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PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE MAY ANTECEDE DIACYLGLYCEROL AS ACTIVATOR OF PROTEIN KINASE C

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SUMMARY: Phosphatidylserine/calcium-dependent protein kinase C (PKC) from rat brain is activated fifty times more efficiently by phosphatidylinositol-4,5-bisphosphate (PIP_2) (K_{app} = 0.04 mole% in Triton-lipid micelles) than by diacylglycerol (DG) (K_{app} = 2 mole%). Both effector lipids appear to bind to the same site but PIP_2 may confer a narrower substrate specificity on the kinase. DG, which together with inositol trisphosphate (IP_3) is generated by hydrolysis from PIP_2 after cell stimulation, has been considered the natural activator of the kinase but it is likely to be anteceded in this function by PIP_2; DG may perhaps retain the function of a back-up activator. The lack of PKC-activation by phosphatidylinositol (PI) or phosphatidylinositol-4-phosphate (PIP) opens the possibility that the Inositide Shuttle, PI $\rightleftharpoons$ PIP $\rightleftharpoons$ PIP_2, has a role in controlling the activity of the kinase.

The activity of protein kinase C (PKC) is thought to be closely linked to the phosphoinositides (1,2). These lipids, which reside mainly in the plasma membrane, partake in an inositol cycle and an inositide shuttle (3).

Inositol Cycle Inositide Shuttle

On cell stimulation, the inositol cycle is set in motion by an activated phospholipase C (PLC) which splits PIP_2 into inositol trisphosphate (IP_3) and diacylglycerol (DG). The "messenger" IP_3 diffuses into the cytosol where it

Abbreviations: PKC, protein kinase C; DG, diacylglycerol (dioleoyl); PIP_2, phosphatidylinositol-4,5-bisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; IP_3, inositol trisphosphate; PS, phosphatidylserine.

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opens calcium storage vesicles and raises the intracellular concentration of this cation (4). DG remains in the plasma membrane to which it translocates the soluble kinase, PKC (5,6), by bonding to the regulatory moiety of that enzyme, hydrophobically and by three H-bonds (7,8). With the further addition of Ca^{2+}, PKC is thus activated. As for the inositide shuttle, its function is still obscure though it has been proposed to serve as an intracellular Ca^{2+} buffering system in which phospholipid "calcium cages" sequester or release the cation as directed by the shuttle (9).

While the connection between inositol cycle and PKC activation seems reasonably explained, there is yet room for some doubts. Cytosol Ca^{2+} increase may result from agents other than IP_3, e.g., diglyceride or phosphatidic acid (10,11), and in a subsecond experiment Ca^{2+} has been found to appear earlier than IP_3 (12). The presumed activation of PKC by DG loses some plausibility when it is considered that the *in vivo* intramembrane concentrations of PIP_2 (the putative precursor of DG) are no higher than 0.2 mole percent of the total lipids, with no more than 1 mole percent for the combined phosphoinositides (rat brain (13-15)), whereas the *in vitro* concentration of DG at half-activation is 2 mole% (Fig. 1a). This would seem to make DG a very inefficient activator. Furthermore, if DG binds and translocates PKC, why cannot the glycerophospholipids, at least in part, fulfill this function since they all include the diglyceride moiety in their molecular structure and can form two H-bonds with their ester CO groups, though not three? It was therefore speculated (8) that the precursor of DG, PIP_2, might already be bound to PKC in the membrane in the resting cell, and that a PIP_2·PKC complex is the proper substrate for phospholipase C. If this is the case it might be expected that PIP_2 will function as an inhibitor of the kinase, by competing with DG, with kinetics similar to those of the PKC-DG-phenobarbital system (16). Experiments, however, showed that PIP_2 is not an inhibitor but an activator of PKC.

MATERIALS AND METHODS

All reagents were purchased from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) was procured from NEN. The purity of lipids was confirmed by thin layer chromatography (17). GF/C filters were from Whatman.

