Activation of PRK1 by Phosphatidylinositol 4,5-Bisphosphate and Phosphatidylinositol 3,4,5-Trisphosphate

A COMPARISON WITH PROTEIN KINASE C ISOTYPES*

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As potential targets for polyphosphoinositides, activation of protein kinase C (PKC) isoforms (β, γ, ε, η, ζ) and a member of the PKC-related kinase (PRK) family, PRK1, has been compared in vitro. PRK1 is shown to be activated by both phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P2) as well as phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3) either as pure sonicated lipids or in detergent mixed micelles. When presented as sonicated lipids, PtdIns 4,5-P2 and PtdIns 3,4,5-P3 were equipotent in activating PRK1, and, furthermore, sonicated phosphatidylinositol (PtdIns) and phosphatidylserine (PtdSer) were equally effective. In detergent mixed micelles, PtdIns 4,5-P2 and PtdIns 3,4,5-P3 also showed a similar potency, but PtdIns and PtdSer were 10-fold less effective in this assay. Similarly, PKC-β, γ, ε, and ζ were all activated by PtdIns 4,5-P2 and PtdIns 3,4,5-P3 in detergent mixed micelles. The activation constants for PtdIns 4,5-P2 and PtdIns 3,4,5-P3 were essentially the same for all the kinases tested, implying no specificity in this in vitro analysis. Consistent with this conclusion, the effects of PtdIns 4,5-P2 and PtdIns 3,4,5-P3 were found to be inhibited at 10 mM MgCl2 and mimicked by high concentrations of inositol hexaphosphate and inositol hexosulfate. The similar responses of these two classes of lipid-activated protein kinase to these phosphoinositides are discussed in light of their potential roles as second messengers.

The phosphatidylinositol 3-kinase family of lipid kinases are responsible for the phosphorylation of inositol lipids at the 3-OH position (reviewed in Ref. 1). In response to various agonists, phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3) accumulates and labeling studies suggest that this is the primary product and as such is the most likely second messenger candidate (2). While this remains an attractive hypothesis, in the absence of a defined intracellular target(s), the operation of the “PtdIns 3-kinase signaling pathway” will remain enigmatic.

By their nature, lipid-dependent protein kinases are attractive candidates as targets for the postulated role of PtdIns-3,4,5-P3 as a second messenger. Protein kinase C (PKC) isoforms constitute the major group of such enzymes, but several recent reports have indicated the existence of an additional class of lipid-activated protein kinases. These kinases, termed PKC-related kinases (PRKs) are closely homologous to PKC isoforms in the catalytic domain while retaining a distinct amino-terminal regulatory domain ((3, 4) see Fig. 6A). Unlike the PKCs which characteristically retain a cysteine-rich C1 domain responsible for effector binding, the PRKs do not encode a C1 domain but show two distinct conserved regulatory domains termed HR1 and HR2 (3). Consistent with the lack of a C1 domain, it has become clear that while the PRKs resemble PKCs in being activated by proteolysis, they differ from PKCs in not being activated by phorbol esters. However, like PKC, various fatty acids and phospholipids have been shown to activate PRKs in vitro (4–6).

In view of the interest in PtdIns 3,4,5-P3 as a second messenger, several studies have addressed the activation of PKC isoforms by this class of lipids (7–9). However, the results show little consistency in which of the different PKC isoforms are activated or in the specificity of different lipids. In this paper we address the specificity of the lipid-kinase interaction in two ways: firstly, with respect to the target enzyme, and secondly, with respect to the phospholipid (head group). In order to do so, members of both lipid-dependent kinase families, PRK and PKC, were compared for activation by polyphosphorylated inositol lipids in the detergent mixed micelle assay. This assay, introduced by Hannun et al. (10) for the study of PKC, allows for specific modulation/stimulation of PKC by phorbol ester and provides a defined and readily controllable context for presentation of other types of lipid to kinases. Our results indicate that there seems to be little specificity for inositol lipid-kinase interactions in vitro.

