Determinants of adenophostin A binding to inositol trisphosphate receptors

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Inositol 1,4,5-trisphosphate (IP_3) receptors from cerebellum and recombinant type 1 IP_3 receptors expressed in Sf9 cells had indistinguishable affinities for IP_3 ($K_d = 6.40 \pm 0.48$ nM) and adenophostin A ($K_d = 0.89 \pm 0.05$ nM). In cytosol-like medium, each of the three mammalian IP_3 receptor subtypes when expressed in Sf9 cells bound adenophostin A with greater affinity than IP_3. It has been suggested that adenophostin A binds with high affinity only in the presence of ATP, but we found that adenophostin A similarly displaced [^{3}H]IP_3 from type 1 IP_3 receptors whatever the ATP concentration. N-terminal fragments of the type 1 receptor were expressed with and without the S1 splice site; its removal had no effect on [^{3}H]IP_3 binding to the 1–604 protein, but abolished binding to the 224–604 protein. The 1–604 fragment and full-length receptor bound adenophostin A with the same affinity, but the fragment had 3-fold greater affinity for IP_3, suggesting that C-terminal residues selectively inhibit IP_3 binding. The 224–604S1$^+$ fragment bound IP_3 and adenophostin A with increased affinity, but as with the 1–604 fragment it bound adenophostin A with only 2-fold greater affinity than IP_3. High-affinity binding of adenophostin A may be partially determined by its 2′-phosphate interacting more effectively than the 1-phosphate of IP_3 with residues within the IP_3-binding core. This may account for the 2-fold greater affinity of adenophostin A relative to IP_3 for the minimal IP_3-binding domain. In addition we suggest that C-terminal residues, which impede access of IP_3, may selectively interact with adenophostin A to allow it unhindered access to the IP_3-binding domain.

Key words: IP_3-binding domain, IP_3 receptor subtype, ligand recognition.

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP_3) receptors are the intracellular Ca^{2+} channels expressed in most mammalian cells that mediate the release of Ca^{2+} from intracellular stores evoked by receptors that stimulate IP_3 formation. The three subtypes (types 1–3) of mammalian IP_3 receptor are closely related and each assembles into homo- or hetero-tetramers to form the functional IP_3-gated Ca^{2+} channel. Although the different subtypes differ in their distributions [1], differ modestly in their affinities for IP_3 and in their abilities to recognize different inositol phosphates [2], and may be differentially modulated [3], the physiological significance of the diversity remains unclear.

Adenophostins, products of the fungus *Penicillium brevicompactum*, were shown in 1993 to be the most potent known agonists of IP_3 receptors [4]. Many subsequent studies using both functional and radioligand-binding assays are consistent with adenophostin A binding to IP_3 receptors with at least 10-fold greater affinity than IP_3 [5–8]. Furthermore, adenophostin A is not degraded by the enzymes that metabolize IP_3, nor does it bind to IP_4-binding sites [9]. These properties have led to the widespread use of adenophostin A in the analysis of many aspects of IP_3-receptor-mediated Ca^{2+} signalling [10–12]. Although adenophostin A is based upon a glucose ring, rather than the *myo*-inositol ring of IP_3, analyses of many structural analogues [5,13] are consistent with the original suggestion [9] that the 3″,4″-bisphosphate and 2″-hydroxyl groups of adenophostin A may effectively mimic the critical 4,5-bisphosphate and 6-hydroxyl groups of IP_3 (see structures in Figures 4B and 4C, below). It is not, however, clear how adenophostin achieves its increased affinity for IP_3 receptors. It may either be that the adenine group of adenophostin serves to improve the positioning of the 2′-phosphate (equivalent to the 1-phosphate of IP_3) or there may be a more direct interaction between the adenine moiety and a site on the receptor. The first explanation would be consistent with a massive decrease in affinity after removal of the 2′-phosphate from adenophostin A [4]. Comparison of the conformations and protonation states of IP_3 and adenophostin A using NMR and molecular modelling also supports the idea that the three phosphate groups of adenophostin A and IP_3 are functionally analogous [13,14]. If specific binding of the adenine moiety of adenophostin A is important for its high affinity, then the recognition site would appear to be very tolerant because even substantial changes to the adenine only modestly decrease the affinity of adenophostin A for IP_3 receptors [5]. Finally, recent evidence suggests that differences in affinity may not alone be sufficient to explain the different interactions between IP_3 receptors and IP_3 relative to adenophostin A [11] and ribophostin, another glucose-based analogue [12].

The IP_3-binding site (one on each subunit of the IP_3 receptor) lies within the N-terminal between residues 226 and 576 [15] and it is formed by two structurally distinct domains [16]. Although the core IP_3-binding site is within this N-terminal region, other residues modulate its properties. The first 225 residues may inhibit IP_3 binding [15]; C-terminal residues, which may associate with the N-terminal [17], can influence IP_3 binding [18,19], and various intracellular signals, Ca^{2+} and calmodulin for example, bind elsewhere and allosterically influence IP_3 binding [20].

Here we examine the interactions between adenophostin A and each of the three recombinant mammalian IP_3 receptor subtypes and, from analysis of IP_3 and adenophostin A binding to bacterially expressed fragments of the IP_3 receptor, we suggest a model to account for the high-affinity binding of adenophostin A to IP_3 receptors.

Abbreviations used: CLM, cytosol-like medium; IP_3, inositol 1,4,5-trisphosphate; K_d, equilibrium dissociation constant; TEM, Tris/EDTA medium.

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EXPERIMENTAL

Materials

IP_3 was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.) and [^{3}H]IP_3 (40 Ci/mmol) was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Adenophostin A was synthesized as previously reported [21]. The immobilized IP_3 matrix was prepared by reacting Affi-gel 10 (Bio-Rad, Hemel Hempstead, Herts., U.K.) with 1D-2-*O*-(2-aminoethyl)-*myo*-IP_3, synthesized by total deprotection of 1D-3,6-di-*O*-benzyl-2-*O*-[2-(2,2,2-trifluoroacetylamino)ethyl]-*myo*-inositol tris-(dibenzylphosphate) [22]. Isopropyl β-D-thiogalactoside was from Medford Labs (Ipswich, Suffolk, U.K.). Insect and bacterial cell culture materials were from Sigma (Poole, Dorset, U.K.) and Difco (Sparks, MD, U.S.A.), respectively.

