3R1 (in TEM), IP2 bound with 282-fold lower affinity than IP3 (\( \Delta K_d = 2.45 \pm 0.10 \)), whereas loss of the 2’-phosphate from AdA caused only a 41-fold decrease in affinity (\( \Delta K_d = 1.61 \pm 0.11 \)) (Figure 2A, Table 1 and Table S1). For the IBC in CLM, removal of the critical phosphate also more substantially reduced the affinity for IP3 relative to AdA: IP3 bound with 60-fold higher affinity than IP2 (\( \Delta K_d = 1.78 \pm 0.08 \)), whereas the affinities of 2’-dephospho-AdA and AdA differed by only 29-fold (\( \Delta K_d = 1.46 \pm 0.12 \)) (Figure 2D, Table 3 and Table S1). Similar results were obtained with the NT in TEM and CLM (Figure 2C and E, Tables 2 and 3 and Table S1). In Ca2+ release assays, and consistent with the binding analyses, removal of the 1-phosphate from IP3 more substantially reduced its potency than did removal...
of the 2′-phosphate from AdA (Figure 2B, Table 1 and Table S1). A previous study suggested that loss of the 2′-phosphate of AdA more profoundly affected the Kd (Takahashi et al., 1994a). However, as stated above, the authors miscalculated the Kd from the IC50, and it is impossible from the data presented to estimate the correct Kd for 2′-dephospho-AdA. We note, although it is unclear whether it contributes to their reported low affinity of 2′-dephospho-AdA for IP3R, that these authors used 2′-dephospho-AdA produced enzymatically rather than by synthesis (Takahashi et al., 1994a).

It is noteworthy that the disparity between the affinities of IP3 and AdA and their dephospho analogues was exaggerated in TEM (Figure 2G). We have not further explored the more pronounced effect of TEM on binding of AdA and IP3 relative to 2′-dephospho-AdA and IP2 (Table 2). The high pH of TEM favours substantial deprotonation of the phosphate groups in all the ligands (Felemez et al., 1999), perhaps thereby enhancing their binding to the IBC. The larger effect of TEM on binding of the trisphosphate ligands (IP3 and AdA) may reflect a greater effect of the different media (pH, ionic strength, counter-ions) on the ionization states of these ligands relative to the bisphosphate ligands. The results do, however, highlight the necessity to examine ligand binding to IP3R in medium resembling that used for functional analyses (e.g. CLM) if the functional and binding analyses are to be compared reliably.

Our demonstration that removal of the 1-phosphate from IP3 reduces both affinity and potency significantly more than does removal of the 2′-phosphate from AdA (Figure 2G) is inconsistent with the notion that the high affinity of AdA results from its 2′-phosphate providing a supra-optimal mimic of the 1-phosphate of IP3 (Takahashi et al., 1994c).

### Contributions of R568 to AdA and IP3 binding

R568 within the α-domain of the IBC is the only residue to interact directly with the 1-phosphate of AdA.
### Table 3

Binding of IP$_3$, AdA and their dephospho analogues to the NT, IBC and IBC mutants assayed in CLM

|                  | IP$_3$       | IBC         | IBC$_{R568Q}$ | IBC$_{R504Q}$ |
|------------------|--------------|-------------|---------------|---------------|
| **K$_d$ (nM)**   |              |             |               |               |
| NT               | 47.0 (7.33 ± 0.16) | 7.23 (8.14 ± 0.05) | 271 (6.57 ± 0.04) | 96.8 (7.01 ± 0.02) |
|                 | 0.8 ± 0.2    | 1.0 ± 0.1   | 1.6 ± 0.3     | 1.3 ± 0.2     |
| **pK$_d$**       |              |             |               |               |
| NT               | 7.42 ± 0.02  | 6.72 ± 0.08 | 7.60 ± 0.08   | 8.40 ± 0.08   |
| **n$_{Hill}$**   |              |             |               |               |
| NT               | 0.8 ± 0.1    | 0.8 ± 0.1   | 0.8 ± 0.1     | 0.8 ± 0.1     |

From equilibrium-competition binding assays, the K$_d$, pK$_d$ and Hill coefficient ($n_{Hill}$) for each ligand are shown for the NT, IBC, IBC$_{R568Q}$ and IBC$_{R504Q}$ fragments of IP$_3$R1 in CLM. Results are means (K$_d$)$_{SEM}$ (pK$_d$ and $n_{Hill}$) from 3–6 independent experiments.

