Mechanism of Interaction of Protein Kinase C with Phorbol Esters

REVERSIBILITY AND NATURE OF MEMBRANE ASSOCIATION*

Marian Mosior and Alexandra C. Newton‡

From the Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0640

A variety of approaches have been employed to demonstrate that the interaction of protein kinase C βII with phorbol ester-containing membranes is reversible, is not accompanied by significant insertion of the protein into the hydrophobic core of the membrane, and is qualitatively similar to the interaction with diacylglycerol (DG). First, we show that under conditions when protein kinase C is bound with equal affinity to membranes containing either DG or phorbol myristate acetate (PMA), increasing ionic strength causes a similar reduction in membrane binding. The similar sensitivity to ionic strength indicates that the forces mediating the binding of protein kinase C to PMA are not significantly different from those mediating the binding to DG. At sufficiently high concentrations of PMA and relatively low concentrations of phosphatidylserine, the binding of protein kinase C to membranes became markedly less sensitive to ionic strength, suggesting that under these conditions direct non-electrostatic interactions with PMA dominate over electrostatic interactions with the lipid headgroups. Importantly, regardless of the strength of the interaction with PMA, protein kinase C exchanges between vesicle surfaces: protein kinase C bound first to phorbol ester-containing multilamellar vesicles exchanged to large unilamellar vesicles upon addition of an excess surface area of the latter. Lastly, the enzyme's intrinsic tryptophan fluorescence was not quenched by bromines located at various positions in the hydrophobic core of the membrane. In contrast, the enzyme's tryptophan fluorescence was significantly quenched by probes positioned at the membrane surface. In summary, our results are consistent with protein kinase C binding reversibly to PMA- or DG-containing membranes primarily via interactions at the membrane interface.

Generation of diacylglycerol (DG)[1] in the plasma membrane is the key signal in activating most members of the lipid-regulated family of protein kinase C isozymes (1, 2). This lipid second messenger has recently been shown to regulate protein kinase Cs function by dramatically increasing the enzyme's affinity for phosphatidylserine (PS)-containing membranes (3–5). The resulting high affinity membrane interaction leads to a conformational change that activates the enzyme: specifically, the autoinhibitory pseudosubstrate domain of the molecule (6) is removed from the active site, thus allowing substrate binding and catalysis (7, 8).

Phorbol esters are functional analogues of DG: they compete for the same binding site on the molecule as DG (9), and they can replace DG in activating the enzyme (10, 11). Unlike DGs, which are metabolized within minutes (12, 13), phorbol esters are long lived in the cell. As a result, these molecules have proved invaluable in activating protein kinase C *in situ*, and a plethora of studies over the past decade have described the phorbol ester-dependent translocation of protein kinase C to cellular membranes (11, 14). However, several reports have questioned whether phorbol esters regulate protein kinase C by the same mechanism as DGs. Notably, it has been proposed that phorbol esters "convert" protein kinase C into a constitutively active form that is irreversibly inserted into the membrane (15–18). Evidence for this derives from observations that the association of protein kinase C with membranes containing phorbol esters, both *in vivo* and *in vitro*, is only partially reversed by chelation of Ca^{2+} (15–19), contrasting with the quantitative dissociation of protein kinase C from vesicles containing DG (19). Direct evidence for the penetration of protein kinase C into the hydrocarbon core of the membranes has also been reported, but this interaction was not unique to phorbol esters (20, 21).

In this contribution, we address the nature of the interaction of protein kinase C with phorbol ester-containing membranes. Using a variety of biophysical and biochemical approaches, we show that the interaction of protein kinase C with phorbol ester-containing membranes occurs primarily via interactions at the membrane interface, in the absence of significant interaction with the hydrocarbon core of the membrane. Furthermore, we show that this interaction is reversible and that the apparent irreversibility noted in the literature results from the remarkably high affinity association with phorbol esters. Thus, DGs and phorbol esters regulate protein kinase C by the same mechanism, with observed differences reflecting primarily differences in the strength of the interaction.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1,2-dipalmitoylphosphatidylcholine (DPPC), egg L-α-phosphatidylcholine (PC), dansylphosphatidylethanolamine (DNS-PE), 1-palmitoyl-2-[(6,7) or (11,12)-dibromo]-stearoyl-phosphatidylcholine and 1,2-*sn*-dioleoylglycerol (DG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). (All lipids were analyzed by thin layer chromatography; only DG displayed a minor contaminant that was identified as 1,3-dioleoylglycerol; this contamination was small and the lipid was used without further purification.) Protamine sulfate, ATP sodium salt, EGTA, Tris (Trizma base),

‡ To whom correspondence should be addressed. Tel.: 619-534-4527; Fax: 619-534-6020.

[1] The abbreviations used are: DG, diacylglycerol; DNS-PE, dansylphosphatidylethanolamine; DODG, 1,2-*sn*-dioleoylglycerol (DG); DPPC, 1,2-dipalmitoylphosphatidylcholine; egg PC, egg L-α-phosphatidylcholine; PDBu, 12,13-dibutyrate phorbol; PMA, 12-myristate 13-acetate phorbol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; P[(6,7) or (11,12)-diBr]SPC, 1-palmitoyl-2-[(6,7) or (11,12)-dibromo]-stearoyl-phosphatidylcholine; BSA, bovine serum albumin.

12-myristate 13-acetate phorbol (PMA), and 12,13-dibutyrate phorbol (PDBu), were supplied by Sigma. The peptides Ac-ERMRPRKRQGS-VRRRV-amide (derived from the pseudosubstrate domain of protein kinase C ε) and Ac-FKKSFKL-amide (derived from the MARCKS protein) were synthesized by the Indiana University Biochemistry Biotechnology Facility. [γ-32P]ATP (3000 Ci mmol⁻¹), [³H]-DPPC (42 Ci mmol⁻¹), and [³H]PMA (20 Ci mmol⁻¹) were supplied by DuPont NEN. Bovine serum albumin, fraction V, fatty acid-free was obtained from Boehringer Mannheim. Protein kinase C βII, from the baculovirus expression system, was purified as described (7) and stored at −20 °C in 10 mM Tris buffer, pH 7.5 (4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol, and 50% (v/v) glycerol. All salts were analytical grade and were supplied by J. T. Baker, Inc. All other chemicals were reagent grade.

