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Membrane-Permeant Esters of Inositol Polyphosphates, Chemical Syntheses and Biological Applications[1]

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Abstract: Membrane-permeant derivatives of inositol polyphosphates are useful tools for biological studies. Racemic 2,3,6-tri-O-butyryl-*myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester(Bt_3IP_3/AM), *myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (IP_3/AM), hexakis(propionyloxymethyl) ester (IP_3/PM) and hexakis(butyryloxymethyl) ester (IP_3/BM) were therefore synthesized. Whereas extracellular application of up to 200 μM of Bt_3IP_3/AM or IP_3/AM to 1321N1 astrocytoma cells failed to mobilize internal calcium, IP_3/PM and IP_3/BM released internal calcium at concentrations as low as 20 μM and 2 μM, respectively. © 1997 Elsevier Science Ltd.

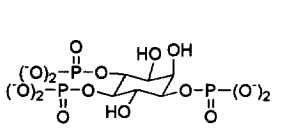
INTRODUCTION

Small molecules lipophilic enough to passively diffuse across plasma membrane of cells and modulate cellular functions serve as important research tools for cell biologists, pharmacologists and neurobiologists. However, many compounds important inside cells are highly charged or hydrophilic molecules. Direct biological administration of these compounds is limited by their poor membrane permeability. Various techniques exist for delivering these membrane-impermeant molecules inside cells, such as microinjection, electroporation, patch-clamp techniques and detergents like saponin. However, these methods have the disadvantage of breaching the plasma membrane and disrupting the intact cell structure, which may jeopardize the more complex functions and long term viability of the cells. Furthermore, microinjection and patch-clamp techniques can only be applied to a few cells at a time. Intracellular delivery of these molecules by converting them to membrane-permeant derivatives overcomes these drawbacks. These compounds diffuse inside cells, undergo cleavage if necessary to regenerate the active messengers, and can be easily applied to cell populations, whole tissues, or intact organisms.

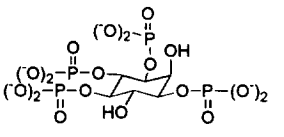
D-*myo*-inositol 1,4,5-trisphosphate (IP_3, Fig. 1) is one of the most ubiquitous intracellular second messengers in eukaryotic cells. It is produced by phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and diacylglycerol. Diacylglycerol binds to and activates protein kinase C, resulting in the phosphorylation of many intracellular proteins. IP_3 binds to a family of receptors and opens Ca^{2+} channels on the endoplasmic reticulum, thus releasing stored Ca^{2+} (reference[1,2]). Since intracellular Ca^{2+} regulates so

many cellular processes such as neuronal signalling, muscle contraction, cell division and fertilization, the regulation of its upstream signal, IP_3, has been under intensive studies. However, classical pharmacological approaches to stimulate cells mostly results in the complete scenario of receptor-stimulated signalling pathways mentioned above. Membrane-permeant derivatives of IP_3 would allow to specifically manipulate this particular intracellular messenger, hence the various pathways could be dissected. The ionic and polar nature of IP_3 and other inositol polyphosphates limits their membrane permeability, so that specialized and disruptive techniques are required to get them into cells. We have therefore attempted to develop a general strategy to deliver inositol polyphosphates into a population of intact cells by designing and synthesizing membrane-permeant derivatives.

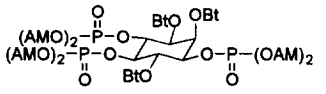
The conversion of carboxylates or phosphates into their acetoxymethyl (AM) esters not only masks the negative charges of these anions but also increases the hydrophobicity of the molecule, hence leading to much improved membrane permeability. Once inside cells, AM esters are rapidly hydrolyzed by ubiquitous intracellular esterases. The hydrolysis leads to a labile hemiacetal intermediate which spontaneously eliminates



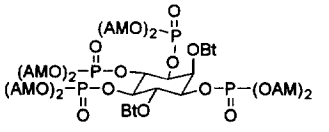
IP_3



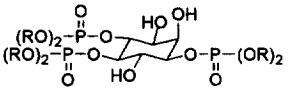
IP_4

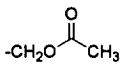


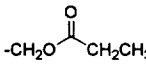
1, Bt_3IP_3/AM



2, Bt_2IP_4/AM



R =  **4**, IP_3/AM

 **5**, IP_3/PM

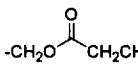
 **6**, IP_3/BM

Figure 1. Chemical structures of 1,4,5-IP_3, 1,3,4,5-IP_4, and their neutral esters: Bt_3IP_3/AM **(1)**, Bt_2IP_4/AM, **(2)** IP_3/AM **(4)**, IP_3/PM **(5)** and IP_3/BM **(6)**.

one molecule of formaldehyde. The parent molecule is regenerated once all the AM groups of the prodrug are hydrolyzed. This strategy has been successfully applied to a variety of carboxylate- or phosphate-containing molecules such as ion indicators[3,4] or nucleotides, cyclic[5,6] or noncyclic[7-9]. Even though more than a dozen inositol polyphosphates have been found in cells, so far only one membrane-permeant derivative, 1,2-di-*O*-butyryl-*myo*-inositol 3,4,5,6-tetrakisphosphate octakis(acetoxymethyl) ester, has been documented to have the expected biological activity[10]. Initially we synthesized the corresponding derivatives of IP_3 and *myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4, Fig. 1), i.e. *rac*-2,3,6-tri-*O*-butyryl-*myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (Bt_3IP_3/AM) and *rac*-2,6-di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate octakis(acetoxymethyl) ester (Bt_2IP_4/AM, Fig. 1), respectively. The butyryl groups masking the free hydroxyls ease the synthesis, increase the hydrophobicity, and prevent phosphate ester migration. Unfortunately, Bt_3IP_3/AM proved to have little or no biological activity, perhaps because the butyryls hydrolyze too slowly and the biologically active intracellular product was probably metabolized faster than it was generated. We therefore synthesized a series of IP_3 esters in which the 2, 3, and 6-hydroxyls remain unprotected, i.e. IP_3 hexakis(acetoxymethyl) ester (IP_3/AM, Fig 1), hexakis(propionyloxymethyl) ester (IP_3/PM) and hexakis(butyryloxymethyl) ester (IP_3/BM). Whereas up to 200 μM IP_3/AM did not mobilize internal calcium, IP_3/PM and IP_3/BM were effective at doses as low as 20 μM and 2 μM respectively.

RESULTS

Esters of IP_3 with hydroxyls esterified by butyryl groups

The synthesis (Fig. 2) of racemic 2,3,6-tri-*O*-butyryl IP_3 hexakis(acetoxymethyl) ester (Bt_3IP_3/AM, **1**) began from 1,4,5,6-tetra-*O*-benzyl-*myo*-inositol (**1a**) [11]. Butyrylation followed by hydrogenolysis of the benzyl groups yielded 2,3-di-*O*-butyryl-*myo*-inositol (**1b**). For brevity, the numbering will be given in terms of the stereochemistry of the biologically active series, with the understanding that the enantiomer is also present. The introduction of the third butyryl group was performed by reaction with one equivalent of butyric anhydride in pyridine. The reaction showed significant regioselectivity[12,13]. Of the four possible products only three were formed under the reaction conditions. The mixture was separated by preparative reverse-phase HPLC with elution in the following order: 2,3,6- (**1c**, 23%), 2,3,5- (5%), and 1,2,3-tri-O-butyryl-*myo*-inositol (50%). Structures of the three structural isomers were assigned by one- and two-dimensional NMR. Phosphitylation of 2,3,6-tri-*O*-butyryl-*myo*-inositol **1c** with dibenzyl N,N-diethylphosphoramidite[14] in the presence of 1H-tetrazole in CH_3CN, followed by oxidation with peracetic acid (30% in AcOH) at -40°C gave the fully protected trisphosphate **1d** in racemic form in 49% yield after preparative HPLC. Hydrogenolysis in acetic acid at room temperature and atmospheric pressure and subsequent lyophilization afforded the desired free acid 2,3,6-tri-O-butyryl-*myo*-inositol 1,4,5-trisphosphate (**1e**) (yield 99%) as a white powder, whose structure

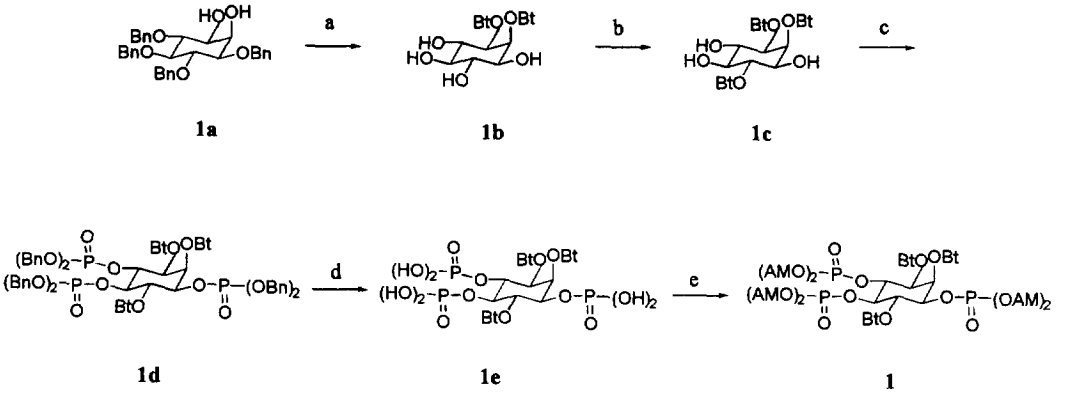
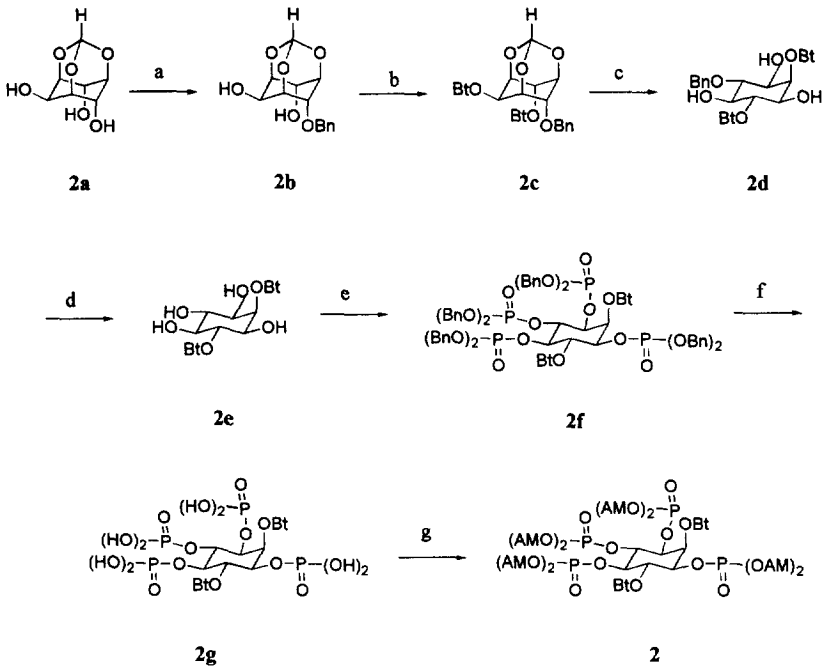


Figure 2. Synthesis of *rac*-Bt_3IP_3/AM.

was verified by one- and two-dimensional ^{1}H-NMR as well as ^{31}P-NMR. This free acid was esterified with AM groups by stirring for 2 days with an excess of diisopropylethylamine (DIEA) and acetoxymethyl bromide (AM-Br) to yield 33% of sufficiently pure Bt_3IP_3/AM (**1**).

