Calcium-Independent Binding to Interfacial Phorbol Esters Causes Protein Kinase C To Associate with Membranes in the Absence of Acidic Lipids†

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Received August 25, 1995; Revised Manuscript Received November 15, 1995⊗

ABSTRACT: The mechanism of interaction of phorbol esters with conventional protein kinase Cs was addressed by examining the direct binding of this class of activators to protein kinase C βII. Binding measurements reveal that the major role of phorbol esters is to increase the affinity of protein kinase C for membranes by several orders of magnitude. The relative increase depends linearly on the mole fraction of phorbol esters in membranes, with the potency illustrated by the finding that 1 mol % phorbol 12-myristate 13-acetate (PMA) increases protein kinase C's membrane association by approximately 4 orders of magnitude. For comparison, diacylglycerol (DG), which also activates protein kinase C by increasing the enzyme's membrane affinity, is 2 orders of magnitude less effective than PMA in altering protein kinase C's membrane affinity. The remarkably high-affinity interaction with phorbol esters allowed us to measure the direct binding of protein kinase C to PMA in neutral membranes and, thus, to evaluate the effect of Ca^{2+} on the phorbol ester interaction in the absence of Ca^{2+} effects on the enzyme's interaction with acidic lipids. Changing the Ca^{2+} concentration over 5 orders of magnitude had no effect on the direct interaction of protein kinase C with PMA immobilized in phosphatidylcholine membranes. Thus, the Ca^{2+}-binding site for membrane association and the phorbol ester-binding site do not interact allosterically. Lastly, a method that does not have the limitations of the Scatchard plot for analysis of amphitropic proteins was used to determine the dissociation constant of protein kinase C from phorbol esters: expressed relative to membrane lipids, the dissociation constant is 1.5×10^{-5} mol %. In summary, our data reveal that (1) the direct binding of protein kinase C to phorbol esters, in the absence of interactions with acidic lipids, provides a major contribution to the free energy change involved in the association of protein kinase C with membranes and (2) this interaction is not regulated by Ca^{2+}.

The family of protein kinase C isozymes transduces the plethora of signals that result in diacylglycerol (DG[1]) production (Nishizuka, 1992). DGs are one of three classes of molecules that are required for the full activation of conventional (Ca^{2+}-regulated) isozymes of protein kinase C *in vivo*, the other two being phosphatidylserine (PS) and Ca^{2+} (Bell & Burns, 1991; Newton, 1993, 1995). Phorbol esters are functional analogues of DG: they compete with (Sharkey et al., 1984), and can substitute for (Castagna et al., 1982), DG in activating conventional and novel (Ca^{2+}-independent) protein kinase Cs both *in vitro* and *in situ* (Ashendel, 1985; Kazanietz et al., 1993).

Much progress has been made in identifying structural determinants involved in the association of protein kinase C with phorbol esters. Through the use of deletion mutants (Ono et al., 1989), the region of protein kinase C involved in binding phorbol esters was narrowed to two conserved Cys-rich motifs that also bind Zn^{2+} (Hubbard et al., 1991; Quest et al., 1992). In support of this, protein constructs containing either of these two Cys-rich motifs have been shown to bind phorbol esters in a lipid-dependent fashion (Burns & Bell, 1991; Quest et al., 1994a; Quest & Bell, 1994a), with critical residues involved in this binding identified by analysis of mutants (Quest et al., 1994b). Similarly, the functional groups on phorbol esters critical for the activation of protein kinase C have been well characterized (Rando & Kishi, 1992). The recent elucidation of the structure of one of the Cys-rich motifs of protein kinase C δ, with bound phorbol ester, has revealed the precise molecular interactions mediating the binding of phorbol esters to a groove in this motif (Zhang et al., 1995). Thus, the structure and molecular interactions of the phorbol ester domain of protein kinase C are well described.

In contrast to structural studies, the mechanism by which phorbol esters activate protein kinase C is not fully established. Several explanations have been offered for early reports demonstrating an increase in the association of protein kinase C with membranes in the presence of phorbol esters (Kraft & Anderson, 1983; May et al., 1985; Wolf et al., 1985b). For example, the increased membrane association induced by phorbol esters has been proposed to arise from conversion of the enzyme into an integral protein (Kraft & Anderson, 1983; Kazanietz et al., 1992), membrane insertion (Nelsestuen & Bazzi, 1991), transformation from "loose" to "tight" binding (Wolf et al., 1985b) or to be mediated by Ca^{2+} bridging (Bell, 1986). The nature of the membrane interaction was recently established in two studies that

† This work was supported by National Institutes of Health Grant GM 43154 (A.C.N.), a Walther Cancer Institute Fellowship (M.M.), and a National Science Foundation Young Investigator Award (A.C.N.).

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

[1] Abbreviations: ATP, adenosine triphosphate; DG, diacylglycerol; DODG, 1,2-*sn*-dioleoylglycerol; DPPC, 1,2-dipalmitoylphosphatidylcholine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; LUV, large unilamellar vesicle; egg PC, egg L-α-phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/96/0435-1612$12.00/0

showed that protein kinase C's interaction with phorbol ester-containing membranes is reversible both in cells (Szallasi et al., 1994) and in lipid bilayers (Mosior & Newton, 1995) and that binding is driven by interactions primarily at the membrane interface (Mosior & Newton, 1995). However, the role of Ca^{2+} in the protein kinase C/phorbol ester interaction is unclear. Apparent synergism between Ca^{2+} and phorbol esters in increasing the association of the enzyme with membranes suggested strong interactions between the binding sites for these two ligands (Wolf et al., 1985b). However, some reports supported a requirement for Ca^{2+} for phorbol ester-dependent membrane association of protein kinase C (Sando & Young, 1983; Huang & Huang, 1986), while others indicated binding even in the presence of chelators (Kikkawa et al., 1983; Tanaka et al., 1986; Bazzi & Nelsestuen, 1989a). Recently, recombinant proteins containing the regulatory domain of protein kinase C or subdomains thereof were shown to bind phorbol esters in a Ca^{2+}-dependent fashion (Quest & Bell, 1994a) and vice versa (Luo et al., 1993). Thus, despite extensive studies on the effects of phorbol esters on protein kinase C function, the molecular mechanism of how these molecules increase protein kinase C's membrane association is unclear.

This contribution presents a quantitative study on how phorbol esters regulate protein kinase C's membrane association. Our data reveal that the major role of phorbol esters is the same as that recently elucidated for DG (Orr & Newton, 1992; Mosior & Epand, 1993): the two activators anchor protein kinase C to the membrane. Specifically, 1 mol % phorbol 12-myristate 13-acetate increases protein kinase C's membrane affinity by over 3 orders of magnitude. In addition, we show that the association of protein kinase C with phorbol esters is Ca^{2+} independent, revealing that there is no allosteric interaction between Ca^{2+} and phorbol ester-binding sites. Lastly, the Appendix describes how we determined the dissociation constant of the PMA/enzyme complex (1.5×10^{-5} mol % relative to membrane lipid) by an alternative method to the Scatchard approach, which has limitations in the study of amphitropic proteins (Burn, 1988) such as protein kinase C.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoylphosphatidylserine (PS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), egg L-α-phosphatidylcholine (PC), and 1,2-*sn*-dioleoylglycerol (DG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). (Analysis of lipids by TLC revealed no detectable contaminants except for DG, which had a minor amount of 1,3-dioleoylglycerol present; this contamination was small and the lipid was used without further purification.) Protamine sulfate, ATP sodium salt, EGTA, Tris (Trizma base), phorbol 12-myristate 13-acetate (PMA), and phorbol 12,13-dibutyrate (PDBu) were purchased from Sigma. The peptides Ac-ERMRPRKRQGSVRRRV-amide (derived from the pseudosubstrate of ϵ PKC) and Ac-FKKSFKL-amide (derived from MARCKS protein) were synthesized by the Indiana University Biochemistry Biotechnology Facility. [γ-^{32}P]ATP (3000 Ci mmol^{-1}), [^{3}H]DPPC (42 Ci mmol^{-1}), and [^{3}H]PMA (20 Ci mmol^{-1}) were purchased from Du Pont-New England Nuclear (Boston, MA). Bovine serum albumin (fraction V, fatty acid free) was from Boehringer Mannheim (Indianapolis, IN). Protein kinase C βII, from the baculovirus expression system, was purified to homogeneity as described in Orr et al. (1992) 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 purchased from J. T. Baker, Inc. All other chemicals were reagent grade.

