Differential Membrane-Binding and Activation Mechanisms of Protein Kinase C-α and -ε†

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ABSTRACT: To elucidate the mechanisms of membrane binding and activation of conventional and novel protein kinase C (PKC), we measured the interactions of rat PKC-α and -ε with phospholipid monolayers and vesicles of various compositions. Besides the established difference in calcium requirement, the two isoforms showed major differences in their membrane-binding and activation mechanisms. For PKC-α, diacylglycerol (DG) specifically enhanced the binding of PKC-α to phosphatidylserine (PS)-containing vesicles by 2 orders of magnitude, allowing PKC-α high specificity for PS. Also, PKC-α could penetrate into the phospholipid monolayer with a packing density comparable to that of the cell membrane only in the presence of Ca^{2+} and PS. When compared to PKC-α, PKC-ε had lower binding affinity for PS-containing vesicles both in the presence and in the absence of DG. As a result, PKC-ε did not show pronounced specificity for PS. Also, PKC-ε showed reduced penetration into PS-containing monolayers, which was comparable to the Ca^{2+}-independent penetration of PKC-α into the same monolayers. Taken together, these results suggest the following: (1) The role of Ca^{2+} in the membrane binding of PKC-α is to expose a specific PS-binding site. (2) Once bound to membrane surfaces, PS specifically induces the partial membrane penetration of PKC-α that allows its optimal interactions with DG, hence the enhanced membrane binding and activation. (3) PKC-ε, due to the lack of Ca^{2+} binding, cannot specifically interact with PS and DG, which implies the presence of other physiological activator(s) for this isoform.

Protein kinases C (PKC)[1] are a family of serine/threonine kinases that transduces the myriad of signals activating cellular functions and proliferation (*1*−*3*). More than 10 members of the PKC family have been identified by molecular cloning. On the basis of common structural features, PKCs are classified into three groups: conventional PKC (α, β_I, β_{II}, and γ subtypes), novel PKC (δ, ε, η, and θ subtypes), and atypical PKC (ζ and ι subtypes). Conventional PKCs are activated by Ca^{2+}-dependent translocation to the membrane containing phosphatidylserine (PS) and diacylglycerol (DG). On the other hand, novel PKCs and atypical PKCs can be activated in a Ca^{2+}-independent way (*4*, *5*), and in particular, atypical PKC might be regulated in a Ca^{2+}- and DG-independent manner (*6*). On the basis of extensive studies on conventional PKCs, it has been generally proposed that the specific binding of conventional PKC to DG- and PS-containing membranes results in an exposure of the pseudosubstrate region that blocks the active site of PKC and hence the enzyme activation (*7*). Conventional PKCs bind anionic phospholipids with little selectivity in the absence of DG but show pronounced specificity for PS in the presence of DG (*8*). Mechanisms of the membrane binding and activation of conventional PKCs, including the mechanism of this synergistic effect between PS and DG, are not fully understood. Furthermore, no systematic analysis of membrane binding and activation of other types of PKCs has been reported to date. To investigate these aspects, we measured the interactions of recombinant rat PKC-α and -ε with phospholipid monolayers and vesicles of various compositions. Results show that specific interactions between the headgroup of PS and PKC-α trigger the penetration of the protein into the membrane, which in turn promotes the binding of PKC-α to DG and the activation. This report also demonstrates that there are major differences in membrane-binding and activation mechanisms between conventional and novel PKCs besides their different calcium requirements.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1,2-*sn*-dioleoylglycerol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Hereinafter, DG refers to 1,2-*sn*-dioleoylglycerol. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-D-serine [POP(D)S] was synthesized as described previously (*9*). Tritiated POPC ([^{3}H]POPC) was prepared from 1-palmitoyl-2-hydroxy-*sn*-

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[1] Abbreviations: ATP, adenosine triphosphate; DG, diacylglycerol (or 1,2-*sn*-dioleoylglycerol); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PG, phosphatidylglycerol; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POP(D)S, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-D-serine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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glycero-3-phosphocholine and [9,10-^{3}H]oleic acid using rat liver microsomes as described (*10*, *11*). Phospholipid concentrations were determined by phosphate analysis (*12*). [γ-^{32}P]Adenosine triphosphate (ATP, 3 Ci/μmol) was from Amersham (Arlington Heights, IL), and cold ATP was from Sigma (St. Louis, MO). Triton X-100 was from Pierce Chemical Co. (Rockford, IL). Restriction endonucleases and enzymes for molecular biology were obtained from either Boehringer Mannheim Biochemicals (Indianapolis, IN) or New England Biolabs (Beverly, MA).

Expression of PKC in Baculovirus-Infected Sf9 Cells. Wild-type rat PKC-α and PKC-ϵ were expressed in baculovirus-infected Sf9 cells (Invitrogen, La Jolla, CA). The cDNAs of these PKCs were kindly provided by Dr. Nishizuka of Kobe University. To subclone the coding sequence of PKC-α into pVL1392 baculovirus transfer vector (Pharmingen, San Diego, CA), new restriction sites (*Not*I and *Eco*RI) were created in the PKC-α gene by polymerase chain reaction mutagenesis using Pfu polymerase (Stratagene, La Jolla, CA). The entire coding region of the PKC-α gene in the resulting vector (designated pVL1392-PKC-α) was verified by sequence analysis using a Sequenase 2.0 kit (Amersham). The same procedure was followed in the construction of the pVL1392-PKC-ϵ vector except that new *Bgl*II and *Not*I restriction sites were created for subcloning. Transfection of Sf9 cells was performed using the BaculoGold Transfection Kit from Pharmingen. This procedure makes use of baculovirus DNA containing a lethal deletion. The cotransfection of the viral DNA with a complementing plasmid, pVL1392-PKC-α or pVL1392-PKC-ϵ, reconstituted viable virus inside insect cells. Prior to transfection, endotoxins were removed from plasmid DNA using an LPS extraction kit (Qiagen, Valencia, CA). Cells were incubated for 4 days at 27 °C, and the supernatant was collected and used to infect more cells for the amplification of virus. After three cycles of amplification, high-titer virus stock solution was obtained. Sf9 cells were maintained as monolayer cultures in TMN-FH medium (Invitrogen) containing 10% fetal bovine serum (GibcoBRL, Grand Island, NY). For protein expression, cells were grown to 2×10^6 cells/mL in 500 mL suspension cultures and infected with the multiplicity of infection of 10. The cells were then incubated for 3 days at 27 °C. For harvesting, cells were centrifuged at 1000*g* for 10 min, washed once with Tris-HCl buffer, pH 7.5, and resuspended in 25 mL of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, 1% Triton X-100, and 0.2 mM phenylmethanesulfonyl fluoride. The suspension was homogenized in a hand-held homogenizer chilled on ice. The extract was centrifuged at 50000*g* and 4 °C for 40 min. The supernatant was loaded onto a 100-mL Q-Sepharose Fast Flow column (Pharmacia). After a wash with 100 mL of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, and 1 mM dithiothreitol), the column was eluted with 200 mL of a linear salt gradient to 0.5 M KCl in buffer A. Active PKC fractions were pooled, adjusted to 2 M KCl, and loaded onto a 10-mL Poros PE column (Boehringer Mannheim) with a flow rate of 4 mL/ min. A linear salt gradient from 2 to 0 M KCl in buffer A (total volume: 60 mL) was applied. Active PKC fractions were concentrated and desalted in an Ultrafree-15 centrifugal filter device (Millipore) and applied to a Mono-Q HR 5/5 column (Pharmacia). The protein was eluted with a linear gradient from 0 to 0.3 M KCl in buffer A (total volume: 15 mL). The PKC peak emerged at 0.15 M KCl and showed a single band with molecular masses of 80 (PKC-α) and 90 kDa (PKC-ϵ) on a SDS−polyacrylamide electrophoresis gel. Protein concentration was determined by the bicinchoninic acid method (Pierce).