The synthesis (Fig. 3) of 2,6-di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate octakis(acetoxymethyl) ester (Bt_2IP_4/AM, **2**) began from *myo*-inositol 1,3,5-orthoformate (**2a**)[15]. The 4-OH group was regioselectively benzylated in 86% yield as described e.g. by Billington et al[16]. Subsequent butyrylation gave the crystalline 4-*O*-benzyl 2,6-di-*O*-butyryl-*myo*-inositol 1,3,5-orthoformate (**2c**) in good yield. The hydrolysis of the orthoester **2c** was performed in 80% CF_3COOH; monitoring by HPLC showed that the desired product 4-*O*-benzyl 2,6-di-*O*-butyryl-*myo*-inositol (**2d**) reached a maximum after 7 h at room temperature. Isolation by preparative HPLC gave a 56% yield. The minor byproducts 4-*O*-benzyl 2-*O*-butyryl *myo*-inositol and 4-*O*-benzyl-6-*O*-butyryl-*myo*-inositol could be isolated as a mixture from which the former crystallized (2% yield). After hydrogenolysis of the benzyl group, the inositol dibutyrate **2e** was phosphitylated as described for the synthesis of **1d**. Oxidation and purification on preparative HPLC yielded the fully protected Ins(1,3,4,5)P_4 derivative **2f** (yield 70%), which was hydrogenated to give 2,6-di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate (**2g**, yield >99%, free acid) as a white powder after freeze-drying. **2g** was reacted with an excess of DIEA and AM-Br in the usual way. The reaction mixture was evaporated to dryness and Bt_2IP_4/AM (**2**) was extracted with toluene in 76% yield. The NMR spectrum indicated that the product was sufficiently pure (>98%) to be used for biochemical purposes.

Bt_3IP_3/AM (**1**) was tested by imaging cytosolic free Ca^{2+} levels in single REF-52 fibroblasts using standard methodology[17]. Cells were loaded with the Ca^{2+}-indicator fura-2 and viewed by fluorescence



a. BnBr, NaH b. Bt_2O, pyridine c. 80% TFA (H_2O) d. H_2, Pd/C
e. (1) Et_2N-P(OBn)$_2$, tetrazole (2) CH_3COOOH f. H_2, Pd/C, HOAc g. AM-Br, DIEA/CH_3CN

Figure 3. Synthesis of *rac*-Bt_2IP_4/AM.

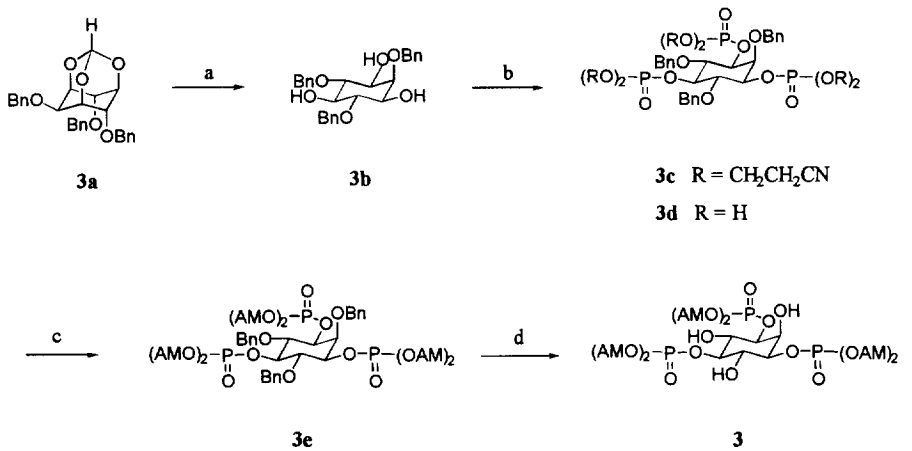
excitation ratioing. Bt_3IP_3/AM was applied extracellularly at concentrations up to 200 μM while monitoring cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$). Although initial experiments with impure samples of Bt_3IP_3/AM gave the expected elevations in $[Ca^{2+}]_i$[18], the effects diminished in amplitude and reproducibility as the purity of the Bt_3IP_3/AM improved. Eventually we were forced to conclude that this ester is biologically ineffective when all the hydroxyls are butyrylated. In particular the ester on the 6-hydroxyl is likely to be problematic, because the flanking 1- and 5-phosphates make it the most hindered ester, yet the 6-hydroxyl must become free to permit binding to the IP_3 receptor (IP_3R). We therefore decided to synthesize IP_3 esters in which the hydroxyls are left nonesterified.

Esters of IP_3 with nonesterified hydroxyls

Synthesis of racemic IP_3/AM (**4**). In the total synthesis of IP_3/AM, the 2, 3, and 6-hydroxyls of *myo*-inositol have to be protected during the synthesis in order to install the phosphate AM esters on 1, 4, and 5 positions. The protecting group for the hydroxyls have to be orthogonal to the phosphate AM esters and

removable without phosphate triester migration, which would proceed rapidly under either basic or acidic conditions. The benzyl group was considered as a candidate because catalytic hydrogenation can be carried out under mild conditions. Again, removal of the protecting group on the 6-hydroxyl, sandwiched between two phosphates and sterically hindered, was expected to be the most troublesome. Since the choice of protecting group had to be made at the beginning of the synthesis, we first tested benzyl groups on a model compound before committing to a multi-step synthesis of IP_3/AM.

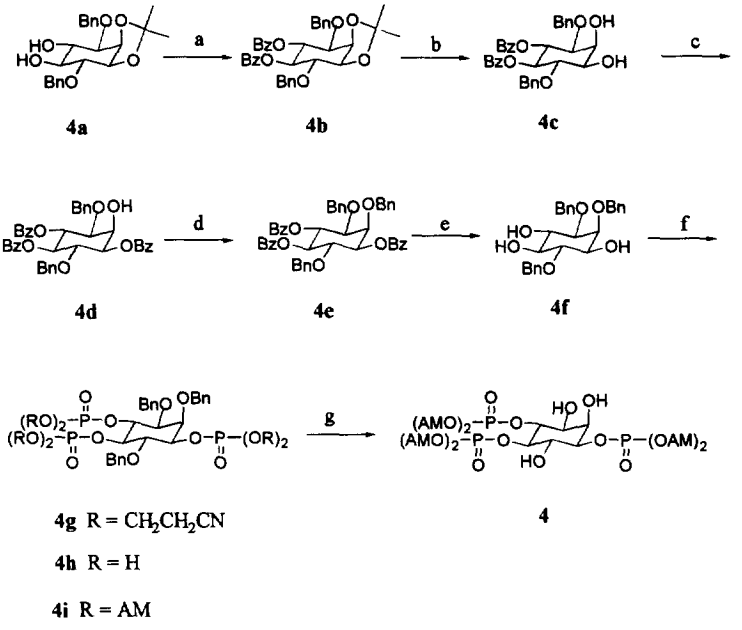
myo-Inositol 1,3,5-trisphosphate hexakis(acetoxymethyl) ester (1,3,5-IP_3/AM, **3**, Fig. 4) was chosen as a model compound. Its precursor 2,4,6-tri-*O*-benzyl-*myo*-inositol 1,3,5-trisphosphate hexakis(acetoxymethyl) ester (**3e**) ought to be accessible in a few steps from the known compound, 1,3,5-methylidyne-2,4,6-tri-*O*-benzyl-*myo*-inositol(**3a**)[15]. Phosphitylation of triol **3b** followed by oxidation with *t*-butyl hydroperoxide afforded trisphosphate **3c**, which was then converted to **3d** after removing β-cyanoethyl groups[19,20]. Esterification of **3d** with excess acetoxymethyl bromide and diisopropylethylamine provided **3e**. Catalytic hydrogenation using palladium on activated carbon (10% Pd/C) failed, but in a mixture of palladium acetate and palladium trifluoroacetate as catalyst and glacial acetic acid as solvent, all the benzyl groups of **3e** were smoothly cleaved in 3 hours at 1 atm of H_2. During the hydrogenation, the temperature was carefully controlled at about 18 °C to minimize phosphate triester migration.



a. HCl, MeOH, reflux b. (1) $(CNCH_2CH_2O)_2PN(i\text{-}Pr)_2$, tetrazole (2) t-BuOOH, **3c** (3) Ammonia, MeOH, 60°C, **3d**
c. AM-Br, DIEA/CH_3CN d. $Pd(OAc)_2$, $Pd(OC(O)CF_3)_2$, HOAc, 18°C

Figure 4. Synthesis of 1,3,5-IP_3/AM

Since the L-enantiomer of IP_3 has negligible binding affinity to the IP_3 receptor[21], synthesis of racemic 1,4,5-IP_3/AM (**4**) was considered adequate for initial experiments. The synthesis started from the known compound **4a**[22]. The 4- and 5-hydroxyls of **4a** were protected as benzoates (Fig. 5). Acid hydrolysis



a. BzCl, pyridine b. MeOH/THF, HCl (cat) c. BzCl (1.1equ.), pyridine
d. Benzyl 2,2,2-trichloroacetimidate, triflic acid (cat) e. K_2CO_3, MeOH
f. (1) $(CNCH_2CH_2O)_2PN(i\text{-}Pr)_2$, tetrazole (2) t-BuOOH (3) Ammonia, MeOH, 60°C (4) AM-Br, DIEA/CH_3CN
g. $Pd(OAc)_2$, $Pd(OC(O)CF_3)_2$, HOAc, 18°C

Figure 5. Synthesis of *rac*-1,4,5-IP_3/AM

of the cis-acetonide and selective esterification of the equatorial 1-hydroxyl in the presence of the axial 2-hydroxyl afforded monoalcohol **4d**[23]. The 2-hydroxyl was then protected with a benzyl group under acidic conditions[24]. Methanolysis removed three benzoates and provided the key intermediate, racemic triol **4f**. Using the same method developed earlier for 1,3,5-IP_3/AM, *rac*-1,4,5-IP_3/AM (**4**) was prepared from **4f** in 5 steps.

