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Activation of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate

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SUMMARY: Phosphatidylinositol 3-kinase (PI 3-kinase) was partially purified from rat liver cytosol and used to synthesize phosphatidylinositol 3,4,5-trisphosphate (PIP_3), using phosphatidylinositol 4,5-bisphosphate (PIP_2) as a substrate. Purified PIP_3 (free of chromatographic oxalate) activated protein kinase C (PKC) in the presence of phosphatidylserine and calcium (PKC -cofactors) in a concentration-dependent manner. In the absence of these cofactors, effect of PIP_3 was not observed. Comparison of the effects of PIP_3 and PIP_2 on PKC activity indicates that PIP_3 is a more potent PKC-activator than PIP_2. The affinity of PKC to PIP_3 was 4 fold higher than that to PIP_2 (K_{PIP3} = 0.022 and K_{PIP2} = 0.087 mol %), while its maximal velocity (V_{max}) was similar to that of PIP_2-stimulated PKC activity (0.4 - 0.5 μmol/mg/min). These results suggest a physiological role for PIP_3 in signal transduction, and support the previous finding (Chauhan et al. (1991) Arch. Biochem. Biophys. 287,283) that PKC-activation by phosphoinositides increases with the state of phosphorylation of these lipids. We propose that PIP_3 by activating PKC may initiate a cascade of events from PIP_3 → PKC-activation → effects on other protein kinases such as MAP-kinase → gene expression.

Phosphoinositides play an important role in signal transduction, synaptic transmission, and other cellular functions (1). There are three major inositol containing lipids: phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 is formed from PI by the sequential action of PI 4-kinase (which forms PIP) and PI 5-kinase. Recently, a phosphatidylinositol 3-kinase (PI 3-

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Abbreviations: PKC, protein kinase C; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, diacylglcerol; PS, phosphatidylserine; MAP-kinase, mitogen activated protein kinase.

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kinase) that phosphorylates phosphoinositides at D-3 position of the inositol ring has been implicated in signal transduction in several cell types (2-5). As a result, the novel lipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is produced from PIP_2. The discovery of PI 3-kinase has uncovered a new pathway of phosphoinositide metabolism with potential intracellular signals. The likely importance of PI 3-kinase in cell signalling is emphasized by the fact that levels of PIP_3 are substantially increased in response to the activation of a variety of cellular tyrosine kinases. These encompass growth factor receptor tyrosine kinases, including the insulin receptor whose signal transduction mechanism has been particularly elusive, and virally encoded proteins whose expression raises protein phosphotyrosine levels and leads to cell transformation (6,7). Thus PI 3-kinase has now emerged as one of the molecular bases of signal transduction through tyrosine phosphorylation.

The involvement of phosphoinositides in the regulation of protein kinase C, a calcium/phospholipid-dependent protein phosphorylating enzyme, is widely accepted. PIP_2 plays a dual role in signal transduction: 1) It gives rise to two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3), a calcium mobilizer; and diacylglycerol (DG), a PKC-activator, generated as a result of receptor-mediated hydrolysis of PIP_2 by phospholipase C. 2) PIP_2 is a potent activator of PKC in presence of phosphatidylserine (PS) and Ca^{2+} (8), as demonstrated by extensive evidence from this laboratory (9-13) and confirmed by others (14,15). We reported that PIP_2 activates PKC in the manner of DG and phorbolesters by increasing the affinity of the kinase for Ca^{2+} (11) and stimulating the translocation of the kinase from soluble to particulate fraction (11). The two activated forms, DG•PKC and PIP_2•PKC, differed in affinity (K_{PIP2} is 50 times smaller than K_{DG}) (8), maximal velocity (V_{DG}/V_{PIP2} = 2.5 at physiological pH, although at alkaline pH, Vmax of PIP_2•PKC and DG•PKC were nearly identical) (13), and pH dependence (pH at half-activation, i.e., pK for DG•PKC is 6.2, and PIP_2•PKC is 7.7) (13). Apart from these kinetic variations, PKC activated by DG or PIP_2 behave remarkably similar. We have suggested that PIP_2-activated PKC may be involved in the down-regulation of the Na^+/H^+ antiport and in cellular proliferation (13).

