Membrane-permeant Esters of Phosphatidylinositol 3,4,5-Trisphosphate*

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Phosphoinositide 3-0H kinases and their products, D-3 phosphorylated phosphoinositides, are increasingly recognized as crucial elements in many signaling cascades. A reliable means to introduce these lipids into intact cells would be of great value for showing the physiological roles of this pathway and for testing the specificity of pharmacological inhibitors of the kinases. We have stereospecifically synthesized di-C₈-PIP₃/AM and di-C₁₂-PIP₃/AM, the heptakis(acetoxymethyl) esters of dioctanoyl- and dilauroylphosphatidylinositol 3,4,5-trisphosphate, in 14 steps from myo-inositol. The ability of these uncharged lipophilic derivatives to deliver phosphatidylinositol 3,4,5-trisphosphate across cell membranes was demonstrated on 3T3-L1 adipocytes and T₈₄ colon carcinoma monolayers. Insulin stimulation of hexose uptake into adipocytes was inhibited by the kinase inhibitor wortmannin and was largely restored by di-C₈-PIP₃/AM, which had no effect in the absence of insulin. Thus phosphatidylinositol 3,4,5-trisphosphate or a metabolite was necessary but not sufficient for stimulation of hexose transport. In T₈₄ epithelial monolayers, di-C₁₂-PIP₃/AM mimicked epidermal growth factor in inhibiting chloride secretion and potassium efflux, suggesting that phosphatidylinositol 3,4,5-trisphosphate was sufficient to modulate these fluxes and mediate epidermal growth factor's action.

The recent discovery of D-3 phosphorylated inositol lipids and their biosynthesis by a family of phosphoinositide 3-0H kinases (PI3K)† has opened a new area in cell signal transduction research (1). These enzymes phosphorylate phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate on the D-3 position of the inositol ring to generate phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃, Fig. 1). Some forms of PI3K such as the yeast Vps34p and homologues produce exclusively PI(3)P. In mammalian cells, PI(3)P is usually constitutively present. PI(3,4)P₂ and PI(3,4,5)P₃ are normally undetectable in unstimulated cells, but can become transiently elevated within seconds to minutes following stimulation with a wide range of growth factors and cytokines. This behavior is indicative of signaling roles for both PI(3,4)P₂ and PI(3,4,5)P₃. Various PI3Ks can be activated through both tyrosine kinase and G-protein dependent pathways and multiple putative downstream targets have been identified including Ca²⁺-independent protein kinase C (PKC) isoforms δ, ε, ζ, and η, proteins with pleckstrin homology domains such as Akt/PKB, as well as other proteins such as synaptotagmin.

Because PI(3,4,5)P₃ seems to play such an important role in signal transduction but is difficult to purify from biological sources, several groups have synthesized PI(3,4,5)P₃ by various synthetic routes and tested its in vitro actions on purified enzymes. However, to reveal the role of PI(3,4,5)P₃ in intact cells, especially when its precise molecular target is uncertain, it would be very helpful to be able to deliver exogenous PI(3,4,5)P₃ to its site of action inside whole cells. Such delivery would also be valuable to test the pharmacological specificity of PI3K blockers such as wortmannin. The ability of wortmannin to inhibit a cellular response is often taken to suggest that the response involves PI3K. The best test to prove the specificity of wortmannin for PI3K would be to deliver PI(3,4,5)P₃ by other means and show that the cellular response is restored. Unfortunately, PI(3,4,5)P₃ has at least 4 negative charges at physiological pH, so it is extremely unlikely to diffuse into cells by itself. Therefore effective administration of PI(3,4,5)P₃ itself to intact cells is problematic. A possible strategy to deliver PI(3,4,5)P₃ across the plasma membrane would be to derivatize the phosphates as acetoxymethyl (AM) esters so that the resulting neutral molecule can cross the plasma membrane by passive diffusion. The desirable feature of AM esters is that they are readily hydrolyzed by intracellular esterases, which should regenerate PI(3,4,5)P₃ inside the cells. This approach has previously been successfully applied to antitumor nucleotides (2, 3), cyclic nucleotides (4, 5), and inositol polyphosphates (6–8), but syntheses of AM esters of phospholipids or other compounds with acyclic phosphodiester bonds have not yet been described. We now report the stereospecific total synthesis of the heptakis(acetoxymethyl) esters of dioctanoyl- and dilauroyl-PIP₃ and tests of their biological effects on insulin-stimulated glucose transport into intact fat cells and epidermal growth factor (EGF)-stimulated chloride transport across monolayers of T₈₄ colon carcinoma cells.

