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SELECTIVE INHIBITION OF PHOSPHATIDYLINOSITOL 3-KINASE BY PHOSPHATIDIC ACID AND RELATED LIPIDS

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Activation of phosphatidylinositol 3-kinase (PI 3-kinase) is necessary for stimulation of cell division and inhibition of apoptosis in several cell types. We report that a synthetic phosphonolipid, 4-(hexadecyloxy)-3-(S)-methoxybutyl phosphonic acid (PoA),as well as the naturally occurring lipids, phosphatidic acid and lyso-phosphatidic acid,are potent and specific inhibitors of PI 3-kinase. The IC_{50}'s for inhibition using phosphatidylinositol as substrate ranged from 10-20 μM. PoA is also the putative primary intracellular metabolite following phospholipase D hydrolysis of the anti-tumour ether lipid, 2'-(trimethylammonio) ethyl-4-(hexadecyloxy)-3-(S)-methoxybutanephosphonate. These results suggest that inhibition of PI 3-kinase following metabolic degradation of ether lipids by phospholipase D may contribute to the cytotoxicity of these compounds. The sensitivity of PI 3-kinase to PA and lyso-PA could imply cross-talk between the phospholipase D and PI 3-kinase signal transduction pathways in vivo.

PI 3-kinase is a heterodimeric lipid kinase that phosphorylates the 3'-position of phosphoinositides resulting in the formation of PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3 [1]. These lipids are not substrates for the classical phospholipases C and are thought to act as second messengers themselves (especially PI 3,4,5 -P_3) [2]. PI 3-kinase consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. Activation of PI 3-kinase and translocation to the plasma membrane is necessary for mitogenesis induced by most growth factors and for cellular neoplastic

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The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; PoA, 4-(hexadecyloxy)-3-(S)-methoxybutyl phosphonic acid; Et-16-OCH_3-PoC, 2'-(trimethylammonio) ethyl-4-(hexadecyloxy)-3-(S)-methoxybutanephosphonate; ET-18-OCH_3, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; TLC, thin-layer chromatography; PI, phosphatidylinositol; PI-3-P, phosphatidylinositol 3-monophosphate; PI-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PI-3,4,5 P_3, phosphatidylinositol 3,4,5-trisphosphate; PA, phosphatidic acid; lyso-PA, lyso phosphatidic acid; PC, phosphatidylcholine.

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transformation by oncogenes of the tyrosine kinase family [3-4]. In addition to mediation of proliferative signals, PI 3-kinase may also be involved in transmission of intracellular signals that prevent programmed cell death (apoptosis). Recently it was shown in NGF stimulated rat pheochromocytoma PC-12 cells that PI 3-kinase activation is necessary for cell survival [5]. Data from our laboratory using hemopoietic cells also supports a role for PI 3-kinase in inhibition of apoptosis by cytokines [M.P. Scheid, R.W. Lauener, and V. Duronio, Biochem. J., in press]. Therefore, PI 3-kinase plays a dual role in cellular growth and transformation by providing signals for both cell division and cell survival. PI 3-kinase exists in several isoforms which may be differentially regulated by protein-tyrosine kinases (via SH2 domains of the p85 subunit), G-protein $\beta\gamma$ subunits, p21 ras and SH3-poly-proline protein-protein interactions [6-9]. The lipid products of PI 3-kinase directly activate the PKC isoforms ε, δ, and η in vitro, hence PKC may be an immediate downstream target of second messengers resulting from PI 3-kinase activation [10].

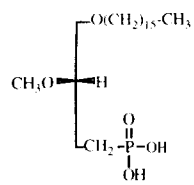
Recently it was shown that the anti-tumour ether lipids, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3) and hexadecylphosphocholine inhibit PI 3-kinase in vitro with IC_{50}'s of 36 μM and 48 μM respectively [11]. Also, in this report, PI (3,4,5)-P_3 formation in PDGF stimulated v-sis NIH 3T3 cells was inhibited by ET-18-OCH_3 with an IC_{50} of 12.5 μM. This discrepancy in IC_{50} values for ET-18-OCH_3 between purified enzyme and whole cells was not addressed.

