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Phospholipid and Ca^{++} Dependency of Phorbol Ester Receptors

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The phospholipid and Ca^{++} dependency of a partially purified phorbol ester apo-receptor from the soluble fraction of mouse brain homogenates was studied. This apo-receptor is believed to be identical with the Ca^{++} and phospholipid-dependent protein kinase C. Binding of phorbol esters to the receptor/kinase C was shown to be entirely dependent on phospholipids. The negatively charged phospholipids phosphatidylserine, phosphatidylinositol, and phosphatidic acid all fully reconstituted binding. The neutral phospholipids were inactive. Among active phospholipids and mixtures of phospholipids, substantial differences (> 100-fold) were observed in the amounts required to achieve reconstitution. Although Ca^{++} was not required for reconstitution of binding activity, it dramatically (up to 100-fold) increased the potency of phospholipids for reconstitution. The phospholipids not only permitted reconstitution of the apo-receptor but also played a major role in determining the binding characteristics of the complex. The K_D values of [^{3}H]phorbol 12,13-dibutyrate were in the range of 0.8 nM for the complex with phosphatidylserine to 30 nM for the complex with dioleoyl-phosphatidic acid. Like the binding affinity, the stimulation of protein kinase C activity by phorbol esters was dependent on the phospholipid into which the receptor/kinase C was reconstituted. The importance of the lipid domain for controlling the receptor/kinase C activity and for modulation of cellular sensitivity to phorbol esters is discussed.

Key words: phorbol ester, tumor promotor, receptor, protein kinase C, phospholipid dependency, Ca^{++}

The phorbol esters are highly potent tumor promoters for mouse skin. In addition, they have profound effects on a wide variety of biological systems. It therefore seemed probable that they interacted with a central regulatory pathway in cells. This laboratory and others have demonstrated the existence of specific, high affinity binding sites for the phorbol esters that appear to mediate their actions. These specific receptors are found both in membranes and, as apo-receptors, in the soluble fraction of cell homogenates (cytosol) [1,2].

Abbreviations used: PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin, DPG, diphosphatidylglycerol; PL-Mix, a phospholipid mixture corresponding to that in human red blood cells; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PDBu, phorbol 12,13-dibutyrate; K_D, dissociation constant; B_{max}, total number of binding sites; ED_{50}, dose which gives 50% of measured effect.

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Recent evidence strongly argues that at least the soluble apo-receptor is the same as the enzyme protein kinase C [3–5]. The binding activity corresponds to the modulatory site on the enzyme, and the phorbol ester binding can be competitively inhibited by diacylglycerols [6]. The postulated normal role of protein kinase C is to mediate one of the response pathways to external stimuli whose action is associated with enhanced breakdown of phosphatidylinositol-4,5-bisphosphate, leading to diacylglycerol formation and intracellular Ca^{++} mobilization [7].

The detection of soluble apo-receptors in cytosol preparations has provided an important tool for manipulating the lipid domain of the functional phospholipid/protein complex. As demonstrated by a number of laboratories, both the enzymatic and phorbol ester binding activities critically depend on the presence of phospholipids [1–9]. Further characterization of the phospholipid requirements for binding and the effects of phospholipids on binding affinity are summarized in this paper and, in addition, it is demonstrated that the phospholipid environment modulates the potency of phorbol esters to activate protein kinase C. We therefore argue that the lipid domain of the active phorbol ester receptor may have a regulatory function and may modulate the cellular sensitivity to phorbol esters and diacylglycerols.

MATERIALS AND METHODS

Materials

L-α-phosphatidyl-L-serine (bovine brain), L-α-phosphatidylinositol (soybean), L-α-phosphatidylinositol-4,5-bisphosphate (bovine brain), L-α-phosphatidylcholine (bovine brain), L-α-phosphatidylethanolamine (bovine brain) and L-α-phosphatidic acid, dioleoyl (synthetic) were obtained from Sigma (St. Louis, MO). Sphingomyelin (bovine) was from Supelco (Bellafonte, PA). A mixture of phospholipids (PL-Mix) corresponding to the phospholipid composition of human red blood cells was purchased from PL-Biochemicals (Milwaukee, WI). The mixture contained PC, PE, PS, SM, PI, and DPG in molar ratios of 30:30:13:25:1:1. Diethylaminoethyl cellulose (DE 52) anion exchanger was from Whatman (Clifton, NJ). Bovine serum albumin (fatty acid poor), leupeptin, phenylmethylsulfonyl fluoride, histone (Type IIIS), PDBu, and bovine gamma globulin were from Sigma. Polyethylene glycol (MW 6000–7500) was purchased from EM Science (Gibbstown, NJ). [^{3}H]PDBu (13.4 Ci/mmol) and [γ-^{32}P]ATP (2 Ci/μmol) were obtained from New England Nuclear (Boston, MA).