Determination of PKC Activity. Activity of PKC was assayed by measuring the initial rate of [^{32}P]phosphate incorporation from [γ-^{32}P]ATP (50 μM, 0.6 μCi/tube) into the peptide substrate (PRKRQGSVRRRVHQVNG, 100 μM in assay), histone III-SS (400 μg/mL), myelin basic protein (200 μg/mL), or protamine sulfate (200 μg/mL) (all from Sigma). The reaction mixture contained Triton X-100 (1.56 mM)/POPS (0.28 mM)/DG (0.02 mM) mixed micelles or large unilamellar vesicles (0.2 mM), 5 mM $MgCl_2$, and 50 μM $CaCl_2$ in 50 μL of 20 mM Tris-HCl, pH 7.5. Since both PKC-α and PKC-ϵ showed high background activity toward the peptide substrate and protamine sulfate in the absence of calcium and PS, these substrates were used mainly for routine assays during purification. Protamine sulfate was also used to assess the free enzyme concentration in vesicle binding measurements (see below) because both PKC isoforms show the highest activity toward this substrate. Free calcium concentration was adjusted using a mixture of EGTA and $CaCl_2$ according to the method of Bers (*13*). Reactions were started by adding the enzyme to the mixture, incubated for a given period (e.g., 5 min for histone) at room temperature, and quenched by adding 50 μL of 1% aqueous phosphoric acid solution. Seventy microliters of quenched reaction mixtures was spotted on P-81 ion-exchange paper (Whatman), washed 4 times with 1% aqueous phosphoric acid solution, and washed once with 95% aqueous ethanol. Papers were transferred into scintillation vials containing 4 mL of scintillation fluid (Sigma), and radioactivity was measured by liquid scintillation counting. The linearity of the time dependence of the reaction was checked by monitoring the degree of phosphorylation at regular intervals.

Protein Kinase C−Vesicle Binding. The binding of PKC to phospholipid vesicles was measured by a centrifugation assay using sucrose-loaded large unilamellar vesicles (100 nm diameter) (*14*). Sucrose-loaded vesicles were prepared as described elsewhere (*15*, *16*). Briefly, the lipid solution was added to a round-bottomed flask, and the organic solvent was evaporated by rotary evaporation. The lipid film was suspended in 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 M sucrose and vortexed vigorously. Unilamellar vesicles were prepared by multiple extrusion through a 0.1-μm polycarbonate filter (Millipore) in a Liposofast microextruder (Avestin, Ottawa, ON). The vesicle solution was diluted 5 times with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl and centrifuged at 100000g for 30 min at 25 °C. The supernatant was removed, and the lipid pellet was resuspended in the same buffer solution. The final concentration of the vesicle solution was determined by measuring the radioactivity of a trace of [^{3}H]POPC (typically 0.1 mol %) included in all phospholipid mixtures. To measure the dependence of PKC binding to vesicles on the anionic phospholipid content, PKC (ca. 15 nM) was incubated for 15 min with sucrose-loaded vesicles (0.1 mM), 1 μM bovine serum albumin, and 50 μM Ca^{2+} (or EGTA; see Results) in 150 μL of 20 mM Tris-HCl, pH 7.5, containing 0.1 M

KCl. Bovine albumin was added to minimize the loss of protein due to nonspecific adsorption to tube walls. Vesicles were pelleted at 100000*g* for 30 min using a Sorvall RCM120EX microultracentrifuge. Aliquots of supernatants were used for protein determination by PKC activity assay using protamine sulfate as a substrate. The fraction of bound enzyme was plotted against the mole percentage of anionic lipid in vesicles. For determination of binding parameters, the enzyme concentration was varied (5−100 nM for PKC-α, 20−200 nM for PKC-ϵ) with a fixed total lipid concentration (100 μM for PKC-α, 50 μM for PKC-ϵ). The bound PKC concentration ($[E]_b$) was plotted as a function of total enzyme concentration ($[E]_T$), and values of n and K_d were determined by nonlinear least-squares analysis of the $[E]_b$ vs $[E]_T$ plot using the standard binding equation:

$$[E]_b = (1/2)\{[E]_T + K_d + [PL]_T/n - \sqrt{([E]_T + K_d + [PL]_T/n)^2 - 4[E]_T[PL]_T/n}\} \quad (1)$$

where $[PL]_T$ represents total phospholipid concentration. Equation 1 assumes that each enzyme binds independently to a site on the interface composed of n phospholipids and with a dissociation constant of K_d.

Monolayer Measurements. Surface pressure (π) of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrobalance (model C-32) as described previously (*17*, *18*). The trough (4 cm diameter × 1 cm deep) has a 0.5 cm deep well for a magnetic stir bar and a small hole drilled at an angle through the wall to allow the addition of protein solution. Phospholipid solution (5−10 μL) in ethanol/hexane (1:9, v/v) or chloroform was spread onto 10 mL of subphase (20 mM Tris-HCl, pH 7.5, containing either 0 or 0.1 mM Ca^{2+}) to form a monolayer with a given initial surface pressure (π_0). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure reading of the monolayer had been stabilized (after ca. 5 min), the protein solution (typically 50 μL) was injected into the subphase and the change in surface pressure ($\Delta\pi$) was measured as a function of time at 23 °C. Typically, the $\Delta\pi$ value reached a maximum after 20 min. The maximal $\Delta\pi$ value depended on the protein concentration and reached saturation when the protein concentration was higher than 1 μg/mL. Protein concentrations were therefore maintained above 1.5 μg/mL to ensure that the observed $\Delta\pi$ represented a maximal value. Under our experimental conditions, $\Delta\pi$ was impractical to measure when $\pi_0 > 30$ dyn/cm. Thus, the $\Delta\pi$ vs π_0 plot was extrapolated to estimate $\Delta\pi$ values in this range.