AdA, adenophostin A; CLM, cytosol-like medium; IBC, IP$_3$-binding core; IP$_2$, inositol 4,5-bisphosphate; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; K$_d$, equilibrium dissociation constant; $n_{Hill}$, Hill coefficient; NT, N-terminal.

### Table 4

Functional responses of mutant IP$_3$R1

|                  | IP$_3$R1   | IP$_3$R1$_{R568Q}$ | IP$_3$R1$_{R504Q}$ |
|------------------|------------|--------------------|-------------------|
| **EC$_{50}$ (µM)** | Release (%) | Release (%) | Release (%) |
| **pEC$_{50}$ ± SEM** |            |            |            |
| NT               | 0.038 (7.42 ± 0.02) | 0.119 (5.92 ± 0.06) | 0.39 (5.42 ± 0.04) |
|                 | 1.1 ± 0.2  | 1.5 ± 0.3 | 1.0 (1.0) |
| **Release (%)**  | 79 ± 3     | 73 ± 7    | 78 ± 3     |
| **EC$_{50}$ (µM)** |            |            |            |
| NT               | 5.01 (5.30 ± 0.08) | 8.05 (5.09 ± 0.07) | 53 ± 5 (4.58 ± 0.14) |
|                 | 1.4 ± 0.2  | 1.1 ± 0.2 | 1.0 ± 0.2 |
| **Release (%)**  | 73 ± 7     | 8.05      | 53 ± 3     |
| **IP$_2$**       |            |            |            |
| **pEC$_{50}$ ± SEM** |            |            |            |
| NT               | 0.003 (8.51 ± 0.05) | 0.02 (7.70 ± 0.05) | 0.60 (6.219 ± 0.002) |
|                 | 1.4 ± 0.1  | 1.0 ± 0.2 | 1.3 ± 0.2 |
| **Release (%)**  | 78 ± 3     | 70 ± 3    | 61 ± 3     |
| **AdA**          |            |            |            |
| **pEC$_{50}$ ± SEM** |            |            |            |
| NT               | 0.16 (6.80 ± 0.02) | 0.26 (6.59 ± 0.07) | 4.21 (5.38 ± 0.05) |
|                 | 1.3 ± 0.2  | 1.7 ± 0.4 | 1.9 ± 0.4 |
| **Release (%)**  | 72 ± 3     | 43 ± 4    | 46 ± 1     |
| **2'-dephospho-AdA** |            |            |            |
| **pEC$_{50}$ ± SEM** |            |            |            |
| NT               | 0.16 (6.80 ± 0.02) | 0.26 (6.59 ± 0.07) | 4.21 (5.38 ± 0.05) |
|                 | 1.3 ± 0.2  | 1.7 ± 0.4 | 1.9 ± 0.4 |
| **Release (%)**  | 72 ± 3     | 43 ± 4    | 46 ± 1     |

From experiments similar to those shown in Figures 2B, 3E and 4F, Ca$^{2+}$ release was measured in DT40 cells expressing only the indicated mutant IP$_3$R. Results show the pEC$_{50}$, EC$_{50}$, $n_{Hill}$ and the maximal Ca$^{2+}$ release evoked by each agonist. Results are presented as means (EC$_{50}$) or means ± SEM (pEC$_{50}$, $n_{Hill}$ and percentage Ca$^{2+}$ release) from 4–6 independent experiments, each with three determinations.

AdA, adenophostin A; IP$_2$, inositol 4,5-bisphosphate; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; K$_d$, equilibrium dissociation constant; $n_{Hill}$, Hill coefficient; NT, N-terminal.
IP3, and it does so via two H-bonds with its side chain (Figure 1A) (Bosanac et al., 2002). We mutated R568 to Q and examined IP3 and AdA binding to the mutant IBC, and Ca2+ release via the mutant full-length IP3R.