Biological tests of IP_3/AM (4). The first tests of IP_3/AM were carried out in 1321N1 astrocytoma cells, which express muscarinic acetylcholine receptors coupled to the PLC-IP_3 signaling pathway[25,26]. Initial assays of the intracellular Ca^{2+} mobilizing activity of IP_3/AM were disappointing. No $[Ca^{2+}]_i$ increase was detected in astrocytoma cells when IP_3/AM was applied extracellularly at a dose as high as 200 μM (Fig. 6). Subsequent stimulation of the cells with the cholinergic agonist carbachol (CCh) gave a normal response. Lower doses of IP_3/AM did not change $[Ca^{2+}]_i$ either (data not shown).

The three free hydroxyls of IP_3/AM may make it too hydrophilic to cross cell membranes efficiently. To check whether membrane permeability was limiting, we needed a system where IP_3/AM could be directly

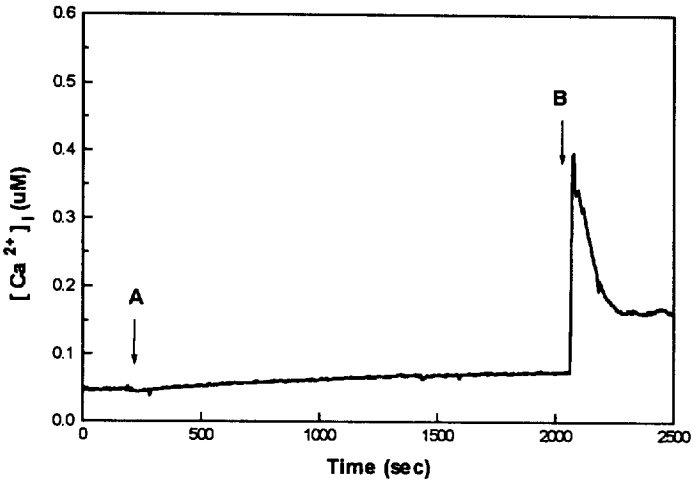


Figure 6. Biological test of IP_3/AM (**4**) in 1321N1 astrocytoma cells. Extracellular application of a high dose of IP_3/AM (200 μM, **A**) had no effect on $[Ca^{2+}]_i$ even after a prolonged period of incubation. Subsequent stimulation with carbachol (200 μM, **B**) gave a normal response.

injected into cells and its calcium releasing activity monitored. *Xenopus* oocytes were chosen because their large size (~1 mm diameter) makes them easy to microinject, and because $[Ca^{2+}]_i$ increases can be readily detected by measuring Ca^{2+}-induced chloride currents[27,28]. Microinjection of nonesterified IP_3 into the oocytes caused an activation of Ca^{2+}-dependent chloride conductance which was biphasic: a fast spike of 200-300 nA was followed by the development of cytosolic Ca^{2+} oscillations as described previously[29,30]. Injection of IP_3/AM also induced a chloride current but with quite different temporal characteristics (Fig. 7). There was about 90 seconds delay before the onset of chloride current was detected, and the current gradually reached its peak of oscillations after another 2 minutes and lasted longer than the current caused by IP_3. The delayed onset of the chloride current probably corresponded to the time that it took to hydrolyze the AM esters of IP_3/AM (6 AM groups per molecule) and to accumulate a "threshold" amount of IP_3[30]. The long-lasting activity of IP_3/AM reflected the prolonged duration of elevated IP_3 levels. Microinjection of orthophosphate AM ester (P_i/AM)[6] as a control produced no chloride current even at higher concentrations (data not shown). This proved that the chloride current induced by IP_3/AM did not result from side products of AM ester hydrolysis such as formaldehyde or acetic acid.The above experiments suggested that IP_3/AM is pharmacologically effective once it reaches the cytoplasm. Thus, if we could increase the membrane-permeability of IP_3/AM and have it diffuse across the membrane at a higher rate, we should be able to raise the $[Ca^{2+}]_i$ simply by extracellular application

of permeant IP_3 derivatives.

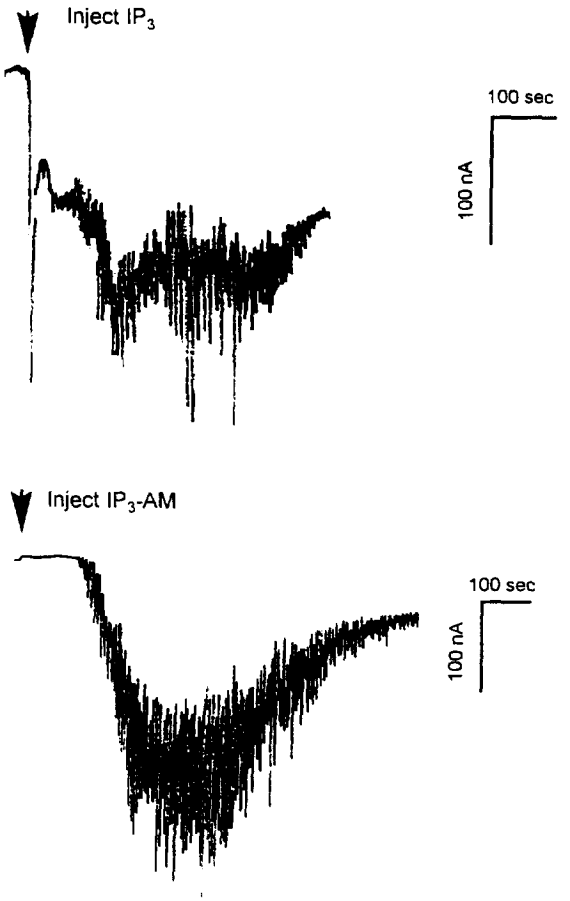


Figure 7. Microinjection of IP_3 (upper trace, ~1 pmol) and IP_3/AM (lower trace, ~10 pmol) in *Xenopus* oocytes activated Ca^{2+} gated chloride channels, but the induced chloride currents displayed different temporal characteristics.

Syntheses of IP_3/PM(5), IP_3/BM (6) and biological tests. A rational approach to improve the membrane permeability of IP_3/AM was to replace AM groups with more hydrophobic homologs such as propionyloxymethyl (PM) or butyryloxymethyl (BM) groups. The resulting prodrugs of IP_3, i.e. IP_3/PM (**5**) and IP_3/BM (**6**, Fig.1), would have six and twelve more methylenes than IP_3/AM, respectively. These changes ought to increase the overall hydrophobicity of the molecule considerably. Intracellular esterases should hydrolyze these esters just like AM esters and regenerate IP_3 by a similar mechanism. Another advantage of

using PM or BM esters was that the same synthetic strategy developed earlier for making IP_3/AM could be used for making these compounds with only slight modifications. Indeed, esterification of intermediate **4h** (Fig. 5) with bromomethyl propionate or bromomethyl butyrate followed by catalytic hydrogenolysis gave **5** and **6** respectively.

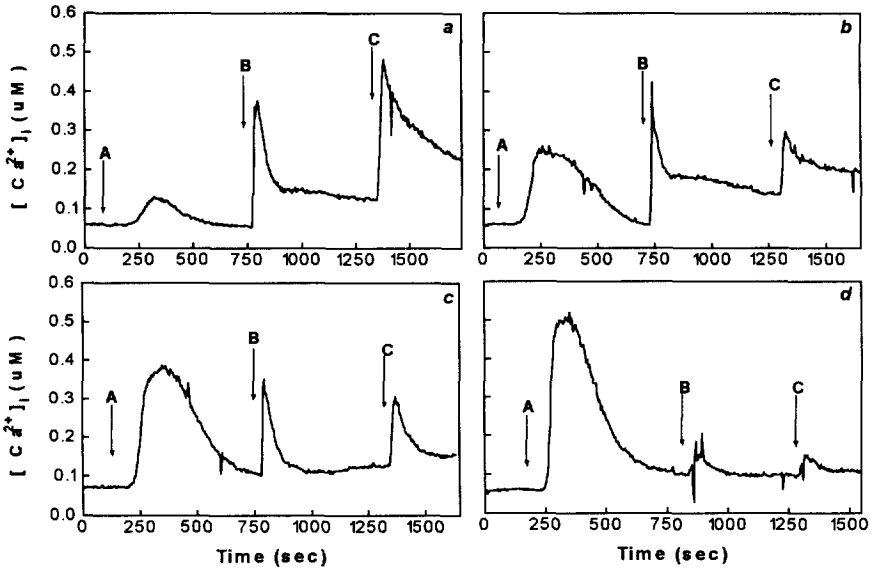


Figure 8. Dose response of racemic IP_3/PM (**5**) on $[Ca^{2+}]_i$ in fura-2/AM loaded 1321N1 astrocytoma cells. (*a*) Extracellular application of **5** (20 μM, **A**) increased cytosolic Ca^{2+} after a lag of 150-200 sec. Carbachol (200 μM, **B**, same in (*b*), (*c*) and (*d*)) and thapsigargin (100 nM , **C**, same in (*b*), (*c*) and (*d*)) added subsequently were still able to release calcium. Higher doses of **5** were used for (*b*) 40 μM, (*c*) 80 μM, and (*d*) 120 μM. In (*d*) , the depletion of internal calcium stores by **5** was nearly complete since CCh and Tg hardly caused any more Ca^{2+} release.

Racemic IP_3/PM (**5**) induced a dose-dependent increase of $[Ca^{2+}]_i$ when applied extracellularly to 1321N1 astrocytoma cells (Fig. 8). 20 μM of IP_3/PM increased cytosolic calcium ($[Ca^{2+}]_i$) after a lag of 150-200 seconds. The emptying of the internal calcium stores was partial at this dose, since CCh and the microsomal Ca^{2+}-ATPase inhibitor thapsigargin (Tg)[31] were still able to release calcium when added after IP_3/PM . CCh did not cause a complete depletion of intracellular calcium stores either, since Tg caused additional Ca^{2+} release after a supramaximal dose of CCh. With higher doses of IP_3/PM, the rate and extent of $[Ca^{2+}]_i$ release increased, and the lag between drug addition and the $[Ca^{2+}]_i$ rise decreased, to about 60 s, but did not disappear completely. This is consistent with the proposed mechanism of IP_3/PM action which requires

time for IP_3/PM to diffuse across the cell membrane, and for intracellular esterases to hydrolyze all six PM groups to yield the active IP_3. The depletion of internal calcium stores by IP_3/PM was nearly complete at 160 μM since CCh and Tg hardly caused any more Ca^{2+} release (Fig. 8-*d*). This indicates that IP_3/PM released calcium from the agonist-mobilizable pool, and therefore supports the conclusion that IP_3/PM generated biologically active IP_3 in the cytosol.