Lipid Vesicles. Sucrose-loaded large unilamellar vesicles containing trace [^{3}H]DPPC were prepared as described in Mosior and Newton (1995). Briefly, mixtures of lipids in chloroform were dried under a stream of nitrogen, evacuated under high vacuum, and then suspended in 20 mM Tris (pH 7.0) containing either 170 mM sucrose or 100 mM KCl. Aliquots (0.5 mL) of 10−50 mM lipid were subjected to freeze−thawing and extrusion using a microextruder Liposofast (Avestin, Inc., Ottawa, Ontario, Canada) to form large unilamellar vesicles (LUVs). Phospholipid concentrations in stock solutions were determined by phosphate analysis (Bartlett, 1959); concentrations after extrusion were calculated from radioactivity.

Incorporation of Phorbol Esters in Vesicles. PMA was incorporated into LUVs as described in Mosior and Newton (1995). The partitioning of PMA into vesicles was complete (>98%) as assessed by centrifugation of sucrose-loaded vesicles and analysis of bound ^{3}H-labeled PMA. Appropriate aliquots of suspensions of vesicles containing PMA were diluted severalfold in either activity or binding assay samples. For vesicles containing PDBu, a solution of this phorbol ester in dimethyl sulfoxide was added directly to binding assay samples. The final concentration 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 interaction of protein kinase C with sucrose-loaded vesicles was measured according to the procedure of Rebecchi et al. (1992) and adopted for protein kinase C (Mosior & Epand, 1993). Membrane-bound enzyme was separated from enzyme in solution by centrifugation of kinase/vesicle incubation mixtures for 15 min at 25 °C. The maximal acceleration varied from 40000*g* for DPPC vesicles to 200000*g* for egg PC or POPC vesicles to ensure ≥95% sedimentation of LUVs. The kinase activity toward protamine sulfate was assayed under identical conditions for both the supernatant and the pellet, as described previously (Mosior & Epand, 1993, 1994; Mosior & Newton, 1995). 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

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

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 ^{3}H-labeled PC between the bottom and top fractions, which was included in trace amounts in all lipid mixtures. The fraction of kinase activity measured in the supernatant in the absence of lipid, β, was equal, within the limits of experimental error, to the value expected for a nonsedimenting protein (*i.e.*, 0.73 under the experimental conditions used). All experiments were performed in a solution containing 20 mM Tris (pH 7.0), 100 mM KCl, and 0.3 mg mL^{-1} BSA. Concentrations of additions that varied depending on the experiment are

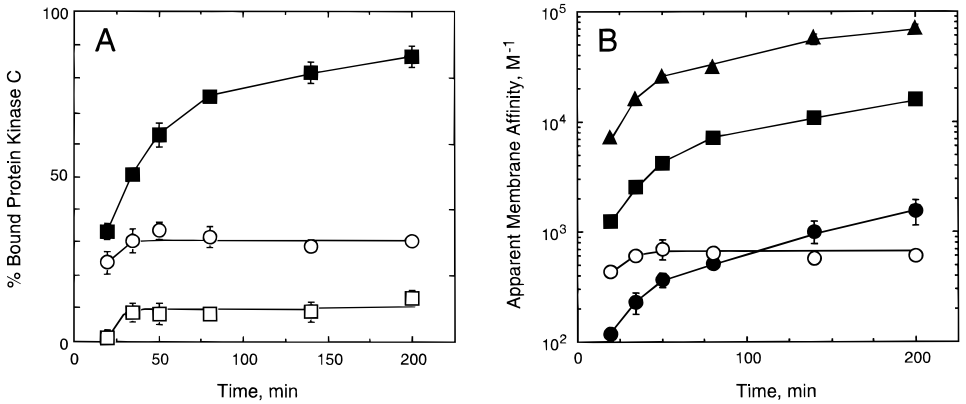


FIGURE 1: Time dependence of protein kinase C's association with membranes containing PMA or DG. (A) The binding of protein kinase C (1 nM) to sucrose-loaded vesicles composed of POPC, 25 mol % POPS, and either 5 mol % DG (○), 0.5 molecule of PMA per large unilamellar vesicle (LUV) (0.0006 mol %; □), or 90 PMA molecules per vesicle (0.1 mol %; ■) was measured. Total lipid concentrations were 2 (□) or 0.5 mM (○, ■). Protein kinase C was incubated with vesicles in the presence of 100 mM KCl, 0.3 mg mL^{-1} BSA, and 20 mM Tris (pH 7.0) for 1−180 min at 22 °C in the presence of 0.5 mM EGTA (■, ○) or 0.3 μM $CaCl_2$ (□). Membrane-bound and free enzyme was separated by centrifugation, as described in Experimental Procedures; this was accomplished in 19 min following the incubation. The time indicated is that elapsed between mixing of protein kinase C and lipid and the end of centrifugation (*i.e.*, incubation time plus separation time). (B) Binding of protein kinase C to vesicles containing 10 PMA per vesicle (0.01 mol %; ●), 100 PMA per vesicle (0.1 mol %; ■), 900 PMA per vesicle (1 mol %; ▲), or 4500 DG per vesicle (5 mol %; ○) is presented as relative membrane affinity calculated from the ratio of bound/free enzyme divided by the total lipid concentration, as described in Experimental Procedures.

indicated in the figure legends. The apparent membrane affinity of the enzyme 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 (0.2 mg mL^{-1}) or synthetic peptides (50 μM) as substrates was measured as described in Orr et al. (1992). The phosphorylation reaction was initiated by the addition of 50 μM [γ-^{32}P]ATP (150 Ci mol^{-1}), 10 mM $MgCl_2$, and indicated concentrations of substrates to an 80 μL reaction volume containing the concentrations of protein kinase C, lipid, phorbol ester, $CaCl_2$, and EGTA noted in the figure legends. Phosphorylation reactions were quenched 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 then washed four times in 0.5 L of 0.4% (v/v) phosphoric acid, followed by one wash in 95% ethanol; associated radioactivity was determined by liquid scintillation counting of papers in Biosafe II.

Calculation of Free Ca^{2+} Concentration. The total concentration of Ca^{2+} 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 Ca^{2+} concentration followed those of Fabiato and Fabiato (1979) and took into account the contamination of all solution components by calcium, the effect of ionic strength, and the interaction of EGTA with Mg^{2+} (when present).

RESULTS

The binding kinetics of protein kinase C to large unilamellar vesicles containing 25 mol % PS and either DG or PMA were investigated. Figure 1A shows that the binding to vesicles promoted by PMA differed from that mediated by DG in two ways. First, it was considerably stronger; for example, after 15 min of incubation (plus separation time), approximately twice as much protein kinase C was membrane associated when incubated with vesicles containing 0.1 mol % PMA than when incubated with a similar concentration of vesicles containing 5 mol % DG. Second, the binding to DG-containing membranes plateaued after 15 min of incubation, whereas the binding to PMA-containing membranes gradually increased with time up to at least 3 h of incubation. However, if membranes contained fewer than 1 PMA molecule per vesicle (0.0006 mol %), binding kinetics were the same as those for DG-containing vesicles. Under the conditions of these assays (no Ca^{2+} present), there was no detectable binding to vesicles in the absence of DG or PMA.

To address whether the lack of equilibrium after extended incubation times would prohibit a quantitative analysis of the effects of phorbol esters on protein kinase C's membrane interaction, we determined how much the membrane affinity actually changed after the initial binding. Because the binding of protein kinase C to membranes depends linearly on the total lipid concentration [see Mosior and Epand (1993) and Figures 2 and 4], an apparent membrane affinity can be calculated from the ratio of bound/free protein kinase C divided by the total lipid concentration (Mosior & Epand, 1993, 1994; Newton & Keranen, 1994). (Note that for vesicles containing anionic lipids with or without DG, or with less than 1 PMA per vesicle, the apparent membrane affinity constitutes an equilibrium constant. Because this quantity increases slowly with time when vesicles contain more than 1 PMA molecule, the apparent membrane affinity is treated only as a parameter reflecting the extent of binding.)

