

Structural Analogues of D-*myo*-Inositol-1,4,5-trisphosphate and Adenophostin A: Recognition by Cerebellar and Platelet Inositol-1,4,5-trisphosphate Receptors

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Received March 28, 1997; Accepted June 20, 1997

SUMMARY

Adenophostins A and B, which are metabolic products of the fungus *Penicillium brevicompactum*, are potent agonists at the D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P₃] receptor. In the current study, adenophostin A was ~50-fold more potent than Ins(1,4,5)P₃ at both releasing Ca²⁺ from the intracellular stores of permeabilized platelets and displacing [³H]Ins(1,4,5)P₃ from its receptor on rat cerebellar membranes. Various analogues bearing structural features found in the adenophostins and/or Ins(1,4,5)P₃ were examined to elucidate the molecular basis for the observed enhanced potency. 2-AMP did not induce Ca²⁺ release from permeabilized platelets or have any effect on Ins(1,4,5)P₃-induced Ca²⁺ release. Two carbohydrate-based analogues, (2-hydroxyethyl)-α-D-glucopyranoside-2',3,4-trisphosphate and α,α'-trehalose-3,4,3',4'-tetrakisphosphate, could induce release of Ca²⁺ and displace [³H]Ins(1,4,5)P₃ from its binding site on rat cerebellar membranes, although both were less potent than Ins(1,4,5)P₃. In

common with adenophostin A, release of Ca²⁺ from the intracellular stores could be inhibited by heparin, and both analogues were metabolically resistant. This study is the first to demonstrate the activity of a synthetic disaccharide at the Ins(1,4,5)P₃ receptor and that the Ins(1,4,5)P₃ receptor is capable of accommodating an increased steric bulk. The minimal importance of the 2-hydroxyl group of Ins(1,4,5)P₃ (occupied by the pyranoside oxygen in adenophostin) was confirmed by comparing the activity of DL-scyllo-Ins(1,2,4)P₃ [which differs from Ins(1,4,5)P₃ solely by the orientation of this hydroxyl group] with that of Ins(1,4,5)P₃. An analogue of this compound, namely, DL-6-CH₂OH-scyllo-Ins(1,2,4)P₃, which possesses an equatorial hydroxymethyl group analogous to the 5'-hydroxymethyl group of adenophostin, was found to be equipotent to Ins(1,4,5)P₃, demonstrating the tolerance of the Ins(1,4,5)P₃ receptor to additional steric bulk at this position.

An elevation in the intracellular levels of Ca²⁺ is known to be a key signaling event coupling cell activation by a wide range of extracellular stimuli to characteristic physiological responses. The ligation of plasma membrane receptors coupled to heterotrimeric G proteins or associated with cytosolic tyrosine kinases causes activation of members of the phospholipase C family of enzymes. Activated phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate to generate the two signaling molecules *sn*-1,2-diacylglycerol and Ins(1,4,5)P₃. *sn*-1,2-Diacylglycerol is the endogenous activator of the serine/threonine-specific family of protein kinases, termed protein kinase C (1), and Ins(1,4,5)P₃ is responsible for mediating the release of Ca²⁺ by binding to specific receptors on specialized intracellular storage sites (2,3). Three

Ins(1,4,5)P₃-R subtypes, together with splice variants of each of these, have been identified, and the genes have been cloned (4, 5). The Ins(1,4,5)P₃-gated Ca²⁺ channel has been demonstrated to exist as a complex of Ins(1,4,5)P₃-R subunits (6) and may be homotetrameric or heterotetrameric (7).

In non-voltage-excitable cells, release of intracellular Ca²⁺ by Ins(1,4,5)P₃ is followed by entry of Ca²⁺ into the cells across the plasma membrane by a mechanism termed "store-operated" Ca²⁺ entry that is dependent on the filling state of the intracellular Ca²⁺ store (8); therefore not only is Ins(1,4,5)P₃ directly responsible for the release of Ca²⁺ from the intracellular stores, but through depletion of these stores, it is indirectly responsible for Ca²⁺ entry (9, 10).

Due to the pivotal role of Ins(1,4,5)P₃ in intracellular signal transduction pathways, there has been much attention directed at determining the structural motifs of Ins(1,4,5)P₃ responsible for its receptor binding capability and Ca²⁺-releasing activity (11). The structure-activity studies per-

This work was supported by the British Heart Foundation (J.W., C.T.M.) and The Wellcome Trust with project (J.W., B.V.L.P.) and program grant (B.V.L.P.) support and the Biotechnology and Biological Research Council, Intracellular Signaling Programme (B.V.L.P.).

ABBREVIATIONS: Ins(1,4,5)P₃, D-*myo*-inositol-1,4,5-trisphosphate; Glc(2',3,4)P₃, (2-hydroxyethyl)-α-D-glucopyranoside-2',3,4-trisphosphate; Trehal(3,4,3',4')P₄, α,α'-trehalose-3,4,3',4'-tetrakisphosphate; DL-scyllo-Ins(1,2,4)P₃, DL-scyllo-inositol-1,2,4-trisphosphate; DL-6-CH₂OH-scyllo-Ins(1,2,4)P₃, DL-6-deoxy-6-hydroxymethyl-scyllo-inositol-1,2,4-trisphosphate; Ins(1,4,5)P₃-R, inositol-1,4,5-trisphosphate receptor.

formed with Ins(1,4,5)P₃ analogues have indicated a key role for the vicinal diequatorial 4,5-bisphosphate system in mediating Ca²⁺ release (12–14), whereas an equatorial 6-OH is thought to be responsible for enhanced binding (15, 16). Both adenophostin A (Fig. 1) and its 6''-O-acetylated homologue adenophostin B possess equivalent features in the form of the glucose-3,4-bisphosphate and the adjacent 2-hydroxyl group, with the pyranoside oxygen acting as a surrogate of C-2 in Ins(1,4,5)P₃. A direct equivalent to the third phosphate group at position 1 of Ins(1,4,5)P₃ is not present in the adenophostins, but they both bear a phosphate group at position 2' of ribose. Removal of this phosphate group has been demonstrated to cause a 1000-fold reduction in binding affinity.(17)

To investigate the molecular basis for the high potency of adenophostin A, we examined the biological activity of several molecules bearing different structural relationships to adenophostin A and/or Ins(1,4,5)P₃ and have compared these with both adenophostin A and Ins(1,4,5)P₃ (Fig. 1). In this study, we report the Ca²⁺-releasing activity of Ins(1,4,5)P₃, adenophostin A, and these structurally related compounds in permeabilized rabbit platelets together with their ability to displace [³H]Ins(1,4,5)P₃ from its receptor in rat cerebellum.

