THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 18, Issue of May 1, pp. 11017–11024, 1998

Printed in U.S.A.

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

(Received for publication, December 19, 1997, and in revised form, February 6, 1998)

Tao Jiang‡§, Gary Sweeney¶, Marco T. Rudolf‖, Amira Klip¶, Alexis Traynor-Kaplan ‡‡, and Roger Y. Tsien‡ §§**

*From the ‡Department of Pharmacology and Howard Hughes Medical Institute, University of California San Diego, La Jolla, California 92093-0647, the ¶Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, the ‖Institute of Organic Chemistry, University of Bremen UFT, 28359 Bremen, Germany, and the **Department of Medicine, University of California San Diego, La Jolla, California 92093*

Phosphoinositide 3-OH 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-C_8-PIP_3/AM and di-C_{12}-PIP_3/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 T_{84} colon carcinoma monolayers. Insulin stimulation of hexose uptake into adipocytes was inhibited by the kinase inhibitor wortmannin and was largely restored by di-C_8-PIP_3/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 T_{84} epithelial monolayers, di-C_{12}-PIP_3/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-OH kinases (PI3K)[1] 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 ($PI(3,4)P_2$), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3, 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. $PI(3,4)P_2$ and PIP_3 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 $PI(3,4)P_2$ and PIP_3. Various PI3Ks can be activated through both tyrosine kinase and G-protein dependent pathways and multiple putative downstream targets have been identified including Ca^{2+}-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 PIP_3 seems to play such an important role in signal transduction but is difficult to purify from biological sources, several groups have synthesized PIP_3 by various synthetic routes and tested its *in vitro* actions on purified enzymes. However, to reveal the role of PIP_3 in intact cells, especially when its precise molecular target is uncertain, it would be very helpful to be able to deliver exogenous PIP_3 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 PIP_3 by other means and show that the cellular response is restored. Unfortunately, PIP_3 has at least 4 negative charges at physiological pH, so it is extremely unlikely to diffuse into cells by itself. Therefore effective administration of PIP_3 itself to intact cells is problematic. A possible strategy to deliver PIP_3 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 PIP_3 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-PIP_3 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 T_{84} 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

* This work was supported by National Institutes of Health Grants NS27177 (to R. Y. T.) and DK47240 (to A. T.-K.), the University-wide AIDS Research Program (to A. T.-K.), and Medical Research Council (Canada) Grant MT-7307 (to A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Aurora Biosciences Corp., 11010 Torreyana Rd., San Diego, CA 92121.

‡‡ Present address: Inologic Inc., 43012 S.E. 108th St., North Bend, WA 98045.

§§ To whom correspondence should be addressed. Tel.: 619-534-4891; Fax: 619-534-5270.

[1] The abbreviations used are: PI3K, phosphoinositide 3-OH kinases; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; PKC, protein kinase C; AM, acetoxymethyl; EGF, epidermal growth factor; IRS, insulin receptor substrate; SH2, Src homology domain 2.

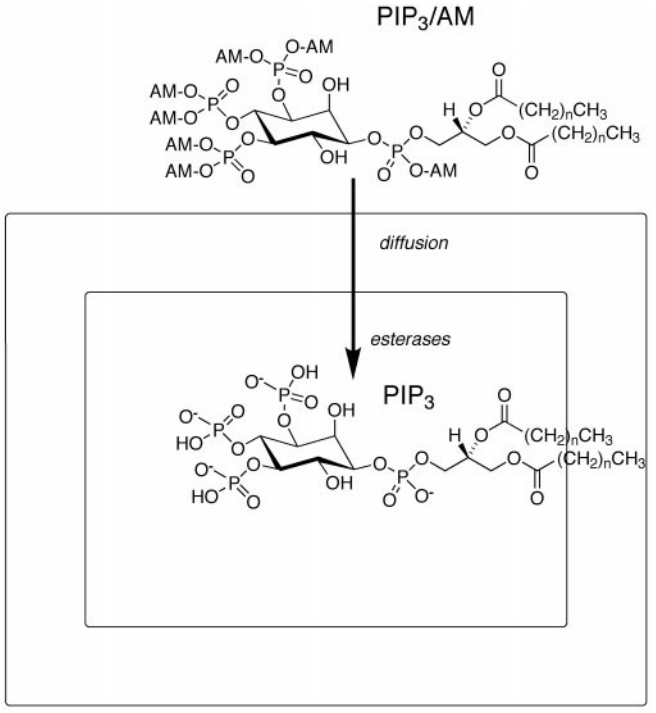

FIG. 1. **Schematic diagram of how PIP_3/AM gets into cells and releases PIP_3.** *AM* denotes acetoxymethyl, CH_3COOCH_2—.

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 (12), thus activating its catalytic p110 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 insulin-stimulated glucose transport in these cells, they are a suitable system to test the effects of the membrane-permeant PIP_3 esters, especially their ability to bypass wortmannin blockade.