Preparation of membranes expressing IP_3 receptor subtypes

Membranes were prepared from baculovirus-infected insect Sf9 cells expressing full-length mammalian type 1, 2 or 3 IP_3 receptors exactly as described earlier [2]. Membranes expressing native type 1 IP_3 receptor were prepared from rat cerebellum [23], and the IP_3 receptor was then purified by sequential heparin and concanavalin A chromatography [24].

Expression of fragments of type 1 IP_3 receptor in bacteria

For expression of N-terminal fragments of the rat type 1 IP_3 receptor in bacteria, appropriate regions were amplified by PCR from the full-length receptor clone lacking the S1 splice region [25]. Fragments are numbered by reference to residues within the full-length (S1$^+$) rat type 1 IP_3 receptor. The fragments were then ligated into the pTrcHis A vector at the *Xho*I/*Eco*RI sites (Invitrogen, Groningen, The Netherlands) to allow expression of N-terminally tagged hexa-His fusion proteins. For the expression construct that included the first 604 residues of the type 1 receptor lacking the S1 splice region (S1$^-$/1–604), the forward primer was 5′-CAACGTCGACATGTCTGACAAAATGTCT-AG-3′ (which inserts a *Sal*I site) and the reverse primer was 5′-CTGGAATTCTCAAAACTGCTTGGCTATGTACTCC-3′ (which inserts a stop codon and an *Eco*RI site). For expression of residues 224–604 from the S1$^-$ type 1 receptor (S1$^-$/224–604), the same reverse primer was used and the forward primer was 5′-AGTGGTCGAATGAAATGGAGTGATAACAAAG-3′ (which inserts a *Sal*I site). Insertion of the S1 splice region (15 amino acid residues) into the N-terminal fragments was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) with forward (5′-CATTACTTGGCA-GCAGAGGTAGACCCTGACTTTGAGGAAGAATGCCT-GGAGTTTCAGCCCTCAGTGGACCCTGATCAGG-3′) and reverse (5′-CTGATCAGGGTCCACTGAGGGCTGAAACT-CCAGGCATTCTTCCTCAAAGTCAGGGTCTACCTCTG-CTGCCAAGTAATGC-3′) primers. The sequences of all constructs were confirmed by sequencing both DNA strands.

For expression of bacterial fusion proteins, the constructs were transformed into *E. coli* strain BL21(DE3) and 1 ml of the culture was grown overnight in Luria–Bertani medium with 50 μg/ml ampicillin at 30 °C. This inoculum was then added to 100 ml of Luria–Bertani medium, cultured at 22 °C and when the D_{600} had reached 1.0–1.5 (about 7 h), isopropyl β-D-thiogalactoside (0.5 mM) was added. After a further 20 h at 15 °C, the cells were harvested by centrifugation (5000 ***g***, 15 min), washed in PBS, and the pellet was rapidly frozen in liquid nitrogen before storage at −80 °C. Bacterial lysates were prepared by re-suspending the frozen pellet in 10 ml of Tris/EDTA medium (TEM; 50 mM Tris/1 mM EDTA, pH 8.3) supplemented with 1 mM β-mercaptoethanol and a protease inhibitor cocktail formulated for purification of poly-His-tagged proteins in bacteria (Sigma). The suspension was incubated with lysozyme (100 μg/ml; Sigma) for 30 min on ice, followed by five cycles of rapid freezing in liquid nitrogen and thawing. The lysate was then sonicated for 20 s (maximal setting on an MSE Soniprep 150) and after centrifugation (30000 ***g***, 60 min), aliquots of the supernatant (typically 4 mg of protein/ml) were frozen in liquid nitrogen and stored at −80 °C.

Protein concentrations were determined [26] using BSA as standard. Standard immunoblotting methods were used to identify the bacterial fusion proteins using a mouse anti-His_6 antibody, followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (both from Sigma) and Super Signal

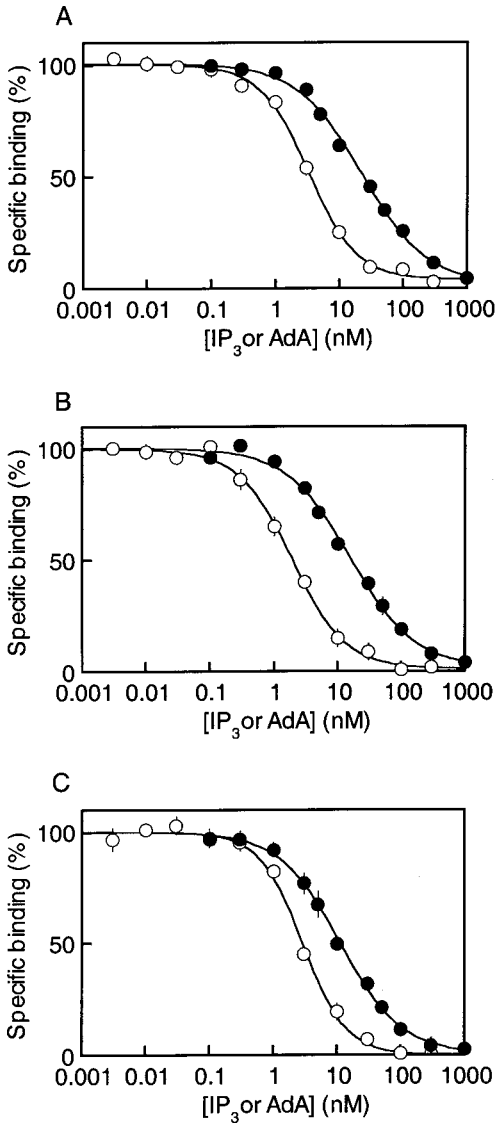

Figure 1 High-affinity binding of adenophostin A to all three subtypes of IP_3 receptor

[^{3}H]IP_3 binding to membranes prepared from Sf9 cells expressing type 1 (**A**), type 2 (**B**) or type 3 (**C**) IP_3 receptors was measured in the presence of the indicated concentrations of IP_3 (●) or adenophostin A (AdA; ○) in CLM. Results are means ± S.E.M. from four independent experiments.

chemiluminescence reagent to visualize immunoreactive bands (Perbio, Tattenhall, Cheshire, U.K.) [2].