EXPERIMENTAL PROCEDURES

Materials—PtdSer, PtdIns, PtdIns 4,5-P2, mixed brain phospholipids, 12-O-tetradecanoylphorbol 13-acetate (TPA), inositol hexaphosphate (InsP6), inositol hexosylphosphate (InsP6), inositol tetraphosphate (InsP4), and myelin basic protein were from Sigma. PtdIns 3,4,5-P3 was made as described (11). [γ-32P]ATP (5000 Ci/mmol) was obtained from Amersham (United Kingdom).

Expression and Purification—PRK1 was overexpressed and purified from COS 7 cells as described previously (5). PKC isoforms were purified using the baculovirus expression system (12), with the exception of both the wild-type and the V12 mutant of PKC-γ (12–137) which were overexpressed and purified from COS 7 cells as described (13).

Protein Kinase Assay—Routinely, 5 μl of enzyme was tested in 40 μl of assay buffer (50 mM HEPES, pH 7.5, 0.75 mM EGTA, 1.625 mM MgCl2, 1.25 mM myelin basic protein, 125 μM ATP, 1 μM of [γ-32P]ATP) for 4 min at 30°C. Activity is linear with incubation time over this period. Reactions were started by addition of ATP and terminated by spotting on Whatman P81 paper and submersing in 30% (v/v) acetic acid followed by three 20-min washes. Incorporation of
orthophosphate into substrate was assessed by scintillation counting. Alterations to effector and substrate concentrations are described in the text and figure legends.

Lipid Preparation—Lipids micelles were dried in a nitrogen stream or under vacuum and then either sonicated in 20 mM HEPES, pH 7.5, on ice for 15 s three times (sonicated micelles) or resuspended in 20 mM HEPES, pH 7.5, in the presence of Triton X-100 at 0.2% (Triton X-100 detergent micelles), yielding the final concentrations stated in the text or figure legends.

Other Procedures—Protein concentrations were determined according to Bradford (14) using a Bio-Rad assay solution. Kinetic calculation was performed using an enzyme kinetics program (Enzfitter; Elsevier Biosoft, Cambridge, UK).

RESULTS

Unlike PKC, the activation of PRKs by PtdIns-3,4,5-P3 and PtdIns-4,5-P2 has not been documented. Initial studies with purified PRK1 were performed with pure lipid vesicles comprised of phosphatidylinerine (PtdSer), phosphatidylinositol (PtdIns), PtdIns-4,5-P2, or PtdIns-3,4,5-P3 (Fig. 1A). When presented as pure lipid vesicles, each of these lipid types were able to activate PRK1 when myelin basic protein was used as a substrate. All lipids displayed similar activation profiles (Fig. 1A).

The concentration-dependent activation of PRK1 observed for lipid vesicles of PtdSer, PtdIns, PtdIns-4,5-P2, and PtdIns-3,4,5-P3 provides limited information on specificity/potency in view of the need to define the surface concentration. In order to work under defined conditions, Triton X-100 mixed micelles were employed (10). In this assay, PtdIns-4,5-P2 and PtdIns-3,4,5-P3 showed similar potency in the activation of PRK1 (Fig. 1B). PtdSer and PtdIns were still able to fully activate PRK1 but only at 10-fold higher concentrations when compared to PtdIns-4,5-P2 and PtdIns-3,4,5-P3.

In employing detergent mixed micelles in the study of PKC, it has been well documented that diacylglycerol-dependent activation of particular PKC isotypes is readily observed in the context of Triton X-100/PtdSer mixed micelles (10). Thus, the effects of PtdIns-3,4,5-P3 and PtdIns-4,5-P2 were studied under similar conditions (in a background of Triton X-100 mixed micelles containing 10 mol % PtdSer). It was found that PtdSer at this concentration has no significant effect on PRK1 activity (Fig. 1B). The presence of PtdSer at 10 mol % in these vesicles does not significantly affect the inositol lipid-dependent activation of PRK1 (Fig. 2).