Here we report, for the first time, that a phospholipid analog, PoA (4-(hexadecyloxy)-3-(S)-methoxy-butylphosphonic acid) as well as the naturally occurring PA and lyso-PA are potent and selective inhibitors of PI 3-kinase. These results suggest that PI 3-kinase may be a target of the anti-tumour ether lipids after metabolic (phospholipase D) conversion of the lipid to the corresponding glycero-phosphates. The possibility of cross-talk between the PI 3-kinase and PLD signalling pathways may be inferred from these data with lyso-PA/PA acting as endogenous inhibitors of PI 3-kinase.

Experimental

Reagents

PoA (Scheme 1) and ET-16-OCH_3-PoC [for synthesis see reference 12] were obtained from Inflazyme Pharmaceuticals (Vancouver, B.C., Canada). Protein A-Sepharose was from Pharmacia. [^{32}P]ATP (6000 Ci/mmole) was from NEN/Dupont. Wortmannin, PC, glycerol, glycerol 2, and 3-phosphates and diacylglycerol were from Sigma. PA and lyso-PA were from Avanti Polar Lipids. TLC plates (Si 60) were from E. Merck (Germany). Anti-PI 3-kinase (p85 subunit) antiserum was from Upstate Biotechnology Inc. (Lake Placid, NY).



Scheme 1. Structure of PoA.

Preparation of anti-p85 immunoprecipitates

Rabbit platelets were used as a source of PI 3-kinase. Platelets were isolated from whole rabbit blood anti-coagulated with citrate-dextrose as described elsewhere [13]. A total detergent lysate was prepared by solubilizing the platelets in Triton X-100 lysis buffer (1 % Triton X-100, 20 mM TrisHCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml aprotinin) for 1 hr on ice. In a batchwise fashion, platelet lysate (100 µg total protein) and 0.5 µl anti-p85 antiserum per assay tube were incubated with mixing for 3 hr at 4 °C, then the immune complexes collected using 50 µl of protein A-Sepharose suspension (50 % v/v). After thorough washing, the beads were re-suspended to 50 % v/v in 10 mM Tris HCl, pH 7.4 then 50 µl of the suspension aliquoted per reaction tube.

In vitro PI 3-kinase assay

PI 3-kinase activity, measured by phosphorylation of PI, was determined exactly as described [14]. Reactions were for 15 minutes at room temperature (linear range, data not shown). Test compounds (sonicated in 10 µl 30 mM Hepes pH 7.4) were incubated for 10 minutes at room temperature with the immunoprecipitates prior to addition of PI.

Effect of PoA on other signaling enzymes

Protein kinase C was assayed as previously described using a mixture of PKC isoforms and myelin basic protein (MBP) as substrate [15]. In vitro protein tyrosine kinase was assayed using recombinant *lck* ($p60^{c\text{-}lck}$)kinase and MBP as substrate [16]. PI-PLC activity was determined as the hydrolysis of [^{3}H]PIP_2 by 20 µg rabbit platelet cytosolic fraction [17]. PI 4-kinase was assayed by quantitating the phosphorylation of exogenous PI by rabbit platelet cytosolic fraction in the presence of 100 nM wortmannin; a modification of a previously described method [18].

Results and discussion

In an in vitro PI 3-kinase assay using PI as substrate, PoA, lyso-PA and PA inhibited formation of phosphatidylinositol 3-phosphate (PI-3-P) in a concentration dependent manner. A representative autoradiogram from an experiment utilizing PoA is shown in Figure 1. The IC_{50}'s for the three lipids ranged from 10-20 µM (Figure 2). Thus, PoA, along with the naturally occurring PA and lyso-PA represent the most potent lipid inhibitors of PI 3-kinase yet found.