The ability of insulin to stimulate uptake of glucose in muscle and fat tissue plays a central role in the maintenance of

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1 The abbreviations used are: PI3K, phosphoinositide 3-0H kinases; PI(3)P, phosphatidylinositol 3-phosphate; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; AM, acetoxymethyl; EGF, epidermal growth factor; IRS, insulin receptor substrate; SH2, Src homology domain 2.

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whole body glucose homeostasis (9). The signal transduction pathway utilized by insulin in promoting glucose transport has been shown to involve autophosphorylation of the insulin receptor with ensuing activation of its intrinsic receptor tyrosine kinase activity and phosphorylation of insulin receptor substrates such as IRS-1, IRS-2, and IRS-3 (10, 11). PI3K interacts with tyrosine-phosphorylated IRS proteins through an SH2 domain on its regulatory p85 subunit (13). The conclusion that PI3K is essential for insulin regulation of glucose transport is based largely on the use of the inhibitors wortmannin (14) and LY294002 (15). These studies have been supported by other approaches, such as overexpression of dominant negative or constitutively active PI3K mutants (16). The impact of PI3K stimulation on glucose transport is mediated either directly by the D-3 phosphorylated inositol phospholipid products of the enzyme, or alternatively by activation of intermediate molecules. Recently, two such downstream effectors of PI3K have been proposed, the serine/threonine kinase Akt or protein kinase B (17) and specific isoforms of protein kinase C (18, 19). Because of the known pharmacology of 3T3-L1 adipocytes and the importance of inositide breakdown, many targets other than PI3K. Therefore we tested whether either PIP3/AM or PIP3 could mimic the effect of EGF.

Fig. 1. Schematic diagram of how PIP3/AM gets into cells and releases PIP3. AM denotes acetoxymethyl, CH2COOCH2-.

1) prolonged stimulation of the muscarinic M3 receptor on T84 cells leads to accumulation of intracellular Ins(3,4,5,6)P4, which, in turn, inhibits transepithelial Cl- efflux by restricting flow through apically located Cl- channels (6). 2) Another pathway, stimulated by EGF and inhibited by wortmannin, also restricts transepithelial Cl- transport (25) by limiting basolateral efflux through K+ channels (26). Moreover, the effects of EGF and carbachol are additive (25), further arguing that the two inhibitory pathways are independent. EGF probably works at least partly through stimulation of PI3K, because EGF treatment elevates PIP3, and the effect of EGF can be ablated by the PI3K inhibitor wortmannin (25, 27). However, these results obtained with standard techniques leave open the questions of whether the wortmannin block is specific and whether EGF might also have other biochemical effects that are also necessary for its inhibition of carbachol-stimulated Cl- flux. Such effects would be plausible because the EGF receptor is a powerful tyrosine kinase with many targets other than PI3K. The signal transduction pathway utilized by insulin in promoting glucose transport has been shown to involve autophosphorylation of the insulin receptor with ensuing activation of its intrinsic receptor tyrosine kinase activity and phosphorylation of insulin receptor substrates such as IRS-1, IRS-2, and IRS-3 (10, 11). PI3K interacts with tyrosine-phosphorylated IRS proteins through an SH2 domain on its regulatory p85 subunit (13). The conclusion that PI3K is essential for insulin regulation of glucose transport is based largely on the use of the inhibitors wortmannin (14) and LY294002 (15). These studies have been supported by other approaches, such as overexpression of dominant negative or constitutively active PI3K mutants (16). The impact of PI3K stimulation on glucose transport is mediated either directly by the D-3 phosphophorylated inositol phospholipid products of the enzyme, or alternatively by activation of intermediate molecules. Recently, two such downstream effectors of PI3K have been proposed, the serine/threonine kinase Akt or protein kinase B (17) and specific isoforms of protein kinase C (18, 19). Because of the known pharmacology of 3T3-L1 adipocytes and the importance of inositide breakdown, many targets other than PI3K. Therefore we tested whether either PIP3/AM or PIP3 could mimic the effect of EGF.

MATERIALS AND METHODS

All chemicals from commercial sources were used without further purification. d-myo-Inositol (Aldrich) was dried at 80 °C under high vacuum overnight before use. sn-1,2-Dioctanoylglycerol and sn-1,2-dilauroylglycerol were purchased from Avanti Polar Lipids, Inc. Reagents were dried by mixing with activated molecular sieves at least 1 day before use. 1H NMR spectra were obtained on Varian 200 MHz or Bruker 300 MHz instruments. 13C NMR were obtained at 50 MHz. Mass spectra were recorded on a electrospray mass spectrometer (Hewlett Packard 5998A). Column chromatography was performed on silica gel (230–400 mesh from EM Science).

