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SHORT COMMUNICATION

Effects of a Water-Soluble Antitumor Ether Phosphonoinositide, D-*myo*-Inositol 4-(Hexadecyloxy)-3(*S*)-methoxybutanephosphonate (C_4-PI), on Inositol Lipid Metabolism in Breast Epithelial Cancer Cell Lines

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ABSTRACT. We have demonstrated previously that D-*myo*-inositol 4-(hexadecyloxy)-3(*S*)-methoxybutanephosphonate (C_4-PI), an isosteric phosphonate analog of phosphatidylinositol developed to inhibit inositol lipid metabolism, was unable to inhibit phosphatidylinositol (PI) 3-kinase activity. We now report the effects of the compound on other aspects of inositol metabolism. We demonstrated that C_4-PI inhibits the activity of purified recombinant PI-phospholipase C-β (PLC-β) at all concentrations tested; it enhanced the activity of PI-PLC-γ and PI-PLC-δ at low concentrations (10 μM), while severely inhibiting their activities at higher concentrations. In the breast cancer cell lines MCF-7 (estrogen receptor positive) and MDA-MB-468 (estrogen receptor negative), C_4-PI had no effect on the uptake of D-*myo*-inositol but severely inhibited its incorporation into PI. In spite of the drastic decrease in PI synthesis, C_4-PI did not affect the levels of inositol incorporated into phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cells. *In vitro* assays showed that C_4-PI inhibited PI synthase activity (inhibition of 35% at 50 μM) but had little effect on PI 4-kinase activity (inhibition of 13% at 150 μM). C_4-PI inhibited the proliferation of MCF-7 and MDA-MB-468 cell lines with IC_{50} values of 12 and 18 μM. Taken together, the results suggest that the accumulation of [^{3}H]inositol in PIP_2 in cells incubated with C_4-PI may be due to the inhibition of PIP_2 hydrolysis in the cells with no effect on its synthesis. The role of these C_4-PI-induced effects in the mechanism of growth inhibition by C_4-PI remains to be established. BIOCHEM PHARMACOL **57**;10:1153–1158, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. phosphonoinositide; ether lipids; phosphoinositide metabolism; phospholipase C

AELs[‖] are a novel class of non-DNA interacting compounds with antitumor properties that currently are under development and undergoing clinical trials [1, 2]. While their mechanism of action is still being debated, there is increasing evidence that perturbation of cell signaling pathways plays an important role in their cytostatic and cytotoxic activity [3]. Intracellular signaling pathways activated by second messengers derived from phosphoinositides are also thought to play important roles in regulating cell proliferation [4–6]. In light of the important role of phosphoinositides in cell signaling pathways, perturbation of pathways leading to their synthesis or inhibition of the second-messenger producing enzymes conceivably could result in inhibition of essential cellular events including proliferation. To target the inositol metabolic pathways more specifically with AELs, an inositol analogue of ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-glycerophosphoinositol (IP-10), was synthesized [7]. The compound was a weak inhibitor of PI 3-kinase activity [8], and was only able to inhibit the proliferation of leukemic and ovarian cancer cell lines at high concentrations [7]. A possible reason for the weak biological effect of IP-10 could be the presence of the C-3 phosphodiester bond that makes it susceptible to hydrolysis by phospholipase C, a situation that can be remedied by substituting a phosphonate bond. Nonhydrolyzable phosphonate analogues of phosphatidylinositol have been synthesized previously [9], but in these compounds the diacylglycerol moiety was replaced with simpler hydropho-

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‖ *Abbreviations*: AEL, antitumor ether lipid; C_4-PI, D-*myo*-inositol 4-(hexadecyloxy)-3(*S*)-methoxybutanephosphonate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-glycerophosphocholine; and C_4-PA, 4-*O*-hexadecyl-3(*S*)-methoxybutanephosphonic acid.

