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CALCIUM-INDEPENDENT ACTIVATION OF PROTEIN KINASE C BY THE DIANIONIC FORM OF PHOSPHATIDIC ACID

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SUMMARY: Phosphatidic acid in the form of small unilamellar vesicles has a dissociation constant of about 8.3 as determined by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. The activation of protein kinase C (PKC) by monovalent phosphatidic acid or phosphatidylserine occurs only in the presence of Ca^{2+}. However, PKC activity on membranes of divalent anionic phosphatidic acid is independent of Ca^{2+} concentration.

Protein kinase C (PKC) plays an important role in the regulation of numerous cellular processes [1]. Most isoforms of the enzymes are activated when membrane-bound and require the presence of phosphatidylserine and Ca^{2+} ions to catalyze the phosphorylation of histone. Other anionic lipids, such as phosphatidic acid [3,4], cardiolipin [3] and phosphatidylinositol-4,5-bisphosphate [5] can replace phosphatidylserine as an activator of PKC. Addition of 1,2 diacylglycerides generally reduces the concentration of Ca^{2+} required to activate PKC [6]. In the case of the phosphatidic acid-dependent activity of PKC, the effects of diacylglycerides on the calcium dependence of the activity are very marked. Low concentrations of diolein cause the activity of the enzyme to be virtually independent of Ca^{2+} concentration, while at higher diolein content in the membrane, Ca^{2+} is actually inhibitory [7]. At pH 7.4, the pH at which the activity of PKC is most commonly assayed, phosphatidic acid is a mixture of singly and doubly ionized forms. To evaluate the role of the state of ionization of phosphatidic acid on its ability to activate PKC in the presence and absence of Ca^{2+}, we have undertaken a study of the pH dependence of the rate PKC-catalyzed reactions.

MATERIALS AND METHODS

Materials: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), purchased from Avanti Polar Lipids (Alabaster, AL),

ABBREVIATIONS

PKC, protein kinase C; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

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were pure on thin layer chromatography and were used without further purification. Histone (type III-S) and protamine sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]-Adenosine-5-triphosphate was obtained from NEN (Montreal, Quebec). Magnesium nitrate was purchased from Alfa Chemical Co. and Ultra Pure Tris was obtained from BRL (Gaithersburg, MD). The enzyme PKC was purified from rat brain [8].

NMR Measurements: ^{31}P NMR spectra of phosphatidic acid in the form of sonicated vesicles suspended in 20 mM Tris-HCl, adjusted to the desired pH were recorded on a Bruker WM-250 NMR spectrometer operating at a frequency of 101.23 MHz and employing broadband proton decoupling. A lock signal was provided by the addition of 10% $^{2}H_2O$. The resonance frequencies were referenced to phosphatidylcholine (-0.84 ppm) and the computer spectral reference was used for calibration of subsequent experiments at varying pH. The accuracy in the chemical shift is $\pm$ 0.09 ppm. After addition of base or acid, the small unilamellar vesicles were sonicated before the pH and NMR spectra were recorded.

Vesicle Assay: PKC activity was measured in the presence of sonicated vesicles using conditions similar to those previously described by Nishizuka and coworkers [9]. Phospholipid films (1.2 μmoles) were suspended in 1 ml buffer (50 mM Tris-HCl, pH=7.4). The lipid suspension was sonicated until visually clear in a bath sonicator (Ultrasonic Cole-Palmer). One 100 μl aliquot of a small unilamellar vesicle suspension was used in a final assay volume of 250 μl containing 20 mM Tris-HCl, 10 mM magnesium nitrate, 200 μg/ml histone III-S or protamine sulfate, 200 μM $CaCl_2$ (when Ca^{2+} was not present, 25 μl 20 mM EGTA was added) and 10 μM [γ-^{32}P]-adenosine-5-triphosphate. The total lipid concentration in the assay was 480 μM. To initiate the reaction, a 25 μl aliquot of PKC (150 ng protein) was added and after briefly vortexing the tubes were incubated for 10 minutes at 30°C. The reaction was terminated by adding 100 μl of cold 5 mg/ml bovine serum albumin and 2 ml of cold 25% trichloroacetic acid. The samples were placed on ice for 15 minutes, then filtered through Whatman GF/C filters which were then washed 5 times with 2 ml each of ice cold trichloroacetic acid. After drying, the filters were counted for their radioactivity.

RESULTS

The ^{31}P NMR chemical shift of the phosphate moiety of phosphatidic acid is sensitive to pH. The ^{31}P NMR spectra of the small unilamellar vesicles of phosphatidic acid exhibits two resonances. We assume that the two signals correspond to those from the inner and outer monolayers of the vesicle bilayer and that the more intense signal comes from the outer monolayer. In these vesicles of POPA, the apparent pK_a for the phosphate group is 8.3 for lipids at either side of the membrane (Fig. 1). Addition of zwitterionic phosphatidylcholine up to 50 mol% does not alter the dissociation behaviour of POPA. However, addition of 50 mol% sphingosine, a cationic PKC inhibitor, decreases the apparent pK_a of POPA significantly to $\sim$7.3 (data not shown).