Lipid Vesicles—Mixtures of lipids in chloroform were dried under a stream of nitrogen and subsequently evacuated under high vacuum. Lipids were then suspended in either 170 mM sucrose or 100 mM KCl, both buffered with 20 mM Tris to pH 7.0. Aliquots (0.5 ml) of 10–50 mM lipid were subjected to five freeze-thaw cycles, followed by 21–51 rounds of extrusion through two stacked 0.1-μm polycarbonate filters in a microextruder Liposofast (Avestin, Inc., Ottawa, Ontario, Canada) to form large unilamellar vesicles. All phospholipid concentrations in stock solutions were determined by phosphate analysis (22). At later stages of experiments, the lipid concentration was calculated from the amount of [³H]DPPC that was routinely included in trace quantities in lipid mixtures. Extrusion of vesicles composed of DPPC and subsequent incorporation of phorbol esters into these vesicles (see below) was accomplished at 45–48 °C under otherwise identical conditions as for vesicles composed of lipids having gel/liquid crystalline phase transition temperatures below the temperature of the experiment (22 °C).

Incorporation of Phorbol Esters in Vesicles—Appropriate aliquots of concentrated PMA or PDBu in dimethyl sulfoxide were injected into vigorously vortexed suspensions of large unilamellar vesicles. Subsequently, vesicles were incubated for 15 min at 22 °C with occasional gentle vortexing. The partitioning of PMA into vesicles was complete (>98%) as assessed by centrifugation of sucrose-loaded vesicles and analysis of bound ³H-labeled PMA. Appropriate aliquots of suspensions of vesicles containing PMA were diluted severalfold in either activity or binding assay samples. PDBu in dimethyl sulfoxide was added directly to binding assay samples. The final content of dimethyl sulfoxide did not exceed 0.5% (v/v) and did not change the association of the enzyme with vesicles to any appreciable degree.

Protein Kinase C Membrane Binding Assay—The sucrose-loaded vesicle assay followed the procedure of Rebecchi *et al.* (23) adopted for protein kinase C, as described elsewhere (4). Membrane-bound enzyme was separated by centrifugation for 15 min at 25 °C. The maximal acceleration varied from 40,000 × g for DPPC vesicles, to 200,000 × g for egg PC or POPC vesicles to assure ≤95% sedimentation of large unilamellar vesicles. Both the supernatant and the pellet were assayed under identical conditions for kinase activity toward protamine sulfate, as described previously (4, 24). For samples incubated at 22 °C for up to 3 h, the total activity was the same regardless of whether vesicles were added immediately prior to the assay or at various times before the assay. The vesicle-associated kinase activity, A_v, was calculated according to Equation 1:

$$A_v = \frac{\beta A_b + (\beta - 1)A_t}{\alpha + \beta - 1}$$ (Eq. 1)

where A_b and A_t are the measured activities of the bottom and top fractions, respectively. The fraction of sedimented vesicles, α, was calculated from the distribution of ³H-labeled PC, which was included in trace amounts in all lipid mixtures. The fraction of kinase activity found in the supernatant in the absence of lipid, β, was equal, within the limits of experimental error, to the value expected for a non-sedimenting protein (*i.e.* 0.73 under the experimental conditions used). All experiments were performed in a standard solution composed of 100 mM KCl, 20 mM Tris, pH 7.0, and 0.3 mg ml⁻¹ BSA. Concentrations of additions that varied depending on experiment are given with appropriate results. The apparent membrane affinity of the enzyme with membranes was defined as the ratio of membrane-bound to free enzyme divided by the total lipid concentration.

Protein Kinase C Activity Assay—The activity of protein kinase C using protamine sulfate or synthetic peptides as substrates followed published procedures (7). The concentration of protamine was 0.2 mg ml⁻¹; the concentration of the peptides was 50 μM. The phosphorylation reaction was initiated by the addition of 50 μM [γ-32P]ATP (150 Ci mol⁻¹), 10 mM MgCl₂, and indicated concentration of substrates, to an
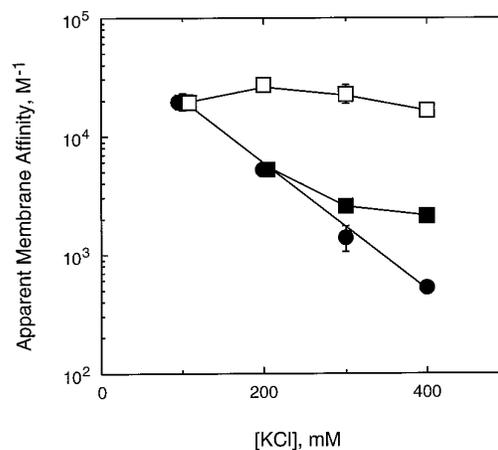

FIG. 1. **Effect of ionic strength on the diacylglycerol or phorbol ester-induced association of protein kinase C with membranes.** The binding of protein kinase C (1 nM) to POPC vesicles (0.5 mM lipid) containing 50 mol % (*closed symbols*) or 25 mol % (*open symbols*) POPS, and either 2 mol % DODG (●), 0.01 mol % PMA (■), or 1 mol % PMA (□) was measured in the presence of 0.5 mM EGTA. Results are expressed as the apparent membrane affinity determined from the amount of membrane-bound and free enzyme, as described under "Experimental Procedures." Data represent the average of at least two independent experiments; *error bars* indicate the range or S.D. Points for 100 and 200 mM KCl were offset slightly to the right and left of the actual concentration to improve their visibility.

80-μl reaction volume containing the concentrations of protein kinase C, lipid, phorbol ester, CaCl₂, and EGTA noted in the figure legends. The phosphorylation reaction was terminated after 8 min at 30 °C by the addition of 25 μl of 5% (v/v) acetic acid. Samples (85 μl) were spotted on P-81 ion-exchange paper and subsequently washed four times in 0.5 liter of 0.4% (v/v) phosphoric acid, followed by one wash in 95% ethanol. Radioactivity associated with papers was determined by liquid scintillation counting in Biosafe II.

Calculation of Free Ca²⁺ Concentration—The total concentration of Ca²⁺ in stock solutions of this cation, as well as stock solutions of other salts and BSA, was measured by plasma emission spectroscopy. The calculations of free Ca²⁺ concentration followed the established protocol (25) and took into account contamination of all solution components by calcium, the effect of ionic strength, and the interaction of EGTA with Mg²⁺ (when present).