Figure 1B shows that PMA caused a dramatic increase in membrane affinity within 15 min and then a much smaller increase with additional incubation time. The initial increase depended linearly on the PMA concentration: a 10-fold increase in PMA caused a 10-fold increase in apparent membrane affinity (*e.g.*, compare second points for 10 PMA/vesicle and 100 PMA/vesicle). In marked contrast, the time-dependent increase depended only slightly on the PMA concentration. For example, between 50 and 200 min, the apparent membrane affinity increased by 3−4-fold for all PMA concentrations in Figure 1B. Thus, the kinetics of the PMA-dependent membrane association are biphasic, with the fast component depending linearly on the mole percent PMA in the membrane and the slow component not being significantly affected by the relative PMA concentration

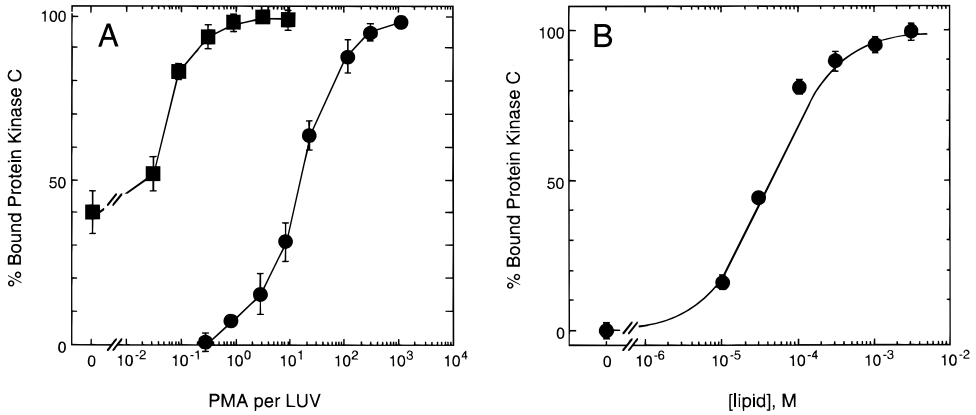


FIGURE 2: PMA dependence of protein kinase C's association with PS in the presence or absence of Ca^{2+}. (A) The binding of protein kinase C to sucrose-loaded vesicles was measured as described in the legend to Figure 1. Binding to vesicles composed of 25 mol % POPS and egg PC as the remaining lipid was measured in the presence of 2 mM total lipid and either 10 μM Ca^{2+} (■) or 0.5 mM EGTA (●). One molecule of PMA per vesicle corresponds to 0.0011 mol % with respect to lipid concentration; PMA was incorporated into vesicles as described in Experimental Procedures. (B) The binding to vesicles composed of 25 mol % PS, 74 mol % PC, and 1 mol % PMA was measured at the indicated concentrations of total lipid. The curve shown is that predicted from the following formula: bound/free enzyme $= (2.2 \times 10^4\ M^{-1})$[lipid].

(except in the cases where membranes contain fewer than 1 PMA per vesicle, in which case the slow component is absent). Thus, despite the lack of equilibrium, it is possible to separate two different phenomena: an initial PMA-dependent binding that changes linearly with PMA concentration and a subsequent slow increase in protein kinase C's membrane association that is not significantly dependent on the PMA concentration. The magnitude of the latter process was relatively small. Note that the lack of equilibrium is a property of the protein kinase C/phorbol ester interaction and not the assay used. Equilibrium is reached within minutes (*i.e.*, of incubation time prior to the separation procedure) for vesicles containing DG or less than 1 PMA/vesicle (see above) or for PS/PC vesicles without DG or PMA (Mosior & Epand, 1993).

The effect of increasing concentrations of PMA on the association of protein kinase C with membranes is explored further in Figure 2. PMA caused an increase from no to complete partitioning of the enzyme with vesicles over an approximately 100-fold concentration range for a given condition. In the presence of Ca^{2+}, the affinity of protein kinase C for PS-containing membranes is sufficiently great that significant binding occurs in the absence of either DG or PMA (Nelsestuen & Bazzi, 1991; Mosior & Epand, 1993; Newton & Keranen, 1994). Figure 2A shows that PMA caused a further increase in the enzyme's affinity for vesicles containing 25 mol % PS in the presence of 10 μM Ca^{2+}. PMA also caused an increase in the interaction with PS vesicles in the absence of Ca^{2+}. In the presence of 0.5 mM EGTA, the PMA-dependent affinity for PS-containing vesicles was approximately 200 times weaker than in the presence of 10 μM Ca^{2+}.

The ratio of membrane-bound to free enzyme was proportional to the total lipid concentration under all conditions tested. Figure 2B shows that the fraction of membrane-bound protein kinase C increased as the concentration of vesicles (25 mol % PS, 1 mol % PMA) was increased; the data fit a curve described by a single constant (see legend to Figure 2B). In the following experiments, apparent membrane affinities were obtained by measuring the binding of protein kinase C, as a function of PMA or Ca^{2+}, at two or three lipid concentrations that differed by at least 1 order of magnitude. This allowed us to calculate an apparent membrane affinity over many orders of magnitude of a particular variable. That is, if binding was of very high affinity, low lipid concentrations were chosen such that protein kinase C was distributed between the solution and the membrane. Conversely, if binding was very weak, high lipid concentrations were chosen to promote association of the enzyme with the membrane.

The association of protein kinase C with membranes containing a 5 orders of magnitude range of PMA was investigated. To obtain accurate relative membrane affinities, binding curves were measured at three lipid concentrations (10 μM, 0.5 mM, and 2.5 mM). The data from these curves are presented in Figure 3. Most strikingly, the affinity of protein kinase C for large unilamellar vesicles increased linearly as the mole percent of phorbol ester in membranes increased, in both the presence or absence of Ca^{2+}. The PMA-induced increase in affinity was remarkably high; for example, 1 mol % PMA in the vesicle membrane (900 molecules per vesicle) resulted in an over 5000-fold increase in the apparent membrane affinity. The data in Figure 3 also reveal that the apparent membrane affinity varies linearly with the PMA/vesicle ratio below 1 PMA per vesicle. At these lower concentrations, the linear dependence reflects the fraction of vesicles with 1 PMA per vesicle. For example, 1 PMA per vesicle increased the membrane affinity 60 times relative to that in the complete absence of PMA, and 2 PMA per 7 vesicles increased the relative affinity by 17-fold ($2/7 \times 60$). Note that the results obtained under equilibrium conditions [*i.e.*, for vesicles with fewer than 1 PMA molecule (first four filled circles in Figure 3] align well with the data obtained after 15 min of incubation under nonequilibrium conditions (subsequent three filled circles). This provides further support that it is possible to separate the initial, mass action-like binding of protein kinase C to phorbol esters from the subsequent time-dependent increase in membrane association. When the binding was measured after 3 h instead of 30 min, the relative affinity increased by a factor of 3−7, consistent with the results in Figure 1B. Importantly, PMA had the same relative effect on binding independent of how long the enzyme was allowed to incubate with vesicles. That is, a 100-fold increase in PMA concen-

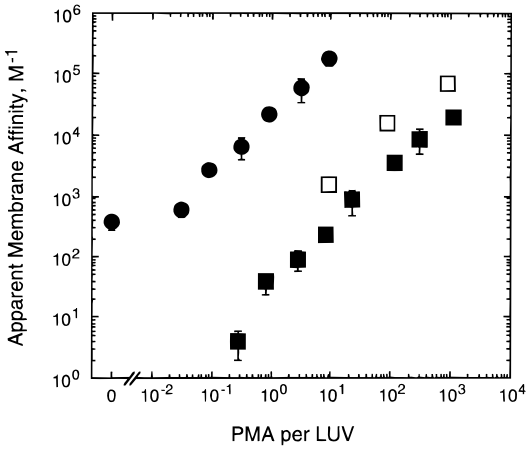


FIGURE 3: Dependence of protein kinase C's membrane affinity on relative membrane concentration of phorbol esters. The binding of protein kinase C to sucrose-loaded vesicles composed of 1/3 POPS/POPC and the indicated concentrations of PMA, expressed as molecules PMA/vesicle, was measured in the presence of 10 μM $CaCl_2$ (●) or 0.5 mM EGTA (■). Protein kinase C (0.5−1 nM) was incubated with vesicles in the presence of 100 mM KCl, 0.3 mg mL^{-1} BSA, and 20 mM Tris (pH 7.0) for 15 min at 22 °C. Total lipid concentrations were 2.5 mM (first five ■), 0.25 mM (fifth to ninth ■), 10 μM (ninth ■), 2 mM (first four ●), 0.2 mM (fourth to sixth ●), and 10 μM (sixth and seventh ●). The results for points measured at different lipid concentrations were averaged. Membrane-bound and free enzyme was separated by centrifugation, as described in Experimental Procedures. The concentration of lipid was varied so that between 20% and 80% of the protein kinase C would be membrane bound, thus allowing the accurate determination of the apparent binding constant. One molecule of PMA per vesicle corresponds to 0.0011 mol % with respect to lipid concentration; PMA was incorporated into vesicles as described in Experimental Procedures. Results are presented as the apparent affinity calculated from the ratio of bound/free enzyme divided by the total lipid concentration, as described in Experimental Procedures. The total incubation of time of enzyme and vesicles, including the centrifugation time, was 30 min. Also shown are results obtained in the presence of 0.5 mM EGTA and a 3 h incubation time (□). Data represent the average of two independent experiments, each in triplicate. Error bars, shown when larger than the symbol size, indicate the range.

tration caused an approximately 100-fold increase in membrane affinity independent of incubation time.