Experimental Procedures

Materials

Chemically synthesized Ins(1,4,5)P₃ was purchased from the Rhode Island Chemical Group (Kingston, RI). Fura-2 (pentapotassium salt) was from Molecular Probes (Eugene, OR). [³H]Ins(1,4,5)P₃ (20–60 Ci/mmol, 10 μCi/ml) and ⁴⁵Ca²⁺ (5–50 mCi/mg Ca²⁺, 2 mCi/ml) were purchased from Amersham International (Buckingham-

shire, UK). FP100 filters were purchased from Whatman (Maidstone, UK). Heparin, oligomycin, creatine phosphokinase, phosphocreatine, saponin A, leupeptin, pepstatin, and ATP were obtained from Sigma Chemical (Poole, Dorset, UK). Ionomycin was purchased from Calbiochem (San Diego, CA). Adenophostin A was a generous gift from Dr. M. Takahashi (Sankyo, Tokyo, Japan). 2'-AMP-free acid purchased from Sigma was converted into the triethylammonium salt to increase its aqueous solubility, and its purity was confirmed by ³¹P NMR and high performance liquid chromatography. Glc(2',3,4)P₃ was synthesized as previously described (18), and Trehal(3,4,3',4')P₄ was synthesized in a similar fashion (19). Racemic 6-CH₂OH-*scyllo*-Ins(1,2,4)P₃ (13) and *scyllo*-Ins(1,2,4)P₃ (20) were synthesized as previously described.

Methods

Preparation of platelets. Washed rabbit platelets were prepared as previously described (21). The resulting platelet pellet from this preparation was resuspended in HEPES-buffered Tyrode's solution (consisting of 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.5 mM Na₂HPO₄, 5.5 mM glucose, 0.25% bovine serum albumin, pH 7.4) before performing the following procedures.

⁴⁵Ca²⁺ release from intracellular stores. Platelets were washed in high-K⁺ buffer A [consisting of 120 mM KCl, 2 mM KH₂PO₄, 5 mM (CH₃COONa)₂O · 6H₂O, 6 mM MgCl₂, 20 mM HEPES, in MilliQ water; 5 mM ATP was added, pH adjusted to 6.9, and free Ca²⁺ concentration adjusted to <150 nM] and then suspended to 3 × 10⁹/ml. The platelets were then permeabilized with 40 μg/ml saponin A, which was removed by further washing in buffer A. The intracellular Ca²⁺ stores were loaded with ⁴⁵Ca²⁺ (2 μCi/ml) for 1 hr in the presence of 10 μg/ml oligomycin. Total release of ⁴⁵Ca²⁺ from the stores was determined by a 3-min incubation with 75 μM ionomycin. Release of ⁴⁵Ca²⁺ from the intracellular stores was determined at 4°, 3 min after the addition of either Ins(1,4,5)P₃, adenophostin A or the

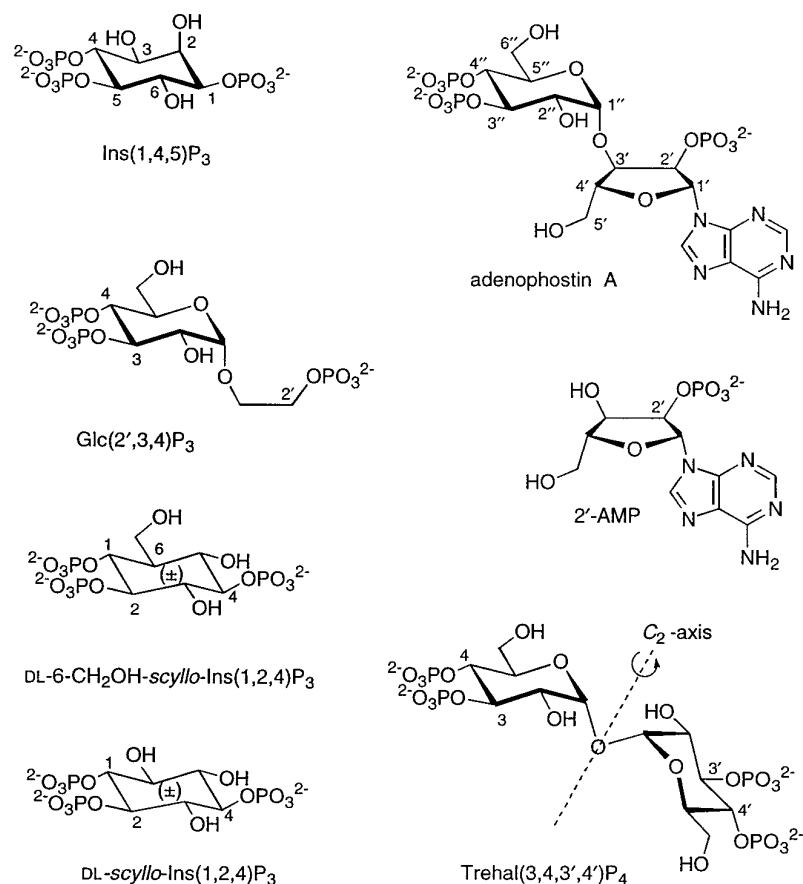


Fig. 1. Molecular structures of Ins(1,4,5)P₃, adenophostin A, and analogues.

structural analogues by separation of free and retained $^{45}\text{Ca}^{2+}$ through filtration of cells using Whatman FP100 filters. $^{45}\text{Ca}^{2+}$ release was determined by liquid scintillation spectroscopy (22).

Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized platelets monitored by spectrophotofluorimetry. Platelets were isolated and washed as above and then resuspended in high K⁺ buffer B (consisting of 100 mM KCl, 20 mM NaCl, 5 mM MgCl₂, 20 mM HEPES, 2 mM EGTA, pH 7.2) at a concentration of $3 \times 10^9/\text{ml}$. After permeabilization with 40 $\mu\text{g}/\text{ml}$ Saponin A (1 min, 20°), the platelets were washed again in buffer B in the absence of EGTA but in the presence of 20 units/ml creatine phosphokinase and 10 $\mu\text{g}/\text{ml}$ oligomycin according to a modification of a previously described method (23). Ca²⁺ uptake into stores was initiated by the addition of 3 mM ATP and 50 mM phosphocreatine. Ca²⁺ release from the stores was monitored using Fura-2 (free acid, 0.5 μM) in the extracellular buffer. Changes in fluorescence were measured using a PTI dual-wavelength spectrophotofluorimeter (excitation, 340 and 380 nm; emission, 510 nm; slit width, 4 nm). Experiments were performed at 20°. The traces shown in the figures represent an increase in fluorescence of Fura-2 that is due to the transient release of Ca²⁺ from the intracellular stores followed by a decrease in fluorescence that is due to Ca²⁺ resequestration. The Ca²⁺/Fura-2-fluorescence was calibrated as previously described (24).

Displacement of [³H]Ins(1,4,5)P₃ binding to specific Ins(1,4,5)P₃-Rs on rat cerebellar membranes. The preparation of rat cerebellar membranes and displacement of [³H]Ins(1,4,5)P₃ bound to the Ins(1,4,5)P₃-Rs on the membranes were performed as previously described (25). Briefly, the cerebella were removed from six rats (200–250 g) and homogenized (twice at 10 sec at 4°) in buffer C (consisting of 20 mM Tris-HCl, 20 mM NaCl, 100 mM KCl, 1 mM EDTA, 1 mg/ml bovine serum albumin, pH 7.7) containing the protease inhibitors 10 μM leupeptin and 10 μM pepstatin. After centrifugation ($50,000 \times g$ for 13 min at 4°), the pellet was resuspended in buffer C and homogenized as described above, and the protein content was adjusted to 5 mg/ml. The cerebellar membranes were either used immediately or frozen (–80°) until use. The binding assay mixture in a total volume of 250 μl contained 1 nM [³H]Ins(1,4,5)P₃ and structural analogues diluted in buffer C at appropriate concentrations. Binding was initiated by the addition of 250 μg of the cerebellar membrane preparation. The assay tubes were incubated (4°) for 10 min before termination of the reaction by centrifugation ($10,000 \times g$ for 4 min at 4°). Nonspecific binding of [³H]Ins(1,4,5)P₃ was assessed as the counts remaining on inclusion of 10 μM nonradiolabeled Ins(1,4,5)P₃ in the assay mixture. After centrifugation, the supernatant was carefully removed, the pellet was resuspended,

and radioactivity bound to the cerebellar membrane was determined by liquid scintillation counting.

Results

Ca²⁺ release from permeabilized platelets. Rabbit platelets permeabilized with saponin and in the presence of oligomycin displayed ATP-dependent $^{45}\text{Ca}^{2+}$ uptake into their nonmitochondrial stores. Uptake reached a steady state by 45 min and was monitored throughout the time course of the experiment and found to remain essentially unchanged. The ionomycin-releasable component of accumulated $^{45}\text{Ca}^{2+}$ was found to be >92%; again, this was not found to change significantly throughout the time course of any of the $^{45}\text{Ca}^{2+}$ -release experiments undertaken.

Treatment of permeabilized platelets with Ins(1,4,5)P₃ (0.003–30 μM) for 3 min (4°) caused a dose-dependent release of $^{45}\text{Ca}^{2+}$ from preloaded intracellular stores (Fig. 2). A time of 3 min was chosen because $^{45}\text{Ca}^{2+}$ release had reached a maximal plateau at this time (results not shown). Adenophostin A (0.0001–0.3 μM) also caused a dose-dependent release of $^{45}\text{Ca}^{2+}$ from the stores of permeabilized platelets; however, it displayed a ~55-fold lower EC₅₀ value than Ins(1,4,5)P₃ (Table 1). Synthetic carbohydrate-based analogues Glc(2',3,4)P₃ and Trehal(3,4,3',4')P₄ were also examined for their ability to release $^{45}\text{Ca}^{2+}$ from permeabilized platelets. Glc(2',3,4)P₃ (0.01–10 μM) dose-dependently released $^{45}\text{Ca}^{2+}$ from the intracellular stores of permeabilized platelets (Fig. 1) with an EC₅₀ value of $2.05 \pm 0.35 \mu\text{M}$, ~5-fold higher than Ins(1,4,5)P₃ (Table 1). Trehal(3,4,3',4')P₄ (0.1–300 μM) was also able to dose-dependently release $^{45}\text{Ca}^{2+}$ from the intracellular stores of permeabilized platelets (Fig. 2), with an EC₅₀ value of 100 μM , ~250-fold higher than Ins(1,4,5)P₃ (Table 1).

The above findings were extended through examination of the kinetics of Ca²⁺ release of Ins(1,4,5)P₃, adenophostin A, Glc(2',3,4)P₃, Trehal(3,4,3',4')P₄, and 2'-AMP. Ca²⁺ release was monitored in the presence of the fluorescent dye Fura-2 (free acid) by spectrophotofluorimetry. The addition of 1 μM Ins(1,4,5)P₃ caused release of Ca²⁺ from the intracellular stores of permeabilized platelets detected as a rapid increase