Fluorescence Measurements—All measurements were carried out on a 4800C Lifetime Spectrofluorometer (SLM Aminco, Urbana, IL). The excitation wavelength was 280 nm. Emission scans from 300 to 360 nm were taken at 1 nm s⁻¹. Excitation and emission slits were set at 4 nm. The emission of dansyl in the resonance energy transfer measurements for the enzyme-vesicle binding kinetics was measured at 520 nm. The total volume of the sample was 1.5 ml. Other details, specific for different experiments, are as described under "Results" and in the figure legends. Correction for inner filter effects followed standard procedures (26); this correction was <13% of the measured emission intensity for all experiments.

Ionic Strength Dependence—A suspension of vesicles and enzyme was incubated for 15 min in a solution containing 0.3 mg ml⁻¹ BSA, 0.5 mM EGTA, 20 mM Tris, pH 7.0, and 100–400 mM KCl. Subsequently, membrane-bound enzyme was separated by the sucrose-loaded vesicle method.

Data Analysis—Rate constants in Fig. 4 were calculated by either single-or double-exponent least-squares fit to the data (changes in fluorescence emission) using the program Sigmaplot.

RESULTS

The primary evidence supporting the hypothesis that phorbol esters cause an irreversible association of protein kinase C with membranes derives from multiple observations that the Ca²⁺-dependent binding of protein kinase C to PMA-containing membranes cannot be completely reversed by chelation of Ca²⁺ (15–19). The possibility that this chelator-dependent binding is reversible was explored in Figs. 1–3.

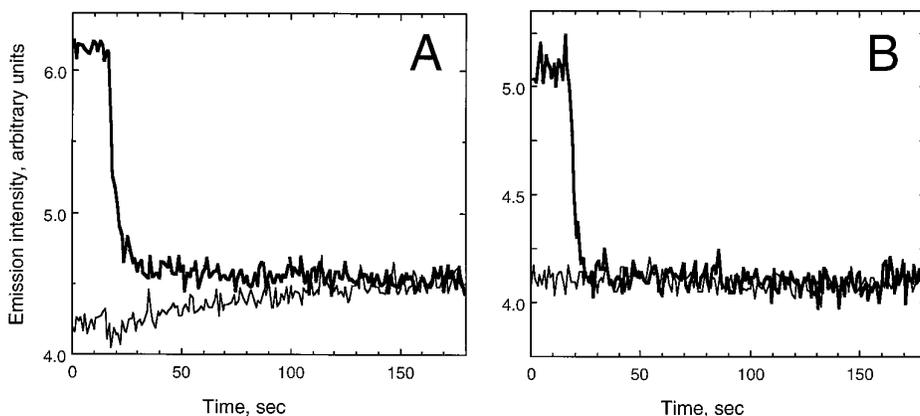Fig. 1 compares the binding of protein kinase C βII to mem-

FIG. 2. **Dissociation of protein kinase C from membranes by chelation of Ca²⁺.** *A*, protein kinase C (60 nM) was incubated with large unilamellar vesicles (5 μM lipid) composed of 25 mol % DNS-PE, 74 mol % POPC, and 1 mol % PMA for 45 min, 22 °C, in a solution containing 0.2 mM CaCl₂, 100 mM KCl, and 20 mM Tris, pH 7.0. A 200-fold concentrated solution of EGTA was injected into a continuously stirred enzyme-lipid mixture at the 15th second of the time scan (*thick line*) to yield a final concentration of 1 mM. The *thin line* represents injection of a concentrated suspension of protein kinase C, at 15 s, into a cuvette containing 1 mM EGTA and vesicles with 1 mol % PMA. Except for Ca²⁺, the final composition of the suspension in which protein kinase C was present before chelation of Ca²⁺ (*i.e. thick line*) was identical to that when protein kinase C was added to a solution with chelator and no Ca²⁺ (*i.e. thin line*). Excitation and emission wavelengths were 280 and 520 nm, respectively; both slits were set at 4 nm. *B*, protein kinase C was incubated as in *A* except that vesicles contained 1 mol % DODG instead of PMA. At 15 s, EGTA was injected into a continuously stirred enzyme-lipid mixture to yield a final concentration of 1 mM (*thick line*). *Thin line* represents the emission in the absence of protein kinase C but otherwise identical conditions.

branes containing either DG or PMA in the presence of excess chelator (0.5 mM EGTA, no added Ca²⁺). Note that the same amount of membrane association can be achieved for membranes containing either activator when the difference in affinity of protein kinase C for each activator is taken into account. Specifically, protein kinase C bound with equal affinity to membranes containing the same amount of PS (50 mol %) and either 2 mol % DG (*filled circles*) or 0.01 mol % PMA (*filled squares*), in the absence of Ca²⁺; the difference in activator concentrations is consistent with the approximately 250-fold increase in protein kinase C's affinity for PMA compared with DG reported recently (27). To test whether the chelator-resistant interaction differed for PMA compared with DG, the ability of increasing ionic strength to reduce the strength of the membrane interaction was compared for both activators. Increasing ionic strength caused a marked reduction in the affinity of protein kinase C for PS membranes containing either 2 mol % DG (*filled circles*) or 0.01 mol % PMA (*filled squares*). The magnitude of this reduction was similar for membranes containing both activators. The similar effect of ionic strength on both interactions suggests that any difference in hydrophobic contributions in the association of protein kinase C with DG-*versus* PMA-containing membranes is small.

The sensitivity of protein kinase C's membrane interaction to ionic strength depended on the membrane content of PS and PMA. When the PMA concentration was increased to 1 mol % and the PS concentration decreased concomitantly from 50 to 25 mol %, protein kinase C's membrane interaction became markedly less sensitive to ionic strength. This ionic strength insensitivity at high concentrations of phorbol esters is consistent with the finding that 1 mol % PMA is sufficient to cause significant association of protein kinase C with membranes in the absence of PS ((27); also see Fig. 7). Thus, the direct association with PMA that can be observed at high concentrations of this ligand likely does not involve the salt-sensitive electrostatic interactions between protein kinase C and PS headgroups that dominate when the PS concentration is relatively high and the DG or PMA concentration relatively low (*filled symbols*).