Partial Purification of the Phorbol Ester Receptor

The soluble fraction of a mouse brain homogenate (cytosol) was prepared as previously described [6]. A partially purified fraction was prepared by ion exchange chromatography of the cytosol on a DE-52 column according to the modified procedure of Kikkawa et al [10]. The column fractions were assayed for protein kinase C activity, and the peak fractions were pooled. Glycerol, phenylmethylsulfonyl fluoride, and leupeptin were added to give final concentrations of 10% (v/v), 1 mM, and 0.5 mM, respectively. This preparation was divided into aliquots and could be stored at −60° C for several weeks without significant loss of activity. Protein was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

[^{3}H]PDBu Binding Assay

Binding of [^{3}H]PDBu to the partially purified receptor or, as in some experiments, to the cytosolic fraction was measured using a polyethylene glycol precipitation assay [6]. The assay mixture (0.25 ml) contained 50 mM Tris-Cl, pH 7.4, 0.1 mM $CaCl_2$, 10–200 μg/ml of protein, 3 mg/ml of bovine gamma globulin, [^{3}H]PDBu and phospholipids, as indicated. Because the receptor preparation contained EGTA and EDTA, the amount of chelators varied with the protein concentration used in individual experiments. Except in a few experiments that were done under conditions of excess Ca^{++} over chelators, the concentration of chelators usually exceeded that of Ca^{++}. In all experiments, a final concentration of 15% (w/w) polyethylene glycol (PEG) was used. Control experiments showed that neither the bovine gamma globulin [11] nor the PEG in the assay had any significant effect on the phorbol ester binding parameters.

Phospholipids were added to the assay mixture in the following way: The lipid solvent was evaporated under a stream of nitrogen; 50 mM Tris-Cl, pH 7.4, was added; and then the phospholipids were dispersed by gentle sonication using a Heat Systems sonicator (Model W 185) with a microtip attachment. Three 20-sec pulses were applied at 1-min intervals while the sample was cooled in ice. The phospholipid solutions were diluted into the assay mixture to give the final concentrations indicated. In experiments with sphingomyelin, a longer sonication time (4 × 30 sec, without cooling) was required to solubilize the lipid homogeneously. Phosphatidic acid and phosphatidylinositol, due to their apparent instability, were solubilized either by gentle vortexing or a very short sonication (3 × 10 sec, with cooling on ice). To measure PDBu saturation curves, the concentration of free [^{3}H]PDBu was varied between 0.1 and 200 nM, depending on the phospholipids used for reconstitution. Nonspecific binding was measured in control samples containing 30 μM cold PDBu in addition to [^{3}H]PDBu.

Protein Kinase C Assay

Protein kinase C activity was measured using the partially purified receptor after it was desalted (to remove EDTA and EGTA) by gel filtration over two 9.1 ml columns of Sephadex G-25M (Pharmacia, Sweden), equilibrated, and eluted with 40 mM Tris-Cl, pH 7.4. The assay mixture (50 μl) contained 40 mM Tris-Cl (pH 7.4), 0.5 mM EGTA, 0.6 mM $CaCl_2$, 0.5 mM dithiothreitol, 7.5 mM magnesium acetate, 0.75 mg/ml histone (IIIS Fraction), 0.25 mg/ml bovine serum albumin, and 25 μM ATP. [γ-^{32}P]ATP was added to give a specific activity of 100–150 cpm/pmol ATP. PDBu and phospholipid solutions (prepared as described above) were added as indicated. After incubation for 5 min at 30°C the tubes were placed on ice, and aliquots (25 μl) from each tube were pipetted onto phosphocellulose paper and washed according to Witt and Roskoski [12].