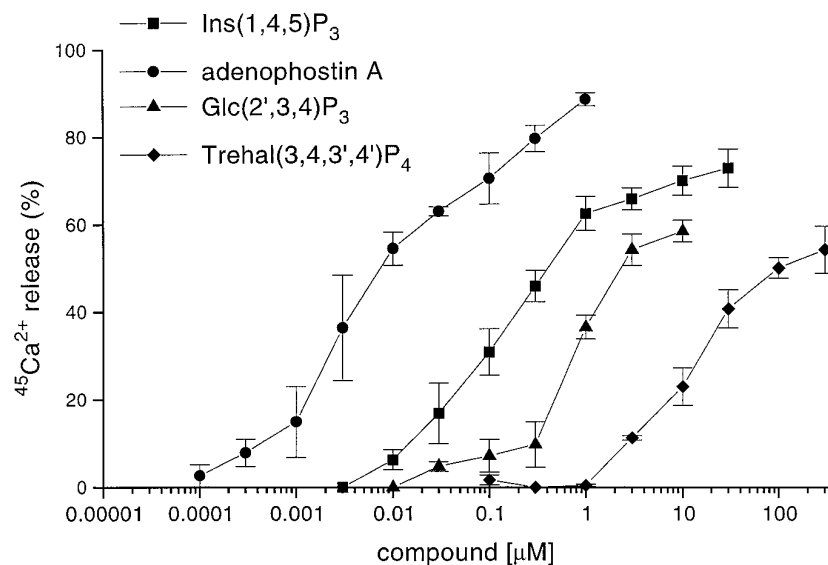


Fig. 2. Ins(1,4,5)P₃, adenophostin A-, Glc(2',3,4)P₃-, and Trehal(3,4,3',4')P₄-induced $^{45}\text{Ca}^{2+}$ release from permeabilized platelets. Permeabilized platelets preloaded with $^{45}\text{Ca}^{2+}$ were treated with Ins(1,4,5)P₃, adenophostin A, Glc(2',3,4)P₃, or Trehal(3,4,3',4')P₄ for 3 min at 4°. Release of $^{45}\text{Ca}^{2+}$ was terminated by rapid filtration and is given as a percentage of maximal $^{45}\text{Ca}^{2+}$ releasable on treatment of platelets with 75 μM ionomycin. Values are mean \pm standard error of six separate experiments performed in triplicate.

TABLE 1

Comparison of Ins(1,4,5)P₃, adenophostin A, 2'-AMP, Glc(2',3,4)P₃, Trehal(3,4,3',4')P₄, DL-scylo-Ins(1,2,4)P₃, and DL-6-CH₂OH-scylo-Ins(1,2,4)P₃ on ⁴⁵Ca²⁺ release and displacement of [³H]Ins(1,4,5)P₃

Compound	⁴⁵ Ca ²⁺ release (EC ₅₀)	Relative potency	Displacement of [³ H]Ins(1,4,5)P ₃ IC ₅₀	Relative potency
	μM		μM	
Ins(1,4,5)P ₃	0.4 ± 0.08	1	0.038 ± 0.005	1
Adenophostin A	0.0073 ± 0.0036	55	0.00074 ± 0.00042	51
2'-AMP	No release (1 mM)			
Glc(2',3,4)P ₃	2.05 ± 0.35	0.20	0.21 ± 0.07	0.18
Trehal(3,4,3',4')P ₄	100 ± 65	0.004	0.37 ± 0.17	0.10
DL-scylo-Ins(1,2,4)P ₃	1.67 ± 0.35	0.24	0.15 ± 0.018	0.25
DL-6-CH ₂ OH-scylo-Ins(1,2,4)P ₃	0.44 ± 0.26	0.91	0.027 ± 0.01	1.4

in the fluorescence of Fura-2 free acid (Fig. 3). The increase in fluorescence was transient, presumably due to the metabolism of Ins(1,4,5)P₃ to give inactive products, resulting in resequestration of Ca²⁺ back into the intracellular stores by Ca²⁺-ATPase activity. The addition of 200 μM 2'-AMP did not stimulate release of Ca²⁺ from the intracellular stores of permeabilized platelets and had no effect on Ins(1,4,5)P₃ binding to its receptor, as determined by the lack of effect on Ca²⁺ release by the subsequent addition of Ins(1,4,5)P₃ (Fig. 3, *top left*).

The addition of adenophostin A to permeabilized platelets caused a dose-dependent release of Ca²⁺ from the intracellular stores. However, unlike the Ca²⁺ release by Ins(1,4,5)P₃, which was transient, Ca²⁺ release by adenophostin A reached a maximum and was then maintained at a plateau phase over the time course of the experiment (Fig. 3, *top right*). Heparin, which is able to compete with Ins(1,4,5)P₃ for its binding site and inhibit Ca²⁺ release, was also found to inhibit release of Ca²⁺ induced by a submaximal concentration of adenophostin A (Fig. 3, *second from top left*). Glc(2',3,4)P₃ was also observed to release Ca²⁺ from the intracellular stores of platelets, detected as an increase in fluorescence of Fura-2 (Fig. 3, *second from top right*). As in the case of adenophostin A, there was a sustained elevation of the fluorescence signal, suggesting that Glc(2',3,4)P₃ is also poorly metabolized in this permeabilized platelet system. Again, Glc(2',3,4)P₃-induced Ca²⁺ release was inhibited by the Ins(1,4,5)P₃-R antagonist heparin (Fig. 3, *second from bottom left*). Trehal(3,4,3',4')P₄ also released Ca²⁺ from permeabilized platelets, and in correlation with the findings for ⁴⁵Ca²⁺ release, no increase in fluorescence (which indicates indicating Ca²⁺ mobilization) was detected until concentrations of Trehal(3,4,3',4')P₄ of >1 μM were applied to the cells (Fig. 3, *second from bottom right*). Trehal(3,4,3',4')P₄ also caused a sustained increase in the fluorescence signal, which, again, indicated poor metabolism of this compound in this permeabilized platelet system compared with Ins(1,4,5)P₃. Trehal(3,4,3',4')P₄-induced Ca²⁺ release was also inhibited by the Ins(1,4,5)P₃-R antagonist heparin (Fig. 3, *bottom left*).

Two racemic scyllo-inositol-based analogues, DL-scylo-Ins(1,2,4)P₃ and its 6-deoxy-6-hydroxymethyl homologue, DL-6-CH₂OH-scylo-Ins(1,2,4)P₃, were also evaluated in the ⁴⁵Ca²⁺-release assay (Fig. 4). Both compounds were relatively potent mobilizers of Ca²⁺, with EC₅₀ values 4-fold higher than and approximately equal to those of Ins(1,4,5)P₃, respectively (Table 1).

Displacement of specific [³H]Ins(1,4,5)P₃ binding to rat cerebellar membranes

[³H]Ins(1,4,5)P₃ was readily displaced from its specific binding site on rat cerebellar membranes by nonradiolabeled Ins(1,4,5)P₃ with an IC₅₀ of 0.038 ± 0.005 μM (Fig. 5, Table 1). Adenophostin A also displaced specifically bound [³H]Ins(1,4,5)P₃ from rat cerebellar membranes, although adenophostin A was around 50 times more potent than Ins(1,4,5)P₃ with an IC₅₀ value of 0.00074 ± 0.00042 μM (Fig. 5, Table 1).

