0026-895X/95/061204-08$3.00/0

MOLECULAR PHARMACOLOGY, 47:1204–1211 (1995).

2-Hydroxyethyl-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

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Received January 11, 1995; Accepted March 14, 1995

SUMMARY

The novel, synthetic, adenophostin A analogue 2-hydroxyethyl-α-D-glucopyranoside-2,3′,4′-trisphosphate [Gluc(2,3′,4′)P_3] was synthesized to probe the structure-activity relationship at the D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] receptor [Ins(1,4,5)P_3R]. This study was stimulated by the recent observation that the fungal isolates adenophostins A and B were very potent, metabolically resistant, Ins(1,4,5)P_3R agonists [*J. Biol. Chem.* **269**:369–372 (1994)]. Gluc(2,3′,4′)P_3 can be visualized as a truncated version of adenophostin A, in which the 2′- and 3′-carbons of the ribose ring, with their terminal phosphate groups, are retained and the remainder of the adenosine residue is excised. Gluc(2,3′,4′)P_3 specifically displaced [^{3}H]Ins(1,4,5)P_3 from pig cerebellar Ins(1,4,5)P_3 binding sites, with an affinity (IC_{50} = 130 nM) only 5-fold weaker than that of Ins(1,4,5)P_3 (IC_{50} = 27 nM). Gluc(2,3′,4′)P_3 was also a full agonist for Ca^{2+} release, being only 10–12-fold less potent than Ins(1,4,5)P_3 in saponin-permeabilized SH-SY5Y neuroblastoma cells [EC_{50} = 647 nM; Ins(1,4,5)P_3 EC_{50} = 52 nM] and Madin-Darby canine kidney cells [EC_{50} = 2484 nM; Ins(1,4,5)P_3 EC_{50} = 247 nM]. Gluc(2,3′,4′)P_3 did not significantly interact with recombinant Ins(1,4,5)P_3 3-kinase and 5-phosphatase enzymes and was also poorly metabolized by saponin-permeabilized SH-SY5Y cells. However, Gluc(2,3′,4′)P_3 was a considerably weaker ligand (~500-fold) and agonist (~1000-fold) than adenophostin A, suggesting that the partial excision of the adenosine residue compromised structural motifs that have favorable interactions with the Ins(1,4,5)P_3R. Indeed, molecular dynamics simulations revealed that the potencies of the three compounds show a correlation with the relative distance of the two vicinal ring phosphates from the remaining phosphate. Gluc(2,3′,4′)P_3, with its α-glucoside ring, is the first synthetic Ins(1,4,5)P_3 analogue that is not structurally based on a phosphorylated inositol isomer and that exhibits potent activity at the Ins(1,4,5)P_3R. This, combined with the metabolic resistance of Gluc(2,3′,4′)P_3, thus affords a novel approach for the investigation of the cellular role of Ins(1,4,5)P_3 and its receptor.

Ins(1,4,5)P_3R are recognized as a homologous family of tetrameric, ligand-gated, Ca^{2+} channels, which allow mobilization of intracellular Ca^{2+} stores in response to activation of cell surface receptors linked to Ins(1,4,5)P_3 generation (1). Although molecular cloning studies have localized the ligand recognition site of the Ins(1,4,5)P_3R to the amino-terminal region (1) and photoaffinity ligands have been used to label the vicinity of the Ins(1,4,5)P_3R binding site (2), little progress has yet been made with respect to modeling the amino acid residues that define the Ins(1,4,5)P_3R binding pocket. In contrast, pharmacological approaches using synthetic analogues of Ins(1,4,5)P_3 to map the ligand binding site of the Ins(1,4,5)P_3R have yielded valuable information (reviewed in Refs. 3–5).

The interaction of Ins(1,4,5)P_3 with its receptor is highly stereospecific, with the vicinal 4,5-phosphate groups being a crucial structural feature and the 1-phosphate of Ins(1,4,5)P_3 further contributing to receptor binding specificity (reviewed in Ref. 3). Partial or total phosphorothioate substitution of

This work was supported by the Wellcome Trust, the Mental Health Foundation (R.A.W., S.R.N.), and the Ministére de la Politique Scientifique (PAIT) and FRSM (C.E.).

ABBREVIATIONS: Ins(1,4,5)P_3R, D-*myo*-inositol-1,4,5-trisphosphate receptor(s); Ins(1,4,5)P_3, D-*myo*-inositol-1,4,5-trisphosphate; Gluc(2,3′,4′)P_3, 2-hydroxyethyl-α-D-glucopyranoside-2,3′,4′-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol-1,3,4,5-tetrakisphosphate; Ins(1,3,4,6)P_4, D-*myo*-inositol-1,3,4,6-tetrakisphosphate; Ins(1,4,5)PS_3, D-*myo*-inositol-1,4,5-trisphosphorothioate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CLB, cytosol-like buffer; MDCK, Madin-Darby canine kidney; Ins(2,4,5)P_3, D-*myo*-inositol-2,4,5-trisphosphate.

the phosphate groups is also well tolerated, producing high affinity ligands that are resistant to metabolism catalyzed by the specific enzymes Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase (6–8). The role of the 2,3,6-hydroxyl groups of Ins(1,4,5)P_3 may be either to donate hydrogen bonds to or to accept hydrogen bonds from the receptor; alternatively, they may act to fix the solution conformation of Ins(1,4,5)P_3 by intramolecular hydrogen bonding to the neighboring phosphates (4). The 6-hydroxyl group appears to make a significant contribution to receptor interaction, because 6-deoxy-Ins(1,4,5)P_3 (9) and 2,3,6-trideoxy-Ins(1,4,5)P_3 (10) are both weak agonists. In contrast, the axial 2-hydroxyl and equatorial 3-hydroxyl groups *per se* do not appear to be as critical for Ins(1,4,5)P_3R binding and Ca^{2+} release, because 2-deoxy-DL-Ins(1,4,5)P_3 (11), 3-deoxy-Ins(1,4,5)P_3, and 2,3-dideoxy-Ins(1,4,5)P_3 (10) are all relatively potent Ins(1,4,5)P_3R agonists. The Ins(1,4,5)P_3R has remarkable tolerance for electronic and steric modification of the 2-position of Ins(1,4,5)P_3 with either axial or equatorial substituents, suggesting that the 2-hydroxyl group is not intimately associated with the receptor binding pocket (reviewed in Ref. 12). In contrast, inversion of the 3-hydroxyl (13, 14) produces loss of activity, whereas increases in the steric bulk of the 3-position substitutent of Ins(1,4,5)P_3 analogues correlate with progressively decreasing activity at the Ins(1,4,5)P_3R (15).