RESULTS

Binding of [^{3}H]PDBu to the partially purified apo-receptor was determined as a function of the concentration of PS added to the assay (Fig. 1, upper panel). Binding was absolutely dependent on added phospholipids, as also observed by others. Under

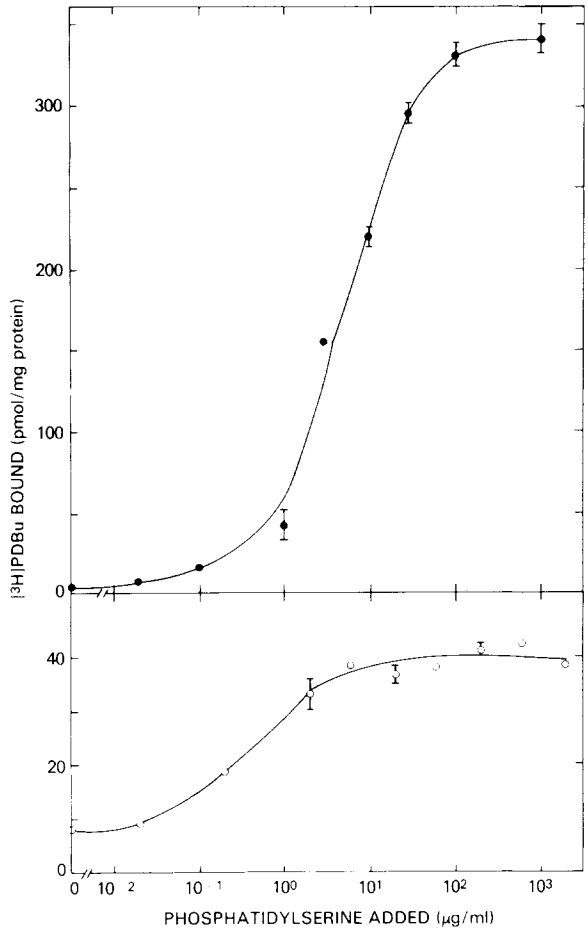

Fig. 1. Specific [^{3}H]PDBu binding as a function of the concentration of phosphatidylserine added. Upper panel: Binding to the partially purified apo-receptor (●). Lower panel: Binding to the cytosolic fraction (○). Specific [^{3}H]PDBu binding was determined as described in Methods. The concentration of free [^{3}H]PDBu was 12–15 nM. Each point represents the mean value for quadruplicate samples in a single experiment (Error bars, ± SEM). Results are representative of at least three independent experiments.

these conditions (an excess of chelators over Ca^{++}), the concentration of phosphatidylserine required for half-maximal binding activity (ED_{50}) was 5–8 μg/ml; an amount of > 100 μg/ml was saturating. The specific activity of the fully reconstituted apo-receptor was approximately 340 pmol/mg protein, depending on the preparation. The phospholipid dependency of the cytosolic preparations (Fig. 1, lower panel) differed in two aspects from that of the partially purified apo-receptor. First, the amount of PS required for reconstitution of half-maximal binding activity was lower (0.3 μg/ml) as was the concentration to achieve saturation, > 10 μg/ml. Secondly, whereas the partially purified apo-receptor showed no binding activity in the absence of added phospholipids, in the cytosolic preparation about 20% of the total binding

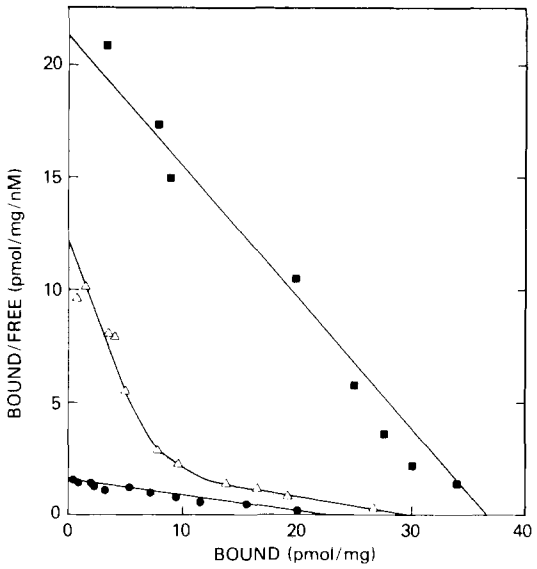

Fig. 2. Scatchard analysis of [^{3}H]PDBu binding to the cytosolic fraction. [^{3}H]PDBu binding was assayed in the absence of added phospholipids (●) or in the presence of phosphatidylserine at 0.2 μg/ml (■) or 0.05 μg/ml (△). Each point represents the mean value for triplicate samples. Results are representative of three independent experiments.

activity still could be measured. This residual binding activity may reflect the presence of endogenous lipids in the cytosol preparation. Lipid extraction of the cytosol [13] followed by two-dimensional thin layer chromatography [14] revealed, upon staining with molybdenum blue, two spots with mobilities similar to those of PC and PE. An additional, unidentified lipid component was detected by staining with iodine (results not shown).