Equilibrium [^{3}H]IP_3 binding

Unless otherwise stated, all equilibrium binding incubations were performed at 4 °C in TEM (final volume, 200 μl) containing [^{3}H]IP_3 (1 nM), membranes (typically 50 μg), bacterial lysate (100 μg) or purified IP_3 receptor (8 μg), and appropriate concentrations of competing ligands. After 5 min, reactions were terminated either by centrifugation alone (membranes; 20000 ***g***, 5 min) or, for soluble proteins, by addition of 200 μl of cold TEM containing 30% poly(ethylene glycol) 8000 and 200 μg of γ-globulin followed by centrifugation. For some experiments with recombinant IP_3 receptors (Figure 1), [^{3}H]IP_3 binding was measured in cytosol-like medium (CLM; 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 1 mM EDTA and 20 mM Pipes, pH 7.0). There was no significant metabolism of [^{3}H]IP_3 by the bacterial lysates under the conditions used for our binding assays (not shown). Pellets were solubilized in 1 ml of EcoScint A scintillation cocktail and their radioactivity was determined by liquid scintillation counting. Total [^{3}H]IP_3 binding was usually more than 2500 d.p.m. and non-specific binding was $< 10\%$ of total binding.

Equilibrium competition binding curves were fitted to logistic equations using non-linear curve fitting (Kaleidagraph; Synergy Software, Reading, PA, U.S.A.) from which equilibrium dissociation constants (K_d) were determined [2].

RESULTS AND DISCUSSION

High-affinity binding of adenophostin A to all three IP_3 receptor subtypes

To allow comparison with native IP_3 receptors, we first compared the binding of IP_3 and adenophostin A to the type 1 IP_3 receptors expressed in cerebellum and the recombinant type 1 IP_3 receptor expressed in Sf9 cells. To maximize specific binding of [^{3}H]IP_3 in these experiments (levels of specific binding are too low in native tissues to be reliably measured in CLM), we used a medium (TEM) similar to that used for all previous analyses of adenophostin A binding [5,7,9,13] in which the pH is high and ionic strength low (see the Experimental section). Our results establish that native cerebellar IP_3 receptors, purified cerebellar IP_3 receptors and recombinant type 1 IP_3 receptors expressed in Sf9 cells have indistinguishable affinities for both IP_3 and adenophostin A (Table 1). These results both justify our use of the Sf9 expression system to explore the ligand-binding properties of IP_3 receptors and suggest that accessory proteins are not required for high-affinity binding of adenophostin A. The latter is consistent with previous work [7] demonstrating that adenophostin A potently stimulates opening of purified IP_3 receptors.

Table 1 Binding of IP_3 and adenophostin A to native, purified and recombinant type 1 IP_3 receptors

Results from 8–9 independent equilibrium competition binding experiments performed in TEM show the K_d and Hill coefficient (h) values for IP_3 and adenophostin A binding to type 1 IP_3 receptors.

	IP_3		Adenophostin A	
	K_d (nM)	h	K_d (nM)	h
Membranes from cerebellum	6.40 ± 0.48	0.83 ± 0.03	0.89 ± 0.05	1.13 ± 0.09
Receptor purified from cerebellum	5.90 ± 0.83	0.71 ± 0.09	0.72 ± 0.18	0.89 ± 0.06
Type 1 receptor in Sf9 membranes	6.71 ± 0.55	0.70 ± 0.03	0.89 ± 0.05	1.08 ± 0.07

Table 2 Binding of IP_3 and adenophostin A to IP_3 receptor subtypes in CLM

Equilibrium competition binding using [^{3}H]IP_3 in CLM was used to define the K_d and Hill coefficient (h) values for IP_3 and adenophostin A binding to types 1, 2 and 3 IP_3 receptors heterologously expressed in Sf9 cells. Results are means ± S.E.M. from 4–9 independent experiments.

	IP_3		Adenophostin A		
	K_d (nM)	h	K_d (nM)	h	K_d (IP_3)/K_d (adenophostin A)
Sf9 type 1	22.7 ± 1.6	0.85 ± 0.05	3.4 ± 0.5	1.16 ± 0.07	6.7 ± 1.1
Sf9 type 2	15.9 ± 1.8	0.85 ± 0.03	1.9 ± 0.3	1.02 ± 0.07	8.4 ± 1.6
Sf9 type 3	11.3 ± 1.9	0.88 ± 0.08	3.5 ± 0.2	1.20 ± 0.07	3.2 ± 0.6

Adenophostin A has been reported to be more potent than IP_3 in stimulating Ca^{2+} release from the intracellular stores of cells expressing predominantly type 1 [9], type 2 [5] or type 3 [8] IP_3 receptors, but its interaction with the three IP_3 receptor subtypes has not been systematically examined. Because adenophostin A is now used extensively in many cell types as a high-affinity agonist of IP_3 receptors, we felt it important to precisely define its affinity for each receptor subtype in CLM. By expressing full-length mammalian IP_3 receptors in Sf9 cells at levels far higher than the endogenous receptor, we can examine the properties of each receptor subtype in an identical cellular context and in medium resembling cytosol [2]. The results shown in Figure 1 confirm our earlier work [2] by demonstrating that, in CLM, type 3 IP_3 receptors bind IP_3 with modestly higher affinity than type 1 receptors, while type 2 receptors have intermediate affinity, although others [27,28] have suggested a different rank order of IP_3-binding affinities. All three receptor subtypes bound adenophostin A with greater affinity than IP_3, but whereas the types 1 and 2 receptors bound adenophostin A with 7–8-fold greater affinity than IP_3, the type 3 receptor bound it with only 3-fold greater affinity (Figure 1, Table 2). These results establish that in Ca^{2+}-free CLM, adenophostin A binds with substantially greater affinity than IP_3 to all three subtypes of mammalian IP_3 receptor. It is noteworthy that for both native and recombinant full-length IP_3 receptors the Hill coefficients (h) of the equilibrium competition binding curves were always slightly steeper for adenophostin A than for IP_3 (Tables 1 and 2). We have no simple explanation for this observation, although others have also reported positively co-operative binding of adenophostin A [4] to IP_3 receptors and even to monomeric IP_3-binding domains [20]. The latter, however, conflicts with our results where Hill coefficients for both IP_3 and adenophostin A binding to N-terminal fragments were not significantly different from unity (Figures 3C and 3D, below).