Regulation of chloride ion flux plays a key role in the control of salt and fluid secretion across mucous membranes in addition to a variety of other functions. An important model system for epithelial transport is the T_{84} colon carcinoma cell line, which forms monolayers that actively transport Cl^- in response to a variety of agonists in much the same way as normal intestinal epithelia. Cl^- secretion in T_{84} cells can be triggered through cyclic AMP (20) and calcium-dependent signaling mechanisms (21). As in many other systems, these pathways interact synergistically in T_{84} (22–24). More recently, we have identified two receptor-activated pathways which limit Cl^- secretion through the calcium-dependent but not through the cyclic AMP-dependent pathway: 1) prolonged stimulation of the muscarinic M_3 receptor on T_{84} cells leads to accumulation of intracellular Ins(3,4,5,6)P_4, 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 PIP_3, 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. Therefore we tested whether either PIP_3/AM or PIP_3 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. ^{1}H NMR spectra were obtained on Varian 200 MHz or Bruker 300 MHz instruments. ^{13}C NMR were obtained at 50 MHz. Mass spectra were recorded on a electrospray mass spectrometer (Hewlett Packard 59987A). 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 CH_2Cl_2 was treated with 3 ml of diisopropylethylamine (17 mmol) and 1 ml of benzyloxymethyl chloride (Fluka, 60% 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 redissolved in CH_2Cl_2 and purified by silica gel chromatography, eluting with 6:4 (v/v) CH_2Cl_2:hexane. 950 mg of colorless oily **5** was obtained, 98% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.72–7.82 (m, 6H), 7.13–7.48 (m, 29H), 6.31 (t, 1H), 5.59 (t, 1H), 5.03 (dd, 1H), 4.92 (s, 4H), 4.80 (d, 1H), 4.68 (s, 4H), 4.52 (dd, 1H), 4.24 (dd, 1H), 1.11 (s, 9H); ^{13}C NMR ($CDCl_3$, δ ppm): 166.2, 159.6, 138.2, 136.4, 133.3, 130.6, 130.4, 130.1, 128.9, 128.7, 128.6, 128.3, 128.2, 127.9, 127.8, 127.7, 97.2, 96.3, 91.8, 74.8, 73.0, 72.0, 71.4, 70.3, 70.1, 27.8, 19.6. MS: calculated for $[C_{59}H_{58}O_{11}Si + H^+]$ 972.2, found 972.2.

Compound **6**: 3.5 g of **5** (3.61 mmol) was dissolved in tetrahydrofuran and 1.2 g of tetrabutylammonium fluoride (4.6 mmol, 1.27 equivalents) was added slowly. After stirring for 20 min at room temperature, the reaction was completed and solvent was removed. Silica gel column chromatography eluting with 98:2 (v/v) $CHCl_3$:CH_3OH furnished 2.48 g of **6**, 94% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.65–7.88 (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, 10H), 4.08 (t, 1H). ^{13}C NMR ($CDCl_3$, δ ppm): 166.0, 165.8, 165.7, 137.3, 137.1, 133.4, 133.2, 133.1, 129.8, 129.7, 128.5, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 96.2, 80.6, 72.5, 72.0, 71.3, 70.5, 70.3, 70.0. MS: calculated for $[C_{43}H_{40}O_{11} + H^+]$ 733.8, found 733.7.

Compound **7**: 732 mg of **6** (1 mmol) was dissolved in dry dimethylformamide with 170 mg of imidazole (2.5 mmol). 250 μl of dimethylisopropylsilyl chloride (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 $CHCl_3$ as eluant. **7** was obtained as 800 mg of colorless oil, 96% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.80–7.98 (m, 6H), 7.25–7.48 (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, 7H), 0.16 (d, 6H). ^{13}C NMR ($CDCl_3$, δ ppm): 166.2, 138.2, 134.0, 133.6, 130.3, 130.1, 128.9, 128.8, 128.7, 128.6, 127.9, 127.8, 96.4, 96.0, 74.3, 72.8, 72.5, 71.3, 70.2, 69.8, 18.4, 16.5, −2.2. MS: calculated for $[C_{48}H_{52}O_{11}Si + H^+]$ 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. Ater removing solvent, the reaction mixture was redissolved in $CHCl_3$ and purified on a silica gel column, eluting with 95:5 (v/v) $CHCl_3$:MeOH. 200

mg of **8** was obtained, 89% yield. ^{1}H NMR ($CDCl_3$, 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). ^{13}C NMR ($CDCl_3$, δ ppm): 129.1, 128.5, 97.3, 97.2, 85.0, 82.8, 74.3, 74.2, 72.6, 71.5, 70.8, 17.5, 15.3, −2.4. MS: calculated for [$C_{27}H_{40}O_8Si$ +H^+] 521.7, found 521.8.

Compound **9:** 0.5 g (2.1 mmol) of 2-cyanoethyl diisopropylchlorophosphoramidite in dry CH_2Cl_2 was mixed with 0.4 ml of diisopropylethylamine (1.1 equivalent) and 160 μl (2.34 mmol) of 2-cyanoethanol. After stirring for 30 min at room temperature, solvent was removed and dry ethyl ether added to precipitate diisopropylethylammonium chloride. The ether extract containing bis(2-cyanoethyl)diisopropylphosphoramidite was mixed with 110 mg of triol **8** (0.17 mmol), then the ether was removed under vacuum. The mixture was redissolved in dry CH_2Cl_2 and 160 mg of 1H-tetrazole (1.1 equivalents) added. After stirring overnight at room temperature under argon, 1 ml of 5 M *tert*-butyl hydroperoxide in hexane was added at 0 °C and stirred 10 min at that temperature and then for 2 h at room temperature. The product was purified on a silica gel column, eluting with 95:5 (v/v) $CHCl_3$:CH_3OH. 220 mg of **9** was obtained, 97% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.32–7.42 (m, 10H), 4.55–5.22 (m, 10H), 4.18–4.50 (m, 15H), 4.08 (t, 1H), 2.82 (t, 12H). MS: calculated for [$C_{45}H_{61}N_6O_{17}P_3Si$ +Na^+] 1102, found 1102.