The affinities of IP3 and AdA for the IBC were similarly reduced (by 37-fold and 31-fold respectively) by the R568Q mutation (ΔpKd = 1.57 ± 0.07 for IP3, and 1.49 ± 0.11 for AdA), whereas the affinities of IP2 and 2′-dephospho-AdA were minimally affected (ΔpKd = 0.40 ± 0.14 and 0.48 ± 0.12 for IP2 and 2′-dephospho-AdA respectively) (Figure 3A and B, Table 3, Table S2). Similar results were obtained with NT568Q in TEM (Figure 3C, Table 2). These

Figure 3
R568 does not selectively enhance AdA binding. Equilibrium-competition binding to IBCNT568Q using 3H-IP3 (1.5 nM) and the indicated ligands in CLM (A). Relative affinities (Kd) of ligands for the IBCNT568Q and IBC (B). Equilibrium-competition binding to the NT568Q using 3H-IP3 (1.5 nM) and the indicated ligands in TEM (C). Representative immunoblot (with anti-IP3R1 antibody, Ab1.5, top panel; and β-adaptin, bottom panel) for DT40-KO cells (KO) and DT40 cells expressing IP3R1 or the indicated mutants (10^5 cells per lane) for DT40-KO cells (KO) and DT40 cells expressing IP3R1 or the indicated mutants (10^5 cells per lane). Molecular weight markers (kDa) are shown. The blot is typical of six similar blots. IP3R expression (corrected for β-adaptin loading) is shown for each mutant relative to DT40-IP3R1 cells (%). Ca2+ release from permeabilized DT40-IP3R1R568Q cells evoked by the indicated ligands (E). Comparison of Ca2+ release for each ligand in normal and mutant IP3R1R568Q (F). Results are means ± SEM, n = 4. AdA, adenophostin A; CLM, cytosol-like medium; IBC, IP3-binding core; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; Kd, equilibrium dissociation constant; NT, N-terminal; TEM, Tris/EDTA medium.

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results suggest that R568 recognizes the 1-phosphate of IP3 and the 2′-phosphate of AdA similarly, and so lends further support to the view that the latter at least partially mimics the 1-phosphate of IP3 (Figure 1B). But why, if IP3 and AdA interact similarly with R568, should removal of the 1-phosphate from IP3 increase its $K_d$ more than does removal of the 2′-phosphate from AdA (Tables 1 and 2)? A likely explanation is that the indirect interaction of the 1-phosphate of IP3 with the backbone of K569 (Figure 1A) (Bosanac et al., 2002) is stronger than the equivalent interaction with the 2′-phosphate of AdA. Unfortunately, mutagenesis using naturally occurring amino acids cannot be used to dissect the role of this backbone interaction. Introduction of a non-natural amino acid, for example replacing K569 with an α-hydroxyl acid residue to replace the backbone NH with O, might address the question (Yang et al., 2004). But the nonsense suppression techniques required to insert the non-natural residue are not presently available to us.

Functional assays of DT40 cells expressing mutant IP3R do not allow absolute sensitivities to be precisely compared between cell lines because it is impossible to establish stable cell lines with identical levels of IP3R expression (Figure 3D). The problem is less severe than might have been anticipated because even a substantial increase in IP3R expression in DT40 cells (>20-fold) caused the sensitivity to IP3 to increase by less than twofold (Dellis et al., 2006). The mutations we have studied caused much larger changes in IP3 sensitivity (~30-fold, Table 4). Furthermore, levels of IP3R expression in the stable DT40 cell lines expressing mutant IP3R differed by no more than 3.5-fold from the line expressing wild-type IP3R1 (Figure 3D).

In functional assays, using DT40 cells expressing only IP3R, R568Q, IP3, and AdA were less potent than in cells expressing wild-type IP3R1: the potencies of IP3 and AdA were reduced by 31-fold ($\Delta pEC_{50} = 1.50 \pm 0.06$) and sixfold ($\Delta pEC_{50} = 0.81 \pm 0.07$) respectively. By contrast, the potencies of IP3 and 2′-dephospho-AdA were only very modestly reduced (1.6-fold, $\Delta pEC_{50} = 0.21 \pm 0.11$ for both ligands) (Figure 3E and F, Table 4 and Table S3). The lesser Ca2+ release with maximally effective concentrations of all four agonists in DT40-IP3R(R568Q) cells (compare Figures 2B and 3E) is probably attributable to the reduced level of expression of IP3R in the mutant cell line (Figure 3D). The selective effect of the R568Q mutation on AdA and IP3, but not the dephospho analogues, is consistent with our analyses of ligand binding (Table 3). But the significantly lesser effect of the mutation on the functional responses to AdA was unexpected because hitherto the interactions between R568 and the critical phosphates may selectively reduce the efficacy of IP3. That conclusion would be consistent with evidence that inositol 2,4,5-trisphosphate is a partial agonist of IP3R (Marchant et al., 1997b).