While the role of PIP_2 is well characterized, the physiological significance of PIP_3 is not yet known, although it is commonly accepted that it is a signal molecule. PIP_3 is certainly not

involved in the production of IP_4, another intracellular messenger, since it not susceptible to phosphodiesteric cleavage (16). We have previously suggested that PKC activation by phosphoinositides depends on the phosphorylation status of these lipids. This is based on the finding that PI is not an activator of PKC; PIP, a weak activator; and PIP_2, a potent PKC-activator (8,11). In the present study, we investigated if PIP_3 can also act as a PKC-effector like PIP_2, or alternatively be a product of PKC·PIP_2-deactivation similar to the IP_3-mediated Ca^{2+}-mobilization and its inactivation by IP_4 formation (17). We report here that PIP_3 activates PKC more efficiently than PIP_2 (K_{PIP2}/K_{PIP3} = 3.9).

MATERIALS AND METHODS

Materials. Phenylsepharose 4B, mono Q and Sephacryl S-200 were purchased from Pharmacia; Triton X-100 from Aldrich. GF/C filters, Partisil 10 Sax, and DE 52 from Whatman. Calf thymus histone type III-S, leupeptin, phenylmethanesulphonyl fluoride, bovine serum albumin, poly-L-Lysine agarose, PS, PIP_2 and DG were purchased from Sigma. The purity of lipids was confirmed by thin layer chromatography (TLC). [γ-^{32}P] ATP (3000Ci/mmol) was procured from New England Nuclear, and Ecoscint A from National Diagnostics.

Purification of protein kinase C. PKC was purified from rats brains by the method of Huang et al (18) using DE 52, phenylsepharose 4B, sephacryl S-200 and polylysine agarose column chromatography. The final preparation, a mixture of α, ß, and γ PKC isozymes, was concentrated by Amicon ultrafiltration, mixed with equal volume of glycerol and stored at -20° C. The specific activity of the enzyme was 0.8 μmol/mg/min at 37°C in a mixed micellar assay (19) with Triton X-100, 0.3%; PS, 9 mol%; DG, 2 mol%; Ca^{2+}, 100 μM, using histone III-S as a substrate.

Micellar assay of PKC activity. Triton X-100 micelles containing PS, PS/DG, PS/PIP_2 or PS/PIP_3 were prepared as described previously (11). Reaction mixtures containing 20 mM Tris-HCl, pH 7.5; lipid micelles; 10 μg of leupeptin; 10 mM $MgCl_2$; 100 μM Ca^{2+}; 80 μg histone III-S; 12.5 μM [γ-^{32}P] ATP in a total volume of 100 μl were incubated at 37°C for 1 minute. The reaction was started by the addition of PKC and terminated after 3 min with 1 ml of ice-cold 20% trichloroacetic acid (TCA) followed by addition of 10 μl of albumin (20 mg/ml). The resulting precipitate was collected on a 2.5 cm GF/C filter, washed 6 times with 1 ml of ice-cold 20% TCA, and counted in 10 ml of Ecoscint A.

Purification of PI 3-kinase. The enzyme was partially purified by the method of Carpenter et al. (20). In brief, rat liver cytosol was subjected to acid-precipitation followed by chromatography on DEAE-Sepharose, S-Sepharose and mono Q.

PI 3-kinase assay. Activity of PI -3 kinase was measured by the method of Carpenter et al. (20). PIP_2/PS (50:50 mol %) liposomes were prepared by evaporating PIP_2 in chloroform/methanol/water (14/7/1) and PS in chloroform/methanol (2/1) under argon, and resuspending the lipid film in 20 mM Tris-HCl containing 1 mM EDTA, pH 7.2, followed by sonication for 5 min under argon atmosphere.

Assay mixtures (25 μl) contained 2 mM $MgCl_2$; 50 μM [γ-^{32}P] ATP; 0.25 mM EDTA; 20 mM Tris-HCl, pH 7.2; 200 μM PIP_2/PS (1:1) liposomes. The reaction was started with PI 3-kinase, and stopped by addition of 105 μl of 1 N HCl, followed by 160 μl of

chloroform/methanol (1:1). Samples were vortexed vigorously and centrifuged to separate aqueous and organic phase. The organic phase was then transferred into a scintillation vial, dried, and counted for radioactive PIP_3.