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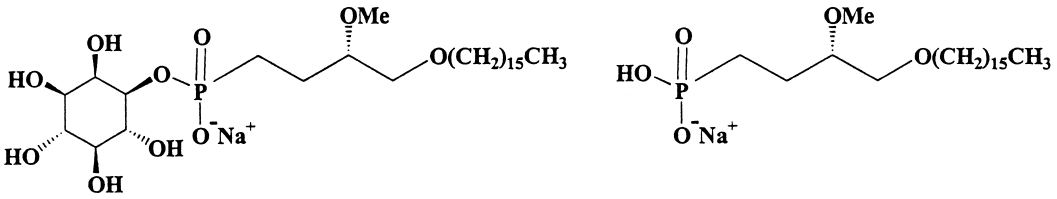

FIG. 1. Chemical structures of C_4-PI and C_4-PA.

bic groups bearing little resemblance to the naturally occurring molecule, and a protected racemic inositol derivative was used instead of D-*myo*-inositol. These inositol phosphonates were very poor inhibitors of *Bacillus cereus* PI-PLC, requiring millimolar concentrations to achieve modest inhibition [9], and their antiproliferative effects are unknown. Recently, more effective phosphonoinositide inhibitors of *B. cereus* PI-PLC were identified [10]; in these lipids, a D-*myo*-inositol headgroup is linked to a di-*O*-alkylglyceryl moiety in which the *sn*-3 oxygen atom is replaced by a methylene group. Moreover, the synthesis of a short-chain diester phosphonate analogue of PIP_2 has been reported [11], which may bind to mammalian PI-PLC enzyme. We report here that an isosteric phosphonate analog of PI, C_4-PI, which closely resembles the naturally occurring compound [12], inhibits [^{3}H]inositol incorporation into PI in intact cells and inhibits PI-PLC.

MATERIALS AND METHODS

Materials

C_4-PI [12] and C_4-PA [13] were synthesized as previously described. The structures of these phosphonates are displayed in Fig. 1. [^{3}H]PI was from Du Pont-New England Nuclear. [γ-^{32}P]ATP was obtained from ICN Biomedical, and [^{3}H]inositol was purchased from Amersham. PI, PIP, and PIP_2 were obtained from Boehringer Mannheim. All tissue culture media and reagents were obtained from Gibco BRL.

Cell Culture

MCF-7 and MDA-MB-468 cell lines from frozen stocks of cultures originally obtained from the American Type Culture Collection were grown in 10% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium. C_4-PI was dissolved in double-distilled water to give a stock solution of 30 mM, which was stored at $-20°$. To investigate the effects of C_4-PI on cell proliferation, C_4-PI (0–30 μM) was added in growth medium to proliferating cells in 24-well plates, and the increase in cell numbers over those at the time of the addition of the compound was determined after 48 hr and expressed as a percentage of the increase in controls [14]. Cell numbers were determined with a Coulter ZM counter.

Effect of C_4-PI on myo-*[^{3}H]Inositol Uptake and the Synthesis of Phosphoinositides*

Medium was removed from proliferating cells in 6-well plates and replaced with a medium containing 2 μCi/mL of [^{3}H]inositol with or without 30 μM C_4-PI, and the cells were incubated for 12 or 24 hr. After detachment with trypsin and washing twice with Hanks' balanced salt solution, the cells were resuspended in a known volume of Hanks' solution. Cells were dispersed using a 21-gauge needle, and aliquots were taken for the determination of cell number with a Coulter ZM counter. The remainder of the cells were pelleted, and lipids were extracted as previously described [15]. Authentic phosphoinositides were added as carriers, followed by separation by TLC on activated silica gel plates impregnated (Merck) with 1% potassium oxalate and 2 mM EDTA with a solvent system of chloroform:acetone:methanol:acetic acid:water (60:23:20:12:8, by vol.). The lipids were visualized by iodine staining. The inositol lipid bands were scraped, and the incorporated radioactivities were determined by scintillation counting.

For uptake studies, proliferating cells in 6-well plates were incubated with growth medium containing 1 μCi/mL of [^{3}H]inositol with or without 30 μM C_4-PI. After 12 hr, the cells were harvested and washed, and aliquots were taken for cell counting as described above. The remainder of the cells were pelleted and lysed in 2 mL of 1% SDS in 0.3 M NaOH, and the associated radioactivity was determined by scintillation counting.

