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Acyclophostin: A Ribose-Modified Analog of Adenophostin A with High Affinity for Inositol 1,4,5-Trisphosphate Receptors and pH-Dependent Efficacy

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ABSTRACT

Adenophostin A is the most potent known agonist of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptors. Equilibrium competition binding studies with ^{3}H-Ins(1,4,5)P_3 showed that the interaction of a totally synthetic adenophostin A with both hepatic and cerebellar Ins(1,4,5)P_3 receptors was indistinguishable from that of the natural product. At pH 8.3, a synthetic analog of adenophostin A (which we named acyclophostin), in which most elements of the ribose ring have been removed, bound with substantially higher affinity (K_d = 2.76 ± 0.26 nM) than Ins(1,4,5)P_3 (K_d = 7.96 ± 1.02 nM) to the ^{3}H-Ins(1,4,5)P_3-binding sites of hepatic membranes. At pH 7, acyclophostin (EC_{50} = 209 ± 12 nM) and Ins(1,4,5)P_3 (EC_{50} = 153 ± 11 nM) stimulated $^{45}Ca^{++}$ release to the same maximal extent and from the same intracellular stores of permeabilized hepatocytes. Comparison of the affinities of a range of Ins(1,4,5)P_3 and adenophostin analogs with their abilities to stimulate Ca^{++} release revealed that although all other agonists had similar EC_{50}/K_d ratios, that for acyclophostin was significantly higher. Similar results were obtained with cerebellar membranes, which express almost entirely type 1 $InsP_3$ receptors. When the radioligand binding and functional assays of hepatocytes were performed under identical conditions, the higher EC_{50}/K_d ratio for acyclophostin was retained at pH 8.3, but it was similar to that for Ins(1,4,5)P_3 when the assays were performed at pH 7. To directly assess whether acyclophostin was a partial agonist of hepatic Ins(1,4,5)P_3 receptors, the kinetics of $^{45}Ca^{++}$ efflux from permeabilized hepatocytes was measured with a temporal resolution of 80 ms using rapid superfusion. At pH 7, the kinetics of $^{45}Ca^{++}$ release, including the maximal rate of release, evoked by maximal concentrations of acyclophostin or Ins(1,4,5)P_3 were indistinguishable. At pH 8.3, however, the maximal rate of $^{45}Ca^{++}$ release evoked by a supramaximal concentration of acyclophostin was only 69 ± 7% of that evoked by Ins(1,4,5)P_3. We conclude that acyclophostin is the highest affinity ribose-modified analog of adenophostin so far synthesized, that at high pH it is a partial agonist of inositol trisphosphate receptors, and that it may provide a structure from which to develop high-affinity antagonists of inositol trisphosphate receptors.

Many extracellular stimuli, including hormones and neurotransmitters, evoke changes in cellular activity by stimulating an increase in cytosolic [Ca^{++}]. In most cells, D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is the cytosolic messenger that links activation of the plasma membrane receptors for these stimuli to the release of Ca^{++} from intracellular stores. In addition to allowing this initial mobilization of Ca^{++} stores, inositol trisphosphate ($InsP_3$) receptors are involved in the regenerative propagation of cytosolic Ca^{++} signals, a feature that probably depends on the ability of cytosolic Ca^{++} itself to regulate $InsP_3$ receptor behavior (Berridge, 1997). $InsP_3$ receptors also have been speculated to be involved in Ca^{++} entry across the plasma membrane, either directly as Ins(1,4,5)P_3-gated Ca^{++} channels within the plasma membrane or as the link between empty intracellular Ca^{++} stores and the Ca^{++} channels through which Ca^{++} enters cells after depletion of intracellular Ca^{++} stores (Putney, 1997). The latter suggestion has been challenged by recent evidence suggesting that even after complete inhibition of expression of each $InsP_3$ receptor subtype, empty Ca^{++} stores remain capable of activating a Ca^{++} entry pathway (Sugawara et al., 1997). Nevertheless, it remains impor-

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ABBREVIATIONS: BM, binding medium; $[Ca^{++}]_c$, medium-free Ca^{++} concentration; CLM, cytosol-like medium; EC_{50}, concentration causing half the maximal effect; *h*, Hill coefficient; Ins(1,4,5)P_3, D-*myo*-inositol-1,4,5-trisphosphate (other inositol phosphates are similarly abbreviated); $InsP_3$, inositol trisphosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

tant to establish the precise roles of the different $InsP_3$ receptor subtypes (Mikoshiba, 1997) in regulating the cytosolic [Ca^{++}]. However, establishment of the roles of $InsP_3$ receptors in generating complex cytosolic Ca^{++} signals (DeLisle et al., 1996) and determination of whether $InsP_3$ receptors are invariably essential elements of the pathways linking stimuli to physiological responses (Acharya et al., 1997) have been limited by the lack of both adequately selective antagonists of $InsP_3$ receptors and of ligands that discriminate between receptor subtypes.

Adenophostin A is the most potent known agonist of type 1 $InsP_3$ receptors (Takahashi et al., 1994a; Hirota et al., 1995), and we recently established that it is similarly potent in causing Ca^{++} release from the intracellular stores of permeabilized hepatocytes (Marchant et al., 1997a), in which type 2 $InsP_3$ receptors are thought to predominate (Wojcikiewicz, 1995; De Smedt et al., 1997) The structure of adenophostin A (Fig. 1) suggests that its 3″,4″-bisphosphate structure with its adjacent 2″-hydroxyl group may mimic the critical 4,5-bisphosphate/6-hydroxy triad of Ins(1,4,5)P_3 and related active analogs. The structures of, and abbreviations for, the adenophostin analogs are shown in Fig. 1, and the abbreviations for the dissaccharide polyphosphates are given in the legend to Fig. 3. As the most potent known ligand of $InsP_3$ receptors that is not metabolized by the enzymes that degrade Ins(1,4,5)P_3 (Takahashi et al., 1994a), adenophostin A provides a structure from which to attempt to devise novel ligands of $InsP_3$ receptors. Such compounds may be more useful than the analogs of Ins(1,4,5)P_3 that have hitherto provided the major source of ligands (Potter and Lampe, 1995). We recently demonstrated that an analog of adenophostin A [3-*O*-(α-D-glucopyranosyl)-β-D-ribofuranoside-2,3′, 4′-trisphosphate; RibP_3](Fig. 1), which lacks the adenine moeity of adenophostin, is less active than adenophostin but as potent as Ins(1,4,5)P_3 in causing Ca^{++} release from permeabilized hepatocytes (Marchant et al., 1997a). In view of this evidence suggesting that the adenine of adenophostin may play an important role in its high-affinity interaction with $InsP_3$ receptors, we examined the behavior of a range of adenophostin A analogs (**1**-**5**) in which the adenine structure was preserved, while the ribose, glucose, and regiochemistry of the phosphate substituents have been altered (Fig. 1). In the present study, we establish that one of these compounds, (2*S*)-9-[1-(α-D-glucopyranosyl-3″,4″-bisphosphate)-2′-monophosphate-prop-3′-yl]adenine, which we have named acyclophostin (**3**), has unusual properties.

Experimental Procedures

Materials. ^{3}H-Ins(1,4,5)P_3 (58 Ci/mmol) was from Amersham (Little Chalfont, UK) and $^{45}CaCl_2$ was from ICN (Thame, UK). Ins(1,4,5)P_3 was from American Radiolabeled Chemicals (St. Louis, MO). Adenophostin A, purified from *Penicillium brevicompactum* (Takahashi et al., 1994a), was a gift from Dr. M. Takahashi (Sankyo Co. Ltd., Japan). The disaccharide analogs of adenophostin (Marchant et al., 1997a), the analogs **1**-**5** (Van Straten et al., 1997a, 1997c), and adenophostin A (Van Straten et al., 1997b) were synthesized and quantified as described previously. All ligands were analyzed by ion-exchange high performance liquid chromatography for isomer purity as fully described by Van Straten et al. (1997a, 1997b, 1997c). Percoll (1.13 g/ml) was from Pharmacia (Uppsala, Sweden). Ionomycin was from Calbiochem (Nottingham, UK), and thapsigargin was from Alamone Laboratories (Jerusalem, Israel). All other reagents were from suppliers listed previously (Marchant et al., 1997a).