Fig. 2 examines the effect of chelation of Ca²⁺ by EGTA on the association of protein kinase C with membranes containing the same concentration of PMA or DG and 25 mol % DNS-PE,

which can substitute for PS in activating protein kinase C (4). Membrane binding was assessed by resonance energy transfer (24, 28). Protein kinase C was incubated for 45 min with vesicles containing 25 mol % DNS-PE and 1 mol % of either PMA or DG in the presence of 200 μM Ca²⁺. Fig. 2 shows that chelation of Ca²⁺ resulted in a rapid dissociation of protein kinase C from membranes, as assessed by a decrease in fluorescence energy transfer from tryptophans to dansyl-PE. The half-time for dissociation was approximately 3 s for protein kinase C bound to vesicles containing either PMA (Fig. 2*A*) or DODG (Fig. 2*B*). Dissociation was complete for DG-containing vesicles as evidenced by the decrease in light emission to the level observed for vesicles in the absence of enzyme (Fig. 2*B*, *thin line*) after compensation for the dilution caused by addition of the EGTA. The same level of emission was observed for vesicles in the presence or absence of EGTA, as well as in the presence of protein kinase C and EGTA (data not shown). For PMA-containing membranes, approximately 10% of the enzyme remained membrane-bound (Fig. 2*A*, *thick line*). However, a comparable level of association was measured within minutes after protein kinase C was added to vesicles of the same composition suspended in a solution containing 0.5 mM EGTA and no exogenous Ca²⁺ (Fig. 5*A*, *thin line*). Thus, the fraction of protein kinase C that remained associated with membranes upon chelation of Ca²⁺ represented the fraction of protein kinase C that associates with such membranes in the absence of Ca²⁺, rather than enzyme that had become irreversibly bound as a result of the initial Ca²⁺-dependent interaction.

At high concentrations of PMA and high lipid/protein ratios, the majority of protein kinase C molecules are vesicle associated, even in the presence of chelator (Fig. 1). To address the reversibility of this Ca²⁺-independent membrane association (*i.e.* the binding to PMA-containing membranes in the absence of Ca²⁺), we asked whether membrane-bound enzyme could translocate to enzyme-free vesicles. In the experiment in Fig. 3, protein kinase C was bound to multilamellar vesicles containing 1 mol % of either DG or PMA, and then incubated with an equal concentration of lipid in the form of large unilamellar vesicles of the same composition. Separation of the multilamellar vesicles from the unilamellar vesicles by centrifugation revealed that approximately 90% of the protein kinase C bound to the DG-containing multilamellar vesicles had now parti-
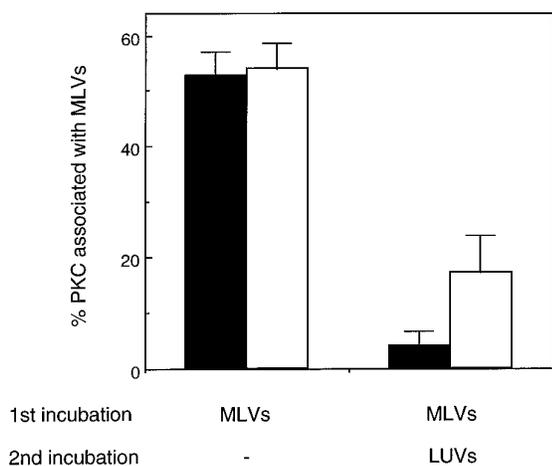
FIG. 3. **Reversibility of protein kinase C's association with membranes containing diacylglycerol or phorbol esters.** Protein kinase C (1 nM) was incubated with multilamellar vesicles composed of 2 mol % DODG, 50 mol % POPS, and 48 mol % POPC (*filled columns*; 2.2 mM total lipid) or 1 mol % PMA, 25 mol % POPS, and 74 mol % POPC (*blank columns*; 5.5 mM total lipid) in the solution described in the legend to Fig. 1. After 15 min of incubation at 22 °C, large unilamellar vesicles of identical composition to that of the multilamellar vesicles were added to the enzyme-lipid mixture to yield a 2-fold increase in the total amount of lipid. Buffer was added to the control samples. After incubation for another 15 min, uni- and multilamellar vesicles were separated by centrifugation at $10,000 \times g$ for 6 min at 22 °C. The percentage of protein kinase C activity associated with the sedimented multilamellar vesicles was calculated as described under "Experimental Procedures."

tioned with the large unilamellar vesicles; control experiments indicated that the unilamellar vesicles had about 10 times more surface area. This result is consistent with complete equilibration of the enzyme between the two types of vesicles. For PMA-containing membranes, protein kinase C did not equilibrate completely between the two types of vesicles: 17 ± 6% remained bound to multilamellar vesicles 15 min after addition of the large unilamellar vesicles. Nonetheless, the majority of the enzyme did exchange from the multilamellar vesicles to the unilamellar vesicles within the course of the assay.

Partial reversibility of protein kinase C's association with membranes containing PMA suggested that the dissociation rate of the enzyme from such membranes could have been a limiting factor over the time scale of the experiment. However, because the association of protein kinase C with vesicles containing more than one molecule of PMA increases with time,[2] prolonging the time of incubation for the exchange experiment described above was unlikely to provide an unambiguous answer. Unfortunately, technical limitations prevented the use of resonance energy transfer to measure the dissociation of protein kinase C from membranes at low occupancy of PMA molecules by the protein; this technique requires substantial occupancy of the membrane surface by protein. As an alternative approach to determining dissociation rates, we compared the rates of initial association of protein kinase C with membranes containing DG or PMA using resonance energy transfer from tryptophans to dansyl-PE. Fig. 4 shows that protein kinase C associated rapidly with DG-containing vesicles (*thick solid line*); the apparent association constant was 0.26 ± 0.04 s^{-1}. Despite the greater than 200-fold higher affinity of protein kinase C for vesicles containing 1 mol % PMA compared with 1 mol % DG, the apparent association constant was 0.16 ± 0.02 s^{-1} for the PMA-containing membranes (*thin solid line*), ap-