**Selective interaction of AdA with R504**

Our previous attempts to predict the binding mode of AdA to the IBC using molecular docking suggested that, in addition to possible interactions with R568, the 2′-phosphate might also interact with the amide NH of K569 and the side chain of R504 (Rosenberg et al., 2003). But our present results (Figure 2) suggest that for AdA, the 2′-phosphate is not a major determinant of its high affinity. One of the possible binding modes also suggested a cation–π interaction between the adenine of AdA and the guanidinium side chain of R504 (Rosenberg et al., 2003) (Figure 4A). Many analyses of synthetic AdA analogues lacking the adenine moiety suggest that the adenine or another aromatic moiety is an important determinant of the high-affinity binding of AdA. The AdA analogues lacking adenine, which retain a phosphate group equivalent to the 2′-phosphate of AdA, typically have $K_d$ values similar to that for IP3 (Table 2) (Jenkins et al., 1997; Tatani et al., 1998; Hotoda et al., 1999; Marwood et al., 2000; Correa et al., 2001). R504 is one of several residues to form a hydrogen bond with the 5-phosphate of IP3, via a bridging water molecule (Bosanac et al., 2002), and probably also with the equivalent 3′-phosphate of AdA (Rosenberg et al., 2003) (Figure 1B). Mutation of R504 inhibits IP3 binding (Yoshikawa et al., 1996) and is likewise expected to disrupt interaction of the 3′-phosphate of AdA. But if the proposed cation–π interaction is important for AdA binding, mutation of R504 might additionally reduce AdA binding by disrupting interactions with its adenine ring. The subsequent experiments were designed to test this hypothesis.

As expected, because each ligand has a phosphate equivalent to the 5-phosphate of IP3, mutation of R504 to Q significantly reduced the affinity of both IP3 and AdA for the IBC (Figure 4B and C, Table 3 and Table S3). However, whereas the affinity of IP3 was reduced by 13-fold ($\Delta pK_d = 1.13 \pm 0.06$), the affinity of AdA was reduced by 353-fold ($\Delta pK_d = 2.55 \pm 0.05$) (Figure 4B and C, Table 3 and Table S2). Binding of the dephospho analogues was less affected by the R504Q mutation: the decrease in affinity was 1.4-fold ($p\Delta = 0.16 \pm 0.07$) for IP3 and
35-fold ($\Delta pK_d = 1.54 \pm 0.14$) for 2'-dephospho-AdA. Similar results were obtained with NT$^{R504Q}$ in TEM (Figure 4D, Table 2).

A further prediction arising from our suggestion that a cation-π interaction involving R504 and the adenine moiety contributes to high-affinity binding of AdA is that adenophostin analogues lacking the adenine moiety should be less affected by mutation of R504. The results shown in Table 2 confirm this prediction for two such analogues, furanophostin...
and ribophostin (Figure 1B). Mutation of R504 to Q reduced the affinity of the NT for furanophostin by 44-fold ($\Delta pK_d = 1.63 \pm 0.10$) and for ribophostin by 21-fold ($\Delta pK_d = 1.33 \pm 0.24$).

Comparison of the effects of the R504Q mutation on each pair of ligands (i.e. IP$_3$ vs. AdA, and IP$_2$ vs. dephospho-AdA) indicates that for each there was a 25-fold greater decrease in the affinity for the AdA analogues (Figure 4C, Table 3 and Table S2). But the lesser effects of the mutation on both dephospho analogues is intriguing because R504 has not been reported to interact with the 1-phosphate of IP$_3$ (Bosanac et al., 2002). However, close inspection of the crystal structure of the IP$_3$-bound IBC also reveals a possible indirect interaction, via water, of R504 with the 1-phosphate (Figure 4E), although this was not discussed in the original report (Bosanac et al., 2002). It is not clear whether a similar interaction could also exist for AdA, but the possibility that both IP$_3$ and AdA interact with R504 via their 1- and 2'-phosphates, respectively, is appealing because it would explain the lesser effects of the R504Q mutation on the dephospho analogues (Figure 4C).

