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The activation of protein kinase C by the polyphosphoinositides phosphatidylinositol 4,5-diphosphate and phosphatidylinositol 4-monophosphate

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Protein kinase C(PKC) is a Ca^{2+}- and phospholipid-dependent protein kinase which can be activated by diacylglycerol, a product of polyphosphoinositide hydrolysis. In this report, we show that the polyphosphoinositides L-α-phosphatidylinositol 4-monophosphate (PI 4P) and L-α-phosphatidylinositol 4,5-diphosphate (PI 4,5DP) can serve as phospholipid cofactors of isolated rat brain PKC. The order of potency of the phosphoinositides in the activation of PKC, PI > PI 4P > PI 4,5DP, shows a negative correlation with the degree of acidity of the phospholipid head group, whether 1 mM Ca^{2+} or 200 nM TPA is present in the reaction assay mixture. Although the polyphosphoinositides are by themselves weaker activators of PKC than PI, small amounts of PI 4,5DP cause a two-fold enhancement of PKC in the presence of Ca^{2+} and PI. While the endogenous phospholipid cofactors of PKC remain to be identified, these results suggest that the small amounts of polyphosphoinositides which are present in cell membranes may play a direct role in the activation of PKC in vivo, by serving as phospholipid cofactors of the enzyme.

Protein kinase C; Polyphosphoinositide; Tumor promoter; Lipid cofactor

1. INTRODUCTION

Polyphosphoinositides are components of the plasma membrane which are metabolized in response to certain growth factors, neurotransmitters, and hormones [1,2]. The hydrolysis of the polyphosphoinositide phosphatidylinositol 4,5-diphosphate by phospholipase C catalysis produces two second messengers, inositol 1,4,5-triphosphate and diacylglycerol [1–3]. Inositol 1,4,5-triphosphate stimulates the release of Ca^{2+} from endoplasmic reticulum stores [1,2] and can be phosphorylated at the three position, forming inositol 1,3,4,5-tetraphosphate, which may facilitate the entry of calcium ions into cells from the external medium [4,5]. Diacylglycerol activates PKC by reducing its requirement for Ca^{2+} [3]. PKC is a Ca^{2+}- and phospholipid-dependent protein kinase which catalyzes the phosphorylation of proteins at serine and threonine residues [3]. PKC catalyzes the phosphorylation of the enzyme inositol 1,4,5-triphosphate 5'-phosphomonoesterase, thereby activating it and facilitating the degradation of inositol 1,4,5-triphosphate [6]. The activation of PKC requires an acidic phospholipid cofactor such as PS or PI, although certain fatty acids can also fulfill the lipid cofactor requirement of the enzyme [7]. Thus, the signal transduction pathway which

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Abbreviations: DMSO, dimethylsulfoxide; PI, phosphatidylinositol; PI 4P, L-α-phosphatidylinositol 4-monophosphate; PI 4,5DP, L-α-phosphatidylinositol 4,5-diphosphate; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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includes the activation of PKC by diacylglycerol also involves the catabolism and resynthesis of potential phospholipid cofactors of PKC, i.e. PI and its polyphosphorylated derivatives.

In this report, we have examined the direct effects that the polyphosphoinositides PI 4,5DP and PI 4P have on isolated rat brain PKC. We have determined the capacities of PI 4P and PI 4,5DP to serve as phospholipid cofactors of PKC, and the effects of variations in the relative concentrations of PI and polyphosphoinositides on PKC activity.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was purchased from Amersham (Arlington Heights, IL), Hydrofluor was from National Diagnostics (Somerville, NJ), and phosphocellulose paper was from Whatman (Clifton, NJ). ATP, PMSF, PS, PI, PI 4P, PI 4,5DP, Tris-HCl and histone IIIS were purchased from Sigma (St. Louis, MO). TPA was purchased from LC Services (Schaumburg, IL). Leupeptin was a gift of the US-Japan Cooperative Cancer Research Program.

2.2. *Methods*

Rat brain PKC was partially purified as described [8] and then chromatographed on a second DEAE Sephacel column with a linear gradient of 0 to 0.3 M NaCl. The resultant PKC preparation had a specific activity of 230 nmol ^{32}P/min per mg and was activated from 10- to 30-fold by 1 mM Ca^{2+} plus 80 μg PS/ml and by 200 nM TPA plus 80 μg PS/ml. PKC was assayed as described [8], by measuring the Ca^{2+}- and phospholipid-dependent phosphotransferase reaction between [γ-^{32}P]ATP and histone IIIS, in a reaction mixture containing 20 mM Tris-HCl at pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 80 μg PS/ml (or indicated amounts of the specified phospholipid), 70 μM [γ-^{32}P]ATP (250–400 cpm/pm), 0.67 mg histone IIIS/ml and 1–4 μg rat brain PKC. Reactions were initiated by the addition of PKC. The reactions proceeded at 30°C and were terminated after 5–10 min (i.e., during the linear phase of the phosphotransferase reaction) on phosphocellulose paper. The radioactivity incorporated into histone was determined as described [8]. The Ca^{2+}- and phospholipid-dependent phosphotransferase activity of PKC was calculated by subtracting the activity observed in the absence of Ca^{2+} and phospholipid from the activity observed in the presence of these cofactors. The TPA-dependent activation of PKC was measured by substituting 200 nM TPA plus 1 mM EGTA for 1 mM Ca^{2+}. TPA in the presence of EGTA has been shown to substitute completely for added Ca^{2+} in the activation of PKC [8–10].