RESULTS

Vesicle Binding and Activation of PKC-α and PKC-ϵ. To determine the differences in membrane-binding and activation mechanisms between PKC-α and -ϵ, we measured the binding of the two PKC isozymes to phospholipid vesicles of various compositions. We also measured the enzyme activities in the presence of these vesicles. We first measured the binding of PKC-α to POPC/POPS and POPC/POPG vesicles as a function of the content of anionic phospholipid in the absence and presence of DG (Figure 1). As reported previously, PKC-α showed a highly sigmoidal dependence on the anionic phospholipid content (*17*−*19*). Also, it showed higher affinity for POPS-containing vesicles than for POPG-containing ones at a given mole percentage of anionic phospholipid (especially in the physiologically relevant range of 20−30 mol %), and the presence of 2.5 mol % DG caused a more pronounced difference in binding affinity. Under our experimental conditions, a half-maximal binding occurred at 13 mol % POPS and at 30 mol % POPG in the presence of 2.5 mol % DG. For a more quantitative comparison of the vesicle binding affinity of PKC-α, we determined the stoichiometry and dissociation constant for binding of PKC-α to POPC/POPS (7:3, mol/mol) and POPC/POPG (7:3) vesicles assuming n noninteracting phospholipid binding sites on a PKC-α molecule. Although assuming partitioning between bulk phase and surface might be an oversimplification given that anionic phospholipids can form domains spontaneously or in the presence of bound PKC, this analysis accounted for the binding of PKC isoforms to various vesicles and yielded binding parameters for quantitative comparison of relative binding affinity. Also, all binding measurements were performed under the same conditions to keep all potential variables constant. The binding isotherms are illustrated in Figure 2, and values of n and K_d determined from curve fitting to eq 1 (see Materials and Methods) are listed in Table 1. Note that K_d was measured under conditions of enzyme crowding on vesicles, and because of possible protein−protein interaction and structural changes of vesicles, the value obtained might be somewhat different from the K_d obtained under conditions of low vesicle coverage. The agreement between theoretical curves and experimental data indicates that the binding follows a simple noncooperative mode in terms of enzyme concentration. The putative cooperative binding of PS to PKC was not taken into account in this analysis because binding isotherms showed no sigmoidal dependency. Since K_d is expressed in

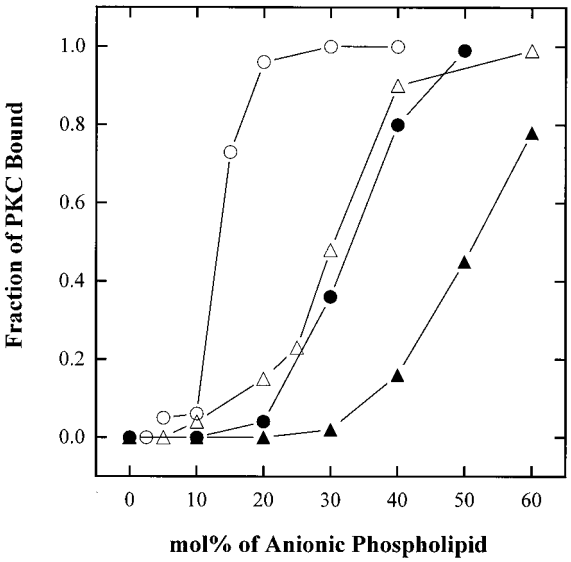

FIGURE 1: The binding of PKC-α to POPC/POPS (●, ○) and POPC/POPG (▲, △) mixed vesicles as a function of the content of POPS and POPG. The fraction of membrane-bound enzyme was determined by the centrifugation of sucrose-loaded vesicles followed by the PKC assay of supernatants. Mixed vesicles contained either no DG (●, ▲) or 2.5 mol % DG (○, △). Total lipid and PKC concentrations were 0.1 mM and 15 nM, respectively, in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, 1 μM bovine serum albumin, and 50 μM free Ca^{2+}. Each data point represents an average of two experiments.

Table 1: Parameters for Binding of PKC-α and -ϵ to Vesicles of Different Compositions

	PKC-α[a]				PKC-ϵ[b]			
vesicles	n[c]	K_d (nM)[c]	nK_d (μM)	PKC/vesicle	n[c]	K_d (nM)[c]	nK_d (μM)	PKC/vesicle
POPC/POPS (70:30)	2200 ± 300	24 ± 8	53	45	470 ± 90	440 ± 90	207	213
POPC/POPS/DG (67.5:30:2.5)	1100 ± 100	0.3 ± 0.1	0.33	91	330 ± 40	40 ± 10	12	303
POPC/POPG (70:30)	3100 ± 900	40 ± 20	118	32	430 ± 80	530 ± 90	228	233
POPC/POPG/DG (67.5:30:2.5)	1900 ± 200	13 ± 3	25	53	350 ± 50	110 ± 30	37	286

[a] Determined in 20 mM Tris-HCl buffer, pH 7.5, containing 50 μM $CaCl_2$. [b] Determined in 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM EGTA. [c] Values represent mean values ± standard errors from a minimum of two measurements.

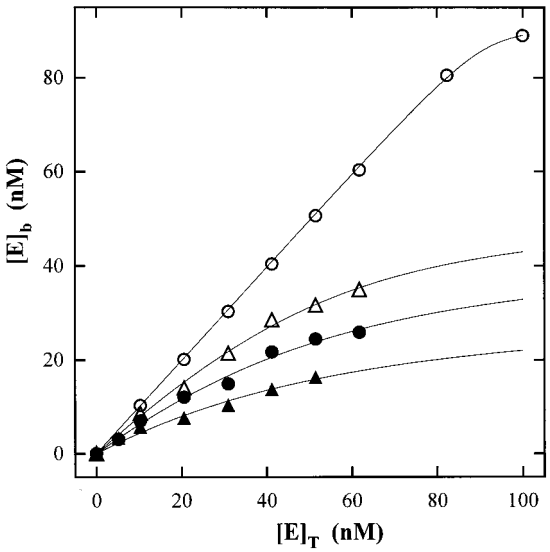

FIGURE 2: Binding isotherms of PKC-α to POPC/POPS and POPC/POPG mixed vesicles. Vesicles used were POPC/POPS (70:30) (●), POPC/POPS/DG (67.5:30:2.5) (○), POPC/POPG (70:30) (▲), and POPC/POPG/DG (67.5:30:2.5) (△). The experimental conditions were the same as those for Figure 1.