Compound **10:** 100 mg of **9** was added to 5 ml of acetonitrile containing 2.5% aqueous HF and stirred at room temperature for 2 h. After removing solvent, the product was purified on a silica gel column eluted with 95:5 (v/v) $CHCl_3$:CH_3OH. 85 mg of **10** was obtained, 94% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.30–7.40 (m, 10H), 5.05 (dd, 2H), 4.86 (s, 2H), 4.84 (d, 2H), 4.82 (s, 2H), 4.78 (t, 1H), 4.64 (s, 2H), 4.63 (d, 2H), 4.22–4.48 (m, 13H), 3.89 (t, 1H), 2.78–2.80 (m, 12H). ^{13}C ($CDCl_3$, δ ppm): 138.2, 137.4, 129.1, 129.0, 128.5, 117.8, 117.6, 117.0, 97.8, 97.5, 82.2, 67.9, 67.6, 67.3, 63.2, 63.1, 20.2, 20.0. MS: calculated for [$C_{40}H_{49}N_6O_{17}P_3$ + H^+]979.4, found 979.4.

Compound **11a:** 80 mg of *sn*-1,2-dioctanoylglycerol (0.23 mmol) in CH_2Cl_2 was mixed with 45 μl of diisopropylethylamine and 55 μl of 2-cyanoethyl diisopropylchlorophosphoramidite. After stirring for 8 h at room temperature, solvent was removed and dry ether was added. The crude ether extract of 2-cyanoethyl (1,2-dioctanoyl)glyceryl diisopropylchlorophosphoramidite was mixed with 100 mg of **5** (0.1 mmol) and 100 mg of tetrazole (1.4 mmol) and kept at room temperature overnight. 150 μl of 5 M *tert*-butyl hydroperoxide was then added. 2 h later, solvent was removed and product was purified on a silica gel column eluted with 98:2 (v/v) $CHCl_3$:CH_3OH. 120 mg of **11a** was obtained, 82% yield. ^{1}H NMR (CDCl3, δ, ppm): 7.32–7.42 (m, 10H), 5.42 (q, 1H), 5.05 (dd, 4H), 4.82 (d, 1H), 4.65–4.80 (m, 7H), 4.20–4.50 (m, 15H), 3.91 (t, 1H), 2.70–2.95 (m, 14H), 2.35–2.48 (m, 4H), 1.50–1.72 (m, 8H), 1.18–1.38 (m, 16H), 0.80–0.96 (m, 6H). ^{13}C (CDCl3, δ ppm): 173.8, 173.6, 138.2, 137.6, 128.9, 128.5, 128.2, 117.7, 117.5, 117.2, 97.6, 97.4, 71.1, 68.7, 66.7, 63.5, 63.2, 62.1, 34.2, 32.0, 29.4, 29.3, 25.2, 22.9, 20.1, 20.0, 19.9, 14.3. MS: calculated for [$C_{62}H_{87}N_7O_{24}P_4$ + Na^+]1461, found 1461.

Compound **13a:** 50 mg of **11a** (35 μmol) in CH_2Cl_2 was stirred overnight with 50 μl of Et_3N at room temperature, then solvent was removed under vacuum. The crude product (**12a**) was used directly for the next step by dissolving in 1 ml of dry acetonitrile and adding 100 μl of bromomethyl acetate (1.02 mmol) and 200 μl of diisopropylethylamine (1.15 mmol). The reaction mixture was stirred overnight at room temperature. Solvent was removed and the residue was extracted with dry ethyl ether. The ether supernatant was evaporated and the resulting yellow oil chromatographed on a silica gel column with 98:2 (v/v) $CHCl_3$:CH_3OH. 30 mg of **13a** was obtained, 55% yield. ^{1}H NMR ($CDCl_3$, δ ppm): 7.28–7.42 (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 [$C_{62}H_{93}O_{38}P_4$ + Na^+]1594, found 1594.

Compound **14a:** 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-C_8-PIP_3/AM) was obtained, 96% yield. ^{1}H NMR: 5.52–5.80 (m, 14H), 5.18 (q, 1H), 4.64–4.98 (m, 5H), 2.06–2.38 (m, 25H), 1.44–1.74 (m, 8H), 1.20–1.42 (m, 16H), 0.84–0.94 (m, 6H). MS: calculated for [$C_{46}H_{79}O_{36}P_4$ + Na^+]1353, found 1353.

Compound **11b** was prepared in the same manner as **11a**, 79% yield. ^{1}H NMR ($CDCl_3$, 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), 4.20–4.48 (m, 15H), 3.92 (t, 1H), 2.68–2.95 (m, 14H), 2.34–2.46 (m, 4H), 1.52–1.72 (m, 12H), 1.17–1.40 (m, 28H), 0.81–0.95 (m, 6H). ^{13}C NMR ($CDC1_3$, δ, ppm): 174.1, 173.8, 138.3, 137.5, 128.8, 128.5, 128.3, 117.8, 117.4, 117.2, 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, 20.0, 19.9, 19.8, 14.3. MS: calculated for [$C_{70}H_{103}N_7O_{24}P_4$ + Na^+]1573, found 1573.

Compounds **12b** and **13b** were prepared in the same manner as **12a** and **13a. 12b** was used directly for synthesis of **13b** without purification. 52 mg of **13b** was obtained in 48% yield. ^{1}H NMR ($CDCl_3$, 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, 8H), 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 [$C_{70}H_{109}O_{38}P_4$ + Na^+]1706, found 1706.