Several compounds containing a glycerol backbone, including PoA, lyso-PA and PA were compared for their ability to inhibit PI 3-kinase in order to determine the minimal structural requirements for the inhibitory effect. Wortmannin (100 nM), a known inhibitor of PI 3-kinase [19], was used as a positive control. None of the lipid (phosphatidylcholine or ET-16-OCH_3-PoC) or non-lipid (glycerol, glycerol 2-phosphate, L- and D, L-glycerol 3-phosphate or diacylglycerol) glycerol derivatives tested significantly inhibited PI 3-kinase at concentrations (200 µM) where PA or its analogues inhibited the enzyme by more than 95 % (Figure 3). Therefore, from this analysis it is concluded that for inhibition of PI 3-kinase by this class of lipids, a free phosphate/phosphonate group is necessary at the sn-3 position; removal of the phosphate (i.e. diacylglycerol) or esterification with choline (i.e. ET-16-OCH_3-PoC or PC) results in loss of activity. At least one long chain alkyl substituent is necessary at the sn-1 or sn-2 position; removal (i.e. glycerol 2- or 3-phosphates) also results in loss of activity. The inhibitory effect is not particularly sensitive to substituent length at the sn-2 position as PoA, PA and lyso- PA are approximately equipotent.

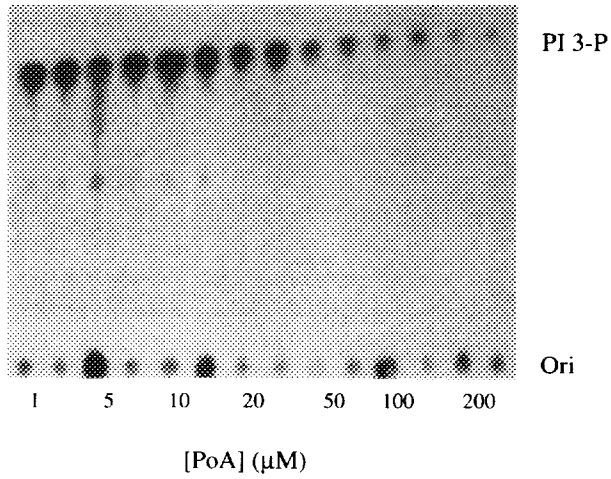


Figure 1. Concentration-dependent inhibition of PI 3-kinase by PoA. Various concentrations of PoA were pre-incubated with the anti-p85 (PI 3-kinase) immunoprecipitates for 10 min. followed by addition of substrate then PI 3-kinase activity was determined as described in Experimental. Representative autoradiogram of TLC plate after exposure to X-ray film for 24 hrs. at -80 °C. Data are from a single experiment in duplicate with similar results obtained on at least three separate occasions.

We also found that an isostere of ET-16-OCH_3-PoC, ET-16-OCH_3-GPC, did not inhibit PI 3-kinase at concentrations up to 200 μM (data not shown). This is in contrast to a recent report indicating that a closely related compound, ET-18-OCH_3-GPC, inhibits PI 3-kinase with an IC_{50} of 36 μM [11]. The reasons for this discrepancy are not known but it could be due to differences in assay conditions from those used in that study (which were not described in detail).

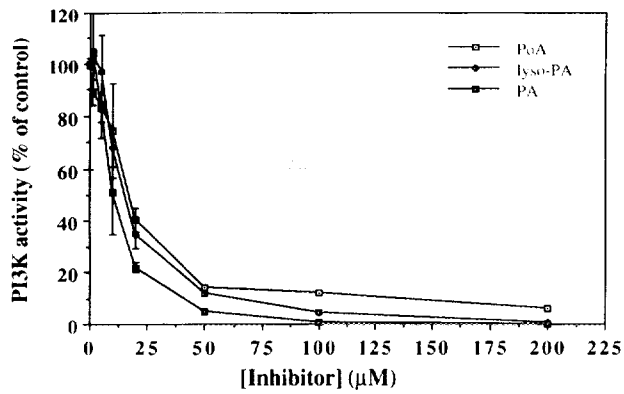


Figure 2. Determination of IC_{50}'s for inhibition of PI 3-kinase by PoA, lyso-PA and PA. PI-3-P spots were excised from the plate (see Figure 1) and radioactivity was quantitated by liquid scintillation counting. PI-3-P count in the control was 2500 cpm. Data are from a single experiment in triplicate (± S.E.M.) representative of at least two separate experiments.