ATP and adenophostin A binding to IP_3 receptors

ATP is known to modulate IP_3 receptor behaviour [29] and adenophostin A has an adenine moiety within its structure (see Figure 4C, below). These observations prompted several groups to consider whether the potency of adenophostin A might reflect an interaction with both the IP_3- and ATP-binding sites of the IP_3 receptor. However, binding of adenophostin A to both sites seems unlikely because the ATP-binding site is rather selective for adenine [29,30], whereas even very substantial changes to the adenine ring of adenophostin A are well tolerated [5]. Furthermore, adenophostin A does not displace [^{32}P]ATP from its

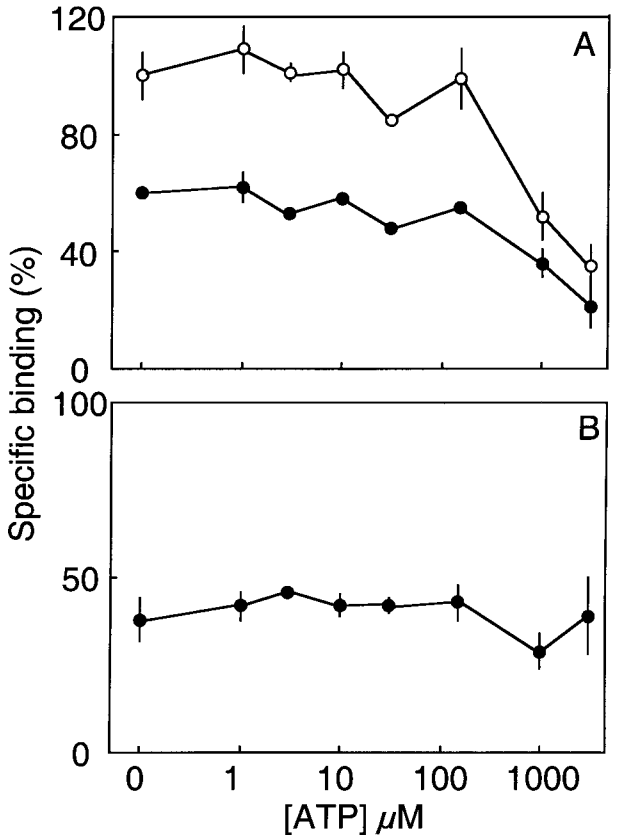

Figure 2 ATP similarly inhibits IP_3 and adenophostin A binding

(**A**) Specific [^{3}H]IP_3 binding (1 nM) to membranes prepared from Sf9 cells expressing type 1 IP_3 receptor was measured in CLM containing the indicated concentrations of ATP (○). In parallel, the effect of adenophostin A [present at a concentration (2 nM) equal to its IC_{50}] on specific [^{3}H]IP_3 binding was measured (●). (**B**) The specific binding of [^{3}H]IP_3 remaining in the presence of adenophostin A (2 nM) and the indicated concentrations of ATP is shown. Results are means ± S.E.M. of four independent experiments.

binding site [30], and nor do adenine nucleotides reduce adenophostin A binding to IP_3 receptors [11,31].

A more complex interaction between ATP and adenophostin A was recently suggested by single-channel analyses of IP_3 receptors from *Xenopus* oocytes [11]. Because adenophostin A appeared to be a potent agonist only in the presence of ATP, these authors suggested that binding of ATP to the adenine nucleotide-binding site was required for the IP_3 receptor to adopt a conformation capable of binding adenophostin A with high affinity. That proposal is difficult to reconcile with the many reports [5,7,9,32] of high-affinity binding of adenophostin A, all of which have excluded ATP. To address the issue more directly, we performed the experiment shown in Figure 2. We argued that if ATP selectively increased the affinity of adenophostin A relative to IP_3, then the ability of a fixed concentration of adenophostin to displace [^{3}H]IP_3 from its receptor should increase as the concentration of ATP is increased. However, in CLM the amount of specifically bound [^{3}H]IP_3 displaced from recombinant type 1 IP_3 receptors by adenophostin A (2 nM) was completely unaffected by ATP, even as the ATP concentration was increased to a level (1 mM) that competed directly with the ligands for binding to the IP_3 receptor [33]. We conclude that ATP does not affect the affinity of type 1 IP_3 receptors for either IP_3 or adenophostin A. All subsequent binding experiments to IP_3 receptor fragments were performed in the absence of ATP.

Binding of IP_3 to N-terminal fragments of the type 1 IP_3 receptor

Immunoblotting (using an antibody to the hexa-His tag) of lysates prepared from bacteria expressing each of the four N-terminal fragments of the type 1 IP_3 receptor confirmed that each was expressed at a similar level (Figure 3A). Although the immunoblots revealed some partial degradation of the 1–604 proteins, only the full-length protein (70 kDa) adhered to an immobilized IP_3 matrix (Figure 3B), indicating that in the [^{3}H]IP_3-binding assays only the full-length protein would be detected. There was no detectable specific binding of [^{3}H]IP_3 to lysates prepared from untransformed bacteria (results not shown), but in keeping with previous work [15] IP_3 bound with high affinity to proteins comprising only the first 604 residues of the IP_3 receptor: their affinity for IP_3 was similar whether (K_d = 2.25 ± 0.38 nM) or not (K_d = 2.86 ± 0.55 nM) they included the S1 splice region. The lack of effect of removal of the S1 splice site on IP_3 binding is consistent with an earlier study of a slightly shorter fragment (residues 1–576) [27]. Mikoshiba and his colleagues have shown that removal of residues from the N-terminal of the IP_3-binding domain increases its affinity for IP_3 [15,34]. Our results confirm that observation with a protein that included only residues 224–604, binding IP_3 with a K_d of 1.1 ± 0.2 nM. However, it is noteworthy that whereas removal of the S1 spice site had no effect on IP_3 binding to the complete N-terminal domain (residues 1–604), we have consistently failed to detect any specific [^{3}H]IP_3 binding in numerous preparations of the shorter domain (224–604) lacking the S1 splice site despite high levels of expression (Figure 3A).