Displacement of [³H]Ins(1,4,5)P₃ from the binding site on rat cerebellar membranes by Glc(2',3,4)P₃ was ~5-fold less effective than displacement by Ins(1,4,5)P₃ and ~280-fold less effective than by adenophostin A, whereas Trehal(3,4,3',4')P₄ was 10-fold less effective than Ins(1,4,5)P₃ and 500-fold less effective than adenophostin A. In agreement with the ⁴⁵Ca²⁺-release data, DL-6-CH₂OH-scylo-Ins(1,2,4)P₃ seemed to be equipotent to Ins(1,4,5)P₃ in displacement of [³H]Ins(1,4,5)P₃, whereas DL-scylo-Ins(1,2,4)P₃ was ~4-fold less potent (Fig. 6 and Table 1).

Discussion

There have been few studies in which the biological activity of the adenophostins has been examined (17, 26–28), and as a consequence, Ca²⁺ release by the adenophostins has been reported in only a small number of tissue types (26, 28). Takahashi *et al.* (26) first demonstrated the high potency of adenophostins A and B at the Ins(1,4,5)P₃-R and showed them to be equipotent at displacing [³H]Ins(1,4,5)P₃ from purified rat cerebellar Ins(1,4,5)P₃-Rs with an IC₅₀ value of 1.3 nM and more potent than Ins(1,4,5)P₃ with an IC₅₀ value of 23 nM. We have also demonstrated, using a rat cerebellar membrane preparation (25), that adenophostin A is more potent than Ins(1,4,5)P₃ at displacing [³H]Ins(1,4,5)P₃ with similar IC₅₀ values of 0.74 and 38 nM, respectively. In rat cerebellar microsomes, Takahashi *et al.* demonstrated the ED₅₀ values for Ca²⁺ release to be 1.4 and 170 nM for adenophostin A and Ins(1,4,5)P₃, respectively, whereas in permeabilized NG108–15 cells, the ED₅₀ values were 53 and 2400 nM, respectively (26). Therefore, in a cell-free system, adenophostin A was ~100-fold more potent at releasing Ca²⁺ than Ins(1,4,5)P₃, whereas in a permeabilized whole-cell system, adenophostin A was found to be ~45-fold more potent at releasing Ca²⁺. In agreement, we found adenophostin A to be ~55-fold more potent than Ins(1,4,5)P₃ at releasing Ca²⁺ from the intracellular stores of permeabilized rabbit platelets. Moreover, in a later study using purified type 1