Compound 5: 730 mg (1 mmol) of diol 4 (28) in dry CH3Cl was treated with 3 ml of diisopropylethylamine (17 mmol) and 1 ml of benzoylcholm chloride (Fluka, 80% purity, 4 mmol) and heated at 60 °C for 30 h. The reaction mixture was allowed to cool and solvent was removed under vacuum. The brown material was dissolved in CH3Cl and purified by silica gel chromatography, eluting with 6:4 (v/v) CH3Cl:hexane. 950 mg of colorless oily compound was obtained, 98% yield. 1H NMR (CDCl3, δ ppm): 7.72–7.82 (m, 6H), 7.13–7.48 (m, 29H), 6.31 (t, 1H), 5.59 (t, 1H), 5.34 (d, 1H), 4.80 (d, 1H), 4.68 (s, 4H), 4.52 (dd, 1H), 4.24 (dd, 1H), 1.11 (s, 9H); 13C NMR (CDCl3, δ ppm): 166.2, 159.6, 138.2, 136.4, 133.3, 130.6, 130.4, 130.1, 129.8, 128.7, 128.8, 127.9, 127.8, 127.5, 96.3, 91.8, 74.8, 73.0, 72.0, 71.4, 70.3, 70.1, 27.1, 19.8. MS: calculated for [C63H72O11Si2]⁺ 972.2, found 972.2.

Compounds 3: 3.5 g of 5 (3.61 mmol) was dissolved in tetrahydrofuran and 1.2 g of tetrabutylammonium fluoride (4.6 mmol) and heated at 60 °C for 30 h. The reaction mixture was allowed to cool and solvent was removed under vacuum. The brown material was dissolved in CH3Cl and purified by silica gel chromatography, eluting with 6:4 (v/v) CH3Cl:hexane. 950 mg of colorless oily compound was obtained, 98% yield. 1H NMR (CDCl3, δ ppm): 7.75–7.83 (m, 6H), 7.01–7.48 (m, 19H), 6.08 (t, 1H), 5.53 (t, 1H), 5.22 (dd, 1H), 4.92 (d, 1H), 4.82 (d, 1H), 4.34–4.72 (m, 19H), 6.08 (t, 1H). 13C NMR (CDCl3, δ ppm): 166.0, 165.8, 165.7, 137.3, 137.1, 133.4, 133.1, 129.8, 129.7, 128.5, 128.4, 127.9, 127.8, 127.5, 96.2, 96.0, 72.5, 72.0, 71.3, 70.5, 70.3, 70.0. MS: calculated for [C59H58O11Si]⁺ 843.7, found 834.0.

Compound 7: 732 mg of 6 (1 mmol) was dissolved in dry dimethylformamide with 170 mg of imidazole (2.5 mmol). 250 μl of dimethylisopropylchloride (1.6 mmol) was added and the reaction mixture stirred under argon at room temperature for 4 h. Dimethylformamide was removed under vacuum and the product purified on a silica gel column with CHCl3 as eluant. 7 was obtained as a white powder, 83% yield. 1H NMR (CDCl3, δ ppm): 7.80–7.82 (m, 6H), 7.01–7.48 (m, 19H), 6.24 (t, 1H), 5.68 (t, 1H), 5.28 (dd, 1H), 5.10 (dd, 2H), 4.91 (s, 1H), 4.84 (d, 1H), 4.79 (d, 1H), 4.66 (s, 2H), 4.57 (s, 2H), 4.31 (s, 2H), 4.12 (dd, 1H), 0.96–0.98 (m, 6H), 0.16 (d, 6H). 13C NMR (CDCl3, δ ppm): 166.2, 165.8, 165.7, 137.3, 137.1, 133.4, 133.2, 133.1, 129.8, 129.7, 128.5, 128.4, 127.9, 127.8, 127.5, 96.2, 96.0, 74.3, 72.8, 72.5, 71.3, 70.2, 69.8, 18.4, 16.5, –22. MS: calculated for [C59H58O11Si2]⁺ 834.0, found 834.0.