Preparation of Rat Liver Membranes. The liver of a male Wistar rat (200–250 g) was perfused in situ with 40 ml of ice-cold buffered saline [116 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 25 mM $NaHCO_3$, 1 mM EGTA, 11 mM glucose, 5%

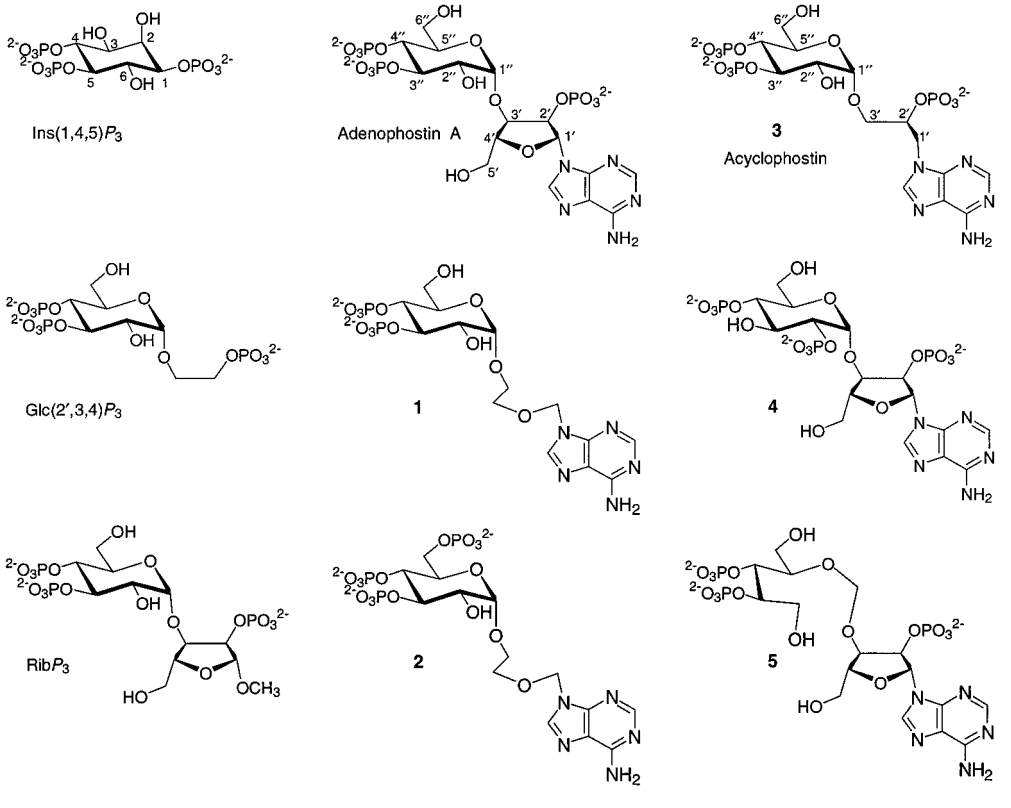

Fig. 1. Structures of Ins(1,4,5)P_3, adenophostin, acyclophostin, and synthetic analogs of adenophostin.

CO_2/95% O_2, pH 7.4, at 2°C]. After excision, the liver was chopped and then homogenized in 25 ml of ice-cold buffered sucrose [250 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4, at 2°C] using a 15-ml glass Dounce homogenizer with 10 strokes of a loose-fitting plunger and 3 strokes with a tighter plunger. The homogenate was made up to 50 ml in ice-cold buffered sucrose, filtered through gauze, and centrifuged (2500*g*, 10 ml), and the pellet then was resuspended in 48 ml of ice-cold buffered sucrose containing Percoll (11.8% final v/v) (Prpic et al., 1984). The suspension was centrifuged (35,000*g*, 30 min), and membranes were harvested as a discrete fluffy band below a fatty layer at the top of each tube. The membranes were resuspended in 50 ml of ice-cold hypo-osmotic buffer (1 mM EGTA, 5 mM HEPES, pH 7.4, at 2°C) to lyse the vesicles and then centrifuged (48,000*g*, 10 min). The final membrane pellet was resuspended in binding medium (BM: 20 mM Tris, 1 mM EDTA, pH 8.3, at 2°C) at ~20 mg protein/ml and stored in liquid nitrogen for up to 14 days. Protein concentrations were measured using the Bradford assay with bovine serum albumin as standard. A single liver typically provided ~70 mg of membrane protein. Although this method produces membranes enriched in markers for plasma membrane, microsomal markers are also present (Prpic et al., 1984), and the membranes are enriched in $InsP_3$ receptors whose characteristics have so far proved indistinguishable from those of permeabilized rat hepatocytes (Marshall and Taylor, 1994).

^{3}H-Ins(1,4,5)P_3 Binding. Liver membranes (0.4 mg protein/tube) were added to BM (pH 7.0 or 8.3) (500 μl) containing ^{3}H-Ins(1,4,5)P_3 (30–60 nCi, final concentration, 1–2 nM) and the appropriate concentration of competing ligand. After 5 min at 2°C, bound and free ^{3}H-Ins(1,4,5)P_3 were separated by centrifugation (20,000*g*, 5 min, 2°C). Previous results established that under these conditions, binding reached equilibrium and degradation of ^{3}H-Ins(1,4,5)P_3 was negligible. Total binding was typically 4000 dpm/tube, and nonspecific binding was approximately 30% of total binding. ^{3}H-Ins(1,4,5)P_3 binding to rat cerebellar membranes was characterized as described previously (Richardson and Taylor, 1993).

$^{45}Ca^{++}$ Release from Permeabilized Rat Hepatocytes. Hepatocytes were isolated by collagenase digestion of the livers of male Wistar rats (200–250 g) as described previously (Richardson and Taylor, 1993) and stored at 4°C in Eagle's medium supplemented with 26 mM $NaHCO_3$ and bovine serum albumin (2 g/100 ml) for up to 24 h. Cells were permeabilized by incubation with saponin (10 μg/ml) in a cytosol-like medium [CLM: 140 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.0, at 37°C] and subsequently loaded to steady-state (1–2 nmol $Ca^{++}/10^6$ cells) by incubation (10^7 cells/ml) for 5 min at 37°C in CLM supplemented with $CaCl_2$ (300 μM, $[Ca^{++}]_c$ = 200 nM), ATP (7.5 mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 10 μM), and $^{45}Ca^{++}$ (7.5 μCi/ml). Unidirectional $^{45}Ca^{++}$ efflux from the intracellular stores was initiated by diluting the cells (5-fold) into Ca^{++}-containing CLM at 37°C supplemented with thapsigargin (final concentration, 1 μM). Appropriate concentrations of Ins(1,4,5)P_3, adenophostin A or related compounds were then added, and 60 s later the $^{45}Ca^{++}$ content of the intracellular stores was determined after quenching in ice-cold medium (310 mM sucrose, 1 mM trisodium citrate) and then rapid filtration through Whatman GF/C filters using a Brandel receptor-binding harvester (Marshall and Taylor, 1994). A similar method was used for experiments in which $^{45}Ca^{++}$ release was measured under different conditions (e.g., pH , temperature) than those used to load the stores.

To compare ^{3}H-Ins(1,4,5)P_3 binding and $^{45}Ca^{++}$ release under identical conditions, permeabilized hepatocytes loaded with Ca^{++} ($[Ca^{++}]_c$ = 200 nM; with 7.5 μCi of $^{45}Ca^{++}$/ml for flux assays) were centrifuged (650*g*, 2 min) and resuspended in BM at 2°C supplemented with 140 mM KCl and 20 mM NaCl to give a final cell density of 2.5×10^6 cells/ml. Equilibrium binding of ^{3}H-Ins(1,4,5)P_3 (3 nM) was measured after 5 min and unidirectional $^{45}Ca^{++}$ release was measured 3 min after the addition of either Ins(1,4,5)P_3 or acyclophostin.

