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Structural Determinants of Adenophostin A Activity at Inositol Trisphosphate Receptors

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ABSTRACT

Adenophostin A is the most potent known agonist of inositol 1,4,5-trisphosphate ($InsP_3$) receptors. Ca^{2+} release from permeabilized hepatocytes was 9.9 ± 1.6-fold more sensitive to adenophostin A (EC_{50}, 14.7 ± 2.4 nM) than to $InsP_3$ (145 ± 10 nM), consistent with the greater affinity of adenophostin A for hepatic $InsP_3$ receptors (K_d = 0.48 ± 0.06 and 3.09 ± 0.33 nM, respectively). Here, we systematically modify the structures of the glucose, ribose, and adenine moieties of adenophostin A and use Ca^{2+} release and binding assays to define their contributions to high-affinity binding. Progressive trimming of the adenine of adenophostin A reduced potency, but it fell below that of $InsP_3$ only after complete removal of the adenine. Even after substantial modifications of the adenine (to uracil or even unrelated aromatic rings, retaining the β-orientation), the analogs were more potent than $InsP_3$. The only analog with an α-ribosyl linkage had massively decreased potency. The 2′-phosphate on the ribose ring of adenophostin A was essential and optimally active when present on a five-membered ring in a position stereochemically equivalent to its location in adenophostin A. *Xylo*-adenophostin, where xylose replaces the glucose ring of adenophostin A, was only slightly less potent than adenophostin A, whereas *manno*-adenophostin (mannose replacing glucose) had similar potency to $InsP_3$. These results are consistent with the relatively minor role of the 3-hydroxyl of $InsP_3$ (the equivalent is absent from *xylo*-adenophostin) and greater role of the equatorial 6-hydroxyl (the equivalent is axial in *manno*-adenophostin). This is the first comprehensive analysis of all the key structural elements of adenophostin A, and it provides a working model for the design of related high-affinity ligands of $InsP_3$ receptors.

Inositol 1,4,5-trisphosphate ($InsP_3$) receptors are intracellular Ca^{2+} channels that are expressed in most mammalian cells, where they are largely responsible for mediating the release of Ca^{2+} from intracellular stores evoked by many cell-surface receptors. Functional $InsP_3$ receptors are homo- or hetero-tetrameric assemblies of subunits encoded by three related mammalian genes and their splice variants (Taylor et al., 1999). Each of these assemblies is capable of forming a Ca^{2+} channel that is regulated by $InsP_3$ and cytosolic Ca^{2+} (Miyakawa et al., 1999), but the subtypes differ in their distributions and in some aspects of their regulation (for review, see Taylor et al., 1999).

Exhaustive analyses of both naturally occurring and synthetic inositol phosphates and their analogs have so far identified only agonists of $InsP_3$ receptors and none has substantially exceeded the affinity of the naturally occurring ligand $InsP_3$. A few, generally low-affinity, partial agonists have been identified (Marchant et al., 1997b; Wilcox et al., 1998; Murphy et al., 2000), but no antagonists. Indeed, the only effective antagonists of $InsP_3$ binding to its receptor are heparin and decavanadate, neither of which has either high affinity or adequate selectivity for $InsP_3$ receptors (Taylor and Richardson, 1991). Caffeine, the xestospongins (Gafni et al., 1997), and 2-aminoethoxydiphenyl borate (Maruyama et al., 1997) also block $InsP_3$ receptors, but at sites distinct from the $InsP_3$-binding site and they too lack either specificity or high affinity (Wilcox et al., 1998; Short and Taylor, 2000).

All known high-affinity agonists of $InsP_3$ receptors include structures analogous to the equatorial 4,5-bisphosphate groups and equatorial 6-hydroxyl of $InsP_3$ (Potter and Lampe, 1995; Wilcox et al., 1998) (Fig. 1). The binding site itself lies within the N-terminal portion of each $InsP_3$ receptor subunit and includes several basic residues, which are conserved between all three receptor subtypes and are likely to interact with the negatively charged phosphate groups of $InsP_3$ (Yoshikawa et al., 1996). More recent studies have shown that the $InsP_3$-binding site of the type 1 $InsP_3$ receptor is formed by two distinct domains of about 120 and 250 residues linked by a loop that includes the SI splice site (Yoshikawa et al., 1999).

The demonstration that adenophostins, products of the fungus *Penicillium brevicompactum*, are the most potent

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ABBREVIATIONS: $InsP_3$, D-*myo*-inositol 1,4,5-trisphosphate; BM, binding medium; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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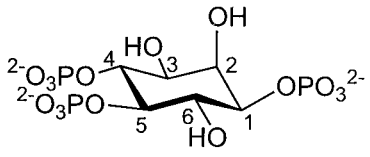
known agonists of $InsP_3$ receptors (Takahashi et al., 1994a) introduced fresh opportunities to develop high-affinity ligands of $InsP_3$ receptors. Adenophostins A and B are not metabolized by the enzymes that degrade $InsP_3$, they do not bind to $InsP_4$ receptors (Takahashi et al., 1994a), and in both functional and radioligand binding assays of all three $InsP_3$ receptor subtypes they bind with about 10-fold greater affinity than $InsP_3$ (Takahashi et al., 1994b; Hirota et al., 1995; Marchant et al., 1997a; Murphy et al., 1997; Missiaen et al., 1998; Shuto et al., 1998; Bird et al., 1999; Marwood et al., 1999).

The structure of adenophostin A suggests that its glucose 3″,4″-bisphosphate structure and adjacent 2″-hydroxyl may mimic the critical 4,5-bisphosphate and 6-hydroxyl of $InsP_3$ (Fig. 1). The adenine group may increase the strength of the binding either by improving the positioning of the 2′-phosphate of adenophostin A (analogous to the 1-phosphate of $InsP_3$) or through a more direct interaction with a residue (possibly aromatic) close to the $InsP_3$-binding site of the receptor (Takahashi et al., 1994a; Marchant et al., 1997a; Hotoda et al., 1999). The charge distribution on the phosphate groups of adenophostin A at physiological pH is virtually identical with that in the equivalent phosphate groups of $InsP_3$ (Felemez et al., 1999).

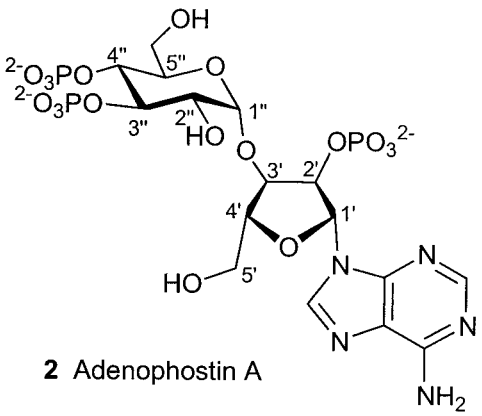
In the present study, we examine the effects of a range of synthetic adenophostin A analogs on $^{45}Ca^{2+}$ release from the intracellular stores of permeabilized hepatocytes. We provide a comprehensive and systematic analysis of the roles of the ribose and purine rings, the glucose ring, and the stereochemistry of the links between these structures in mediating the high-affinity interaction between adenophostin A and $InsP_3$ receptors.