Compound 8: 290 mg of 7 (0.35 mmol) was dissolved in dry methanol. 100 mg of KCN (1.53 mmol, dried over KOH under vacuum) was added and the reaction mixture stirred at room temperature for 9 h. After removing solvent, the reaction mixture was redissolved in CHCl3 and purified on a silica gel column, eluting with 95:5 (v/v) CHCl3:MeOH. 200
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mg of 8 was obtained, 98% yield. 1H NMR (CDCl3, δ ppm): 7.34–7.37 (m, 10H), 5.01 (d, 1H), 4.93(d, 1H), 4.85 (d, 1H), 4.83 (s, 2H), 4.78 (m, 6H), 4.63 (d, 1H), 4.57 (d, 1H), 4.09 (d, 1H), 3.3–3.7 (m, 3H), 0.96 (s, 3H), 0.93 (s, 3H), 0.85 (m, 1H), 0.06 (s, 6H). 13C NMR (CDCl3, δ ppm): 129.1, 128.5, 97.5, 97.2, 85.0, 92.5, 74.3, 74.2, 72.5, 71.5, 70.8, 15.3, –2.4. MS: calculated for [C62H93O38P4 Na]+ 1573, found 1573.

Compound 8: 2-methyl of 5 mg of palladium black for room temperature and atmosphere pressure. After filtering off the catalyst and removing solvent, 4 mg of 14a (di-C25-PIP-Ac) was obtained, 96% yield. 1H NMR (CDCl3, δ ppm): 7.25–7.42 (m, 10H), 5.52–5.82 (m, 14H), 5.18 (q, 1H), 4.62–4.98 (m, 5H), 4.04–4.42 (m, 8H), 2.04–2.38 (m, 25H), 1.46–1.72 (m, 8H), 1.20–1.42 (m, 16H), 0.82–0.98 (m, 6H). MS: calculated for [C62H93O38P4 Na]+ 1573, found 1573.

Compound 11a: 15 mg of 13a in tetrahydrofuran was hydrogenated with 5 mg of palladium black for 4 h at room temperature and atmospheric pressure. After filtering off the catalyst and removing solvent, 4 mg of 14a (di-C25-PIP-Ac) was obtained, 96% yield. 1H NMR (CDCl3, δ ppm): 7.31–7.42 (m, 10H), 5.41 (q, 1H), 5.06 (dd, 4H), 4.80 (d, 1H), 4.64–4.82 (m, 7H), 4.20–4.50 (m, 15H), 3.92 (t, 1H), 2.68–2.95 (m, 14H), 2.34–2.64 (m, 4H), 1.52–1.72 (m, 12H), 1.17–1.40 (m, 28H), 0.81–0.93 (m, 6H). 13C NMR (CDCl3, δ ppm): 174.1, 173.8, 138.3, 137.5, 128.5, 128.3, 113.7, 117.4, 117.8, 97.5, 97.3, 71.2, 68.8, 66.8, 63.4, 63.2, 62.1, 34.1, 31.9, 29.6, 29.4, 25.3, 22.9, 20.2, 20.1, 19.9, 19.8, 14.3. MS: calculated for [C62H93O38P4 Na]+ 1573, found 1573.

Compound 12b and 13b were used for synthesis of 13b without purification. 1H NMR (CDCl3, δ ppm): 7.26–7.41 (m, 10H), 5.52–5.83 (m, 14H), 5.17 (q, 1H), 4.63–5.00 (m, 5H), 4.05–4.45 (m, 2H), 2.05–2.40 (m, 25H), 1.44–1.74 (m, 12H), 1.19–1.44 (m, 28H), 0.82–0.98 (m, 6H). MS: calculated for [C62H93O38P4 Na]+ 1706, found 1706.

Compound 14b was prepared in the same manner as 14a. 40 mg of 14b (di-C25-PIP-Ac) was obtained in 95% yield. 1H NMR: 5.51–5.79 (m, 25H), 4.65–5.00 (m, 10H), 4.55–5.15 (m, 15H), 1.20–1.42 (m, 16H), 0.84–0.94 (m, 6H). MS: calculated for [C62H93O38P4 Na]+ 1465, found 1465.

Adipocyte Cell Culture—All cell culture solutions and supplements were obtained from Life Technologies, Inc. (Burlington, ON, Canada). 3T3-L1 cells were a kind gift from Dr. G. Holman (University of Bath, United Kingdom) and were grown in monolayer culture in 12-well plates, batEagle’s medium/10% (v/v) calf serum in Dulbecco’s modified Eagle’s medium (GIBCO; Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% (v/v) calf serum and 1% (v/v) antibiotic solution (10,000 units/ml penicillin and 10 mg/ml streptomycin) in an atmosphere of 5% CO2 at 37 °C and this medium was refreshed every 48 h. Prior to experimental manipulation, the cells were depleted of serum for 3 h.