The Ca^{2+}-mobilizing action of Ins(1,4,5)P_3 is rendered transient by the action of two specific enzymes, Ins(1,4,5)P_3 5-phosphatase and Ins(1,4,5)P_3 3-kinase (3). The 5-phosphatase catalyzes the removal of the 5-phosphate group to produce the inactive inositol-1,4-bisphosphate (3), whereas 3-kinase catalyzes the phosphorylation of Ins(1,4,5)P_3 to generate Ins(1,3,4,5)P_4, which is a full but weaker agonist at the Ins(1,4,5)P_3R (15–17). Ins(1,4,5)P_3 3-kinase resembles the Ins(1,4,5)P_3R in exhibiting significant stereoselectivity and positional selectivity. In contrast, Ins(1,4,5)P_3 5-phosphatase appears to be considerably less selective, possessing affinity for most of the inositol phosphates and analogues yet described (3, 18).

Until very recently, interaction with the Ins(1,4,5)P_3R appeared to be limited to Ins(1,4,5)P_3, some inositol tetrakisphosphates such as Ins(1,3,4,6)P_4 (12) and Ins(1,3,4,5)P_4 (15), a limited number of synthetic *myo*-, *chiro*-, and *scyllo*-inositol trisphosphates or trisphosphorothioate analogues (3–5), and the nonselective antagonists heparin (19, 20) and decavanadate (21, 22). However, recently two very potent Ins(1,4,5)P_3R agonists, adenophostins A and B, exhibiting chemical structures distinct from that of Ins(1,4,5)P_3, were isolated from the culture broth of the fungus *Penicillium brevicompactum* (23–25). Indeed, adenophostins A and B are approximately 100-fold more potent at the Ins(1,4,5)P_3R than is the native ligand, while also being very resistant to metabolism via either Ins(1,4,5)P_3 3-kinase or 5-phosphatase activities (23). Here we report the initial characterization of Gluc(2,3′,4′)P_3 (Fig. 1), a novel synthetic Ins(1,4,5)P_3/adenophostin A analogue. Gluc(2,3′,4′)P_3 is structurally based on the α-glucoside moiety of adenophostin A and can be visualized as adenophostin A with the majority of the adenosine residue excised. Because Gluc(2,3′,4′)P_3 (and the α-glucoside moiety of adenophostin A) efficiently mimic many of the pertinent structural features required for Ins(1,4,5)P_3R interaction, our primary rationale for synthesizing Gluc(2,3′,4′)P_3 was to evaluate the significance of the adenosine residue of adenophostin A in conferring enhanced potency at the Ins(1,4,5)P_3R and metabolic resistance to the enzymes 3-kinase and 5-phosphatase.

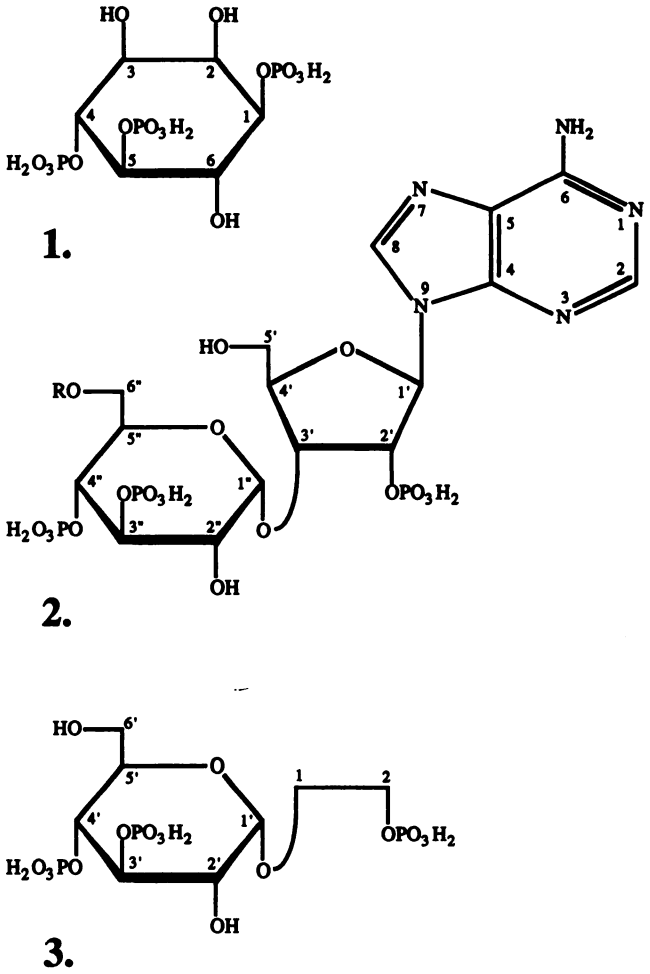

Fig. 1. Structures of Ins(1,4,5)P_3 (1), adenophostins A and B (2), and Gluc(2,3′,4′)P_3 (3). Note that Gluc(2,3′,4′)P_3 and adenophostins A and B can be visualized in a binding orientation that efficiently mimics many of the crucial structural features defining the interaction of Ins(1,4,5)P_3 with its receptor. For adenophostin A, R is H; for adenophostin B, R is $COCH_3$.