Enzyme Assays

PI-PLC isozymes β-1, γ-1, and δ-1 were purified from HeLa cells transfected with recombinant vaccinia virus containing the entire coding sequence of each enzyme [16, 17]. The activities of the PLC isozymes were measured as previously described [18]. Briefly, the assays were performed in a reaction volume of 200 μL containing [^{3}H]PI (20,000 cpm/assay), 150 μM soybean PI, 3 mM $CaCl_2$, 2 mM EGTA, and 0.1% (w/v) sodium deoxycholate. C_4-PI was added to the assay at the required final concentration, and the reaction was started by the addition of 20 ng of the purified enzyme. The reaction was allowed to proceed at

37° for 5 min and was terminated as previously described [19].

PI synthase was assayed as previously described [20]. Microsomes were prepared from proliferating MCF-7 cells [21]. A standard assay mixture (100 μL) contained 100 mM glycylglycine/NaOH (pH 9.0), 0.2 mM CDP-diacylglycerol, 2 mM Triton X-100, 5 mM D-*myo*-[^{3}H]inositol (2 μCi/nmol), 2 mM $MnCl_2$, 50 mM $MgCl_2$, and 50 μg of MCF-7 microsomal protein. After a 10-min incubation period at 37°, the reaction was stopped by the addition of 2 mL of chloroform:methanol:10 M HCl (200:100:1, by vol.) followed by vigorous mixing. After centrifugation to separate the phases, 1 mL of the chloroform phase was removed into a vial, and the solvent was evaporated by air. Scintillant was added, and radioactivity was determined by scintillation counting.

PI 4-kinase was assayed in the presence or absence of C_4-PI with MCF-7 cell microsomal extracts as the source of both enzyme and substrate, essentially as described [22]. The reaction products were separated by TLC on oxalate-impregnated plates with chloroform:acetone:methanol:acetic acid:water (60:23:20:12:8, by vol.) as the developing solvent. Phosphorylated lipids identified by autoradiography were scraped and counted.

Analytical Procedures

The protein concentration of soluble extracts was determined by the Coomassie protein assay (Pierce); microsomal protein was determined by a modification [21] of the Lowry method.

RESULTS AND DISCUSSION

The results of studies on the effect of C_4-PI on the activity of purified recombinant PI-PLC β-1, γ-1, and δ-1 are displayed in Fig. 2A. At a C_4-PI concentration of 10 μM, the activities of the γ-1 and δ-1 isoforms were enhanced, whereas the activity of the β-1 isoform was inhibited slightly. At 30 μM, PI-PLC β-1 activity was inhibited about 84%, while the γ-1 and δ-1 isoforms were inhibited by 75 and 65%, respectively. Further decreases in the activities of each isoform were observed at a 100 μM concentration of the phosphonoinositide. The specificity of the inhibitory effects of C_4-PI was demonstrated by the observation that C_4-PA (Fig. 2B), an analogue of C_4-PI that lacks the inositol ring, slightly inhibited (15%) the β-1 isomer, had no effect on γ-1, and activated δ-1 (Fig. 2B). These results suggest that changes in the headgroups of the AELs could result in targeting different processes in cells that regulate cell growth. The ability of C_4-PI to inhibit the catalytic activity of representatives of all three isoforms of mammalian PI-PLC indicates its greater cytotoxicity over the phosphonates synthesized by Shashidhar *et al.* [9]. These differences in potency reflect the importance of utilizing groups that closely resemble the naturally occurring molecules. The reasons for the activation of the γ-1 and δ-1 PI-PLC isoforms by low concentrations of C_4-PI are not known, nor is the reason for the activation by all the tested concentrations of C_4-PA.