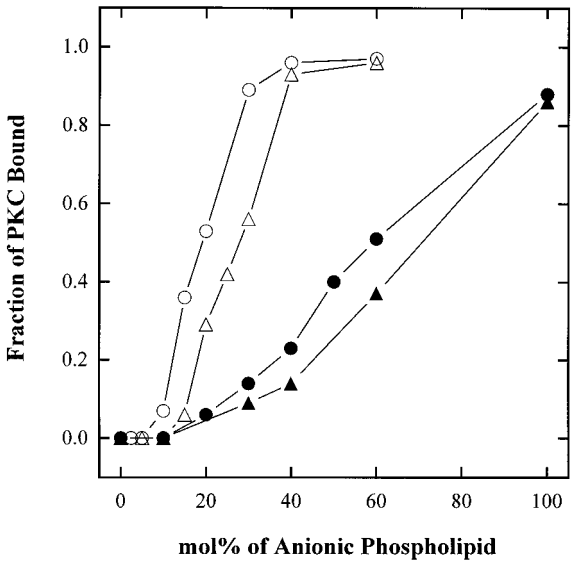

FIGURE 3: Binding of PKC-ϵ to POPC/POPS (●, ○) and POPC/POPG (▲, △) mixed vesicles as a function of the content of POPS and POPG. The fraction of membrane-bound enzyme was determined by the centrifugation of sucrose-loaded vesicles followed by the PKC assay of supernatant. Mixed vesicles contained either no DG (●, ▲) or 2.5 mol % DG (○, △). Total lipid and PKC concentrations were 0.1 mM and 15 nM, respectively, in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, 1 μM bovine serum albumin, and 0.2 mM EGTA. Each data point represents an average of two experiments.

terms of molarity of enzyme binding sites composed of n phospholipids, nK_d is the dissociation constant in terms of molarity of single lipid molecules. Also, dividing the number of phospholipids per vesicle (ca. 1×10^5 for 100-nm vesicles) by n yields the number of PKC molecules bound per vesicle. As shown in Figure 2, PKC-α showed exceptionally high affinity for POPC/POPS/DG (67.5:30:2.5) vesicles. This, in conjunction with the minimum requirement of phospholipid concentration for complete pelleting of liposomes ($[PL]_T = 50$ μM), made it necessary to measure the binding of PKC-α under the conditions in which a majority of enzyme molecules were bound to vesicles (i.e., $[E]_b \approx [E]_T$). Due to this limitation, the K_d value for POPC/POPS/DG (67.5:30:2.5) vesicles might represent an upper limit estimate. Taking this into account, 2.5 mol % DG enhanced the binding of PKC-α to POPC/POPS (7:3) vesicles at least 160-fold. In comparison, the same concentration of DG caused only a 5-fold increase in binding to POPC/POPG (7:3) vesicles. As a result, in the presence of physiologically relevant 2.5 mol % DG and 30 mol % anionic phospholipid, PKC-α prefers PS-containing vesicles to PG-containing vesicles by 2 orders of magnitude. In the absence of DG, the difference is only 2-fold. This compares well with the reported PS preference for other conventional PKCs estimated from the dependence of their vesicle binding on the anionic phospholipid content (*8*, *19*).

The n values listed in Table 1 provide additional information about how differently PKC-α binds to different vesicles. Assuming a spherical protein molecule, one can estimate from the molecular weight of PKC-α (ca. 77 000) and the average density of protein (1.37 g/cm^3) (*20*) that its molecular radius and maximal cross-sectional area are 28 Å and 2500 Å^2, respectively. Thus, it is expected from the known surface area of 100 nm diameter vesicles that up to ca. 1240 PKC molecules can bind per vesicle. Experimental values (30−90 molecules) calculated by dividing 1×10^5 by the n values in Table 1 are much smaller than this estimate even after taking into account the fact that only 30% of surfaces are occupied by anionic phospholipids in these vesicles. Thus, it appears that PKC-α is bound to the vesicle surfaces in a rather extended conformation. Also, the difference in n value between POPC/POPS (7:3) and POPC/POPG (7:3) vesicles implies that PKC-α might bind to these vesicles in different conformations (or different orientations). Finally, a significant reduction of n value in the presence of DG might suggest that the protein undergoes a conformational change into a more compact form in the presence of DG.

We then measured the binding of PKC-ϵ to these vesicles under the same conditions except that Ca^{2+} was absent from the binding mixture (Figure 3). When compared to PKC-α, PKC-ϵ showed a lower binding affinity for PS-containing vesicles and, as a result, a markedly lower degree of

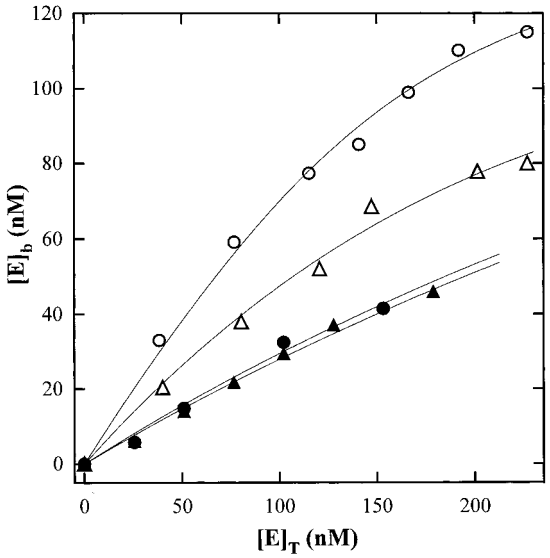

FIGURE 4: Binding isotherms of PKC-ϵ to POPC/POPS (●, ○) and POPC/POPG (▲, △) mixed vesicles. Vesicles used were POPC/POPS (70:30) (●), POPC/POPS/DG (67.5:30:2.5) (○), POPC/POPG (70:30) (▲), and POPC/POPG/DG (67.5:30:2.5) (△). The experimental conditions was the same as those for Figure 3 except that the concentration DG-containing vesicles in this experiment was 50 μM.

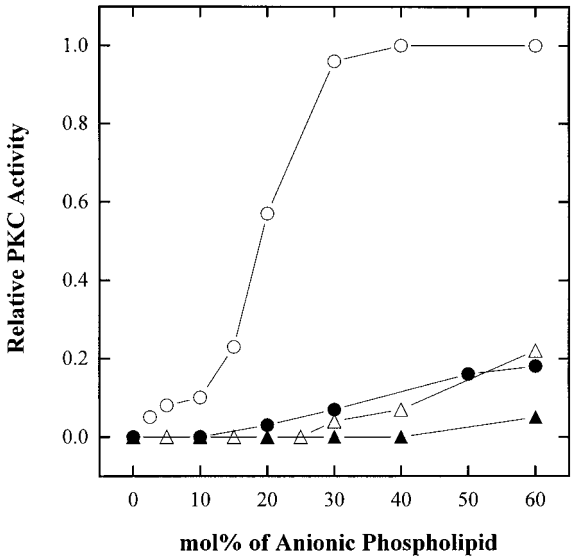

FIGURE 5: Dependence of activity of PKC-α toward histone substrate on the content of POPS (●, ○) or POPG (▲, △) in vesicles. Mixed vesicles contained either no DG (●, ○) or 2.5 mol % DG (○, △). Total lipid and PKC concentrations were 0.2 mM and 15 nM, respectively, in 20 mM Tris-HCl, pH 7.5, containing 0.1 M KCl, 5 mM $MgCl_2$, and 50 μM free Ca^{2+}. Each data point represents an average of two experiments. The absolute value of maximal activity was 0.35 nmol/(μg·min).

selectivity between PS and PG both in the presence and in the absence of DG. As described above, we determined values of n and K_d for PKC-ϵ from curve fitting of binding isotherms to eq 1 (Figure 4). The binding parameters listed in Table 1 show that 2.5 mol % DG enhances the binding of PKC-ϵ to POPC/POPS (7:3) vesicles by 16-fold, which is much lower than the ca. 160-fold increase seen for PKC-α. For POPC/POPG (7:3) vesicles, DG enhances the binding ca. 7 fold, which is comparable to the increase observed for PKC-α. As a result, the selectivity of PKC-ϵ for PS over PG is only ca. 3-fold. In terms of relative vesicle-binding affinity, PKC-ϵ binds ca. 40 times less tightly to POPC/POPS/DG (67.5:30:2.5) vesicles than PKC-α, whereas both PKC isoforms have comparable affinity for POPC/POPG/DG (67.5:30:2.5) vesicles. On the other hand, smaller n values indicate that PKC-ϵ binds to the vesicles in a more compact conformation than that of PKC-α. Also, the presence of DG and the vesicle composition do not appear to affect the binding mode of PKC-ϵ.