Adenophostin A binding to IP_3-binding domains

Two previous studies have examined adenophostin A binding to N-terminal fragments (residues 1–580 or 1–581) of the IP_3 receptor. One suggested that adenophostin A bound with about 9-fold greater affinity than IP_3 [35], although the affinity for IP_3 was remarkably low ($K_d \approx 580$ nM), while the other found adenophostin A to bind with only 1.5-fold greater affinity than IP_3 [20]. Our results demonstrate that the 1–604S1$^+$ protein has the same affinity for adenophostin A as the full-length receptor although it binds IP_3 with 3-fold greater affinity: it therefore binds adenophostin A with only (2.78 ± 0.39)-fold greater affinity than IP_3. The shorter protein (224–604S1$^+$) binds both IP_3 and adenophostin A with greater affinity, such that it too binds adenophostin A with only (2.75 ± 0.60)-fold greater affinity than IP_3 (Figure 3D).

Conclusions

Residues 226–576 form the minimal IP_3-binding core of the type 1 IP_3 receptor and within this region several conserved Arg and Lys residues are essential [15,27]. Arg-568 appears to selectively interact with the 1-phosphate of IP_3 [15], which is known to be important for its high-affinity binding [2], and one model for adenophostin A binding proposes that its 2′-phosphate behaves as a super-optimal mimic of the 1-phosphate of IP_3 (see the Introduction). A few residues that immediately precede the IP_3-binding core inhibit IP_3 binding to type 1 IP_3 receptors [15]. In our experiments, removal of the N-terminal residues caused a 2-

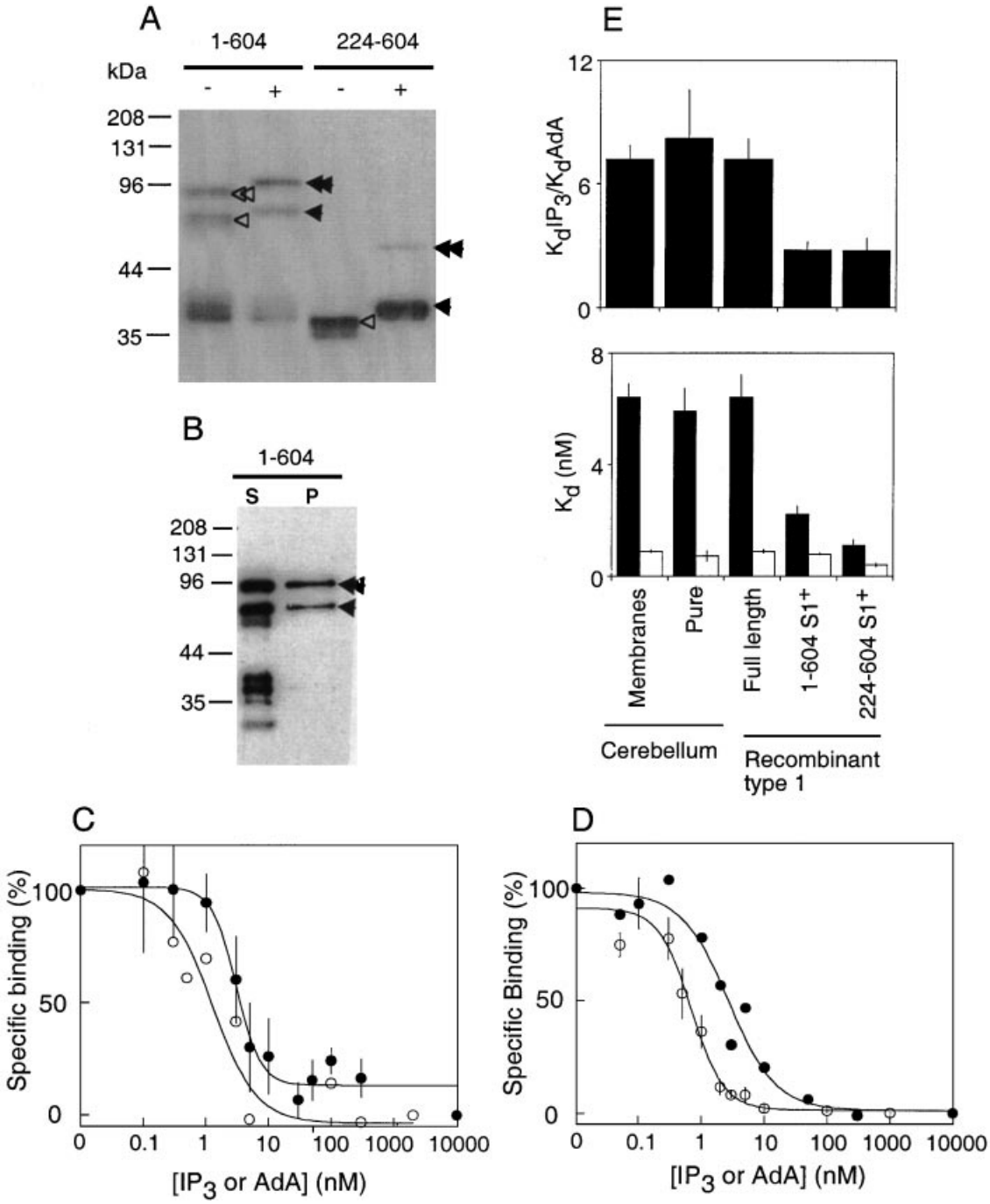

Figure 3 Binding of IP_3 and adenophostin A to N-terminal fragments of type 1 IP_3 receptors

