

# Biphenyl 2,3',4,5',6-pentakisphosphate, a novel inositol polyphosphate surrogate, modulates $\text{Ca}^{2+}$ responses in rat hepatocytes

Fabrice Vandeput,\* Laurent Combettes,<sup>†</sup> Stephen J. Mills,<sup>‡</sup> Katrien Backers,\* Alexandre Wohlkönig,<sup>§</sup> Jan B. Parys,<sup>||</sup> Humbert De Smedt,<sup>||</sup> Ludwig Missiaen,<sup>||</sup> Geneviève Dupont,<sup>¶</sup> Barry V. L. Potter,<sup>‡</sup> and Christophe Erneux\*<sup>1</sup>

\*Institut de Recherche Interdisciplinaire (IRIBHM), Université Libre de Bruxelles, Campus Erasme, Brussels, Belgium; <sup>†</sup>INSERM UMR-S757, Université de Paris-Sud, Orsay, France; <sup>‡</sup>Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom; <sup>§</sup>CNRS-UMR8161, Institut de biologie de Lille, France; and <sup>||</sup>Laboratorium voor Fysiologie, Dept. Molecular Cell Biology, KULeuven, Campus Gasthuisberg, Leuven, Belgium; and <sup>¶</sup>ULB, Faculté des Sciences, Belgium

**ABSTRACT** Benzene polyphosphates containing phosphate groups on one ring are  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase inhibitors when evaluated against type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase. A novel biphenyl derivative, biphenyl 2,3',4,5',6-pentakisphosphate, with five phosphate groups on two rings was synthesized: It inhibited the activity of two inositol 5-phosphatases: type I and SHIP2 with  $\text{Ins}(1,3,4,5)\text{P}_4$  as substrate. The inhibition was competitive with respect to the substrate.  $\text{IC}_{50}$  value measured in rat hepatocytes, which contains the native  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase, was in the micromolar range at 1.0  $\mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$  as substrate. Biphenyl 2,3',4,5',6-pentakisphosphate did not affect the activity of  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase A in the 5–100  $\mu\text{M}$  range. Surprisingly, experimental evidence supports an effect of biphenyl 2,3',4,5',6-pentakisphosphate at the level of the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. Finally, when injected into rat hepatocytes, the analog affected the frequency of  $\text{Ca}^{2+}$  oscillations in a positive or negative way depending on its concentration. At very high concentrations of the analog,  $\text{Ca}^{2+}$  oscillations were even suppressed. These data were interpreted as a dual effect of the biphenyl 2,3',4,5',6-pentakisphosphate on cytosolic  $[\text{Ca}^{2+}]$  increases: an activation effect through an increase in  $\text{Ins}(1,4,5)\text{P}_3$  level via  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase inhibition and an inhibitory effect, which was exerted directly on the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. Thus, our data show for the first time that the frequency of  $\text{Ca}^{2+}$  oscillations in response to a  $\text{Ca}^{2+}$ -mobilizing agonist can be controlled by inhibitors of type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase.—Vandeput, F., Combettes, L., Mills, S. J., Backers, K., Wohlkönig, A., Parys, J. B., De Smedt, H., Missiaen, L., Dupont, G., Potter, B. V. L., Erneux, C. Biphenyl 2,3',4,5',6-pentakisphosphate, a novel inositol polyphosphate surrogate, modulates  $\text{Ca}^{2+}$  responses in rat hepatocytes. *FASEB J.* 21, 1481–1491 (2007)

**Key Words:** inositol 5-phosphatase • SHIP2 •  $\text{Ins}(1,4,5)\text{P}_3$  receptor •  $\text{Ca}^{2+}$  signaling • benzene polyphosphates

DISTINCT FORMS OF inositol and phosphatidylinositol polyphosphate 5-phosphatases selectively remove the phosphate from the 5-position of the inositol ring from both soluble and lipid substrates, that is, inositol 1,4,5-trisphosphate ( $\text{Ins}(1,4,5)\text{P}_3$ ), inositol 1,3,4,5-tetrakisphosphate ( $\text{Ins}(1,3,4,5)\text{P}_4$ ), phosphatidylinositol 4,5-bisphosphate ( $\text{PtdIns}(4,5)\text{P}_2$ ), or phosphatidylinositol 3,4,5-trisphosphate ( $\text{PtdIns}(3,4,5)\text{P}_3$ ) (1, 2). In mammalian cells, this family contains a series of distinct genes and splice variants (2, 3). All inositol polyphosphate 5-phosphatases share an inositol 5-phosphatase catalytic domain and various protein modules probably responsible for specific cell localization or recruitment (SH2 domain, proline-rich sequences, prenylation sites). Type-I and the SH2-domain-containing inositol 5-phosphatase 2 (SHIP2) are two members of this family of proteins involved in  $\text{Ca}^{2+}$  (4, 5) and insulin signaling, respectively, as well as the control of obesity (6, 7).

We initiated this study in order to identify novel inositol 5-phosphatase inhibitors as molecular tools for studies using intact cells. The availability of phosphatase inhibitors of this family of enzymes could facilitate a comparison of their catalytic domains. It will also allow us to initiate intact-cell studies with suitable phosphate-masked derivatives by modulating phosphatase activities, as previously shown for the various isoforms of cyclic nucleotide phosphodiesterases (8), for example. This would allow a better understanding of their individual functions in the control of intracellular levels of inositol phosphates and of phosphoinositides.

On the basis of modeling studies, we hypothesized that  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase activity plays an impor-

<sup>1</sup> Correspondence: Institut de Recherche Interdisciplinaire (IRIBHM), Université Libre de Bruxelles, Campus Erasme, Bldg. C, 808 Route de Lennik, 1070 Brussels, Belgium. E-mail: cerneux@ulb.ac.be

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tant role in the control of the frequency of  $\text{Ca}^{2+}$  oscillations in response to  $\text{Ca}^{2+}$ -mobilizing agonists (9). This hypothesis could be tested using the model of connected hepatocytes by injecting the phosphatase into one cell. Connected hepatocytes display quasi-identical  $\text{Ca}^{2+}$  oscillations in response to stimulation by noradrenaline or vasopressin (10). Therefore, the non-injected cell provides the control situation, allowing for a direct visualization of the effect of 5-phosphatase (11).

In the present study, one of our aims was to inject an inositol 5-phosphatase inhibitor, which might increase the intracellular level of  $\text{Ins}(1,4,5)\text{P}_3$ , producing a new steady-state level of  $\text{Ins}(1,4,5)\text{P}_3$  that could control the frequency of  $\text{Ca}^{2+}$  oscillations.

Benzene polyphosphates are polyanionic compounds that can accommodate phosphate groups around a six-membered planar ring with a similar, but more rigid phosphate regiochemistry to a natural inositol phosphate and can potentially interact with inositol polyphosphate-binding proteins. Additionally, the nature of the benzene ring may encourage the formation of new intermolecular ligand-protein interactions such as  $\pi$ -cation interactions that cannot be observed in inositol polyphosphates. These criteria make the benzene ring an interesting replacement for the conformationally mobile *myo*-inositol ring. Benzene 1,2,4-trisphosphate ( $\text{Bz}(1,2,4)\text{P}_3$ ) was the first simple benzene polyphosphate to be modeled on the  $\text{Ins}(1,4,5)\text{P}_3$  structure and has an arrangement of phosphate groups similar to 2,3,6-trideoxy  $\text{Ins}(1,4,5)\text{P}_3$ , a weak agonist for  $\text{Ca}^{2+}$  release at the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. Poitras *et al.* found that  $\text{Bz}(1,2,4)\text{P}_3$  interacted with the  $\text{Ins}(1,4,5)\text{P}_3$  receptor of adrenal-cortex microsomes, albeit with a 10,000 fold weaker binding affinity than  $\text{Ins}(1,4,5)\text{P}_3$  (12).  $\text{Bz}(1,2,4)\text{P}_3$  did not release  $\text{Ca}^{2+}$ , but weakly inhibited  $\text{Ca}^{2+}$  release in a dose-dependent way. We recently prepared a novel series of benzene polyphosphates that inhibit type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase with different potency. Interestingly, most of the analogs were not dephosphorylated, one exception being 3-hydroxybenzene 1,2,4-trisphosphate ( $(\text{OH})\text{Bz}(1,2,4)\text{P}_3$ ), which was a very efficient substrate of type-I 5-phosphatase (13).