Experimental Procedures

Materials. Most reagents used were as described previously (16). Disodium ATP, EGTA, and low molecular weight heparin (M_r 4000–6000) were from Sigma, and fluo-3 was from Calbiochem. All other reagents were of the highest purity available. Chemically synthesized Ins(1,4,5)P_3 (26), as the hexapotassium salt, was obtained from the University of Rhode Island Foundation Chemistry Group. $^{45}CaCl_2$ (approximately 1000 Ci/mmol; Amersham) and [^{3}H]Ins(1,4,5)P_3 (41 Ci/mmol; Amersham) were used throughout. Gluc(2,3′,4′)P_3 was used as the hexasodium salt; its full synthesis will be described elsewhere.

Computer-assisted molecular modeling. Energy minimizations of structures and molecular dynamics simulations were run using the program Amber, within the software package Discover 2.9 (Biosym Technologies, San Diego, CA). The conformational freedom of Gluc(2,3′,4′)P_3 and adenophostin A were explored by a molecular dynamics simulation at 1000°K for 10 psec after initial equilibration for 0.1 psec. Constraints were applied to force the adjacent phosphates on the glucose ring to maintain their equatorial-equatorial conformations in both cases. These were set by applying a penalty

force of 100 kcal/mol/Å^2 if the phosphorus-phosphorus distance moved beyond the bounds of 5.2–5.4 Å [Gluc(2,3′,4′)P_3] or 5.4–5.6 Å (adenophostin A). The total energy and the interatomic distance between the three phosphates of each molecule were monitored in 0.05-psec steps throughout the dynamic simulation.

Cell culture. SH-SY5Y human neuroblastoma cell monolayers (passages 70–90), initially a gift from Dr. J. L. Biedler (Sloane-Kettering Institute, New York, NY), were subcultured and maintained as described (16), except that the culture medium was supplemented with 10% (v/v) newborn calf serum and 1% fetal calf serum (GIBCO). MDCK cells, initially a gift from the Virology Department at the National Institute for Medical Research (Mill Hill, UK), were passaged (1/10) weekly using a trypsin/EDTA solution (GIBCO), maintained in 175-cm^2 plastic flasks (Nunc), and fed with modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (GIBCO). Cell monolayers were harvested for experiments using a buffer consisting of 0.02% (w/v) Na_2EDTA (537 μM), 0.9% (w/v) NaCl (154 mM), and 10 mM HEPES (free acid), pH 7.2.

Ins(1,4,5)P_3 binding studies. Preparation of P_2 membranes from pig cerebellum and the [^{3}H]Ins(1,4,5)P_3 binding and displacement assays were performed as described (27). After incubation for 30 min at 4°, bound and free [^{3}H]Ins(1,4,5)P_3 were separated by high speed centrifugation (28,000 × *g* for 3 min at 4°) in a refrigerated microfuge (Heraeus Sepatech, Germany). Specifically bound [^{3}H]Ins(1,4,5)P_3 was routinely 2500 dpm/assay, and nonspecific binding (approximately 150–200 dpm/assay) was defined by addition of 10 μM Ins(1,4,5)P_3.

Ca^{2+} mobilization assay. $^{45}Ca^{2+}$ mobilization was assessed in saponin-permeabilized SH-SY5Y and MDCK cells at 20–22°, as described previously for SH-SY5Y cells (16), with each experiment performed in duplicate. Cells were used at a final protein concentration of 0.5–0.6 mg/ml in a CLB consisting of 120 mM KCl, 2 mM KH_2PO_4, 5 mM $(CH_2COONa)_2 \cdot 6H_2O$, 2.4 mM $MgCl_2 \cdot 6H_2O$, 2 mM Na_2ATP, and HEPES free acid, adjusted to pH 7.2 with 20% (w/v) KOH. In each assay ionomycin (5 μM, free acid; Calbiochem) was used to define the total releasable $^{45}Ca^{2+}$ pool and Ins(1,4,5)P_3 (20–30 μM) was used to define the Ins(1,4,5)P_3-sensitive $^{45}Ca^{2+}$ pool.

Ins(1,4,5)P_3 3-kinase and 5-phosphatase enzyme assays. Rat brain 3-kinase was expressed in *Escherichia coli* as β-galactosidase fusion product starting from clone C5, as described previously (28). The assay used 10 μM Ins(1,4,5)P_3 in the presence of 10 μM free Ca^{2+} and 0.1 μM calmodulin. Human brain Ins(1,4,5)P_3 5-phosphatase type 1 (clone D1 from Ref. 29) was expressed in *E. coli* after subcloning in the pMal vector (New England Biolabs). The properties of the recombinant enzyme are the same as previously reported by De Smedt *et. al* (29). The assay used 10 μM Ins(1,4,5)P_3 with 2,3-diphosphoglycerate and Ins(1,3,4,5)P_4 as positive controls, as described previously (30).

Fluo-3 measurement of Ca^{2+} transients. The metabolism of Ca^{2+}-mobilizing inositol polyphosphates was indirectly measured by monitoring Ca^{2+} transients in permeabilized SH-SY5Y cells using CLB supplemented with 1 μM fluo-3, as described previously (12). SH-SY5Y cells were harvested as described above, centrifuge-washed twice in fluo-3/CLB (500 × *g*, for 1 min), and then resuspended in prewarmed (37°) fluo-3/CLB, at a high cell protein concentration of 4–5 mg/ml. Cells (1.5 ml) were placed in a thermostatted fluorimetry cuvette containing a magnetic stirrer bar and were allowed to equilibrate at 37° for 2–3 min. The cell suspension was then permeabilized with 50 μg/ml saponin, which allowed loading of the ATP-dependent intracellular calcium stores. Ins(1,4,5)P_3 and Gluc(2,3′,4′)P_3 were pretreated with Chelex-100 (Bio-Rad) to remove any contaminating Ca^{2+} and were added to the cell suspensions as 100–200-fold aqueous stocks. Experiments were conducted in a LS-50β fluorimeter (Perkin-Elmer, UK), collecting fluorescence intensities once each second, using excitation and emission wavelengths of 505 nm and 530 nm, respectively (both with 5-nm slit width).