To compare the inositol lipid activation of PRK1 with that of representative PKC isotypes, a Triton X-100/10% PtdSer mixed micelle assay was employed (Fig. 3A). It is evident that at 16 mol % PtdIns-4,5-P2 or PtdIns-3,4,5-P3, the phorbol ester-responsive PKC isotypes were all activated to an extent comparable with that seen in the presence of TPA. In the case of PKC-γ, which is insensitive to TPA (see Fig. 3A (15)), activation was also observed, albeit only about 2-fold above basal activity. Consistent with the previous data (Figs. 1 and 2), PRK1 also showed a robust activation at 16 mol % PtdIns-4,5-P2 or PtdIns-3,4,5-P3.

The activation observed by all the PKCs and PRK1 is extremely sensitive to the concentration of Mg2+ and Ca2+ employed in the assay (Fig. 3B). Excess of these divalent cations negates the activation observed by PtdIns-4,5-P2 and PtdIns-3,4,5-P3, further enforcing the idea that the anionic charge of these lipids is important for activation. For PRK1, the effect of Mg2+ on PtdIns-4,5-P2 and PtdIns-3,4,5-P3 activation is shown in Fig. 4. The effect of Mg2+ is clearly biphasic, being optimum at physiological Mg2+ concentrations (~1 mM). The observation that activation by PtdIns-3,4,5-P3 is more sensitive to inhibition by high concentrations of Mg2+ is consistent with the notion that the lipid effects are charge-dependent. This idea is supported by the observation that Mg2+ inhibition of PtdIns-4,5-P2 is less pronounced than that of PtdIns-3,4,5-P3 (Fig. 4A).
further supported by the observation that at high concentrations the inositol polyphosphate InsP₆ and the related synthetic sulfated compound InsS₆ are also able to cause activation of PRK1 in the 20–100 μM range (Fig. 5). While there is a lack of distinction in comparing PRK1 and the phorbolester-responsive PKC isotypes, there is a domain in PRK1 (HR2) that is related to the Vo domains of PKC-e and -h (see Fig. 6). In order to test whether this domain contributed to the inositol lipid activation observed, a deletion mutant of PKC-h (Fig. 6A (13)) was expressed and partially purified. Comparison of PKC-h with PKC-h D (2–137) showed that there was no difference in lipid activation (Fig. 6B). The apparent nonselectivity of protein kinase activation in vitro by these inositol lipids was further studied in the context of mixed brain phospholipids. As shown in Fig. 7 for PKC-β₁, PKC-ε, PKC-η, and PRK1, there is little distinction in either potency or efficacy for PtdIns-3,4,5-P₃ and PtdIns-4,5-P₂ with any one of these kinases.

**DISCUSSION**

The data presented here indicate that PRK1 can be activated by pure lipid vesicles of PtdIns-4,5-P₂ and PtdIns-3,4,5-P₃, to an extent comparable with vesicles of PtdSer and PtdIns. This is consistent with the activation of PRK1 by a number of different phospholipids (4–6). Activation of PRK1 by these pure lipid vesicles may or may not reflect a true activator role. In the case of PKC, PtdSer is a potent activator when presented alone; however, in detergent (or mixed phospholipid) micelles, activation of PKC becomes dependent upon diacylglycerol or phorbol esters (10). In view of the wealth of literature on the (physiological) activation of PKC by diacylglycerol or phorbol esters in intact cells, it can be surmised that the physical status of the pure phospholipid vesicles poorly reflects the situation in vivo. By contrast, while not physiological, the detergent mixed micelles support an in vitro behavior of PKC in keeping with that observed in vivo.
Employing the Triton X-100 mixed micelle assay revealed that PtdSer and PtdIns activation of PRK1 was not achieved until 10-fold higher effector concentrations were employed compared with PtdIns-4,5-P$_2$ and PtdIns-3,4,5-P$_3$. A similar effect, comparable with that seen with PtdSer and PtdIns, was also observed for mixed brain phospholipids (5). The dependence and extent of PRK1 activation was indistinguishable for the two polyphosphoinositides (PtdIns-4,5-P$_2$ and PtdIns-3,4,5-P$_3$) tested; given the uniform context in which they are presented (i.e. Triton X-100), this similarity is likely to reflect a similar mode of action. In principle, there may be some absolute nonspecific requirement for phospholipid in order to observe selective activation by one of these phosphoinositides. However, similar activation profiles were observed for PRK1 whether Triton X-100, Triton X-100/PtdSer, or Triton X-100/mixed brain phospholipids were employed. In each case, the A$_{0.5}$ values for the polyphosphoinositides were between 4 and 6 mol %. This represents 7 molecules of phosphoinositide per Triton X-100 micelle (140 molecules total (10)). As a point of reference, 1–2 mol % diacylglycerol (i.e. 2–3 molecules/micelle) is required for PKC activation under similar conditions (10).