Compound **14b** was prepared in the same manner as **14a.** 40 mg of **14b** (di-C_{12}-PIP_3/AM) was obtained in 95% yield. ^{1}H NMR: 5.51–5.79 (m, 14H), 5.16 (q, 1H), 4.65–4.96 (m, 5H), 2.02–2.41 (m, 25H), 1.45–1.74 (m, 12H), 1.17–1.46 (m, 28H), 0.83–0.92 (m, 6H). MS: calculated for [$C_{54}H_{93}O_{36}P_4$ + 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, bathed in Dulbecco's modified Eagle's medium supplemented with 10% (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% CO_2 at 37 °C and this medium was replenished 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 mM NaCl, 2.4 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$, and 20 mM Na-HEPES, pH 7.4. Glucose uptake was measured in 0.25-ml incubation volumes using 10 μM 2-[^{3}H]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 0.05 N NaOH, and the radioactivity of a 0.75-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.

T_{84} Colon Carcinoma Cell Culture—T_{84} cells (passages 15–45) were grown and maintained as described previously (30) in Dulbecco's modified Eagle's medium/F-12 media (JRH Biosciences, Lexena, 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 T_{84} monolayers were incubated for 0.5 h at 37 °C with 0.1 ml of PIP_3 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% O_2, 5% CO_2 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 (I_{sc}) and conductances were recorded at 4-s intervals using Acquire and Analyze Software 1.1. (Physiologic Instruments, San Diego, CA). Increased I_{sc} in T_{84} has been demonstrated to reflect transepithelial Cl^- secretion (21).

Rb^+ Efflux Measurements—Rb^+ flux 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; $H_2PO_4^-$, 0.44; HPO_4^{2-}, 0.34; Ca^{2+}, 1; Mg^{2+}, 1; HEPES (pH 7.2), 15; and D-glucose, 10. The cells were loaded for 30 min with 5 μCi/0.25 ml at 37 °C added to the basolateral surface. Simultaneously, the apical surface was bathed with cell permeant esters of PIP_3 (200 μM) or vehicle. Following 4 rinses of apical and basolateral surfaces with Hank's buffer over a period of 6 min, the inserts were sequentially transferred at 2-min intervals to fresh wells of a 24-well culture dish floating in a 37 °C water bath. After 12 min, the inserts were transferred to wells containing 0.1 mM carbachol for the remainder of the experiment. After the experiment, the contents of the wells and the inserts were transferred to vials containing Ecoscint which were counted in a Packard scintillation counter. The data was analyzed as

described by Venglarik *et al.* (31) yielding rate constants of nuclide efflux at 2-min intervals.

RESULTS

Synthesis—The synthesis of acetoxymethyl esters of PIP_3 (Fig. 2) started with enantiomerically pure D-1-*O*-(tert-butyldiphenylsilyl)-3,4,5-*O*-tribenzoyl-*myo*-inositol (**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 benzyloxymethyl 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*-butyldiphenylsilyl ether, whose bulk was essential for regioselectivity in the synthesis of **4**. But we found that *tert*-butyldiphenylsilyl could not be removed at a late stage without cleavage of other protecting groups, so it had to be replaced in **7** by dimethylisopropylsilyl. The benzoate groups on **7** were removed by KCN in methanol to give triol **8**, which was phosphitylated on the 3, 4, and 5 positions and oxidized to **9** with phosphates protected as β-cyanoethyl 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 phosphitylated and linked to *sn*-1,2-dioctanoylglycerol or the analogous dilauroylglycerol to **11a** or **11b**, respectively. The β-cyanoethyl protection on the phosphates was removed with anhydrous triethylamine to **12a** or **12b** without affecting the diacylglycerol esters. The PIP_3's with 2,6-benzyloxymethyl ethers were esterified with bromomethyl acetate to **13a** or **13b** to mask all seven potential negative charges as acetoxymethyl (AM) esters. The final products **14a** and **14b** were obtained by hydrogenolysis of the benzyloxymethyl groups to free the 2,6-hydroxyls. For biological comparison, the corresponding di-C_8-PIP_3 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 PIP_3/AM. The di-C_8 version (**14a**) restored a greater percentage (87%) of the insulin stimulation than that (56%) produced by di-C_{12}-PIP_3/AM (**14b**). Interestingly, neither PIP_3/AM had a significant effect on hexose uptake in the absence of insulin.

Effects on Cl^- Transport Across T_{84} Monolayers—In contrast to the above results with adipocytes, PIP_3/AM by itself was able to mimic the action of EGF on a model of colonic epithelia. The di-C_{12} version (**14b**) was more effective than the di-C_8 (**14a**), the opposite ranking from that seen with the adipocytes. Extracellular nonesterified PIP_3 had no effect, a finding that confirmed that the site of action is intracellular and that esterification is necessary for effective transmembrane delivery of PIP_3 in this system. Fig. 4 shows the large I_{sc} 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-C_{12}-PIP_3/AM (**14b**) for 30 min (*dashed line*). EGF and PIP_3/AM were equally effective in reducing carbachol-stimulated peak I_{sc} to 15% of control. The combination of maximal doses of both EGF and PIP_3/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 PIP_3 or a metabolite thereof, which is not only necessary but sufficient to mediate the effects of EGF in this response.