In this study, we report the synthesis of a related, but highly novel, derivative with five phosphate groups on two rings (a biphenyl moiety) biphenyl 2,3',4,5',6-pentakisphosphate ( $\text{biphenyl}(2,3',4,5',6)\text{P}_5$ ), **Fig. 1**. It inhibits the activity of two inositol 5-phosphatases: type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase and SHIP2. It is the most potent inhibitor of the current series. Surprisingly, we found that it also binds to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor type 1 ( $(\text{IP})_3\text{R1}$ ) and inhibits  $\text{Ins}(1,4,5)\text{P}_3$ -

induced  $\text{Ca}^{2+}$  release in permeabilized cells. When injected into intact hepatocytes, it was able to affect both positively and negatively the frequency of  $\text{Ca}^{2+}$  oscillations depending on its concentration and on the sensitivity of the cell.

## MATERIALS AND METHODS

### Materials

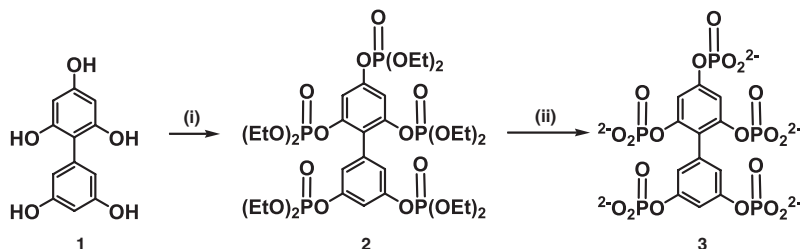
The synthesis of  $\text{Ins}(1,3,4,5)\text{P}_4$  (14), as well as the synthesis of three- and four-phosphorylated analogs, has been reported ((13) and unpublished results). Compete™ (Protease inhibitor cocktail) was from Roche Diagnostics (Mannheim, Germany). ProBond™ Nickel-Chelating Resin was purchased from Invitrogen (Carlsbad, CA). Hyperfilm-MP, enhanced chemoluminescence (ECL®), Western blot analysis reagents, and HiLoad Superdex 75 prep grade were from Amersham. [ $^3\text{H}$ ] $\text{Ins}(1,4,5)\text{P}_3$  (22 Ci/mmol) and [ $^3\text{H}$ ]  $\text{Ins}(1,3,4,5)\text{P}_4$  (22 Ci/mmol) were from DuPont NEN-PerkinElmer. Dowex 1-X8 (formate form) was from Bio-Rad Laboratories (Hercules, CA). A bacterial construct encoding the catalytic domain of  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase A was expressed and purified as reported in Poinas *et al.* (15). The cloning and expression of human brain type-I  $\text{InsP}_3$  5-phosphatase were carried out as described previously (16). A glutathione S-transferase (GST)-SHIP2 construct encoding the catalytic domain of SHIP2 was expressed as previously reported (17). R23-11 cells used in this study are triple  $\text{Ins}(1,4,5)\text{P}_3$ -receptor-knockout cells derived from chicken DT40 chicken B lymphoma cells, which are stably transfected with mouse  $\text{IP}_3\text{R1}$  (18).

### Synthesis of biphenyl-2,3',4,5',6-pentakisphosphate

#### 2,3',4,5',6-pentakis(diethylphosphoryloxy)-biphenyl (2)

A mixture of dry  $\text{CH}_2\text{Cl}_2$  (10 ml) and diethyl chlorophosphate (1.0 ml, 7.0 mmol) and dry *N,N*-diisopropylethylamine (2.1 ml, 12 mmol) was stirred at room temperature. Biphenyl 2,4,6,3',5'-pentanol (1) (234 mg, 1.0 mmol) was slowly added to the mixture, and ultrasound was used to dissolve the solid. As the compound dissolved, the solution became a deeper yellow color, which was cooled using dry ice and stirred for 30 min. 3-Chloroperoxybenzoic acid (2.0 g, 11.58 mmol) dissolved in  $\text{CH}_2\text{Cl}_2$  (25 ml) was added quickly, and the solution was stirred for a further 30 min. The mixture was purified by flash chromatography [ $\text{EtOAc}$  then  $\text{EtOAc-EtOH}$  (5:1)],  $R_f = 0.34$ ,  $\text{EtOAc-EtOH}$  (5:1), to give the pure title compound (2) as a yellow oil 699 mg (76%).  $^1\text{H}$  NMR (270 MHz,  $\text{CDCl}_3$ ) 1.16–1.22 (12 H, m,  $2 \times \text{ArOPO}(\text{OCH}_2\text{CH}_3)_2$ ), 1.31–1.39 (18 H, m,  $3 \times \text{ArOPO}(\text{OCH}_2\text{CH}_3)_2$ ), 3.88–3.96 (8 H, m,  $2 \times \text{ArOPO}(\text{OCH}_2\text{CH}_3)_2$ ), 4.16–4.28 (12 H, m,  $3 \times \text{ArOPO}(\text{OCH}_2\text{CH}_3)_2$ ), 7.02–7.03 (2 H, m, Ar), 7.16–7.18 (1 H, m, Ar) 7.20–7.21 (2 H, m, Ar);  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ) -6.80 (2 P,  $2 \times \text{ArOPO}(\text{OCH}_2\text{CH}_3)_2$ ), -6.97 (1 P,

**Figure 1.** Structures of biphenyl-2,4,6,3',5'-pentaol (1), 2,3',4,5',6-pentakis(diethylphosphoryloxy)-biphenyl (2), and biphenyl 2,3',4,5',6-pentakisphosphate (3). The scheme of the synthesis is: i) Diisopropylethylamine,  $\text{Cl-P}(\text{OEt})_2$ ,  $\text{CH}_2\text{Cl}_2$ , (reverse addition), then *m*CPBA, (76%); ii)  $\text{CH}_2\text{Cl}_2$  TMSBr, then purification on Q-Sepharose ion exchange resin, triethylammonium bicarbonate  $0 \rightarrow 2.0$  M (91.5%).



ArOPO(OCH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>, -7.38 (2 P, 2 × ArOPO(OCH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>); (MS, FAB<sup>+</sup>) Calcd for C<sub>32</sub>H<sub>56</sub>O<sub>20</sub>P<sub>5</sub> [M + H]<sup>+</sup> 915.2053. Found 915.2066. Calcd for C<sub>32</sub>H<sub>55</sub>O<sub>20</sub>P<sub>5</sub> C, 42.02, H, 6.06. Found C, 41.6, H, 5.98.

### Biphenyl 2,3',4,5',6-pentakisphosphate (3)

Compound (2) (227 mg, 248 μmoles), was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (10 ml). Bromotrimethylsilane (2.0 ml, 15.15 mmol) was added, and the solution was stirred for 22 h at room temperature. The solvents were evaporated off, and the remaining syrup was stirred in MeOH (10 ml) for 30 min. Final purification of compound (3) was accomplished over Q-Sepharose Fast Flow using a linear gradient of 0→2.0 M TEAB buffer. The title compound eluted at a buffer concentration of 1.3–2.0 M and produced (3), a glassy triethylammonium salt, 227 μmoles (91.5%). <sup>1</sup>H NMR (270 MHz, D<sub>2</sub>O) 6.87 (1 H, m, Ar), 6.96 (2 H, m, Ar), 7.05 (2 H, m, Ar); <sup>31</sup>P NMR (109 MHz, D<sub>2</sub>O) -1.33 (1 P, ArOPO<sub>3</sub><sup>2-</sup>), -1.76 (2 P, 2 × ArOPO<sub>3</sub><sup>2-</sup>), -2.66 (2 P, 2 × ArOPO<sub>3</sub><sup>3-</sup>); (MS, FAB<sup>-</sup>) 633.1, Calcd for C<sub>12</sub>H<sub>14</sub>O<sub>20</sub>P<sub>5</sub> [M - H]<sup>-</sup> 632.8772. Found 632.8783.

### Preparation of a lysate of rat hepatocytes

Hepatocytes (10<sup>7</sup> cells) were lysed in 1 ml of ice-cold lysis buffer containing 10 mM Tris pH 8.0, 0.15 M KCl, 0.5% Nonidet P-40, 2 mM 2-mercaptoethanol, 0.1 M NaF, protease and phosphatase inhibitors (5 μM leupeptin, 0.1 mM pefabloc, 2.5 μM okadaic acid, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM EDTA).

### Preparation of hepatocytes for [Ca<sup>2+</sup>]<sub>i</sub> measurements

Isolated rat hepatocytes were prepared from fed female Wistar rats by limited collagenase digestion of rat liver, as described previously (11). Under these conditions, ~20% of the cells were associated by two (doublet) or three (triplet) and were distinguished from aggregates of nonconnected cells in conventional light microscopy by screening for dilated bile canaliculi, indicators of maintained functional polarity. After isolation, rat hepatocytes were maintained (5 × 10<sup>5</sup> cells/ml) at 4°C in Williams' medium E supplemented with 10% fetal calf serum, penicillin (100,000 U/ml), and streptomycin (100 μg/ml). Cell viability, assessed by trypan blue exclusion, remained greater than 96% for 4–5 h.