Purification of protein kinase C. The enzyme was purified from rat brains essentially by the method of Woodgett and Hunter (18) with DE52 and phenyl-sephrose 4B column chromatography. The final preparation was concentrated (1 mg/ml) by reverse-dialysis against solid polyethylene glycol 8000 and dialyzed overnight at 4°C into 20 mM Tris-Cl, pH 7.5 containing 0.1% (v/v) mercaptoethanol, 100 μM EGTA and 10% glycerol, and stored at 4°C. The specific activity of PKC was 30 nmol/mg/min at 37°C in a mixed micellar assay system of 0.3% Triton X-100 (19) containing 9 mol% PS and 2 mol% dioleoyl-glycerol of Triton, and 50 μM Ca^{2+}.

Phosphorylation of histones by protein kinase C. The total reaction volume of 0.1 ml contained 20 mM Tris-Cl, pH 7.5, 0.8 mg/ml histone III-S, 40 μg/ml leupeptin, 12.5 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 50 μM Ca^{2+}, and 10 μl of lipid micelles containing phosphatidylserine (PS) and other specified lipids in 3% Triton X-100 (final concentration of Triton = 0.3% and PS = 445 μM in the reaction mixture). The lipid micelles were prepared by drying the required amount of lipids under a stream of nitrogen in a glass tube followed by solubilization in 3% Triton X-100 by vortexing and incubating for 5 min at 37°C (19). The concentration of lipids is expressed as mol% of Triton X-100. Controls were run with 2 mM EGTA. The reaction, at 37°C, was started with 10 μl of enzyme and terminated after 3 min with 1 ml of 25% ice cold trichloro-acetic acid (TCA) and 1 ml of ice-cold bovine serum albumin (400 μg/ml). The resulting precipitate was collected on a 2.5 cm GF/C filter and washed 6 times with 1 ml of ice-cold 25% TCA, then counted in 10 ml of hydrofluor.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of DG and PIP_2 on PKC activity. PIP_2 activates PKC around 50 times more efficiently than DG, with a K_{app} of ca. 0.04 mol% (Fig. 1b) against a K_{app} of ca. 2 mol% for DG (Fig. 1a). It can also be seen that the maximal velocity of phosphorylation is 2-3 times higher for DG·PKC than for PIP_2·PKC, though this velocity is achieved only at unphysiologically high concentrations of DG. The reason is, possibly, a narrower substrate specificity of PIP_2·PKC. This is plausible to expect for the true activator, and such a difference in specificity is not without precedent; it has been found between DG-activated and phorbol ester-activated PKC (20).

Several assays were performed in order to show that the PKC-activating role of PIP_2 is not an accident of the particular assay system (histone and a micellar lipid matrix containing PS and PIP_2): autophosphorylation (21); phosphorylation of erythrocyte ghosts; and phosphorylation of histone in a liposomal rather than a micellar system (22). PIP_2 proved to be an effector in all three cases, with K_{PIP_2} effective at concentrations at least one order of magnitude lower than K_{DG}.