Determination of 2-Deoxyglucose Uptake in 3T3-L1 Adipocytes—3T3-L1 adipocyte monolayers were rinsed with 140 μM NaCl, 2.4 mM MgSO4, 5 mM KCl, 1 mM CaCl2, and 20 mM Na-HEPES, pH 7.4. Glucose uptake was measured in 0.25-ml incubation volumes using 10 μM 2-[3H]deoxyglucose (1 μCi/ml, NEN Life Science Products) for 5 min. Previous studies have demonstrated 2-deoxyglucose uptake to be linear in this time period. The radioactive solution was aspirated, and the cells were rinsed three times with ice-cold isotonic saline solution. Cells were disrupted with 1.0 ml of 0.5% NaOH and the radioactivity of a 0.5-ml aliquot of the cell lysate was quantitated by liquid scintillation counting using an LKB 1217 β-counter. Protein concentration of the lysate was determined using the Bradford method (29). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B (Sigma) and was subtracted from total uptake.

T84 Colon Carcinoma Cell Culture—T84 cells (passages 15–45) were grown and maintained as described previously (30) in Dulbecco’s modified Eagles medium/F12 (JRH Biologicals, Lenexa, KS) supplemented with 5% newborn calf serum, 2 mM glutamine, and 50 units/ml each of penicillin/streptomycin (Core Cell Culture Facility, University of California, San Diego). Cells used in experiments were plated on Costar “snap-well” inserts and maintained in culture for 6–10 days to allow for formation of tight junctions prior to the experiment.

Short Circuit Current Measurements—Snapwell inserts containing confluent T84 monolayers were incubated for 0.5 h at 37 °C with 0.1 ml of PIP3 derivatives (200 μM) or vehicle applied to the apical side. The monolayers were then mounted into modified Ussing chambers (Physiologic Instruments, San Diego, CA), whose basolateral side was bathed with Ringers solution warmed to 37 °C and gassed continuously with 95% O2, 5% CO2 at a rate of 30–35 ml/min. The spontaneous potential difference across the monolayer was short-circuited with a voltage clamp (Model VCC MC6, Physiologic Instruments, San Diego, CA). Short circuit current (Isc) and conductances were recorded at 4-s intervals using Acquire and Analyze Software 1.1. (Physiologic Instruments, San Diego, CA). Increased Isc in T84 was demonstrated to reflect transcellular CI secretion (21).

Rb+ Efflux Measurements—Rb+ efflux measurements were a modification of a method previously published by Venglarik et al. (31). Monolayers grown on Costar snap-well inserts (Cambridge, MA) were rinsed in Hank’s balanced salt solution containing (in mM) Na+, 137.6; Cl–, 146.3; K+ 5.8; H2PO4–, 0.44; HPO42–, 0.34; Ca2+, 1; Mg2+, 1; HEPES (pH 7.2), 15; and d-glucose, 10. The cells were loaded for 30 min with 5 μCi/25 ml at 37 °C added to the basolateral surface. Simultaneously, the apical surface was bathed with cell permeant esters of PIP3 (200 μM) or vehicle applied to the apical side. The monolayers were then mounted into modified Ussing chambers (Physiologic Instruments, San Diego, CA), whose basolateral side was bathed with Ringers solution warmed to 37 °C and gassed continuously with 95% O2, 5% CO2 at a rate of 30–35 ml/min. The spontaneous potential difference across the monolayer was short-circuited with a voltage clamp (Model VCC MC6, Physiologic Instruments, San Diego, CA). Short circuit current (Isc) and conductances were recorded at 4-s intervals using Acquire and Analyze Software 1.1. (Physiologic Instruments, San Diego, CA). Increased Isc in T84 was demonstrated to reflect transcellular CI secretion (21).

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RESULTS

Synthesis—The synthesis of acetoxyethyl esters of PIP₃ (Fig. 2) started with enantiomerically pure 0-1-O-(tert-butylidiphenylsilyl)-3,4,5-O-tribenzoyl-myoinositol (4) which was prepared from myo-inositol in 4 steps with 30% yield. The synthesis of 4 was developed by Bruzik and Tsai (28), and their method offers many advantages including enantiomeric purity and good yield. The NMR spectra, MS, and optical rotation of the diol 4 that we prepared are all consistent with their report. Diol 4 was then protected with benzylxymethyl ethers, which can be easily removed by hydrogenolysis at the end of the synthesis without affecting the other groups on the product. At this stage, the myo-inositol 1-hydroxyl was still protected as a tert-butylidiphenylsilyl ether, whose bulk was essential for regioselectivity in the synthesis of 4. But we found that tert-butylidiphenylsilyl could not be removed at a late stage without cleavage of other protecting groups, so it had to be replaced in 7 by dimethylisopropylsilyle. The benzate groups on 7 were removed by KCN in methanol to give triol 8, which was phosphorylated on the 3, 4, and 5 positions and oxidized to 9 with phosphates protected as β-cyanoethoxy esters. At this stage, the dimethylisopropylsilyl chloride protecting the 1-hydroxyl could be cleaved by 2.5% HF to give 10 without affecting the other protecting groups. The 1-hydroxyl of 10 was phosphorylated and linked to sn-1,2-dioctanoylglycerol or the analogous dilauroylglycerol to 11a or 11b, respectively. The β-cyanoethoxy protection on the phosphates was removed with anhydrous triethylamine to 12a or 12b without affecting the diaclcoylglycerol esters. The PIP₃₄’s with the 2,6-benzoxymethyl ethers were esterified with bromomethyl acetate to 13a or 13b to mask all seven potential negative charges as acetoxyethyl (AM) esters. The final products 14a and 14b were obtained by hydrogenolysis of the benzoxymethyl groups to free the 2,6-hydroxyls. For biological comparison, the corresponding di-C₈-PIP₃ lacking the AM esters was prepared by hydrogenolysis of 12a.