Functional analyses with DT40 cells expressing IP$_3$R$_{1R504Q}$ confirmed the results with binding. The sensitivity of the mutant IP$_3$R$_{1R504Q}$ was significantly decreased for all ligands, although again the effect on the dephospho analogues was more modest than that on IP$_3$ and AdA (Figure 4F and G, Table 4 and Table S3). Most importantly, whereas the EC$_{50}$ for IP$_3$ was reduced by 23-fold ($\Delta pEC_{50} = 1.35 \pm 0.17$), that for AdA was reduced by 196-fold ($\Delta pEC_{50} = 2.29 \pm 0.06$). These $\Delta pEC_{50}$ values for IP$_3$ and AdA are significantly different. Together, the binding and functional analyses establish that R504 is more important for binding of AdA than for IP$_3$. The selective effect of R504 on AdA binding does not result from interaction with the 2'-phosphate of AdA, but is instead likely to reflect the contribution of a cation-$\pi$ interaction between the adenine of AdA and the guanidinium side chain of R504 (Figure 4A).

Discussion

Inositol 1,4,5-trisphosphate and AdA are full agonists of IP$_3$R (Rossi et al., 2009), but the latter binds with substantially greater affinity than does IP$_3$ (Takahashi et al., 1994b; Correa et al., 2001) (Figure 2F). We have established that the IBC, to which IP$_3$ binds to initiate activation of the IP$_3$R, is alone capable of binding AdA with ~20-fold greater affinity than IP$_3$ (Table 3). Presently available evidence suggests that once AdA or IP$_3$ has bound to the IBC, each causes indistinguishable activation of the IP$_3$R (Rossi et al., 2009). The increased potency of AdA, relative to IP$_3$, in evoking Ca$^{2+}$ release via IP$_3$R is therefore likely to be entirely attributable to the stronger interactions between AdA and the IBC. This conclusion provided the impetus for resolving the interactions between AdA and the IBC that mediate its high-affinity binding.

The 4″- and 3″-phosphates of AdA mimic the essential 4,5-bisphosphate moiety of IP$_3$ (Figure 1B) and the 2'-phosphate of AdA has been thought to at least partially mimic the 1-phosphate of IP$_3$ (Takahashi et al., 1994b). None of our present results challenges this interpretation. Hitherto, an appealing explanation for the high affinity of AdA has been the suggestion that its 2'-phosphate is better placed than the 1-phosphate of IP$_3$ to interact with residues in the IBC (Takahashi et al., 1994c; Wilcox et al., 1995). Our results establish that this is not the basis of the high-affinity binding of AdA. First, removal of the 2'-phosphate...
from AdA has significantly less effect on its activity than does removal of the 1-phosphate from IP3 (Figure 2G, Tables 1–3 and Table S1). Second, mutation of R568, one of the key residues with which the 1-phosphate of IP3 interacts (Bosanac et al., 2002), similarly reduces the affinity of the IP3R for IP3 and AdA, while minimally affecting binding of IP2 or 2′-dephospho-AdA (Figure 3A and B, Table 3 and Table S1). These results establish that the 2′-phosphate of AdA partially mimics the 1-phosphate of IP3, but the latter probably interacts more strongly with the backbone of K569. We conclude that the high affinity of AdA for IP3R does not result from its 2′-phosphate behaving as a supra-optimally positioned mimic of the 1-phosphate of IP3 (Figure 5).

Considerable evidence suggests that the adenine group of AdA contributes significantly to its high-affinity binding (Tatani et al., 1998; Hotoda et al., 1999; Marwood et al., 2000; Correa et al., 2001; Rosenberg et al., 2003) and our molecular modelling has suggested that this might result from a cation-π interaction between the adenine and the guanidinium side chain of R504 (Rosenberg et al., 2003) (Figure 4A). Our present results are consistent with this suggestion. Because R504 interacts with the 5-phosphate of IP3 (Figure 1A) and, almost certainly, in similar fashion with the equivalent 3″-phosphate of AdA (Figures 1B and 5), mutation of this residue (R504Q) significantly decreased the affinity of the IP3R for both ligands. But more importantly, the effects, in both functional and binding assays, were significantly greater for AdA than for IP3 (Figure 4D and G, Tables 2–4, Tables S2 and S3). These results establish the greater importance of R504 for AdA binding, which would be consistent with AdA, but not IP3, forming a cation-π interaction with this residue (Figure 5). We conclude that the high affinity of AdA for IP3R is not due to its 2′-phosphate, and that AdA interacts more strongly than IP3 with R504, most likely reflecting a cation-π interaction between the adenine group and R504. This interaction may provide opportunities for synthesis of less polar ligands of IP3R (Sureshan et al., 2009).

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**Conflicts of interest**

None.

**References**

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels, 4th edition. Br J Pharmacol 158: S1–S254.