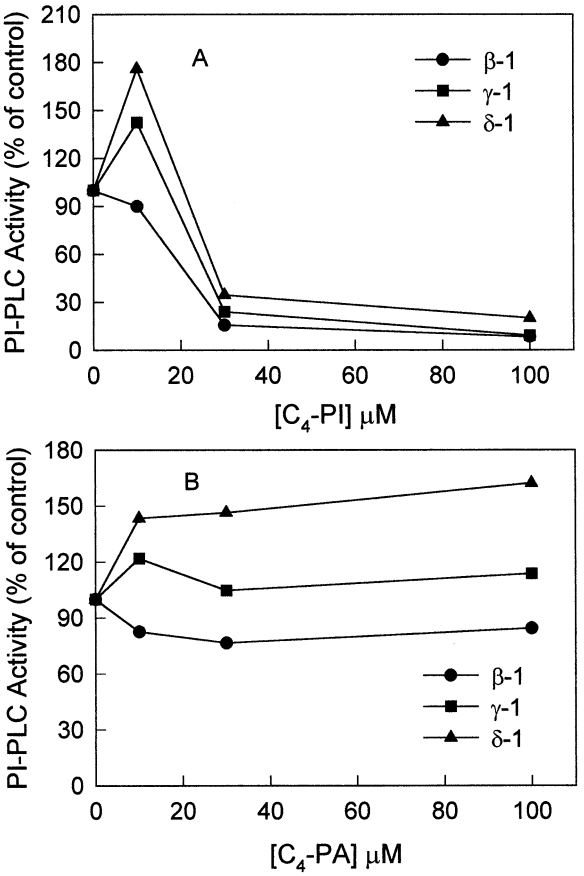

FIG. 2. Effects of C_4-PI and C_4-PA on the activity of PLC-β, PLC-γ, and PLC-δ. The activities of purified recombinant PLC-β1, PLC-γ1, and PLC-δ1 were assayed in the presence of various concentrations of C_4-PI (A) or C_4-PA (B). The results are expressed as a percentage of controls without the drugs and represent the average of quadruplicate determinations. The control values were 1300, 1900, and 2000 cpm for PLC-β1, PLC-γ1, and PLC-δ1, respectively.

Previous studies had indicated that AELs had little effect on the synthesis of inositol lipids. For example, hexadecylphosphocholine, the prototype alkylphosphocholine, severely inhibited (90%) the uptake of D-*myo*-inositol into a number of cancer cells without affecting the incorporation of label into the inositol lipids [23, 24], whereas ET-18-OCH_3, the prototype alkyllysophospholipid, also had no effect on the synthesis of PI in MCF-7 and A549 cells [21]. Since the rationale for synthesizing C_4-PI was to target inositol metabolism, we investigated its effects on the synthesis of inositol lipids in MCF-7 and MDA-MB-468 cell lines. Incubation with C_4-PI for 12 hr caused a large decrease in the quantity of label incorporated into PI. Figure 3 shows that inositol incorporation into PI was inhibited by 30% in MDA-MB-468 cells and by 70% in MCF-7 cells. After 24 hr of incubation with 30 μM C_4-PI, the decrease of inositol incorporation into the PI pool in