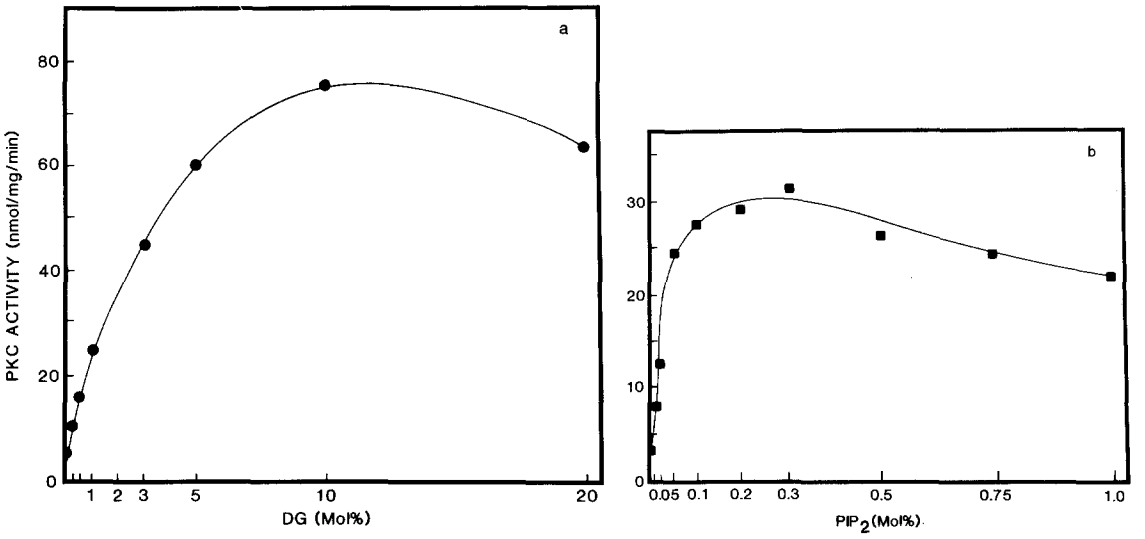

Fig. 1. Activation of PKC by DG (a) or PIP_2 (b) in lipid micelles containing 9 mol% PS of Triton X-100. Protein kinase C activity was measured by mixed micellar assay with 0.3% Triton X-100 (19).

It may be mentioned at this point that the PKC-activating ability of PIP_2 has recently been investigated (23), but in a system not containing the obligatory matrix lipid, phosphatidylserine. Under such condition the inositides can assume, in part, the enabling role of this phospholipid but not the activating role of DG or PIP_2 in the complete system. In another study PIP_2 has been shown to activate the phosphorylation of a protein, profilin, but at very high concentrations of the lipid (K_{PIP_2} = 45 mole%), and probably as the result of a modification of the substrate-membrane matrix (24).

In Figure 2, evidence is presented that DG and PIP_2 act on the same enzyme and occupy the same site on it. If they activated different enzyme species, e.g. isoenzymes, then the addition of PIP_2 to an enzyme mixture already maximally activated by DG (Fig.2,A) should result in a further increase in V_{max}; but this does not take place (B). At activator concentrations sufficiently below saturation, on the other hand, the activators are additive (Fig. 2, C,D,E and Fig. 3), as compatible with a common binding site. The data also rule out the possibility of multiple activator binding sites on the same enzyme molecule: in that case, a synergistic effect should ensue.

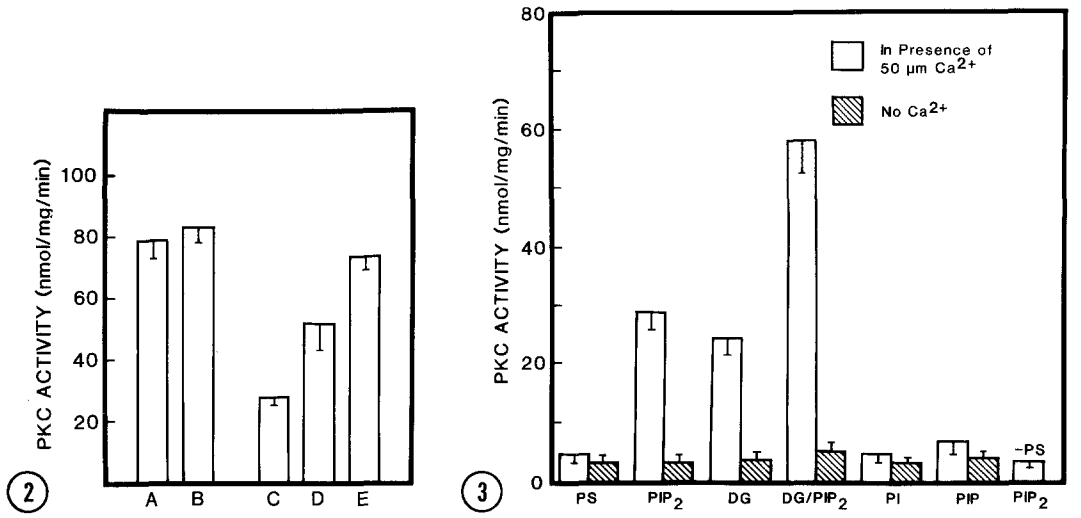

Fig. 2. Additivity of DG- and PIP_2-dependent activation of protein kinase C. All assay mixtures included 9 mole% phosphatidylserine in 0.3% Triton X-100, (i.e., a final concentration of PS = 445 μM). A) activity of PKC saturated with DG = 10 mole%; B) saturation with DG, as in A, plus 0.2 mole% PIP_2; no increase of activity is observed; C) saturation with PIP_2 = 0.2 mole%; D) activation by submaximal concentration of DG = 3 mole%; E) combination of C and D, i.e., 0.2 mole% PIP_2 plus 3 mole% DG; additivity is observed.