### Microinjection

Microinjection was performed using an Eppendorf microinjector (5242), as described previously (10). Micropipettes with an internal tip diameter of 0.5 μm (Femtotips, Eppendorf) were filled with biphenyl(2,3',4,5',6)P<sub>5</sub> at the indicated concentration together with 5 mM Fura-2 in a buffer solution containing 100 mM KCl, 20 mM NaCl, 10 mM HEPES adjusted to pH 7.1. After microinjection, cells were allowed to recover for at least 10 min. The success of microinjection was assessed by monitoring the morphology of the cells before and after manipulation and checking the ability of the cell to retain injected Fura-2 and low [Ca<sup>2+</sup>]<sub>i</sub>.

### Determination of [Ca<sup>2+</sup>]<sub>i</sub> changes in hepatocytes

Ca<sup>2+</sup> imaging was carried out as described previously (11). Briefly, the excitation light was supplied by a high-pressure xenon arc lamp (75 W), and the excitation wavelengths were selected using 340- and 380-nm filters (10-nm bandwidth) mounted in a processor-controlled rotating filter wheel (Sut-

ter, Novato, CA) between the UV lamp and the microscope. Fluorescence images were collected by a CCD camera (Princeton Instruments, Evry, France), digitized and integrated in real time by an image processor (Metafluor, Princeton Instruments).

### Malachite green phosphatase assay

The enzyme activity of the 5-phosphatases was measured with a phosphate-release assay using an acidic malachite green dye. Di-C<sub>8</sub> phosphoinositides, inositol phosphates or analogs were diluted in 30-μl assay buffer (50 mM HEPES (pH 7.4), 2 mM MgCl<sub>2</sub>, 1 mg/ml BSA). The phosphatase reaction was initiated by adding the enzyme diluted in assay buffer (15 μl) to the substrates, and samples were incubated at 37°C. After 7 min, reactions were stopped by the addition of 15 μl 0.1 M EDTA. 75 μl of malachite green reagent was added to 50 μl of the reaction solution. Samples were allowed to stand for 10 min for color development before measuring absorbance at 650 nm. Inorganic-phosphate release was quantified by comparison to a standard curve of KH<sub>2</sub>PO<sub>4</sub> in dH<sub>2</sub>O.

### Ins(1,4,5)P<sub>3</sub>/Ins(1,3,4,5)P<sub>4</sub> phosphatase assay

Inositol phosphatase activity was assayed at 37°C, using [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> or [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> in 50 mM HEPES (pH 7.4), 2 mM MgCl<sub>2</sub>, 1 mg/ml BSA, and the appropriate enzyme dilution in a final volume of 50 μl. The assay was initiated by adding the substrate, stopped after 7 min by addition of 1 ml ice-cold 0.4 M ammonium formate/0.1 M formic acid or 0.7 M ammonium formate/0.1 M formic acid (for Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> phosphatase assay, respectively), and the resulting solution was immediately applied to Dowex columns. [<sup>3</sup>H]Ins(1,4)P<sub>2</sub>, the product of [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> 5-phosphatase, and [<sup>3</sup>H]Ins(1,3,4)P<sub>3</sub>, the product of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> 5-phosphatase, were separated on 0.5 ml and 1 ml Dowex columns, respectively. [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> 3-kinase assay was performed as described before (19).

### [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding

The [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding assay was performed on microsomes of Sf9 cells overexpressing IP<sub>3</sub>R1 by a rapid filtration method, exactly as described previously (20). Incubation (0.15 mg protein/sample) was performed on ice at pH 7.4 in the presence of 7 nM [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub>. In control samples, the specific binding amounted to 97 ± 1% of the total binding.

### Ca<sup>2+</sup>-release studies in permeabilized R23–11 cells

Cell pellets of R23–11 cells stably expressing IP<sub>3</sub>R1 were resuspended in intracellular medium (120 mM KCl, 30 mM HEPES, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM ATP, 25 mM phosphocreatine, 50 U of creatine kinase and 5 μM Fluo-3) and transferred to a 4-ml fluorescence quartz cuvette thermostatically maintained at 37°C. Cell density was 5 × 10<sup>7</sup> cells/ml. Mild treatment of the cells with digitonin (50 μM) disrupted the plasma membrane. The Fluo-3 fluorescence (λ<sub>ex</sub>=503 nm and λ<sub>em</sub>=530 nm) was measured with an Aminco-Bowman Series 2 spectrometer (Spectronic Unicam, Rochester, NY). A23187 (8 μM) was added at the end of each experiment to measure the total releasable Ca<sup>2+</sup>. The fluorescence signal *F* was calibrated by first adding 0.5 mM Ca<sup>2+</sup> (*F*<sub>max</sub>) and then adding 5 mM EGTA (*F*<sub>min</sub>). The free [Ca<sup>2+</sup>]<sub>i</sub> was calculated using the equation:

$$[\text{Ca}^{2+}] \text{ (nM)} = 864 \cdot (F - F_{\min}) / (F_{\max} - F). \quad (1)$$

### Description of the mathematical model

We used a previously published mathematical model to describe  $\text{Ca}^{2+}$  dynamics and  $\text{Ins}(1,4,5)\text{P}_3$  metabolism (9). Given the predominant role played by 5-phosphatase with respect to 3-kinase in  $\text{Ins}(1,4,5)\text{P}_3$  metabolism,  $\text{Ins}(1,3,4,5)\text{P}_4$  was not considered. The model was modified to take into account the inhibitions of both the  $\text{Ins}(1,4,5)\text{P}_3$  receptor and the 5-phosphatase by biphenyl(2,3',4,5',6) $\text{P}_5$ . Thus, the fraction of activatable channels (Eq. 1 in (9)) became

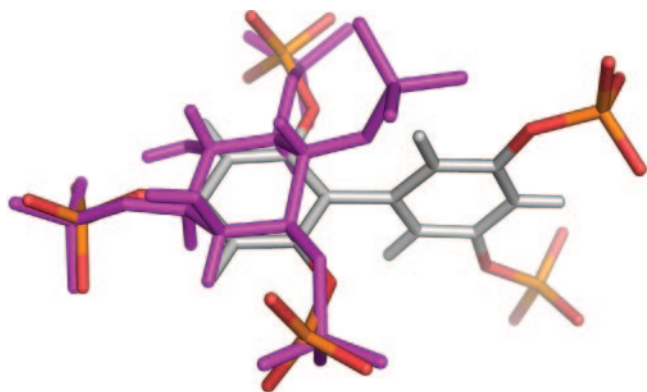
$$IR_{\text{able}} = (1 - R_{\text{des}}) \frac{[\text{Ins}(1,4,5)\text{P}_3]}{K_{\text{IP}} + [\text{Ins}(1,4,5)\text{P}_3]} \frac{K_{\text{BP}}}{K_{\text{BP}} + [\text{BP}]}$$

where [BP] stands for the concentration of biphenyl(2,3',4,5',6) $\text{P}_5$ , and  $K_{\text{BP}}$  is the inhibition constant of the  $\text{InsP}_3\text{R}$  by biphenyl(2,3',4,5',6) $\text{P}_5$ . The rate of 5-phosphatase (eq. 3 in (9)) became

$$V_{5\text{P}} = V_{\text{P}} \frac{[\text{Ins}(1,4,5)\text{P}_3]}{K_{\text{P}} + [\text{Ins}(1,4,5)\text{P}_3]} \frac{K_{\text{IBP}}}{K_{\text{IBP}} + [\text{BP}]}$$

where  $K_{\text{IBP}}$  represents the inhibition constant of the 5-phosphatase by biphenyl(2,3',4,5',6) $\text{P}_5$ .

Parameter values were the same as reported earlier in Fig. 2 of (9), except for  $k_1 = 14.4606 \text{ s}^{-1}$ ,  $k_- = 0.217 \text{ s}^{-1}$ ,  $K_{\text{act}} = 0.6 \text{ }\mu\text{M}$  and  $K_{\text{IP}} = 0.2 \text{ }\mu\text{M}$ . Moreover,  $K_{\text{BP}} = 7 \text{ }\mu\text{M}$  and  $K_{\text{IBP}} = 0.5 \text{ }\mu\text{M}$ . These latter values were arbitrary, the only constraint being the fact that 5-phosphatase had a higher affinity for biphenyl(2,3',4,5',6) $\text{P}_5$  than the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. In Fig. 8, the values for biphenyl(2,3',4,5',6) $\text{P}_5$  concentrations are 0, 1.5, 25, and 40  $\mu\text{M}$  from top to bottom. A low stimulus corresponded to a rate of phospholipase C activity ( $V_{\text{PLC}}$ ) equal to  $0.03 \text{ }\mu\text{Ms}^{-1}$ , and a high stimulation to  $0.3 \text{ }\mu\text{Ms}^{-1}$ .