In parallel to assessing the behavior of PRK1, various PKC isotypes were also analyzed. With respect to the polyphosphoinositides, the PKC isotypes studied (aside from PKC-ζ) behaved essentially as PRK1. Activation of PKC-β$_1$, PKC-ε, and PKC-η was observed for both PtdIns-4,5-P$_2$ and PtdIns-3,4,5-P$_3$. Furthermore, the extent of activation was comparable with that observed for optimal Triton X-100/PtdSer/TPA concentrations. Once again, as with PRK1, no specificity was observed.

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Fig. 4. Activation of PRK1 by PtdIns-4,5-P$_2$ and PtdIns-3,4,5-P$_3$ is sensitive to [Mg$^{2+}$]. The effect of titrating [Mg$^{2+}$] on the activity of PRK1 was measured. Duplicate assays were carried out in Triton X-100 micelles containing either 16 mol % PtdIns-4,5-P$_2$ (● ● ●), 16 mol % PtdIns-3,4,5-P$_3$ (● ● ●), or no effector (Δ Δ Δ).

Fig. 5. Effect of InsP$_4$, InsP$_6$, and InsS$_6$ on PRK1 activity. A, PRK1 was assayed with various concentrations of the inositols polyphosphates InsP$_4$ (● —●) and InsP$_6$ (● — ●) in the absence of detergent. B, as A except in the presence of Triton X-100 micelles as described under "Experimental Procedures."

Inositol Lipid Activation of Multiple PKCs and PRK1

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between these two polyphosphoinositides, each showing similar A_0.5 values in both Triton X-100/PtdSer and Triton X-100/ mixed brain phospholipids. This lack of distinction between PKC isotypes and between these two lipids is in variance with some previously published data (7–9). However, these studies have not assessed activation in the context of detergent mixed micelles and, as such, variations between phosphoinositides may have reflected in part alterations to the vesicle structures, these considerations have been discussed extensively for PKC activation (see, for example, Ref. 16). Furthermore, there appears to be a significant variation in regulatory properties of PKC isotypes that is a function of source, purity, and storage which may vary in the different studies. For example, we have found this to be a particularly acute problem for PKC-\(\zeta\) which when expressed in SF9 or H15 insect cells progressively loses its lipid/TPA dependence on purification and storage (discussed in Ref. 17).

The modest activation of PKC-\(\zeta\) under the conditions tested here (2-fold) is similar to the extent of activation we have observed for PKC-\(\zeta\) when pure PtdSer vesicles are employed (data not shown). Thus, in a sense, PKC-\(\zeta\) behaves as all the other kinases studied here, it is only the absolute extent of activation above basal that varies. The variation with the responsiveness (9) and nonresponsiveness (8) described by others may reflect distinct basal activities from the different enzyme sources.