By what means does PIP_3 inhibit I_{sc}? One possibility might be an inhibition of the carbachol-stimulated rise in $[Ca^{2+}]_i$, but direct imaging of $[Ca^{2+}]_i$ in fura-2-loaded T_{84} monolayers failed to reveal any such effect of PIP_3/AM. Transepithelial Cl^- fluxes are known to require opening of basolateral K^+ channels, whose function can be assayed by measuring efflux of preloaded ^{86}Rb as a K^+ surrogate. As shown in Fig. 5, di-C_{12}-PIP_3/AM did inhibit carbachol-stimulated ^{86}Rb efflux by >50%, so these K^+ channels are a likely target for the PIP_3 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*-butyldiphenylsilyl-*myo*-inositol-3,4,5-tri-*O*-benzoate, in which the extreme steric bulk of the *tert*-butyldiphenylsilyl 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*-butyldiphenylsilyl group had to be replaced by a less hindered analog, dimethylisopropylsilyl. We initially chose dioctanoyl groups in the diacylglycerol moiety because of the pioneering work of Reddy *et al.* (32), who showed that di-C_8-PIP_3 was more soluble and tractable than PIP_3's with more physiological fatty acids on the order of C_{18} or C_{20}. Because di-C_8-PIP_3/AM proved easy enough to handle and because longer chain lengths might well simulate the natural PIP_3 more closely, we eventually also synthesized di-C_{12}-PIP_3/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 C_8 version proved more effective than its C_{12} analog on adipocytes, perhaps because the prominent fat droplets in those cells provided a competitive sink for the more hydrophobic analog. By contrast, the C_{12} version was more potent than C_8 on the colonic epithelia, where the stronger membrane binding and more physiological chain length of the C_{12} might be decisive.

Advantages and Potential Problems of Masking the Polar Groups of PIP_3—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 PIP_3/AMs, it was reported that di-C_{16}-$PI(3,4)P_2$, di-C_8-PIP_3, and di-C_{16}-PIP_3 activate the kinase Akt and stimulate motility and chemotaxis when added extracellularly as sonicated vesicles to intact NIH 3T3 fibroblasts (33, 34). It was suggested that the $PI(3,4)P_2$ or PIP_3-containing vesicles fuse with the plasma membrane and deliver the free lipid to the intracellular leaflet. In our hands, di-C_8-PIP_3 was ineffective at mimicking PI3K activation in T_{84} epithelia, whereas the AM ester was fully effective. Thus in cell types in which fusion with PIP_3 liposomes is not as facile as in NIH 3T3, the uncharged hydrolyzable PIP_3 esters may be a more reliable means of delivery.

One important choice in the design of a membrane-permeant PIP_3 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