The characteristics of the [^{3}H]PDBu binding to the cytosolic preparation in the absence of added PS or in the presence of limiting amounts of PS were determined by Scatchard analysis [15] of binding data (Fig. 2). Addition of PS at 0.2 μg/ml yielded a straight Scatchard plot, consistent with a homogenous class of binding sites possessing a K_D of 1–2 nM. This K_D value is close to that of 0.8 nM for the partially purified apo-receptor at saturating PS concentrations (see below). The B_{max} value, 37 pmol/mg, compared to 62 pmol/mg obtained for this specific cytosolic preparation at saturating PS concentrations. In the absence of added PS, a straight Scatchard plot for [^{3}H]PDBu binding again was obtained. The affinity, however, was substantially lower (K_D = 16 nM) than in the presence of added PS, and the B_{max} value was somewhat lower (23 pmol/mg). The cytosolic binding activity measured in the absence of added phospholipids is not detectable if insufficient polyethylene glycol (10% rather than 15%) is used for precipitation of the receptor [3]. Addition of a very low concentration of PS (0.05 μg/ml) yielded a curved Scatchard plot, indicating generation of heterogeneity in the reconstituted apo-receptor. Because of the presence of residual lipids in the cytosol, the partially purified apo-receptor preparation was used for detailed characterization. Although the possibility remains that contaminating

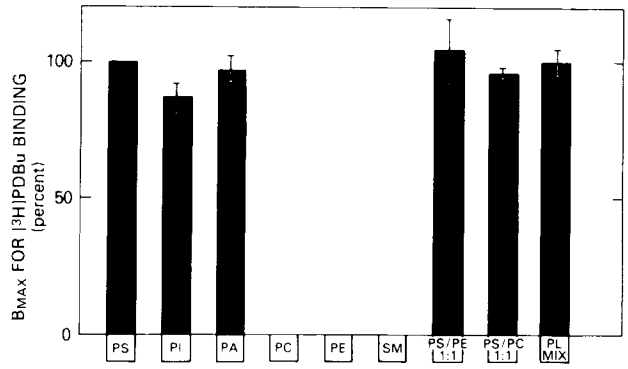

Fig. 3. Efficacy of different phospholipids for reconstitution of specific [^{3}H]PDBu binding activity of the partially purified apo-receptor. B_{max} values were determined from Scatchard analysis of the binding data. Indicated are the mean values ± SE of at least two independent experiments normalized to the B_{max} of the receptor preparations reconstituted with a saturating amount of phosphatidylserine.

enzymatic activities in the partially purified preparations may affect the quantitative values, they are unlikely to affect the overall conclusions. In addition, limited recent studies by the present authors and others using apo-receptors purified to homogeneity agree with the results obtained with the less purified preparations.

Different phospholipids were compared for their abilities to reconstitute [^{3}H]PDBu binding activity. Three aspects of the reconstitution were determined: First, what were the maximal extents of reconstitution? Second, how much phospholipid was required to reconstitute? Third, to what degree did the [^{3}H]PDBu binding affinities depend on the specific phospholipid used for reconstitution?

It was found that the neutral phospholipids PC, PE, and SM failed to reconstitute binding activity at concentrations up to 1 mg/ml (Fig. 3). In contrast, the negatively charged phospholipids PS, PI, and dioleoyl-PA all were able to reconstitute binding fully. Likewise, mixtures of PS with neutral phospholipids also gave complete reconstitution.

Among active phospholipids, substantial differences in the amounts required to achieve reconstitution were observed. This difference is illustrated in Figure 4 for PS and for PS/PC (1:2) mixed liposomes. ED_{50} values measured under conditions of an excess of chelators over Ca^{++} ranged from 1–2 μg/ml for dioleoyl-PA to 200–300 μg/ml for PL-Mix.

The presence of free Ca^{++} dramatically increased the potency of phospholipids for reconstitution of binding activity. For example, the ED_{50} value for PS was decreased 30-fold in the presence of excess Ca^{++} (0.5 mM EGTA, 0.6 mM Ca^{++}) from about 7 μg/ml to 0.2 μg/ml. For PL-Mix, the ED_{50} value was shifted about 100-fold to 2 μg/ml under those conditions (Fig. 5). In contrast to its effect on the potency of phospholipids, in most cases Ca^{++} did not substantially affect the binding affinity of the receptor-lipid complex for [^{3}H]PDBu.