$^{45}Ca^{++}$ Release from Cerebellar Microsomes. Microsomes were prepared from rat cerebella as described previously (Patel et al., 1997). Briefly, cerebella from five male Wistar rats were homogenized in a Teflon-glass homogenizer in 9 volumes of ice-cold medium containing 250 mM sucrose, 5 mM HEPES (pH 7.05), 10 mM KCl, 1 mM $MgCl_2$, and a cocktail of protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.02 unit/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, 100 μM captopril). After two centrifugations (1000*g*, 5 min), the pellet was resuspended in homogenization medium and centrifuged again (9000*g*, 10 min). The supernatants from each of the three centrifugation steps were pooled and centrifuged (100,000*g*, 75 min), and the resulting microsomal pellet was resuspended in homogenization medium (~15 mg protein/ml) and stored in liquid nitrogen. Microsomes (50 μl) were diluted into loading medium (2.73 ml) containing 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.0), 240 μM EGTA, 64 μM $CaCl_2$ ($[Ca^{++}]_c$ ~200 nM), 1.5 mM ATP, 5 mM phosphocreatine, 1 unit/ml creatine phosphokinase, 8 μCi/ml $^{45}Ca^{++}$, and the protease inhibitor cocktail. After 5 min at 20°C, microsomes (20 μl) were added to appropriate agonists, and after 45 s, their $^{45}Ca^{++}$ contents were determined by stopping the incubations in ice-cold medium (100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 1 mM EGTA) and rapid filtration through Whatman GF/C filters (Nunn and Taylor, 1990).

Rapid Kinetics of $^{45}Ca^{++}$ Release from Permeabilized Rat Hepatocytes. Permeabilized hepatocytes loaded with $^{45}Ca^{++}$ were immobilized between the filters of our superfusion apparatus, and media (20°C) were delivered to the cells (2 ml/s) from pressurized cylinders regulated by computer-controlled solenoid valves (Marchant et al., 1997b). The effluent containing the $^{45}Ca^{++}$ released from the cells was collected into vials (80 ms/fraction) arranged around the circumference of a programmable turntable. Details of the equipment and superfusion methods have been described previously (Marchant et al., 1997b). The equipment allows rapid (half-time = 46 ± 6 ms) exchange of the medium surrounding the permeabilized cells while measuring unidirectional $^{45}Ca^{++}$ efflux from them with high temporal resolution (80 ms) and under conditions where the composition of the medium (including its $[Ca^{++}]_c$) is rigorously controlled. At the end of each run, cells were superfused with CLM containing Triton X-100 (0.05%) to release all of the $^{45}Ca^{++}$ remaining within the stores. Radioactivity ($^{45}Ca^{++}$ and the inert marker, ^{3}H-inulin) within each sample was determined by liquid scintillation counting after the addition of EcoScint-A scintillation cocktail (National Diagnostics, Aylesbury, UK).

All traces were corrected for the unstimulated rate of $^{45}Ca^{++}$ efflux, and the amount of $^{45}Ca^{++}$ released into each vial was then expressed as a fraction of the total $^{45}Ca^{++}$ content of the intracellular stores.

Analysis. Equilibrium-competition binding curves were fitted to four-parameter logistic equations using a nonlinear curve-fitting program (Kaleidagraph, Synergy Software, PA)

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

where B is the total amount of ^{3}H-Ins(1,4,5)P_3 bound in the presence of the competing ligand, [L]; T and N are the total and nonspecific ^{3}H-Ins(1,4,5)P_3 binding, respectively; h is the Hill coefficient; and IC_{50} is the concentration of competing ligand causing 50% displacement of specific ^{3}H-Ins(1,4,5)P_3 binding from which the K_d for each ligand was calculated. Concentration-response relationships were fitted to an analogous equation from which the maximal effect, EC_{50}, and h for each ligand were determined. Student-Newman-Keuls multiple range test was used to establish the statistical differences between ratios of numbers. All results are reported as mean ± S.E.M.

Results and Discussion

Effects of Adenophostin A on Liver. Maximally effective concentrations of Ins(1,4,5)P_3 (10 μM) and adenophostin A (1 μM) released Ca^{++} from the same intracellular stores of permeabilized hepatocytes: alone or in combination they released ~55% of the intracellular Ca^{++} pool. Adenophostin A, however, was significantly more potent than Ins(1,4,5)P_3, with the half-maximal response to it (EC_{50} = 12.3 ± 0.3 nM) occurring at an ~12-fold lower concentration than that to Ins(1,4,5)P_3 (EC_{50} = 153 ± 11 nM) (Table 1). In equilibrium competition binding studies to hepatic membranes, adenophostin A (K_d = 1.60 ± 0.37 nM) bound to a single class of ^{3}H-Ins(1,4,5)P_3-binding site with substantially greater affinity than Ins(1,4,5)P_3 (K_d = 7.96 ± 1.02 nM) (Table 1). We conclude, in keeping with results from other cells (Takahashi et al., 1994a; Hirota et al., 1995; Murphy et al., 1997; Missiaen et al., 1998), that in permeabilized hepatocytes, adenophostin A is the most potent agonist of $InsP_3$ receptors yet identified. From both functional and radioligand binding analyses of liver and cerebellum, the properties of natural and totally synthetic adenophostin A (Van Straten et al., 1997b) were very similar (Table 1); the synthetic compound was used for most experiments. In subsequent experiments, we examined the effects of several structural analogs (Fig. 1; **1–5**) of adenophostin A on hepatic $InsP_3$ receptors.

Adenophostin A has within its structure an adenine moeity (Fig. 1) that is apparently required for optimal binding to $InsP_3$ receptors (Marchant et al., 1997a), and because adenine nucleotides also bind to $InsP_3$ receptors (Missiaen et al., 1997), the high-affinity binding of adenophostin A might have resulted from it simultaneously binding to the $InsP_3$- and adenine nucleotide-binding sites. Our results suggest that such an explanation is unlikely because preincubation (3 min) of cerebellar membranes with adenine, adenosine, ADP, AMP, or adenosine-2′-monophosphate (1 mM) (Missiaen et al., 1997) did not selectively inhibit binding of adenophostin A relative to Ins(1,4,5)P_3 (not shown).

Effects of Modified Adenophostin Analogs in Liver. The effects of five modified analogs of adenophostin A (Fig. 1) on Ca^{++} release from the intracellular stores of permeabilized hepatocytes are summarized in Table 1. Two of the analogs (**2**, **5**) were inactive at concentrations of ≤100 μM. Three related analogs (**1**, **3**, **4**) were active, with each causing release of the entire Ins(1,4,5)P_3-sensitive Ca^{++} store as revealed by the inability of a subsequent addition of Ins(1,4,5)P_3 (10 μM) to cause further Ca^{++} release. The most potent of the analogs (**3**) (EC_{50} = 209 ± 12 nM), which we have named acyclophostin, was almost as potent as Ins(1,4,5)P_3 (Table 1). In equilibrium competition binding studies to hepatic membranes, four of the analogs (**1**-**4**) completely displaced specifically bound ^{3}H-Ins(1,4,5)P_3, although the affinity of **2** was extremely low (K_d = 4.2 μM), in keeping with its lack of functional effect. Acyclophostin bound with substantially greater affinity (K_d = 2.76 ± 0.26 nM) than Ins(1,4,5)P_3 (K_d = 7.96 ± 1.02 nM) and with only marginally lower affinity than adenophostin A (K_d = 1.60 ± 0.37 nM) (Fig. 2). Acyclophostin has the highest affinity for $InsP_3$ receptors of any adenophostin analog with a modified ring structure yet synthesized.