In order to prepare phospholipid stock solutions, phospholipids were dried under nitrogen for the removal of chloroform, resuspended in water by vortexing, and sonicated for 1 min on ice. Stock solutions of TPA (1 mg/ml) were made up in DMSO and stored at −20°C.

3. RESULTS

Fig.1 illustrates the relative capacities of PI, PI 4P and PI 4,5DP to activate PKC in the presence of 1 mM Ca^{2+}. While all three compounds activated PKC under these conditions, it is clear that the activation potencies of the polyphosphoinositides were significantly weaker than that of PI, throughout a range of phospholipid concentrations from 1 μg/ml to 500 μg/ml. PI 4,5DP was

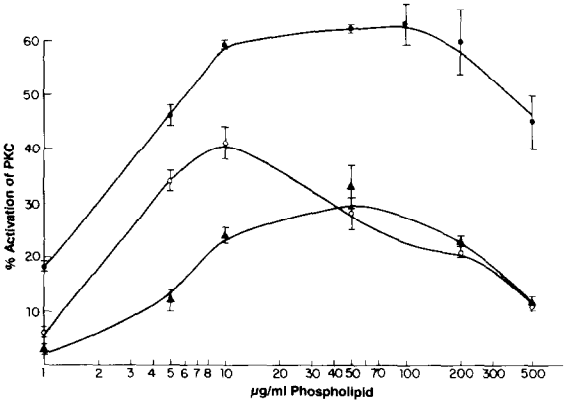

Fig.1. The Ca^{2+}-dependent activation of PKC by PI, PI 4P and PI 4,5DP. PKC activity was assayed as described in section 2.2, in the presence of 1 mM Ca^{2+} and the indicated phospholipid. '% activation of PKC' represents the percentage of activation of PKC relative to that observed in the presence of 1 mM Ca^{2+} and 200 μg PS/ml. The latter condition produced maximal activity and, therefore represents the 100% value. Its actual value was 230 ± 9 nmol ^{32}P transferred/min per mg. (●)PI, (○) PI 4P and (▲) PI 4,5DP.

substantially weaker than PI 4P as a PKC cofactor at phospholipid concentrations of 1–10 μg/ml, although at higher phospholipid concentrations PI 4,5DP and PI 4P activated PKC with similar efficacies. Thus the relative potencies of PI, PI 4P and PI 4,5DP in the activation of PKC in the presence of Ca^{2+} correlated with the degree to which each phospholipid was phosphorylated, with the most potent PKC phospholipid cofactor being the least phosphorylated.

In order to investigate whether the potencies of the polyphosphoinositides in the activation of PKC were lower than that of PI due to Ca^{2+} chelation by the anionic phosphate groups of the polyphosphoinositides, we examined the activation of PKC by these phospholipids in the presence of 200 nM TPA and 1 mM EGTA. Previous reports have indicated that the phorbol ester TPA can completely overcome the requirement for added Ca^{2+} in the in vitro activation of PKC [8–10]. Fig.2 shows that the relative potencies of PI and the polyphosphoinositides in the TPA- and phospholipid-mediated activation of PKC were PI > PI 4P > PI 4,5DP, over a range of phospholipid concentrations from 1 μg/ml to 50 μg/ml. PI was a more potent PKC phospholipid cofactor than either polyphosphoinositide at all phospholipid concentrations tested (1–500 μg/ml phospholipid). Thus the relative capacities of PI and the polyphosphoinositides to activate PKC were similar whether the enzyme was under the regulation of Ca^{2+} and phospholipid or TPA and phospholipid, providing strong evidence that the relative potencies which were observed in the Ca^{2+}- and phospholipid-dependent reaction do not simply reflect Ca^{2+} chelation by the polyphosphoinositides.

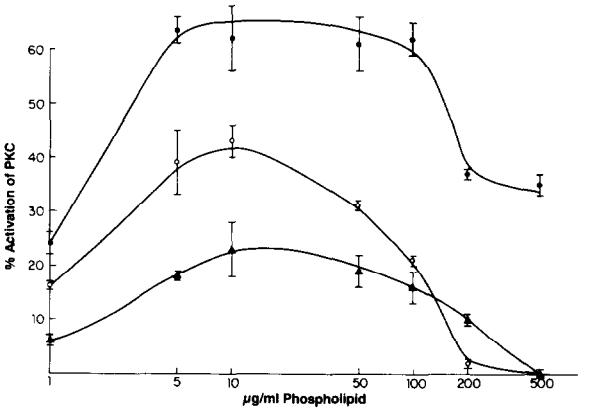

Fig.2. The TPA-dependent activation of PKC by PI, PI 4P and PI 4,5DP. PKC activity was assayed as described in section 2.2, in the presence of 200 nM TPA plus 1 mM EGTA and the indicated phospholipid. '% activation of PKC' represents the percentage of activation of PKC relative to that observed in the presence of 200 nM TPA and 200 μg PS/ml. In this experiment the latter value was 300 ± 4 nmol ^{32}P transferred/min per mg. (●) PI, (○) PI 4P and (▲) PI 4,5DP.