The marked increase in membrane affinity caused by phorbol esters led us to explore whether protein kinase C could directly bind to, and be activated by, phorbol esters in the absence of acidic lipids, if sufficiently high concentrations of phospholipid and phorbol ester were chosen. Figure 4A shows that protein kinase C bound to egg PC vesicles (4 mM total lipid) in a PMA-dependent manner. Binding of protein kinase C to PMA-containing PC vesicles depended linearly on the PMA concentration and, thus, reflected specific binding to the vesicles rather than nonspecific sedimentation.

Figure 4B shows that the binding to PC/PMA vesicles displayed the same characteristics as the binding to vesicles containing acidic lipids. Specifically, the ratio of bound/free enzyme increased linearly with vesicle concentration.

Figure 4C shows that the PMA-dependent binding to PC vesicles was accompanied by the activation of protein kinase C. PMA in PC vesicles stimulated protein kinase C to phosphorylate the heptapeptide FKKSFKL-NH_2 derived from the MARCKS proteins (Chakravarthy et al., 1991) and the hexadecapeptide ERMRPRKRQGSVRRRV derived from the pseudosubstrate of protein kinase C ϵ (Schaap et al., 1989) in the absence of Ca^{2+}. This PMA-dependent phosphorylation was not stimulated by Ca^{2+} (not shown), in contrast to the marked effect of Ca^{2+} on the PS-stimulated activation of protein kinase C. The degree of activation was sensitive to the substrate: the rate of phosphorylation of the ϵ peptide mirrored the fraction of enzyme that was vesicle associated. In contrast, the rate of phosphorylation of a smaller, less charged peptide based on the MARKS protein lagged behind binding (*e.g.*, only 5% of V_{max} when 25% bound). Thus, the interaction of protein kinase C with PMA is sufficiently strong that membrane binding and activation can occur in the complete absence of acidic lipids.

The effect of Ca^{2+} on the PMA-dependent binding of protein kinase C to PC vesicles is examined in Figure 5. Strikingly, the apparent membrane affinity was independent of Ca^{2+} concentration over a 5 orders of magnitude range (from 0.5 nM to 0.1 mM). This reveals that there is no allosteric interaction between the primary phorbol ester-binding site and Ca^{2+}-binding site involved in membrane association. In contrast to the binding to PC vesicles, the binding to vesicles containing 25 mol % PS depended on the Ca^{2+} concentration above 0.1 μM Ca^{2+}. The inset shows the dependence of binding on Ca^{2+} concentration in the presence of 50 μM lipid; the apparent membrane affinity is plotted in the main figure. The Ca^{2+} dependence on binding to PS-containing vesicles is consistent with previous reports showing that Ca^{2+}- and PS-binding sites interact allosterically (Mosior & Epand, 1994; Newton & Keranen, 1994). The lack of a Ca^{2+} effect below 0.1 μM Ca^{2+} indicates that this concentration is around that of the apparent dissociation constant of Ca^{2+} from the PMA/PS/enzyme complex. Note that because protein kinase C has a relatively high affinity for PS, a much lower concentration of PMA (0.03 mol %) was chosen for the binding to vesicles composed of PC and PS.

To test whether DG, which also increases the affinity of protein kinase C for membranes, binds to the same site on protein kinase C as phorbol esters, we examined whether DG affected the PMA-induced increase in membrane affinity. Figure 6 shows the effect of increasing concentrations of PMA on the affinity of protein kinase C for vesicles containing 0.5 or 5 mol % DODG. For vesicles containing 0.5 or 5 mol % DG, the presence of 1 molecule of PMA per vesicle caused 10- and 2-fold increases, respectively, in the affinity of protein kinase C for membranes relative to no PMA. In the absence of DG, 1 molecule of PMA increased the affinity of protein kinase C for membranes by 60-fold (see Figure 3). Thus, DG decreases the PMA-induced increase in membrane affinity, suggesting that these two activators compete for the same site on protein kinase C.

Figure 7 compares the effect of PMA to that of DG on the catalytic activity of protein kinase C. Maximal activity required 3 orders of magnitude more DG than PMA. Importantly, the maximal activity was the same when both DG and PMA were present in the vesicles. Note that under the conditions of the assay with 1 mol % PMA (2 mM lipid), over 99% of the protein kinase C was membrane bound even without DG. Thus, when protein kinase C was maximally bound to membranes, its catalytic activity was the same with either DG or PMA and was unaltered when both activators were present. Thus, no synergistic action of DG and PMA was observed. Curiously, PMA was about 4-fold more potent in activating protein kinase C than in increasing the

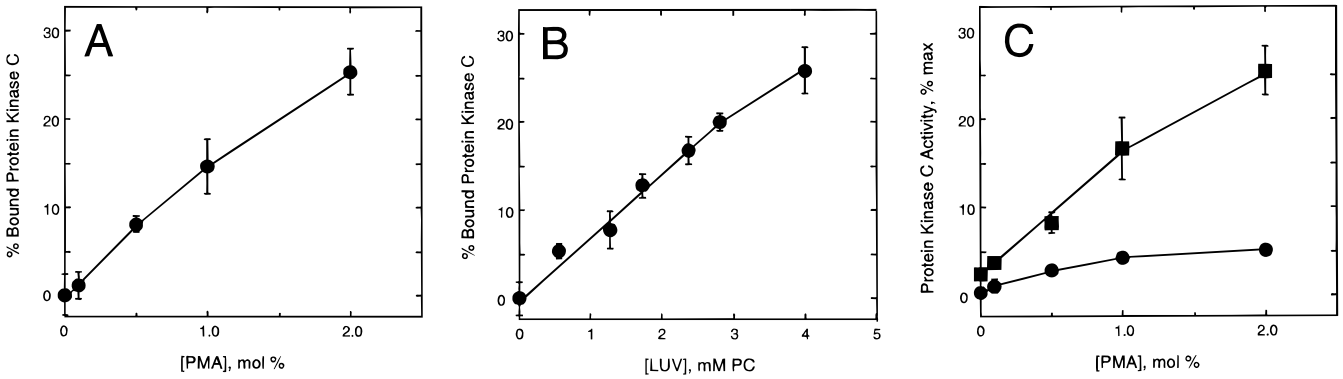


FIGURE 4: Binding and activation of protein kinase C by phorbol esters in the presence of POPC vesicles. (A) The binding of protein kinase C (0.5 nM) to POPC vesicles (4 mM lipid) containing 0−2 mol % PMA was measured in the presence of 0.5 mM EGTA, 0.3 mg mL^{-1} BSA, 100 mM KCl, and 20 mM Tris (pH 7.0) after 15 min of incubation, as described for Figure 1. (B) The binding to POPC vesicles containing 2 mol % PMA was measured as a function of the indicated lipid concentrations. (C) The activity of protein kinase C toward phosphorylation of a protein kinase C-selective peptide based on the MARCKS protein (50 μM) (●) or a peptide based on the pseudosubstrate of protein kinase C ϵ (50 μM) (■) was measured as described in Experimental Procedures. Activity is expressed relative to the maximal activity of protein kinase C toward each peptide measured in the presence of PS, DG, and Ca^{2+}; 1 unit corresponds to 1 nmol of phosphate incorporated in 1 min at 30 °C per microgram of protein kinase C. Maximal activities were 1.3 and 5.2 units for the MARCKS- and ϵ-derived peptides, respectively.

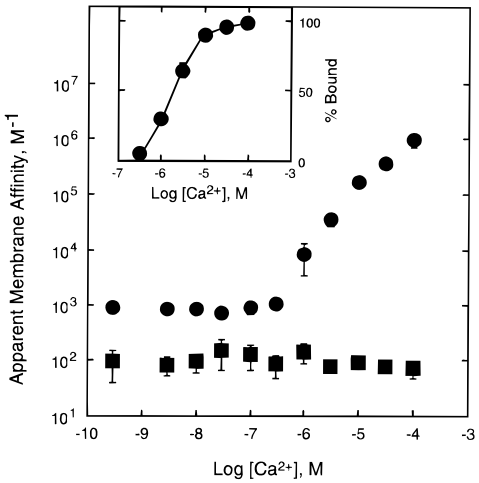


FIGURE 5: Dependence of protein kinase C's affinity for phorbol ester-containing membranes as a function of Ca^{2+} concentration and PS content. The binding of protein kinase C (1 nM) to sucrose-loaded vesicles composed of egg PC (98 mol %) and PMA (2 mol %) (■) or PS/PC (1/3, mol ratio) and PMA (0.03 mol %) (●) was measured at the indicated concentrations of free Ca^{2+}, as described in the legend to Figure 1, except that 0.5 mM EGTA was present in all incubations. Lipid concentrations were 2−5 mM and 5−500 μM for PC and PS/PC vesicles, respectively. Results are presented as the apparent membrane affinity calculated from the binding data, as described in Experimental Procedures. Data represent the average and range of two independent experiments, each in triplicate. The inset shows the binding of protein kinase C to vesicles containing PS (●), from which the data in the main figure were obtained in part. The total lipid concentration was 50 μM.