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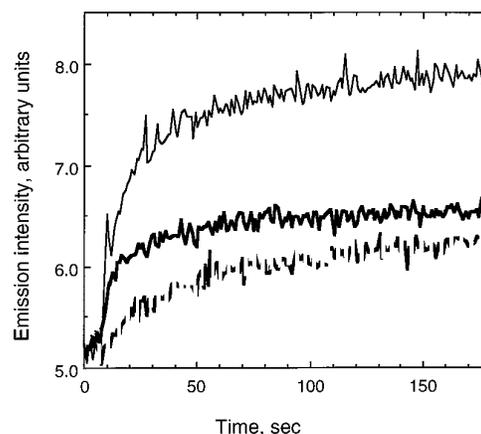FIG. 4. **Kinetics of the association of protein kinase C with vesicles.** A concentrated solution of protein kinase C (1.5 μM) was injected into a rapidly stirring suspension of large unilamellar vesicles (8 μM lipid) to yield a final concentration of 60 nM. The vesicles were suspended in a solution containing 100 mM KCl, 10 mM Tris, pH 7.0, 0.5 mM EGTA, and either 0.45 or 0.7 mM CaCl$_2$. The final concentration of free Ca^{2+} was 1 μM (*dashed line*) or 200 μM (*thin and thick solid lines*). Vesicles were composed of 25 mol % POPS, 74 mol % egg PC, and either 1 mol % PMA (*thin solid and dashed lines*) or 1 mol % DODG (*thick solid line*). Samples were excited at 280 nm and emission was recorded at 520 nm. Excitation and emission slits were set at 4 nm.

proximately half that for association with the DG-containing membranes. Thus, the dissociation of protein kinase C from vesicles containing PMA is likely to be at least 2 orders of magnitude slower than that from vesicles containing an equal concentration of DG. To measure the association rate under conditions resulting in comparable membrane association of protein kinase C, the Ca^{2+} concentration was reduced 200-fold for the incubation with PMA-containing vesicles. In the presence of 1 μM Ca^{2+}, the fluorescence emission in the presence of vesicles containing 1 mol % PMA (*dashed line*) reached the level obtained in the presence of vesicles containing 1 mol % DG and 200 μM Ca^{2+} within 10 min, and did not change significantly afterward (data not shown). However, the apparent association constant of protein kinase C under low Ca^{2+} conditions decreased only by a factor of 6 to 0.026 ± 0.001 s^{-1}, despite the 200-fold lower membrane affinity. This observation suggests that the association of protein kinase C with phorbol esters may be rate limiting in the binding of enzyme to membranes containing phorbol esters. In summary, under conditions when the apparent association constants were identical for vesicles containing the same mol % DG or PMA, the association of the enzyme with the latter proceeded at a significantly slower rate. In the presence of 0.2 mM Ca^{2+} and 1 mol% of either PMA or DG in the membrane, biphasic kinetics were observed in the association of protein kinase C with membranes. The apparent rate constant for the slower component was the same (0.023 ± 0.002 s^{-1}) for both PMA- and DG-containing vesicles, suggesting that it did not reflect an irreversible membrane insertion which is thought to require phorbol esters and not DG (19). The slower component in Fig. 4 likely reflects steric saturation of the membrane surface because of the high ratio of enzyme/lipid.

The sensitivity of protein kinase C's intrinsic tryptophan fluorescence to either surface-positioned (Fig. 5) or membrane-inserted quenchers (Fig. 6) was used as an independent and direct method to address the degree of penetration of the enzyme into the hydrophobic core of the membrane. Protein kinase C βII has 9 tryptophans (29–31); 5 of these are in the membrane-interacting regulatory domain. Fig. 5 shows that the presence of dansyl-labeled PE in PC vesicles caused a 40%
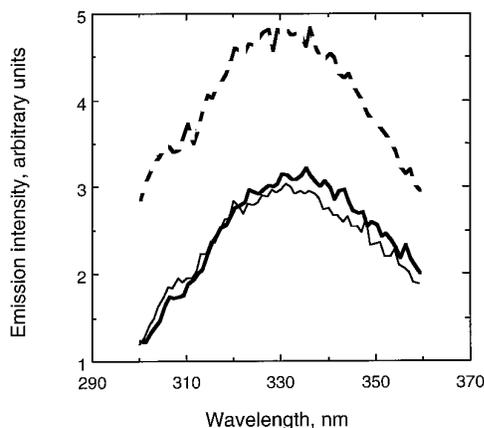
FIG. 5. **Quenching of the intrinsic tryptophan fluorescence of protein kinase C by dansyl fluorophores localized at the water-lipid interface.** Protein kinase C (60 nM) and large unilamellar vesicles (150 μm lipid) composed of 74 mol % POPC and either 25 mol % POPS and 1 mol % DODG (*dashed line*), 25 mol % DNS-PE and 1 mol % DODG (*thick solid line*), or 25 mol % DNS-PE and 1 mol % PMA (*thin solid line*) were incubated in a solution composed of 0.2 mM CaCl$_2$, 100 mM KCl, and 20 mM Tris, pH 7.0. Samples were excited at 280 nm and the emission from 300 to 360 nm recorded. Excitation and emission slits were set at 4 nm; spectra were corrected for inner filter effect.
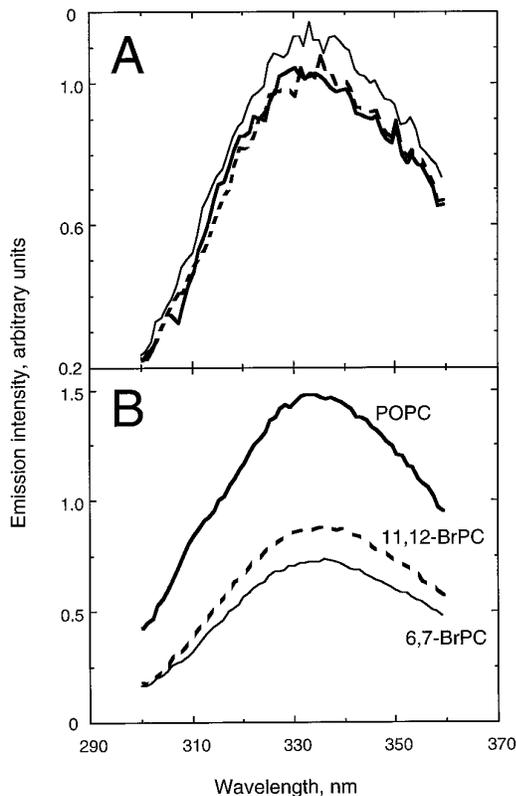