Figs 1 and 2 clearly indicate that PI 4,5DP is a much weaker phospholipid cofactor for PKC than is PI. Since PI 4,5DP is a minor constituent of cell membranes relative to PI [1], we next examined whether low concentrations of PI 4,5DP might modify the activation of PKC by PI. Table 1 shows that 1 μg PI 4,5DP/ml, which by itself had negligible effects on PKC activity in the presence of Ca^{2+} (fig.1), caused an almost 2-fold enhancement of the activation of PKC by 1 mM Ca^{2+} and 10 μg/ml PI. Similar enhancements of the Ca^{2+}- and PI-dependent activation of PKC were observed with 5 μg and 10 μg PI 4,5DP/ml (table 1). Table 2 shows that, at concentrations of 1–10 μg PI 4P/ml, PI 4P also enhanced the activation of PKC by 1 mM Ca^{2+} and 10 μg PI/ml. However, PI 4P was weaker than PI 4,5DP in the enhancement of the Ca^{2+}- and PI-dependent activity of the enzyme.

Table 1

The enhancement of the Ca^{2+}- and PI-dependent activity of PKC by PI 4,5DP

μg PI 4,5DP/ml	pmol ^{32}P/min	Fold-enhancement of PKC activity
0	12 ± 1	1.0
1	23 ± 1	1.9
5	25 ± 1	2.0
10	26 ± 1	2.2

Ca^{2+}- and PI-dependent PKC activity was measured as described in section 2.2 in the presence of 1 mM Ca^{2+} and 10 μg PI/ml. The effects of PI 4,5DP on the Ca^{2+}- and PI-dependent activity of PKC were determined by including the indicated amounts of PI 4,5DP in the PKC assay mixtures

Table 2

The enhancement of the Ca^{2+}- and PI-dependent activity of PKC by PI 4P

μg PI 4P/ ml (activity)	pmol ^{32}P/ min	Fold-enhancement of PKC
0	15 ± 1	1.0
1	20 ± 2	1.3
5	25 ± 1	1.7
10	23 ± 1	1.5

Ca^{2+}- and PI-dependent PKC activity was measured as described in section 2.2 in the presence of 1 mM Ca^{2+} and 10 μg PI/ml. The effects of PI 4P on the Ca^{2+}- and PI-dependent activity of PKC were determined by including the indicated amounts of PI 4P in the PKC assay mixtures

4. DISCUSSION

Polyphosphoinositide hydrolysis occurs in response to the interactions of certain growth factors, neurotransmitters and hormones with their receptors and results in the production of diacylglycerol, an activator of PKC [1–3]. Since PKC is activated by acidic phospholipids [7] and since polyphosphoinositides are acidic phospholipids whose metabolism is intimately linked with the in vivo activation of PKC [1–3], it is important to characterize direct effects of the polyphosphoinositides on PKC activity. In this report, we show that PI 4P and PI 4,5DP can, in fact, activate isolated PKC, although at optimal concentrations they are only about one-third to one-half as potent as PI. The order of potency of the phosphoinositides in the activation of PKC (PI > PI 4P > PI 4,5DP) shows a negative correlation with respect to the degree of phosphorylation of the lipids. The capacity of each of these lipids to serve as a PKC cofactor is similar whether 1 mM Ca^{2+} or 200 nM TPA plus 1 mM EGTA is present, demonstrating that the reduced capacity of polyphosphoinositides to activate PKC is not simply a consequence of Ca^{2+} chelation by the phosphorylated head group of the lipid. Thus, the capacities of these phospholipids to serve as PKC substrates appear to be greatly affected by the acidity of their head groups.

Although PS is the most effective of the known phospholipid activators of isolated PKC [7], it is not clear at this time which phospholipids are important in the activation of PKC in vivo. Presumably, in intact cells PKC is activated by association with cell membranes which have a very complex composition. In most cells, polyphosphoinositides comprise 10–20% of the inositol lipids [1]. In this report, we found that the polyphosphoinositide PI 4,5DP was a weak PKC activator in the absence of other phospholipids, but a minor amount of it, which by itself had negligible effects on PKC activity, caused a 2-fold enhancement of the activation of isolated PKC by 1 mM Ca^{2+} and 10 μg PI/ml. Thus, in our in vitro system, when the phospholipid composition was about 10% PI 4,5DP and 90% PI, the activation of PKC was 2-fold greater than that obtained with PI alone. PI 4P enhanced the activity of the enzyme in a similar fashion, although to a lesser extent. While the endogenous phospholipid cofactors of PKC remain to be identified, these results suggest that the minor amounts of PI 4,5DP and PI 4P which are present in the cell membranes may play a direct role in the activation of PKC in vivo, by serving as phospholipid cofactors for the enzyme.

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