Data analysis. EC_{50} and IC_{50} values (concentrations producing half-maximal stimulation and inhibition, respectively) and slope factors were estimated by computer-assisted curve-fitting using Prism version 1.0 (GraphPad Software). Combined data from the independent experiments were expressed as means ± standard errors for three or four experiments. Statistical comparison of the $\log_{10}$ (IC_{50}) and $\log_{10}$ (EC_{50}) values was performed with Excel (Microsoft), using unpaired Student's *t* tests (assuming unequal variances) at the $p \leq 0.05$ level.

Results

Recognition by the Ins(1,4,5)P_3R. Ins(1,4,5)P_3 displaced [^{3}H]Ins(1,4,5)P_3 from the Ins(1,4,5)P_3 binding sites of pig cerebellar membranes, producing a monophasic profile with an IC_{50} of 27 nM (Fig. 2; Table 1). Gluc(2,3′,4′)P_3 also completely displaced specifically bound [^{3}H]Ins(1,4,5)P_3 from a single population of binding sites, as revealed by computer-assisted curve-fitting. Indeed, Gluc(2,3′,4′)P_3 was a potent ligand (IC_{50} = 130 nM) with an affinity only 5-fold lower than that of Ins(1,4,5)P_3 (Fig. 2; Table 1).

$^{45}Ca^{2+}$ release from saponin-permeabilized SH-SY5Y neuroblastoma cells. Ins(1,4,5)P_3 potently mobilized 75.4 ± 0.3% and 76.6 ± 1.1% of the total preloaded $^{45}Ca^{2+}$ from SH-SY5Y and MDCK cells, respectively, with EC_{50} values of 52 and 247 nM (Fig. 3; Table 1). Gluc(2,3′,4′)P_3 was tested at concentrations up to 100 μM and was apparently a full agonist for Ca^{2+} release in both SH-SY5Y and MDCK cells, releasing Ca^{2+} with a 10–12-fold lower potency than Ins(1,4,5)P_3. Additionally, heparin (50 μg/ml), a potent but nonselective Ins(1,4,5)P_3R antagonist, significantly inhibited both Ins(1,4,5)P_3- and Gluc(2,3′,4′)P_3-induced Ca^{2+} release in SH-SY5Y cells (Table 1).

Recognition by recombinant Ins(1,4,5)P_3 5-phosphatase and 3-kinase enzymes. Gluc(2,3′,4′)P_3 was poorly recognized by 5-phosphatase, exhibiting significant inhibi-

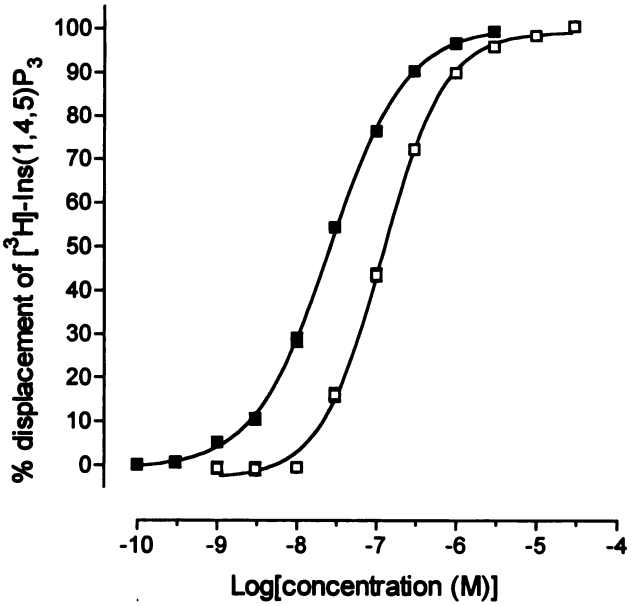

Fig. 2. Percentage displacement by Ins(1,4,5)P_3 (■) and Gluc(2,3′,4′)P_3 (□) of specific [^{3}H]Ins(1,4,5)P_3 binding to pig cerebellar membrane Ins(1,4,5)P_3 binding sites. Results are shown as mean ± standard error of three or four experiments. Nonspecific binding was defined using 10 μM Ins(1,4,5)P_3 (100% displacement); see Experimental Procedures for further details.

TABLE 1

$^{45}Ca^{2+}$ release (four or more experiments) and Ins(1,4,5)P_3R binding (three or more experiments) profiles for Ins(1,4,5)P_3 and Gluc(2,3′,4′)P_3

Results are expressed as log (IC_{50}) or log (EC_{50}) (mean ± standard error). See Experimental Procedures for a complete description of the method.

	Ins(1,4,5)P_3R binding, log (IC_{50})	
	Ins(1,4,5)P_3	Glc(2,3′,4′)P_3
Pig cerebellar membranes	−7.577 ± 0.019	−6.888 ± 0.022
	$^{45}Ca^{2+}$ release, log (EC_{50})	
	Ins(1,4,5)P_3	Gluc(2,3′,4′)P_3
SH-SY5Y cells		
Control	−7.287 ± 0.019	−6.190 ± 0.025
+50 μg/ml Heparin	−6.481 ± 0.074	−5.572 ± 0.037
MDCK cells		
Control	−6.608 ± 0.067	−5.605 ± 0.028
+50 μg/ml Heparin	−5.618 ± 0.111	−4.652 ± 0.037

tion of dephosphorylation activity only at concentrations exceeding 300 μM (Table 2). Its inhibitory properties were similar to those of the nonspecific inhibitor 2,3-diphosphoglycerate but ~1000-fold weaker than those of the potent and specific inhibitor Ins(1,3,4,5)P_4 (29). Ins(1,4,5)P_3 5-phosphatase is considerably less selective than 3-kinase, binding to some degree many inositol phosphates and some phosphorylated compounds (3, 18), so this weak interaction with Gluc(2,3′,4′)P_3 was not surprising. In contrast, Gluc(2,3′,4′)P_3 failed to inhibit Ins(1,4,5)P_3 3-kinase activity even at concentrations up to 1 mM. Thus, Gluc(2,3′,4′)P_3 was poorly recognized by Ins(1,4,5)P_3 5-phosphatase and particularly 3-kinase.