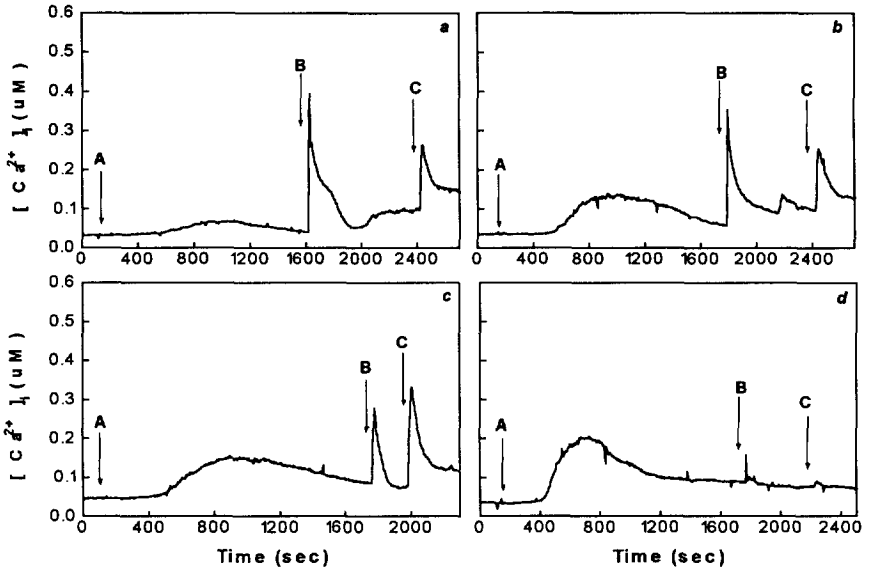


Figure 9. Dose response of racemic IP_3/BM (**6**) on $[Ca^{2+}]_i$ in fura-2/AM loaded 1321N1 astrocytoma cells. (*a*) Extracellular application of **6** (2 μM, **A**) slightly increased $[Ca^{2+}]_i$. Carbachol (200 μM, **B**, same in (*b*), (*c*) and (*d*)) and thapsigargin (100 nM, **C**, same in (*b*), (*c*) and (*d*)) added subsequently were still able to release calcium. Higher doses of **6** were used for (*b*) 4 μM, (*c*) 10 μM, and (*d*) 25 μM. In (*d*), the depletion of internal calcium stores by **6** was nearly complete since CCh and TG hardly caused any more $[Ca^{2+}]_i$ release.

IP_3/BM (**6**) was even more potent in inducing $[Ca^{2+}]_i$ increase when applied extracellularly in astrocytes (Fig. 9). A $[Ca^{2+}]_i$ rise was noticeable at a dose as low as 2 μM (Fig. *9-a*). The depletion of intracellular Ca^{2+} stores by IP_3/BM was nearly complete at 25 μM since CCh and Tg hardly caused any more $[Ca^{2+}]_i$ increase (Fig. 9-*d*). The increased potency of IP_3/BM very likely resulted from improved membrane permeability. The $[Ca^{2+}]_i$ rise induced by IP_3/BM was more gradual and sustained than that from IP_3/PM. Also, the lag between drug addition and the onset of $[Ca^{2+}]_i$ rise increased to over 6 minutes. The esterase hydrolysis rate of BM

esters is probably slower than that of PM esters. It was reasonable that it would take longer to generate equal amount of active IP_3 species from IP_3/BM than from IP_3/PM.

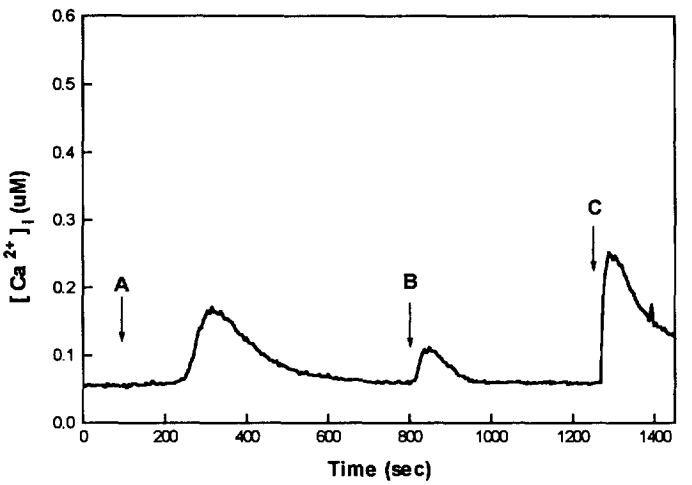


Figure 10. IP_3/PM released Ca^{2+} from internal stores in 1321N1 astrocytoma cells. HBS was replaced with Ca^{2+} free DPBS (0 Ca^{2+}, 0.2 mM EGTA, 20 mM Hepes, pH 7.4, 2g/L glucose) right before the Ca^{2+} imaging experiment started. Extracellular application of **5** (60 μM, **A**) released Ca^{2+} from internal stores after a lag of about 150 sec. Subsequent stimulation with carbachol (200 μM, **B**) gave much less internal Ca^{2+} release. Ionomycin (5 μM, **C**) released an additional amount of Ca^{2+}.

In calcium free medium, extracellular application of IP_3/PM also caused $[Ca^{2+}]_i$ increase in 1321N1 astrocytoma cells (Fig. 10). This confirmed that IP_3/PM induced $[Ca^{2+}]_i$ increase was due to the release of calcium from internal stores, which was consistent with the mechanism of IP_3 induced calcium release. Subsequent stimulation with a high dose of CCh released little Ca^{2+}, indicating that the first dose of IP_3/PM (60 μM) already released a fair amount of Ca^{2+} from IP_3- sensitive stores. Finally, ionomycin further released Ca^{2+}, possibly from IP_3- insensitive stores.

Control experiments using orthophosphate PM ester (P_i/PM) showed no detectable $[Ca^{2+}]_i$ increase even at 100 μM (Fig. 11). This supports the conclusion that the $[Ca^{2+}]_i$ increase induced by IP_3/PM or IP_3/BM resulted from the production of active IP_3 species, rather than from some unspecific effects caused by PM ester hydrolysis. However, 1,3,5-IP_3/PM caused a similar response as IP_3/PM when applied extracellularly to astrocytes (data not shown). In permeabilized bovine aortic smooth muscle cells, 1,3,5-IP_3 was nearly 2000

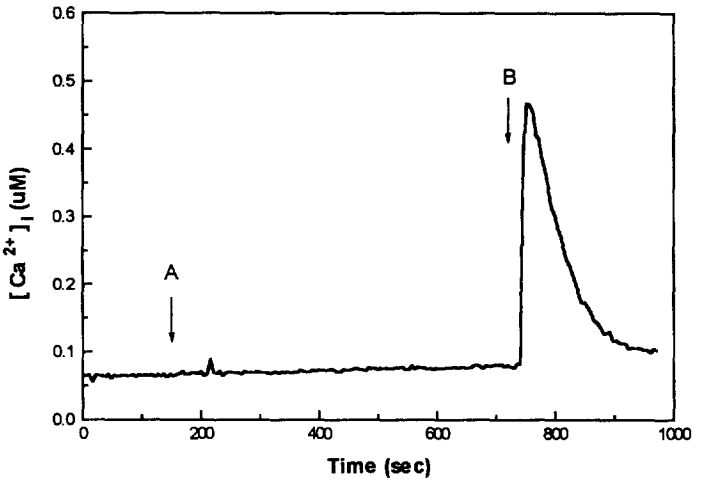


Figure 11. Test of inorganic phosphate PM ester (P_i/PM) on $[Ca^{2+}]_i$ in fura-2/AM loaded 1321N1astrocytoma cells. Extracellular addition of P_i/PM (100 μM, **A**) had no effect on $[Ca^{2+}]_i$. Subsequent stimulation with carbachol (100 μM, **B**) gave a normal response.

times less potent than D-1,4,5-IP_3 in mobilizing internal Ca^{2+} (reference[32]). The observed Ca^{2+} mobilizing activity of 1,3,5-IP_3/PM most likely resulted from phosphate triester migration during the delivery and hydrolysis process, which led to the production of D-1,4,5-IP_3 after hydrolysis and caused $[Ca^{2+}]_i$ increase.

DISCUSSION

Our results show the multiple pitfalls and iterations required to create membrane-permeant esters of IP_3. The initial molecule had butyryl groups esterifying the 2, 3, and 6-hydroxyls to increase hydrophobicity and prevent phosphate migration. Analogous butyryl groups on the 2′-hydroxyl of adenosine 3′,5′-cyclic monophosphate and the 1, 2-hydroxyls of *myo*-inositol 3,4,5,6-tetrakisphosphate were quite helpful for both ease of synthesis and biological efficacy[5,6,10]. However, the butyryl esters in Bt_3IP_3/AM prevented reproducible biological activity. $[Ca^{2+}]_i$ elevations were only observed on impure samples in which some of the butyryl groups were probably missing. The most likely explanation why butyryls were forbidden is that the 6-*O*-butyryl must be hydrolyzed to regain binding to the IP_3 receptor. Such hydrolysis would need to be fairly fast if regeneration of IP_3 is to compete with the rapid breakdown of this messenger, yet the butyrate ester is especially hindered by its two flanking phosphates. By contrast, the butyryls on the 1, 2-hydroxyls of *myo*-inositol 3,4,5,6-tetrakisphosphate are much less hindered, and that messenger can build up more easily because

its metabolism is much slower than that of 1,4,5-IP_3 [10].

Rather than try to deprotect the 6-hydroxyl selectively, we chose to synthesize IP_3/AM, in which all three phosphates still have AM esters but all three hydroxyls are left nonesterified. Benzyl ethers were chosen to protect the hydroxyls, so that deprotection by catalytic hydrogenation could avoid cleavage of the sensitive AM esters or phosphate migration. Therefore the phosphates had to be protected as β-cyanoethyl esters rather than benzyl esters as in the Bt_3IP_3/AM synthesis. Unfortunately, IP_3/AM was also poorly active but for a different probable reason, insufficient lipophilicity leading to low membrane permeability. Evidently the three free hydroxyl groups were enough to keep the polarity high even though the phosphates were completely esterified. When IP_3/AM was directly microinjected, bypassing the membrane barrier, it was quite able to regenerate IP_3 and cause a $[Ca^{2+}]_i$ response.

More hydrophobic neutral esters of IP_3, racemic IP_3/PM (**5**) and IP_3/BM (**6**), were therefore synthesized. When applied extracellularly in 1321N1 astrocytoma cells at concentration as low as 20 μM, IP_3/PM released Ca^{2+} from internal stores, whereas IP_3/BM was active in elevating $[Ca^{2+}]_i$ at 2 μM. Both compounds mobilized $[Ca^{2+}]_i$ in a dose dependent manner. The peak height of the $[Ca^{2+}]_i$ transient was variable, often not as high as can be produced by a saturating dose of an agonist such as carbachol that activates endogenous IP_3 production. In addition, the temporal characteristics of the $[Ca^{2+}]_i$ transient can be rationalized by the intracellular mode of action of IP_3/PM or IP_3/BM. Both prodrugs have to undergo hydrolysis of up to six protecting groups, so that the concentration of the active species probably rises not as a step function but rather with a sigmoidal time course. Therefore the Ca^{2+} should trickle out relatively gradually. The ability of IP_3/PM or IP_3/BM to abolish almost all of the response to carbachol added subsequently confirms that both compounds do act on agonist- and IP_3-sensitive pool of Ca^{2+}. Although IP_3/BM is effective at lower concentrations than those of IP_3/PM, IP_3/BM also hydrolyzes much more slowly and is therefore unable to produce $[Ca^{2+}]_i$ increases as large as those of IP_3/PM. For these reasons it is unlikely that a further increase in the fatty acid chain length would be of great value.