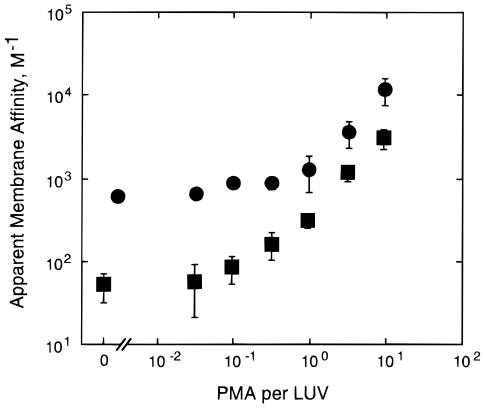


FIGURE 6: Effect of diacylglycerol on the phorbol ester-dependent binding of protein kinase C to membranes. The binding of protein kinase C to sucrose-loaded vesicles containing 25 mol % POPS, 74.5 or 70 mol % PC, and 0.5 (■) or 5 mol % (●) DODG was measured in the presence of 0.5 mM EGTA, as described in Experimental Procedures and the legend to Figure 1. Lipid concentrations were 2 (0.5 mol % DODG) or 0.2 mM (5 mol % DODG). Results are presented as the apparent membrane affinity calculated from the ratio of bound/free enzyme divided by the total lipid concentration, as described in Experimental Procedures. Data represent the mean ± SD of three independent experiments.

enzyme's membrane affinity relative to DG. Activation could lag behind binding for DG as a result of the marked differences in surface concentration of the two ligands needed to effect the same level of binding (*i.e.*, the residency time on a PMA molecule would be greater than that on a DG molecule for two membranes containing equal levels of bound protein kinase C because 100-fold more DG molecules would be present than PMA molecules).

The phorbol ester-dependent increase in protein kinase C's membrane affinity depends on the surface concentration of the phorbol ester rather than its absolute concentration. Figure 8 examines the effect of increasing the mole percent of phorbol ester while maintaining the same absolute phorbol ester concentration (achieved by adjusting the total lipid concentration) on the ratio of membrane-bound to free protein kinase C. In the presence of 0.01 μM PMA, the increase in this ratio stimulated by PMA was 10 times greater when the phorbol ester was present at a relative concentration of 0.02 mol % than at 0.002 mol %. Similarly, the increase in the ratio of membrane-bound to free protein kinase C induced by 0.05 mol % PDBu (25 nM) was approximately 10 times greater than that induced by 0.005 mol % PDBu (also 25 nM). Under the conditions in Figure 8, PMA and PDBu were approximately 250 and 10 times, respectively, more effective than DG in increasing the affinity of protein kinase C for membranes.

DISCUSSION

The foregoing results reveal the molecular mechanism by which phorbol esters increase protein kinase C's membrane affinity. Specifically, phorbol esters bind directly to protein

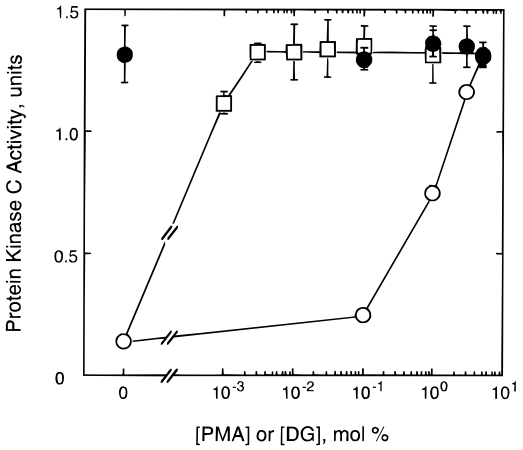


FIGURE 7: Effect of diacylglycerol on phorbol ester-stimulated activity of protein kinase C. The activity of protein kinase C (0.2 nM) was measured in the presence of 200 μM $CaCl_2$ and large unilamellar vesicles (2 mM) composed of 25 mol % PS and either 5 mol % DODG (○), 1 mol % PMA (□), or 5 mol % DODG and 1 mol % PMA (●); POPC constituted the remaining component. The substrate was a synthetic peptide based on MARCKS (50 μM). Data represent the mean ± SD of two independent experiments, each in triplicate.

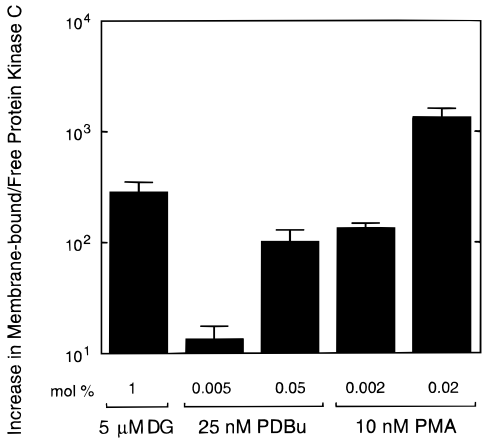


FIGURE 8: Dependence of the phorbol ester-induced increase in protein kinase C's membrane affinity on the membrane concentration of the phorbol ester. The binding of protein kinase C (1 nM) to large unilamellar vesicles composed of 25 mol % POPS, the indicated mol % of either DG, PDBu, or PMA, and POPC as the remaining component was measured in the presence of 0.2 mM $CaCl_2$, as described in Experimental Procedures and the legend to Figure 1. Lipid concentrations were varied from 10 (*e.g.*, highest concentration of PMA) to 500 μM (*e.g.*, no DG or phorbol esters), so that between 10% and 90% of the protein kinase C was membrane bound for accurate determination of the ratio of bound/free protein kinase C. Results are presented as the ratio of bound/free protein kinase C measured in the presence of DG or phorbol ester divided by the ratio of bound/free protein kinase C measured under identical conditions, except that DG or phorbol ester was not in the membranes. Thus, the number reflects the DG- or phorbol ester-dependent increase in protein kinase C's membrane partitioning. Data represent the average and range or mean ± SD of at least two experiments.

kinase C, in the absence of acidic lipids and in a Ca^{2+}-independent manner, thus providing a membrane anchor independent of the occupancy of the PS/Ca^{2+}-binding domain (C2 domain). The linear dependence of the PMA-induced membrane binding on the mole fraction of this molecule, rather than its absolute concentration, is consistent with the apparent increase in affinity arising from the direct binding of the enzyme to membrane-embedded PMA. Thus, the major role of phorbol esters in the regulation of protein kinase C is similar to that recently discovered for DG (Orr & Newton, 1992; Mosior & Epand, 1993, 1994; Newton & Keranen, 1994). Remarkably, the affinity for PMA is so high that membrane association can occur in the absence of acidic lipids. In summary, our data indicate that the direct binding of protein kinase C to phorbol esters provides a major contribution to the free energy change involved in the association of protein kinase C with membranes that is separate from the Ca^{2+}-dependent binding to PS.

Affinity. Since the discovery that phorbol esters activate protein kinase C (Castagna et al., 1982), the interaction between these two types of molecules has been treated as a bimolecular interaction whose stoichiometry and characteristic dissociation constant can be derived from the Scatchard plot (Sharkey et al., 1984; Castagna et al., 1982; Kazanietz et al., 1993; Kikkawa et al., 1983; Tanaka et al., 1986; Bazzi & Nelsestuen, 1989a; Hannun & Bell, 1986). For example, such an approach was used recently to calculate the dissociation constants of phorbol esters from recombinant domains of protein kinase C expressed as GST fusion (Burns & Bell, 1991; Quest & Bell, 1994a). However, experimental and theoretical considerations reveal that Scatchard analysis is not appropriate for the calculation of the dissociation constant of the phorbol ester/lipid/membrane complex. Specifically, the Scatchard plot describes the interaction between soluble molecules, as well as the interaction between soluble ligands and receptors that are an integral part of the membrane (Lauffenburger & Sinderman, 1993). Neither of these conditions describes the interaction of protein kinase C and phorbol esters, both of which partition between the solution and membranes. Furthermore, the association of protein kinase C with membranes depends strongly on the composition of the membrane (Bazzi & Nelsestuen, 1991; Orr & Newton, 1992; Mosior & Epand, 1993) and, for conventional protein kinase C's, the Ca^{2+} concentration (May et al., 1985; Wolf et al., 1985b; Bazzi & Nelsestuen, 1991; Orr & Newton, 1992; Mosior & Epand, 1993, 1994). As a result, the concentration of phorbol esters causing half the protein kinase C molecules to be complexed to phorbol esters depends on the PS content of the membranes and the Ca^{2+} concentration. For example, the addition of 1 ppm phorbol esters with respect to lipid (2 nM PMA) doubled the amount of protein kinase C associated with membranes composed of PS/PC (1/3 mol ratio) in the presence of 10 μM Ca^{2+} (Figure 2A). For comparison, 40 000 times more PMA (80 μM) resulted in only 25% of protein kinase C binding to membranes composed of PC (Figure 4A). Equally striking effects of Ca^{2+} on the PMA-dependent association of protein kinase C with membranes are illustrated in Figures 2A and 3. Thus, the apparent association constant for the protein kinase C/phorbol ester complex is a composite of the actual dissociation constant of phorbol esters from the enzyme and the dissociation constant of the enzyme from other components such as PS. As shown in the Appendix, the apparent dissociation constant derived from the Scatchard plot is a composite of six independent dissociation constants. As a result, the dissociation constants of phorbol esters from protein kinase C/lipid complexes reported in the literature are specific to a particular set of experimental conditions.