Fluo-3 assessment of Glc(2,3′,4′)P_3-induced Ca^{2+} transients. We previously demonstrated that the addition of Ins(1,4,5)P_3 [or other Ins(1,4,5)P_3 analogues] to permeabilized SH-SY5Y cells resulted in a transient Ca^{2+} mobilization profile, which correlated well with active metabolic removal by 3-kinase and 5-phosphatase activities (8). Here, in SH-SY5Y cells addition of 10 μM Ins(1,4,5)P_3 produced a Ca^{2+} transient that returned to base-line within 3 min. However, 10 μM Gluc(2,3′,4′)P_3 produced a sustained Ca^{2+} release that exhibited only moderate decay over 30 min, allowing further stimulation with 10 μM Ins(1,4,5)P_3 to release Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} pools (Fig. 4). The second Ins(1,4,5)P_3 stimulation was also transient, confirming our earlier data with the recombinant 3-kinase and 5-phosphatase enzymes, which indicated that Gluc(2,3′,4′)P_3 did not significantly affect either enzyme activity. The slow decay of the Gluc(2,3′,4′)P_3-induced Ca^{2+} transient suggests that the compound is not specifically metabolized by enzymes in the permeabilized cells but undergoes nonspecific and slow metabolic degradation. Similarly, in preliminary experiments Gluc(2,3′,4′)P_3 also produced a sustained Ca^{2+} release in saponin-permeabilized MDCK cells.

Computer-assisted structural analysis. Molecular modeling was used to assess the conformational flexibility of Ins(1,4,5)P_3, Gluc(2,3′,4′)P_3, and adenophostin A (Fig. 5). The chair conformation of the fully minimized structure of Ins(1,4,5)P_3 was almost perfectly superimposable on the energy-minimized α-glucoside ring of both Gluc(2,3′,4′)P_3 and adenophostin A. Thus, the 6-hydroxyl and 4,5-bisphosphate moieties crucial for the interaction of Ins(1,4,5)P_3 with its

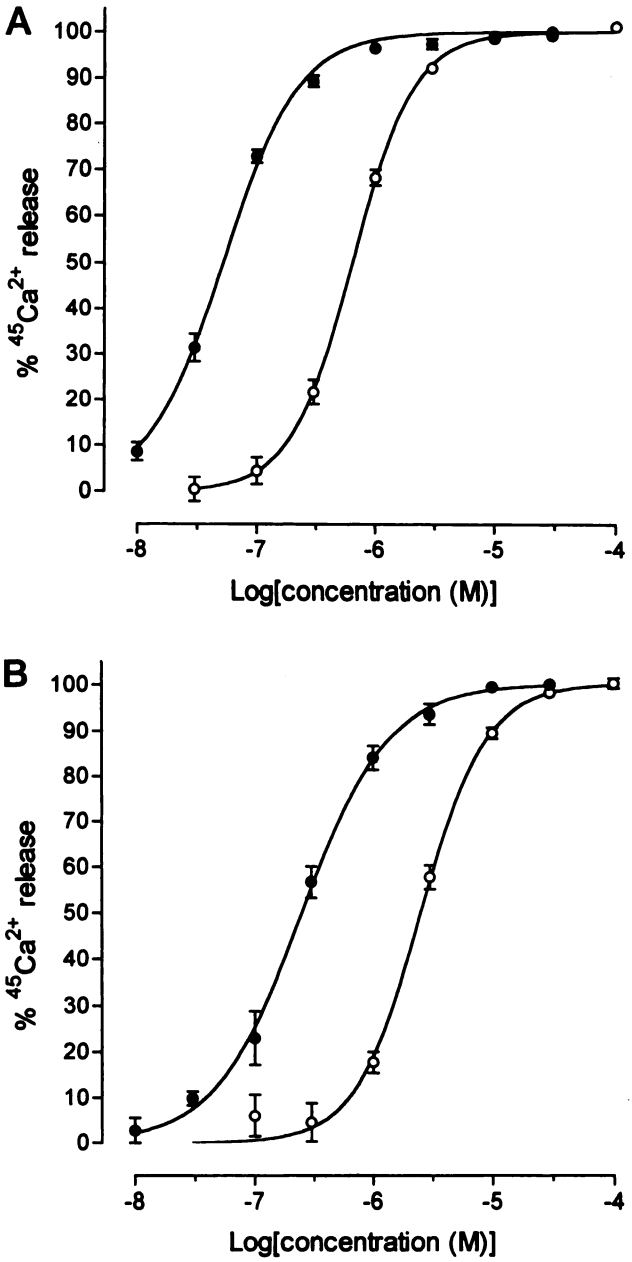

Fig. 3. Intracellular Ca^{2+} mobilization induced by Ins(1,4,5)P_3 and Gluc(2,3′,4′)P_3. Data indicate the percentage of $^{45}Ca^{2+}$ released at 20–22° from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores of saponin-permeabilized SH-SY5Y cells (A) and MDCK cells (B), in the presence of increasing concentrations of Ins(1,4,5)P_3 (●) and Gluc(2,3′,4′)P_3 (○). Results are shown as mean ± standard error of four experiments. Maximal Ca^{2+} release (100% release) from intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores was defined using an internal standard of 30 μM Ins(1,4,5)P_3; see Experimental Procedures for a complete description of the method.

receptor were efficiently mimicked by Gluc(2,3′,4′)P_3 and adenophostin A (Fig. 5). The fully energy-minimized structure of Ins(1,4,5)P_3 was determined to have the following phosphate-phosphate distances (all measurements were taken between phosphorus atoms of the indicated phosphate groups): 8.0 Å (1- and 4-positions), 7.3 Å (1- and 5-positions), and 5.5 Å (4- and 5-positions).