Experimental Procedures

Materials. $InsP_3$ (**1**) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Thapsigargin was from Alamone Laboratories (Jerusalem, Israel), and ionomycin was from Calbiochem (Nottingham, UK). The analogs of adenophostin A were synthesized as follows: adenophostin A (**2**) (Marwood et al., 2000a,c); purinophostin (**3**) and imidophostin (**5**) (Marwood et al., 2000b); ribophostin (**6**) (Jenkins et al., 1997); Glc(2′,3,4)P_3 (8) (Jenkins and Potter, 1996); **27-29** (Marchant et al., 1997a); furanophostin (**7**) (Marwood et al., 1999); **17**, **18**, and **26** (Shuto et al., 1998); **9** and **10** (Rosenberg et al., 2000); acyclophostin (**19**) and **25** (Van Straten et al., 1997); **20-23** (Rosenberg et al., 2001); *xylo*-adenophostin (**11**) and *manno*-adenophostin (**12**) (Marwood et al., 2000c); uridophostin (**13**); and **4**, **14**, and **30** (Marwood et al., 2000d). The syntheses of **15**, **16**, and furanophostin 3,4-bisphosphorothioate (**24**, furanophostin-PS_2) will be reported elsewhere. The structures of the analogs used, together with their abbreviations, are shown in Fig. 2.



1 Ins(1,4,5)P_3



2 Adenophostin A

Fig. 1. Structures of $InsP_3$ and adenophostin A. The structures are drawn to highlight the means whereby the 3″- and 4″-phosphates, 2″-hydroxyl, and 2′-phosphate of adenophostin A may mimic the crucial 4- and 5-phosphates, 6-hydroxyl, and 1-phosphate of $InsP_3$.

$^{45}Ca^{2+}$ Release from the Intracellular Stores of Permeabilized Cells. Hepatocytes were isolated from the livers of male Wistar rats (200–300 g) as described previously (Marchant et al., 1997a) and permeabilized in Ca^{2+}-free cytosol-like medium (140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM PIPES, pH 7.0) by incubation with saponin (10 μg/ml) at 37°C for 8 min. The cells were resuspended (2.2×10^6 cells/ml) in cytosol-like medium supplemented with 300 μM $CaCl_2$ (free $[Ca^{2+}]$ = 200 nM), ATP (1.5 mM), creatine phosphate (5 mM), creatine phosphokinase (1 unit/ml), the mitochondrial inhibitor *p*-trifluoromethoxyphenylhydrazone (10 μM), and $^{45}CaCl_2$ (5 μCi/ml). After 5 min at 37°C, during which the intracellular stores loaded to steady state with $^{45}Ca^{2+}$, $InsP_3$, adenophostin, A or an analog were added, together with thapisgargin (1 μM) to inhibit further Ca^{2+} uptake. After a further 1 min, the $^{45}Ca^{2+}$ contents of the stores were determined by filtration through Whatman GF/C filters followed by washing with ice-cold sucrose (310 mM) and sodium citrate (1 mM). The actively accumulated $^{45}Ca^{2+}$ content of the stores was defined as that which could be released by ionomycin (10 μM).

[^{3}H]$InsP_3$ Binding to Hepatic Membranes. Membranes were prepared from perfused rat livers using Percoll-gradient centrifugation as described previously (Beecroft et al., 1999). Briefly, after perfusion in situ with cold buffered saline, the liver was removed, homogenized with a Dounce homogenizer in cold buffered sucrose (25 ml; 250 mM sucrose, 5 mM Hepes, 1 mM EGTA, pH 7.4), filtered through gauze, and then centrifuged (25,000*g*, 10 min). The pellet was resuspended in buffered sucrose (48 ml) containing Percoll (11.8% final v/v; Amersham Pharmacia Biotech, Uppsala, Sweden), recentrifuged (35,000*g*, 30 min), and the membranes harvested as a fluffy band just beneath the fatty layer at the top of each tube. The membranes in hypo-osmotic medium (1 mM EGTA, 5 mM HEPES, pH 7.4, 2°C) were centrifuged (48,000*g*, 10 min) and the pellet resuspended in binding medium (BM: 50 mM Tris, 1 mM EDTA, pH 8.3, 2°C) at ~20 mg of protein per millilter, before freezing in liquid nitrogen and storage at −80°C.

For measurements of [^{3}H]$InsP_3$ binding, liver membranes (0.1 mg of protein in 500 μl of BM) were added to [^{3}H]$InsP_3$ (final concentration 1.3–1.8 nM, specific activity 35–60 Ci/mmol) and the appropriate concentration of competing ligand. After 5 min at 2°C, during which equilibrium was attained (Beecroft et al., 1999), bound and free ligand were separated by centrifugation (20,000*g*, 5 min, 4°C). Total binding was typically 3000 dpm/tube, of which ~30% was specific.

Analysis. Equilibrium-competition binding curves were fitted to logistic equations using nonlinear curve-fitting routines (Kaleidagraph; Synergy Software, Reading, PA):

$$B = \frac{(T - N)}{1 + \left(\frac{[L]}{IC_{50}}\right)^{n_H}} + N$$

where B is the amount of [^{3}H]$InsP_3$ bound in the presence of a concentration of the competing ligand, [L]; T and N are the total and nonspecific [^{3}H]$InsP_3$ binding, respectively; n_H is the Hill coefficient; and IC_{50}

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is the concentration of competing ligand causing 50% displacement of specific [^{3}H]InsP$_3$ binding. The K_d value for each ligand was then calculated from the IC_{50} value. A similar equation was used to analyze concentration-effect relationships for agonist-evoked Ca^{2+} mobilization from which the maximal effect, EC_{50}, and n_H of each ligand were determined.

The potencies of analogs are expressed relative to the potency of InsP$_3$ determined in exactly parallel experiments (see below). The variance of the potency ratio (with mean K_d values of a and b for the two agonists) was calculated from the variances (var) of each using the following equation (Colquhoun, 1971):

$$var\left(\frac{a}{b}\right) = \left(\frac{a}{b}\right)^2\left(\frac{var(a)}{a^2} + \frac{var(b)}{b^2}\right)$$

All results are shown as means ± S.E.M.

Results and Discussion

Adenophostin A Is a Potent Agonist of Hepatic InsP$_3$ Receptors. The results shown in Fig. 3 confirm previous work (Marchant et al., 1997a) by demonstrating that adenophostin A is a full agonist of hepatic InsP$_3$ receptors with about 10-fold greater affinity than InsP$_3$. In assays of Ca^{2+} mobilization, adenophostin A was 9.9 ± 1.6-fold more potent than InsP$_3$, and in radioligand binding assays it had 6.4 ± 1.1-fold greater affinity (Table 1). Similar results have been obtained with other tissues (see above). Whereas our previous work (Marchant et al., 1997a) used adenophostin A purified from *P. brevicompactum* (Takahashi et al., 1994a), the