There have been few attempts to define the structural determinants of the high-affinity interaction of adenophostin A with $InsP_3$ receptors, although each active adenophostin analog has so far proved to be a full agonist. Most results are consistent with the 3′′,4′′-bisphosphate and 2′′-hydroxyl of adenophostin mimicking the essential 4,5-bisphosphate and 6-hydroxyl of Ins(1,4,5)P_3 (Potter and Lampe, 1995) and with the 2′-phosphate of adenophostin mimicking the 1-phosphate of Ins(1,4,5)P_3 (Fig. 1). The latter is supported by the observation that deletion of the 2′-phosphate from the ribose of adenophostin A massively reduced its binding affinity (Takahashi et al., 1994b). Glc(2′,3,4)P_3 (Fig. 1), which was recently synthesized by two groups (Wilcox et al., 1995; Jenkins and Potter, 1996), is a low affinity agonist of $InsP_3$ receptors (Marchant et al., 1997a), but its affinity is increased to almost match that of Ins(1,4,5)P_3 when the flexible side chain to which the 2′-phosphate is attached is conformationally restricted in an anhydroerythritol derivative (Tatani et al., 1998).

Conformational restriction and phosphate positioning were also shown to be important in our previous study of the activity of disaccharide polyphosphates related to adenophostin (Marchant et al., 1997a). Ribophostin ($RibP_3$), for example, essentially is adenophostin A without an adenine group (Fig. 1) and is as potent as Ins(1,4,5)P_3 in evoking Ca^{++} release; until the present work, it represented the most potent synthetic analog known (Marchant et al., 1997a). Conformational restriction together with a nucleoside-type base

TABLE 1

Effects of Ins(1,4,5)P_3, adenophostin, and its analogs on ^{3}H-Ins(1,4,5)P_3 binding and Ca^{2+} mobilization

Experiments similar to those shown in Fig. 2 were used to determine the K_d from equilibrium competition binding experiments with ^{3}H-Ins(1,4,5)P_3 (pH 8.3, 2°C) and the EC_{50} from $^{45}CA^{++}$ flux assays (pH 7, 37°C) for each of the indicated compounds. Results are expressed as means ± S.E.M. of 3 to 16 (n = 1, for binding with **2**) independent experiments with duplicate determinations for each. The results of similar equilibrium competition binding experiments with rat cerebellar membranes performed under identical conditions to those used for hepatic membranes are shown for some of the compounds (n = 3–16).

	Cerebellum Binding		Liver Binding		Liver Ca^{++} Mobilization	
	K_d	h	K_d	h	EC_{50}	h
	nM		*nM*		*nM*	
Natural adenophostin A	0.92 ± 0.08	1.44 ± 0.1	0.86 ± 0.20	1.28 ± 0.37	10.9 ± 0.7	2.18 ± 0.39
Synthetic adenophostin A	1.22 ± 0.11	1.25 ± 0.0	1.60 ± 0.37	1.18 ± 0.09	12.3 ± 0.3	2.36 ± 0.13
Ins(1,4,5)P_3	6.83 ± 0.78	0.91 ± 0.03	7.96 ± 1.02	1.02 ± 0.08	153 ± 11	2.25 ± 0.20
Acyclophostin (**3**)	3.83 ± 0.48	0.97 ± 0.10	2.76 ± 0.26	1.12 ± 0.17	209 ± 12	2.48 ± 0.18
4	N.D.[a]		161 ± 24	0.98 ± 0.1	1870 ± 66	2.51 ± 0.14
1	N.D.[a]		2,650 ± 490	0.54 ± 0.03	33,300 ± 3,500	3.05 ± 0.39
2	N.D.[a]		4,220	0.43	Inactive at 100 μM	
5	N.D.[a]		Inactive at 100 μM		Inactive at 100 μM	

[a] N.D., not determined.

therefore seems to be essential for analogs to be more potent than Ins(1,4,5)P_3. The analogs used in the present study were designed to further explore the determinants of the increased potency of adenophostin by introducing modifications in the ribose and glucose rings and the regiochemistry of phosphate substitution.

It is not surprising that **5** was inactive: opening of the cyclic structure of the glucose-related ring presumably allows too much conformational mobility around the essential vicinal bisphosphate and neighboring hydroxyl group, which are essential elements of the pharmacophore (Potter and Lampe, 1995). A similar acyclic analog of Ins(1,4,5)P_3 had previously been shown to be inactive (unpublished observation).

The substantial decrease (>200-fold) in the affinity of **1** for InsP_3 receptors parallels the massive decrease in the affinity of adenophostin A after removal of its 2′-phosphate (Takahashi et al., 1994b) and of Ins(1,4,5)P_3 after removal of its 1-phosphate (Potter and Lampe, 1995). These results suggest that a nucleoside alone, in this case an acyclo-type nucleoside, does little to augment the activity of the glucose bisphosphate motif of adenophostin. The addition of a phosphate group to the 5′′-position of the glucose ring of **1** to give **2** further reduces biological activity. There is an inexact parallel with the much lesser affinity of Ins(1,3,4,5)P_4 relative to Ins(1,4,5)P_3 for InsP_3 receptors (Burford et al., 1997), part of which may result from the additional 3-phosphate completely altering the ionization behavior of the 4,5-bisphosphate (Guédat et al., 1997).

Compound **4** appeared to be a relatively potent agonist, being only 10- to 20-fold less potent than Ins(1,4,5)P_3. This was surprising because every known agonist of Ins(1,4,5)P_3 receptors has a vicinal bisphosphate motif, but there is no such motif in **4** (Fig. 1). A possible explanation might be to suggest that the 2′- and 2′′-phosphates of **4** may be close enough together to mimic the vicinal 4,5-bisphosphate of Ins(1,4,5)P_3. This would suggest a novel orientation within the receptor in which the 4′′-phosphate and 3′′-hydroxyl of **4** might correspond to the 1-phosphate and 6-hydroxyl of Ins(1,4,5)P_3. We cannot, however, exclude the possibility that migration of a protecting group during a prephosphorylation stage in the synthesis of **4** may have lead to minor contamination with adenophostin A. It is difficult to eliminate such an explanation because it would require <1% contamination of **4** with adenophostin A to account for its observed activity,