Finally, we measured the activity of PKC-α and PKC-ϵ in the presence of the same vesicles that were used for binding studies. PKC-α activity was measured using histone as a substrate because most commercial synthetic peptide substrates showed high background phosphorylation in the absence of Ca^{2+} and lipid cofactors. As shown in Figure 5, the activity of PKC-α toward histone also showed a highly sigmoidal dependency on the PS content in the presence of DG. In general, PKC activity reflected the binding of the protein to the vesicles, although full activity required a higher PS content than did the complete vesicle binding of protein to the same vesicles (see Figure 1). This has been observed for other conventional PKCs and ascribed to several factors including histone-induced vesicle precipitation, the requirement of a higher PS content for the binding of substrate to the vesicle, and the presence of Mg^{2+}, which lowers the membrane affinity of PKC in the binding measurements (*19, 21*). In agreement with previous reports, DG further enhanced the PKC-α activity even when all the protein molecules were bound to vesicles (*8, 19*). Under our experimental conditions, DG was able to increase the activity of PS-bound PKC-α by a factor of ca. 5, thereby corroborating the notion that the role of DG in PKC-α activation is not only to enhance the membrane binding of protein but also to increase the rate of phosphorylation. This is higher than the 2−3-fold increase in activity reported for other conventional PKCs (*8, 19*). The difference might derive from different assay conditions being employed; for example, much lower calcium and phospholipid concentrations were used in our assay. We then measured the activity of PKC-ϵ toward myelin basic protein in the same manner. Myelin basic protein was mainly used for PKC-ϵ because of its low activity toward histone. Unlike the case of PKC-α, the PKC-ϵ activity essentially reflected the extent of binding for both POPS and POPG vesicles both in the absence and in the presence of DG (Figure 6). Again a smaller degree of selectivity between PS and PG was apparent. For PKC-ϵ, DG enhanced the activity of PS-bound protein by a factor of ca. 2. Thus, the effects of DG on both the vesicle binding and the catalytic activity of PKC-ϵ are smaller than those observed for PKC-α.

Interaction of PKC-α and -ϵ with Phospholipid Monolayers. To gain molecular insights into the different effects of DG and anionic phospholipids on the membrane binding and activation of PKC-α and -ϵ, we measured the penetration of these proteins into phospholipid monolayers of various compositions. Penetration of a protein into a phospholipid monolayer at the air−water interface can be sensitively monitored in terms of the change in surface pressure ($\Delta\pi$). It was previously reported that the injection of a mixture of conventional PKCs partially purified from rat brain beneath a PS monolayer caused an increase in surface pressure (*22, 23*). The magnitude of the increase was dependent on the phospholipid composition and the initial surface pressure (π_0)

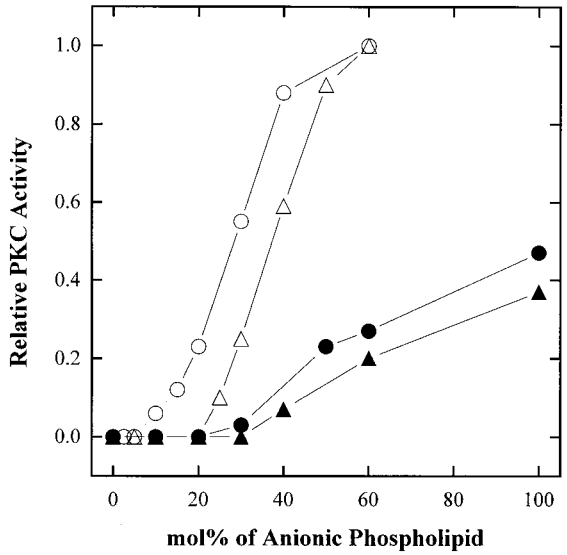

FIGURE 6: Dependence of activity of PKC-ϵ toward myelin basic protein on the content of POPS (●, ○) or POPG (▲, △) in vesicles. Mixed vesicles contained either no DG (●, ▲) or 2.5 mol % DG (○, △). Total lipid and PKC concentrations were 0.2 mM and 15 nM, respectively, in 20 mM Tris-HCl, pH 7.5, containing 0.1 M KCl, 5 mM $MgCl_2$, and 0.2 mM EGTA. Activity assay was conducted as described in Materials and Methods. The absolute value of maximal activity was 0.32 nmol/(μg·min). Activity measured using histone III-SS (30-min incubation) yielded a similar result. Each data point represents an average of two experiments.

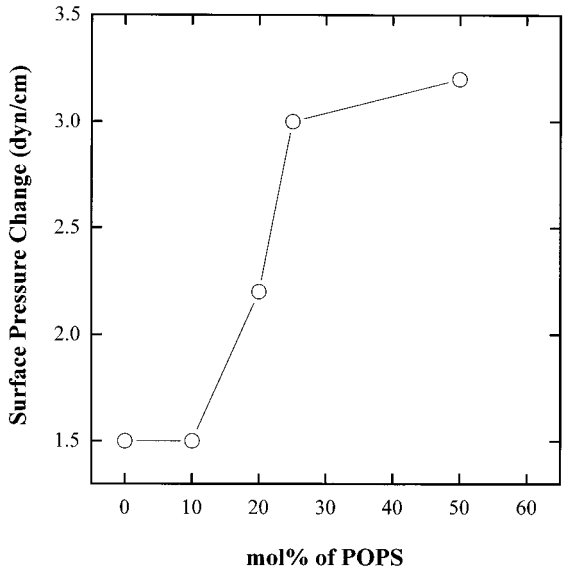

FIGURE 7: Effect of POPS content of POPC/POPS mixed monolayers on the penetration of PKC-α. The initial surface pressure was kept constant (25 ± 0.2 dyn/cm). The protein concentration in the subphase (20 mM Tris-HCl, pH 7.5, containing 0.1 mM free Ca^{2+}) was 1.5 μg/mL (20 nM). Each data point represents an average of two independent measurements.