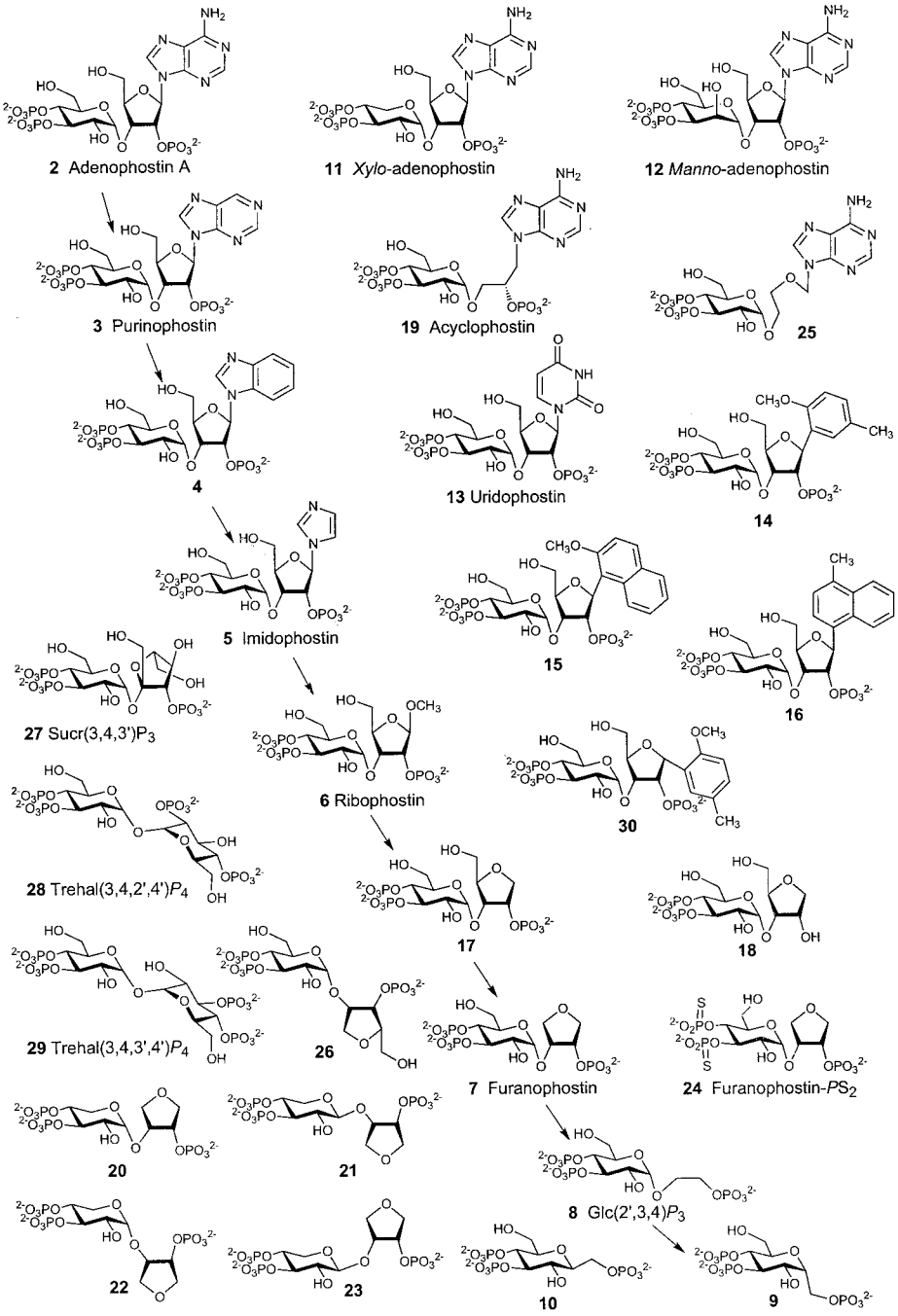

Fig. 2. Structures of adenophostin A and its analogs.

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present results were obtained with synthetic adenophostin A (Marwood et al., 2000a). However, in parallel comparisons synthetic (EC_{50} = 9.6 ± 1.0 nM, n_H = 2.46 ± 0.19, n = 3) and natural (EC_{50} = 9.2 ± 1.7 nM, n_H = 2.99 ± 0.32, n = 3) adenophostin A had indistinguishable effects on Ca^{2+} release from permeabilized hepatocytes (Marwood et al., 2000a). The similarity is important in providing the justification for subsequent analyses of the effects of synthetic analogs of adenophostin A.

All the adenophostin A analogs used in the present study (Fig. 2) that were sufficiently potent to evoke a maximal response at concentrations ≤10 μM released the same fraction of the intracellular stores as a maximal concentration of $InsP_3$ (~50%). Furthermore, when a maximally effective concentration of any one of the analogs was combined with 10 μM $InsP_3$, the response was no greater than that evoked by the $InsP_3$ alone (data not shown). Although the fraction of the stores released by a maximally effective concentration of $InsP_3$ varied somewhat between experiments (29–55%), when averaged across all the experiments reported here, 50 ± 4% of the actively accumulated $^{45}Ca^{2+}$ was released by 10 μM $InsP_3$ (Table 1). An indistinguishable response was evoked by a maximal concentration (1 μM) of adenophostin A (Table 1). The responses to $InsP_3$, adenophostin A, and each of the active analogs were positively cooperative (Hill coefficients, $n_H > 1$) (Table 2). None of the inactive (**18**, **23**) or very weakly active (**10**, **22**, **25**, **26**) analogs behaved as competitive antagonists: the response to a submaximal concentration of $InsP_3$ (200 nM) was undiminished in the presence of 10 μM of any of these analogs (data not shown).

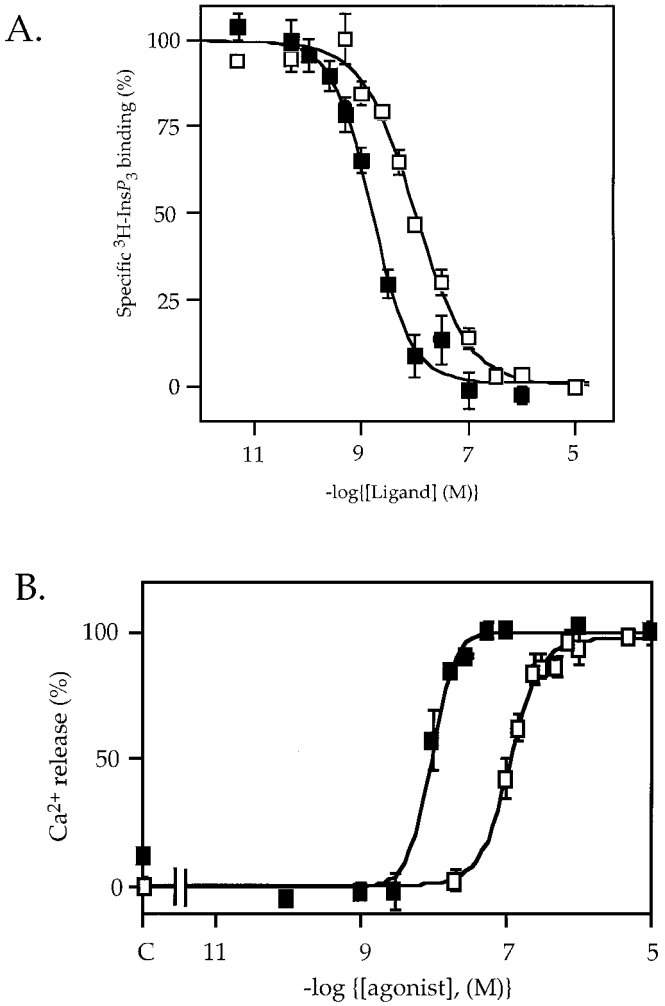

Fig. 3. Adenophostin A is a high-affinity full agonist of hepatic $InsP_3$ receptors. A, specific binding of [^{3}H]$InsP_3$ to hepatic membranes is shown in the presence of the indicated concentrations of $InsP_3$ (□) and adenophostin A (■). B, effects of the indicated concentrations of $InsP_3$ (□) and adenophostin A (■) on Ca^{2+} release from the intracellular stores of permeabilized hepatocytes are shown as percentages of the $InsP_3$-sensitive Ca^{2+} stores. Results are means ± S.E.M. of four independent experiments.

The results shown in Table 2 summarize experiments performed over a lengthy period during which there were modest variations in the sensitivity of the permeabilized hepatocytes to $InsP_3$ (the EC_{50} value varied between 90 ± 7 and 201 ± 20 nM). Similar variations were observed in the binding experiments (the K_d value for $InsP_3$ varied between 2.72 ± 0.88 and 9.65 ± 0.89 nM). To be confident that even the small (although statistically significant) differences in potency (or affinity) observed between some of the analogs were meaningful, we have compared the potency of each analog with the potency of $InsP_3$ measured in exactly parallel experiments. All figures (Figs. 4–8) show results analyzed in this way (i.e., with potencies expressed relative to $InsP_3$), whereas Table 2 shows the actual EC_{50} values determined for each analog.