**Figure 2.** Overlay of the structures of two inositol phosphatase inhibitors  $\text{Ins}(1,3,4,5)\text{P}_4$  and biphenyl(2,3',4,5',6) $\text{P}_5$ .  $\text{Ins}(1,3,4,5)\text{P}_4$  (in purple) and biphenyl(2,3',4,5',6) $\text{P}_5$  (in gray) were built in Sybyl7.1 (Tripos associates). Charges were calculated by the Gasteiger-Huckel method, and each ligand was optimized to convergence using the MMFF94s method. Atoms of phosphate-1, phosphate-3, and phosphate-5 of  $\text{Ins}(1,3,4,5)\text{P}_4$  were overlaid on the phosphate-4, phosphate-2, and phosphate-6 of the biphenyl compound using the fit atoms command in Sybyl7.1. This gave the best fit root mean square deviation of 1.32 Å between equivalent atoms.

## RESULTS

### Biphenyl(2,3',4,5',6) $\text{P}_5$ is a potent inhibitor of two inositol 5-phosphatases

In a screening of several tris- and tetrakis-phosphorylated benzene derivatives, we observed that they were potent inhibitors of recombinant type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase (13). The inhibition was very much influenced by the number and position of the phosphate groups on the benzene ring. To establish the specificity of these new inhibitors of inositol 5-phosphatase, we compared two members of this family of enzymes: type-I inositol 5-phosphatase and SHIP2. Type-I inositol 5-phosphatase controls the degradation of  $\text{Ins}(1,4,5)\text{P}_3$  (4), and SHIP2 has been shown to control  $\text{PtdIns}(3,4,5)\text{P}_3$  levels both *in vitro* (21) and *in vivo* (22, 23). Both enzymes could use  $\text{Ins}(1,3,4,5)\text{P}_4$  as substrate, which was therefore used in comparative studies (Table 1).  $\text{Ins}(1,4,5)\text{P}_3$  was not a substrate of SHIP2, and the related truncated lipid analog di-C8  $\text{PtdIns}(3,4,5)\text{P}_3$  was not a substrate of type-I inositol 5-phosphatase (Table 1).

In this study, in addition to the various regioisomeric tetraphosphorylated analogs on one benzene ring, a novel pentakisphosphate derivative with phosphates spread over two benzene rings was also evaluated (Table 2). It appeared to be a rather potent inhibitor of the two inositol 5-phosphatases with  $\text{IC}_{50}$ -values in the low micromolar range using 10  $\mu\text{M}$   $\text{Ins}(1,3,4,5)\text{P}_4$  as a substrate; it was also a better inhibitor compared to the tetrakis-phosphorylated analogs on one benzene ring for both enzymes (Table 2). The mechanism of 5-phosphatase inhibition appears to be competitive with respect to the substrate (data not shown). When SHIP2 was compared to type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase, biphenyl(2,3',4,5',6) $\text{P}_5$  was a better inhibitor of SHIP2 ( $\text{IC}_{50} = 1.8 \text{ }\mu\text{M}$ ) compared to type I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase ( $\text{IC}_{50} = 7.9 \text{ }\mu\text{M}$ , Fig. 3). The biphenyl molecule also inhibited the crude  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase in a lysate of rat hepatocytes ( $\text{IC}_{50} = 1 \text{ }\mu\text{M}$  at 1  $\mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$ , Fig. 3). When incubated in the presence of recombinant type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase or SHIP2, none of the benzene tetrakisphosphate molecules nor biphenyl(2,3',4,5',6) $\text{P}_5$  at 100  $\mu\text{M}$  was dephosphorylated, as measured in a phosphate-release assay (the assays were run at different concentrations of enzyme and  $\text{Ins}(1,3,4,5)\text{P}_4$  used as positive control, was very significantly dephosphorylated as expected from our radiolabeled assay resolved on Dowex columns, data not shown).

### Biphenyl(2,3',4,5',6) $\text{P}_5$ is not an inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase A

To further characterize the properties of biphenyl(2,3',4,5',6) $\text{P}_5$ , we tested its effect on two different proteins that use  $\text{Ins}(1,4,5)\text{P}_3$  either as substrate or ligand:  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase and the  $\text{IP}_3\text{RI}$ . In the study of Poitras *et al.*, benzene 1,2,4-trisphosphate was an inhibi-

TABLE 1. Substrate specificity of type-I inositol 5-phosphatase and SHIP2 (assayed by malachite green phosphatase assay)

Substrate	Type-I 5-phosphatase	SHIP2
Ins(1,4,5)P <sub>3</sub>	207 ± 19	ND
Ins(1,3,4,5)P <sub>4</sub>	36 ± 12	57 ± 14
Ins(1,4,5,6)P <sub>4</sub>	ND	ND
di-C8 PtdIns(5)P	ND	ND
di-C8 PtdIns(3,5)P <sub>2</sub>	ND	ND
di-C8 PtdIns(4,5)P <sub>2</sub>	143 ± 29	15 ± 6
di-C8 PtdIns(3,4,5)P <sub>3</sub>	ND	115 ± 27

The substrates were at 100 μM. Data are expressed as P<sub>i</sub> produced (pmol/min) ± SD ND, non detectable.

tor of the activity of Ins(1,4,5)P<sub>3</sub> 3-kinase of bovine adrenal cortex (IC<sub>50</sub> = 0.4 μM at 6 μM Ins(1,4,5)P<sub>3</sub>). This result prompted us to evaluate the effect of the biphenyl analog on recombinant Ins(1,4,5)P<sub>3</sub> 3-kinase A, the major isoenzyme expressed in brain (24). Our data in this study were compared with several inositol phosphate analogs, some of which being potent inhibitors, such as D-2-deoxy Ins(1,4,5)P<sub>3</sub> and D-3-deoxy Ins(1,4,6)P<sub>3</sub> (15). At concentrations up to 30 μM of biphenyl(2,3',4,5',6)P<sub>5</sub>, Bz(1,2,3,4)P<sub>4</sub>, Bz(1,2,3,5)P<sub>4</sub> or Bz(1,2,4,5)P<sub>4</sub>, the inositol 3-kinase activity was not affected (Fig. 4). Significant inhibition of activity only started at 100 μM. We conclude that biphenyl(2,3',4,5',6)P<sub>5</sub> and the tetrakis-phosphorylated one-ring analogs are rather poor inhibitors of the Ins(1,4,5)P<sub>3</sub> 3-kinase A.

#### Direct effect of biphenyl(2,3',4,5',6)P<sub>5</sub> on IP<sub>3</sub>R1

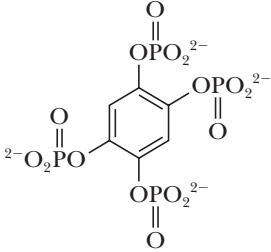
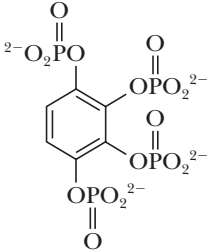
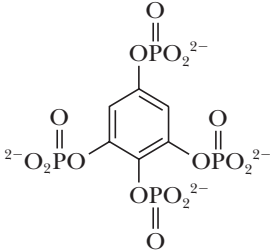
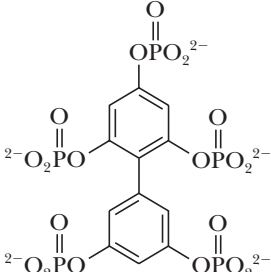
It was reported that several inositol 5-phosphatase inhibitors that are Ins(1,3,4,5)P<sub>4</sub> derivatives are also able to inhibit the binding of Ins(1,4,5)P<sub>3</sub> to IP<sub>3</sub>R1 (25). A series of tri- and tetra-phosphorylated analogs was tested on [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding on microsomes of Sf9 cells overexpressing IP<sub>3</sub>R1: biphenyl(2,3',4,5',6)P<sub>5</sub> was clearly the most potent inhibitor of Ins(1,4,5)P<sub>3</sub> binding (50% displacement being observed at 1.4 μM). Bz(1,2,4)P<sub>3</sub> and Bz(1,2,3,4)P<sub>4</sub> were not capable of displacing bound [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> in the 1–10 μM range (Fig. 5). The displacement of [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding by the benzene tetrakisphosphates was also studied at a fixed concentration of 10 μM (Table 3). The most potent inhibitor of the tetra-phosphorylated analogs was Bz(1,2,4,5)P<sub>4</sub> (47% of the control at 10 μM), which was also the most potent inhibitor of type-I inositol 5-phosphatase activity. In the same experiment, 10 μM biphenyl(2,3',4,5',6)P<sub>5</sub> decreased the binding of [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> to 14% of its control value (Table 3). Our data indicate that the number and position of the phosphate groups on the benzene ring influence the recognition pattern of phosphorylated benzene molecules at the IP<sub>3</sub>R1.