(**A**) Lanes were loaded with lysate (10 μg of protein) from bacteria expressing the indicated N-terminal fragments of the type 1 IP_3 receptor and then detected with a hexa-His antibody. Arrows (filled for $S1^+$, open for $S1^-$) denote the bands corresponding to the full-length construct; double arrows show bands of higher molecular mass, which may be oligomers. (**B**) After incubation with the IP_3 matrix (see the Experimental section), 1–$604S1^+$ protein was separated into supernatant (S; proteins not adhering to the matrix) and pellet (P; proteins adhering to the matrix) and the two fractions were then subjected to immunoblotting using the hexa-His antibody. (**C**, **D**) Specific binding of [^{3}H]IP_3 to lysates from bacteria expressing the 1–$604S1^+$ (**C**) or 224–$604S1^+$ (**D**) construct is shown in the presence of the indicated concentrations of adenophostin A (AdA; ○) or IP_3 (●). Results are means ± S.E.M. from six (**C**) or three (**D**) experiments using three independent bacterial preparations. For clarity, error bars are shown on only one curve in each trace. (**E**) The lower panel shows the affinities of full-length IP_3 receptors and the various N-terminal fragments for IP_3 (solid bars) and adenophostin (open bars). The upper panel shows the ratio of two affinities for each of the proteins. All binding experiments (**C**–**E**) were performed in TEM.

fold increase in the affinity of the receptor for both IP_3 and adenophostin A (Figure 3D), suggesting that this inhibitory domain similarly inhibits binding of both agonists (Figure 4). The binding pocket itself is comprised of two domains: domain 1 (residues 226–317), separated from domain 2 (residues 346–576) by a loop that includes the S1 splice region (residues 318–332)

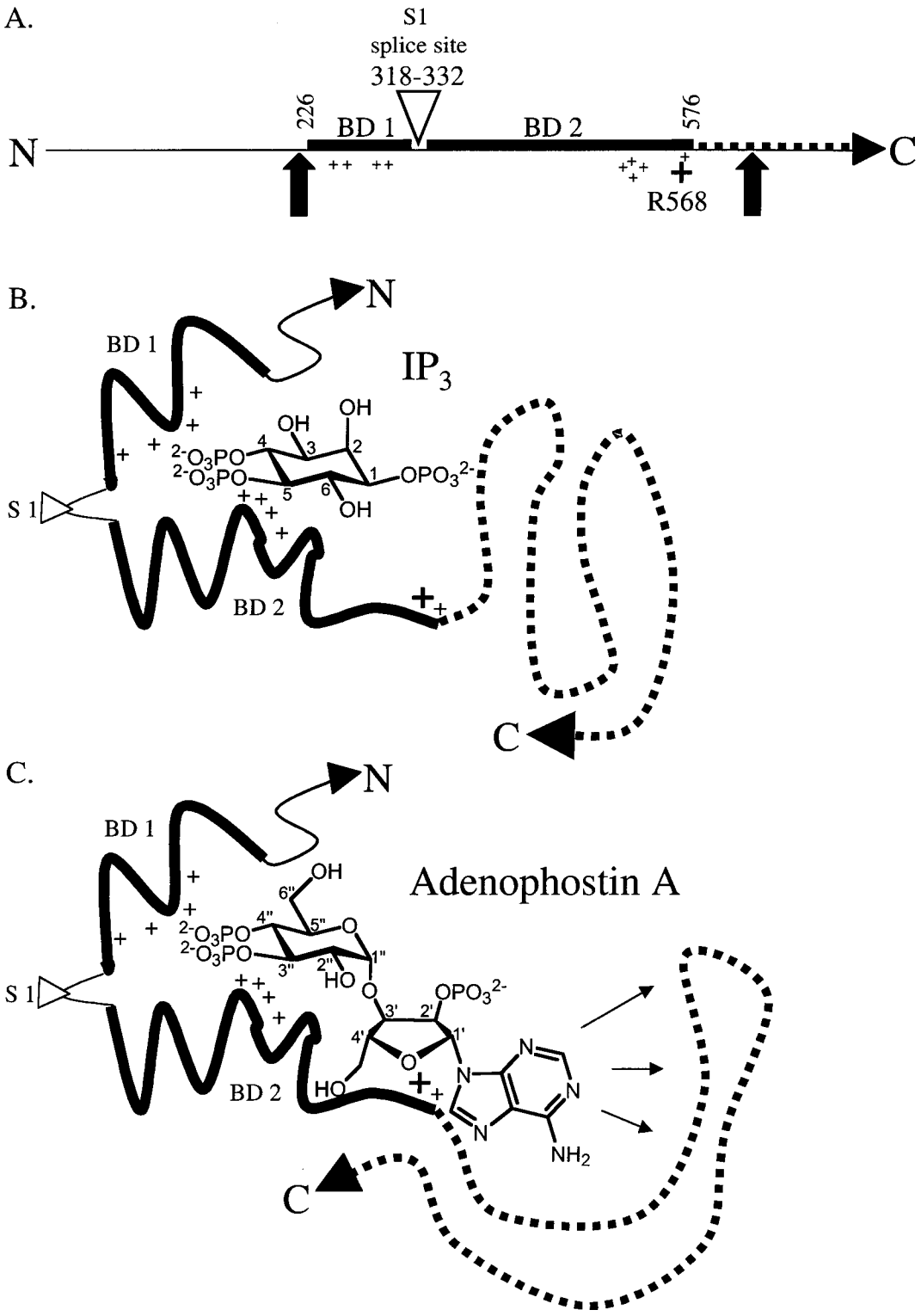

Figure 4 A possible model for adenophostin A binding to IP_3 receptors

(**A**) Both IP_3 and adenophostin A bind to the same minimal structure, formed by two domains (BD 1 and BD 2; bold lines) lying between residues 226 and 576 and linked by the short S1 splice site. Several conserved positively charged residues in each domain are likely to interact with the phosphate groups of the ligands. Arg-568 is probably essential for recognition of the 1-phosphate of IP_3 and the analogous 2′-phosphate of adenophostin A. Arrows denote the positions of residue 604 (the C-terminus of the IP_3-binding domains) and residue 224 (the N-terminus of the shortest IP_3-binding domain). (**B**, **C**) We propose that access of both IP_3 and adenophostin A to the binding pocket is partially occluded by residues towards the N-terminal (thin line); their removal therefore increases the affinity of both ligands. C-terminal residues (dashed line) also occlude access to the receptor, but we suggest that the adenosine moiety of adenophostin A can bind to this region to displace it and allow fast access of the remainder of the molecule to the core binding domain. IP_3 cannot displace this second inhibitory domain and so has less ready access to the binding pocket. Removal of C-terminal residues therefore selectively increases the affinity for IP_3.