Isolation of PIP_3. In another experiment, PIP_3 was isolated from the organic phase by TLC using 1% oxalated silica 60 plates and 2-propanol:2 M acetic acid (65:35, V/V) as solvent system. PI, PIP and PIP_2 were used as standard markers and visualized by iodine vapors. The PIP_3 spot which always ran behind PIP_2 spot was identified by autoradiography. PIP_3 was also characterized by deacylation (21) and deglyceration (22) procedure using partisil 10 Sax HPLC column. Initially, we extracted PIP_3 from TLC plates with chloroform:methanol: H_2O (14:7:1, V/V), and found that oxalic acid from oxalated TLC plates coeluted with PIP_3. In order to obtain oxalic acid free PIP_3, it was necessary to partition the extraction solvent into aqueous and organic phase. The PIP_3 spot was scrapped and suspended in 1.05 ml of 1 N HCl, followed by the addition of 1.6 ml of chloroform:methanol (1:1, V/V). The sample was vortexed vigorously and centrifuged to separate organic phase from aqueous phase. The organic phase containing PIP_3 was carefully removed and passed through 0.22 μm millipore filter to remove silica particles. In most experiments, freshly prepared PIP_3 was used.

RESULTS AND DISCUSSION

For the initial experiments, PIP_3 was extracted from oxalated TLC plates by chloroform: methanol:water (14:7:1, V/V). When this PIP_3 preparation was used for PKC assay, it inhibited PKC in concentration-dependent manner (data not shown). Testing the solvent system used for the extraction of PIP_3 from TLC plates showed that we had extracted oxalic acid along with PIP_3. To check out if the observed PKC-inhibitory effect was due to oxalic acid or to PIP_3, we studied the effect of oxalic acid on PKC-mediated histone phosphorylation (Fig. 1). Oxalic acid was indeed found to inhibit PKC activity in a concentration-dependent manner. Therefore, for further experiments PIP_3 and oxalic acid were separated by partitioning the extract into water and organic phases. The organic phase containing PIP_3 was dried and used for its effect on PKC activity. As shown in Fig.2, purified PIP_3 completely free of oxalic acid activated PKC in presence of the cofactors (PS and Ca^{2+}). PIP_3 alone, i.e., in the absence of PS and Ca^{2+}, did not have any effect on the kinase. While our work was in progress, it was reported (23) that PIP_3 can stimulate the activity of the PKC ζ isozyme, but not the conventional PKCs, i.e., α, β, and γ isozymes. Our results, on the other hand, clearly demonstrate that PIP_3 is a very potent activator of PKC (the mixture of α, β and γ isozymes). The reason for this discrepancy is unknown but the possibility exists that in the contrary report (23) a contamination of PIP_3 with oxalic acid may have counteracted the PIP_3-mediated activation of PKC.

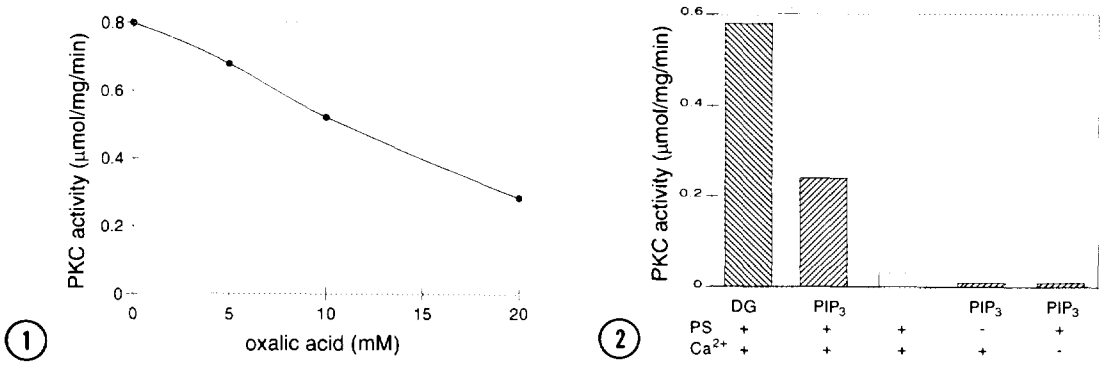

Fig. 1. Inhibition of protein kinase C activity by oxalic acid. DG-stimulated protein kinase C activity was measured in presence of different concentrations of oxalic acid using mixed micellar assay (19). The concentrationsof lipids in the reaction (with respect to Triton X-100) were: PS 9 mol % or 445 μM; DG, 2 mol % or 99 μM.