Ins(1,3,4,5)P<sub>4</sub>, a well characterized inhibitor of type-I inositol 5-phosphatase, is also an inhibitor of [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding (50% displacement at 4.8 μM, Fig. 5).

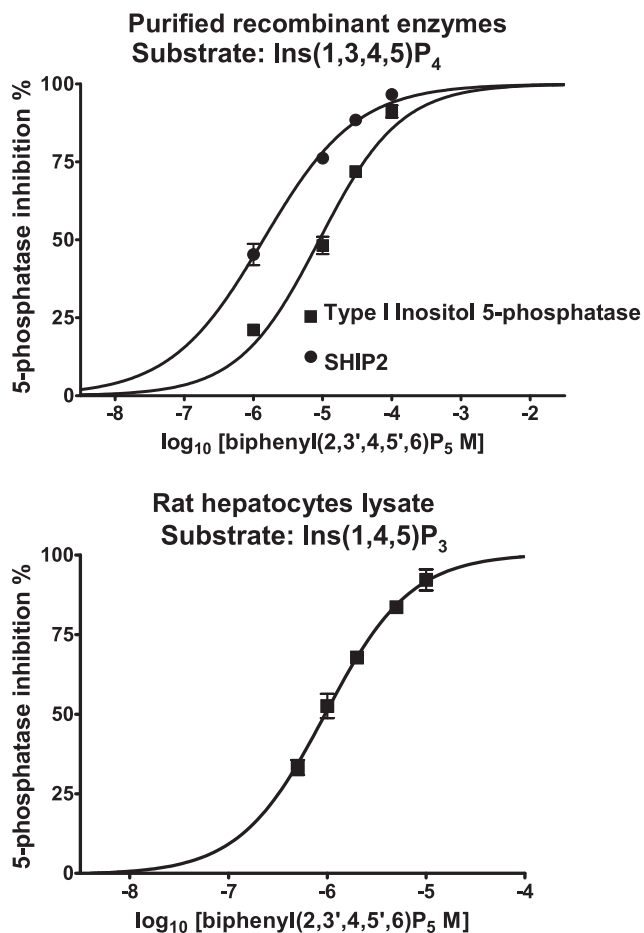
#### Biphenyl(2,3',4,5',6)P<sub>5</sub> is an inhibitor of Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release

As biphenyl(2,3',4,5',6)P<sub>5</sub> competed with Ins(1,4,5)P<sub>3</sub> for the binding to IP<sub>3</sub>R1, we examined its functional effect on Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release. An ideal model for this analysis are the permeabilized triple Ins(1,4,5)P<sub>3</sub>-receptor-knockout R23–11 cells expressing IP<sub>3</sub>R1 (18). Biphenyl(2,3',4,5',6)P<sub>5</sub> inhibited the Ins(1,4,5)P<sub>3</sub> 5-phosphatase activity in lysates of the

TABLE 2. Inhibitory potency of benzene polyphosphate analogs

Compound	IC <sub>50</sub> , μM	
	Type I	SHIP2
	14.4 ± 1.8	11.2 ± 3.7
Bz(1,2,4,5)P <sub>4</sub>		
	46.8 ± 9.7	19.6 ± 5.0
Bz(1,2,3,4)P <sub>4</sub>		
	41.1 ± 6.6	40.0 ± 11.9
Bz(1,2,3,5)P <sub>4</sub>		
	7.9 ± 0.7	1.8 ± 0.2
Biphenyl(2,3',4,5',6)P <sub>5</sub>		

Data were obtained with 10 μM Ins(1,3,4,5)P<sub>4</sub> as substrate. Data are expressed as means of triplicates ± SD. Bz(1,2,3,4)P<sub>4</sub>, Benzene 1,2,3,4-tetrakisphosphate; Bz(1,2,4,5)P<sub>4</sub>, Benzene 1,2,4,5-tetrakisphosphate; Bz(1,2,3,5)P<sub>4</sub>, Benzene 1,2,3,5-tetrakisphosphate.

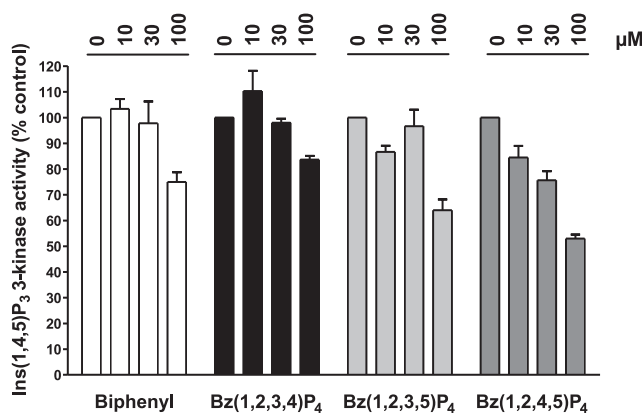


**Figure 3.** Effect of biphenyl(2,3',4,5',6)P<sub>5</sub> on recombinant enzymes and crude rat hepatocyte lysate. Effect of 1–100 μM of biphenyl(2,3',4,5',6)P<sub>5</sub> on recombinant enzymes at 10 μM Ins(1,3,4,5)P<sub>4</sub> as substrate (top panel). Effect of 0.5–10 μM of biphenyl(2,3',4,5',6)P<sub>5</sub> on rat hepatocyte lysate at 1 μM Ins(1,4,5)P<sub>3</sub> (bottom panel). The data are representative of three different experiments and are means of triplicates ± SD.

same cells also with the same potency compared to recombinant Ins(1,4,5)P<sub>3</sub> 5-phosphatase (data not shown). The addition of Ins(1,4,5)P<sub>3</sub> at concentrations of 50, 100, or 150 nM, induced Ca<sup>2+</sup> release from the nonmitochondrial intracellular stores as previously reported (18). This Ca<sup>2+</sup> release was inhibited by 1 or 4 μM biphenyl(2,3',4,5',6)P<sub>5</sub>, added 1 min before 100 or 300 nM Ins(1,4,5)P<sub>3</sub> (Fig. 6). A23187 was added at the end of the experiment to evaluate the total amount of releasable Ca<sup>2+</sup>. Therefore, the interaction between the analog and the IP<sub>3</sub>R1 is functionally relevant in this cell model since at higher concentrations, it overrules the effect of the analog on Ins(1,4,5)P<sub>3</sub> 5-phosphatase, which is also present.

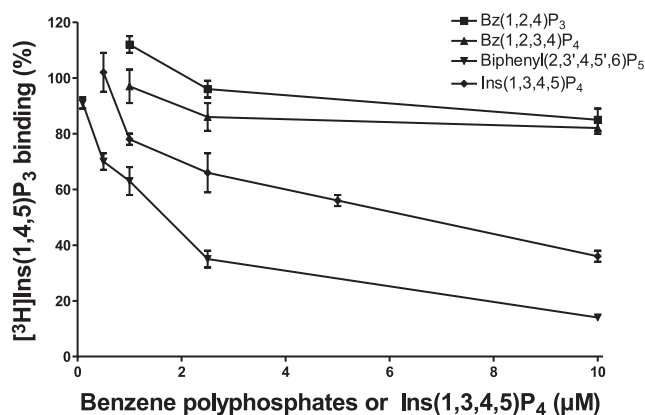
### Dual effect of biphenyl(2,3',4,5',6)P<sub>5</sub> on Ca<sup>2+</sup> oscillations in hepatocytes

On the basis of the experimentally determined kinetic parameters of the Ins(1,4,5)P<sub>3</sub> 5-phosphatase and 3-kinase, it was predicted that the 5-phosphatase primarily



**Figure 4.** The effect of benzene polyphosphate analogs on recombinant Ins(1,4,5)P<sub>3</sub> 3-kinase A. Effect of 0–100 μM of the various analogs on Ins(1,4,5)P<sub>3</sub> 3-kinase activity at 1 μM Ins(1,4,5)P<sub>3</sub> substrate level. Data are expressed as a percentage of control value (activity in the presence of water) ± SD.

controls the levels of Ins(1,4,5)P<sub>3</sub> and, thus the occurrence and frequency of Ca<sup>2+</sup> oscillations (9). We have used the model of doublets of hepatocytes that are tightly coupled by gap junctions. Coordination of Ca<sup>2+</sup> oscillations among connected hepatocytes is fully dependent on Ins(1,4,5)P<sub>3</sub> diffusion through gap junctions. It was previously shown that Ca<sup>2+</sup> signals in the two cells were different when active 5-phosphatase (as compared to inactive enzyme) had been injected into one cell of the doublet at low (0.1 μM) and maximal (10 μM) noradrenaline concentrations (26). In response to 0.1 μM noradrenaline, [Ca<sup>2+</sup>]<sub>i</sub> remained at a low basal level in the phosphatase-injected cell and oscillated at low frequency in the adjacent cell (26). This was interpreted by a reduction in the concentration of Ins(1,4,5)P<sub>3</sub> in the injected cells and by an intermediate concentration in the noninjected cell. We therefore compared the Ca<sup>2+</sup> oscillations between the hepatocyte doublets, which had been injected with



**Figure 5.** Effect of various concentrations of analogs on [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding. [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> binding on microsomes of Sf9 cells overexpressing IP<sub>3</sub>R1 was performed in the presence of various concentrations of the indicated compounds. Values are expressed as a percentage of the binding observed in control conditions and are presented as means ± SEM.