Berridge MJ, Bootman MD, Roderick HL (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4: 517–529.

Borissow CN, Black SJ, Paul M, Tovey SC, Dedos SG, Taylor CW et al. (2005). Adenophostin A and analogues modified at the adenine moiety: synthesis, conformational analysis and biological activity. Org Biomol Chem 3: 245–252.

Bosanac I, Alattia J-R, Mal TK, Chan J, Talarico S, Tong FK et al. (2002). Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. Nature 420: 696–700.

Cardy TJ, Traynor D, Taylor CW (1997). Differential regulation of types 1 and 3 inositol trisphosphate receptors by cytosolic Ca2+. Biochem J 328: 785–793.

Cheng Y-C, Prusoff WH (1973). Relationship between the inhibition constant (K) and the concentration of inhibitor causing 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108.

Colquhoun D (1971). Lectures in Biostatistics. Clarendon Press: Oxford.

Correa V, Riley AM, Shuto S, Horne G, Nerou EP, Marwood RD et al. (2001). Structural determinants of adenophostin A activity at inositol trisphosphate receptors. Mol Pharmacol 59: 1206–1215.

Dellis O, Dedos S, Tovey SC, Rahman T-U-, Dubel SJ, Taylor CW (2006). Ca2+ entry through plasma membrane IP3 receptors. Science 313: 229–233.

Desai T, Gigg J, Gigg R, Martin-Zamora E (1994). The preparation of intermediates for the synthesis of 1-D-myo-inositol 1,4,5- and 2,4,5-trisphosphates, 1,4-bisphosphate 5-phosphorothioate, and 4,5-bisphosphate 1-phosphorothioate from 1D-3,6-di-O-benzyl-1,2-O-isopropylidene-myo-inositol. Carbohydr Res 262: 59–77.

Felemex M, Marwood RD, Potter BVL, Spiess B (1999). Inframolecular studies of the protonation of adenophostin A: comparison with 1-D-myo-inositol 1,4,5-trisphosphate. Biochem Biophys Res Commun 266: 334–340.

Foskett JK, White C, Cheung KH, Mak DO (2007). Inositol trisphosphate receptor Ca2+ release channels. Physiol Rev 87: 593–658.

Glouchankova L, Krishna UM, Potter BVL, Falck JR, Bezprozvanny I (2000). Association of the inositol-(1,4,5) trisphosphate receptor ligand binding
Selective recognition of inositol phosphates by subtypes of inositol trisphosphate receptor. Biochem J 355: 59–69.

Ott RL, Longnecker M (2010). An introduction to statistical methods and data analysis. Brooks/Cole, Cengage Learning.

Parekh AB, Riley AM, Potter BVL (2002). Adenophostin A and ribophostin, but not inositol 1,4,5-trisphosphate or mannophostin, activate a Ca2+ release-activated Ca2+ current, I_Ca(Ca), in weak intracellular Ca2+ buffer. Biochem J 361: 133–141.

Potter BVL, Lampe D (1995). Chemistry of inositol lipid mediated cellular signaling. Angew Chem Int Ed Engl 34: 1933–1972.

Ramos-Franco J, Galvan D, Mignery GA, Fill M (1999). Location of the permeation pathway in the recombinant type-1 inositol 1,4,5-trisphosphate receptor. J Gen Physiol 114: 243–250.

Rosenberg HJ, Riley AM, Laude AJ, Taylor CW, Potter BVL (2003). Synthesis and Ca2+-mobilizing activity of purine-modified mimics of adenophostin A: a model for the adenophostin-Ins(1,4,5)P3 receptor interaction. J Med Chem 46: 4860–4871.

Rossi AM, Riley AM, Tovey SC, Rahman T, Dellis O, Taylor EJA et al. (2009). Synthetic partial agonists reveal key steps in IP3 receptor activation. Nat Chem Biol 5: 631–639.

Rossi AM, Riley AM, Potter BVL, Taylor CW (2010). Adenophostins: high-affinity agonists of IP3 receptors. Curr Top Membr 66 (in press)

Shuto S, Tatani K, Ueno Y, Matsuda A (1998). Synthesis of adenophostin analogues lacking the adenine moiety as novel potent IP3 receptor ligands: some structural requirements for the significant activity of adenophostin A. J Org Chem 63: 8815–8824.

Sugawara H, Kurosaki M, Takata M, Kurosaki T (1997). Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. EMBO J 16: 3078–3088.