Fig. 2. Effect of PIP_3 and diacylglycerol on PKC activity. PKC-mediated histone III-S phosphorylation was measured by mixed micellar assay (19). Total reaction volume (0.1 ml) contained 20 mm Tris-HCl, pH 7.5, 80 μg histone III-S, 10 μg leupeptin, 12.5 μM ATP, 10 mM $MgCl_2$, 25 μM EDTA, 25 μM EGTA, 100 μM Ca^{2+} (where indicated), and 10 μl of lipid micelles. The lipids in the reaction mixture (with respect to Triton X-100) were: DG, 1 mol % or 49.5 μM; PIP_3, 0.032 mol % or 1.58 μM, and PS 9 mol % or 445 μM (where indicated).

We have reported earlier that PIP_2 is a potent activator of PKC in the presence of PS and Ca^{2+} (8). This finding was further supported by several reports from this laboratory (9-13) and other workers (14,15). A Lineweaver-Burk plot of 1/PKC activity vs 1/phosphoinositide concentration (in mole % of Triton X-100) from the present study shows that the affinity of PKC to PIP_3 is 4 times higher than that of PIP_2, i.e., (K_{PIP2}/K_{PIP3} = 3.9), but the maximal velocity (Vmax) of PIP_3•PKC (0.4 μmol/mg/min) is essentially similar to that of PIP_2•PKC (0.5 μmol/mg/min) (Fig. 3). We suggested earlier that activation of PKC by phosphoinositides depends on the phosphorylation status of these molecules based on our previous results that activation is in the order PIP_2 > PIP > PI (11). This hypothesis is further strengthened by the results of this study that PIP_3 activates PKC more efficiently than PIP_2.

The PIP_3 content in unstimulated neutrophils is approximately 0.05 μM and increases to approximately 2 μM after the stimulation of the cells (24). The effective local concentration of PIP_3 in the membrane may be even higher than 2 μM. Thus the PIP_3 concentrations used in this study for the activation of PKC fall in physiological range. We propose a role of PIP_3 in signal transduction, possibly a second messenger role in the cell since it is generated as a

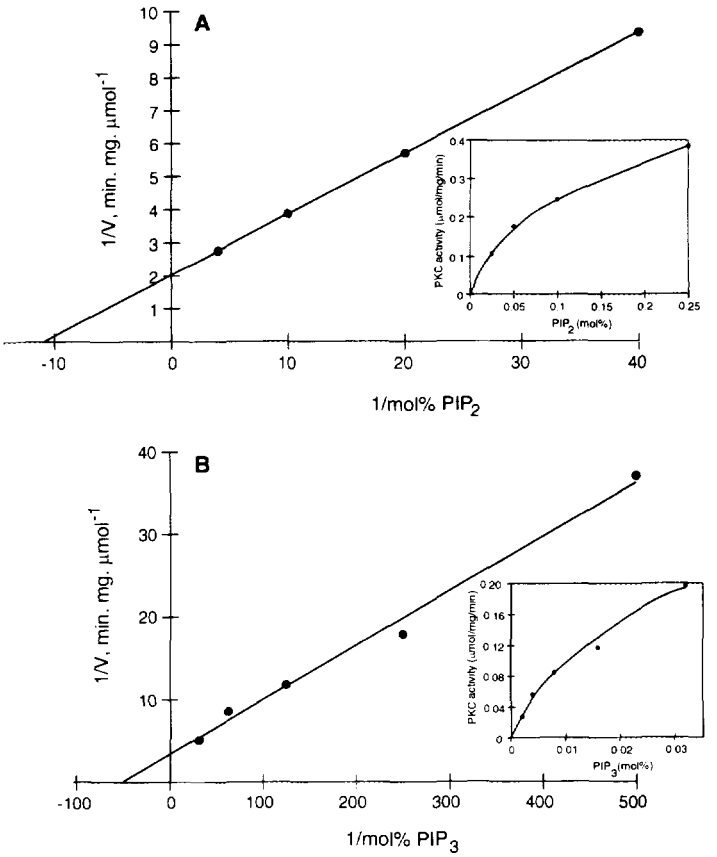

Fig. 3. Lineweaver-Burk plot of 1/PKC activity vs. 1/phosphoinositide concentration. PKC-mediated histone III-S phosphorylation was measured by mixed micellar assay (19) using different concentrations of phosphoinositides, i.e., PIP_2, 0-0.25 mol % (Fig. 3A) and PIP_3, 0-0.32 mol% (Fig. 3B).