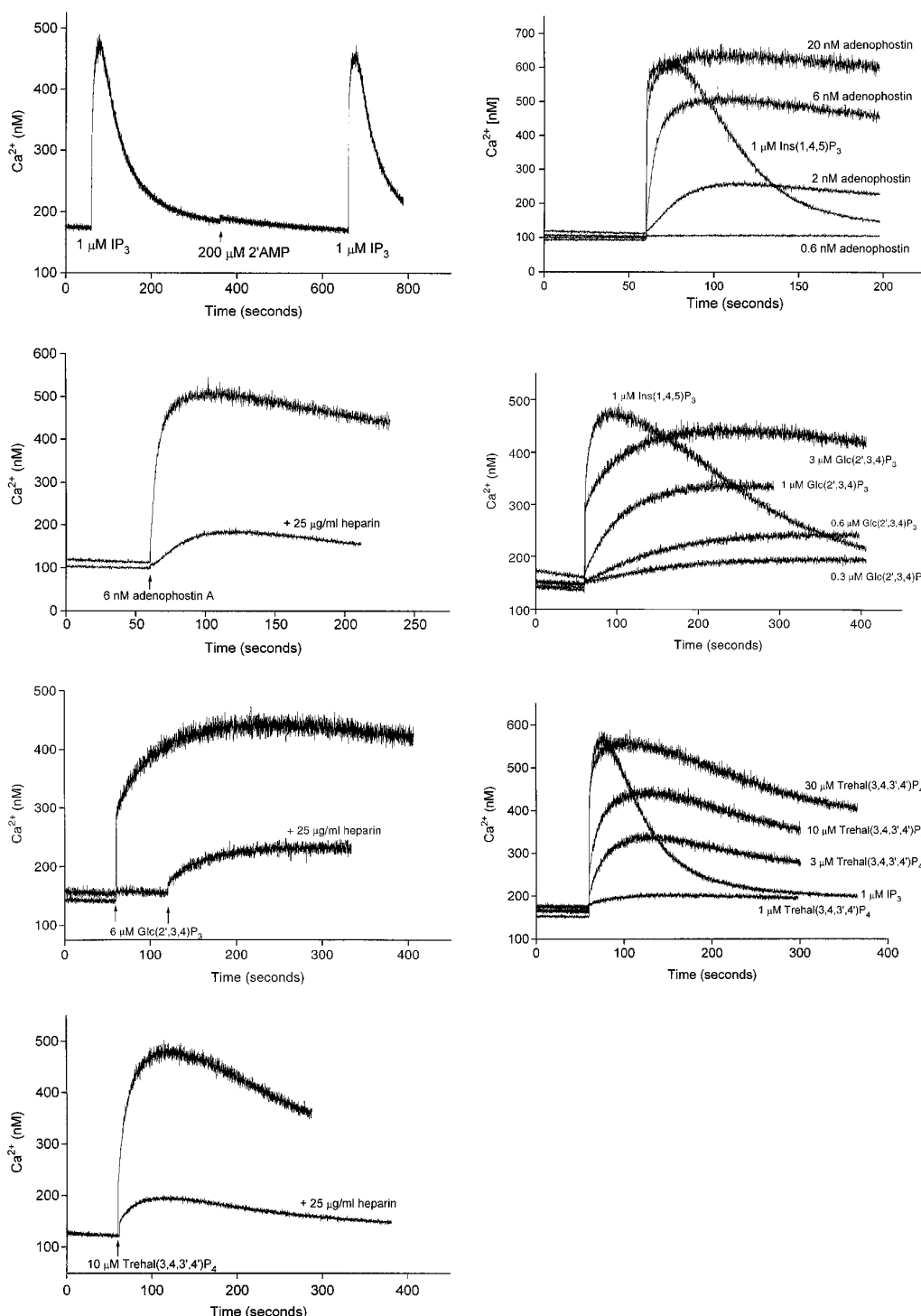


Fig. 3. Ca^{2+} mobilization induced by $\text{Ins}(1,4,5)\text{P}_3$, 2'-AMP, adenophostin A, $\text{Glc}(2',3,4)\text{P}_3$, and $\text{Trehal}(3,4,3',4')\text{P}_4$, as monitored by spectrophotofluorimetry. Release of Ca^{2+} from the intracellular stores of permeabilized platelets in the presence of Fura-2 (free acid) was monitored by spectrophotofluorimetry. Platelets were treated with $1\ \mu\text{M}\ \text{Ins}(1,4,5)\text{P}_3$, followed by $200\ \mu\text{M}\ 2'\text{-AMP}$ and then $1\ \mu\text{M}\ \text{Ins}(1,4,5)\text{P}_3$ (top left), adenophostin A (top right), heparin ($25\ \mu\text{g/ml}$, 2-min pretreatment) followed by 6 nM adenophostin A (second from top left), $\text{Glc}(2',3,4)\text{P}_3$ (second from top right), heparin ($25\ \mu\text{g/ml}$, 2-min pretreatment) followed by 6 $\mu\text{M}\ \text{Glc}(2',3,4)\text{P}_3$ (second from bottom left), $\text{Trehal}(3,4,3',4')\text{P}_4$ (second from bottom right), and heparin ($25\ \mu\text{g/ml}$, 2-min pretreatment) followed by $10\ \mu\text{M}\ \text{Trehal}(3,4,3',4')\text{P}_4$ (bottom left). Values are from a single experiment representative of three individual experiments.

$\text{Ins}(1,4,5)\text{P}_3$ -Rs, adenophostin had only a 10-fold higher potency than $\text{Ins}(1,4,5)\text{P}_3$. Release of $^{45}\text{Ca}^{2+}$ from permeabilized rabbit platelets by adenophostin was inhibited by the $\text{Ins}(1,4,5)\text{P}_3$ -R antagonist heparin, indicating interaction of adenophostin with the $\text{Ins}(1,4,5)\text{P}_3$ -R. Unlike $\text{Ins}(1,4,5)\text{P}_3$, however, adenophostin A caused a sustained, rather than transient, release of Ca^{2+} when added to permeabilized platelets, indicating that adenophostin A is resistant to the metabolizing enzymes located in permeabilized platelets.

To further elucidate the structural features responsible for the high potency of adenophostin A, we investigated the

biological activity of several compounds whose structures represent different aspects of the construction of adenophostin A. Two of the major differences between adenophostin A and $\text{Ins}(1,4,5)\text{P}_3$ are the adenosine component and the hydroxymethyl substituent, and compounds were prepared in an attempt to examine the contribution of each of these moieties.

The finding that 2'-AMP alone was inactive at releasing Ca^{2+} from the intracellular stores of permeabilized platelets supports the assumption that the activity of the adenophostins originates in the phosphorylated glucose compo-

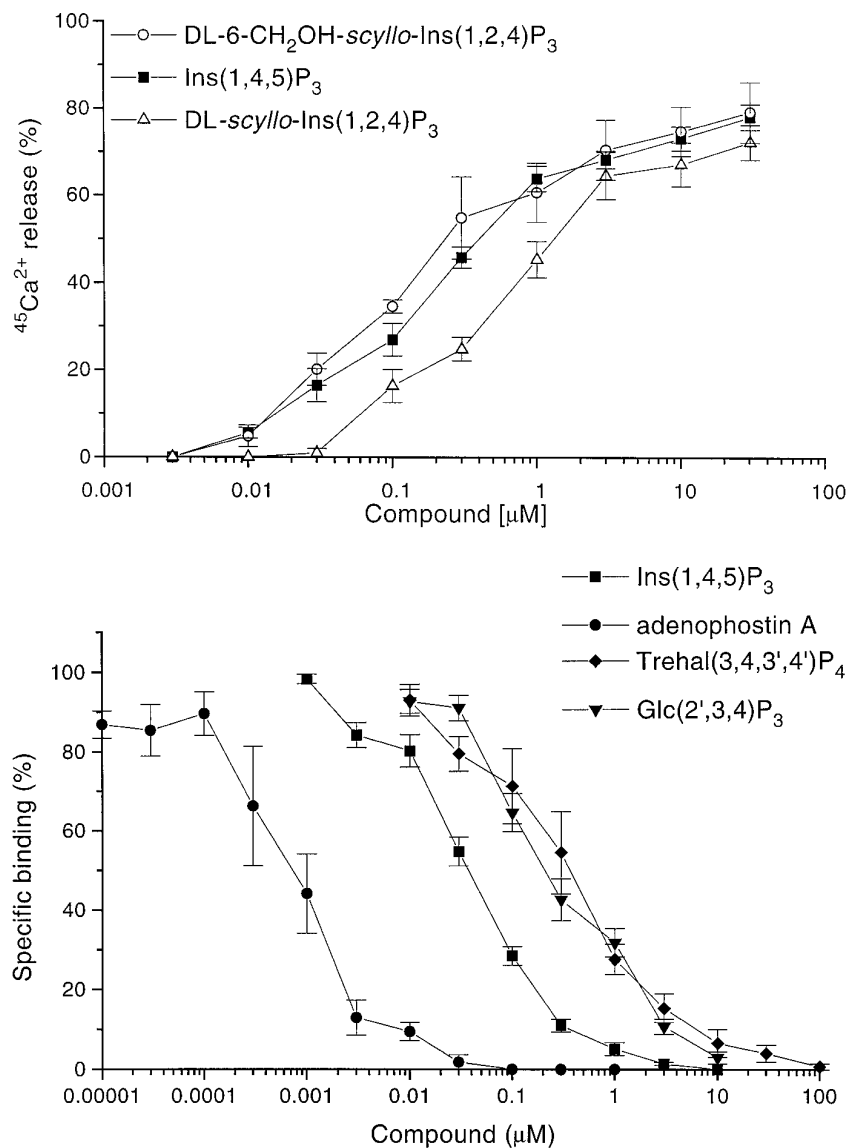


Fig. 4. Ins(1,4,5)P₃-, scyllol-Ins(1,2,4)P₃-, and 6-CH₂OH-scyllol-Ins(1,2,4)P₃-induced ⁴⁵Ca²⁺ release from permeabilized platelets. Permeabilized platelets preloaded with ⁴⁵Ca²⁺ were treated with Ins(1,4,5)P₃, DL-scyllol-Ins(1,2,4)P₃, or DL-6-CH₂OH-scyllol-Ins(1,2,4)P₃ for 3 min at 4°. Release of ⁴⁵Ca²⁺ was terminated by rapid filtration and is given as a percentage of maximal ⁴⁵Ca²⁺ releasable on treatment of platelets with 75 μM ionomycin. Values are mean ± standard error of six separate experiments, each performed in triplicate.