The similar behavior of all these lipid-activated kinases toward the two polyphosphoinositides tested suggests that the interaction in vitro may be nonphysiological since: (i) PtdIns-3,4,5-P_3 would itself be derived from PtdIns-4,5-P_2 and yet both show a similar potency in these assays, i.e. the activation status of the putative target would not change and (ii) there is no conservation of potential ligand binding sites when comparing the PKC family with that of the PRKs. The latter point is worthy of further comment since there is similarity between the PKC-\(\eta\)/V_o domain and that of the PRK1/2 HR2 domain (3).

In order to address the idea that this domain may be of importance, we tested a V_o deletion mutant of PKC-\(\eta\) (13) see Fig. 6) and observed that it displays the same polyphosphoinositide response as wild-type PKC-\(\eta\). This lack of an identifiable, conserved binding site is consistent with the lack of distinction between these polyphosphoinositides and suggests that the interaction reflects a more general anionic phospholipid requirement of the respective lipid binding domains of these kinases. The activation observed for all the PKCs and PRK1 is extremely sensitive to the concentration of Mg^{2+} and Ca^{2+} employed in the assay and the observation that at high concentrations the inositol polyphosphate In_{Sp} and the related In_{S0} are able to cause activation of PRK1 (Fig. 5) further enforce the idea that the charge effects in these activations are important. The fact that the polyphosphoinositide (PtdIns-3,4,5-P_3 and PtdIns-4,5-P_2) effects are able to activate within the 4–8 mol % range in Triton X-100 indicates that there is some property of these lipids that promotes their interaction when compared with PtdSer and PtdIns, the clustered phospho groups being the obvious candidates.

In conclusion, PRK1 and members of the PKC family are activated with a similar potency by PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3 when presented in a uniform detergent mixed micelle context. While these lipids might share a common specific binding site, no such discernible site is obvious when comparing domains of PRK1 with those of the PKC isotypes. The implication is that if either lipid is involved in the specific activation of either class of protein kinases in vivo then other cofactors and/or distinct conditions concern. This issue will only be resolved in a physiological context.

REFERENCES
1. Downes, C. P., and Carter, A. N. (1991) Cell Signalling 3, 501–513
2. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) Nature 351, 33–39
3. Palmer, R. H., Riddell, J., and Parker, P. J. (1995) Eur. J. Biochem. 227, 344–351
4. Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hirao, K., and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 204, 348–356
5. Palmer, R. H., and Parker, P. J. (1995) Biochem. J. 309, 315–320
6. Morrice, N. A., Fecondo, J., and Wettenhall, R. E. H. (1994) FEBS Lett. 351, 171–175
7. Singh, S. S., Chauhan, A., Brookerhoff, H., and Chauhan, V. P. S. (1993) Biochim. Biophys. Acta. 1195, 104–112
8. Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Anjja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367
9. Nakanoishi, H., Brewster, K. A., and Extom, J. H. (1993) J. Biol. Chem. 268, 13–16
10. Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1985) J. Biol. Chem. 260, 10039–10043
11. Stabel, S., Schaap, D., and Parker, P. J. (1991) Methods Enzymol. 670–673
12. Dekker, L. V., McIntyre, P., and Parker, P. J. (1993) J. Biol. Chem. 268, 19498–19504
13. Epand, R., and Lester, D. (1990) Trends Pharmacol. Sci. 11, 317–320
14. Epand, R., and Lester, D. (1990) Trends Pharmacol. Sci. 11, 317–320
15. Pears, C. J., Kour, G., House, C., Kemp, B. E., and Parker, P. J. (1998) Eur. J. Biochem. 269, 89–94

Fig. 7. Activation of PRK1, PKC-\(\beta_1\), PKC-\(\epsilon\) and PKC-\(\eta\) by PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3 in Triton X-100 micelles containing 10 mol % mixed brain phospholipids. The effect of titrating PtdIns-4,5-P_2 (●) and PtdIns-3,4,5-P_3 (●) was measured on the activity of PRK1, PKC-\(\beta_1\), PKC-\(\epsilon\), and PKC-\(\eta\) in 10 mol % mixed brain phospholipid/Triton X-100 micelles.