Equilibrium analysis is further impaired by the apparent lack of equilibrium in the phorbol ester/protein kinase C interaction observed under most conditions (Figure 1). This can be circumvented by limiting measurements to equilibrium

conditions (*i.e.*, fewer than 1 PMA per vesicle). Because of the extraordinarily high affinity of protein kinase C for PMA, this is possible: the dissociation constant can be readily determined by using the first four experimental points in Figure 3 (*i.e.*, fewer than 1 PMA per vesicle) and eq A5 in the Appendix. Our results suggest, however, that it is possible to separate the equilibrium binding of protein kinase C from the slow association component under all conditions. First, the kinetics of the slow component for the association of enzyme with vesicles containing >1 PMA is approximately 1 order of magnitude slower than that of the equilibrium component measured under equilibrium conditions (*i.e.*, no or fewer than 1 PMA/vesicle). Second, the dependence of the fast and slow components of the association kinetics display qualitative differences in the dependence on the mole fraction PMA in the membrane. Third, results obtained for vesicles with >1 PMA per vesicle at the time required to reach equilibrium for all other types of vesicles (*i.e.*, ±DG) align well with those obtained under equilibrium conditions. Fourth, the strict linear dependence on the membrane-bound/free protein ratio on either the total lipid concentration or the mole fraction PMA in the membrane is consistent with equilibrium binding in the association of protein kinase C with PMA-containing vesicles. Thus, the equilibrium analysis proposed in the second part of the Appendix (eqs 4−6) may be extended to all results obtained after 15 min of incubation.

Our data obtained under equilibrium conditions (Figure 3) are, however, sufficient to determine the dissociation constant of the protein kinase C/PMA complex. Thus, by fitting the results for the binding of protein kinase C to PMA in the presence of PS and Ca^{2+} (Figure 3) to eq A5 in the Appendix, we obtained a value for the dissociation constant of membrane-bound protein kinase C from membrane-bound PMA (K_6 in the Appendix): 1.5×10^{-5} mol % with respect to lipid. This value can be converted into more conventional units by using the Guggenheim approach (Aveyard & Haydon, 1973; Lauffenburger & Sinderman, 1993), which has been successfully applied to the description of the binding of basic peptides to anionic lipids (Kim et al., 1991; Mosior & McLaughlin, 1992). Following the approach described in these papers, we assumed the thickness of the solute/lipid interface to be 1 nm and the surface area of a lipid molecule to be 0.7 nm^2 and arrived at the value of 0.4 μM PMA in the membrane.[2] This particular dissociation constant dominates over all of the other ones even for "soluble" PDBu, because the association of protein kinase C with the membrane depends on the interfacial concentration of this molecule rather than its bulk concentration (Figure 8). This dependence on interfacial concentration was also noted by Blumberg and co-workers (Sharkey et al., 1984). The importance of the interfacial concentration of ligand has also been demonstrated for other lipid-binding proteins, such as phospholipase A2 (Hendrickson & Dennis, 1984). Because the effect of DG on protein kinase C's association with membranes is 2 orders of magnitude smaller than the effect of PMA, the same dissociation constant for DG has a value on the order of 10^{-3} mol %.

The second dissociation constant, that of soluble protein from membrane-bound PMA (K_1 in the Appendix), was too weak to determine directly. However, extrapolation of the results obtained with vesicles composed of PC and PMA only (Figure 4A) suggests a value of at least 100 μM.[3] The question arises whether the dissociation constant of a putative soluble protein/phorbol ester complex can be determined from our results. In this regard, a recent study demonstrated that the binding of Ca^{2+} to protein kinase C in solution can be determined (Mosior & Epand, 1994). Specifically, if the enzyme associates with its ligand with two different association constants, higher for the membrane-bound enzyme and lower for the soluble enzyme, then the association of the enzyme with the membrane ceases to depend on the ligand concentration at concentrations that are above the dissociation constant of the lower affinity interaction. In this study, the membrane association depended linearly on PMA concentration even at the highest concentrations of PMA employed, suggesting that enzyme in solution was not interacting detectably with PMA in solution. Note that under all conditions more than 98% of the PMA was lipid associated. Thus, association of soluble protein kinase C with PMA in solution was unlikely to be significant under our experimental conditions. Recently, however, the association of the more readily water-soluble PDBu with protein kinase C δ, as well as its second cysteine-rich domain, was observed in the absence of lipids (Kazanietz et al., 1995). Addition of PS increased this affinity by 2 orders of magnitude, similar to the increase we observed in the PMA-dependent binding to pure PC versus PC/PS membranes. Large differences in experimental conditions preclude direct comparison between these two sets of data.

Stoichiometry. The preceding results are consistent with earlier findings (Sharkey et al., 1984; Kikkawa et al., 1983; Tanaka et al., 1986; Bazzi & Nelsestuen, 1989a; Hannun & Bell, 1986; Fisher & Yanagimoto, 1989) showing that protein kinase C binds only one phorbol ester molecule with high affinity. If the equilibrium analysis can be extended to all PMA concentrations, as suggested by our results, then the association constant of the putative second binding site is at least 3 orders of magnitude lower than that for the first one (Figure 3). This is somewhat surprising considering that both Cys-rich regions of Ca^{2+}-dependent protein kinase C γ have been reported to display very similar affinities for phorbol esters (Burns & Bell, 1991; Quest & Bell, 1994a). However, the orientation of one of these domains with regard to the plane of the membrane when the enzyme is associated with PS, or the masking by other domains in the protein, may severely limit access of phorbol esters to the second binding site. In this regard, when a neighboring domain was attached to the Cys-2 motif in a recombinant protein, the association with phorbol esters was almost 2 orders of magnitude weaker (Quest & Bell, 1994a). In the present study, protein kinase C associated with vesicles in a time-dependent manner when vesicles contained more than 1 PMA per vesicle. A similar

[2] Note that the bulk (*i.e.*, total) concentration of PMA in the test tube typically ranges from nanomolar to micromolar, depending on the total lipid concentration. Thus, numbers reported in the literature as the dissociation constant of phorbol ester from protein kinase C that are based on the bulk concentration of the former molecule would be relevant only in the context of the particular experimental setup. See Discussion.

[3] This number was calculated by assuming that (i) PMA distributes evenly between both bilayer leaflets and (ii) protein kinase C does not associate with PC. The equilibration of PMA between the opposing leaflets of the bilayer has been demonstrated to occur in less time than the 15 min (Fisher & Yanagimoto, 1989) incubations of lipid vesicles with PMA in the experiments reported here.

time dependence observed in the activation of protein kinase C by phorbol esters and other molecules led to the proposal that a second low-affinity phorbol ester-binding site might be involved (Kazanietz & Blumberg, 1992). Curiously, our results indicate that this second phase of membrane association that occurs in the presence of PMA shows little change with phorbol ester concentrations differing by 2 orders of magnitude. Thus, it is not clear whether the slow time-dependent increase in affinity for vesicles containing more than 1 PMA molecule reflects the occupancy of a second phorbol ester site.

Alternatively, the time-dependent changes could reflect the irreversible insertion of protein kinase C into the hydrophobic core of the membrane, as suggested by several studies (Kraft & Anderson, 1983; Kazanietz et al., 1992; Bazzi & Nelsestuen, 1989b; Nelsestuen & Bazzi, 1991). Two recent reports suggest that this is unlikely: first, Blumberg and coworkers have shown that membrane-translocated protein kinase C redistributes to the cytosol when phorbol ester-treated cells are washed to remove the phorbol esters (Szallasi et al., 1994), and second, biophysical measurements reveal that protein kinase C's association with phorbol ester-containing membranes is reversible and does not involve detectable membrane insertion (Mosior & Newton, 1995). Thus, although our results suggest that at least 2 PMA molecules per vesicle are required to observe the second, slow component of the PMA-induced membrane association of protein kinase C, the mechanism of this phenomenon is unclear.