Suresh KM, Truselle M, Tovey S, Taylor CW, Potter BVL (2008). 2-Position base-modified analogs of adenophostin A as high-affinity agonists of the d-myo-inositol trisphosphate receptor: in vitro evaluation and molecular modeling. J Org Chem 73: 1682–1692.

Suresh KM, Riley AM, Ross AM, Tovey SC, Dedos SG, Taylor CW et al. (2009). Activation of IP3 receptors by synthetic bisphosphate ligands. Chem Commun 14: 1204–1206.

Takahashi M, Kagasaki T, Hosoya T, Takahashi S (1994a). Adenophostins A and B: potent agonists of inositol-1,4,5-trisphosphate receptor produced by Penicillium brevicompactum. Taxonomy, fermentation, isolation, physico-chemical and biological properties. J Antibiot 46: 1643–1647.
Takahashi M, Tanzawa K, Takahashi S (1994b). Adenophostins, newly discovered metabolites of *Penicillium brevicompactum*, act as potent agonists of the inositol 1,4,5-trisphosphate receptor. J Biol Chem 269: 369–372.

Takahashi S, Takeshi K, Takahashi M (1994c). Adenophostins A and B: potent agonists of inositol-1,4,5-trisphosphate receptors produced by *Penicillium brevicompactum*. Structure elucidation. J Antibiot 47: 95–100.

Tatani K, Shuto S, Ueno Y, Matsuda A (1998). Synthesis of 1-O-[35S]-4-hydroxytetrahydrofuran-4-yl]-α-D-glucopyranoside 3,4,3′-trisphosphate as a novel potent IP₃ receptor ligand. Tetrahedron Lett 39: 5065–5068.

Taylor CW, Genazzani AA, Morris SA (1999). Expression of inositol trisphosphate receptors. Cell Calcium 26: 237–251.

Taylor CW, Fonseca PCA, Morris EP (2004). IP₃ receptors: the search for structure. Trends Biochem Sci 29: 210–219.

Tovey SC, Sun Y, Taylor CW (2006). Rapid functional assays of intracellular Ca²⁺ channels. Nat Protocol 1: 259–263.

Wilcox RA, Erneux C, Primrose WU, Gigg R, Nahorski SR (1995). 2-Hydroxy-α-D-glucopyranoside-2,3′,4′-trisphosphate, a novel, metabolically resistant, adenophostin A and myo-inositol-1,4,5-trisphosphate analogue, potently interacts with the myo-inositol-1,4,5-trisphosphate receptor. Mol Pharmacol 47: 1204–1211.

Yang X, Wang M, Fitzgerald MC (2004). Analysis of protein folding and function using backbone modified proteins. Bioorg Chem 32: 438–449.

Yoshida M, Sensui N, Inoue T, Morisawa M, Mikoshiba K (1998). Role of two series of Ca²⁺ oscillations in activation of ascidian eggs. Dev Biol 203: 122–133.

Yoshikawa F, Morita M, Monkawa T, Michikawa T, Furuuchi T, Mikoshiba K (1996). Mutational analysis of the ligand binding site of the inositol 1,4,5-trisphosphate receptor. J Biol Chem 271: 18277–18284.

**Supporting information**

Additional Supporting Information may be found in the online version of this article:

Table S1 Relative potencies and affinities of IP₃ and AdA analogues interacting with native IP₃R1 and its N-terminal fragments. The table shows the relative effectiveness for each pair of ligands in evoking Ca²⁺ release from DT40-IP₃R1 cells (ΔpEC₅₀) and in binding (in TEM) to full-length IP₃R1 and NT, and binding (in CLM) to the NT or IBC (ΔpKd). Results are shown as means ± SEM, from at least three independent experiments. The data from which these values are derived are shown in Figure 2.

Table S2 Relative affinities of IP₃ and AdA analogues interacting with wild-type and mutant IBC. For each ligand, the relative affinity (ΔpKd) is shown for mutant and wild-type IBC (in CLM). Results are shown as means ± SEM, from at least three independent experiments. The data from which these values are derived are shown in Figures 2, 3 and 4.

Table S3 Relative potencies of IP₃ and AdA analogues interacting with mutant IP₃R1. For each ligand the relative potency in evoking Ca²⁺ release (ΔpEC₅₀) is shown for wild-type and mutant IP₃R. Results are shown as means ± SEM, from at least three independent experiments. The data from which these values are derived are shown in Figures 2, 3 and 4.

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