During the course of the dynamic simulation, the distances between the 2-phosphate and the 3′- and 4′-phosphates of Gluc(2,3′,4′)P_3 varied between 8.2 and 11.6 Å (2- and 3′-

TABLE 2

Interaction of Gluc(2,3′,4′)P_3 with recombinant Ins(1,4,5)P_3 5-phosphatase and 3-kinase enzymes

The inhibition of Ins(1,4,5)P_3 metabolism by each compound is expressed as a percentage of a control value (100%) obtained in the presence of water. 2,3-Diphosphoglycerate and Ins(1,3,4,5)P_4 were used as controls for the 5-phosphatase experiments. See Experimental Procedures for a complete description of the method.

Compound	5-Phosphatase	3-Kinase
	% of control	*% of control*
2,3-Diphosphoglycerate, 1 mM	48 ± 3	
Ins(1,3,4,5)P_4		
1 μM	64 ± 10	
10 μM	21 ± 4	
20 μM	6 ± 1	
Gluc(2,3′,4′)P_3		
100 μM	97 ± 32	104 ± 4
300 μM	91 ± 16	104 ± 17
1000 μM	55 ± 10	100 ± 5

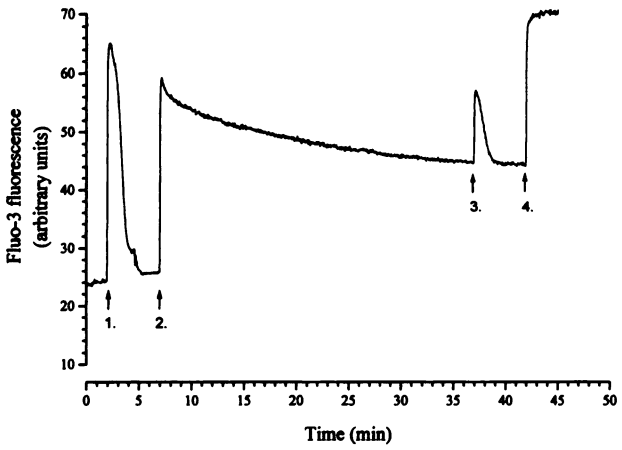

Fig. 4. Profiles of Ca^{2+} release and Ca^{2+} uptake induced by Ins(1,4,5)P_3 (10 μM) (*arrows 1* and *3*), Gluc(2,3′,4′)P_3 (10 μM) (*arrow 2*), and ionomycin (5 μM) (*arrow 4*), detected fluorimetrically using saponin-permeabilized SH-SY5Y cells in fluo-3/CLB at 37°. A trace representative of three independent experiments is presented. See Experimental Procedures for a complete description of the method.

positions) and 9.1 and 11.0 Å (2- and 4′-positions). The distances between the 2′-ribose phosphate of adenophostin A and the two glucose ring phosphates varied between 7.5 and 11.5 Å (2′-ribose and 3″-positions) and between 6.7 and 10.7 Å (2′-ribose and 4″-positions).

Discussion

Very recently the fungal culture isolates adenophostins A and B (24, 25) were found to be very potent full agonists at the Ins(1,4,5)P_3R (23). Significantly, the adenophostins have chemical structures distinct from that of Ins(1,4,5)P_3 (Fig. 1) (23, 25), but the 2″-hydroxyl group and the equatorial 3″- and 4″-phosphate groups on the α-glucoside ring of these compounds can be visualized in a binding orientation that efficiently mimics the receptor interaction of the crucial 4,5-bisphosphate/6-hydroxyl motif of Ins(1,4,5)P_3 (23). The 2′-phosphate on the ribose ring of the adenophostins also appeared to enhance activity, because a derivative of adenophostin A dephosphorylated at this position exhibited an approximately 2000-fold lower affinity at the purified rat cerebellar Ins(1,4,5)P_3R (25).

We recently assessed a number of 3-position-substituted

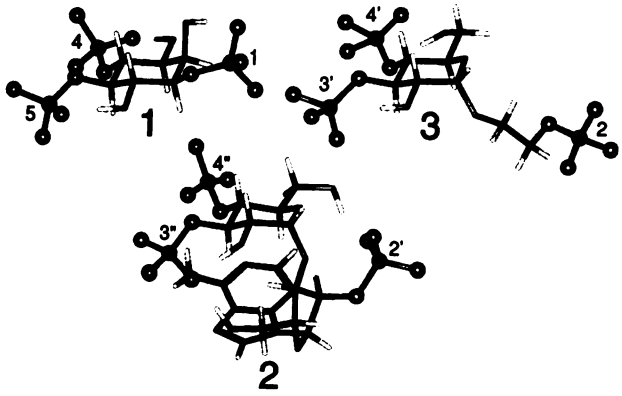

Fig. 5. Structures of Ins(1,4,5)P_3 (**1**), adenophostin A (**2**), and Gluc(2,3′,4′)P_3 (**3**). Structures **1** and **3** are the fully energy-minimized structures, whereas structure **2** is one representative conformation taken from a molecular dynamics simulation (see Experimental Procedures). The three compounds are shown with the inositol (**1**) or glucose (**2** and **3**) rings in the same relative conformations and orientations (with the ring and the equatorial vicinal phosphates on the *left*), to highlight the possible significance of the distance between these groups and the remaining phosphate group in determining receptor binding affinity. The positions of the phosphate groups (drawn uniquely as ball-and-stick representations) are numbered to allow comparison with Fig. 1.