of the monolayer, the Ca^{2+} concentration in the subphase, and the amount of injected enzyme. To systematically analyze the PKC−phospholipid monolayer interactions, we measured surface pressure changes caused by the penetration of PKC-α and -ϵ into a variety of phospholipid monolayers. First, we measured the penetration of PKC-α into POPC/POPS mixed monolayers with varying compositions. As shown in Figure 7, at a given π_0 of monolayer (25 dyn/cm) and a saturating protein concentration (1.5 μg/mL), $\Delta\pi$ was dependent on the mol percentage of POPS and showed a

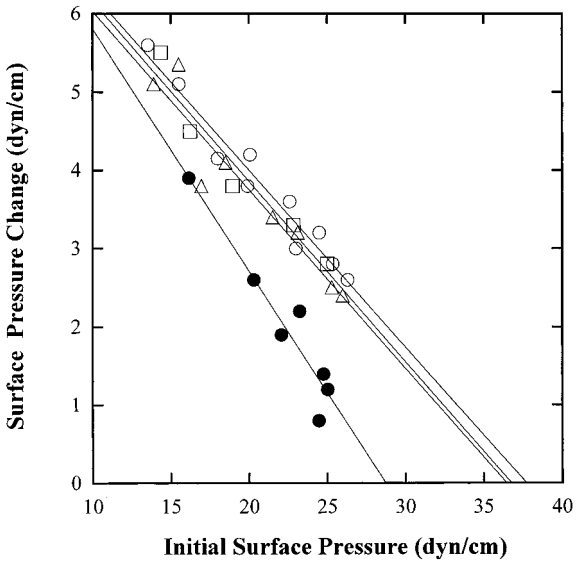

FIGURE 8: Effect of initial surface pressure of POPS-containing mixed monolayers on the penetration of PKC-α. The mixed monolayers were POPC/POPS (7:3) (○), POPE/POPS (7:3) (△), and POPC/POPS/DG (7:3:0.5) (□). The protein concentration in the subphase was 20 nM. The subphase was 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM free Ca^{2+}. For the POPC/POPS (7:3) monolayer, the penetration was also measured using the subphase containing no free Ca^{2+} (1 mM EGTA) (●). Each data point is from a single measurement.

maximum at ≥25 mol % PS. All ensuing measurements were therefore performed with phospholipid monolayers containing 30 mol % POPS (and other anionic phospholipids). Second, we measured the penetration of PKC-α into the POPC/POPS (7:3, mol/mol) monolayer as a function of π_0. Qualitatively, a higher π_0 indicates a higher surface packing density of monolayer. Those proteins (e.g., apolipoproteins) whose actions involve partial or full penetration of membranes have an ability to penetrate into the phospholipid monolayer with π_0 comparable to or higher than that of biological membranes (ca. 31 dyn/cm) (*24*−*27*), and vice versa. In general, $\Delta\pi$ is inversely proportional to π_0 of the phospholipid monolayer, and an extrapolation of the $\Delta\pi$ vs π_0 plot yields the critical surface pressure (π_c) (*28*) which specifies an upper limit of π_0 of a monolayer into which a protein can penetrate. Therefore, π_c should be above 31 dyn/cm if the protein is able to penetrate into the membrane under physiological conditions. As illustrated in Figure 8, the π_c value estimated for PKC-α and the POPC/POPS (7:3) monolayer was 38 dyn/cm at 0.1 mM Ca^{2+} and 29 dyn/cm in the absence of Ca^{2+}. When π_0 > 30 dyn/cm, the estimated $\Delta\pi$ for the POPC/POPS (7:3) monolayer was below 2 dyn/cm, suggesting that only partial penetration of protein would occur under these conditions. In conjunction with previous monolayer data for conventional PKCs, this result suggested that the Ca^{2+}-dependent binding of PKC-α to the cell membrane containing PS involves the partial penetration of the protein into the membrane. Although 0.1 mM is higher than the cellular Ca^{2+} concentration necessary for the activation of PKC-α, this concentration was used for the monolayer measurements facilitate the binding of PKC-α to monolayers. Lower Ca^{2+} concentrations (e.g., 5−10 μM) resulted in essentially the same degree of $\Delta\pi$ but at a slower rate (data not shown). When POPE was substituted for POPC in the mixed monolayer [i.e., POPE/POPS (7:3)

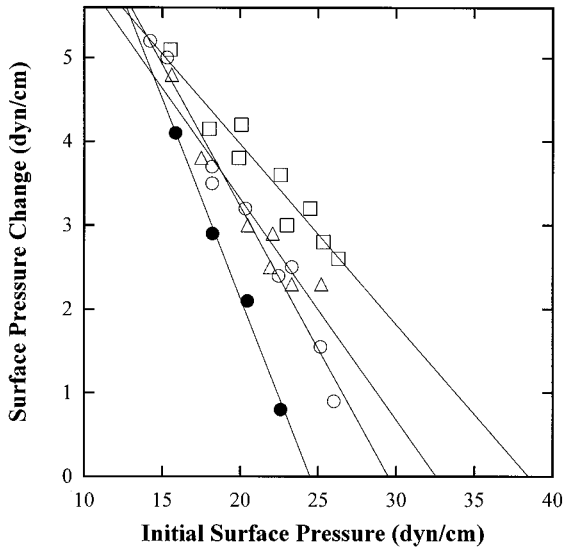

FIGURE 9: Effect of initial surface pressure of non-POPS-containing mixed monolayers on the penetration of PKC-α. The mixed monolayers were POPC/POPG (7:3) (○) and POPC/POP(D)S (7:3) (△). A plot for POPC/POPS (7:3) (□) is also shown for comparison. The protein concentration in the subphase was 20 nM. The subphase was 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM free Ca^{2+}. For the POPC/POPG (7:3) monolayer, the penetration was also measured using the subphase containing no free Ca^{2+} (1 mM EGTA) (●). Each data point is from a single measurement.

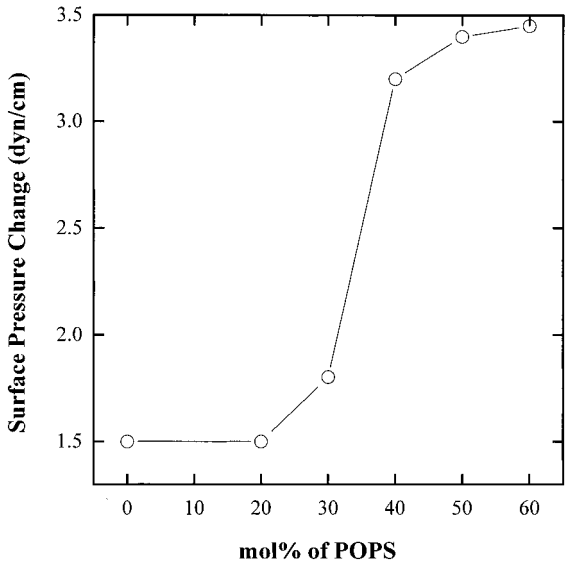

FIGURE 10: Effect of POPS content of POPC/POPS mixed monolayers on the penetration of PKC-ϵ. The initial surface pressure was kept constant (25 ± 0.2 dyn/cm). The protein concentration in the subphase (20 mM Tris-HCl, pH 7.5, and 1 mM EGTA) was 20 nM. Each data point represents an average of two independent measurements.