Effects on Hexose Uptake into 3T3-L1 Adipocytes—Hexose uptake into adipocytes was markedly stimulated by a maximally effective dose of insulin (Fig. 3). As shown previously, this increase could be mostly prevented by the PI3K inhibitor wortmannin. The crucial new result is that wortmannin inhibition could be largely circumvented by PIP₃/AM. The di-C₈ version (14a) restored a greater percentage (87%) of the insulin stimulation than that (56%) produced by di-C₁₂-PIP₃/AM (14b). Interestingly, neither PIP₃/AM had a significant effect on hexose uptake in the absence of insulin.

Effects on Cl⁻ Transport Across T₈₄ Monolayers—In contrast to the above results with adipocytes, PIP₃/AM by itself was able to mimic the action of EGF on a model of colonic epithelia. The di-C₁₂ version (14b) was more effective than the di-C₈ (14a), the opposite ranking from that seen with the adipocytes. Extracellular nonesterified PIP₃ had no effect, a finding that confirmed that the site of action is intracellular and that esterification is necessary for effective transmembrane delivery of PIP₃ in this system. Fig. 4 shows the large Iₖc stimulated by carbachol (dotted line) and its nearly complete inhibition by pretreatment either with 1 µM EGF for 15 min (circles) or with 200 µM di-C₁₂-PIP₃/AM (14b) for 30 min (dashed line). EGF and PIP₃/AM were equally effective in reducing carbachol-stimulated peak Iₖc to 15% of control. The combination of maximal doses of both EGF and PIP₃/AM (solid line) was no more effective than either alone. These results argue that both agents are working through the same mechanism, namely generation of intracellular PIP₃ or a metabolite thereof, which is not only necessary but sufficient to mediate the effects of EGF in this response.

By what means does PIP₃ inhibit Iₖc? One possibility might be an inhibition of the carbachol-stimulated rise in [Ca²⁺]ᵢ, but direct imaging of [Ca²⁺]ᵢ in fura-2-loaded T₈₄ monolayers failed to reveal any such effect of PIP₃/AM. Transepithelial Cl⁻ fluxes are known to require opening of basolateral K⁺ channels, whose function can be assayed by measuring efflux of preloaded ⁸⁶Rb as a K⁺ surrogate. As shown in Fig. 5, di-C₁₂-PIP₃/AM did inhibit carbachol-stimulated ⁸⁶Rb efflux by >50%, so these K⁺ channels are a likely target for the PIP₃ effect.

DISCUSSION

Synthetic Strategy and Choice of Fatty Acid Chain Length—The synthesis proceeded fairly smoothly from the cheap starting material myo-inositol via 1-O-tert-butylidiphenylsilyl-myoinositol-3,4,5-tri-O-benzoate, in which the extreme steric bulk of the tert-butylidiphenylsilyl group was used to differentiate the 2- and 6-positions from the 3-, 4-, and 5-positions. Unfortunately, the very same bulk prevented deprotection under conditions that preserved the protecting groups on the phosphates. Therefore once the 2,6-positions were blocked, the tert-butylidiphenylsilyl group had to be replaced by a less hindered analog, dimethylisopropylsilyle. We initially chose dicytanol groups in the diacylglycerol moiety because of the pioneering work of Reddy et al. (32), who showed that di-C₁₆-PIP₃ was more soluble and tractable than PIP₃’s with more physiological fatty acids on the order of C₁₈ or C₂₀. Because di-C₁₆-PIP₃/AM proved easy enough to handle and because longer chain lengths might well simulate the natural PIP₃ more closely, we eventually also synthesized di-C₁₂-PIP₃/AM. A virtue of the present synthetic route is that the diacylglycerol group is added intact at a late stage, so that variations in this part of the molecule are relatively easy. The C₆ version proved more effective than its C₁₂ analog on adipocytes, perhaps because the prominent fat droplets in those cells provided a competitive sink for the more hydrophobic analog. By contrast, the C₁₂ version was more potent than C₆ on the colonic epithelia, where the stronger membrane binding and more physiological chain length of the C₁₂ might be decisive.