Ins(1,4,5)P_3 analogues and found that compounds with substituents with steric bulk greater than that of the native 3-hydroxyl group exhibited progressively reduced affinity at the Ins(1,4,5)P_3R (15). Significantly, the 5″-position of adenophostins A and B can be visualized in a binding conformation that corresponds to the 3-position of Ins(1,4,5)P_3. However, adenophostins A and B are essentially equipotent and 100-fold more potent than Ins(1,4,5)P_3, despite the 5″-position being occupied by CH_2OH or CH_2OCOCH_3 groups, respectively. This may indicate that adenophostins A and B interact with the Ins(1,4,5)P_3R binding pocket in a subtly different orientation than does Ins(1,4,5)P_3, because no steric selection against the relatively large 5′-position substituents was apparent. Here we sought to further elucidate the interaction of the adenophostins and Ins(1,4,5)P_3 by synthesizing Gluc(2,3′,4′)P_3, which can be modeled as adenophostin A with the adenosine residue largely excised. Thus, the 1′-position of Gluc(2,3′,4′)P_3 is linked via an α-glucosidic bond to the 1- and 2-position carbons, with the 2-position carbon possessing a terminal phosphate group. This configuration mimics both the native structure connecting the base of the ribose ring and the α-glucoside ring of adenophostin A and presumably, at least to some degree, the 1-phosphate of Ins(1,4,5)P_3. Significantly, in Gluc(2,3′,4′)P_3 the terminal 2-position phosphate group is not as spatially constrained as the analogous 2″-phosphate on the ribose ring of adenophostin A.

Gluc(2,3′,4′)P_3 was found to be a potent full agonist in SH-SY5Y and MDCK cells, being only approximately 10–12-fold weaker than Ins(1,4,5)P_3. Additionally, both Gluc(2,3′,4′)P_3- and Ins(1,4,5)P_3-induced Ca^{2+} release were inhibited by heparin, a potent but nonselective Ins(1,4,5)P_3R antagonist. Furthermore, using the well characterized pig cerebellum Ins(1,4,5)P_3R preparation, it was demonstrated that Gluc(2,3′,4′)P_3 specifically displaced [^{3}H]Ins(1,4,5)P_3 with only 5-fold lower affinity, compared with Ins(1,4,5)P_3. Thus, Gluc(2,3′,4′)P_3 is the first small synthetic compound

not based on a phosphorylated inositol isomer to exhibit high affinity and efficacy at the Ins(1,4,5)P_3R.

In contrast, Gluc(2,3′,4′)P_3 did not appear to significantly interact with or inhibit the specific metabolic enzymes 3-kinase and 5-phosphatase. Gluc(2,3′,4′)P_3 began to significantly inhibit 5-phosphatase only at concentrations exceeding 300 μM, whereas it failed to inhibit 3-kinase activity even at 1 mM. The lack of interaction with Ins(1,4,5)P_3 3-kinase was interesting because this enzyme usually exhibits a structure-activity profile similar to that of the Ins(1,4,5)P_3R with respect to Ins(1,4,5)P_3 analogues (3–5). Furthermore, studies in permeabilized SH-SY5Y cells indicated that, whereas the Ca^{2+}-mobilizing action of Ins(1,4,5)P_3 was transient (3 min), Gluc(2,3′,4′)P_3 was able to produce a sustained release (>30 min) of intracellular Ca^{2+}, suggesting that Gluc(2,3′,4′)P_3 exhibited significant metabolic resistance. A slow progressive decline in the Ca^{2+} mobilization profile was apparent, perhaps indicating that nonspecific metabolism was occurring; this was not surprising, given the simple glucose-based structure of Gluc(2,3′,4′)P_3.

Thus, Gluc(2,3′,4′)P_3 resembles adenophostin A in its metabolic resistance but is a weaker full agonist at the Ins(1,4,5)P_3R (23). Given that the 2″-phosphate on the ribose ring of adenophostin A significantly increases receptor affinity (23), our findings suggest two receptor binding scenarios, i.e., that both the presence of the 2′-phosphate on the ribose ring and the structural constraint dictated by the ribose ring are important for optimal receptor interaction or, alternatively, that the adenosine and ribose residues of the adenophostins we synthetically excised to form Gluc(2,3′,4′)P_3 also have favorable interactions with either a specific or nonspecific site in the vicinity of the Ins(1,4,5)P_3R binding pocket, which act in a concerted fashion to further stabilize the receptor interaction. For example, enhanced binding energy might be provided if adenophostin A could simultaneously interact both with the Ins(1,4,5)P_3 binding pocket (via its phosphoryated glucose and ribose residues) and with one of the ATP binding sites on the Ins(1,4,5)P_3R (via its adenosine residue). The latter hypothesis is perhaps unlikely, given that cytosolic ATP is present at millimolar levels, but direct testing of the hypothesis will require a more subtle synthetic excision of the adenosine residue from the adenophostin A structure. Nevertheless, the first hypothesis has been addressed here using computer-assisted molecular modeling.