monolayer], essentially the same pattern was observed. Furthermore, the presence of up to 5 mol % DG in POPC (or POPE)/POPS (7:3) monolayer did not cause any detectable changes in the membrane penetrating power of PKC-α, thereby indicating that DG itself plays no role in enhancing the membrane penetration of PKC-α. In addition, we measured the effect of other anionic phospholipids on the PKC-α−monolayer interactions. As shown in Figures 1 and 5, PS is the only anionic phospholipid that can fully activate PKC-α in a physiologically relevant concentration range of 20−30 mol %. Since our data show that the Ca^{2+}-dependent binding of PKC-α to a PS-containing monolayer induces the penetration of protein into the monolayer, we explored the possibility that the inability of other anionic phospholipids such as PG to activate PKC-α is due to the inability of PKC-α to penetrate into membranes containing these phospholipids. As shown in Figure 9, PKC-α showed a lesser degree of penetration into the POPC/POPG (7:3) monolayer than into the POPC/POPS (7:3) monolayer at π_0 in the range 15−25 dyn/cm. Perhaps most important, π_c for the POPC/POPG (7:3) monolayer was found to be 29 dyn/cm at 0.1 mM Ca^{2+} and 24 dyn/cm in the absence of Ca^{2+}. This result points to the direct correlation between the remarkable PS specificity in PKC-α activation and the unique ability of PS to induce the Ca^{2+}-dependent penetration of PKC-α into the membrane. To further test this notion, we measured the interaction of PKC-α with a mixed monolayer containing a synthetic anionic phospholipid, POP(D)S whose efficiency in PKC-α activation lies between those of PS and PG. It was reported that conventional PKC isoforms, due to their stereoselectivity for the L-serine headgroup of PS, had about 30% activity in the presence of this D-serine analogue of PS in in vitro activity assays (*9*). We also found that toward histone, PKC-α showed ca. 50% activity in the presence of POPC/POP(D)S/DG (67.5:30:2.5) vesicles when compared to POPC/POPS/DG (67.5:30:2.5) vesicles under the same conditions. As shown in Figure 9, POP(D)S had a reduced ability to induce the monolayer penetration of PKC-α when compared to POPS but was slightly more effective than POPG: π_c for the POPC/POP(D)S (7:3) monolayer was 33 dyn/cm at 0.1 mM Ca^{2+}. Thus, the efficacy of different anionic phospholipids to activate PKC-α is correlated well, albeit qualitatively, with their ability to induce the membrane penetration of PKC-α. Taken together, these monolayer data clearly show that high the PS specificity of PKC-α in the presence of DG derives from the ability of PS to trigger the partial penetration of the protein into the cell membrane or phospholipid vesicles.

Finally, we measured the interactions of novel PKC-ϵ with phospholipid monolayers with different compositions. The dependence of $\Delta\pi$ on the mole percentage of POPS in POPC/POPS mixed monolayers at a given π_0 (25 dyn/cm) showed that, compared to PKC-α under the same condition, PKC-ϵ caused a smaller degree of $\Delta\pi$ (Figure 10). As a result, a higher mole percentage of POPS (>40 mol %) was required for the maximal penetration of PKC-ϵ. Similarly, the $\Delta\pi$ vs π_0 plot for the POPC/POPS (7:3) monolayer yielded a π_c value of 30 dyn/cm, which was considerably lower than that for PKC-α (Figure 11). Only when a higher PS content of the mixed monolayer was used, did PKC-ϵ show a degree of monolayer penetration comparable to that of PKC-α; for example, π_c for POPC/POPS (5:5) was 38 dyn/cm. As expected, Ca^{2+} had no enhancing effect on the monolayer penetration of PKC-ϵ. As a matter of fact, the presence of 0.1 mM Ca^{2+} in the subphase decreased the π_c value (26 dyn/cm) for the POPC/POPS (7:3) monolayer. This was due mainly to reduced electrostatic potential at the monolayer surface, as 0.1 mM Mg^{2+} showed essentially the same effect (data not shown). As is the case with PKC-α, DG had no effect on the π_c value. Finally, PKC-ϵ showed essentially the same degree of penetration into the POPC/POPS (7:3) monolayer and the POPC/POPG (7:3) monolayer. This is

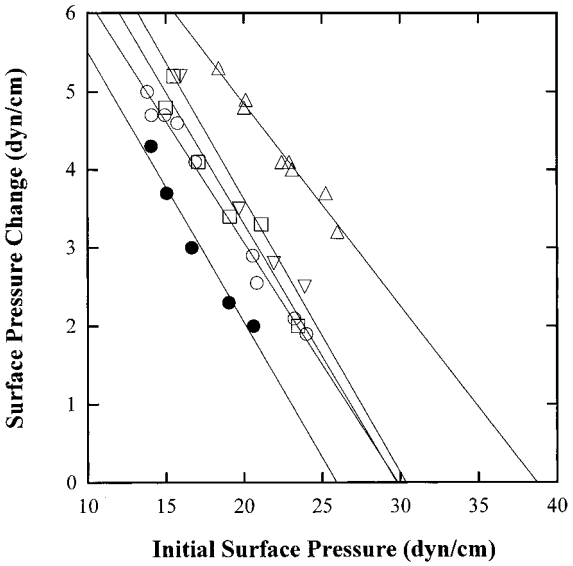

FIGURE 11: Effect of initial surface pressure of various mixed monolayers on the penetration of PKC-ϵ. Monolayers were POPC/POPS (7:3) (○), POPC/POPS (5:5) (△), POPC/POPS/DG (7:3:0.5) (□) and POPC/POPG (7:3) (▽). The subphase was 20 mM Tris-HCl, pH 7.5, containing 1 mM EGTA. For the POPC/POPS (7:3) monolayer, the penetration was also measured using the subphase containing 0.1 mM free Ca^{2+} (●). Protein concentration was 20 nM. Each data point is from a single measurement.

consistent with the vesicle-binding and activation data for PKC-ϵ. Thus, the monolayer data again demonstrate distinct membrane-binding properties of PKC-ϵ.