TABLE 3. [ $^3\text{H}$ ]Ins(1,4,5)P<sub>3</sub> binding in control conditions and in the presence of the indicated compounds (10  $\mu\text{M}$ )

	[ $^3\text{H}$ ]Ins(1,4,5)P <sub>3</sub> binding
Control	473 $\pm$ 21
Bz(1,2,4)P <sub>3</sub>	429 $\pm$ 20
3-BnO-Bz(1,2,4)P <sub>3</sub>	364 $\pm$ 19
Bz(1,2,3,4)P <sub>4</sub>	394 $\pm$ 30
Bz(1,2,3,5)P <sub>4</sub>	369 $\pm$ 15
Bz(1,2,4,5)P <sub>4</sub>	223 $\pm$ 7
Biphenyl(2,3',4,5',6)P <sub>5</sub>	65 $\pm$ 5

Values are expressed in fmol Ins(1,4,5)P<sub>3</sub> bound/mg protein and are presented as mean  $\pm$  SE for 3–5 experiments, each performed in four-fold. Bz(1,2,4)P<sub>3</sub>, Benzene 1,2,4-trisphosphate; 3-BnO-Bz(1,2,4)P<sub>3</sub>, 3-Benzyloxybenzene 1,2,4-trisphosphate.

biphenyl(2,3',4,5',6)P<sub>5</sub>. These experiments were designed at two different concentrations of the analog (10  $\mu\text{M}$  and 300  $\mu\text{M}$ ) in the injection pipette. We assumed the intracellular concentration of the benzene polyphosphate inside the cell to be at least 10-fold lower. At 10  $\mu\text{M}$  (injected concentration), we observed two different responses: either an increase or a decrease in the frequency of Ca<sup>2+</sup> oscillations. An increase in the frequency of the oscillations is shown in Fig. 7A at 0.1–1  $\mu\text{M}$  noradrenaline (the injected cell being the bottom trace and is compared to the upper trace). For 17 doublets in which one cell was injected, 4 cells showed an increase and 4 cells a decrease in the frequency of Ca<sup>2+</sup> oscillations; 9 cells showed no effect. At 300  $\mu\text{M}$  of the biphenyl analog in the injection pipette, the Ca<sup>2+</sup> response in the injected cell was inhibited either partially (depending on the agonist concentration) or completely (Fig. 7B, C, respectively). At 1  $\mu\text{M}$  noradrenaline, low-frequency Ca<sup>2+</sup> oscillations were detected in the injected cell, whereas high-frequency oscillations were observed in the adjacent cells, suggesting that the biphenyl compound was mainly

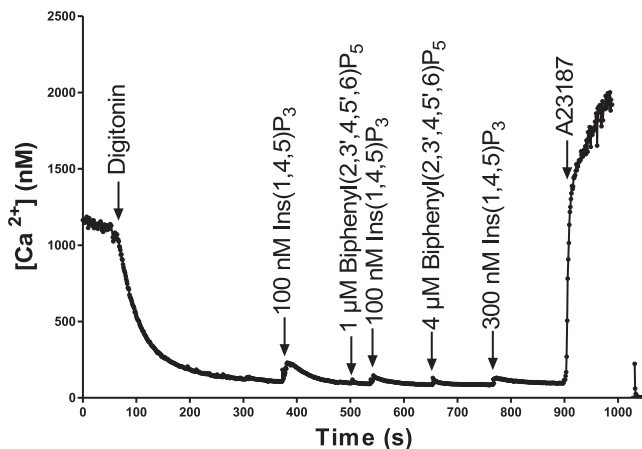


Figure 6. Ca<sup>2+</sup> release in permeabilized R23–11 cells expressing IP<sub>3</sub>R1. Ca<sup>2+</sup> release was measured as described in Materials and Methods. Biphenyl(2,3',4,5',6)P<sub>5</sub> was added 1 min before Ins(1,4,5)P<sub>3</sub>. The inhibitory effect of biphenyl(2,3',4,5',6)P<sub>5</sub> on Ca<sup>2+</sup> release induced by 100 and 300 nM Ins(1,4,5)P<sub>3</sub> was reproduced in three independent experiments.

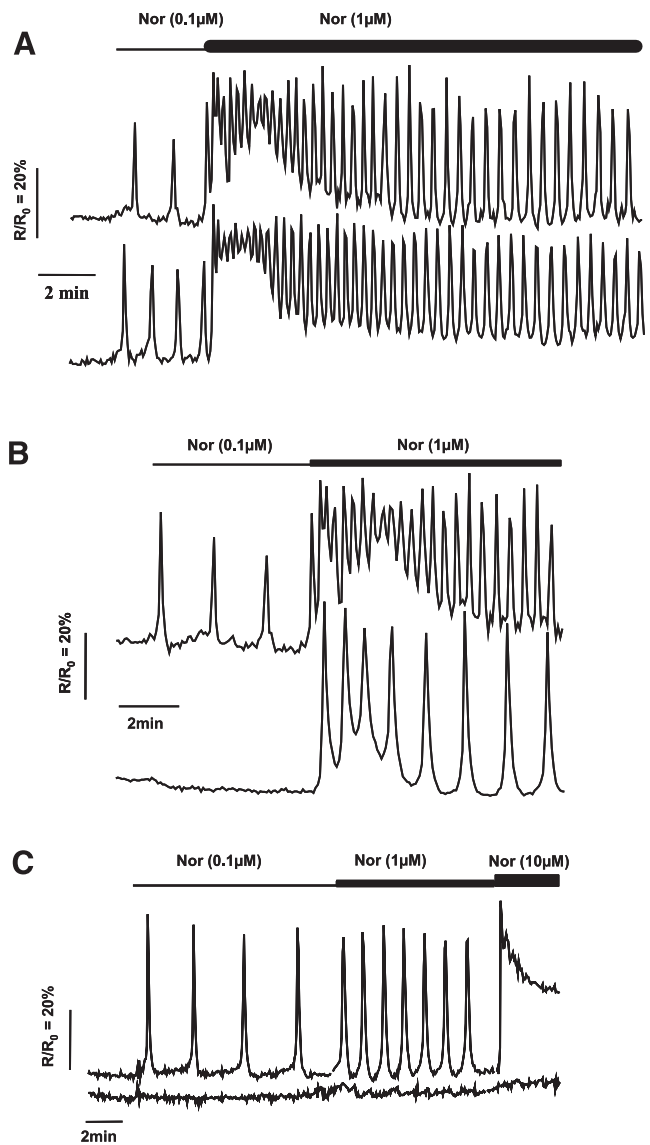


Figure 7. Ca<sup>2+</sup> signals in response to 0.1–10  $\mu\text{M}$  noradrenaline in hepatocytes. One cell of the doublet (lower trace in each panel) was microinjected with Fura-2 and 10  $\mu\text{M}$  (A) and 300  $\mu\text{M}$  (B and C) biphenyl(2,3',4,5',6)P<sub>5</sub> in the injection pipette. Hepatocytes doublets were challenged with noradrenaline (Nor, 0.1, 1, or 10  $\mu\text{M}$ ) for the time shown by the horizontal bar. Results are representative of a large number of injected cells as indicated in the text.

active in the injected cells (Fig. 7B at 1  $\mu\text{M}$  noradrenaline). In 21 doublets from which one cell was injected, 11 cells showed a partial inhibition and only responded at 1.0  $\mu\text{M}$  noradrenaline, whereas 10 cells showed a total inhibition at 0.1–10  $\mu\text{M}$  noradrenaline (as shown in Fig. 7C).