FIG. 6. **Effect of bromines located at different membrane depths on the tryptophan fluorescence of cytochrome b_5 and protein kinase C.** Protein kinase C (60 nM) (*A*) or cytochrome b$_5$ (0.5 μM) (*B*) was incubated with large unilamellar vesicles (150 μm lipid) composed of 25 mol % POPS, 1 mol % PMA, and 74 mol % of either POPC (*thick solid line*), P(6,7-diBr)SPC (*thin solid line*), or P(11,12-diBr)SPC (*dashed line*) as described in the legend to Fig. 3. The excitation wavelength was 280 nm; excitation, and emission slits were set at 4 nm. Spectra were corrected for inner filter effect.

decrease in the intrinsic emission of the enzyme (*solid lines*) relative to vesicles that did not have the probe but had equimolar PS (*dashed line*). No quenching was observed when neither DG nor PMA was included in vesicles, in the presence of 0.5 mM

EGTA (*i.e.* under conditions where little protein kinase C was vesicle-associated) (not shown). Under this latter condition (*i.e.* free protein kinase C but vesicles present), or in the presence of only protein kinase C (no vesicles), the fluorescence emission at 300 nm was approximately 15% lower than in the presence of vesicles with bound protein kinase C but without the quencher (*dashed line*). For these experiments, an excess of lipid was used so that >99% of the protein kinase C was membrane-bound. An almost identical degree of quenching was observed for vesicles containing 1 mol % of either DG (*thick solid line*) or PMA (*thin solid line*) and 25 mol % DNS-PE. Despite the 200-fold difference in protein kinase C's affinity for membranes containing 1 mol % PMA compared with 1 mol % DG, the spectra for both membranes had similar shapes indicating that protein kinase C's tryptophans are in similar environments whether bound to DG- or PMA-containing membranes. Both the quenching by DNS-PE (Fig. 5) and the resonance energy transfer from tryptophan to the dansyl moiety (Fig. 2) indicate that at least some of the tryptophans of protein kinase C are proximal to the interface.

In contrast to the significant quenching of protein kinase C's tryptophans by the surface-positioned dansyl probe, bromines situated at either the 6 and 7 or 11 and 12 carbons on the acyl chain had no detectable effect on tryptophan fluorescence. Fig. 6*A* shows that the tryptophan emission of protein kinase C was the same whether bound to vesicles containing 25 mol % PS and 1 mol % PMA and either 74 mol % POPC (*thick solid line*), P(6,7-DiBr)SPC (*thin solid line*), or P(11, 12-DiBr)SPC (*dashed line*). Identical results were obtained when PMA was substituted by DG (data not shown). In marked contrast, the tryptophan fluorescence of cytochrome b_5, which does insert into the hydrophobic core of the membrane (32, 33), was quenched by 57% when bound to vesicles with PC brominated at acyl chain positions 6 and 7, and by 48% when bound to vesicles with PC brominated at acyl chain positions 11 and 12 (Fig. 6*B*). Note that the quenching of cytochrome b_5 with PC/PS vesicles observed in Fig. 6*B* was qualitatively and quantitatively similar to that reported in assays using pure PC vesicles (33, 34). In summary, unlike cytochrome b_5, the tryptophan fluorescence of protein kinase C is insensitive to quenchers at positions 6 or greater on lipid acyl chains.

Most of the differences in the association of protein kinase C with vesicles containing either DG or PMA can be accounted for by the quantitative differences in the affinity of the enzyme for these two activators and the differences in association rate constants, rather than by invoking irreversible membrane insertion mediated by phorbol esters. However, we have found that the association of protein kinase C with vesicles containing DG reaches equilibrium within minutes whereas the association with PMA-containing vesicles increases slowly with time (Fig. 7).[2] This slow component might result from irreversible penetration of protein kinase C into the hydrophobic core of the membrane. To test this possibility, we took advantage of the finding that the interaction of protein kinase C with PMA is strong enough to cause protein kinase C to associate with vesicles containing only PC and no PS (Fig. 7).[2] If the slow increase in membrane association reflects membrane penetration, then a slower association with lipids in the gel state *versus* liquid crystalline state might be expected. Fig. 7 shows that protein kinase C associated with similar kinetics to PMA-containing vesicles in the gel phase (DPPC; T^m = 41.5 °C (35); *squares*) as to vesicles in the liquid crystalline phase (egg PC; Tm<0 °C; *circles*). Curiously, the enzyme bound with approximately three times higher apparent affinity to the lipids in the gel phase compared with those in the liquid crystalline phase. Consistent with no significant differences in the interaction
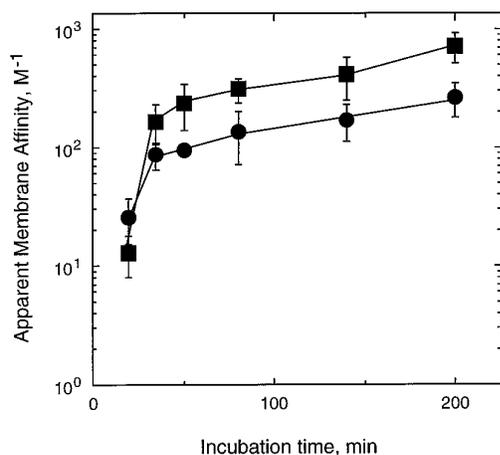
FIG. 7. **Association of protein kinase C with phorbol ester-phosphatidylcholine membranes in liquid crystalline or gel phase.** Protein kinase C (1 nM) was incubated with vesicles (5 mM lipid) composed of 2 mol % PMA and either 98 mol % POPC (●) or 98 mol % DPPC (■) for the indicated times at 22 °C; the incubation medium contained 0.5 mM EGTA, 0.3 mg ml^{-1} BSA, 100 mM KCl, and 20 mM Tris, pH 7.0. Membrane-bound enzyme was separated from free enzyme by centrifugation, as described under "Experimental Procedures." The indicated times include the 19-min separation procedure. Data represent the mean and range of results from two independent experiments.

with both membranes, the PMA-dependent activation of protein kinase C was similar in the presence of DPPC or egg PC vesicles: PMA (1 mol %) in DPPC membranes activated protein kinase C to 0.12 nmol μg^{-1} min^{-1} (10% of V_{max}) and 1.8 nmol μg^{-1} min^{-1} (34% of V_{max}) using as substrates a peptide based on the MARCKS protein and a peptide based on the pseudosubstrate of protein kinase C ϵ, respectively. For vesicles composed of egg PC, corresponding values were 0.07 and 2.2 nmol μg^{-1} min^{-1}, respectively. The lack of significant sensitivity in the kinetics of membrane association and activation to the lipid phase suggests that any putative penetration of protein kinase C into the bilayer does not involve significant interaction with the acyl chains.