[16] (Figure 4). Neither functional [36] nor binding [27] assays have previously found any effect of removing the S1 splice region, indicating that it is not an essential part of the IP_3-binding pocket. Our results with the 1–604 fragment confirm those observations (Figure 3C), but results with the shorter fragment, where [^{3}H]IP_3 binding was detected only in the $S1^+$ construct (Figure 3C), suggest that when pruned too close to the minimal IP_3-binding core the S1 region is essential to stabilize the interaction between the two domains of the IP_3-binding site.

We conclude that high-affinity binding of adenophostin A is an intrinsic property of the IP_3 receptor that does not depend upon simultaneous binding of ATP (Figure 2). All three IP_3 receptor subtypes bind adenophostin A with high affinity, but the subtype with highest affinity for IP_3 (type 3) does not have a correspondingly higher affinity for adenophostin A (Table 2). Likewise, N-terminal fragments of the type 1 receptor bind IP_3 with significantly higher affinity than the full-length receptor, but this is not matched by a proportionate increase in their affinity for adenophostin A (Figure 3E). It is clear therefore that removal of C-terminal residues from the IP_3 receptor increases its affinity for IP_3 without affecting its affinity for adenophostin A. In the full-length receptor, therefore, residues lying C-terminal to the IP_3-binding core are likely to impede access of IP_3 to its receptor, a conclusion that is consistent with evidence indicating an interaction between the N- and C-terminal regions of the receptor [17,37]. The simplest explanation for our results is to suggest that adenophostin A may be capable of deflecting this inhibitory region to allow less restricted access to the binding pocket and so bind with high affinity by virtue of more rapid association with the receptor (Figure 4). That interpretation would be consistent with evidence from analyses of the rate of channel closure of the IP_3 receptor after removal of adenophostin A, which indicated that despite a 10-fold higher affinity than IP_3 for hepatic IP_3 receptors, adenophostin A dissociated from the receptor only 2-fold more slowly than IP_3 [38].

We suggest, therefore, that the high affinity of adenophostin A for full-length IP_3 receptors may, as previously suggested [9], be partially determined by its 2′-phosphate interacting more effectively than the 1-phosphate of IP_3 with residues (probably including Arg-568) within the IP_3-binding core. This may account for the 2-fold greater affinity of adenophostin A relative to IP_3 for even the minimal IP_3-binding domain (Figure 3D). In addition, residues lying C-terminal to the IP_3-binding domain (though clearly not the ATP-binding site), which normally impede the access of IP_3 to the receptor, may selectively interact with adenophostin A to allow it unhindered access to the IP_3-binding domain.

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REFERENCES

1 Taylor, C. W., Genazzani, A. A. and Morris, S. A. (1999) Expression of inositol trisphosphate receptors. Cell Calcium **26**, 237–251

2 Nerou, E. P., Riley, A. M., Potter, B. V. L. and Taylor, C. W. (2001) Selective recognition of inositol phosphates by subtypes of inositol trisphosphate receptor. Biochem. J. **355**, 59–69

3 Patel, S., Joseph, S. K. and Thomas, A. P. (1999) Molecular properties of inositol 1,4,5-trisphosphate receptors. Cell Calcium **25**, 247–264

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

5 Correa, V., Riley, A. M., Shuto, S., Horne, G., Nerou, E. P., Marwood, R. D., Potter, B. L. and Taylor, C. W. (2001) Structural determinants of adenophostin A activity at inositol trisphosphate receptors. Mol. Pharmacol. **59**, 1206–1215

6 Shuto, S., Tatani, K., Ueno, Y. and Matsuda, A. (1998) Synthesis of adenophostin analogues lacking the adenine moiety as novel potent IP_3 receptor ligands: some structural requirements for the significant activity of adenophostin A. J. Org. Chem. **63**, 8815–8824

7 Hirota, J., Michikawa, T., Miyawaki, A., Takahashi, M., Tanzawa, K., Okura, I., Furuichi, T. and Mikoshiba, K. (1995) Adenophostin-medicated (*sic*) quantal Ca^{2+} release in the purified and reconstituted inositol 1,4,5-trisphosphate receptor type 1. FEBS Lett. **368**, 248–252

8 Missiaen, L., Parys, J. B., Sienaert, I., Maes, K., Kunzelmann, K., Takahashi, M., Tanzawa, K. and De Smet, H. (1998) Functional properties of the type-3 $InsP_3$ receptor in 16HBE14o-bronchial mucosal cells. J. Biol. Chem. **273**, 8983–8986

9 Takahashi, M., Tanzawa, K. and Takahashi, S. (1994) 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

10 Broad, L. M., Armstrong, D. L. and Putney, Jr, J. W. (1999) Role of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} feedback inhibition of calcium release-activated calcium current I_{crac}. J. Biol. Chem. **274**, 32881–32888

11 Mak, D.-O., McBride, S. and Foskett, J. K. (2001) ATP-dependent adenophostin activation of inositol 1,4,5-trisphosphate receptor channel gating: kinetic implications for the duration of calcium puffs in cells. J. Gen. Physiol. **117**, 299–314

12 Parekh, A. B., Riley, A. M. and Potter, B. V. L. (2002) Adenophostin A and ribophostin, but not inositol 1,4,5-trisphosphate or *manno*-adenophostin, activate a Ca^{2+} release-activated Ca^{2+} current, I_{CRAC}, in weak intracellular Ca^{2+} buffer. Biochem. J. **361**, 133–141