Fig. 5. Displacement of specific [³H]Ins(1,4,5)P₃ binding to Ins(1,4,5)P₃-Rs on rat cerebellar membranes by Ins(1,4,5)P₃, adenophostin A, Glc(2',3,4)P₃, and Trehal(3,4,3',4')P₄. Values are mean ± standard error of three experiments performed in duplicate.

ment, with the 3,4-bisphosphate/2-hydroxyl on the glucopyranose ring mimicking the key structures of Ins(1,4,5)P₃ responsible for Ca²⁺ release. However, the fact that the adenophostins are more potent than Ins(1,4,5)P₃ suggests not only that the Ins(1,4,5)P₃-R is able to accommodate the bulk of the adenosine component at the 1'' position of the glucopyranose ring but also that this structure is necessary for the high potency of the adenophostins.

The 1-phosphate group of Ins(1,4,5)P₃, although not essential for its activity, is thought to enhance its potency (15). It has been suggested that the 2'-phosphate on the ribose ring of the adenophostins may be positioned to fit the Ins(1,4,5)P₃-R more effectively than the 1-phosphate of Ins(1,4,5)P₃ and therefore further enhance the affinity of the adenophostins for the Ins(1,4,5)P₃-R (26). Indeed, the importance of this phosphate group at the 2'-position of the ribose ring was confirmed when its removal caused a 1000-fold reduction in the activity of adenophostin A (17). If a binding pocket for the adenosine moiety of the adenophostins exists at the Ins(1,4,5)P₃-R, it is possible that 2'-AMP alone might bind to the Ins(1,4,5)P₃-R, interfere with Ins(1,4,5)P₃ bind-

ing, and thus inhibit Ins(1,4,5)P₃-induced Ca²⁺ release. However, 2'-AMP had no effect on Ins(1,4,5)P₃-induced Ca²⁺ release in permeabilized platelets, indicating that when separated from the glucopyranose bisphosphate motif, 2'-AMP alone has no significant affinity for the Ins(1,4,5)P₃-R or, if able to bind, it does not interfere with Ins(1,4,5)P₃ binding or Ca²⁺-release activity.

Given that features from both the glucopyranose ring and the adenosine moiety of adenophostin A are necessary for its potent binding and Ca²⁺-releasing activity, it remains to be established which of these features are sufficient for such activity. Glc(2',3,4)P₃ possesses the glucopyranose ring of adenophostin, whereas all except C2 and C3 of the ribose ring and the 2'-phosphate group of the adenosine component have been removed (18). In common with adenophostin, Glc(2',3,4)P₃ was metabolically resistant when added to a permeabilized cell preparation, and Ca²⁺ release from the intracellular stores of permeabilized platelets was inhibited by the Ins(1,4,5)P₃-R antagonist heparin. However, although Glc(2',3,4)P₃ was found to both release Ca²⁺ from the intracellular stores of permeabilized rabbit platelets (EC₅₀ = 2.05

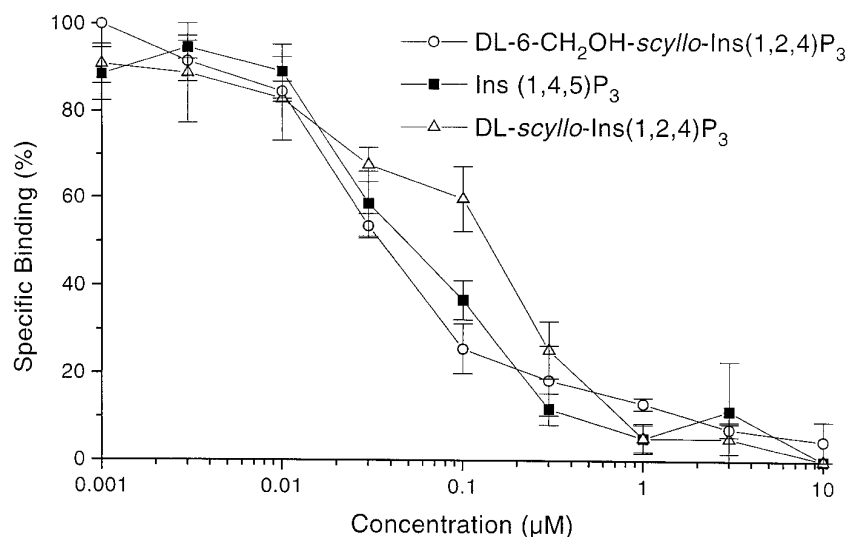


Fig. 6. Displacement of specific [³H]Ins(1,4,5)P₃ binding to rat cerebellar membranes by Ins(1,4,5)P₃, DL-scyлло-Ins(1,2,4)P₃, or DL-6-CH₂OH-scyлло-Ins(1,2,4)P₃. Values are mean ± standard error of three experiments performed in duplicate.

μM) and displace [³H]Ins(1,4,5)P₃ from the Ins(1,4,5)P₃ binding sites of rat cerebellar membranes (IC₅₀ = 0.21 μM), it was ~5-fold lower in potency than Ins(1,4,5)P₃ and ~280-fold weaker than adenophostin in both binding and Ca²⁺-release studies. Similarly, Wilcox *et al.* (29) reported a 5-fold lower affinity for Glc(2',3,4)P₃ compared with Ins(1,4,5)P₃ in binding studies using pig cerebellar membranes and a 10–12-fold lower potency for Glc(2',3,4)P₃ in Ca²⁺-release studies using SH-SY5Y and MKCK cells compared with Ins(1,4,5)P₃.