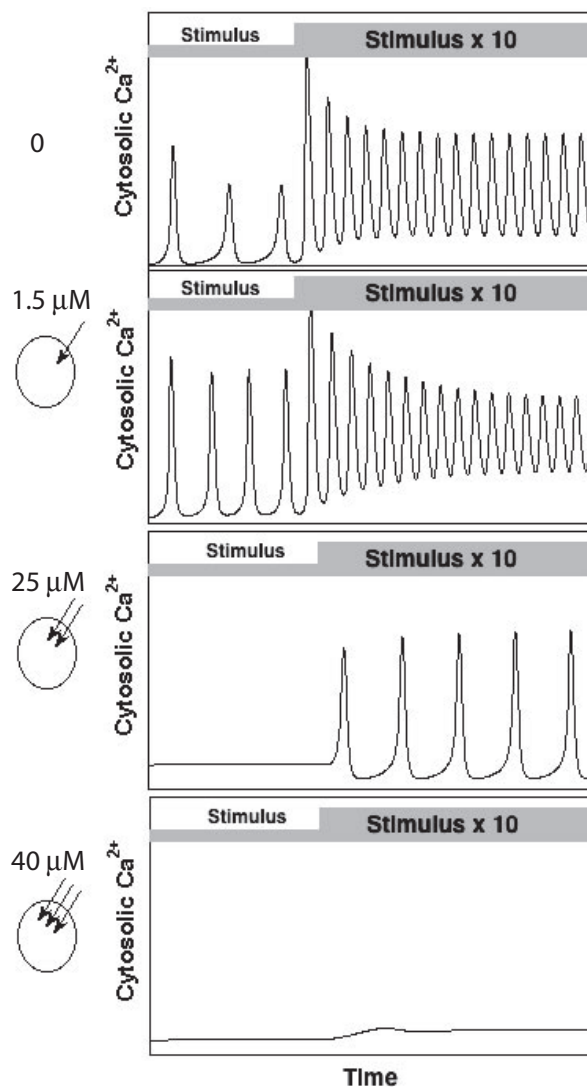
#### Simulation of the behavior of biphenyl(2,3',4,5',6)P<sub>5</sub> in intact hepatocytes

Using a model for Ca<sup>2+</sup> oscillations and the different parameters of both the synthesis and metabolism of Ins(1,4,5)P<sub>3</sub>, we previously simulated the presence of Ca<sup>2+</sup> oscillations in agonist-stimulated cells. It was sug-

gested that the phosphatase primarily controls the levels of  $\text{Ins}(1,4,5)\text{P}_3$  and thereby the occurrence and frequency of  $\text{Ca}^{2+}$  oscillations (9). We used the same model and introduced biphenyl(2,3',4,5',6) $\text{P}_5$  as a competitive inhibitor at both the level of the 5-phosphatase and at the  $\text{Ins}(1,4,5)\text{P}_3$  binding site of the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. We introduced a lower dissociation constant for the phosphatase as compared to the binding to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. When a low concentration of the analog was present inside the cells, the model predicted an increase in the frequency of  $\text{Ca}^{2+}$  oscillations in response to a stimulus (1.5  $\mu\text{M}$  as compared to control cell in Fig. 8). At higher concentrations, the  $\text{Ca}^{2+}$  response was totally lost.  $\text{Ca}^{2+}$  oscillations were recovered when the concentration of the stimulus was 10-fold higher (Fig. 8 at 25  $\mu\text{M}$  of biphenyl(2,3',4,5',6) $\text{P}_5$ ). The fact that in our experimental observations, the frequency of  $\text{Ca}^{2+}$  oscillations could either decrease or increase at low concentration of biphenyl(2,3',4,5',6) $\text{P}_5$ , depending on the injected cell, was interpreted as the consequence of its interaction at two proteins: First, on  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase, thereby increasing the intracellular levels of  $\text{Ins}(1,4,5)\text{P}_3$  and second, on the  $\text{Ins}(1,4,5)\text{P}_3$  receptor itself by an inhibitory effect on  $\text{Ins}(1,4,5)\text{P}_3$  binding. This dual effect depended on the dissociation constant of the analog with respect to the 5-phosphatase as compared to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. In this framework, at high concentrations of biphenyl(2,3',4,5',6) $\text{P}_5$ , the concentration of  $\text{Ins}(1,4,5)\text{P}_3$  in the cell would be high (because of 5-phosphatase inhibition), but this would not allow any significant  $[\text{Ca}^{2+}]_i$  increase, because the receptor would also be inhibited. Our simulation data suggest that in the cell, biphenyl(2,3',4,5',6) $\text{P}_5$  had a higher apparent affinity for the phosphatase as compared to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. Indeed, if the affinity for the  $\text{Ins}(1,4,5)\text{P}_3$  receptor would be higher than for the phosphatase, an increase in the frequency of  $\text{Ca}^{2+}$  oscillations at low agonist concentrations (as shown in Fig. 7A) could never be observed.

## DISCUSSION

Inositol and lipid phosphatases can act at the 5-, 4-, and 3-position of the inositol ring, generating either inactive compounds or novel second messengers (27). Several soluble inositol phosphates and phosphoinositides are dephosphorylated at the 5-position by the large family of inositol 5-phosphatases, which in mammalian cells consist of 10 different enzymes (1). Their primary structures show the presence of a catalytic domain and a series of protein interaction motives which target the enzymes to different parts of the cell. Inositol 5-phosphatase inhibitors could be useful tools in order to evaluate the role of individual enzymes in any cell model and compare their relative activities and eventual redundancy in cells. Type-I inositol 5-phosphatase appears to be a membranous enzyme as shown initially in liver or brain tissue (28, 29). Cloning studies have shown that it is the shorter enzyme in the mam-



**Figure 8.** Numerical simulations of  $\text{Ca}^{2+}$  oscillations in response to agonist stimulation at different concentrations of biphenyl(2,3',4,5',6) $\text{P}_5$  injected to the cell. The different panels show simulated  $\text{Ca}^{2+}$  oscillations in the absence (upper panel) or in the presence of biphenyl(2,3',4,5',6) $\text{P}_5$  (three lower panels, increasing concentrations as indicated). Traces have been obtained with a classical inhibition term for type I inositol 5-phosphatase and  $\text{Ins}(1,4,5)\text{P}_3$  receptor by biphenyl(2,3',4,5',6) $\text{P}_5$ . A critical assumption of the model is that the 5-phosphatase has a higher affinity for the analog than the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. See Materials and Methods for a description of the equations and parameter values. The data were obtained at one- and at a ten-fold higher concentration of agonist as indicated by the horizontal line.

malian inositol 5-phosphatase family (412 amino acids) and that it contains a C-terminal prenylation site (30). It controls the levels of  $\text{Ins}(1,4,5)\text{P}_3$  and the occurrence of  $\text{Ca}^{2+}$  oscillations in intact cells. A mutation of the prenylation site also abolished its effect on  $[\text{Ca}^{2+}]_i$  (4).

We looked for inositol 5-phosphatase inhibitors using human type-I inositol 5-phosphatase produced in bacteria. Initial studies of Poitras *et al.* using a relatively crude enzyme have suggested that benzene polyphosphate analogs could be of interest in that respect (12).



A wider study of benzene polyphosphate analogs containing two, three, and four phosphate groups, show that they may act as type-I inositol 5-phosphatase inhibitors (13). They were not substrates at 100  $\mu\text{M}$ , indicating that if they interact at the active site, they do not completely adopt the structure of  $\text{Ins}(1,4,5)\text{P}_3$  or  $\text{Ins}(1,3,4,5)\text{P}_4$ , the two natural substrates of that enzyme. One exception is 3-hydroxybenzene 1,2,4-trisphosphate, which was dephosphorylated by type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase to 2,3-dihydroxybenzene 1,4-bisphosphate (13).

Surprisingly, we show here that the most potent inhibitor was biphenyl(2,3',4,5',6) $\text{P}_5$ . Compared to bisphosphoglycerate, the first type-I inositol 5-phosphatase inhibitor identified ( $K_i$  of approx. 0.35 mM with  $\text{Ins}(1,4,5)\text{P}_3$  as substrate (31)), biphenyl(2,3',4,5',6) $\text{P}_5$  is much more potent, having an  $\text{IC}_{50}$ -value in the low micromolar range, and was not a substrate using the phosphate release assay when tested at 100  $\mu\text{M}$ . A possible explanation for the potency of biphenyl(2,3',4,5',6) $\text{P}_5$  is that the phosphate groups of this molecule can nearly overlay with the phosphate groups of  $\text{Ins}(1,3,4,5)\text{P}_4$ , (Fig. 2) a very potent inhibitor of type I inositol 5-phosphatase. However, in contrast to this molecule, it is not a substrate.