Role of the Adenosine Moiety in High-Affinity Binding of Adenophostin A. Adenophostin A (**2**, Fig. 1) can be viewed as a phosphorylated glucose glycosidically linked at its 1″-position to an adenosine phosphorylated at the 2′-position. In the first series of experiments, we examined the functional consequences of systematically trimming the adenosine (Fig. 4). The results show that as elements of the adenine (**2**-**6**, **17**) and then of the ribose (**7**-**10**) ring were successively removed, there was a progressive decrease in the potency of the analog to evoke Ca^{2+} mobilization. Analogs (ribophostin, **6**; furanophostin, **7**; and **17**) without an adenine moiety were only slightly less potent than $InsP_3$, but after complete removal of the adenine and opening of the five-membered ring [Glc(2′,3,4)P_3, **8**] the potency fell to 12 ± 1-fold less than that of $InsP_3$. Shortening of the flexible O-C-C chain in **8** by two atoms to give the α-*C*-glycoside **9** caused a further slight reduction in potency. Interestingly, the isomeric β-*C*-glycoside **10** was much weaker, despite its apparent resemblance to $InsP_3$. Also, in relation to **6** (ribophostin), some of us have recently reported that elaboration of the methyl group in **6** to propyl or phenylpropyl groups did not appreciably affect potency (De Kort et al., 2000).

The importance of the 2′-phosphate of adenophostin A is apparent from the massive difference in potency between compounds **17** and **18**. Both ligands lack the adenine of adenophostin A, but **17** had only 5.46 ± 0.88-fold lesser potency than $InsP_3$ (Fig. 4), whereas **18**, which lacks the 2′-phosphate, was inactive (Fig. 5C). These results are consistent with those from radioligand binding analyses of cerebellar membranes where **18** bound with ~300-fold lesser affinity than **17** (Shuto et al., 1998), and removal of the 2′-phosphate from adenophostin A reduced affinity by about 100-fold (Takahashi et al., 1994b). There is a similar 100- to 300-fold difference in the affinities of Ins(4,5)P_2 and Ins(1,4,5)P_3 for $InsP_3$ receptors (Nerou et al., 2001), in keeping with the idea that the 2′-phosphate of adenophostin A mimics the 1-phosphate of Ins(1,4,5)P_3 (Fig. 1). The conclusion gains further support from the 159 ± 19-fold lesser

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potency of **25** relative to acyclophostin (**19**) (Fig. 5B); the major difference between them being the loss of the 2′-phosphate from **25** (Table 2).

Effects of Modifying the Nucleoside and Its Links with the Glucose Ring. The effects of modifying the base (adenine) of adenophostin A on biological activity are shown in Fig. 5A. Removal of the amino group from the adenine (to give purinophostin, **3**) had a minimal effect. Replacement of the pyrimidine ring of the adenine by a benzene ring (**4**) reduced potency by 4.0 ± 1.0-fold, and its complete removal reduced the potency of the analog (imidophostin, **5**) to almost that of $InsP_3$ (Fig. 5A). Even replacement of the purine moiety (adenine) of adenophostin A with a much smaller pyrimidine (uracil, to give uridophostin, **13**) caused the potency to decrease by only 2.31 ± 0.40-fold. Finally, replacement of the entire adenine moiety of adenophostin A with unrelated single (**14**) or double (**15**, **16**) aromatic rings with a β-*C*-ribosyl glycosidic linkage produced ligands with activities comparable with that of **4** and significantly greater than that of $InsP_3$ (Fig. 5A). As expected, changing the stereochemistry of the *C*-glycosidic linkage of **14** from β to α (**30**) massively decreased potency (by 29 ± 11-fold relative to **14**, and to 11.8 ± 2.8-fold less than that of $InsP_3$). Hence, although large aromatic groups attached to the 1′-position in a β-orientation improve potency (**14** is 10.2 ± 1.9-fold *more* potent than **17**), they have the opposite effect when attached in the α-orientation (**30** is 2.8 ± 0.7-fold *less* potent than **17**). The effect may be caused by steric hindrance around the important 2″-OH and/or the 2′-phosphate group, which may occur with the α- but not the β-oriented rings.

These results indicate that although an adenine attached to the 1′-position of the ribose ring through a β-glycosidic linkage gives the most potent agonist (adenophostin A), even radical changes to the structure of the attached group are tolerated. Each of the analogs with a β-glycosidically linked aromatic system (**3**-**5** and **13**-**16**) is significantly more potent than **17** in which there is no substitution at the 1′-position.

In the adenophostins, the 2′-phosphate group is separated

TABLE 1

Effects of adenophostin A and $InsP_3$ on [^{3}H]$InsP_3$ binding and on Ca^{2+} release from the intracellular stores of permeabilized hepatocytes

Results from the many analyses of $InsP_3$- and adenophostin A-evoked Ca^{2+} mobilization used in this study have been combined (where n = number of independent sets of experiments, and (n) the total number of concentration-effect curves analyzed). The concentration causing half the maximal effect (EC_{50}), the Hill coefficient (n_H), and the fraction of the total Ca^{2+} stores released by a maximal concentration of each agonist are shown (means ± S.E.M.). Results from equilibrium competition binding experiments show the K_d and n_H determined in n independent experiments.

	Ca^{2+} Release EC_{50}	n_H	Maximal Response	n	Binding	
					K_d	n_H
	nM		%		*nM*	
Ins*P*$_3$, **1**	145 ± 10	2.20 ± 0.16	50 ± 4	10 (57)	3.09 ± 0.33	1.05 ± 0.08
Adenophostin A, **2**	14.7 ± 2.4	2.26 ± 0.15	51 ± 1	6 (26)	0.48 ± 0.06	1.2 ± 0.10

TABLE 2

Effects of analogs of adenophostin A on Ca^{2+} release from the intracellular stores of permeabilized hepatocytes

The EC_{50} and Hill coefficient (n_H) for Ca^{2+} mobilization evoked by each of the ligands is shown for n independent experiments. Because the experiments were performed over a substantial period, each assay was accompanied by a parallel measurement of $InsP_3$-evoked Ca^{2+} release to allow the activity of each analog to be calibrated relative to the response to $InsP_3$ (see under *Experimental Procedures*). The data shown in this table show the results before calibration to the relevant $InsP_3$ response.