These findings initially suggested that the excised region of adenosine was important for conferring the extreme potency of the adenophostins. Alternatively, however, it may be that because the terminal 2'-position phosphate group of Glc(2',3,4)P₃ is not as spatially constrained as the 2'-phosphate on the ribose ring of adenophostin, it is not able to confer the same increased potency to Glc(2',3,4)P₃ (29); the 2'-position phosphate may be required to be held in a precise position with respect to the glucopyranose ring to increase its potency (26).

The tetrakisphosphate Trehal(3,4,3',4')P₄ is larger and conformationally more rigid than Glc(2',3,4)P₃ and consists of two copies of the phosphorylated glucopyranose component of adenophostin in a C₂ symmetrical molecule. This molecule possesses all the key features of Glc(2',3,4)P₃ believed to be important for Ca²⁺ release but, in addition, holds the phosphates of the second glucopyranose ring in a more rigid conformation. Again, like Glc(2',3,4)P₃ and adenophostin, Trehal(3,4,3',4')P₄ was demonstrated to be metabolically resistant, and release of Ca²⁺ was inhibited by heparin. Trehal(3,4,3',4')P₄ was found to be ~10-fold less potent than Ins(1,4,5)P₃ at displacing [³H]Ins(1,4,5)P₃ from its receptor on rat cerebellar membrane but ~250-fold less potent at releasing ⁴⁵Ca²⁺ from the stores of permeabilized platelets, a finding confirmed using dynamic measurements of Ca²⁺ release monitored in the presence of the Ca²⁺-specific fluorescent dye Fura-2 by spectrophotofluorimetry. This study is the first to demonstrate the activity of a synthetic disaccharide derivative at the Ins(1,4,5)P₃-R and to demonstrate that as predicted, the Ins(1,4,5)P₃-R is capable of accommodating the steric bulk of the second glucose residue of Trehal(3,4,3',4')P₄. A preliminary molecular modeling study of Trehal(3,4,3',4')P₄ and adenophostin A suggested that al-

though either one of the two equivalent glucopyranose-3,4-bisphosphate components of this molecule would be a good mimic of the equivalent structure in adenophostin A, neither phosphate group on the second ring would occupy a position in space equivalent to that of the 2'-phosphate in the adenophostins (19). The reason for the disparity in the Ca²⁺-release activity and [³H]Ins(1,4,5)P₃ displacement is not immediately clear, but it seems unlikely that it is due to the assay conditions because Glc(2',3,4)P₃ and adenophostin A gave the same relative potency in binding and Ca²⁺-release assays compared with Ins(1,4,5)P₃. Thus, it may be that one or both of the phosphate groups on the second ring of Trehal(3,4,3',4')P₄ somehow reduces the ability of this analogue to cause opening of the integral ion channel. This unusual aspect of the activity of Trehal(3,4,3',4')P₄ may have implications for the design of Ins(1,4,5)P₃-R antagonists.

Finally, the differences in activity between DL-scyлло-Ins(1,2,4)P₃ and DL-6-CH₂OH-scyлло-Ins(1,2,4)P₃ may have implications for the effect of the 5'-hydroxymethyl group in adenophostin A. The observation that DL-6-CH₂OH-scyлло-Ins(1,2,4)P₃ is equipotent with Ins(1,4,5)P₃ implies that the CH₂OH component, which is not present in Ins(1,4,5)P₃ itself, is tolerated by the Ins(1,4,5)P₃-R despite the additional steric bulk. The presence of an analogous structure in adenophostin A is in accordance with this finding. This observation is significant because previous studies of position 3-substituted Ins(1,4,5)P₃ analogues seemed to show that large substituents at this position were not tolerated by the receptor, and this led some researchers to suggest that adenophostin A must bind in a different orientation to Ins(1,4,5)P₃ to overcome the sterically handicapping CH₂OH group (29). Our results also imply that at least in scyлло-analogues of Ins(1,4,5)P₃, replacement of the secondary hydroxyl group at this position with an hydroxymethyl group enhances potency at the Ins(1,4,5)P₃-R. This motif could therefore be of interest in the design of Ins(1,4,5)P₃-R ligands.

On the basis of the activities of the analogues examined in the current study, we were able to draw several conclusions regarding the structural basis for the activity of the adenophostins. As we and others have previously suggested (18, 26, 29), the Ca²⁺-releasing activity of adenophostin A does indeed seem to reside in the 3,4-phosphorylated glucopyranose

motif, which mimics the most important parts of Ins(1,4,5)P₃. This activity is somehow augmented by the 2'-AMP component, although this structure, in isolation, cannot cause Ca²⁺-release or antagonize Ins(1,4,5)P₃-mediated Ca²⁺-release. The addition of a third phosphate group, as in Glc(2',3,4)P₃, which is theoretically able to access the area of the receptor available to the 2'-phosphate of the adenophostins, was not sufficient to confer adenophostin-like potency when this group was conformationally highly mobile. A phosphorylated disaccharide such as Trehal(3,4,3',4')P₄ can be accommodated by the receptor binding site, but again, it is likely that accurate placement of a phosphate group on the accessory residue would be required for high potency. Whether optimal placing of this third phosphate is all that is required for potency greater than that of Ins(1,4,5)P₃ remains to be seen. The presence of a 5''-CH₂OH component in adenophostin A did not require the glucopyranose component to interact with the receptor in a different way to Ins(1,4,5)P₃. Indeed, a molecule closely related to Ins(1,4,5)P₃ yet possessing this component [6-CH₂OH-*scyllo*-Ins(1,2,4)P₃] showed Ins(1,4,5)P₃-like potency; therefore, at least in *scyllo*-inositol analogues, a CH₂OH group at this position may give rise to a modest increase in potency, and a similar effect may apply to adenophostin A. Interestingly, in adenophostin B, the 5''-CH₂OH group is acetylated, giving even greater steric bulk yet no decrease in potency. It is not clear whether adenophostin-like potency can be attained by a simple disaccharide framework with appropriately placed phosphate and hydroxyl groups or whether something resembling the adenine ring system is also required. It may be that this structure serves to orient the third phosphate in a particular way at the receptor or that the adenine itself has favorable interactions with a region close to the Ins(1,4,5)P₃-binding site. Given the current state of knowledge, either or both of these alternatives are possible. A definitive resolution of this last point may be attainable by the synthesis and evaluation of disaccharide-like adenophostin analogues lacking the adenine structure.

Acknowledgments

We thank Dr. M. Takahashi for an authentic sample of adenophostin A.

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