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COUNTER-REGULATORY EFFECTS OF PHOSPHATIDIC ACID ON PROTEIN KINASE C ACTIVITY IN THE PRESENCE OF CALCIUM AND DIOLEIN

Richard M. Epand and Alan R. Stafford

Department of Biochemistry, McMaster University, Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada, L8N 3Z5

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Phosphatidic acid can replace phosphatidylserine in the activation of protein kinase C. However, in the presence of diolein, the addition of Ca^{2+} results in the inhibition of the enzyme. This phenomenon could lead to a negative feedback regulation of protein kinase C activity as a result of stimulation of the cycling of phosphatidylinositol.

Protein kinase C plays an important role in the regulation of numerous cellular processes including exocytosis as well as growth and differentiation [1]. The enzyme is most active when bound to membranes and it requires the presence of an anionic lipid. Calcium is also generally required for activity, although some protein kinase C isoforms do not require calcium for activation by unsaturated fatty acids [2]. Phosphatidylserine is thought to be the anionic lipid that activates this enzyme physiologically in the presence of calcium. However, other anionic lipids such as phosphatidic acid [3], cardiolipin [3], and phosphatidylinositol 4,5-bisphosphate [4] function as activators. Activation of this enzyme by phosphatidic acid is of particular interest because of the many effects this lipid has on cells including increasing DNA synthesis and inducing platelet aggregation. Although phosphatidic acid is known to activate protein kinase C [5,6], the characteristics of this activation have not been investigated.

MATERIALS AND METHODS

Materials: All phospholipids were purchased from Avanti Polar Lipids, Pelham, AL. 1,2-diolein was purchased from Nu-Chek Prep., Elysian, MN. Histone (type III-S) was from the Sigma Chem. Co., St. Louis, MO and [γ-^{32}P] adenosine 5'-triphosphate was from NEN, Montréal, Québec. $Mg(No_3)_2$ was from Alfa Chem. Co. Protein kinase C was purified from rat brain as previously described [7]. For assays of protein kinase C in vesicles, special care was taken to remove Ca^{2+} contamination. Double distilled water was further treated with Chelex to remove trace ions. All salts used were the

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highest purity commercially available. Phospholipids were made into small unilamellar vesicles by sonication and then passed through a column of G-50 Sephadex. The void volume fractions containing lipid were collected, pooled and lyopholyzed.

Mixed micelle assay for protein kinase C: We used the Triton X-100 micelle assay as previously described by Bell and coworkers [8]. Briefly, phospholipid and 1,2-diolein (when present) were deposited as a lipid film by solvent evaporation from a solution of lipid in chloroform/methanol (2/1, v/v). The lipid films were then solubilized by the addition of 3% Triton X-100. Enzyme assays were performed at 30° in the presence of 5 μM [γ-^{32}P] adenosine 5'-triphosphate, 150 ng protein kinase C, 20 mM Tris-HCl, 25 mM $MgCl_2$, 200 μg/mL histone III-S, 1 mM $CaCl_2$ or 20 mM EGTA, 2.75 mM Triton X-100 with phospholipids and diolein as specified. The reaction was initiated by the addition of the enzyme. After 10 min incubation at 30°, the reaction was terminated by adding 100 μL of 5 mg/mL of ice cold BSA and 2 ml of cold 25% trichloroacetic acid. The samples were briefly vortexed, placed on ice for 15 min, then filtered through GF/C Whatman filters which were then washed 4 times with 2 mL each of ice-cold 25% trichloroacetic acid. The filters were allowed to dry and then counted with 6 mL ACS scintillation fluid. Assays were performed in triplicate.

Vesicle assay for protein kinase C: The assay was carried out essentially as described by Nishizuka and coworkers [5]. Lipid films were suspended and sonicated into 20 mM Tris-HCl, pH 7.5. The assay mixture contained 5 mM $Mg(NO_3)_2$ (ultra pure grade), 200 μg/mL histone III-S, EDTA or $CaCl_2$ as indicated, 5 μM [γ-^{32}P] ATP and 150 ng protein kinase C. The incubation was carried out for 10 min at 30° and the phosphorylated protein precipitated, collected on filters and counted as described above.

RESULTS

The activation of protein kinase C by phosphatidic acid in Triton micelles in the presence of 1,2-diolein is dependent on calcium concentration (Table I). Increased activation is observed at low Ca^{++} but it decreases at higher Ca^{++} concentrations. Similar results have been observed with egg phosphatidic acid, distearoyl phosphatidic

TABLE I

Calcium Dependence of the Activation of Protein Kinase C by 1-Palmitoyl-2-oleoyl Phosphatidic Acid in Triton Micelles

$[Ca^{2+}]$ (μM)	Protein kinase C activity % Basal (absence of Ca^{2+} and phospholipid)
0	173 ± 2
2	380 ± 10
20	324 ± 20
200	267 ± 15

Micelle composition: Triton X-100 with 10 mol % phosphatidic acid and 2 mol % 1,2-diolein.