Migration of phosphates is evidently still a problem, because 1,3,5-IP_3/PM unexpectedly mobilized intracellular Ca^{2+} as well as racemic 1,4,5-IP_3/PM. Since 1,3,5-IP_3 itself is known to have very little affinity for the IP_3 receptor, the most probable explanation is that 1,3,5-IP_3/PM undergoes migration of phosphate triesters to vicinal hydroxyls at a rate competitive with or faster than PM ester hydrolysis. This phosphate scrambling is undesirable for pharmacological application because it reduces the efficiency of delivery and causes the loss of specificity. Ultimately one would like to use analogous esters to explore the physiological effects of inositol polyphophates other than 1,4,5-IP_3. Phosphate migration would complicate the outcome of the experiments when attempting to distinguish between isomers.

Among the three free hydroxyls, only the 6-hydroxyl interacts specifically with the IP_3R and plays an important role in releasing Ca^{2+}. The 2- and 3-hydroxyls play only minor roles in binding to the IP_3R[33,34]. IP_3

analogues with 2- and 3-hydroxyls masked with certain protecting groups bind to the IP_3R nearly as well as IP_3 does[35,36]. Thus, new permeant IP_3 derivatives could have their 2- and 3- hydroxyls blocked and should still be able to exert their activities like IP_3/PM or IP_3/BM. The 6-hydroxyl could be covered by a photolabile caging group while the phosphates are masked with PM esters. The PM esters would still confer membrane permeability and intracellular trapping and could be allowed to hydrolyze completely, after which photolysis of the single photolabile group would give a sudden release of active IP_3 species like that produced by agonist stimulation. The synthesis of these caged and membrane-permeant derivatives of inositol polyphosphates is in progress.

ACKNOWLEDGEMENTS

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EXPERIMENTAL PROCEDURES

Organic syntheses

^1H- and ^{31}P- NMR spectra were taken on either a Varian-200 (200MHz) or a General Electric QE-300 (300MHz) spectrometer. Chemical shifts of ^1H-NMR were reported as values relative to tetramethylsilane. ^{31}P-NMR was run with broad-band proton decoupling and 85% phosphoric acid was used as an external standard. Fast atom bombardment mass spectroscopy (FAB-MS) and exact mass determinations were performed by the mass spectroscopy facilities at the Scripps Research Institute (La Jolla, CA). Unit mass determinations were performed at the HHMI mass spectrometry facility (University of California, San Diego) on a Hewlett-Packard 5989B single quadruple mass spectrometer. The instrument was used in the electrospray ionization mode. Flash chromatography was performed using Silica Gel 60 (230-400 mesh, Merck). Thin-layer chromatography (TLC) was carried out on Analtech Silica Gel G-250 glass plate. General detection was by exposing to iodine vapor or spraying the plates with 2M sulfuric acid, followed by charring on a hot plate. Preparative reverse phase HPLC separations were done on a 10 μm RP-8 RsiL column (Biorad, 22x250, flow rate 9.9 mL/min).

Methylene chloride, N,N-diisopropylethylamine(DIEA) and pyridine were distilled from CaH_2, and acetonitrile was distilled from P_2O_5. These and other solvents (purchased in highest purity available) were stored over 4-Å molecular sieves. All of the reagents were purchased from Aldrich. Unless specified, all the reactions were done under an atmosphere of argon. Bromomethyl acetate, bromomethyl propionate and bromomethyl butyrate were synthesized following known procedures[37,38]. N,N-diisopropyl-bis(β-

cyanoethyl)phosphoramidite was synthesized as described[39].

***rac*-2,3-Di-*O*-butyryl-*myo*-inositol (1b)** : A solution of 1,4,5,6-tetra-O-benzyl-*myo*-inositol[11] (**1a**) (10g, 18.6 mmol) and n-butyric anhydride (15.4 mL, 100 mmol) in 50 mL of dry pyridine was stirred at room temperature for 4 days. After evaporation at reduced pressure the resulting oil was dissolved in 100 mL glacial acetic acid and 1.06 g (1 mmol) Pd/C (10%) was added. H_2 was bubbled through the reaction mixture for 4h at room temperature. The catalyst was removed by ultrafiltration and crude 2,3-di-O-butyryl-*myo*-inositol (**1b**) was obtained by direct lyophilization. Recrystallization from acetone yielded 4.34 g (73%); m.p. 141-142°C; ^{1}H-NMR (CD_3CN/D_2O) δ 1.01 (t, 3H, J = 7.5 Hz), 1.06 (t, 3H, J = 7.5 Hz), 1.69 (m, 2H), 1.74 (m, 2H), 2.34 (m, 2H), 2.45 (t, 2H, J = 7.5 Hz), 3.50 (dd, 1H, J = 9.2, 9.2 Hz), 3.74 (dd, 1H, J = 9.5, 9.5 Hz), 3.80 (dd, 1H, J = 3.0, 9.6 Hz), 3.84 (dd, 1H, J = 9.2, 10.0 Hz). 4.92 (dd, 1H, J = 3.0, 10.1 Hz), 5.57 (dd, 1H, J = 3.0, 3.0 Hz); MS *m/z* $(M-H)^-$ 319. Anal. Calcd for $C_{14}H_{24}O_8$: C, 52.48; H, 7.57. Found: C, 52.36; H, 7.46.

***rac*-2,3,6- (1c), 2,3,5-, and 1,2,3-Tri-*O*-butyryl-*myo*-inositol** : A solution of the dibutyrate **1b** (2.0 g, 6.25 mmol) in pyridine was stirred with 0.98 mL (6.0 mmol) n-butyric acid anhydride for 24h at room temperature. After evaporation under reduced pressure, the oily residue was dissolved in MeOH and purified by flash chromatography (RP-8, 45% MeOH) to yield 0.53 g (23%) **1c**. ^{1}H-NMR ($CDCl_3$) δ 0.85 - 1.05 (m, 9H), 1.65 (m, 6H), 2.30 - 2.43 (m, 7H), 2.47 (d, 1H, J = 3.3, -OH), 2.61 (d, 1H, J = 4 Hz, -OH), 3.58 (ddd, 1H, J = 3.5, 9.5, 9.9 Hz), 3.84 (ddd, 1H, J = 2.7, 7.0, 10.0 Hz), 3.92 (ddd, 1H, J = 4.0, 10.0, 10.0 Hz), 4.84 (dd, 1H, J = 3.0, 10.1 Hz), 5.14 (dd, 1H, J = 10.0, 10.0 Hz), 5.57 (dd, 1H, J = 3.0, 3.0 Hz); MS *m/z* $(M-H)^-$ 389. Anal. Calc. for $C_{18}H_{30}O_9$: C, 55.36; H, 7.76. Found: C, 55.28; H, 7.64 0.12 g (5%).

0.12 g (5%) of ***rac*-2,3,5-Tri-O-butyryl-*myo*-inositol** (^{1}H-NMR ($CDCl_3$) δ (m, 9H), 1.67 (m, 6H), 2.29 (t, 2H, J = 7.5 Hz), 2.39 (m, 4H), 3.81 (m, 2H), 3.92 (dd, 1H, J = 10.5, 10.5 Hz), 4.94 (dd, 1H, J = 9.6, 9.6 Hz), 4.94 (dd, 1H, J = 2.6, 10.0 Hz), 5.55 (dd, 1H, J = 2.6, 2.6 Hz). MS m/z $(M-H)^-$ 389) and 1.16 g (50%) of ***rac*-1,2,3-Tri-O-butyryl-*myo*-inositol** (^{1}H-NMR ($CDCl_3$) δ 0.90 - 1.03 (m, 9H), 1.63 (m, 6H), 2.28 (m, 2H), 2.35 (t, 4H, J = 7.2 Hz), 3.59 (dd, 1H, J = 9.9, 9.9 Hz), 3.88 (m, 2H), 4.98 (m, 2H), 5.53 (dd, 1H, J = 3.2, 3.2 Hz). MS *m/z* $(M-H)^-$ 389) were also purified.

***rac*-2,3,6-Tri-*O*-butyryl-*myo*-inositol 1,4,5-trisphosphate hexakisbenzyl ester (1d)** : 2,3,6-Tri-O-butyryl-*myo*-inositol (**1c**) (70 mg, 180 µmol) was dissolved in 6 mL dry CH_3CN and added to 1.5 mmol freshly prepared dibenzyl N,N-diethyl phosphoramidite. 1*H*-tetrazole (105 mg, 1.5 mmol) was added and the mixture was stirred overnight. Oxidation took place at -40°C by slowly adding 340 µL CH_3COOOH (30% w/w in AcOH, 1.5 mmol). The solution was allowed to warm up to room temperature. The solvent was removed, and the resulting oil as redissolved in 20 mL ether and subsequently washed with 5% $NaHSO_3$ (pH 4), 10% $NaHCO_3$ and H_2O (2x20 mL each). After drying with Na_2SO_4, the organic layer was evaporated to give a clear oil. Chromatography by preparative HPLC (RP-8, 91% MeOH) yielded 100 mg (48%) of the desired fully protected inositol trisphosphate **1d** as a white powder; m.p. 75°C. ^{1}H-NMR ($CDCl_3$) δ 0.66, 0.78, 0.95 (3t, 3H

each, all J = 7.4 Hz), 1.24 - 1.70 (m, 6H), 2.02 (t, 2H, J = 7.5 Hz), 2.10 (m, 2H), 2.36 (t, 2H, J = 7.5 Hz), 4.45 (ddd, 1H, J = 3.5, 8.5, 9.5 Hz), 4.49 (ddd, 1H, J = 9.0, 9.0, 10.0 Hz), 4.80 - 5.10 (m, 14H), 5.59 (dd, 1H, J = 9.5, 10.0 Hz), 5.71 (dd, 1H, J = 3.2, 3.2 Hz), 7.28 (m, 30H). ^{31}P-NMR (145.8 MHz) δ -0.9, -1.3, -1.5 (1:1:1). MS *m/z* $(M\text{-}C_7H_7)^-$ 1079.

***rac*-2,3,6-Tri-*O*-butyryl-*myo*-inositol 1,4,5-trisphosphate (1e)** : A mixture of **1d** (76 mg, 65 µmol) and Pd/C (10%) (106 mg, 100 µmol) in glacial acetic acid was hydrogenated at room temperature and atmospheric pressure for 3h. After filtration (Whatman GF/A) and lyophilization the product was obtained as a white powder in 99% yield (free acid, 40.6 mg). ^{1}H-NMR (D_2O) δ 0.89 (m, 9H), 1.60 (m, 6H), 2.32 (m, 2H), 2.42 (m, 4H), 4.38 (ddd, 1H, J = 10.0, 10.0, 10.0 Hz), 4.42 (ddd, 1H, J = 3.0, 9.5, 10.0 Hz), 4.61 (ddd, 1H, J = 9.7, 10.0, 10.0 Hz), 5.14 (dd, 1H, J = 3.0, 10.0 Hz), 5.36 (dd, 1H, J = 10.0, 10.0 Hz), 5.68 (dd, 1H, J = 3.0, 3.0 Hz). ^{31}P-NMR (DMSO-d_6, 145.8 MHz) δ -0.7, 0.0, 0.4 (1:1:1). MS *m/z* $(M\text{-}H)^-$ calcd 629.0802, obsd 629.0826.