	EC_{50}	n_H	n	Reference
	nM			
1, $InsP_3$	145 ± 10	2.20 ± 0.16	10	
2, Adenophostin A	14.7 ± 2.4	2.26 ± 0.15	6	
3, Purinophostin	18 ± 1	2.04 ± 0.24	10	Marwood et al., 2000b
4	47 ± 7	1.82 ± 0.21	4	
5, Imidophostin	108 ± 20	2.51 ± 0.64	7	Marwood et al., 2000b
6, Ribophostin	329 ± 20	2.88 ± 1.40	7	
7, Furanophostin	329 ± 48	2.18 ± 0.10	5	Marwood et al., 1999
8, Glc(2′,3,4)P_3	1867 ± 64	2.76 ± 0.10	4	Marchant et al., 1997a
9	2414 ± 173	4.99 ± 1.84	3	Rosenberg et al., 2000
10	~10 μM	nd	5	Rosenberg et al., 2000
11, *xylo*-Adenophostin	28 ± 8	2.50 ± 0.77	8	
12, *manno*-Adenophostin	180 ± 16	2.85 ± 0.55	8	
13, Uridophostin	34 ± 2	2.45 ± 0.29	10	
14	48 ± 6	1.55 ± 0.14	4	
15	99 ± 3	2.47 ± 0.07	4	
16	62 ± 3	2.03 ± 0.09	4	
17	491 ± 69	2.32 ± 0.35	4	
18	Inactive[a]	nd	3	
19, Acyclophostin	209 ± 12	2.48 ± 0.18	6	Beecroft et al., 1999
20	487 ± 58	2.51 ± 1.00	3	Rosenberg et al., 2001
21	2694 ± 276	1.78 ± 0.43	3	Rosenberg et al., 2001
22	>10 μM[b]	nd	5	Rosenberg et al., 2001
23	Inactive[a]	nd	5	Rosenberg et al., 2001
24, FuranophostinPS_2	1682 ± 346	2.30 ± 0.27	3	
25	33 ± 3 μM	3.05 ± 0.39	3	Beecroft et al., 1999
26	~20 μM	nd	6	
27, Sucr(3,4,3′)P_3	5409 ± 110	2.60 ± 0.08	3	Marchant et al., 1997a
28, Trehal(3,4,2′,4′)P_4	1271 ± 46	2.53 ± 0.43	3	Marchant et al., 1997a
29, Trehal(3,4,3′,4′)P_4	2466 ± 29	2.37 ± 0.27	3	Marchant et al., 1997a
30	1372 ± 291	2.29 ± 0.36	4	

[a] No significant release of Ca^{2+} with 10 μM of the analog.
[b] 27 ± 5% of the Ins*P*$_3$-sensitive Ca^{2+} stores released by 10 μM of **22**.
nd, not determined.

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from the α-glucopyranosyl ring by a three-atom -O-C-C- chain. It seems that some conformational restraint of this chain by incorporation of its two C atoms into a five-membered ring of appropriate stereochemistry (e.g., ribose) is necessary for optimal activity. However, removal of part of the ribose ring to give an apparently flexible structure (acyclophostin, **19**) produces an analog with significant activity, providing the adenine is retained. Acyclophostin is unusual in that its efficacy is affected by pH (Beecroft et al., 1999), but under the conditions used to compare analogs in this study, it was only 1.37 ± 0.13-fold less potent than $InsP_3$. Acyclophostin (**19**) certainly has much greater potency than **8** (which lacks the adenine moiety of **19**) or **25**, (which lacks the 2′-phosphate) (Fig. 5B). These results suggest that even when the critical 2′-phosphate is positioned on a flexible link between the adenine and glucose ring, it can still contribute significantly to binding affinity.

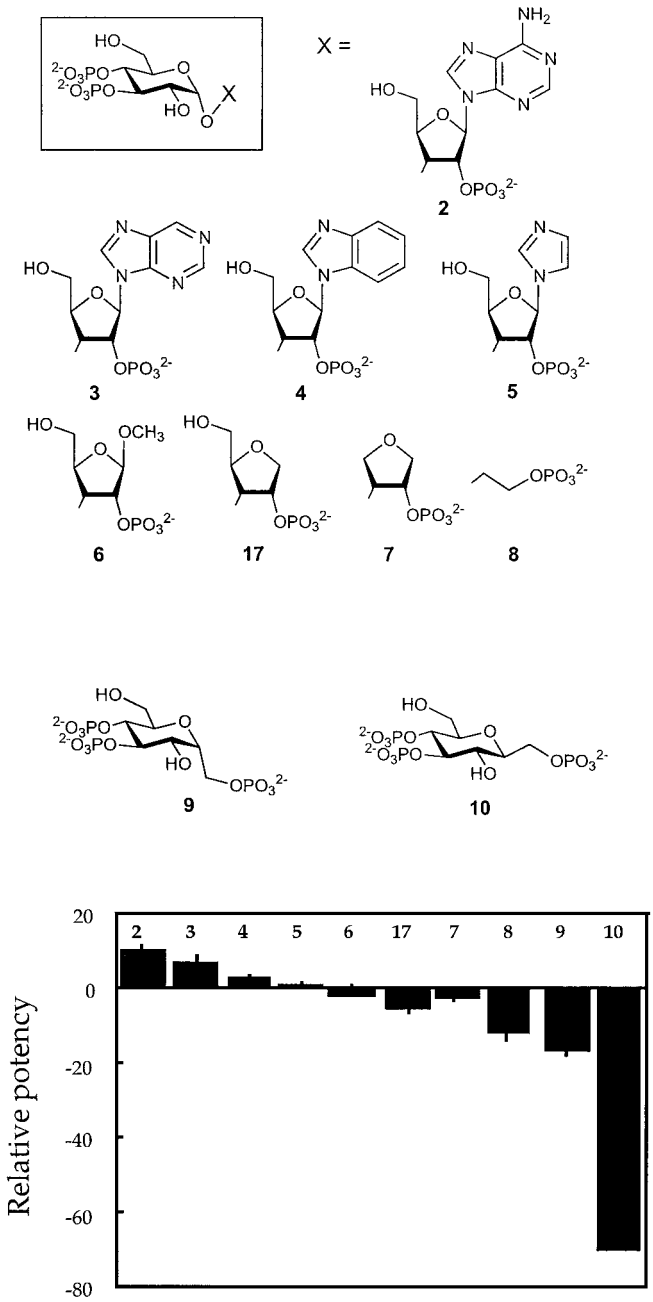

Fig. 4. Effects of modifying the adenosine moiety of adenophostin A. In this and subsequent figures, the EC_{50} value of each of the analogs is compared with that determined for $InsP_3$ in parallel experiments to give a potency ratio. Ratios >1 denote agonists more potent than $InsP_3$, and ratios <−1 denote agonists less potent than $InsP_3$. The relative potencies, calculated as described under *Experimental Procedures*, are shown as means ± S.E.M.; the sample sizes are the same as those in Table 2.

Other ring structures can effectively replace the ribose of adenophostin A to give analogs (**27**-**29**) (Fig. 2) more potent than **18**. However, even the most active of these analogs (**28**), which is more potent than **8**, is still 6.0 ± 0.3-fold less potent than ribophostin (**6**), where the 2′-phosphate is attached to a ribose ring (Fig. 5C).

It is interesting that analog **26**, which is a regioisomer of the relatively potent **17**, shows very low activity. The likely reason is that the stereochemical differences between **26** and **17** at the positions corresponding to C-2′ and -3′ of adenophostin A result in a different three-dimensional positioning of the 2′-phosphate group at the receptor binding site. Steric hindrance from the differently orientated hydroxymethyl group and five-membered ring may also be involved. Similar factors may account for the low activity of **27**, the fructose component of which retains the required stereochemistry, but has a different type of glycosidic linkage to glucose and an extra hydroxymethyl group.

We conclude that of the analogs examined so far, a ribose ring (or close relative) between the pyranosyl and adenine moieties positions the 2′-phosphate most effectively for optimal binding.

Modifications to the Glucose Ring of Adenophostin A. Whereas $InsP_3$ is based on a *myo*-inositol ring structure, the analogous 4,5-bisphosphate and 6-hydroxyl groups in adenophostin A are attached to a glucose ring. The functional consequences of modifying this sugar are shown in Fig. 6A.

Xylo-adenophostin (**11**), in which the glucose of adenophostin A has been replaced by xylose, was only 1.9 ± 0.6-fold less potent than adenophostin A. The structures of the two analogs differ only in that adenophostin (**2**) has a CH_2OH attached to the 5″-position, which presumably mimics the 3-hydroxyl of $InsP_3$ (Fig. 1). The small difference in potency between **2** and **11** is therefore consistent with the relatively minor contribution of the 3-hydroxyl of $InsP_3$ to binding (Kozikowski et al., 1993; Nerou et al., 2001).