DISCUSSION

This report describes the first systematic comparison of membrane-binding and activation mechanisms of conventional (PKC-α) and novel (PKC-ϵ) PKCs. It is evident from this study that the differences are beyond the requirement of Ca^{2+} as once thought. For rigorous quantitative analysis of binding, we determined the parameters n and K_d for the two PKC isoforms at physiologically relevant concentrations of 2.5 mol % DG and 30 mol % anionic phospholipids. As described in Results, accurate determination of n and K_d was hampered by two experimental limitations. First, the binding of PKC-α to PS vesicles was so tight that the binding was measured under the condition $E_T \approx E_b$. Second, the binding of PKC-ϵ to vesicles in the absence of DG was not tight enough to give saturation in the enzyme concentration range used in this study. Despite these limitations, theoretical curves constructed using the best-fit values of n and K_d were generally in good agreement with experimental data. Further, a simulated curve drawn using n and K_d values beyond the range of standard error of each best-fit value noticeably deviated from the experimental data. Thus, the binding parameters listed in Table 1 should allow reasonably accurate comparison of relative membrane-binding affinities of PKC isoforms for various vesicles. In agreement with previous reports, DG specifically enhances the binding of PKC-α to PS-containing vesicles by 2 orders of magnitude, allowing PKC-α high specificity for PS (*19*, *29*). Also, DG improves the catalytic efficiency of the enzyme. Furthermore, the enzyme activity in the presence of different vesicles largely reflects the binding affinity for the vesicles. Finally, n values for different vesicles reveal that PKC-α might bind to these vesicles in different conformations (or orientations). Besides these findings, this study provides an important new insight into the origin of PS−DG synergism. As pointed out by Newton and Keranen (*8*), the high PS specificity of conventional PKC in the presence of DG can be accounted for by two different models. One model assumes that the PS binding of PKC exposes its DG binding site, and the other holds that DG binding exposes a specific PS binding site. Our monolayer data clearly show that the high PS specificity of PKC-α in the presence of DG derives from the ability of PS to trigger the penetration of the protein into the membrane. On the other hand, the inability of DG to induce the penetration of PKC-α into the membrane containing either PS or PG makes the alternative model seem unlikely. Other anionic phospholipids with reduced ability to enhance the membrane binding and activity of PKC-α are incapable of inducing the penetration of the protein into compactly packed monolayers. Although DG is known to form a hexagonal phase, DG at 2.5 mol % would not cause a lateral phase separation of vesicle surfaces but would be randomly embedded in the phospholipid matrix (*30*, *31*). Because DG lacks a large polar headgroup, it would be slightly buried from the polar surface of the phospholipid membrane, and as a result, it might not be readily accessible to the surface-bound PKC. Thus, the PS-induced partial penetration would allow PKC to interact more efficiently with DG. Because the binding of DG to PKC was shown to generate a new hydrophobic surface on PKC (*32*), the DG-bound PKC would have stronger hydrophobic interactions with the membrane core, and this should account for the large enhancement of the membrane-binding affinity of PKC-α. The notion that DG binding of PKC-α requires the penetration of protein into the membrane is supported by a recent NMR study on the interactions of the PKC−γ DG-binding domain with lipid micelles which showed that half of the domain was capable of inserting into membranes (*33*). Mosior and Newton (*34*) argued that the binding of PKC-β_{II} to DG or phorbol esters does not involve significant penetration of the enzyme into the hydrophobic core of the membrane on the basis of the lack of fluorescence quenching of PKC tryptophans by bromines located at various positions in the hydrophobic core of the membrane. It should be noted, however, that at the compactly packed membrane surface (i.e., $\pi_0 > 30$ dyn/cm), the protein would only partially penetrate into the membrane (i.e., $\Delta\pi < 2$ dyn/cm), and this should be sufficient for making contact with DG. Indeed, Souvignet et al. (*23*) found that conventional PKC showed the highest activity toward a substrate in the monolayer at the surface pressure which allowed only partial penetration of protein into the monolayer. This partial penetration might not lead to the quenching of tryptophan fluorescence by brominated phospholipids even if tryptophans are involved in penetration. Our monolayer data provide additional insights into the presence of a specific PS binding site in PKC-α and the role of Ca^{2+} in the membrane binding of PKC-α. At a given surface pressure, POPC/POPS (7:3), POPC/POP(D)S (7:3), and POPC/POPG (7:3) monolayers should have essentially the same physical properties. Thus, the selective penetration of PKC-α into the POPC/POPS (7:3) monolayer when $\pi_0 > 30$ dyn/cm points to the presence of a specific PS binding site in the protein. In that case, selective enhancing effects of Ca^{2+} on the PKC-α penetration into PS monolayers would

suggest that the role of Ca^{2+} is to cause a conformational change to expose a PS binding site rather than simply to anchor the protein to the anionic membrane surface.

Compared to PKC-α, PKC-ϵ shows several distinct properties besides different Ca^{2+} requirements. First, in the absence of DG, PKC-ϵ has a lower binding affinity for PS-containing vesicles and shows reduced penetration into PS-containing monolayers. Since PKC-α would not bind vesicles with detectable affinity in the absence of Ca^{2+}, it is difficult to directly compare the vesicle-binding behaviors of PKC-α and PKC-ϵ in the absence of Ca^{2+}. The monolayer measurements, however, allow this direct comparison because both isoforms show significant monolayer penetration at lower initial surface pressures even in the absence of Ca^{2+}. The finding that the penetration of PKC-ϵ into POPC/POPS (7:3) and POPC/POPG (7:3) monolayers is comparable to the Ca^{2+}-independent penetration of PKC-α into the same monolayers in conjunction with lower membrane-binding affinity of PKC-ϵ suggests that PKC-ϵ lacks an alternative apparatus that effectively replaces the role of Ca^{2+}. In particular, the inability of PKC-ϵ to recognize PS-containing monolayers is consistent with the notion that the role of Ca^{2+} in the membrane binding of conventional PKCs is to expose a PS binding site. Second, DG enhances the binding of PKC-ϵ to PS-containing vesicles to a much lesser degree and improves the activity of membrane-bound enzyme to a lesser extent. As a result, PKC-ϵ does not show pronounced specificity for PS either in the presence or in the absence of DG. The smaller effects of DG can be explained in terms of lower membrane penetrating ability of PKC-ϵ. As described above, the optimal interactions between PKC-α and DG appear to require a partial penetration of the protein into the membrane. When PKC cannot achieve this partial penetration, DG will have a much smaller effect on the binding and the enzyme activity. Indeed, the enhancing effects of DG on the binding of PKC-α to PG-containing vesicles and on the binding of PKC-ϵ to both PS- and PG-containing vesicles are all comparable and much smaller than the effect of DG on the membrane binding of PKC-α to PS-containing vesicles. Finally, these results raise a question as to whether PS and DG are genuine physiological activators of PKC-ϵ. It is conceivable that there are one or more yet unknown physiological activators for PKC-ϵ and other novel PKC isoforms. For instance, it is conceivable that a highly anionic phospholipid (e.g., a phosphoinositide) could greatly enhance the recruitment of enzyme to membrane surfaces, given that at higher concentrations of anionic phospholipids PKC-ϵ can bind vesicles and monolayers as well as PKC-α does.

In summary, this study provides new insights into the mechanism of membrane binding and activation of conventional (PKC-α) and novel (PKC-ϵ) PKC. For PKC-α, the role of Ca^{2+} appears to be to expose a specific PS binding site. Once bound to membrane surfaces, PS specifically induces the partial membrane penetration of PKC-α that allows its optimal interactions with DG, hence the enhanced membrane binding and activation. PKC-ϵ, due to the lack of Ca^{2+} binding, cannot specifically interact with PS, and thus DG has relatively lower and nonselective enhancing effects on its binding and activation. The mechanism by which PS specifically induces the partial membrane penetration of PKC-α remains to be elucidated.

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