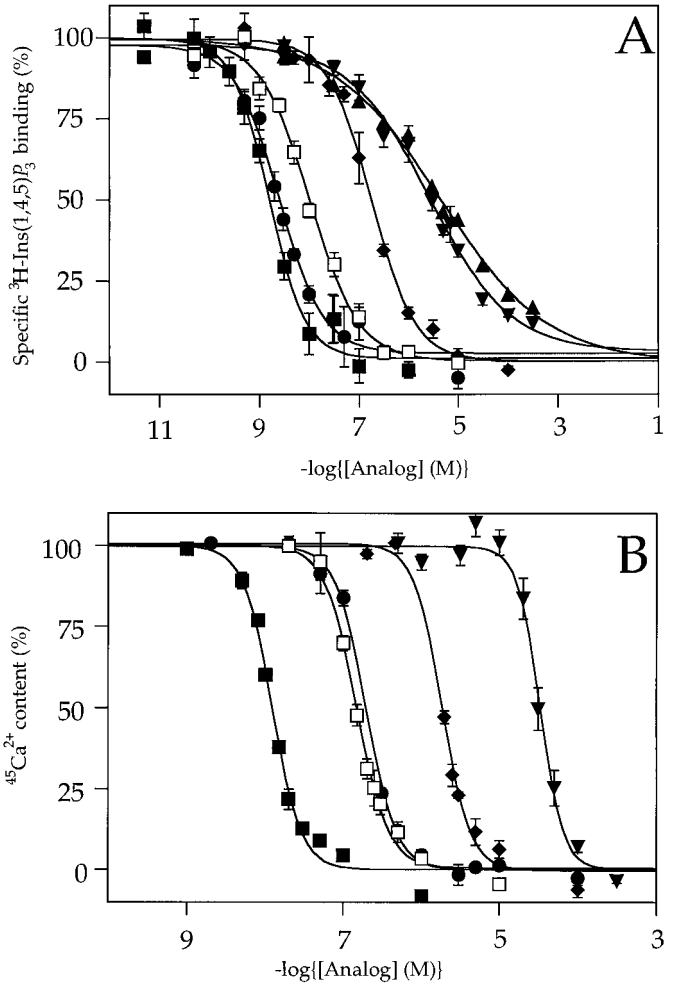

Fig. 2. Effects of Ins(1,4,5)P_3, synthetic adenophostin A, and its analogs on ^{3}H-Ins(1,4,5)P_3 binding and Ca^{++} mobilization in liver. A, Specific ^{3}H-Ins(1,4,5)P_3 binding to rat hepatic membranes is shown in the presence of the indicated concentrations of Ins(1,4,5)P_3 (□), adenophostin A (■), acyclophostin (●), **4** (♦), **1** (▼), and **2** (▲). Results (percent specific binding in the absence of competing ligand) are expressed as mean ± S.E.M. of three or more independent experiments (except for **2**, $n = 1$) with duplicate determinations in each. B, The results show the effects of the indicated concentrations of Ins(1,4,5)P_3 (□), adenophostin A (■), acyclophostin (●), **4** (♦), and **1** (▼) on the Ca^{++} content of the Ins(1,4,5)P_3-sensitive Ca^{++} stores of permeabilized hepatocytes. Results [percent Ins(1,4,5)P_3-sensitive Ca^{++} stores] are expressed as mean ± S.E.M. of three or more independent experiments.

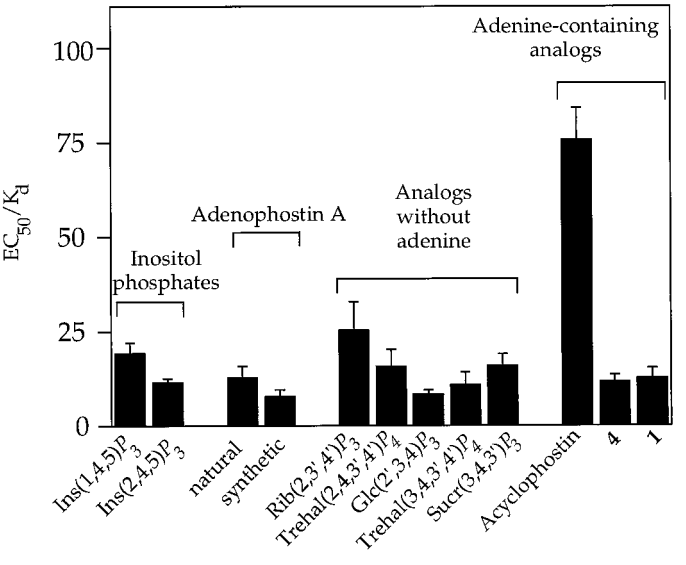

Fig. 3. Comparison of the ratios of EC_{50}/K_d for different agonists of Ins(1,4,5)P_3 receptors. From experiments similar to those shown in Fig. 2, the K_d of each of the ligands for the InsP_3 receptors of hepatic membranes was determined by equilibrium competition binding with ^{3}H-Ins(1,4,5)P_3 and the EC_{50} for Ca^{++} mobilization was determined in permeabilized hepatocytes. The figure shows the ratio (derived from at least three independent experiments for both binding and functional assays) of the EC_{50}/K_d for each agonist (mean ± S.E.M.). Within each of the four categories of agonist, the most potent is presented first. Data for the phosphorylated disaccharides are taken from Marchant et al. (1997a); their structures are abbreviated as Glc(2′,3,4)P_3, 2-hydroxyethyl-α-D-glucopyranoside-2′,3,4-trisphosphate; Rib(2,3′,4′)P_3, 3-*O*-(α-D-glucopyranosyl)-β-D-ribofuranoside-2,3′,4′-trisphosphate; Sucr(3,4,3′)P_3, sucrose 3,4,3′-trisphosphate; Trehal(2,4,3′,4′)P_4, α, α′-trehalose-2,4,3′,4′-tetrakisphosphate; and Trehal(3,4,3′,4′)P_4, α,α′-trehalose-3,4,3′,4′-tetrakisphosphate. There is no statistical difference between the EC_{50}/K_d ratio for Ins(1,4,5)P_3 and any of the other agonists except acyclophostin (*$p < .05$).

and such contamination cannot be excluded by standard spectroscopic and analytical techniques.

Acyclophostin (**3**) was almost as potent as $Ins(1,4,5)P_3$ and similar to ribophostin (EC_{50} = 213 nM) (Marchant et al., 1997a) in causing Ca^{++} release from hepatic Ca^{++} stores (Table 1) and significantly more potent than $Glc(2',3,4)P_3$ (EC_{50} = 1867 nM) (Marchant et al., 1997a). The adenosine of acyclophostin is connected to the glucose bisphosphate via a short linker with an attached phosphate group, and the stereochemistry at this center is the same as at the 2′-phosphate of adenophostin. These results therefore suggest that conformational restriction via a second ring structure (as in ribophostin and adenophostin) is not the only way to improve the biological activity of adenophostin analogs relative to $Glc(2',3,4)P_3$. Presumably, both the 2′-phosphate and the adenine of acyclophostin contribute to its activity at $InsP_3$ receptors but rather less effectively than for adenophostin. Despite some attempts at molecular modeling of adenophostin analogs (Wilcox et al., 1995), the apparently flexible nature of acyclophostin presents considerable difficulties in identifying a single low-energy conformation, making it unlikely that modeling of acyclophostin alone will provide unambiguous information. This approach may become more practicable after synthesis and biological evaluation of related conformationally restricted ligands.

Efficacy of Acyclophostin in Liver and Cerebellum. Acyclophostin bound to hepatic $InsP_3$ receptors with appreciably higher affinity than $Ins(1,4,5)P_3$, but it was marginally less potent in causing Ca^{++} mobilization (Table 1), suggesting that acyclophostin might be a partial agonist. Although the radioligand binding and functional analyses were performed under different conditions to optimize the signals obtained from them, comparison of the EC_{50}/K_d ratio for each of the agonists provides an index of their relative efficacies. The EC_{50}/K_d ratio was similar for each of 11 different agonists, including adenophostin A and two of the active ribose-modified adenophostin analogs (**1**, **4**), but the ratio for acyclophostin was significantly ($p < .05$) higher (by ~4-fold) than that for any other agonist (Fig. 3). The results suggest that acyclophostin may be a partial agonist of $InsP_3$ receptors. A similar comparison of EC_{50}/K_d ratios, although with binding and functional analyses performed under different conditions and with different cell types, was previously used to suggest that $Ins(1,2,3,5)P_4$ might be a partial agonist of $InsP_3$ receptors (Burford et al., 1997).

An alternative explanation for the higher EC_{50}/K_d ratio for acyclophostin is that the permeabilized hepatocytes used for functional assays and the liver membranes used for radioligand binding might express different $InsP_3$ receptor subtypes with different relative affinities for acyclophostin and $Ins(1,4,5)P_3$. Both reverse transcription polymerase chain reaction analysis (De Smedt et al., 1997) and subtype-selective antibodies (Wojcikiewicz, 1995) of whole liver homogenates concur in suggesting that >80% of the $InsP_3$ receptors are type 2 and the remainder are type 1. However, the exact distribution of subtypes is unknown within either our hepatocyte preparation (>95% hepatocytes) or the hepatic membranes prepared from whole liver (in which ~65% of cells are hepatocytes). Unfortunately, with the $InsP_3$ receptor subtype-selective antibodies currently available and the relatively low density of $InsP_3$ receptors in permeabilized hepatocytes, we were unable to directly compare levels of expression of receptor subtypes in the two preparations. However, other evidence suggests that receptor heterogeneity is unlikely to be the cause of the difference in the EC_{50}/K_d ratio for acyclophostin and $Ins(1,4,5)P_3$.