This result prompted us to consider whether biphenyl(2,3',4,5',6) $\text{P}_5$  could also inhibit the activity of the lipid phosphatase SHIP2. Because this enzyme was shown to accept  $\text{Ins}(1,3,4,5)\text{P}_4$  as a substrate, we used that substrate in our comparative studies. Although we clearly showed that  $\text{Ins}(1,4,5)\text{P}_3$  was not a substrate of SHIP2, the biphenyl analog was an inhibitor of SHIP2 and was even more potent compared to its effect on type-I enzyme. The rank order of potency (biphenyl >  $\text{Bz}(1,2,4,5)\text{P}_4$ ) was conserved between the two enzymes, suggesting that they share some conserved structural characteristics. In another study, although not tested as an inhibitor,  $\text{Ins}(1,2,3,4,5)\text{P}_5$  was reported to be a good substrate of SHIP2 (32). Therefore, highly phosphorylated molecules such as biphenyl(2,3',4,5',6) $\text{P}_5$  could be very well tolerated at the active site of SHIP2. We have observed that assay conditions could largely influence the lipid phosphatase activity of SHIP2: the presence of phosphatidylserine vesicles stimulated the activity of SHIP2 with  $\text{PtdIns}(3,4,5)\text{P}_3$  di-C8 as substrate, whereas type-I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase was not sensitive to the presence of this anionic lipid (17). The inhibitory effect of biphenyl(2,3',4,5',6) $\text{P}_5$  was also seen with  $\text{PtdIns}(3,4,5)\text{P}_3$  di-C8 as substrate but only in the absence of phosphatidylserine. In the presence of the anionic lipid at 200  $\mu\text{M}$ , the inhibitory effect was no longer observed (data not shown). Therefore, although we have established that biphenyl(2,3',4,5',6) $\text{P}_5$  shows an affinity for SHIP2, the potency of inhibition of its natural lipid substrate may still rely on assay conditions. A high-throughput assay for SHIP2 of a 91,060-member compound library resulted in the identification of SHIP2 inhibitors, although no chemical structures were presented in this study (33). A comparison of the best inhibitors of that study with biphenyl(2,3',4,5',6) $\text{P}_5$  will

be interesting and may provide a route to more potent novel inhibitors.

The biphenyl analog was not an inhibitor of the  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase A produced in bacteria. Because  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase A is very specific in the recognition of  $\text{Ins}(1,4,5)\text{P}_3$  and tolerates only very few modifications of its structure, thus, this result was not unexpected (15, 34). Data in the study of Poitras *et al.* indicated that benzene 1,2,4-trisphosphate competitively blocked  $\text{Ins}(1,4,5)\text{P}_3$  binding to adrenal cortex microsomes with a half-maximal concentration at 34  $\mu\text{M}$  (12). Our conditions for measuring [ $^3\text{H}$ ] $\text{Ins}(1,4,5)\text{P}_3$  binding have the advantage of using a single  $\text{Ins}(1,4,5)\text{P}_3$  receptor subtype, and the assay was performed at pH of 7.4, which is more physiologically relevant than in many other studies. Among the phosphorylated benzene analogs we have tested, biphenyl(2,3',4,5',6) $\text{P}_5$  was by far the most potent competitor of [ $^3\text{H}$ ] $\text{Ins}(1,4,5)\text{P}_3$  binding on  $\text{IP}_3\text{R1}$ . We suggest that the displacement is not just electrostatic: competition of [ $^3\text{H}$ ]  $\text{Ins}(1,4,5)\text{P}_3$  binding by nonradioactive  $\text{Ins}(1,4,5)\text{P}_3$  occurs at much lower concentrations with a 50% displacement around 50 nM and a nearly complete displacement observed at 100 nM (20). Moreover, when the trisphosphorylated benzene regioisomers were compared,  $\text{Bz}(1,2,4,5)\text{P}_4$  was always more efficient as compared to  $\text{Bz}(1,2,3,4)\text{P}_4$  or  $\text{Bz}(1,2,3,5)\text{P}_4$  (Table 3). Given the fact that  $\text{Ins}(1,3,4,5)\text{P}_4$ , a potent type-I 5-phosphatase inhibitor, also inhibited the binding of  $\text{Ins}(1,4,5)\text{P}_3$  to the receptor (25), this would indicate that the receptor and the phosphatase must share some common structural requirements. It was suggested in another study that  $\text{Ins}(1,3,4,5)\text{P}_4$  acted at two steps: at low concentrations, it facilitates store-operated  $\text{Ca}^{2+}$  influx by inhibition of  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase (25). At higher concentrations, it was as an inhibitor of  $\text{Ins}(1,4,5)\text{P}_3$  receptors, enabling  $\text{Ins}(1,3,4,5)\text{P}_4$  to act as a potent bimodal regulator of cellular sensitivity to  $\text{Ins}(1,4,5)\text{P}_3$ . Thus, it provides both facilitatory and inhibitory feedback on  $\text{Ca}^{2+}$  signaling (25).

High concentrations of biphenyl(2,3',4,5',6) $\text{P}_5$  were shown to inhibit the  $\text{Ca}^{2+}$  response in two different cell models: in B cells overexpressing the  $\text{IP}_3\text{R1}$  and in hepatocytes. This is supported by the fact that it inhibits the binding of  $\text{Ins}(1,4,5)\text{P}_3$  to  $\text{IP}_3\text{R1}$ , thereby preventing any  $\text{Ca}^{2+}$  response. The most important feature of our study is the observation that biphenyl(2,3',4,5',6) $\text{P}_5$  may positively or negatively affect the frequency of  $\text{Ca}^{2+}$  oscillations stimulated by noradrenaline. Because we injected the analog into hepatocytes, the result is probably dependent on its effective concentration that is experienced by the  $\text{Ins}(1,4,5)\text{P}_3$  receptor. It affects the phosphatase activity in a very restricted window of concentration of both agonist and analog. In our injections, this has implied the use of 10  $\mu\text{M}$  of biphenyl(2,3',4,5',6) $\text{P}_5$  in the injection pipette. The data presented here highlight two important properties of a type-I phosphatase inhibitor such as  $\text{Ins}(1,3,4,5)\text{P}_4$  and the biphenyl polyphosphate molecule:

1) at high concentrations, it inhibits the receptor and therefore the  $\text{Ca}^{2+}$  response and 2) at low concentrations, it provides a window where it increases the level of  $\text{Ins}(1,4,5)\text{P}_3$  producing a new steady-state level of this important molecule. This is essentially the result of its interaction with the 5-phosphatase and the enzymatic properties of that enzyme compared to those of the  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase. Consequently, this increase in  $\text{Ins}(1,4,5)\text{P}_3$  concentration activates the frequency of  $\text{Ca}^{2+}$  oscillations. This interpretation is supported by our model of  $\text{Ca}^{2+}$  oscillations, taking into account the fact that the dissociation constant is lower for the  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase than for the  $\text{Ins}(1,4,5)\text{P}_3$  receptor (Fig. 8).

In conclusion, our data suggest that biphenyl(2,3',4,5',6) $\text{P}_5$  could be an interesting core molecule in order to control intracellular levels of  $\text{Ins}(1,4,5)\text{P}_3$  and its effect in  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release. At low concentrations, it inhibits the  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase activity and participates in the generation of a new steady-state level of  $\text{Ins}(1,4,5)\text{P}_3$ . At high concentration, it primarily acts by inhibiting the binding of  $\text{Ins}(1,4,5)\text{P}_3$  to its receptor and, by this mechanism, the  $\text{Ca}^{2+}$  response. The phosphatase appears to be a key enzyme in the control of  $\text{Ca}^{2+}$  oscillations, and further study will be needed to investigate the physiological consequences of this control in hepatocytes and other cells. The close functional relationship between the phosphatase and the  $\text{Ins}(1,4,5)\text{P}_3$  receptor converging in the control of  $\text{Ca}^{2+}$  oscillations is consistent with the fact that in yeast, it is another pathway that is used in the metabolism of  $\text{Ins}(1,4,5)\text{P}_3$ : an inositol phosphate multikinase, controls the level of  $\text{Ins}(1,4,5)\text{P}_3$  and the synthesis of inositol pentakisphosphate (35). Yeast also does not have an  $\text{Ins}(1,4,5)\text{P}_3$  receptor nor an  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase (36).

Biphenyl(2,3',4,5',6) $\text{P}_5$  could be a lead molecule in the synthesis of cell-permeable  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase inhibitors that could be exploited as biological tools. It is achiral and easier to synthesize than a chiral inositol polyphosphate or related derivative. Furthermore, the synthesis of the masked lipophilic phosphate derivative which can be used without microinjection should be easy to accomplish since only the phosphate groups have to be protected. These compounds may also inhibit other isoenzymes within the inositol 5-phosphatase family. They could be evaluated as competitors with respect to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor, and as we have shown here, could positively or negatively affect the frequency of  $\text{Ca}^{2+}$  oscillations in response to a  $\text{Ca}^{2+}$ -mobilizing agonist. **FJ**

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