DISCUSSION

The foregoing results indicate that protein kinase C's high affinity membrane interaction induced by phorbol esters is reversible and does not involve significant penetration of the enzyme in the hydrophobic core of the membrane. Several lines of evidence are consistent with protein kinase C-binding membranes via an electrostatic interaction that dominates at relatively low concentrations of phorbol esters, and a non-ionic interaction that dominates at relatively high concentrations of PMA. Each type of interaction is sufficient for membrane binding: high concentrations of acidic lipids result in membrane association in the absence of molecules that bind the phorbol ester domain, and high concentrations of PMA result in protein kinase C binding to neutral lipids. Importantly, both interactions are reversible.

Chelator-resistant, but Reversible, Association of Protein Kinase C with Membranes—A number of groups have reported that phorbol esters and similar molecules cause a chelator-irreversible association of protein kinase C with PS-containing membranes (15, 16, 18). This apparent lack of reversibility has been proposed to arise from a phorbol ester-induced insertion of protein kinase C into the hydrophobic core of the membrane (15–19). In contrast, when DGs were present in the membrane, protein kinase C was shown to dissociate readily from the membranes upon addition of chelator (19). However, until recently (3, 4), DGs were thought to have no effect on the association of protein kinase C with membranes (28). Thus, quali-

tatively different modes of interaction with protein kinase C were ascribed to DGs and phorbol esters (15–19). We have found that this apparent irreversibility arises because the combined free energy of association of protein kinase C with phorbol esters and PS is sufficient to cause substantial levels of membrane association even in the presence of chelator (Figs. 1 and 3). Specifically, PMA is 250-fold more potent than DG in increasing protein kinase C's membrane affinity (Fig. 1). As a consequence, considerably less PS in the membrane is required to achieve the same level of membrane association for PMA-containing membranes as for DG-containing membranes. Similarly, because Ca^{2+} and PS act allosterically (24), considerably less Ca^{2+} is required to achieve the same level of binding to PMA-containing membranes compared with DG-containing membranes. Indeed, the affinity for PS, in the presence of DG, is sufficiently high that significant association occurs in the absence of Ca^{2+} (24, 36). Thus, the chelator resistance reflects the extraordinarily high affinity interaction with PMA-containing membranes. This membrane affinity can be markedly reduced by increasing the ionic strength, which reduces the electrostatic component from the PS-protein kinase C contribution to binding. Note that when the association of protein kinase C with PMA dominates over that with PS (*e.g.* 1 mol % PMA, 25 mol % PS, 0.5 mM EGTA), the association of the enzyme with membranes displays little sensitivity to ionic strength; this likely results from the direct interaction of protein kinase C with phorbol esters that is biochemically identified by the ability of protein kinase C to bind phorbol esters in phosphatidylcholine membranes (Fig. 7). However, even when the interaction is driven primarily by direct binding to PMA, protein kinase C's membrane interaction is reversible. Under these conditions, protein kinase C exchanges between vesicle surfaces (Fig. 3). That is, even though the interaction is insensitive to increasing ionic strength or chelation of Ca^{2+}, protein kinase C molecules exchange between vesicle surfaces indicating that the affinity is very high, but that the binding is nonetheless reversible.

Membrane Association without Significant Interaction with the Hydrophobic Core—The insensitivity of protein kinase C's intrinsic fluorescence to quenching by bromines located in the bilayer (Fig. 6A) suggests that regions of the regulatory domain containing tryptophan do not penetrate significantly into the hydrophobic core of the membranes, even in the presence of high concentrations of PMA. The insensitivity of tryptophan fluorescence to quenchers in the core of the bilayer is consistent with recent structural data indicating that tryptophans in the C1 and C2 domains are likely to be at, or away from, the membrane interface. Specifically, the crystal structure of the second Cys-rich domain in protein kinase C δ reveals that the equivalent Trp[58] in the first Cys-rich domain of protein kinase C βII would be at the base of a hydrophobic cap, near an interface of charged residues, and might thus be expected to lie in close proximity to the polar membrane surface rather than in the hydrophobic core (37). Secondly, modeling of protein kinase C's C2 domain[3] based on the crystal structure of the C2 domain of synaptotagmin (38) suggests that two tryptophans (Trp[245] and Trp[247]) also lie at the membrane-protein interface because they are next to a positively charged β-sheet that is a good candidate for interaction with phosphatidylserine headgroups. A third tryptophan in the C2 domain (Trp[223]) would be distal to the membrane-interacting surface; no structural information exists on the position of the fifth Trp (Trp[274]), but its proximity to the relatively polar hinge region would suggest that it, too, is not involved in hydrophobic membrane interac-

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[3] A. C. Newton, unpublished data.

tions. Thus, fluorescence quenching data are consistent with existing structural information suggesting the tryptophans in protein kinase C's regulatory domain do not penetrate significantly the hydrophobic component of the membrane. Note, however, that the hydrophobic cap on the top third of the Cys-rich domain, apparent in the crystal structure, is about 8 Å, suggesting that penetration up to this depth may occur (37).

An alternative possibility to account for the lack of effects of brominated PCs on protein kinase C's fluorescence is that the enzyme preferentially binds PS, thus decreasing the local concentration of brominated PC to levels too low to allow interaction with membrane-inserted tryptophans. However, other proteins that preferentially bind anionic lipids are sensitive to brominated PCs, indicating that the highly dynamic behavior of acyl chains precludes this possibility. For example, the tryptophan fluorescence of SecA (39) and the putative membrane-binding domain of cytidylyltransferase (40) were quenched by up to 60% by brominated PCs even though bilayers contained 50 mol % anionic lipids (twice the concentration used in the above experiments); the latter example is particularly relevant because binding of this peptide to anionic lipids displays sigmoidal kinetics consistent with enrichment of the local environment of the peptide with anionic lipids (40).