Advantages and Potential Problems of Masking the Polar Groups of PIP₃—Our strategy of esterifying all the phosphate negative charges was based on extensive prior experience with the transmembrane delivery of phosphate-containing second messengers. Masking of all charges is highly beneficial for cyclic nucleotides (4, 5), which carry only one charge, and are essential for inositol polyphosphates (6–8), which have multiple charges. However, after the completion of our synthesis of PIP₃/AMs, it was reported that di-C₁₆-P(3,4)P₂, di-C₁₀-P(3,4,5)P₃, and di-C₁₆-P(3,4,5)P₃ activate the kinase Akt and stimulate mitolity and chemotaxis when added extracellularly as sonicated vesicles to intact NIH 3T3 fibroblasts (33, 34). It was suggested that the P(3,4,5)P₃ or PIP₃-containing vesicles fuse with the plasma membrane and deliver the free lipid to the intracellular leaflet. In our hands, di-C₁₀-P(3,4,5)P₃ was ineffective at mimicking PI3K activation in T₈₄ epithelia, whereas the AM ester was fully effective. Thus in cell types in which fusion with PIP₃ liposomes is not as facile as in NIH 3T3, the uncharged hydroxylizable PIP₃ esters may be a more reliable means of delivery.

One important choice in the design of a membrane-permeant PIP₃ derivative is whether to protect the 2- and 6-hydroxyls and if so, with what. In the present molecules, those hydroxyls have been left free. The advantage is that neither the cell nor the experimenter need to do anything to unmask the OH
groups on those positions. The main disadvantage of free hydroxyls is that they permit extensive migration of phosphate triesters. Such migration produces unwanted isomers and necessitates much higher concentrations of the permeant ester (7). Based on previous problems with hydrolysis of 6-O-butyrate esters of inositol-1,4,5-trisphosphate, we feared that esters on the 2- and 6-positions of PIP3 would similarly refuse to hydrolyze quickly enough because they are similarly sandwiched between flanking phosphate groups. However, 2,6-di-O-butyryl-PIP3/AM does have biological activity (27), so this concern may have been overcautious. Yet another possibility would be to mask the 2- and 6-hydroxyls with UV-photolyzable caging groups such as 3,4-dimethoxy-2-nitrobenzyl ethers. This group has proven to be the ideal way to protect the 6-hydroxyl of inositol 1,4,5-trisphosphate because it prevents migration yet can be instantaneously removed with a flash of UV, ideal for unleashing the immediate actions of this fast-acting, rapidly metabolized messenger (8). Although it is not clear whether any important physiology may require such rapid delivery of PIP3, a caged membrane-permeant PIP3 would be an ideal way to find out.

Generalization to Other Polyphosphoinositides and Acyclic Phosphodiester—Now that permeant esters have been shown to deliver the extremely polar phospholipid PIP3 across the plasma membrane, it would be interesting to synthesize analogous esters of related phospholipids such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 4,5-bisphosphate. PI(3,4)P2 could be produced intracellularly by dephosphorylation of PIP3 and may be more potent than PIP3 at activating certain isoforms of protein kinase C. The 4,5-isomer is not only the classical substrate for phospholipase C but also is important for membrane-cytoskeletal interaction. Esters of such phospholipids might help reveal which interconversions occur inside cells and which lipids are the proximal

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FIG. 2. Synthesis of PIP3/AM. The steps from myo-inositol to 4 were as described by Bruzik and Tsai (28). The abbreviations used are: TMS-OTf, trimethylsilyl trifluoromethanesulfonate; TBDPS, tert-butyldiphenylsilyl; Bz, benzoyl; py, pyridine; BOM, benzoxymethyl; TBAF, tetraethylammonium fluoride; THF, tetrahydrofuran; DMIPS, dimethylisopropylsilyl; IPr, isopropyl; R, 2-cyanoethyl. Ak, α-alkyl, —(CH2)nCH3, where n = 6 or 10. R', acetoxymethyl.
agonists for the many downstream targets. Thiophosphate analogs might be particularly helpful because the thiophosphate groups are generally nonmetabolizable.