The competition experiment in Figure 6 is consistent with earlier findings that DGs and phorbol esters appear to compete for the same binding site (Sharkey et al., 1984; Hannun & Bell, 1986; Bazzi & Nelsestuen, 1989b). That is, the presence of DG in vesicles reduced the binding of protein kinase C to PMA. Curiously, the presence of DG seemed to increase the apparent association constant of the enzyme with PMA by a factor of 10−30. On the basis of the ability of PMA to compete with DG, Sharkey et al. (1984) reported that the relative binding constant of protein kinase C for PMA was considerably larger than that for DG than what we report. This discrepancy could result from DG altering the affinity of protein kinase C for PMA, as suggested from our own competition study.

In contrast to this work and previous reports (Sharkey et al., 1984; Bazzi & Nelsestuen, 1989b), a recent report suggested that phorbol esters and DGs bind to separate binding sites because phorbol esters seem to increase further the activity of DG-stimulated enzyme (Slater et al., 1994). However, only the activity of the enzyme was reported, so that the degree of membrane association was unclear. Analysis of the effect of PS, DG, Ca^{2+}, and Mg^{2+} on the association of protein kinase C with phospholipid membranes (Mosior & Epand, 1993, 1994) suggests that, under the experimental conditions chosen by Slater et al. (1994), the enzyme was not fully associated with membranes containing DG and no PMA. Addition of PMA, which has an over 200 times higher affinity for protein kinase C, would have resulted in complete association of the enzyme with the membrane, thus accounting for the observed increase in activity. This contribution shows that if over 99% of the enzyme is membrane bound in the presence of either DG and/or PMA, the activity of protein kinase C is identical (Figure 7).

Interaction of Ca^{2+}- and PMA-Binding Sites. Remarkably, the PMA-induced association of protein kinase C with PC membranes was unaffected by changes in Ca^{2+} concentration over 5 orders of magnitude. Similarly, alteration of the Ca^{2+} concentration over 5 orders of magnitude had no significant effect on enzymatic activity supported by PMA in PC membranes. This insensitivity to Ca^{2+} demonstrates that the Ca^{2+}- and phorbol ester-binding sites do not interact allosterically: occupancy of one does not alter the affinity of the other for its ligand. The same conclusion was reached for DG on the basis of the insensitivity of the DG-dependent increase in membrane affinity to Ca^{2+} (Mosior & Epand, 1994; Newton & Keranen, 1994). Several different explanations were offered to explain the apparent synergism between phorbol esters and Ca^{2+} in the association of protein kinase C with membranes (May et al., 1985; Wolf et al., 1985a,b; Nelsestuen & Bazzi, 1991; Bell, 1986). Here (Figure 5) and elsewhere (Mosior & Epand, 1994; Newton & Keranen, 1994) we demonstrate that this apparent effect is a consequence of the allosteric interaction of PS- and Ca^{2+}-binding sites (*i.e.*, Ca^{2+} increases the affinity for PS) and the fact that both PS and phorbol ester (or DG) contribute to the association of the enzyme with membranes. The apparent association constant of protein kinase C with membranes containing PS is a linear function of the free Ca^{2+} concentration over a wide range of Ca^{2+} concentrations (Mosior & Epand, 1994). Because either phorbol esters or DG contributes significantly to the association of protein kinase C with membranes, the same level of membrane association is achieved in their presence at lower Ca^{2+} concentrations as is achieved in their absence but at higher Ca^{2+} concentrations. At Ca^{2+} concentrations below the dissociation constant of this cation from membrane-bound protein kinase C (Mosior & Epand, 1994), the extent of association of the enzyme with membranes solely depends on the PS and PMA content (Figures 1, 3, and 5). Thus, the molecular mechanism that accounts for why phorbol esters reduce protein kinase C's Ca^{2+} requirement for activity is because phorbol esters cause tighter binding to the membranes; as a consequence, less Ca^{2+} is required.

Model of Activation of Ca^{2+}-Dependent Protein Kinase C. On the basis of the preceding results and previously published work (Orr & Newton, 1992; Mosior & Epand, 1993, 1994; Newton & Keranen, 1994), specific roles can be assigned to four classes of activators: anionic lipids, phorbol esters and DGs, Ca^{2+}, and peptide (or protein) substrates. The first two classes provide the free energy change required for the association of protein kinase C with membranes. Whether the binding sites for PS and phorbol esters/DGs affect the affinity of the other has not yet been determined. Entropy considerations (Dwyer & Bloomfield, 1981; Finkelstein & Janin, 1989; Perutz, 1990) would suggest that the direct binding to PS facilitates the binding to PMA or DG because of the reduction in the number of degrees of freedom and the correct alignment of the protein and ligand; however, additional allosteric interactions may also play a role in allowing binding to either PS or PMA/DG to increase the affinity for the other class of activator. As described in this paper for phorbol esters and presented previously for DG (Orr & Newton, 1992; Mosior & Epand, 1993; Newton & Keranen, 1994), the role of these ligands is to increase the affinity of protein kinase C for membranes by approximately 4 or 2 orders of magnitude, respectively, when

present at membrane concentrations of 1 mol %. Importantly, the association of the enzyme with phorbol esters/DGs is Ca^{2+} independent. Thus, the binding of Ca^{2+} has no functional effect on the conformation of the phorbol ester-/DG-binding sites in the C1 domain.

In marked contrast, the affinity of protein kinase C for PS is a linear function of Ca^{2+} concentration in the range between the dissociation constants of this cation from membrane-bound and soluble enzyme (Mosior & Epand, 1994). Thus, the role of Ca^{2+} is to alter the conformation of protein kinase C so that it has a higher affinity for acidic lipids. Substrate binding also regulates the activity of protein kinase C. Notably, while the combined interaction with PS, DG, and Ca^{2+}, in the absence of substrate, has been shown to activate protein kinase C by releasing the pseudosubstrate from the active site (Orr et al., 1992), strongly interacting substrates such as protamine are able to cause this conformational change in the absence of lipid and Ca^{2+} (Orr & Newton, 1994). This contribution shows that certain peptide substrates and PMA immobilized in PC membranes, in the absence of PS, are sufficient to activate protein kinase C, presumably by also removing the pseudosubstrate from the active site. Similarly, PS/PC vesicles and Ca^{2+} alone (Mosior & Epand, 1993) or PS/DG/PC vesicles in the absence of Ca^{2+} (Mosior & Epand, 1994) activate the enzyme to phosphorylate peptide substrates. Taken together, these results suggest that either PS or strongly interacting substrates are necessary and sufficient to cause the conformational change leading to the activation of protein kinase C. Ongoing studies are aimed at elucidating the individual contributions of PS, DG/PMA, and peptide substrates to conformational changes that accompany protein kinase C's membrane binding and activation.

CONCLUSIONS

The foregoing results reveal that the mechanism of interaction of protein kinase C with membranes that is promoted by phorbol esters is qualitatively similar to the binding induced by DG. The major difference between the two activators is that phorbol esters are "super" DGs in that one phorbol ester has the same effect on protein kinase C's membrane interaction as 100 DGs. Association of protein kinase C with phorbol esters depends on their mole percent in the interface and is Ca^{2+} independent. Note that it is the allosteric interaction between PS- and Ca^{2+}-binding sites that causes the apparent Ca^{2+} sensitivity in the association of protein kinase C with phorbol esters. The remarkably high-affinity binding to PMA accounts for the ability of PMA to cause conventional protein kinase C's to translocate to membranes *in vivo* in the absence of increased intracellular Ca^{2+}. The dramatic increase in protein kinase C activity induced by phorbol ester arises primarily from the translocation of this enzyme to membranes, which results from the additional free energy change provided by the direct association of the enzyme with phorbol esters.

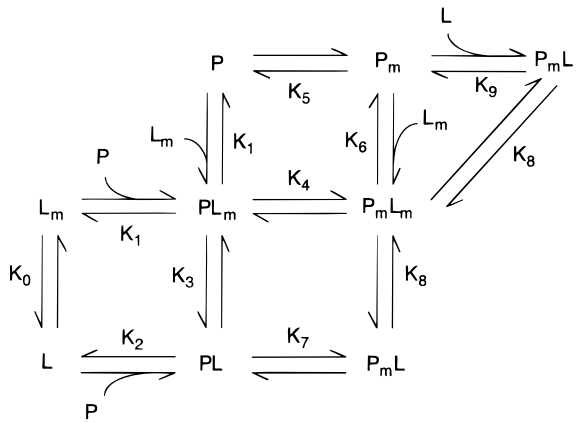
ACKNOWLEDGMENT

We thank Beth Gooding and Sherry Cai for purifying protein kinase C, Lisa Wright for measuring Ca^{2+} concentrations, and Avanti Polar Lipids for participating in the National Science Foundation Young Investigator program.