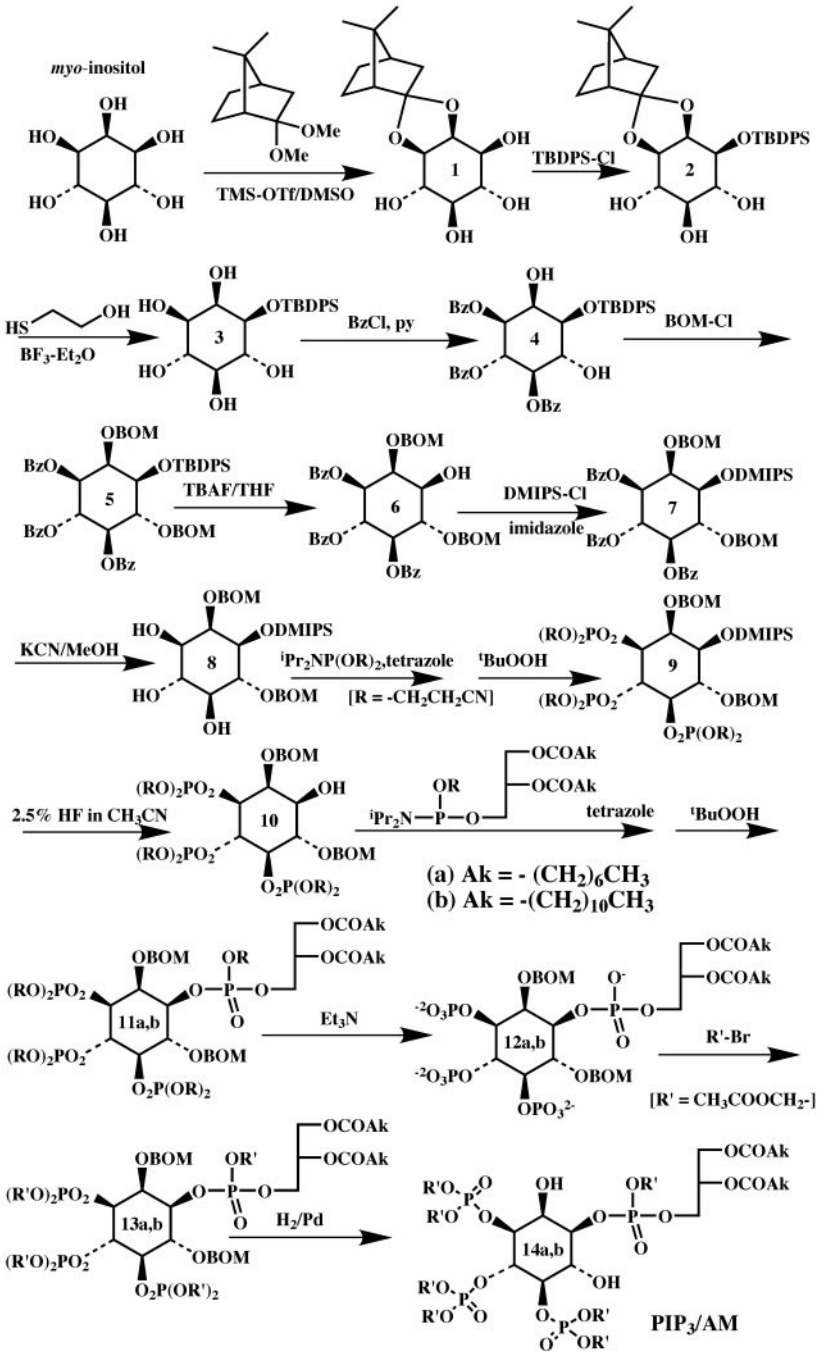

FIG. 2. **Synthesis of PIP_3/AM.** The steps from *myo*-inositol to **4** were as described by Bruzik and Tsai (28). The abbreviations used are: *TMS-OTf*, trimethylsilyl trifluoromethanesulfonate; TBDPS, *tert*-butyldiphenylsilyl; *Bz*, benzoyl; *py*, pyridine; *BOM*, benzyloxymethyl; *TBAF*, tetrabutylammonium fluoride; *THF*, tetrahydrofuran; *DMIPS*, dimethylisopropylsilyl; ^{i}Pr, isopropyl; *R*, 2-cyanoethyl. *Ak*, *n*-alkyl, $—(CH_2)_mCH_3$, where $m = 6$ or 10. *R′*, acetoxymethyl.

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 PIP_3 would similarly refuse to hydrolyze quickly enough because they are similarly sandwiched between flanking phosphate groups. However, 2,6-di-*O*-butyryl-PIP_3/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 PIP_3, a caged membrane-permeant PIP_3 would be an ideal way to find out.

Generalization to Other Polyphosphoinositides and Acyclic Phosphodiesters—Now that permeant esters have been shown to deliver the extremely polar phospholipid PIP_3 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. $PI(3,4)P_2$ could be produced intracellularly by dephosphorylation of PIP_3 and may be more potent than PIP_3 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

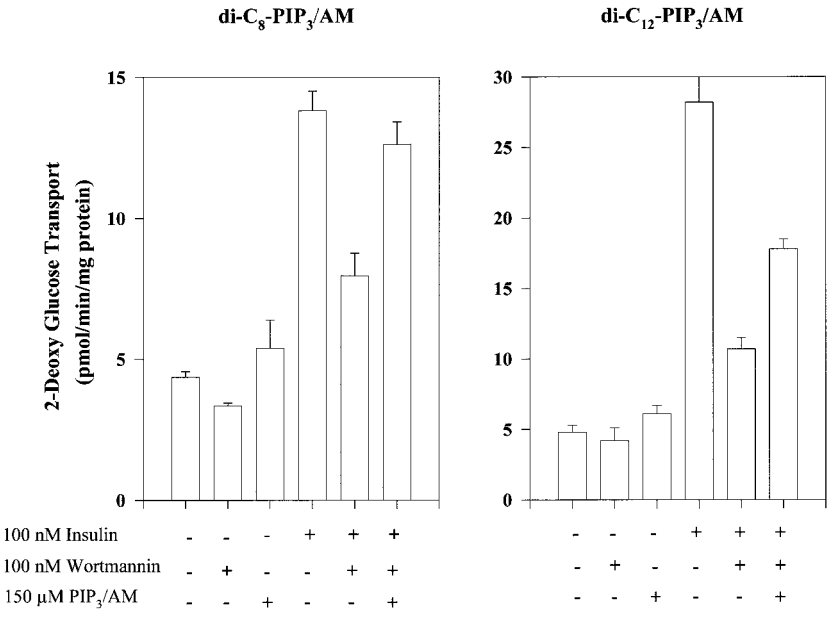

FIG. 3. **Effects of insulin, wortmannin, and PIP_3/AM on hexose uptake.** Adipocytes were incubated in the presence (+) or absence (−) of the agents listed *below* each *bar*. Insulin (100 nM) was added 30 min before measurement of deoxyglucose uptake as described under "Materials and Methods." PIP_3/AM (150 μM) and wortmannin (100 nM) were added for a 15-min preincubation before insulin treatment. The *left* and *right panels* indicate experiments using di-C_8-PIP_3/AM and di-C_{12}-PIP_3/AM, respectively.