At present we do not have analytical means to quantify how much PIP3 is actually being delivered inside the cell. Probably the PIP3/AM would have to be radiolabeled, which would be a significant synthetic challenge for the future, or better analytical methods to measure unlabeled PIP3 would have to be developed.

Mechanism of Insulin Signaling in Adipocytes and Additional Signals Provided by Insulin Receptors—PIP3/AMs of two different chain lengths were capable of partially overcoming the inhibitory effect of wortmannin on insulin-stimulated glucose transport. However, PIP3/AMs alone did not stimulate basal glucose transport. Thus, PIP3 appears to be necessary but not sufficient for maximal insulin-stimulated glucose transport. We propose that a bifurcation of the insulin induced signal may occur: one signal involves generation of PIP3, while the other is independent of this product. Full stimulation of glucose transport would require activation of both signals.

The nature of the signal that is dependent on PIP3 is currently being investigated, and could include two enzymes whose activity was recently shown to depend on prior PI3K activation: the protein kinase c-Akt (also known as PKB) and protein kinase C-ζ. Akt was recently characterized (35) and found to be rapidly stimulated by insulin and other growth factors (36). Importantly, overexpression of Akt in 3T3-L1 adipocytes resembled insulin action in that it caused an insulin-like elevation of glucose transport (37). Activation of Akt requires its phosphorylation on two residues, Thr-308 and Ser-473. The kinase responsible for phosphorylation of Thr-308 is stimulated by micromolar concentrations of PIP3 and is thus named PIP3-dependent protein kinase-1 (38). PIP3 acts both by activating PIP3-dependent protein kinase-1 and binding to the PH domain of Akt which is required to allow phosphorylation by the kinase (39). The kinase that phosphorylates Akt on Ser-473 is not known and may be independent of prior PI3K
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activation. If so, it is conceivable that full activation of Akt could suffice to stimulate glucose transport in response to insulin.

An alternative possibility is that the signal from PIP₃ that relieves the wortmannin-imposed block on insulin stimulation of glucose transport is the activation of PKC-ζ. Indeed, it was recently shown that PKC-ζ is activated in response to insulin in 3T3-L1 adipocytes, and that overexpression of a kinase-dead PKC-ζ reduces the insulin-dependent stimulation of glucose transport (40). PIP₃ may act by activating PKC-ζ. The downstream substrate of this enzyme is not known but it is plausible that it may lead to the phosphorylation of Ser-473 on Akt, thereby converging on this latter kinase. Alternatively, the product of PKC-ζ may be independent from and complementary to the action of Akt.

The results presented also show that PIP₃/AM did not restore the full stimulation of glucose transport caused by wortmannin. This could be due to a need for higher local concentrations of the product, for additional products of PI3K, or to the participation of functions inhibited by wortmannin which are independent of the inhibition of PI3K. Whereas there are no clear candidates, the fungal metabolite has been shown to inhibit enzymes such as mitogen-activated protein kinase (41), phospholipase A₂ (42), and PI 4-kinase (43). This highlights the usefulness of PIP₃/AM in dissecting out the steps that are strictly dependent on this PI3K product in the study of insulin action.

In conclusion, the results presented demonstrate that: (a) a lipid product of PI3K, PIP₃, or a metabolite thereof, participates in the stimulation of glucose transport by insulin; (b) PIP₃ is required but not sufficient to elicit full stimulation of glucose transport; and (c) a second insulin signal independent of PIP₃ participates in the stimulation of glucose transport. EGF Signaling via PIP₃ in T₈⁴ Cells—Membrane permeant esters of PIP₃ mimic the inhibitory effects of EGF both on Cl⁻ secretion and efflux through K⁺ channels. Moreover, the EGF- and PIP₃/AM-induced inhibitions, but not that due to carba-

chol, could be reversed by pretreatment with 3′,5′-Ins(1,4,5,6)-P₁/AM (27). Although di-C₁₂- and di-C₁₂-PIP₃/AM had the same basic effects, the latter was somewhat more potent. We attempted to test whether PIP₃/AM would overcome wortmannin reversal of EGF inhibition of Cl⁻ secretion, but unfortunately this experiment is greatly complicated by the ability of wortmannin to augment calcium-dependent Cl⁻ secretion in the absence of EGF. However, pretreatment with PIP₃/AM dramatically reduced calcium-mediated chloride secretion in the presence of wortmannin, consistent with actions of PIP₃/AM that bypass wortmannin. Together, these data strongly suggest that a lipid product of PI-3 kinase mediates EGF-induced inhibition of Cl⁻ secretion in T₈⁴ colonic epithelia. However, the current studies do not exclude the possibility that PI(3,4)P₂ or other PIP₃ metabolite is the ultimate signal.

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