First, cerebellum expresses almost exclusively type 1 $InsP_3$ receptors (Wojcikiewicz, 1995), yet under identical equilibrium competition binding conditions, cerebellar and hepatic membranes had very similar affinities for $Ins(1,4,5)P_3$ and acyclophostin (Table 1). Acyclophostin is unlikely, therefore, to discriminate between the two $InsP_3$ receptor subtypes (1 and 2) expressed in liver.

Second, when radioligand and functional assays were performed under identical conditions using permeabilized hepatocytes in Ca^{++}-free medium at 2°C and pH 8.3 (see Experimental Procedures), the EC_{50}/K_d ratio for acyclophostin was again higher than that for $Ins(1,4,5)P_3$ (Table 2). Similar experiments with membranes (for radioligand binding) and microsomes (for Ca^{++} release) from cerebellum established

TABLE 2

Effects of acyclophostin and $Ins(1,4,5)P_3$ on Ca^{++} release and ^3H-$Ins(1,4,5)P_3$ binding measured under identical conditions at pH 8.3 in permeabilized hepatocytes

Permeabilized hepatocytes loaded with Ca^{++} and resuspended in modified BM at 2°C (see *Experimental Procedures*) were used to measure the effects of acylophostin and $Ins(1,4,5)P_3$ on Ca^{++} release and ^3H-$Ins(1,4,5)P_3$ binding under identical conditions. Results are mean ± S.E.M. of four or five independent determinations; *h* are shown in parentheses. Bold text denotes the acylophostin/$Ins(1,4,5)P_3$ ratio.

	K_d	EC_{50}	EC_{50}/K_d	
	nM			
$Ins(1,4,5)P_3$	43 ± 8 (0.83 ± 0.05)	33 ± 2 (2.69 ± 0.14)	0.77 ± 0.15	**2.2**
Acyclophostin	19 ± 6 (0.78 ± 0.08)	32 ± 5 (2.90 ± 0.84)	1.69 ± 0.62	

TABLE 3

Effects of pH on EC_{50}/K_d ratio for acyclophostin and $Ins(1,4,5)P_3$ in liver

Permeabilized hepatocytes were loaded with $^{45}Ca^{++}$ before 5-fold dilution into CLM (including 1 μM thapsigargin) at 2°C; the final pH was either 8.3 or 7.0, and $[Ca^{++}]_c$ was maintained at 200 nM. The $^{45}Ca^{++}$ contents of the stores were assessed 180 s after the addition of agonist. Equilibrium competition binding to liver membranes was performed in BM (pH 8.3 or 7.0, 2°C). The EC_{50} and K_d for $Ins(1,4,5)P_3$ and acyclophostin are shown as mean ± S.E.M. of 3–16 independent experiments. The final column denotes the EC_{50}/K_d ratios, and the bold text indicates the acyclophostin/$Ins(1,4,5)P_3$ ratio.

		EC_{50}	K_d	EC_{50}/K_d	
			nM		
pH 8.3	$Ins(1,4,5)P_3$	23 ± 1	8.0 ± 1.0	2.9 ± 0.3	**3.1**
	Acyclophostin	25 ± 1	2.8 ± 0.3	9.0 ± 0.9	
pH 7.0	$Ins(1,4,5)P_3$	10 ± 1	10.4 ± 0.2	1.0 ± 0.2	**1.4**
	Acyclophostin	17 ± 1	11.8 ± 0.2	1.4 ± 0.1	

that here, too, the EC_{50}/K_d ratio was higher for acyclophostin (not shown).

We conclude that acyclophostin does not discriminate between the $InsP_3$ receptor subtypes (types 1 and 2) expressed in liver and that its high EC_{50}/K_d ratio therefore indicates that at pH 8.3 it behaves as a partial agonist of both hepatic and cerebellar $InsP_3$ receptors.

Acyclophostin Is a pH-Dependent Partial Agonist. When the comparison of the effects of acyclophostin and $Ins(1,4,5)P_3$ on $^{45}Ca^{++}$ mobilization from permeabilized hepatocytes and binding to hepatic membranes was performed at pH 7, there was no significant difference in the EC_{50}/K_d ratio for acyclophostin and $Ins(1,4,5)P_3$ (Table 3). It was impracticable to perform both assays on permeabilized hepatocytes at pH 7 because the density of $InsP_3$-binding sites was too low to permit their characterization at this suboptimal pH . However, because the relative affinities of acyclophostin and $Ins(1,4,5)P_3$ for the receptors in permeabilized hepatocytes and hepatic membranes are indistinguishable at pH 8.3, we are confident that radioligand binding to hepatic membranes provides a valid comparison with permeabilized cells. These results therefore suggest that at pH 7, acyclophostin and $Ins(1,4,5)P_3$ may be similarly efficacious in evoking Ca^{++} release from permeabilized hepatocytes. We conclude that when radioligand and functional assays are performed under similar conditions, acyclophostin appears to be a partial agonist at high pH and a full agonist at pH 7.

Rapid Kinetics of Ca^{++} Mobilization. Most analogs of $Ins(1,4,5)P_3$ are suggested to be full agonists (Potter and Lampe, 1995; Marchant et al., 1997b), but conventional measurements of the extent of Ca^{++} release are ill-suited to reliable measurement of efficacy. Even a partial agonist may be capable, as shown for acyclophostin (Fig. 2), of releasing the entire $Ins(1,4,5)P_3$-sensitive Ca^{++} store but would be expected to do so more slowly (Safrany et al., 1993; Marchant et al., 1997b). The efficacy of agonists of $InsP_3$ receptors can best be resolved by measuring initial rates of $^{45}Ca^{++}$ mobilization, which more closely reflect the extent to which $InsP_3$ receptors have opened (Marchant et al., 1997b). In the final experiments, we used rapid superfusion of immobilized cells to determine the rates of $^{45}Ca^{++}$ release evoked by acyclophostin and $Ins(1,4,5)P_3$. For practical reasons, our rapid superfusion experiments are restricted to 20°C, but because the discrepant EC_{50}/K_d ratio for acyclophostin is similar whether $^{45}Ca^{++}$ release is measured at 2°C or 37°C (not shown), the need to perform the kinetics experiments at 20°C should not compromise their utility.

Figure 4A illustrates the typical response to a maximal concentration of $Ins(1,4,5)P_3$ (5 μM): a rapid acceleration toward a peak rate of $^{45}Ca^{++}$ release is abruptly followed by a biphasic decay in the rate of $^{45}Ca^{++}$ release. The mechanisms underlying this pattern of $Ins(1,4,5)P_3$-evoked Ca^{++} release have been addressed previously (Marchant et al., 1997b; Marchant and Taylor, 1998), and the kinetic characteristics of the behavior observed in the present study were indistinguishable from those published previously. With the limited amounts of acyclophostin available, it was impracticable to complete such a detailed analysis, which would have required maximal stimulation with acyclophostin for several seconds. Instead, we examined only the initial responses to supramaximal concentrations of acyclophostin (25 μM) and $Ins(1,4,5)P_3$ (5 μM). At pH 7, the responses to acyclophostin and $Ins(1,4,5)P_3$ were indistinguishable: the time taken to reach the peak rate of $^{45}Ca^{++}$ release (320 ± 25 ms, $n = 5$; 293 ± 27 ms, $n = 9$, respectively), the peak rate of $^{45}Ca^{++}$ release (Fig. 4B), and the decaying phase of the response (not shown) were all similar. These results are entirely consistent with our suggestion that at pH 7, acyclophostin and $Ins(1,4,5)P_3$ are each a full agonist of hepatic $InsP_3$ receptors. At pH 8.3, however, the time taken to reach the peak rate of $^{45}Ca^{++}$ release was longer for acyclophostin (500 ± 30 ms, $n = 4$) than for $Ins(1,4,5)P_3$ (420 ± 25 ms, $n = 4$), and the peak rate evoked by acyclophostin was only 69 ± 7% of that evoked by $Ins(1,4,5)P_3$ (Fig. 4B). Analysis of the rapid kinetics of $^{45}Ca^{++}$ release therefore confirms our suggestion that