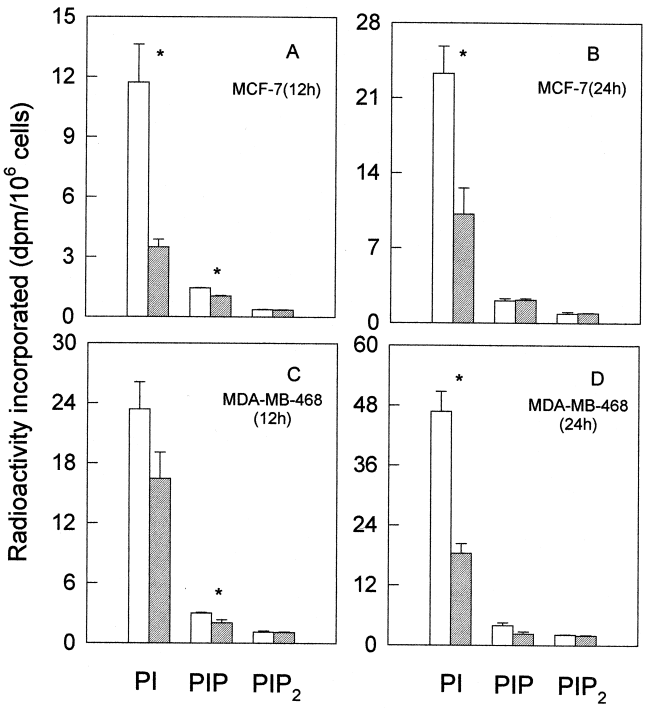

FIG. 3. Effects of C_4-PI on the incorporation of [^{3}H]inositol into phosphoinositides. Proliferating MCF-7 (A, B) and MDA-MB-468 (C, D) cells were incubated with [^{3}H]inositol in the presence (shaded bars) or absence (open bars) of 30 μM C_4-PI for 12 (A, C) or 24 (B, D) hr. The medium was removed, and the cells were washed and detached with trypsin. Aliquots were taken for determination of the cell numbers. Inositol lipids were extracted with organic solvents, as described in Materials and Methods, followed by the addition of authentic phosphoinositide standards. The phosphoinositides were separated by TLC on oxalate impregnated silica gel plates, the plates were visualized by I_2 vapor and scraped, and the associated radioactivity was determined. The data shown are the means ± SD of three different experiments. Key: (*) $P < 0.05$ compared with the corresponding controls (by Student's *t*-test).

both cell lines was still significantly different from controls. Examination of the effect of C_4-PI on the levels of inositol taken up revealed no differences in the uptake of inositol between the untreated and drug-treated cells (data not shown), indicating that the decreased PI synthesis was not due to decreased uptake of label.

As C_4-PI had no effect on the uptake of inositol but significantly decreased its incorporation into PI, we examined the possibility that the compound might inhibit the synthesis of PI via inhibition of PI synthase catalytic activity. The results showed that the addition of 10, 20, or 50 μM C_4-PI inhibited PI synthase activity by 14, 28, and 35%, respectively. The degree of inhibition obtained in these *in vitro* assays is not as high as might be expected given the severe inhibition of PI synthesis observed in the cells. The reason for this is unclear, but since C_4-PI is expected to accumulate in cell membranes, it may be able to interact more favourably with the membrane-bound PI synthase than with the solubilized enzyme in the *in vitro* aqueous-based assay that contained 2 mM detergent. Thus, the inhibition of PI synthesis by C_4-PI in the cells could be due to the inhibition of PI synthase by C_4-PI.

Surprisingly, the large drug-induced decrease in label incorporated into PI was not reflected in decreased incorporation of label into PIP_2 (Fig. 3), the direct precursor of cellular second messengers. To further analyze the results, the distribution of label in PI, PIP, and PIP_2 was expressed as a percentage of the total label in the phosphoinositide fraction (Table 1). The results showed that after 24 hr of incubation with 30 μM C_4-PI there was an increase in the proportion of label accumulating in the PIP_2 fraction in both cell types. Thus, C_4-PI had caused a redistribution of the label such that there was a decrease in the proportion of [^{3}H]inositol in PI but significant elevation in the proportion in PIP_2. This redistribution was apparent in MCF-7 even after 12 hr but was less evident in MDA-MB-468. These results are consistent with C_4-PI having minimal effects on PI 4-kinase and PIP 5-kinase while inhibiting PI-PLC activity. *In vitro* studies confirmed that C_4-PI had very little effect on PI 4-kinase activity. The assay of PI 4-kinase in the presence of 150 μM C_4-PI demonstrated that activity was inhibited by only 13%. As discussed earlier, C_4-PI did inhibit the activity of all isoforms of PI-PLC.

Examination of the effects of C_4-PI on the proliferation of MCF-7 and MDA-MB-468 cell lines revealed that C_4-PI inhibited the proliferation of both breast cancer cell lines (Fig. 4) with IC_{50} values of 12 and 18 μM, respectively.