Manno-adenophostin (**12**) in which mannose replaces the glucose of adenophostin A, is substantially less potent (12.2 ± 2.3-fold) than the parent compound, although still only 1.51 ± 0.15-fold less potent than $InsP_3$ (Fig. 6A). The potency of **12** is surprising because although it differs from adenophostin only in the orientation of the hydroxyl equivalent to the 6-hydroxyl of $InsP_3$ (Fig. 1), inversion or removal of the 6-hydroxyl in inositol phosphates invariably reduces potency by at least 100-fold (Kozikowski et al., 1993; Wilcox et al., 1994; Nerou et al., 2001). There are no other known high-affinity agonists of $InsP_3$ receptors lacking a hydroxyl equivalent to the equatorial 6-hydroxyl of $InsP_3$. It has been suggested that the 6-OH group of $InsP_3$ may donate an H-bond to the receptor (Kozikowski et al., 1993), an interaction that is presumably disrupted when this group is axial. However, an additional role for the equatorial 6-OH has been proposed, in which it may influence the behavior of the crucial bisphosphate by mediating its interaction with the 1-phosphate (Felemez et al., 2000). Changing the orientation of the 6-OH to axial ($InsP_3$ to *epi*-$InsP_3$) alters the commu-

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nication between the 4,5-bisphosphate and 1-phosphate so that they behave effectively as if isolated from one another. This "inframolecular" effect may partly underlie the greatly decreased activity of *epi*-$InsP_3$ (Felemez et al., 2000). In adenophostin A, the analogous 3″,4″-bisphosphate and 2′-phosphate group are located on separate rings. The effect of modifying the mediating hydroxyl group (2″-OH) may therefore be different from that in $InsP_3$.

Stereochemistry of the *O*-Glycosidic Linkage. Because *xylo*-adenophostin (**11**) is almost as potent as adenophostin A (**2**), and xylose-based analogs are both simpler and require fewer chemical steps for their synthesis than glucose-based analogs, we chose to explore the functional consequences of modifying the stereochemistry of the *O*-glycosidic linkage between the pyranosyl and furanosyl moieties using xylose-based analogs (Fig. 6B).

As shown above, **11** is only 1.9 ± 0.6-fold less potent than **2**. Just as furanophostin (**7**), which lacks the adenine and 4′-CH_2OH of adenophostin A, is 22 ± 5-fold less potent than its parent (**2**), so **20**, the xylose-based analog of furanophostin, is 17 ± 3-fold less potent than its parent (**11**). The similarities establish that modifications to the adenosine moiety have similar consequences for glucose- and xylose-based analogs. These results provide the justification for using **20** to explore the functional consequences of changing the stereochemistry of the *O*-glycosidic linkage between the furanosyl and pyranosyl rings (Fig. 6B).

Changing the stereochemistry of the *O*-glycosidic linkage to xylose from α (**20**) to β (**23**) massively decreased potency, so that **23** was effectively inactive. It is likely that in low-energy conformations of **23**, the three-dimensional location of the third phosphate group is very different from that in **20**, and that it is therefore unable to mimic the 1-phosphate of $InsP_3$ or the 2′-phosphate of adenophostin A. Analog **22** is stereochemically similar to the glucose-based **26**, and they show similar low potency. Unlike **26**, however, **22** has no hydroxymethyl groups and so it is likely that its weak activity (and by implication that of **26**) is again simply attributable to incorrect spatial positioning of its nonvicinal phosphate group. It is interesting that analog **21**, which combines the stereochemical changes to the tetrahydrofuranyl ring of **22** with a change from an α- to a β-*O*-glycosidic linkage shows significant activity (19 ± 2-fold weaker than $InsP_3$). Molecular models suggest that the combined effect of the stereochemical changes in **21** is to return the nonvicinal phosphate group to a spatial orientation more closely approaching that in **20** than in **22**, **23**, or **26** (Rosenberg et al., 2001).

Phosphorothioate Modifications to the Phosphate Groups. Substitution of phosphates by phosphorothioate groups in certain inositol phosphates can give partial ago-

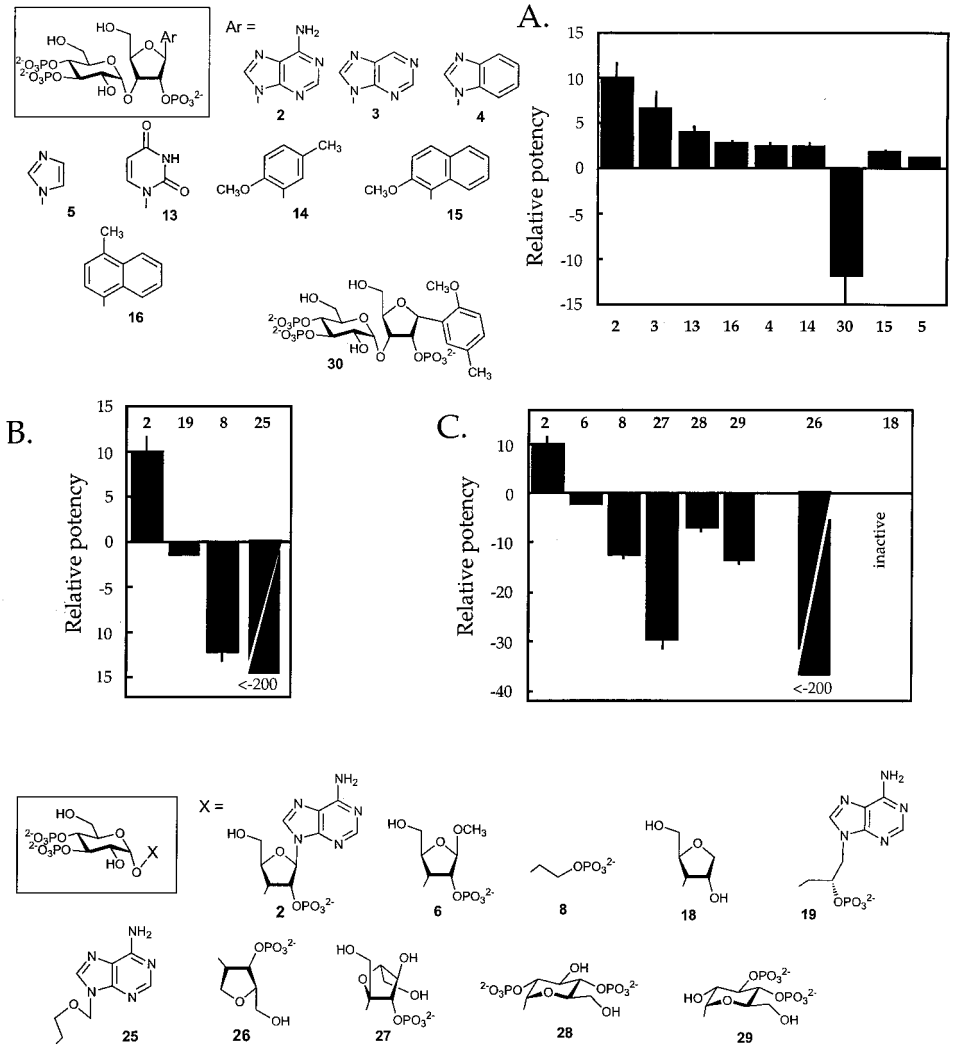

Fig. 5. Effects of modifying the nucleotide motif of adenophostin A and its linkage with the glucose ring. A, modifications to the base. B, flexible analogs. C, effects of replacing the ribose ring of ribophostin (**6**) with other ring structures. The results are presented in the same format as Fig. 4.