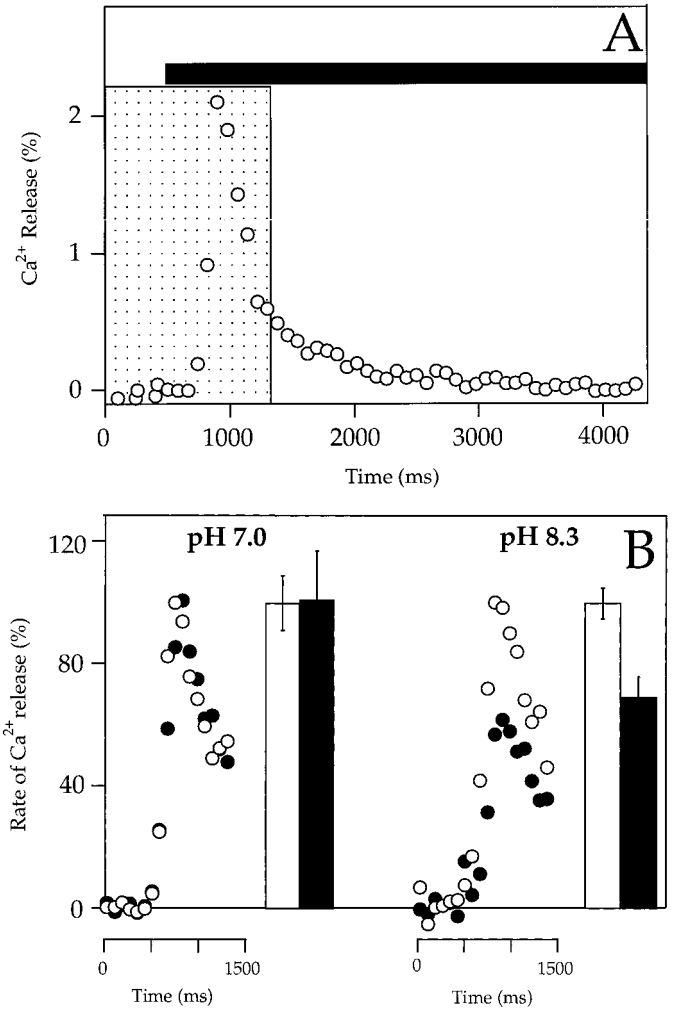

Fig. 4. Rapid kinetics of $^{45}Ca^{++}$ efflux stimulated by acyclophostin and $Ins(1,4,5)P_3$. A, Permeabilized hepatocytes loaded with $^{45}Ca^{++}$ were immobilized within the rapid superfusion apparatus and then rapidly ($t_{1/2}$ = 46 ± 6 ms) superfused with CLM at pH 7 containing 5 μM $Ins(1,4,5)P_3$. The $^{45}Ca^{++}$ released from the cells was collected at 80-ms intervals, and the stimulated rate of release is shown as a percent of the total $^{45}Ca^{++}$ content of the stores. The trace shows results from a single experiment represenative of three similar experiments. The stippled area denotes the component of the response used to compare the behavior of acyclophostin and $Ins(1,4,5)P_3$ in B. B, Permeabilized hepatocytes were stimulated with acyclophostin (25 μM) or $Ins(1,4,5)P_3$ (5 μM) in CLM buffered at pH 7 or 8.3, and the rate of $^{45}Ca^{++}$ release [percent of the maximal rate evoked by $Ins(1,4,5)P_3$] was recorded during 1.5 s of rapid superfusion. The traces are from single experiments, each typical of four other experiments. The histograms show (mean ± S.E.M., $n = 4$) the peak rates of $^{45}Ca^{++}$ release (percent) evoked by $Ins(1,4,5)P_3$ (open) and acyclophostin (solid).

at pH 7, acyclophostin is a full agonist, but at pH 8.3, it is a partial agonist of $InsP_3$ receptors.

Conclusions. Most active inositol phosphate analogs appear to be full agonists of $InsP_3$ receptors (Potter and Lampe, 1995), although it must be recognized that the assays used in most laboratories do not measure rates of Ca^{++} release and may thereby fail to detect a partial agonist unless it has very low intrinsic activity.

Several phosphorothioate analogs of $InsP_3$ (Safrany et al., 1993), including 3-deoxy-3-fluoro-inositol-1-phosphate 4,5-bisphosphorothioate (Wilcox et al., 1997) and D-*myo*-inositol-1,4,6-trisphosphorothioate (Mills et al., 1995), and perhaps some deoxy analogs (Mezna and Michelangeli, 1996), Ins(2,4,5)P_3 (Marchant et al., 1997b), Ins(1,2,3,5)P_4 (Burford et al., 1997), and Ins(1,3,4,6)P_4 (Gawler et al., 1991) have all been reported to be partial agonists of $InsP_3$ receptors. However, each of these partial agonists has a substantially lesser affinity than Ins(1,4,5)P_3 for the receptor; even the highest affinity analog has 10-fold lower affinity than Ins(1,4,5)P_3 (Wilcox et al., 1997). Acyclophostin is the highest affinity partial agonist of $InsP_3$ receptors so far identified.

The interaction between acyclophostin and $InsP_3$ receptors is unusual in that acyclophostin is a full agonist at pH 7 but a partial agonist at pH 8.3 (Fig. 4). Another analog, 3-amino-3-deoxy-Ins(1,4,5)P_3, has also been reported to show pH-dependent efficacy: it is a full agonist at pH 7.2 but a partial agonist at pH 6.8 (Kozikowski et al., 1994). It is not, however, clear whether under the conditions used for those comparisons, the decrease in pH was not also accompanied by a substantial increase in free [Ca^{++}], which would inhibit the type 1 $InsP_3$ receptors of the SH-SY5Y cells used for the assays. We cannot yet resolve whether the pH-dependent ability of acyclophostin to activate $InsP_3$ receptors results from an effect of pH on the receptor or the ligand. The former would be intriguing because it would suggest that from among the many analogs examined, acyclophostin is uniquely dependent on specific pH-sensitive residues within the receptor to cause channel opening subsequent to its binding to the receptor.

Unfortunately, there have been only a few systematic studies of the pK_a values of the phosphate groups of inositol phosphates; furthermore, values measured in aqueous media may differ substantially from those in less polar solvents (Tribolet and Sigel, 1987), which may more closely mimic the environment within the binding site of the receptor. The pK_a values for Ins(1,3,4,5)P_4 and Ins(1,2,4,5)P_4 have been determined (Guédat et al., 1997), as have those for a conformationally restricted $InsP_3$ analog (Riley et al., 1998). In light of these data, the phosphate groups of adenophostin are unlikely to be fully ionized at pH 7 or pH 8.3, and by analogy with Ins(4,5)P_2, the bisphosphate motif might be expected to have third and fourth pK_a values at about 6 and 8 (Schmitt et al., 1993) (i.e., within the pH range that affects the efficacy of acyclophostin). Although the 3-hydroxyl of Ins(1,4,5)P_3 and the 5″-hydroxymethyl group of acyclophostin may have different effects on the pK_a of the neighboring (4 or 4″)-phosphate, it is difficult to envisage large differences in the phosphate pK_a values for adenophostin and acyclophostin. The conformational restriction imposed by the furanoside ring of adenophostin may influence the pK_a of its 2′-phosphate, and that ring is, of course, missing from acyclophostin, where the 2′-phosphate may interact directly with the base (Fig. 1). In short, without directly determining the pK_a values of acyclophostin and comparing them with the other analogs, it is impossible to eliminate the possibility that the pH-dependent efficacy of acyclophostin results from a conformational change in the ligand. An alternative explanation would be that although Ins(1,4,5)P_3 and acyclophostin share substantially overlapping binding sites, they may differ in the receptor residues they contact to cause channel opening.

We conclude that acyclophostin has the highest affinity of any adenophostin analog with a modified ring structure so far synthesized and that its unusual pH-dependent efficacy may result from effects of pH on either the ligand or the receptor. The latter would suggest that different agonists may use different residues within a receptor to cause its activation.