APPENDIX: ANALYSIS OF ASSOCIATION CONSTANTS FOR MEMBRANE-PARTITIONING, AMPHITROPIC MOLECULES

The Scatchard plot is routinely used for assessing the affinity and stoichiometry of intermolecular interactions. Its usefulness has been experimentally verified and theoretically confirmed for soluble ligands interacting with membrane-bound receptors [for a review see Lauffenburger and Sinderman (1993)]. In the last several years, this approach has also been widely used to investigate the interaction of protein kinase C, an amphitropic protein (Burn, 1988) that transiently associates with membranes, with its ligands, phorbol esters (Sharkey et al., 1984; Burns & Bell, 1991; Quest & Bell, 1994a; Kazanietz et al., 1992; Fisher & Yanagimoto, 1989) and Ca^{2+} (Luo & Weinstein, 1993). Because both protein kinase C and phorbol esters partition between the solution and membrane, the usefulness of the Scatchard plot is limited.

Here we derive a modification of the Scatchard equation that describes the interaction of an amphitropic molecule, P, that partitions into the membrane with a partition coefficient, K_5, and binds either soluble, L, or membrane-bound ligand, L_m. The partitioning of the latter is described by the partitioning coefficient, K_0. The subscript m identifies the molecule that partitions in the membrane (*i.e.*, the part of bimolecular complex that provides the membrane attachment). The membrane association of all species involved in this scheme is treated as a simple partitioning described by an appropriate partition coefficient. [This is not true in general because, for example, association of protein kinase C with membranes is a function of the molar fraction of anionic lipid(s) and the total lipid concentration. Furthermore, there are two classes of ligands, cations (*e.g.*, Ca^{2+}) as well as DG and phorbol esters. It is possible, however, to set the physicochemical parameters of the experiment constant and fix all but the protein and one of the ligand concentrations. Under such conditions, protein appears to partition into the membrane in the presence of an excess of lipid.] The association constants (*i.e.*, K_1, K_2, K_6, and K_9) are identified readily from partition coefficients (*i.e.*, K_0, K_3, K_4, K_5, K_7, and K_8) by the presence of a ligand or macromolecule that "comes" into reaction. Only six of these constants are independent.



By using elementary algebra, one arrives at the following

formula, presented analogously to the Scatchard equation:

$$\frac{\dfrac{[PL_m + P_mL + P_mL_m]}{[L]}}{[P^t]} = K' - K''\frac{[PL_m + P_mL + P_mL_m]}{[P^t]} \quad \text{(A1)}$$

$$K' = \frac{K_0K_1 + K_5K_9 + K_0K_5K_6}{1 + K_5}$$

$$K'' = \frac{K_0K_1 + K_5K_9 + K_0K_5K_6 + K_2}{1 + K_5}$$

where $[P^t]$ denotes the total concentration of the macromolecule P.

Two points are noteworthy. Firstly, under the experimental conditions used for the Scatchard analysis of the binding of phorbol esters to protein kinase C, the apparent association constant derived from the equation comprises association constants for three different bimolecular complexes (PL_m, P_mL_m, and P_mL); these are lumped into one category because the binding assays used do not distinguish between them. Secondly, the equation (A1) can be rewritten in the form of three independent linear equations for each of the bimolecular complexes; these would have the same tangents and three different "intercept" terms, which now constitute elements in the sum of products constituting the intercept term, K', in eq A1. The tangent, K'', always contains constants describing all possible bimolecular complexes, whereas the intercept term, K', contains only the constants describing the complex(es) under consideration.

Two limiting cases of eq A1 are described here. First, we consider the case when most of the protein is membrane bound under experimental conditions (*i.e.*, $K_5 \gg 1$). Thus, the tangent K'' simplifies to

$$K'' = (K_0K_1 + K_2)/K_5 + K_9 + K_0K_6 \quad \text{(A2)}$$

If the ligand does not partition into the membrane and associates much more weakly with soluble protein than with membrane-bound protein, then K'' is equal to the association constant of membrane-bound protein with the soluble ligand, K_9. The tangent K'' represents an average of three different association constants (assuming, once more, that K_2 is small), additionally modified by the partitioning coefficient of the phorbol ester. Because of a smaller number of degrees of freedom for membrane-bound molecules, the association constant for the membrane-bound protein kinase C with membrane-bound phorbol ester, K_6, should be much larger than that for the same molecules in solution [for discussion see Finkelstein and Janin (1989), Perutz (1990), and Dwyer and Bloomfield (1981)]; therefore, it is not possible to determine which constant is actually measured when the partitioning of phorbol esters (*e.g.*, PDBu) is very weak.

A second limiting case is when the macromolecule associates with membranes with a low affinity, such that $K_5 \ll 1$. In the case of protein kinase C, this occurs at either low mole percent anionic lipid or total lipid concentration or low Ca^{2+} concentrations. Under such conditions, K'' simplifies to

$$K'' = K_0K_1 + K_2 + K_5(K_9 + K_0K_6) \quad \text{(A3)}$$

If K_0 and $K_2 \ll 1$, then K'', once again, represents an average of association constants for soluble and membrane-bound phorbol esters multiplied by the partitioning coefficient of the protein, K_5. Since the latter is $\ll 1$, the apparent association constant of the ligand with the macromolecule found from the Scatchard plot is always an underestimate.

The approach used in this paper takes advantage of (1) the strong membrane partitioning of the ligand, PMA, and (2) the fact that the association of protein kinase C with membranes can be measured in the absence of phorbol esters. Specifically, we measure the association of protein kinase C with membranes in the absence of PMA (K_5) and then in the presence of a known surface concentration of PMA, $[L_m]$. The ratio of the apparent association constants, r, in the presence and absence of the ligand (PMA) is equal to

$$r = 1 + (K_9/K_0 + K_6 + K_1)[L_m] \quad \text{(A4)}$$

This equation is simplified further for two reasons. First, we have measured the association constant of soluble protein with membrane-bound ligand, K_1, in an independent experiment and found that it is 2 orders of magnitude smaller than K_6. This likely results from the smaller entropy changes required for the association of membrane-bound protein with immobilized and oriented phorbol ester than for the association of soluble, randomly oriented phorbol ester. Second, because the partitioning of PMA in membranes is very strong, $K_0 \gg 1$, then $K_9/K_0 \ll K_6$. Thus, eq A4 simplifies to

$$r = 1 + K_6[L_m] \quad \text{(A5)}$$

Therefore, the tangent of the plot of r against $[L_m]$ yields the association constant of membrane-bound protein with membrane-bound ligand. If there were an additional binding site for the same ligand, then

$$r = 1 + K_{61}[L_m](1 + K_{62}[L_m]) \quad \text{(A6)}$$

If $K_{62} < K_{61}$, then for $[L_m] \gg 1/K_{62}$, r will change approximately with $[L_m]^2$ and have a slope of 2 on a double-logarithmic plot.

It is possible that the interaction of a macromolecule with a ligand changes the affinity of the protein for the membrane allosterically, as is the case for the regulation of protein kinase C's membrane association with PS by Ca^{2+}. Under such circumstances, the products of association constants such as K_5K_6 are substituted with a single association constant for the bimolecular complex with the membrane (Wyman & Gill, 1990). However, because the change in the affinity for the membrane is equal to the change in the affinity for the ligand in the case of allosteric interactions, then the association constant of the ligand with the protein/membrane complex can be determined. Such an application was used to calculate the dissociation constant of Ca^{2+} from protein kinase C in the presence of PS (Mosior & Epand, 1994). Thus, the current contribution is an extension of the same approach for another class of ligands of protein kinase C. Two-step binding to ligands embedded in the membrane was also proposed earlier for phospholipase A2 (Hendrickson & Dennis, 1984). The method we describe is similar to linkage analysis approaches, which allow one to determine the stoichiometry and dissociation constants for more than one ligand interacting with a soluble macromolecule (Wyman & Gill, 1990).

In summary, the partitioning of amphitropic proteins between solution and the membrane presents limitations to analysis by Scatchard plots to elucidate binding constants with ligands. Rather, the analysis presented here allows the dissection of the apparent association constant of the direct interaction of protein kinase C with phorbol esters.

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BI952031Q