***rac*-2,3,6-Tri-*O*-butyryl-*myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (Bt_3IP_3, 1)** : A solution of the free acid **1e** (9.5 mg, 15 µmol) in 1 mL of dry CH_3CN was evaporated to dryness, DIEA (22 mg, 170 µmol) and 1 mL dry CH_3CN was added, and the suspension was dried again in high vacuum. Subsequently, dry CH_3CN (0.5 mL), DIEA (44 mg, 340 µM), and acetoxymethyl bromide (150 mg, 950 µM) were added. The mixture was stirred under argon for 2 days at room temperature, the solvent was evaporated in high vacuum, and the hexakis(acetoxymethyl) ester **1** was extracted from the solid residue with toluene to yield 33%. ^{1}H-NMR (toluene-d_8) δ 0.70 (t, 3H, J = 7.0 Hz), 0.85 (t, 3H, J = 7.5 Hz), 0.99 (t, 3H, J = 7.5 Hz), 1.50 (m, 4H), 1.65 -1.90 (m, 2H), 1.74 (s, 3H), 1.75 (s, 3H), 1.78 (2s, 6H), 1.79 (s, 3H), 2.20 - 2.70 (m, 6H), 4.85 (ddd, 1H, J = 7.2, 7.2, 8.0 Hz), 4.90 (m, 1H), 5.25 - 5.85 (m, 15H), 6.00 (dd, 1H, J = 3.2, 3.2 Hz); ^{31}P-NMR (121.5 MHz) δ -4.00, -4.10, -4.25 (1:1:1); MS *m/z* 989 $(M\text{-}CH_2OAc)^-$.

***rac*-4-*O*-Benzyl-*myo*-inositol 1,3,5-orthoformate (2b)** : **2b** was prepared from *myo*-inositol-1,3,5-orthoformate (**2a**, 0.5g, 2.63mmol) by benzylation of the monoanion according to known procedures[16]. Analysis data are consistant with those of Baudin et al[40].

***rac*-4-*O*-Benzyl-2,6-di-*O*-butyryl-*myo*-inositol 1,3,5-orthoformate (2c)** : The monobenzyl ether **2b** (400 mg, 1.79 mmol) was butyrylated under standard conditions (n-butyric anhydride/pyridine at room temperature) to yield 438 mg (73% yield, colourless crystals) of the dibutyrate **2c** after recrystallization from MeOH. m.p.: 97°C; ^{1}H-NMR ($CDCl_3$) δ 0.83 (t, 3H, J = 7.5 Hz), 1.00 (t, 3H, J = 7.5 Hz), 1.50 (m, 2H), 1.72 (m, 2H), 2.12 (m, 2H), 2.47 (t, 2H, J = 7.5 Hz), 4.30 (m, 1H), 4.38 (m, 2H), 4.59 (AB, 2H, J = 11 Hz), 4.64 (m, 1H), 5.33 (ddd, 1H, J = 1.1, 2.0, 2.0 Hz), 5.42 (ddd, 1H, J = 1.8, 4.0, 5.0 Hz), 5.56 (d, 1H, J = 1.1 Hz), 7.43 (m, 5H). MS *m/z* $(M\text{-}H)^-$ calcd 419.1706, obsd 419.1689.

***rac*-4-*O*-Benzyl-2,6-di-*O*-butyryl-*myo*-inositol (2d)** : The fully protected inositol derivative **2c** was dissolved in 10 mL CF_3COOH (80% in water) and stirred for 7h at room temperature. After evaporation to

dryness, the crude product was purified on preparative HPLC (RP-8) to collect three fractions. The main fraction (80% by HPLC) gave the desired product **2d** (210 mg, 56% yield) in high purity; ^{1}H-NMR ($CDCl_3$) δ 1.00 (m, 6H), 1.69 (m, 4H), 2.40 (m, 4H), 3.68 (m, 4H), 4.83 (s, 2H), 5.12 (m, 1H), 5.50 (dd, 1H, J = 2.5, 2.5 Hz), 7.34 (s, 5H); MS *m/z* $(M-H)^-$ calcd 409.1862, obsd 409.1881.

An earlier eluting fraction contained a mixture of ***rac*-4-O-benzyl-6-O-butyryl-*myo*-inositol** and ***rac*-4-O-benzyl-2-O-butyryl-*myo*-inositol**. The former product could be purified by recrystallization (8 mg, 2% yield); m.p. 115°C; ^{1}H-NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 7.3 Hz), 1.55 (m, 2H), 2.26 (t, 2H, J = 7.4 Hz), 2.78 (m, 3H, OH), 3.02 (d, 1H, J = 2.4 Hz, OH), 3.40 (m, 3H), 3.55 (dd, 1H, J = 9.5, 9.5 Hz), 3.94 (ddd, 1H, J = 2.4, 2.4, 2.4 Hz), 4.75 (AB, 2H, J = 11.5 Hz), 5.01 (dd, 1H, J = 10.0, 10.0 Hz), 7.20 (m, 5H); MS *m/z* $(M-H)^-$ calcd 339.1444, obsd 339.1450.

***rac*-2,6-Di-*O*-butyryl-*myo*-inositol (2e)** : A mixture of **2d** (0.46 mmol) and 106 mg Pd/C (10%) (0.1 mmol Pd) in glacial acetic acid was stirred under H_2 for 3h at room temperature. Filtration (Whatman GF/A) and lyophilization yielded 148 mg (>99% yield) of the inositol dibutyrate **2e** as a clear oil. ^{1}H-NMR (CD_3OD) δ 0.99 (m, 6H), 1.70 (m, 4H), 2.41 (m, 4H), 3.35 (m, 1H), 3.55 (m, 1H), 3.62 (dd, 1H, J = 10.0, 10.0 Hz), 3.72 (dd, 1H, J = 2.5, 10.5 Hz), 5.11 (dd, 1H, J = 10.5, 10.5 Hz), 5.47 (dd, 1H, J = 2.5, 2.5 Hz); MS *m/z* $(M-H)^-$ calcd 319.1393, obsd 319.1388.

***rac*-2,6-Di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate octakis(benzyl) ester (2f)** : A solution of **2 e** (136 mg, 0.44 mmol) and 1H-tetrazole (350 mg, 5mmol) was added to freshly prepared dibenzyl N,N-diethyl phosphoramidite (1.59 g, 5 mmol). The mixture was stirred under argon overnight and then cooled down to -40°C. CH_3COOOH (1.12 mL, 5 mmol, 32% w/w in acetic acid) was added and the solution was allowed to warm up to room temperature. The solvents were removed, and the resulting oil was dissolved in 30 ml ether and washed twice with 20 mL of each of the following solutions: 5% $NaHSO_3$ (pH 4), 10% $NaHCO_3$, H_2O. After drying with Na_2SO_4 the organic layer was evaporated to yield a clear oil. The crude product **2f** was purified by preparative HPLC on RP-8, eluting with 93% MeOH. Yield: 436 mg, 72%. ^{1}H-NMR ($CDCl_3$) δ 0.63 (t, 3H, J = 7.0 Hz), 0.94 (t, 3H, J = 7H), 1.31 (m, 2H), 1.64 (m, 2H), 2.08 (m, 2H), 2.38 (t, 2H, J = 7 Hz), 4.27 - 4.51 (m, 3H), 4.82 - 5.14 (m, 17H), 5.56 (dd, 1H, J = 10.0, 10.0 Hz), 6.06 (dd, 1H, J = 3.0, 3.0 Hz), 7.08 - 7.40 (m, 40H); ^{31}P-NMR (121.5 MHz) δ -0.62, -0.99, -1.07, -1.36 (1:1:1:1); MS *m/z* $(M - C_7H_7)^-$ 1271 and $(M-H)^-$ 1361.

***rac*-2,6-Di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate (2g)** : The fully protected inositol tetrakisphosphate **2f** (390 mg, 0.29 mmol) was dissolved in 10 mL glacial acetic acid, and 1g of 10% Pd/C was added. The mixture was hydrogenated at atmospheric pressure and room temperature for 3h. After filtration, the solution was lyophilized to yield **2g** as the free acid (183 mg, 98% yield) in form of a white powder; ^{1}H-NMR (DMSO-d_6) δ (t, 3H, J = 7 Hz), 0.94 (t, 3H, J = 7 Hz), 1.53 (m, 2H), 1.63 (m, 2H), 2.38 (m,4H), 4.25 - 4.55 (m, 4H), 5.20 (dd, 1H, J = 9.0, 9.0 Hz), 5.62 (m, 1H); ^{31}P-NMR (121.5 MHz) δ +0.9, -0.1, -0.9,-1.4

(1:1:1:1); MS *m/z* $(M-H)^-$ calcd 639.0046, obsd 639.0053.

***rac*-2,6-Di-*O*-butyryl-*myo*-inositol 1,3,4,5-tetrakisphosphate octakis(acetoxymethyl) ester (Bt_2InsP_4/AM, 2)** : A solution of **2g** (9.6 mg, 15 μmol) in 1 mL of dry CH_3CN was evaporated to dryness. DIEA (22 mg, 170 μmol) was added and again evaporated to dryness. Final dissolution of the salt in dry CH_3CN under argon was followed by addition of acetoxymethyl bromide (30 mg, 200 μmol) and more DIEA (23 mg, 180 μmol). The solution was stirred for 4 days and subsequently evaporated to dryness in high vacuum. The resulting oily mixture of salts was extracted with 1 mL of toluene to yield 12 mg (73%) of the desired octakis(acetoxymethyl) ester **2** in over 98% purity, determined by NMR. ^{1}H-NMR (toluene-d_8) δ 1.03 (t, 3H, *J* = 7.5 Hz), 1.28 (t, 3H, *J* = 7.5 Hz), 1.79 (m, 2H), 1.95 - 2.10 (m, 26H), 2.29 (m,2H), 2.91 (dt, 2H, *J* = 4.0, 7.5 Hz), 4.85 (ddd, 1H, *J* = 9.3, 9.3, 9.3 Hz), 4.90 - 5.08 (m, 2H), 5.28 (ddd, 1H, *J* = 9.5, 9.5, 9.5 Hz), 5.60 - 6.15 (m, 17H), 6.37 (dd, 1H, *J* = 3.0, 3.0 Hz); ^{31}P-NMR (121.5 MHz) δ -3.45, -3.80, -4.15 and -4.55 (1:1:1:1). MS *m/z* 1143 ($M-CH_2OAc$).