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References

Acharya JK, Jalink K, Hardy RW, Hartenstein V and Zuker CS (1997) $InsP_3$ receptor is essential for growth and differentiation but not for vision in *Drosophila*. *Neuron* **18:**881–887.

Berridge MJ (1997) Elementary and global aspects of calcium signalling. *J Physiol* **499:**291–306.

Burford NT, Nahorski SR, Chung S-K, Chang Y-T and Wilcox RA (1997) Binding and activity of the nine possible regioisomers of *myo*-inositol tetrakisphosphate at the inositol 1,4,5-trisphosphate receptor. *Cell Calcium* **1:**301–310.

De Smedt H, Missiaen L, Parys JB, Henning RH, Sienaert I, Vanlingen S, Gijsens A, Himpens B and Caseels R (1997) Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *Biochem J* **322:**575–583.

DeLisle S, Blondel O, Longo FJ, Schnabel WE, Bell GI and Welsh MJ (1996) Expression of inositol 1,4,5-trisphosphate receptors change the Ca^{2+} signal of *Xenopus* oocytes. *Am J Physiol* **270:**C1255–C1261.

Gawler DJ, Potter BVL, Gigg R and Nahorski SR (1991) Interactions between inositol tris- and tetrakis-phosphates: Effects on Ca^{2+} mobilization in SH-SY5Y cells. *Biochem J* **276:**163–167.

Guédat P, Schlewer G, Krempp E, Riley AM, Potter BVL and Speiss B (1997) Investigation of the inframolecular acid-base properties of D-*myo*-inositol 1,3,4,5-tetrakisphosphate and DL-*myo*-inositol 1,2,4,5-tetrakisphosphate. *J Chem Soc Chem Commun* **625:**626.

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

Jenkins DJ and Potter BVL (1996) A Ca^{2+}-mobilising carbohydrate-based polyphosphate: Synthesis of 2-hydroxyethyl α-D-glucopyranoside-2′,3.4-trisphosphate. *Carb Res* **287:**169–182.

Kozikowski AP, Fauq AH, Wilcox RA, Challiss RAJ and Nahorski SR (1994) The novel Ins(1,4,5)P_3 analogue 3-amino-3-deoxy-Ins(1,4,5)P_3: A pH-dependent Ins(1,4,5)P_3 receptor partial agonist in SH-SY5Y neuroblastoma cells. *J Med Chem* **37:**868–872.

Marchant JS, Beecroft MD, Riley AM, Jenkins DJ, Marwood RD, Taylor CW and Potter BVL (1997a) Disaccharide polyphosphates based on adenophostin A activate hepatic *d-myo*-inositol 1,4,5-trisphosphate receptors. *Biochemistry* **36:**12780–12790.

Marchant JS, Chang Y-T, Chung S-K, Irvine RF and Taylor CW (1997b) Rapid kinetic measurements of $^{45}Ca^{2+}$ mobilization reveal that Ins(2,4,5)P_3 is a partial agonist of hepatic $InsP_3$ receptors. *Biochem J* **321:**573–576.

Marchant JS and Taylor CW (1998) Rapid activation and partial inactivation of inositol trisphosphate receptors by inositol trisphosphate. *Biochemistry* **37:**11524–11533.

Marshall ICB and Taylor CW (1994) Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochem J* **301:**591–598.

Mezna M and Michelangeli F (1996) The effects of inositol 1,4,5-trisphosphate ($InsP_3$) analogues on the transient kinetics of Ca^{2+} release from cerebellar microsomes. *J Biol Chem* **271:**31818–31823.

Mikoshiba K (1997) The $InsP_3$ receptor and intracellular Ca^{2+} signaling. *Curr Opin Neurobiol* **7:**339–345.

Mills SJ, Riley AM, Murphy CT, Bullock AJ, Westwick J and Potter BVL (1995) *myo*-Inositol 1,4,6-trisphosphorothioate and *myo*-inositol 1,3,4-trisphosphorothioate: New synthetic Ca^{2+}-mobilizing partial agonists at the inositol 1,4,5-trisphosphate receptor. *Bioorg Med Chem Lett* **5:**203–308.

Missiaen L, Parys JB, De Smedt H, Sienaert I, Sipma H, Vanlingen S, Maes K and Casteels R (1997) Effect of adenine nucleotides on *myo*-inositol-1,4,5-trisphosphate-induced calcium release. *Biochem J* **325:**661–666.

Missiaen L, Parys JB, 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.

Murphy CT, Riley AM, Lindley CJ, Westwick J and Potter BVL (1997) Structural analogues of D-*myo*-inositol-1,4,5-trisphosphate and adenophostin A: Recognition by cerebellar and platelet inositol-1,4,5-trisphosphate receptors. *Mol Pharmacol* **52:**741–748.

Nunn DL and Taylor CW (1990) Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptors. *Biochem J* **270:**227–232.

Patel S, Morris SA, Adkins CE, O'Beirne G and Taylor CW (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 USA* **94:**11627–11632.

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

Prpic V, Green KC, Blackmore PF and Exton JH (1984) Vasopressin-, angiotensin II-, and α_1-adrenergic-induced inhibition of a Ca^{2+} transport by rat liver plasma membrane vesicles. *J Biol Chem* **259:**1382–1385.

Putney JW Jr (1997) *Capacitative Calcium Entry*. R. G. Landes Company, Austin.

Richardson A and Taylor CW (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.

Riley AM, Guédat P, Schlewer G, Spiess B and Potter BVL (1998) A conformationally restricted cyclic phosphate analogue of inositol trisphosphate: Synthesis and physicochemical properties. *J Org Chem* **63:**295–305.

Safrany ST, Wilcox RA, Liu C, Dubreuil D, Potter BVL and Nahorski SR (1993) Identification of partial agonists with low intrinsic activity at the inositol-1,4,5-trisphosphate receptor. *Mol Pharmacol* **43:**499–503.

Schmitt L, Bortmann P, Schlewer G and Spiess B (1993) *myo*-Inositol 1,4,5-trisphosphate and related compounds' protonation sequence: Potentiometric and ^{31}P-NMR studies. *J Chem Soc Perkin Trans* **2:**2257–2263.

Sugawara H, Kurosaki M, Takata M and 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.

Takahashi M, Tanzawa K and Takahashi S (1994a) 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 and Takahashi M (1994b) 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 and Matsuda A (1998) Synthesis of 1-*O*-[3*S*,4*R*)-3-hydroxytetrahydrofuran-4-yl]-α-D-glucopyranoside 3,4,3′-trisphosphate as a novel potent IP_3 receptor ligand. *Tetrahedron Lett* **39:**5065–5068.

Tribolet R and Sigel H (1987) Self-association and protonation of adenosine 5-monophosphate in comparison with its 2- and 3-analogues and tubercidin 5-monophosphate (7-deaza-AMP). *Eur J Biochem* **163:**353–363.

Van Straten NCR, Van der Marel GA and Van Boom JH (1997a) Synthesis of 3′′,4′′-bisphosphate-containing analogs of adenophostin A. *Tetrahedron* **53:**6539–6554.

Van Straten NCR, Van der Marel GA and Van Boom JH (1997b) An expeditious route to the synthesis of adenophostin A. *Tetrahedron* **53:**6509–6522.

Van Straten NCR, Van der Marel GA and Van Boom JH (1997c) Synthesis of 2′,3′′,4′′-trisphosphate-containing analogs of adenophostin A. *Tetrahedron* **53:**6523–6538.

Wilcox RA, Erneux C, Primrose WU, Gigg R and 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.

Wilcox RA, Fauq A, Kozikowski AP and Nahorski SR (1997) Defining the minimal structural requirements for partial agonism at the type I *myo*-inositol 1,4,5-trisphosphate receptor. *FEBS Lett* **402:**241–245.

Wojcikiewicz RJH (1995) Type I, II and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J Biol Chem* **270:**11678–11683.

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