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nists (Potter and Lampe, 1995; Murphy et al., 2000), and might thereby provide a step toward the development of antagonists. However, the strategy has so far been limited by the relatively low affinity of these phosphorothioate analogs. It seems that partial agonists of this type can be generated by combining a slight structural perturbation of the $InsP_3$ molecule (particularly at position 3) with phosphorothioate substitution (Potter and Lampe, 1995). Replacement of the 4- and 5-phosphate groups of 3-deoxy-3-fluoro-Ins(1,4,5)P_3 with phosphorothioates, for example, produced a partial agonist with an affinity only 10-fold less than $InsP_3$ for type 1 $InsP_3$ receptors (Wilcox et al., 1997). Thus, it seems that higher affinity partial agonists may be created by limiting phosphorothioate substitution to the bisphosphate, leaving the 1-phosphate group, with its ability to enhance affinity, unchanged.

We reasoned that adenophostin analogs such as **7** (furanophostin) might be promising candidates for this approach in that they are related to position 3-modified $InsP_3$ analogs (hydroxymethyl of glucose replacing 3-hydroxyl of $InsP_3$), and retain $InsP_3$-like affinity for the receptor. Furthermore, their structure (nonvicinal phosphate located on a separate ring) has advantages in terms of synthetic strategy for creating novel ligands with the 2′-phosphate and 3″,4″-bisphosphorothioate pattern.

In **24**, the two phosphate groups on the glucose ring of furanophostin (**7**) have been replaced by phosphorothioates to give the first phosphorothioate-containing adenophostin analog, furanophostin-PS_2 (Fig. 2). The result is a substantial decrease in potency (5.1 ± 1.3-fold relative to **7**) (Table 2). The decrease is consistent with similar decreases in potency after phosphorothioate substitution of $InsP_3$: inositol 1,4,5-trisphosphorothioate was 2.8-fold less potent than $InsP_3$ (Taylor et al., 1989). However, maximal concentrations of $InsP_3$ and furanophostin-PS_2 caused similar amounts of Ca^{2+} to be released from the intracellular stores (41 ± 2%, $n = 3$ for each). Furthermore, in both functional and binding assays furanophostin-PS_2 was about 12-fold less potent than $InsP_3$ (Tables 2 and 3). Although rapid measurements of rates of Ca^{2+} release would be required to unequivocally establish the efficacy of furanophostin-PS_2 (Marchant et al., 1997b), our results are certainly consistent with the conclusion that furanophostin-PS_2 is a full agonist of hepatic $InsP_3$ receptors. A possible explanation for the unexpectedly high efficacy of furanophostin-PS_2 may be that its 5″-hydroxymethyl group rather closely mimics the 3-hydroxyl of $InsP_3$ and therefore fails to provide sufficient structural perturbation around the pseudo-3 position to significantly reduce the efficacy of furanophostin-PS_2. Future attempts to develop high-affinity ligands with low intrinsic activity may therefore

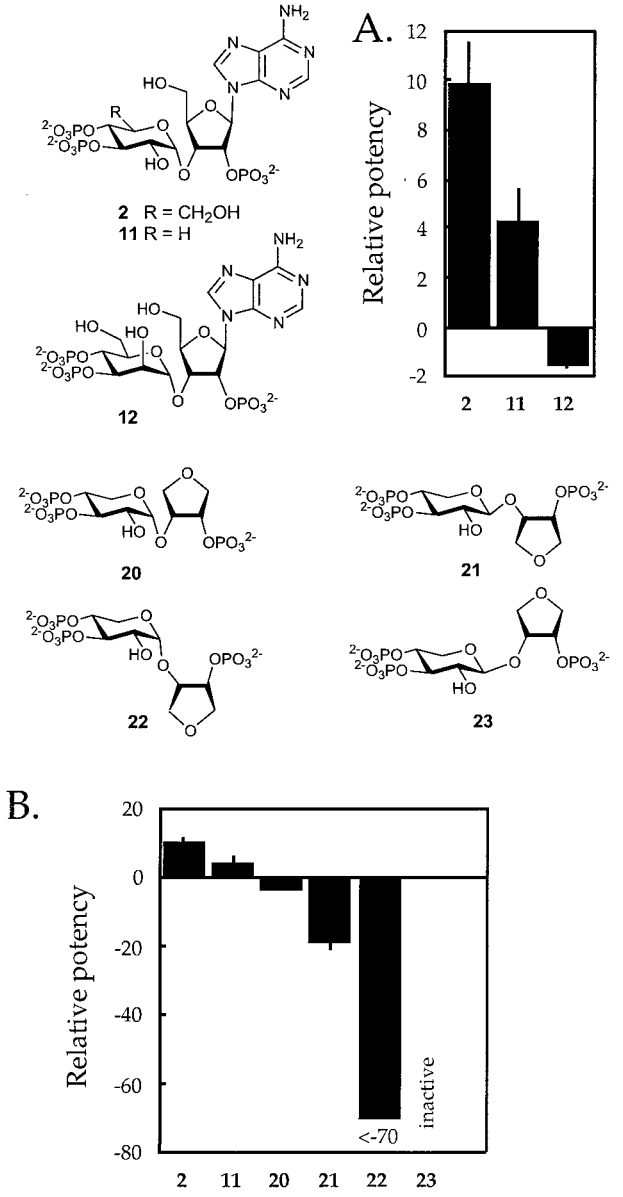

Fig. 6. Modifications to the pyranosyl ring (A) and the stereochemistry of the glycosidic linkage (B). The results are presented in the same format as Fig. 4.

TABLE 3

Binding of selected analogs of adenophostin A to the $InsP_3$ receptors of hepatic membranes

The K_d and Hill coefficient (n_H) determined from equilibrium competition binding with [^{3}H]$InsP_3$ are shown for selected ligands. To correct for variability between experiments performed over a substantial period, each binding assay was accompanied by a parallel measurement of $InsP_3$ binding. For Fig. 7, the affinity of each analog was then expressed relative to that for $InsP_3$ (see under *Experimental Procedures*). The data in this table show the results (from *n* independent experiments) before calibration to the relevant $InsP_3$ affinity.

	K_d	n_H	*n*	Reference
	nM			
1, $InsP_3$	3.09 ± 0.33	1.05 ± 0.08	24	
2, Adenophostin A	0.48 ± 0.06	1.29 ± 0.10	10	
3, Purinophostin	0.80 ± 0.25	1.12 ± 0.18	4	Marwood et al., 2000b
5, Imidophostin	2.19 ± 0.80	1.06 ± 0.06	4	Marwood et al., 2000b
6, Ribophostin	4.40 ± 0.80	0.99 ± 0.05	3	
12, *manno*-Adenophostin	4.66 ± 0.62	0.83 ± 0.03	5	
13, Uridophostin	1.20 ± 0.27	0.98 ± 0.07	3	
24, Furanophostin-PS_2	39.5 ± 5.9	1.09 ± 0.14	4	

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require more substantial modifications to the 5″-position of adenophostin analogs.

Binding of Adenophostin A Analogs to Hepatic $InsP_3$ Receptors. Because $InsP_3$-binding sites are present at low density in hepatocytes and the only available radioligand ($[^3H]InsP_3$) has relatively low specific activity, radioligand binding analyses are more demanding and costly than functional assays; they must also be performed in a medium (BM) with high pH and low ionic strength. For these reasons, our radioligand binding analyses of adenophostin A analogs have focused on only the key ligands.