Fig. 3. Effect of activators on PKC in the presence and absence of Ca^{2+}. All samples contained 9 mole% phosphatidylserine of Triton X-100 except where indicated. Other lipids in the reaction mixture (with respect to Triton X-100) were: PIP_2, 0.1 mole% or 4.95 μM; diacylglycerol (dioleoyl), DG, 1 mole% or 49.5 μM; phosphatidylinositol, PI, 0.2 mole% or 9.89 μM; phosphatidylinositol-4-phosphate, PIP, 0.2 mole% or 9.89 μM.

Figure 3 shows that DG·PKC and PIP_2·PKC have the same cofactor requirements: phosphatidylserine and Ca^{2+} are indispensible. The activities obtained with each effector are again seen to be additive. Finally, phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PIP) show little, if any, capability of activating the kinase. This indicates that the inositol-5-phosphate group of PIP_2 is ionically linked to a cationic ligand on the enzyme and that the regulatory geometries of DG·PKC and PIP_2·PKC must be quite different: another reason to expect different substrate specificities. It also appears that the inositide shuttle, i.e., the reversible phosphorylation-dephosphorylation of the inositides, PI $\rightleftharpoons$ PIP $\rightleftharpoons$ PIP_2, may have a role in controlling cellular protein phosphorylation.

This work has been presented as an abstract (25).

REFERENCES

1. Berridge, M.J., and Irvine, R.F. (1984) Nature 312, 315-321.
2. Nishizuka, Y. (1986) Science 233, 305-312.
3. Brockerhoff, H., and Ballou, C.E. (1962) J. Biol. Chem. 237, 1764-1766.
4. Joseph, S.K., Williams, R.J., Corkey, B.E., Matschinsky, F.M., and Williamson, J.R. (1984) J. Biol. Chem. 259, 12952-12955.
5. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348.
6. Besterman, J.M., Duronio, V., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci, USA 83, 6785-6789.
7. Rando, R.R., and Young, N. (1984) Biochem. Biophys. Res. Commun. 122, 818-823.
8. Brockerhoff, H. (1986) FEBS Lett. 201, 1-4.
9. Brockerhoff, H. (1986) Chem. Phys. Lipids 39, 83-92.
10. Farese, R.V., Konda, T.S., Davis, J.S., Standaert, M.L., Pollet, R.J., and Cooper, D.R. (1987) Science 236, 586-589.
11. Brass, L.F., and Laposata, M. (1987) Biochem. Biophys. Res. Commun. 142, 7-14.
12. Tashjian, Jr., A.H., Heslop, J.P., and Berridge, M.J. (1987) Biochem. J. 243, 305-308.
13. Wells, M.A., and Dittmer, J.C. (1965) Biochemistry 4, 2459-2468.
14. Dittmer, J.C., and Douglas, M.G. (1969) Ann. N.Y. Acad. Sci. 165, 515-525.
15. Soukup, J.F., Friedel, R.O., and Shanberg, S.M. (1978) J. Neurochem. 30, 635-637.
16. Chauhan, V.P.S., and Brockerhoff, H. (1987) Life Sci. 40, 89-93.
17. Deshmukh, D.S., Kuizon, S., and Brockerhoff, H. (1981) J. Neurochem. 36, 594-601.
18. Woodgett, J.R., and Hunter, T. (1987) J. Biol. Chem. 262, 4836-4843.
19. Hannun, Y.A., Loomis, C.R., and Bell, R.M. (1986) J. Biol. Chem. 261, 7184-7190.
20. Ramsdell, J.S., Pettit, G.R., and Tashjian, A.H. (1986) J. Biol. Chem. 261, 17073-17080.
21. Newton, A.C., and Koshland, D.E. (1987) J. Biol. Chem. 262, 10185-10188.
22. Boni, L.T., and Rando, R.R. (1985) J. Biol. Chem. 260, 10819-10825.
23. O'Brian, C.A., Arthur, W.L., and Weinstein, I.B. (1987) FEBS Lett. 214, 339-342.
24. Hansson, A., Skoglund, G., Lassing, I., Lindberg, U., and Ingelman-Sundberg, M. (1988) Biochem. Biophys. Res. Commun. 150, 526-531.
25. Chauhan, V.P.S., and Brockerhoff, H. (1988) FASEB J. 2, A349.