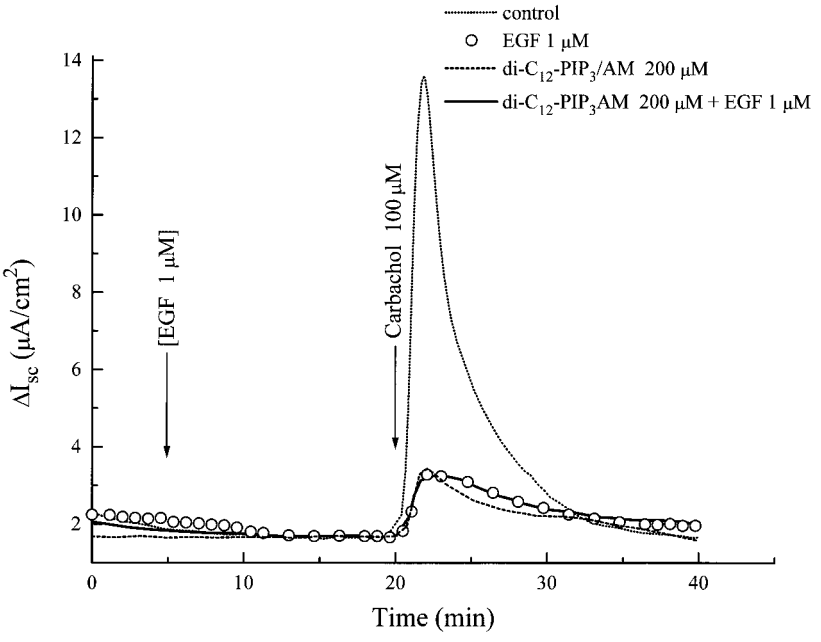

FIG. 4. **The inhibitory effects of EGF and PIP_3/AM on Cl^- secretion are nonadditive.** T_{84} monolayers were incubated for 30 min with 200 μM di-C_{12}-PIP_3/AM or vehicle prior to mounting in Ussing chambers. Chloride secretion was assessed as short circuit current (I_{sc}), which was measured continuously at 4-s intervals as described under "Materials and Methods." EGF (1 μM) was added to the basolateral surface of some chambers followed 15 min later by carbachol (100 μM). Controls were also stimulated with carbachol but not pretreated with EGF. Each *trace* represents the mean of six experiments.

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 PIP_3 is actually being delivered inside the cell. Probably the PIP_3/AM would have to be radiolabeled, which would be a significant synthetic challenge for the future, or better analytical methods to measure unlabeled PIP_3 would have to be developed.

Mechanism of Insulin Signaling in Adipocytes and Additional Signals Provided by Insulin Receptors—PIP_3/AMs of two different chain lengths were capable of partially overcoming the inhibitory effect of wortmannin on insulin-stimulated glucose transport. However, PIP_3/AMs alone did not stimulate basal glucose transport. Thus, PIP_3 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 PIP_3, 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 PIP_3 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 PIP_3 and is thus named PIP_3-dependent protein kinase-1 (38). PIP_3 acts both by activating PIP_3-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

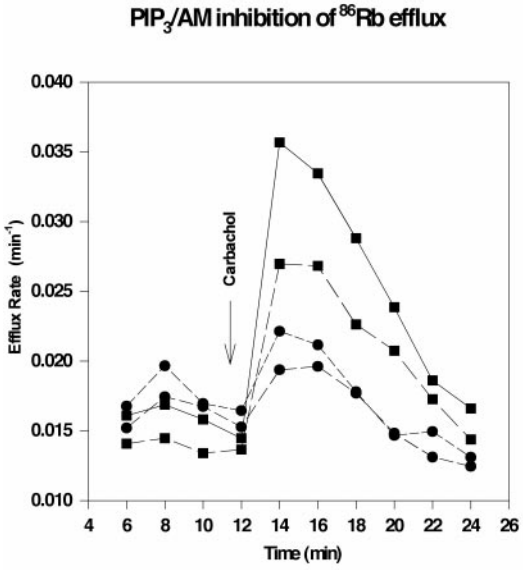

FIG. 5. **PIP_3/AM inhibits ^{86}Rb efflux from preloaded T_{84} colonic epithelia.** T_{84} monolayers grown on Millipore inserts were pretreated with either di-C_{12}-PIP_3/AM (200 μM, *circles*) or vehicle (*squares*) for 30 min during labeling. The two traces for each condition are replicate experiments. Monolayers were washed and the buffer in the basolateral reservoir was replaced every 2 min. Carbachol (100 μM) was added to the basolateral reservoir at the indicated time. Values are the calculated rate constants at the indicated time points for individual experiments.

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 PIP_3 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-LI adipocytes, and that overexpression of a kinase-dead PKC-ζ reduces the insulin-dependent stimulation of glucose transport (40). PIP_3 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 PIP_3/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 A_2 (42), and PI 4-kinase (43). This highlights the usefulness of PIP_3/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, PIP_3, or a metabolite thereof, participates in the stimulation of glucose transport by insulin; (*b*) PIP_3 is required but not sufficient to elicit full stimulation of glucose transport; and (*c*) a second insulin signal independent of PIP_3 participates in the stimulation of glucose transport.

EGF Signaling via PIP_3 in T_{84} Cells—Membrane permeant esters of PIP_3 mimic the inhibitory effects of EGF both on Cl^- secretion and efflux through K^+ channels. Moreover, the EGF- and PIP_3/AM-induced inhibitions, but not that due to carbachol, could be reversed by pretreatment with $Bt_2Ins(1,4,5,6)P_4$/AM (27). Although di-C_8- and di-C_{12}-PIP_3/AM had the same basic effects, the latter was somewhat more potent. We attempted to test whether PIP_3/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 PIP_3/AM dramatically reduced calcium-mediated chloride secretion in the presence of wortmannin,[2] consistent with actions of PIP_3/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 T_{84} colonic epithelia. However, the current studies do not exclude the possibility that $PI(3,4)P_2$ or other PIP_3 metabolite is the ultimate signal.

Acknowledgment—We thank Eleanor Wolfson for expert technical assistance.