The results are summarized in Table 3. In Fig. 7 the affinity of each analog is expressed relative to the affinity of $InsP_3$ measured in parallel experiments (under *Experimental Procedures*); the analogous comparisons from functional assays are shown alongside. Other than acyclophostin (**19**), which we previously suggested was a partial agonist at pH 8.3 (the pH used for binding assays) but a full agonist at pH7 (Beecroft et al., 1999), there were no substantial disparities between the binding and functional assays. The rank orders of the analogs shown, which included modifications to each of the major elements of adenophostin A, were similar in binding and functional assays (Fig. 7). The results are therefore consistent with each of the analogs (**1-3**, **5**, **6**, **12**, **13**, and **24**) being a full agonist, differing from adenophostin A only in its affinity for hepatic $InsP_3$ receptors.

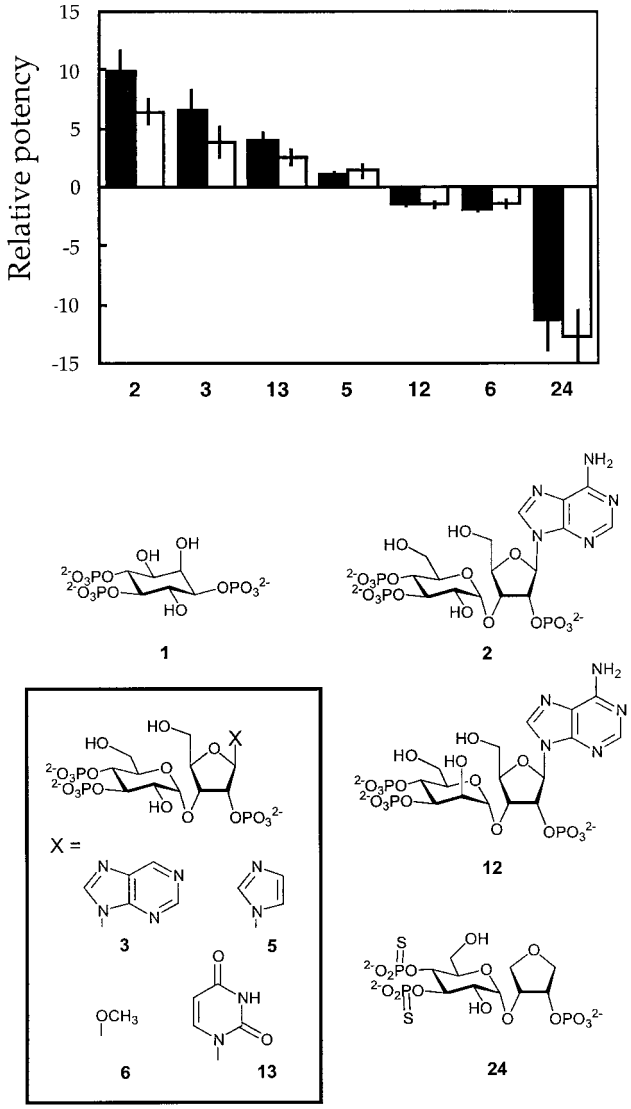

Fig. 7. Binding of selected adenophostin A analogs to hepatic $InsP_3$ receptors. A, K_d values of selected analogs were determined using equilibrium competition binding with $[^3H]InsP_3$ (Table 3) and the results (□) then expressed relative to the affinity for $InsP_3$ measured in exactly parallel experiments (means ± S.E.M.; under *Experimental Procedures*). Ratios >1 denote ligands with greater affinities than $InsP_3$, and ratios <−1 denote ligands with affinities less than $InsP_3$. Solid columns (■) show the relative potencies of the same analogs in assays of $^{45}Ca^{2+}$ release.

Conclusions

Adenophostin A is the most potent known agonist of $InsP_3$ receptors. None of the modifications we have made to the structure of adenophostin A has succeeded in either increasing its affinity for $InsP_3$ receptors or in changing its efficacy. We have, however, designed new ligands based upon adenophostin A with potencies higher than that of $InsP_3$. Using both these ligands and simplified analogs of adenophostin A, we have established which structural elements of adenophostin A determine its high affinity for $InsP_3$ receptors (Fig. 8) in hepatocytes, which express predominantly type 2 $InsP_3$ receptors (Taylor et al., 1999).

The glucose 3″,4″-bisphosphate and adjacent 2″-hydroxyl of adenophostin A are thought to mimic the critical 4,5-bisphosphate and 6-hydroxyl of $InsP_3$ (Fig. 1) (Takahashi et al., 1994a). The 2′-phosphate of adenophostin A is thought to mimic the 1-phosphate of $InsP_3$ and for both ligands removal of this phosphate causes the affinity to decrease by at least 100-fold (Fig. 4) (Shuto et al., 1998). The 3-hydroxyl of $InsP_3$ contributes little to binding (Kozikowski et al., 1993; Nerou et al., 2001), and likewise removal of the analogous hydroxyl from adenophostin A (to give *xylo*-adenophostin, **11**) has minimal effect. These results suggest that structure-activity relationships derived from inositol phosphates can be used to predict the activity of adenophostin analogs, at least for some substituents of the pyranosyl ring. However, the unexpectedly high potency of *manno*-adenophostin (**12**) suggests that the roles of the 2′-hydroxyl of adenophostin A and the 6-hydroxyl of $InsP_3$ are not exactly analogous: this hydroxyl group is clearly more important for $InsP_3$ than adenophostin A binding.

We have explored in detail the contribution of the adenine group to the high-affinity binding of adenophostin A to $InsP_3$ receptors. Progressive removal of elements of the adenine cause progressive decreases in binding affinity (Fig. 4), but

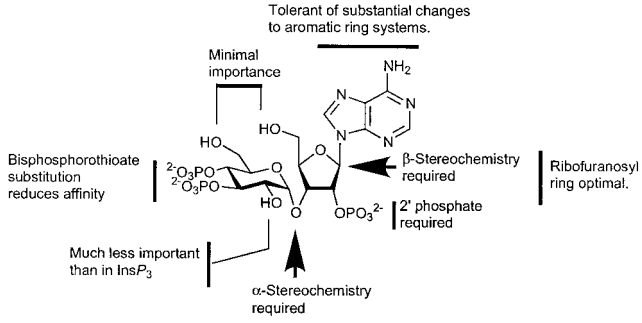

Fig. 8. Key features contributing to the high-affinity binding of adenophostin A to $InsP_3$ receptors. The scheme summarizes the key determinants of adenophostin A binding, some of which are inferred from the activities of simplified analogs.

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the adenine can be replaced by completely unrelated aromatic ring systems (**13**, **14,** and **16**) with only modest decreases in potency (Fig. 5A). The stereochemistry of the link between the furanosyl ring and the aromatic rings is, however, crucial: β-1′-aromatic substituent (as occurs in adenophostin A) enhances affinity, whereas a similar group in the α-orientation has the opposite effect (Fig. 5A). A phosphate group at the position equivalent to the 1-position of $InsP_3$ (2′ in adenophostin A) is essential (see above) and optimally active when attached to a furanosyl ring (Fig. 5). The stereochemistry of the *O*-glycosidic link between the pyranosyl and furanosyl rings is crucial, with an α-glycosidic linkage (as occurs in adenophostin A) optimally positioning the pseudo 1-phosphate.

As the most potent stable agonist of $InsP_3$ receptors, adenophostin A is already widely used to examine the mechanisms underlying intracellular Ca^{2+} regulation. Our analyses of the key determinants of the high-affinity interaction between adenophostin A and $InsP_3$ receptors should facilitate development of further related analogs with properties tailored to meet specific biological requirements.

Acknowledgments

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