result of receptor-mediated agonist stimulation of the cells. Activation of PKC by PIP_3 may provide a novel link between the tyrosine kinase activity (of certain oncogene products and growth factor receptors) and the serine/threonine phosphorylation cascades that they initiate (25).

Comparison of the known structures of the PKC isozymes reveals four regions of homology that are conserved (C regions) and five regions of variable, unconserved sequences (26). The C1 region is known to lie near the amino terminus and within the lipid-binding, regulatory domain of PKC and to be necessary for high-affinity phorbolester (or DG) binding (27). Another conserved sequence in the regulatory domain, C2, has been suggested to confer Ca^{2+} dependency on phorbol ester (or DG) binding (27). We suggest that PIP_3, like DG, may bind at the C1 region though not exactly at the same positions because of dissimilarities in the nature of these lipids;

PIP_3 is a highly charged molecule while DG is a neutral lipid. Interaction of PIP_3 or PIP_2 with PKC may be mediated through Ca^{2+} since direct interaction of phosphoinositides with PKC in absence of divalent cations results in the inactivation of kinase. This phosphoinositide-mediated inactivation of protein kinases parallels the charge density of the phosphoinositides (PIP_2>PIP>PI) (28). Thus, in PIP_3•PKC activation, both C1 and C2 region may be involved; i.e., PIP_3 may hydrogen-bind to the C1 region through its carbonyl groups, while its phosphate groups may interact electrostatically in the C2 region with the collaboration of Ca^{2+}. If PKC is allowed to interact with phosphoinositides in the absence of Ca^{2+}, acyl carbonyls may still bind to C1 region of the enzyme but the phosphate groups probably get engaged to the basic groups on the enzyme. Thereby, conformational changes of PKC occur in such a way that the enzyme is irreversibly inactivated (28). It is possible that Mg^{2+} binding sites may also be involved in stabilizing the active PIP_3•PKC complex.

It has been reported that NGF and EGF in PC12 pheochromocytoma cells interact with receptor tyrosine kinases (29) and stimulate PI 3-kinases (30). These growth factors share common early biochemical responses; in particular, they both activate MAP kinases (mitogen or messenger activated protein kinases) in PC 12 cells (31). MAP kinases are a series of a serine/threonine protein kinases that must be phosphorylated on both threonine and tyrosine to become activated (32). However, they appear not to be direct substrates of receptor tyrosine kinases but are downstream targets of a protein kinase cascade (33). In this cascade of signal transduction, growth factors activate MAP kinase kinase kinase that phosphorylates at a serine/threonine site of MAP kinase kinase which is capable of phosphorylating MAP kinase on both serine/threonine and tyrosine residues, thereby activating this kinase (34). Activation of MAP kinase kinase kinase is itself dependent upon serine/threonine phosphorylation. Therefore, one additional component, the kinase which phosphorylates at serine/threonine residues of MAP-kinase - kinase - kinase must exist linking the growth factor/tyrosine kinase to MAP kinase in this cascade. Activation of PI 3-kinase and accumulation of PIP_3 in NGF and EGF- stimulated PC12 cells parallels the activation of MAP kinase (30). An intriguing hypothesis is that MAP kinase kinase kinase may be a substrate of PIP_3•PKC (active form of PKC) and get activated upon phosphorylation at serine/threonine residues.

In conclusion, PIP_3 is a potent PKC-activator in presence of PS and Ca^{2+}. PIP_3 may play a role as an intracellular signal molecule that may help cross talk between two or more signals, e.g., interaction between serine/threonine kinase and threonine/tyrosine kinase. The high phosphate charge density of PIP_3 also suggests that the lipid may partake in a pH maintenance system, such as the Na^+/H^+ antiport mechanism (13).

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