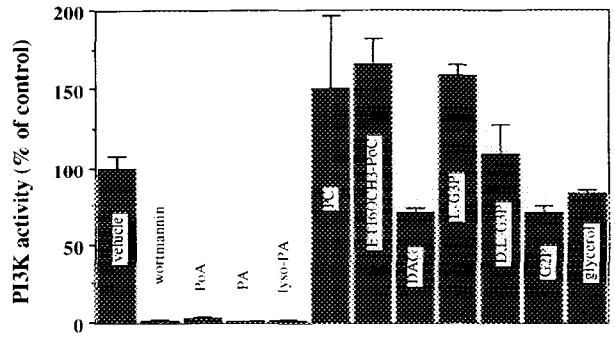


Figure 3. Effect of various glycerol derivatives, including PoA, lyso-PA and PA, on PI 3-kinase. Compounds at 200 µM were compared for their ability to inhibit PI 3-kinase activity. All compounds were sonicated for 1 minute prior to inclusion in the assay. Wortmannin (100 nM) was used as a positive control. PI 3-kinase activity is expressed as percentage relative to control (vehicle only). Data are from a single experiment in triplicate (+/- S.E.M.) representative of three similar experiments.

The ability of PoA and PA to inhibit several other important signaling enzymes was assessed (Figure 4). Neither PKC, *lck* tyrosine kinase, PI-PLC or PI 4-kinase was significantly inhibited in vitro at concentrations of PoA or PA (200 µM) which completely (>95%) inhibited PI 3-kinase. Thus PoA and PA are highly specific for PI 3-kinase.

To the best of our knowledge this is the first report describing inhibition of PI 3-kinase by phosphatidic acid and related lipids. The ramifications of this novel activity displayed by phosphatidic acid and analogues are numerous. Ether lipids targeting signal transduction pathways, either directly or after metabolism, is likely an important component of their selective cytotoxicity toward tumour cells [20]. From these data we suggest that a significant proportion of

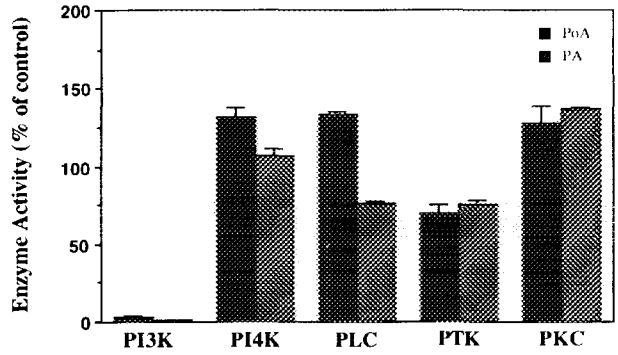


Figure 4. Effect of PoA and PA on other enzymes involved in signal transduction. PI-PLC (phosphoinositide-specific phospholipase C), PKC (protein kinase C), PI 4-kinase (phosphatidylinositol 4-kinase) and lck protein tyrosine kinase were compared with PI 3-kinase for their sensitivity to inhibition by PoA and PA. The lipids were used at 200 µM. The values represent the percentage activity of each enzyme remaining in the presence of the test compound relative to control (vehicle). At 200 µM, 2.81 +/- 1.0 % and 0.95 +/- 0.3 % PI 3-kinase activity remained compared to control for PoA and PA, respectively. Data are from a single experiment in triplicate (+/- S.E.M.)

the cytotoxicity of ether lipids may be due to intracellular cleavage by phospholipase D followed by inhibition of PI 3-kinase. Metabolism of ether lipids to the corresponding glycero-phosphates by phospholipase D has been demonstrated in several cell and tissue types [21]. The capacity of cells to metabolize ether-linked lipids via phospholipase D may then dictate their sensitivity to these compounds. The phosphonate analogues of ether lipids (e.g. ET-16-OCH3-PoC) may be more effective as cytotoxic antit-tumour compounds due to their resistance to phospholipase C degradation [22-23].

It is tempting to speculate that there is cross-talk between the phospholipase D and PI 3-kinase pathways in-vivo since, as demonstrated here, the PLD products PA and lyso-PA inhibit PI 3-kinase in vitro. Verification of this hypothesis will require demonstration of these effects in whole cells by measuring the 3'-phosphoinositide products of PI 3-kinase and correlating with PLD activation.

Acknowledgments

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