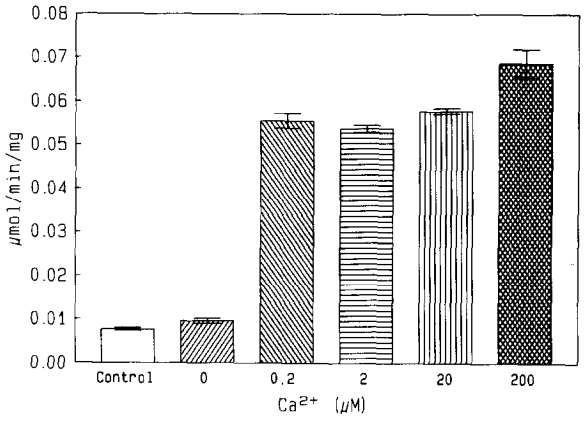

Figure 1 - Effect of calcium concentration on protein kinase C activity in vesicles of 1-palmitoyl-2-oleoyl phosphatidic acid. Activity is expressed as µmoles of histone phosphorylated per minute per mg protein kinase C. Control is in the absence of lipid vesicles and calcium. Zero is in the presence of EGTA plus trace calcium contamination.

acid and dioleoyl phosphatidic acid. With bovine brain phosphatidylserine maximal activation required calcium but showed no dependence on calcium concentration between 2 and 200 µM. In the presence of calcium, phosphatidylserine activated the enzyme about 4-fold more than did phosphatidic acid.

Further studies were done with a vesicle assay, since this system is closer to a biological membrane and has been found more sensitive to calcium concentration. Activation of protein kinase C by 1-palmitoyl-2-oleoyl phosphatidic acid requires the presence of Ca^{++} (Fig. 1). Activation is not greatly altered by higher concentrations of calcium up to 200 µM (Fig. 1). In contrast, in the presence of 2 mol % 1,2-diolein,

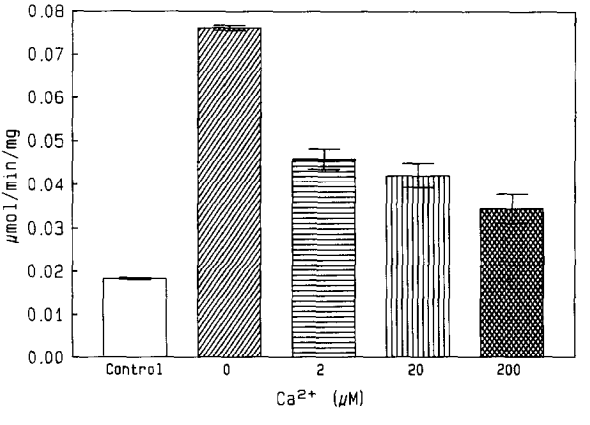

Figure 2 - Effect of calcium concentration on protein kinase C activity in vesicles of 1-palmitoyl-2-oleoyl phosphatidic acid containing 2 mol % diolein (also see legend for Fig. 1).

higher calcium concentrations begin to inhibit the enzyme (Fig. 2). This is not observed with phosphatidylserine-diolein stimulated activity which is independent of calcium concentration below 200 μM. Diolein is known to markedly reduce the concentration f calcium required for activation. We assume that at zero added Ca^{2+} there is sufficient calcium contamination present to fully activate the enzyme. We also observed maximal activation of protein kinase C with phosphatidylserine and diolein without the addition of Ca^{2+}. The results in Fig. 1 as well as earlier results of others [5,6] confirm that calcium is required for phosphatidic acid activation of protein kinase C.

DISCUSSION

Increasing calcium concentration results in the inhibition of phosphatidic acid-stimulated protein kinase C activity in Triton micelles (Table I) or in vesicles containing diolein (Fig. 2). This observation is of particular interest because it suggests a mechanism for the feedback regulation of protein kinase C. Agonist occupancy of certain receptors leads to the activation of phospholipase C. The products of phospholipase C-catalyzed hydrolysis of phosphatidylinositol diphosphate are diacylglycerol and inositol triphosphate. The diacylglycerol is an activator or protein kinase C and this activation combined with the intracellular action of inositol triphosphate leads to a rise in intracellular calcium concentrations. Some of the diacylglycerol is phosphorylated to produce phosphatidic acid. This phosphatidic acid in the presence of diacylglycerol and elevated calcium concentrations can reduce the activity of protein kinase C. Agonists which stimulate inositol phosphate cycling lead to the production of diacylglycerols and elevate intracellular calcium concentrations resulting in protein kinase C activation. However, subsequently phosphatidic acid is formed which, in the presence of calcium and diacylglycerol will inhibit the activity of protein kinase C. Hence phosphatidic acid can serve as a negative feedback modular or protein kinase C activity.

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