2,4,6-Tri-*O*-benzyl-*myo*-inositol (3b): 1,3,5-O-Methylidyne-2,4,6-tri-*O*-benzyl-*myo*-inositol (**3a**, 1 g, 2.2 mmol) was refluxed with 80 mL MeOH and 2 mL 15% HCl for 20 min. After cooling down to room temperature, the solution was neutralized to pH 8 with concentrated NH_4OH (30%). The solvent was removed under vacuum and the left residue was extracted with ethyl acetate. The organic layer was concentrated and purified on Si 60 column (Hexane/EtOAc, 1:1, v/v). 0.95g (95%) white solid was obtained. ^{1}H-NMR ($CDCl_3$): d 3.5-3.74(m, 5H), 3.98(t, J=2.6 Hz, 1H), 4.85(m, 6H), 7.38(m, 15H).

2,4,6-Tri-*O*-benzyl-*myo*-inositol 1,3,5-trisphosphate hexakis(β-cyanoethyl) ester (3c): To a solution of 0.15g (0.33 mmol) of triol **3b** and 0.53g (2 mmol) of N,N-diisopropyl-bis(β-cyanoethyl)phosphoramidite in 2 mL CH_2Cl_2 was added 0.16g (2.3 mmol) of 1H-tetrazole dissolved in CH_3CN (6 mL). After stirring at room temperature for 2 h, the mixture was filtered through a sintered glass filter. The filtrate was concentrated and loaded on a short silica gel column. Eluting with hexane/ethyl acetate (2:1 to 1:1, v/v) yielded 0.18g of clear glass after drying in vacuo. This phosphite triester intermediate was then dissolved in 2mL CH_2Cl_2 and cooled down to -20°C. 0.4 mL tert-butyl hydroperoxide (3M solution in 2,2,4-trimethylpentane) in 1 mL CH_2Cl_2 was added dropwise at this temperature. After addition, the solution was warmed up to room temperature in 0.5 h and the reaction was continued for another 1 h. The mixture was loaded directly onto the silica gel column and eluted with 20 mL ethyl acetate. The eluant was then changed to ethyl acetate/methanol (9:1, v/v) and the product was collected. Evaporation of solvent provided 0.18g (55% for 2 steps) of product as a clear glass. ^{31}P-NMR ($CDCl_3$, 121.5 MHz): δ -2.7, -2.2 (2:1).

2,4,6-Tri-*O*-benzyl-*myo*-inositol 1,3,5-trisphosphate (3d): 0.1g (0.1 mmol) trisphosphate **3c** was suspended in 1.5 mL methanol and 6 mL concentrated ammonia. The solution was heated at 60°C for 3 h and the solvent was removed under vacuum. The resulting pale yellow solid was used directly for the next step.

2,4,6-Tri-*O*-benzyl-*myo*-inositol 1,3,5-trisphosphate hexakis(acetoxymethyl) ester (3e): The

trisphosphate **3d** (from 50 mg of **3c**) was mixed vigorously with 0.5 mL CH_3CN and 0.1 mL DIEA. The mixture was then dried under vacuum. This procedure was repeated at least three times until a homogenous solution was obtained after adding CH_3CN/DIEA (If necessary, sonication may help solubilization). At this time, the counter-ion of phosphate was exchanged from ammonium ion to diisopropylethylammonium ion. After a final round of drying, the pale yellow solid was suspended in 2 mL CH_3CN and 0.25 mL DIEA. 100 mg (0.65 mmol) of bromomethyl acetate was added to this solution. After stiring for 1 day, another 80 mg of bromomethyl acetate was added and the reaction was continued for another 24 h. The solvent and excess reagent were evaporated under vacuum. The remaining mixture was purified on silica gel column using ethyl acetate as eluant. 38 mg of relatively pure product was obtained. This material was further purified on reverse phase HPLC (C-18) and 17 mg (31% based on **3c**) pure product was collected as a clear glass. ^{1}H-NMR ($CDCl_3$): δ 2.1(m, 18H), 4.0(t, 2H), 4.4(m, 4H), 4.8(m, 6H), 5.2-5.6(m, 12H), 7.3-7.4(m, 15H). ^{31}P-NMR ($CDCl_3$): δ -4.0, -4.3 (1:2).

***myo*-Inositol 1,3,5-trisphosphate hexakis(acetoxymethyl) ester (3)**: 17 mg of pure **3e** was dissolved in 1.5 mL glacial acetic acid. 20 mg of palladium(II) acetate and 10 mg of palladium(II) trifluoroacetate was added. The benzyl groups were removed by catalytic hydrogenation at 1 atm of H_2. To minimize the phosphate triester migration during the reaction, the temperature of the solution was carefully controlled at about 18°C using a water bath. After 3 h, the catalyst was filtered. The filtrate was freeze-dried and 9 mg (95%) of pure product was obtained as a clear glass. ^{1}H-NMR (CD_3OD): δ 2.1(m, 18H), 4.0(t, 2H), 4.2(q,1H), 4.4(m, 4H), 5.3-5.5(m, 12H). ^{31}P-NMR: -4.4, -4.8 (1:2). MS: exact mass calcd for ($C_{24}H_{39}O_{27}P_3$ + Na^+) 875.0789, obsd 875.0772.

***rac*-1,2-O-Isopropylidene-3,6-di-*O*-benzyl-4,5-di-*O*-benzoyl-*myo*-inositol (4b)**: 3g of diol **4a** (7.49 mmol, prepared as described in ref [22]) was dissolved in 30 mL of dry pyridine. At 0°C, 2.26 mL of benzoyl chloride (19.5 mmol) was added dropwise. After 2 hours , pyridine was removed under vacuum. The resulting syrup was dissolved in methylene chloride and washed with diluted HCl and saline solution. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel with hexane/ethyl acetate (5:1, v/v). Evaporation and drying in vacuo gave 4.34g (95%) of colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.41(s, 3H), 3.92-4.02(m, 2H), 4.34(t, J=6.01 Hz, 1H), 4.5(dd, J=4.1, 3.7 Hz, 1H), 4.71-4.76(m, 4H), 5.41(t, J=8.51 Hz, 1H), 5.86(t, J=8.79 Hz, 1H), 7.15-8.0(m, 20H).

***rac*-3,6-Di-*O*-benzyl-4,5-di-*O*-benzoyl-*myo*-inositol (4c)**: 4.34g (7.13 mmol) of compound **4b** was dissolved in 50 mL of methanol and 20 mL of THF. 50 μL of concentrated HCl was added to the solution and the stirring was continued at room temperature overnight. The reaction was quenched by adding 50 μL of concentrated NH_4OH. After removal of the solvent in vacuo, the residue was purified by silica gel column using hexane/ethyl acetate as eluant. (2:1 to 1:2, v/v). 3.69g (91%) of white solid was obtained . ^{1}H-NMR ($CDCl_3$): δ 3.64-3.77(m, 2H), 4.11(t, J=9.48 Hz, 1H), 4.34(t, J=2.81 Hz, 1H), 4.51-4.7(m, 4H), 5.49(t, J=9.68 Hz, 1H),

5.91(t, J=9.9 Hz, 1H), 7.15-8.0(m, 20H).

***rac*-1,4,5-Tri-*O*-benzoyl-3,6-di-*O*-benzyl-*myo*-inositol (4d)**: At 0°C, to a solution of 1.1g of diol **4c** (1.93 mmol) in 15 mL (2.37 mmol) of dry pyridine was added 0.275 mL of benzoyl chloride. 3 h later, pyridine was removed under vacuum and the resulting syrup was dissolved in methylene chloride. This was washed with diluted HCl and saturated saline solution. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column using hexane/ethyl acetate as eluant. (3:1, v/v). 1.04g (80%) of white solid was obtained after drying the product in vacuo. ^{1}H-NMR ($CDCl_3$): δ .383(dd, J=9.73 , 2.56 Hz, 1H), 4.46-4.72(m, 6H), 5.28(dd, J=10.03 , 2.64 Hz, 1H), 5.61(t, J=9.64 Hz), 5.98(t, J=9.98 Hz), 7.0-8.1(m, 25H).

***rac*-1,4,5-Tri-*O*-benzoyl-2,3,6-tri-*O*-benzyl-*myo*-inositol (4e)**: 0.8g (1.19 mmol) of compound **4d** was mixed with 12 mL of cyclohexane , 6 mL of methylene chloride and 0.88 mL (4.76 mmol) of benzyl 2,2,2-trichloroacetimidate. 30 μL (23 umol) of trifluoromethanesulfonic acid was then added at room temperature. After more than 12 h, the solvent was removed under vacuum and the product was purified by silica gel column. (hexane/ethyl acetate, 3:1, v/v). 0.64 g (70%) of clear glass was obtained after drying the product in vacuo. ^{1}H-NMR ($CDCl_3$): δ 3.82(dd, J=5.07, 2.16 Hz,1H), 4.4-5.0(m, 8H), 5.2(dd, J=5.11, 2.52 Hz, 1H), 5.61(t, J=9.64 Hz, 1H), 6.09(t, J=10.1 Hz, 1H), 7.0-8.1(m, 30H).

***rac*-2,3,6-Tri-*O*-benzyl-*myo*-inositol (4f)**: 0.6 g (0.79 mmol) of **4e** was dissolved in minimum amount of methylene chloride and mixed with 15 mL of methanol. Potassium carbonate (300 mg) catalyzed methanolysis completely removed the benzoyl groups at room temperature after 24h. The mixture was filtered through a glass filter paper and concentrated. The product was purified using silica gel chromatography (CH_2Cl_2/MeOH, 15:1, v/v). 0.31g (86%) of white solid was collected. ^{1}H-NMR ($CDCl_3$): δ 2.34(d, 1H), 2.53(m, 2H), 3.3(dd, 1H), 3.41-3.75(m, 3H), 3.95-4.1(m, 2H), 4.6-5.0(m, 6H), 7.4(m, 15H).

***rac*-2,3,6-Tri-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis(β-cyanoethyl) ester (4g)**: **4g** was prepared from **4f** (60%) in the same manner as making **3c** from **3b**. ^{31}P-NMR ($CDCl_3$): -2.74, -2.59 (1:2).

***rac*-2,3,6-Tri-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate (4h)**: 0.2g (0.2 mmol) of trisphosphate **4g** was suspended in 1.5 mL methanol and 6 mL concentrated ammonia. The solution was heated at 60°C for 3 h and concentrated. The resulting pale yellow solid was used directly for the next step.

***rac*-2,3,6-Tri-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (4i)**: This was synthesized from trisphosphate **4h** in the same manner as making **3e** from **3d.** ^{1}H-NMR ($CDCl_3$): δ 2.1(m, 18H), 3.51(d, J=9.9 Hz), 4.07(t, J=9.6 Hz), 4.36-4.92(m, 10H), 5.3-5.68(m, 12H), 7.28-7.43(m, 15H). ^{31}P-NMR (121.5 Hz): δ -3.9, -4.2, -4.6 (1:1:1).