Modeling studies by Takahashi *et al.* (23) have indicated that the vicinal equatorial 4″- and 3″-bisphosphates and the α-glucoside ring of adenophostin A can be superimposed on the vicinal equatorial 4- and 5-bisphosphates and the *myo*-inositol ring of Ins(1,4,5)P_3. Significantly, our modeling studies indicate that the vicinal equatorial 4′- and 3′-bisphosphates and the α-glucoside ring of Gluc(2,3′,4′)P_3 can be almost perfectly superimposed on the analogous structural moieties of both adenophostin A and Ins(1,4,5)P_3 (Figs. 1 and 5). Because for receptor interaction the nonvicinal 1-phosphate of Ins(1,4,5)P_3 appears to be less critical than the vicinal 4- and 5-phosphates, it has been hypothesized that the 2′-phosphate on the ribose ring of adenophostin A may be in a better position for interaction with the Ins(1,4,5)P_3R, thus giving rise to the greater potency of adenophostin A (23). Given the accepted importance of the vicinal 4- and 5-bisphosphates for Ins(1,4,5)P_3R binding and the suggested importance of the 2′-phosphate group of adenophostin A, the possible conformations of Gluc(2,3′,4′)P_3 and adenophostin A were explored by using a molecular dynamics simulation. In particular, the consequences of the greater flexibility of the hydroxyethyl phosphate moiety of Gluc(2,3′,4′)P_3 [which is structurally analogous to both the 1-phosphate of Ins(1,4,5)P_3 and the 2′-phosphate of adenophostin A] were investigated.

The vicinal phosphates on the glucose or inositol rings were fixed in their diequatorial conformation, and the distance from these to the third phosphate was monitored throughout the trajectory. The ribose 2′-phosphate of adenophostin A was constrained throughout, remaining closer to the glucose ring phosphates than does the corresponding 2-position phosphate of Gluc(2,3′,4′)P_3 (Fig. 5). During the course of the simulation the adenophostin A structure remained quite compact (data not shown), in a hairpin-like conformation (see Fig. 5, structure **2**), and was apparently unable to achieve an extended conformation. The total energy of the molecule remained correspondingly higher than that of Gluc(2,3′,4′)P_3 throughout. Precise positioning of the 1-phosphate of Ins(1,4,5)P_3 and the corresponding phosphates of Gluc(2,3′,4′)P_3 and adenophostin A may significantly affect binding affinity. Within the relatively inflexible Ins(1,4,5)P_3 molecule, the 1-phosphate may not be as appropriately positioned as the slightly more extended 2′-ribose phosphate of adenophostin A. Indeed, compared with both Ins(1,4,5)P_3 and adenophostin A, the analogous 2-phosphate of Gluc(2,3′,4′)P_3 may be too extended, requiring a larger expenditure of binding energy to adopt a more favorable binding conformation. In adenophostin A, presumably the relative position of the 2′-phosphate is optimal for binding, being constrained in a moderately extended conformation by the ribose ring and thus requiring little additional energy to adopt an efficient binding conformation. Thus, the exact positions of the nonvicinal phosphates of Ins(1,4,5)P_3 (1-phosphate), adenophostin A (2′-phosphate), and Gluc(2,3′,4′)P_3 (2-phosphate) may play a critical role in determining affinity at the Ins(1,4,5)P_3R.

Gluc(2,3′,4′)P_3 should be a useful, metabolically resistant, Ins(1,4,5)P_3 analogue. Historically, Ins(2,4,5)P_3, with a potency 10–15-fold weaker than that of Ins(1,4,5)P_3 (11, 31), has been used as a metabolically resistant analogue because it is a weak substrate for both 5-phosphatase and, in particular, 3-kinase (31, 32). Similarly, Ins(1,4,5)PS_3 is 5–20-fold weaker than Ins(1,4,5)P_3, is very metabolically resistant to both 5-phosphatase and 3-kinase activities (6–8), and thus has been even more widely used. However, although the phosphorothioate group has a molecular volume only slightly greater than that of a phosphate group (15), it does exhibit a distinctive chemistry. The replacement of oxygen by sulfur to form the phosphorothioate group results in considerable disturbance in charge distribution, with increased negative charge on the sulfur and reduced negative charge on the remaining oxygens (33, 34). Indeed, Challiss *et al.* (35) recently demonstrated that Ins(1,4,5)PS_3 interacts with equal affinity at two sites in rat cerebellar membranes, one of which is the Ins(1,4,5)P_3R and the other a site that displays low affinity for Ins(1,4,5)P_3. Thus, these changes in charge distribution appear to subtly affect interactions with the Ins(1,4,5)P_3R binding pocket, and it is significant that many of the Ins(1,4,5)P_3R partial agonists so far identified contain the 1,4,5-trisphosphorothioate motif (12, 36). Furthermore,

phosphorothioates are also generally less stable than the corresponding phosphates (33, 37), and we have observed that aqueous solutions of Ins(1,4,5)PS_3 rapidly lose activity even when stored at −70°. Gluc(2,3′,4′)P_3 has a potency comparable to that of Ins(2,4,5)P_3 and Ins(1,4,5)PS_3 but offers substantial advantages, because it is both chemically and metabolically stable and, being based on an α-glucoside ring, would not be metabolized in cells to form inositol polyphosphates with potentially independent second messenger actions.

Gluc(2,3′,4′)P_3, the first synthetic glucose-basedIns(1,4,5)P_3 analogue, potently interacts with Ins(1,4,5)P_3R populations from pig cerebellum (38) and SH-SY5Y neuroblastoma cells (39), systems known to predominantly express the neuronal type I Ins(1,4,5)P_3R. However, we also used MDCK cells, which are known to express the type I, II, and III Ins(1,4,5)P_3R isoforms and in which semiquantitative polymerase chain reaction analysis has revealed type III as the most abundantly expressed isoform (40). Significantly, although Ins(1,4,5)P_3 was less active in MDCK cells, compared with SH-SY5Y cells, the relative potency of Gluc(2,3′,4′)P_3 was very similar to that of Ins(1,4,5)P_3, indicating that no differential interaction between Gluc(2,3′,4′)P_3 and Ins(1,4,5)P_3R isoforms was apparent. Thus, due to its novel structure Gluc(2,3′,4′)P_3 will be an important lead compound and tool for the further investigation of the Ins(1,4,5)P_3R binding pocket, especially for defining the structural features that produce the supra-agonist activity of the adenophostin fungal isolates.

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