Quenching of the intrinsic fluorescence emission of protein kinase C by spin and fluorescently labeled fatty acids has been reported (20). However, the degree of the reported quenching by 1:1 PC/PS vesicles was identical in the presence and absence of Ca^{2+}. Because the association of protein kinase C with such vesicles in the absence of Ca^{2+} is too weak to be detected (4, 41), the observed changes in the intrinsic fluorescence of the enzyme under those conditions are not likely to reflect membrane association and hence, even less likely, membrane penetration by the enzyme. Labeling of protein kinase C with a photoactivable probe residing in the hydrocarbon core of the membrane has also been reported (21). However, this labeling was observed at very low mol % PS, under conditions where no significant association of protein kinase C with vesicles occurs (4, 41), was decreased with increasing Ca^{2+} concentration, and was weaker in the presence of phorbol esters compared with DG. A second study examining whether protein kinase C in chromafin granule membranes is labeled with a photoactivatable probe revealed no significant labeling of protein kinase C, whether binding was Ca^{2+} or PMA mediated.[4] This latter result with natural membranes is consistent with our lack of evidence for penetration of protein kinase C into model membranes.

Kinetics of Protein Kinase C's Membrane Interaction—The kinetics describing the association of protein kinase C with membranes containing PMA is more complex than observed for membranes containing DG. At low concentrations of PMA (not shown) or at high saturation levels of PMA by bound protein (Fig. 4), an apparent equilibrium is reached within minutes, similar to the rapid reaching of equilibrium with DG-containing membranes. In the presence of a large excess of free PMA molecules over protein kinase C, the initial high affinity membrane association is followed by a slow and small magnitude increase in the fraction of membrane-bound protein (Fig. 7). A similar slow, time-dependent increase in protein kinase C activity induced by phorbol esters has been interpreted as evidence for an irreversible membrane insertion of protein kinase C (18, 19). Our results reveal that the slow increase in the association of protein kinase C with PMA membranes shows no significant dependence on the physical state of the bilayer; some difference in association kinetics might be expected if

protein kinase C penetrated the hydrocarbon core. In addition to similar binding kinetics, protein kinase C was activated to a similar extent by membranes present in the liquid crystalline phase (egg PC at 22 °C; $T^m < 0$ °C) or membranes present in the gel phase (DPPC at 22 °C; $T^m = 41.5$ °C). However, slightly tighter binding to lipids in the gel phase was observed; the latter might result from PMA being immobilized, and thus possibly better "docked" for protein kinase C, in the gel phase *versus* the liquid crystalline phase. In marked contrast to protein kinase C, the peripheral membrane-partitioning viral coat protein M13 (42) displays over an order of magnitude increased affinity for DOPC in the liquid crystalline phase *versus* DPPC in the gel phase; similar to this peripheral protein, the trans-membrane $(Ca^{2+}-Mg^{2+})$ATPase displays a similar preference for DOPC in the liquid-crystalline phase *versus* DPPC in the gel phase (43). Note that the long term changes in the degree of association with PMA membranes (severalfold increase in affinity over hours) are small from an energetic view point (on the order of 1 kcal mol^{-1}), and it is difficult to speculate about the nature of this process (note that transfer of two methylene groups from a hydrophilic to hydrophobic environment involves 1.5 kcal mol^{-1}) (44). In summary, little, if any, change in the putative hydrophobic interaction of protein kinase C with lipid bilayers is induced by phorbol esters compared with DG.

The phorbol ester-induced activity of protein kinase C also displayed little dependence on the state of the membrane. This finding is inconsistent with models of activation of protein kinase C that invoke membrane penetration in order to render the enzyme catalytically competent (17, 45, 46).

Conclusions—This contribution reveals that protein kinase C associates with membranes, and becomes activated, as a result of interactions with lipid components primarily at the membrane-solute interface. Most importantly, these interactions are reversible, even in the presence of PMA and in the presence of chelator. Previous studies suggesting irreversibility in the PMA-protein kinase C interaction can be accounted for the by the 2-orders of magnitude increase in affinity of protein kinase C for PMA compared with DG. In addition, our data suggest that protein kinase C does not insert significantly into the hydrocarbon core of the membrane. Taken together with the finding that 1 mol % phorbol esters increases protein kinase C's membrane affinity by over 4 orders of magnitude (27), the following model for protein kinase C's regulation by phorbol esters is suggested: binding of phorbol esters to the phorbol ester-binding domain on protein kinase C alters the equilibrium partitioning of the enzyme for membranes by many orders of magnitude, with the degree depending on the relative concentration of the phorbol esters in the membrane (for example, 0.01 mol % increases the membrane affinity 600-fold). This high affinity binding to PMA membranes is fundamentally the same as the high affinity binding to DG membranes; the major difference is the strength of the interaction. Specifically, the interaction is reversible and the enzyme exchanges freely between membranes. The apparent irreversibility noted in the literature can be accounted for by the formidable increase in membrane affinity caused by phorbol esters.

The preceding model accounts for the effects of phorbol esters on protein kinase C *in situ*. In particular, the well-documented increase in the amount of protein kinase C recovered in membrane fractions of cells stimulated with phorbol esters (*e.g.* 15, 47, 48) reflects the several orders of magnitude increase in membrane affinity caused by phorbol esters. This binding is resistant to chelation because it is of such high affinity. In addition, the nature of the high affinity interaction indicates that there may be many cases *in vivo* where PMA or DG is sufficient to activate Ca^{2+}-dependent protein kinase Cs in the

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[4] N. Merezhinskaya, personal communication.

absence of an increase in the intracellular Ca^{2+} level. Either high mol % PS or high lipid concentrations would favor the partitioning of protein kinase C with membranes. A striking example is provided by rod outer segments, where the lipid concentrations are so high (0.2 M (49)) that the interaction of a Ca^{2+}-regulated protein kinase C with PS and DG would be sufficient for activation of the enzyme at Ca^{2+} levels below the dissociation constant of Ca^{2+} from membrane-bound protein kinase C (24). In summary, DG and PMA have similar effects on protein kinase C: they induce an extraordinarily high affinity interaction of protein kinase C with membranes that is reversible, but can be of such high affinity that it occurs in the absence of Ca^{2+}, or, in the case of PMA, absence of PS.

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