13 Hotoda, H., Murayama, K., Miyamoto, S., Iwata, Y., Takahashi, M., Kawase, Y., Tanzawa, K. and Kaneko, M. (1999) Molecular recognition of adenophostin, a very potent Ca^{2+} inducer, at the D-myo-inositol 1,4,5-trisphosphate receptor. Biochemistry **38**, 9234–9241

14 Felemez, M., Marwood, R. D., Potter, B. V. and 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

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

16 Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1999) Cooperative formation of the ligand-binding site of the inositol 1,4,5-trisphosphate receptor by two separable domains. J. Biol. Chem. **274**, 328–334

17 Joseph, S. K., Pierson, S. and Samanta, S. (1995) Trypsin digestion of the inositol trisphosphate receptor: implications for the conformation and domain organization of the protein. Biochem. J. **307**, 859–865

18 Mignery, G. A. and Südhof, T. C. (1990) The ligand binding site and transduction mechanism in the inositol-1,4,5-trisphosphate receptor. EMBO J. **9**, 3893–3898

19 Nakade, S., Maeda, N. and Mikoshiba, K. (1991) Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. Biochem. J. **277**, 125–131

20 Vanlingen, S., Sipma, H., De Smet, P., Callewaert, G., Missiaen, L., De Smedt, H. and Parys, J. B. (2000) Ca^{2+} and calmodulin differentially modulate *myo*-inositol 1,4,5-trisphosphate (IP_3)-binding to the recombinant ligand-binding domains of the various IP_3 receptor isoforms. Biochem. J. **346**, 275–280

21 Marwood, R. D., Correa, V., Taylor, C. W. and Potter, B. V. L. (2000) Synthesis of adenophostin A. Tetrahedron Asymmetry **11**, 397–403

22 Riley, A. M., Potter, B. V. L. (2000) Poly(ethylene glycol)-linked dimers of D-*myo*-inositol 1,4,5-trisphosphate. Chem. Commun. 983–984

23 Richardson, A. and Taylor, C. W. (1993) Effects of Ca^{2+} chelators on purified inositol 1,4,5-trisphosphate ($InsP_3$) receptors and $InsP_3$-stimulated Ca^{2+} mobilization. J. Biol. Chem. **268**, 11528–11533

24 Patel, S., Morris, S. A., Adkins, C. E., O'Beirne, G. and Taylor, C. W. (1997) Ca^{2+}-independent inhibition of inositol trisphosphate receptors by calmodulin: redistribution of calmodulin as a possible means of regulating Ca^{2+} mobilization. Proc. Natl. Acad. Sci. U.S.A. **94**, 11627–11632

25 Mignery, G. A., Südhof, T. C., Takei, K. and De Camilli, P. (1989) Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature (London) **342**, 192–195

26 Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

27 Newton, C. L., Mignery, G. A. and Südhof, T. C. (1994) Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate ($InsP_3$) receptors with distinct affinities for $InsP_3$. J. Biol. Chem. **269**, 28613–28619

28 Wojcikiewicz, R. J. H. and Luo, S. G. (1998) Differences among type I, II, and III inositol-1,4,5-trisphosphate receptors in ligand-binding affinity influence the sensitivity of calcium stores to inositol-1,4,5-trisphosphate. Mol. Pharmacol. **53**, 656–662
29 Bezprozvanny, I. and Ehrlich, B. E. (1993) ATP modulates the function of inositol 1,4,5-trisphosphate-gated channels at two sites. Neuron **10**, 1175–1184
30 Maes, K., Missiaen, L., Parys, J. B., Sienaert, I., Bultynck, G., Zizi, M., De Smet, P., Casteels, R. and De Smedt, H. (1999) Adenine-nucleotide binding sites on the inositol 1,4,5-trisphosphate receptor bind caffeine, but not adenophostin A or cyclic ADP-ribose. Cell Calcium **25**, 143–152
31 Beecroft, M. D., Marchant, J. S., Riley, A. M., Van Straten, N. C. R., Van der Marel, G. A., Van Boom, J. H., Potter, B. V. L. and Taylor, C. W. (1999) Acyclophostin: a ribose-modified analog of adenophostin A with high affinity for inositol 1,4,5-trisphosphate receptors and pH-dependent efficacy. Mol. Pharmacol. **55**, 109–117
32 Marchant, J. S., Beecroft, M. D., Riley, A. M., Jenkins, D. J., Marwood, R. D., Taylor, C. W. and Potter, B. V. L. (1997) Disaccharide polyphosphates based upon adenophostin A activate hepatic D-*myo*-inositol 1,4,5-trisphosphate receptors. Biochemistry **36**, 12780–12790
33 Nunn, D. L. and Taylor, C. W. (1990) Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptors. Biochem. J. **270**, 227–232
34 Yoshikawa, F., Uchiyama, T., Iwasaki, H., Tomomori-Satoh, C., Tanaka, T., Furuichi, T. and Mikoshiba, K. (1999) High efficient expression of the functional ligand binding site of the inositol 1,4,5-trisphosphate receptor in *Escherichia coli*. Biochem. Biophys. Res. Commun. **257**, 792–797
35 Glouchankova, L., Krishna, U. M., Potter, B. V. L., Falck, J. R. and Bezprozvanny, I. (2000) Association of the inositol-(1,4,5) trisphosphate receptor ligand binding site with phosphatidylinositol (4,5)-bisphosphate and adenophostin A. Mol. Cell Biol. Res. Commun. **3**, 153–158
36 Ramos-Franco, J., Caenepeel, S., Fill, M. and Mignery, G. (1998) Single channel function of recombinant type-1 inositol 1,4,5-trisphosphate receptor ligand binding domain splice variants. Biophys. J. **75**, 2783–2793
37 Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1999) Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. Structural and functional coupling of cleaved ligand binding and channel domains. J. Biol. Chem. **274**, 316–327
38 Adkins, C. E., Wissing, F., Potter, B. V. L. and Taylor, C. W. (2000) Rapid activation and partial inactivation of inositol trisphosphate receptors by adenophostin A. Biochem. J. **352**, 929–933

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