REFERENCES

1. Toker, A., and Cantley, L. C. (1997) *Nature* **387,** 673–676
2. Sastry, J. K., Nehete, P. N., Khan, S., Nowak, B. J., Plunkett, W., Arlinghaus, R. B., and Farquhar, D. (1992) *Mol. Pharmacol.* **41,** 441–445
3. Freed, J. J., Farquhar, D., and Hompton, A. (1989) *Biochem. Pharmacol.* **38,** 3193–3198
4. Schultz, C., Vajanaphanich, M., Harootunian, A. T., Sammak, P. J., Barrett, K. E., and Tsien, R. Y. (1993) *J. Biol. Chem.* **268,** 6316–6322
5. Schultz, C., Vajanaphanich, M., Genieser, H. G., Jastorff, B., Barrett, K. E., and Tsien, R. Y. (1994) *Mol. Pharmacol.* **46,** 702–708
6. Vajanaphanich, M., Schultz, C., Rudolf, M. T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S. B., Tsien, R. Y., Barrett, K. E., and Traynor-Kaplan, A. E. (1994) *Nature* **371,** 711–714
7. Li, W., Schultz, C., Llopis, J., and Tsien, R. Y. (1997) *Tetrahedron* **53,** 12017–12040
8. Li, W., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R. Y. (1998) *Nature*, in press
9. Kahn, C. R. (1996) *Diabetes* **45,** 1644–1654
10. Cheatham, B., and Kahn, C. R. (1995) *Endocr. Rev.* **6,** 117–142
11. Yenush, L., and White, M. F. (1997) *BioEssays* **19,** 491–500
12. Myers, M. G., Jr., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354
13. Shoelson, S. E., Sivaraja, M., Williams, K. P., Hu, P., Schlessinger, J., and Weiss, M. A. (1993) *EMBO J.* **12,** 795–802
14. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3568–3573
15. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Biol. Cell* **14,** 4902–4911
16. Shepherd, P. R., Nave, B. T., and O'Rahilly, S. (1996) *J. Mol. Endocrinol.* **17,** 175–184
17. Downward, J. (1995) *Nature* **376,** 553–554
18. Farese, R. V. (1996) *Proc. Soc. Exp. Biol. Med.* **213,** 1–12
19. Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) *J. Biol. Chem.* **269,** 32358–32367
20. Weymer, A., Huott, P., Liu, W., McRoberts, J. A., and Dharmsathaphorn, K. (1985) *J. Clin. Invest.* **76,** 1828–1836
21. Dharmsathaphorn, K., Cohn, J., and Beuerlein, G. (1989) *Am. J. Physiol.* **256,** C1224–C1230
22. Cartwright, C. A., McRoberts, J. A., Mandel, K. G., and Dharmsathaphorn, K. (1985) *J. Clin. Invest.* **76,** 1837–1842
23. MacVinish, L. J., Pickles, R. J., and Cuthbert, A. W. (1993) *Br. J. Pharmacol.* **108,** 462–468
24. Vajanaphanich, M., Schultz, C., Tsien, R. Y., Traynor-Kaplan, A. E., Pandol, S. J., and Barrett, K. E. (1995) *J. Clin. Invest.* **96,** 386–393
25. Uribe, J. M., Keely, S. J., Traynor-Kaplan, A. E., and Barrett, K. E. (1996) *J. Biol. Chem.* **271,** 26588–26595
26. Barrett, K. E., Smitham, J., Traynor-Kaplan, A. E., and Uribe, J. M. (1998) *Am. J. Physiol.* **43,** C958–C965
27. Eckmann, L., Rudolf, M. T., Ptasznik, A., Schultz, C., Jiang, T., Wolfson, N., Tsien, R. Y., Fierer, J., Shears, S. B., Kagnoff, M. F., and Traynor-Kaplan, A. E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 14456–14460
28. Bruzik, K. S., and Tsai, M. D. (1992) *J. Am. Chem. Soc.* **114,** 6361–6374
29. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 247–254
30. Dharmsathaphorn, K., Mandel, K. G., McRoberts, J. A., Tisdale, L. D., and Masui, H. (1984) *Am. J. Physiol.* **264,** G204–G208
31. Venglarik, C. J., Bridges, R. J., and Frizzell, R. A. (1990) *Am. J. Physiol.* **259,** C358–C364
32. Reddy, K. K., Saady, M., Falck, J. R., and Whited, G. (1995) *J. Org. Chem.* **60,** 3385–3390

[2] A. Traynor-Kaplan, unpublished observations.

33. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) *Science* **275,** 665–668
34. Derman, M. P., Toker, A., Hartwig, J. H., Spokes, K., Falck, J. R., Chen, C. S., Cantley, L. C., and Cantley, L. G. (1997) *J. Biol. Chem.* **272,** 6465–6470
35. Coffer, P. J., and Woodgett, J. R. (1991) *Eur. J. Biochem.* **201,** 475–481
36. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) *EMBO J.* **14,** 4288–4295
37. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378
38. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol.* **7,** 261–269
39. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) *Science* **277,** 567–570
40. Bandyopadhyay, G., Sandaert, M. L., Zhao, L., Yu, B., Avignon, A., Galloway, L., Karnam, P., Moscat, J., and Farese, R. V. (1997) *J. Biol. Chem.* **272,** 2551–2558
41. Ferby, I. M., Waga, I., Hoshino, M., Kume, K., and Shimizu, T. (1996) *J. Biol. Chem.* **271,** 11684–11688
42. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995) *J. Biol. Chem.* **270,** 25352–25355
43. Balla, T., Downing, G. J., Jaffe, H., Kim, S., Zolyomi, A., and Catt, K. J. (1997) *J. Biol. Chem.* **272,** 18358–18366