TABLE 1. Effect of C_4-PI on the distribution of [^{3}H]inositol in phosphoinositides

		% Distribution of radioactivity			
		12 hr		24 hr	
Cell line	Phosphoinositide	Control	+ C_4-PI	Control	+ C_4-PI
MCF-7	PI	86.4 ± 1.3	71.2 ± 2.8	88.7 ± 1.0	76.2 ± 4.5
	PIP	10.8 ± 0.9	21.6 ± 2.0	7.9 ± 0.5	16.6 ± 3.2
	PIP_2	2.8 ± 0.5	7.3 ± 0.8	3.3 ± 0.5	7.2 ± 1.3
MDA-MB-468	PI	84.9 ± 1.8	84.0 ± 1.2	88.6 ± 0.5	81.4 ± 1.4
	PIP	11.0 ± 1.2	10.4 ± 0.5	7.5 ± 0.6	10.0 ± 1.6
	PIP_2	4.0 ± 0.6	5.7 ± 0.9	3.9 ± 0.3	8.6 ± 0.5

The radioactivity in PI, PIP, and PIP_2 in the experiments described in Fig. 3 is expressed as a percentage of the total radioactivity in all three phosphoinositides. Data are means ± SD of three independent experiments.

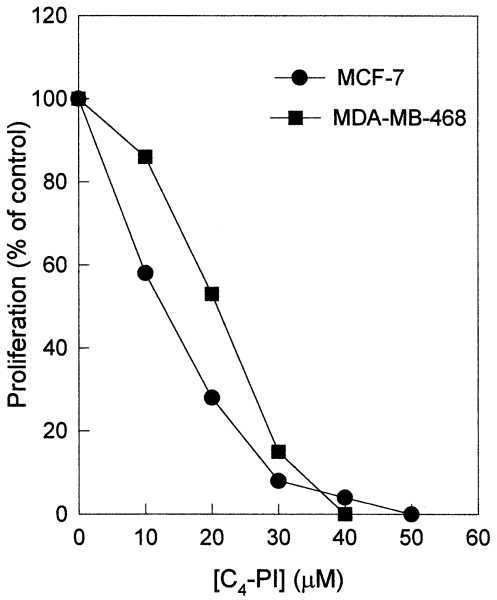

FIG. 4. Inhibition of cell proliferation by C_4-PI. Proliferating cells growing in 24-well plates were incubated with 10% FBS-supplemented medium containing C_4-PI (0–60 μM). The cells in representative wells were counted on day 0 prior to the addition of the compound. Forty-eight hours after the incubation with C_4-PI, the cells were detached and the cell numbers were determined by electronic counting. The increase in cell numbers over day 0 for each concentration was expressed as a percentage of that in control wells that did not receive the compound. Results are the means of three different experiments with quadruplicate wells in each experiment. The standard deviation from the means was less than 9%. The numbers of cells on day 0, prior to the addition of C_4-PI, were 85,000 ± 3,500 for MCF-7 and 97,000 ± 10,000 for MDA-MB-468. After 48 hr of incubation, the final cell numbers in the controls were 184,000 ± 20,000 for MCF-7 and 213,000 ± 14,000 for MDA-MB-468.

Cells treated with C_4-PI concentrations as high as 40 μM did not round up or detach from the wells; they were morphologically similar to the controls. While it is tempting to speculate that the inhibition of enzymes participating in inositol lipid metabolism, PI-PLC and PI synthase, is responsible for the inhibition of cell growth, one would need to establish a key role of inositol metabolites in these cells as well as to demonstrate that inhibitory concentrations of C_4-PI in quiescent cells inhibit the generation of inositol phosphates. In addition, since C_4-PI is phosphorylated by PI 3-kinase [12] and PI 4-kinase (Richard C and Arthur G, unpublished observations), one would also need to demonstrate that phosphorylated products of C_4-PI do not perturb signaling pathways activated by the generation of naturally occurring phosphoinositide metabolites in cells. We would also like to point out that we have not excluded the possibility that the mechanism of inhibition of cell proliferation by C_4-PI could be unrelated to the effects of the compound on inositol lipid metabolism.

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