***rac-myo*-Inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (4)**: This was made from **4i** by using the same conditions as described above. ^{1}H-NMR (CD_3OD): δ 2.1(m, 18H), 3.7(dd, J=4.95, 2.4 Hz, 1H), 4.03(t, J=9.3 Hz, 1H), 4.2(t, J=2.1 Hz, 1H), 4.26-4.41(m, 2H), 4.64(q, J=8.4 Hz, 1H), 4.89(m, 12H). ^{31}P-NMR: δ -4.8, -4.5 (1:2). MS: exact mass calcd for ($C_{24}H_{39}O_{27}P_3 + Na^+$) 875.0789, obsd 875.0818.

***rac*-2,3,6-Tri-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis(propionyloxymethyl) ester**: This was synthesized from **4h** and bromomethyl propionate in the same manner as making **4i**. MS: exact mass calcd for ($C_{51}H_{69}O_{27}P_3$ + Cs^+) 1339.2293, obsd 1339.2261.

***rac-myo*-Inositol 1,4,5-trisphosphate hexakis(propionyloxymethyl) ester (5)** : **5** was synthesized from the above compound using the same hydrogenation conditions as making IP_3/AM. ^{1}H-NMR (CD_3OD): δ 1.15(t, J=7.5 Hz, 18H), 2.45(m, 12H), 3.7(dd, J=4.95 , 2.4 Hz, 1H), 4.03(t, J=9.3 Hz, 1H), 4.2(t, J=2.1 Hz, 1H), 4.26-4.41(m, 2H), 4.64(q, J=8.4 Hz, 1H), 4.89(m, 12H).

***rac-myo*-Inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester (6)**: The compound was synthesized from **4h** in 2 steps using the same procedure as making **4** (except using bromomethyl butyrate as esterification reagent). ^{1}H-NMR (CD_3OD): δ 0.98(t, J=7.5 Hz, 18H), 1.71(m, 12H), 2.4(m, 12H), 3.7(dd, J=4.95 , 2.4 Hz, 1H), 4.03(t, J=9.3 Hz, 1H), 4.2(t, J=2.1 Hz, 1H), 4.26-4.41(m, 2H), 4.64(q, J=8.4 Hz, 1H), 4.89(m, 12H). MS: exact mass calcd for ($C_{36}H_{63}O_{27}P_3$ + Cs^+) 1153.1824, obsd 1153.1850.

Intracellular Ca^{2+} measurements

1321N1 astrocytoma cells (a gift of Dr. J.H. Brown, Department of Pharmacology, University of California, San Diego) were grown in DMEM supplemented with 5% (v/v) fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate. Fura-2/AM was from Molecular Probes (Eugene, OR). Carbachol was purchased from Sigma Chemical Co. (St.Louis, MO). Thapsigargin and ionomycin were from Calbiochem (La Jolla, CA).

For Ca^{2+} imaging experiments, cells were seeded onto 25 mm glass coverslips and cultured for at least 24 hours before use. The culturing medium was then replaced with Hanks' buffered saline (HBS, Gibco) supplemented with 2 g/L glucose and 20 mM Hepes (pH 7.4) immediately before the Ca^{2+} measurements. Cells were loaded with the Ca^{2+} indicator fura-2/AM (0.5 μM) at room temperature in the dark with gentle shaking for 0.5 h. After loading, cells were gently washed, and incubated for another 20 min in HBS. For experiments done in Ca^{2+} free medium, HBS was replaced with Dulbecco's phosphate buffered saline (Gibco, 0 Ca^{2+}, 0.2 mM EGTA, 20 mM Hepes, pH 7.4, 2 g/L glucose) right before the actual Ca^{2+} imaging experiment started. Fluorescence ratio images using pseudocolor hues to represent the range of fluorescence excitation ratios (350/380 nm) of fura-2 were obtained as previously described[17,41]. The images were analyzed by taking average $[Ca^{2+}]_i$ values within the area of a spot placed over each cell in a field. Typically at least 12 cells were averaged and the results shown for each figure are representative of at least 3 experiments. Activation of Ca^{2+} release from internal stores was initiated by adding ionomycin, carbachol, thapsigargin or neutral esters of IP_3 predissolved in HBS (containing less than 1% DMSO) to the fura-2/AM loaded cells.

Electrophysiology on Xenopus laevis oocytes

Oocytes (stage V and VI) were obtained as described[27]. Electrophysiological experiments were performed under two-electrode voltage-clamp using a Dagan CA-1a oocyte clamp (Dagan Corporation, MN)[42]. The bath solution contained (mM) NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; $NaHCO_3$, 1; HEPES, 10; $MgCl_2$, 1; pH 7.4. Intracellular injections were performed during continued electrophysiological recording using a third pipette, broken manually and filled with 150 mM KCl and 10 mM HEPES (pH 7.0), containing either 20 μM IP_3, 200 μM IP_3/AM or 400 μM orthophosphate AM ester. For the latter two, DMSO concentration was 1% in the pipette.

Oocytes were clamped at -60 mV. Voltage step protocols (-60 to +10 mV) were perfomed before and during current development to determine the reversal potential of the IP_3 or IP_3/AM stimulated currents. The reversal potential was -20 to -25 mV, and the current was abolished by injection of EGTA (data not shown), which is compatible with Ca^{2+}-activated Cl^- current[27].

REFERENCES

1. Berridge, M. J. *Nature* **1993**, 361, 315-325.
2. Berridge, M. J.; Irvine, R. F. *Nature* **1989**, 341, 197-205.
3. Tsien, R. Y. *Nature* **1981**, 290, 527-528.
4. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, 260, 3440-3450.
5. Schultz, C.; Vajanaphanich, M.; Genieser, H. G.; Jastorff, B.; Barrett, K. E.; Tsien, R. Y. *Mol. Pharmacol.* **1994**, 46, 702-708.
6. Schultz, C.; Vajanaphanich, M.; Harootunian, A. T.; Sammak, P. J.; Barrett, K. E.; Tsien, R. Y. *J. Biol. Chem.* **1993**, 268, 6316-6322.
7. Srivastva, D. N.; Farquhar, D. *Bioorg. Chem.* **1984**, 12, 118-129.
8. Sastry, J. K.; Nehete, P. N.; Khan, S.; Nowak, B. J.; Plunkett, W.; Arlinghaus, R. B.; Farquhar, D. *Mol. Pharmacol.* **1992**, 41, 441-445.
9. Freed, J. J.; Farquhar, D.; Hampton, A. *Biochem. Pharmacol.* **1989**, 38, 3193-3198.
10. Vajanaphanich, M.; Schultz, C.; Rudolf, M. T.; Wasserman, M.; Enyedi, P.; Craxton, A.; Shears, S. B.; Tsien, R. Y.; Barrett, K. E.; Traynor-Kaplan, A. *Nature* **1994**, 371, 711-714.
11. Angyal, S. J.; Tate, M. E. *J. Chem. Soc.* **1965**, 6949-6955.
12. Watanabe, Y.; Shinohara, T.; Fujimoto, T.; Ozaki, S. *Chem. Pharm. Bull.* **1990**, 38, 562-563.
13. Tegge, W.; Ballou, C. E. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 94-98.
14. Perich, J. W.; Johns, R. B. *Tetrahedron Lett.* **1987**, 28, 101-102.
15. Lee, H. W.; Kishi, Y. *J. Org. Chem.* **1985**, 50, 4402-4404.
16. Billington, D. C.; Baker, R.; Kulagowski, J. J.; Mawer, I. M.; Vacca, J. P.; deSolms, S. J.; Huff, J. R. *J.*

Chem. Soc. Perkin Trans. 1 **1989**, 1423-1429.

17. Tsien, R. Y.; Harootunian, A. T. *Cell Calcium* **1990**, 11, 93-109.
18. Schultz, C.; Tsien, R. Y. *FASEB J.* **1992**, *6(5)*, A1924abstract.
19. Cooke, A. M.; Gigg, R.; Potter, B. V. *J. Chem. Soc. ,Chem. Commun.* **1987**, 1525-1526.
20. Sinha, N. D.; Biernat, J.; McManus, J.; Koster, H. *Nucleic. Acids. Res.* **1984**, 12, 4539-4557.
21. Willcocks, A. L.; Cooke, A. M.; Potter, B. V. L.; Nahorski, S. R. *Biochem. Biophys. Res. Commun.* **1987**, 146, 1071-1078.
22. Gigg, J.; Gigg, R.; Payne, S.; Conant, R. *J. Chem. Soc. Perkin Trans. 1* **1987**, 423-429.
23. Gigg, R.; Warren, C. D. *J. Chem. Soc. (C)* **1969**, 2367-2371.
24. Iversen, T.; Bundle, K. R. *J. Chem. Soc. ,Chem. Commun.* **1997**, 1240
25. Martinson, E. A.; Goldstein, D.; Brown, J. H. *J. Biol. Chem.* **1989**, 264, 14748-14754.
26. Nakahata, N.; Ishimoto, H.; Kurita, M.; Ohmori, K.; Takahashi, A.; Nakanishi, H. *Brain Res.* **1992**, 583, 100-104.
27. Barish, M. E. *J. Physiol. ,Lond.* **1983**, 309-325.
28. Miledi, R.; Parker, I. *J. Physiol. (Lond)* **1984**, 357, 173-183.
29. Parker, I.; Miledi, R. *Proc. R. Soc. Lond. B. Biol. Sci.* **1987**, 231, 27-36.
30. Parker, I. *J. Physiol. ,Lond.* **1988**, 407, 95P
31. Thastrup, O.; Dawson, A. P.; Scharff, O.; Foder, B.; Cullen, P. J.; Drobak, B. K.; Bjerrum, P. J.; Christensen, S. B.; Hanley, M. R. *Agents Actions* **1989**, 27, 17-23.
32. Polokoff, M. A.; Bencen, G. H.; Vacca, J. P.; Jane deSolms, S.; Young, S. D.; Huff, J. R. *J. Biol. Chem.* **1988**, 263, 11922-11927.
33. Kozikowski, A. P.; Fauq, A. H. *J. Am. Chem. Soc.* **1990**, 112, 7403-7404.
34. Kozikowski, A. P.; Ognyanov, V. I.; Fauq, A. H.; Nahorski, S. R.; Wilcox, R. A. *J. Am. Chem. Soc.* **1993**, 115, 4429-4434.
35. Safrany, S. T.; Sawyer, D.; Wojcikiewicz, R. J.; Nahorski, S. R.; Potter, B. V. *FEBS Lett.* **1990**, 276, 91-94.
36. Hirata, M.; Yanaga, F.; Koga, T.; Ogasawara, T.; Watanabe, Y.; Ozaki, S. *J. Biol. Chem.* **1990**, 265, 8404-8407.
37. Neuenschwander, M.; Iseli, R. *Helv. Chim. Acta.* **1977**, 60, 1061
38. Grynkiewicz, G.; Tsien, R. Y. *Polish J. Chem.* **1987**, 61, 443-447.
39. Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta.* **1987**, 70, 175-186.
40. Baudin, G.; Glänzer, B. I.; Swaminathan, K. S.; Vasella, A. *Helv. Chim. Acta* **1988**, 71, 1367-1378.
41. Tsien, R. Y. *Methods Cell Biol.* **1989**, 30, 127-156.
42. Stuhmer, W. *Methods in Enzymology* **1992**, 207, 319

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