Changes in the pH have opposite effects on the phosphatidic acid-dependent activity of PKC in the presence and absence of 200 μM $CaCl_2$ (Fig. 2). As a result, at pH 8.5 the activity of PKC in the presence of POPA is virtually independent of the presence of Ca^{2+}. In contrast to POPA, Ca^{2+} is required at all pH values for the activation of PKC in the presence of POPS

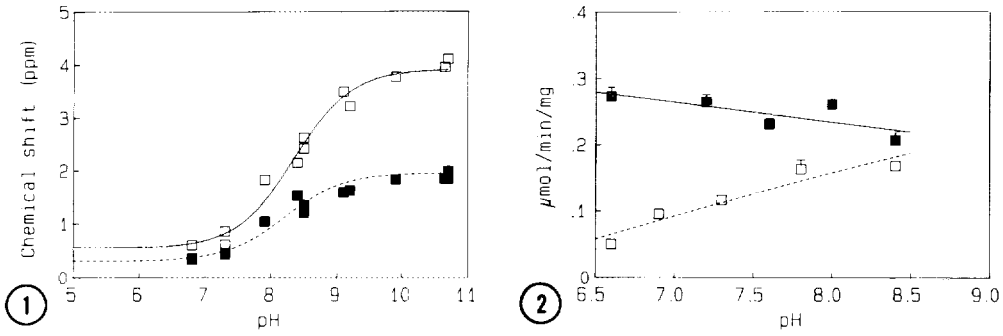

Figure 1. Dissociation of POPA in small unilamellar vesicles with the aqueous pH as measured by ^{31}P NMR spectroscopy. Open squares represent POPA dissociation curve in the outer monolayer and filled squares in the inner monolayer. No $CaCl_2$ was added to the NMR samples.

Figure 2. PKC activity determined with the vesicle assay using small unilamellar vesicles consisting of POPA at various aqueous pH in the presence (■) and absence (□) of 200 μM $CaCl_2$.

(Fig. 3). The pH dependence of PKC in the presence of POPS and Ca^{2+} is not very different from that previously reported using a Triton-micelle assay [10].

There is a high rate of PKC-catalyzed phosphorylation of protamine sulfate in the absence of lipid or calcium cofactors [11]. The pH dependence of the rate of PKC-catalyzed phosphorylation of protamine sulfate (Fig. 4) is different from the pH dependencies measured in the presence of lipid. Therefore, changes in enzyme rates as a function of pH for the lipid-activated enzyme are not due solely to direct effects of pH on the state of protonation of PKC but rather likely represent changes in lipid-protein interaction.

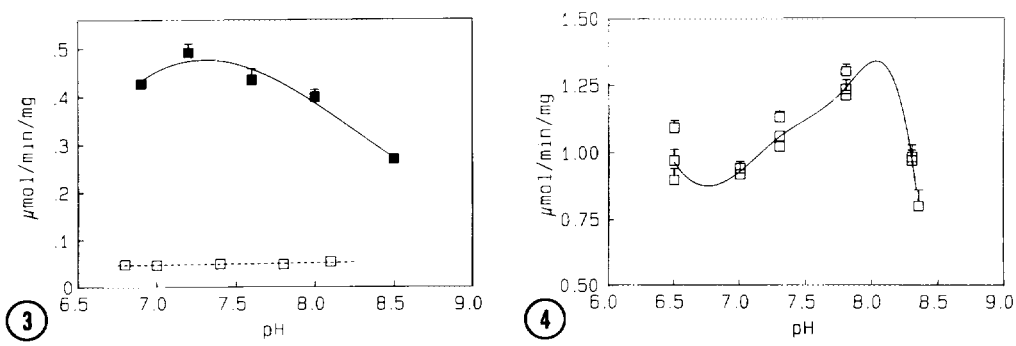

Figure 3. PKC activity determined with the vesicle assay using small unilamellar vesicles consisting of POPS at various aqueous pH in the presence (■) and absence (□) of 200 μM $CaCl_2$.

Figure 4. Protamine sulfate phosphorylation by PKC in the absence of phospholipid and Ca^{++}.

DISCUSSION

The intrinsic pK for the second ionization of phosphatidic acid is expected to be about 7.2. However, because of electrostatic repulsion among phosphate groups on the membrane and because the negatively charged membrane surface will attract protons, the surface pH will not equal the bulk pH. As a result the titration curve of phosphatidic acid is broadened and shifted to higher pH values (Fig. 1). At pH 7.4, the singly ionized form of phosphatidic acid predominates but there is also a significant concentration of the doubly ionized form.

Increasing the fraction of the doubly ionized form of phosphatidic acid by raising the pH eliminates the calcium dependence of PKC-catalyzed phosphorylation of histones (Fig. 2). This loss of the calcium requirement at high pH does not occur with vesicles composed of phosphatidylserine (Fig. 3). We have shown previously [7] that diolein readily induces calcium-independent PKC-catalyzed phosphorylation with phosphatidic acid. We suggested that this effect may be a consequence of the small size of the phosphatidic acid headgroup, which is further spread apart by the presence of diolein. In this regard, higher pH will also spread the headgroups of POPA further apart as a result of forming a doubly charged lipid which will incur greater electrostatic repulsion among the headgroups and also reduce intermolecular hydrogen bonding. This destabilized membrane may be required for the insertion of PKC in the absence of calcium.

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