The phospholipids determine not only whether or not the apo-receptor can bind [^{3}H]PDBu but also appear to play a major role in determining the binding character-

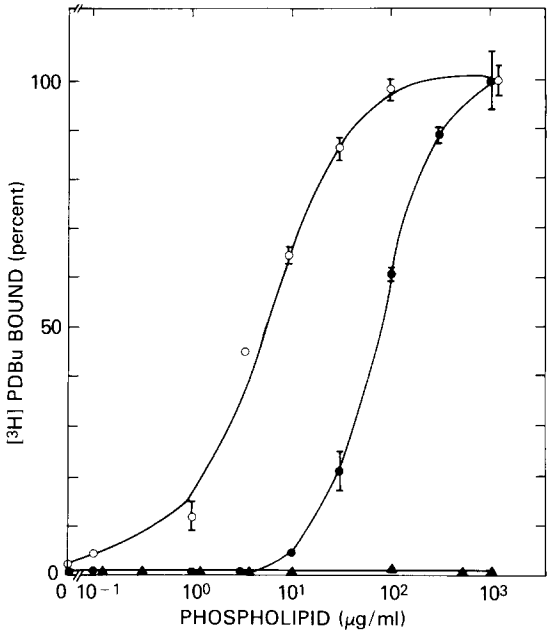

Fig. 4. Concentration dependency of different phospholipids for reconstitution of specific [^{3}H]PDBu binding activity of the partially purified apo-receptor. [^{3}H]PDBu binding was measured in the presence of phosphatidylserine (○), phosphatidylcholine (▲), or a 1:2 (w/w) mixture of phosphatidylserine/phosphatidylcholine (●). The concentration of free [^{3}H]PDBu was 12–15 nM. Each point is the mean ± SE determined for quadruplicate samples. Results are representatives of at least two independent experiments.

istics of the complex. [^{3}H]PDBu binding affinities to the apo-receptor were determined in the presence of saturating concentrations of various phospholipids (Fig. 6). PS plus the apo-receptor yielded a complex with the highest affinity for [^{3}H]PDBu binding (K_D = 0.8 ± 0.09 nM, mean ± SE, n = 7). Reconstitution of the apo-receptor with dioleoyl-PA led to a holo-receptor complex with a K_D value for [^{3}H]PDBu of 30 ± 6 nM (mean ± SE, n = 5). Reconstitution into the other phospholipids and phospholipid mixtures gave intermediate K_D values (Fig. 6).

As expected from the modulation of the binding affinity of the receptor for PDBu, phospholipids also determined the degree of stimulation of protein kinase C by the phorbol esters. As shown in Figure 7, kinase C activity was stimulated about fourfold by PDBu. However, the stimulation of protein kinase C required much higher concentrations of PDBu when the enzyme was reconstituted with the PL-Mix rather than with PS. For example, PDBu at a 3×10^{-8} M concentration led to a 1.8-fold stimulation in the presence of PL-Mix and a 2.8-fold stimulation in the presence of PS. At a PDBu concentration of 10^{-7} M, the stimulation was 2.7-fold and 3.3-fold, respectively. Only at high concentrations of PDBu ($> 10^{-6}$ M) was the stimulation of protein kinase C not differentially affected by the phospholipid environment.

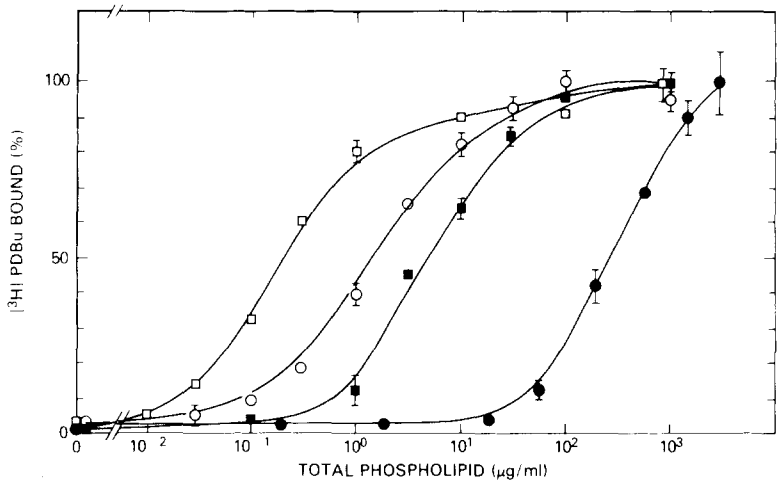

Fig. 5. Dose-response curves of different phospholipids for reconstitution of specific [^{3}H]PDBu binding activity at different Ca^{++} concentrations. [^{3}H]PDBu binding of the partially purified receptor was measured in the presence of phosphatidylserine (□,■) or PL-Mix (○,●) under conditions of "low Ca^{++}" (PS [■], PL-Mix [●]) and "high Ca^{++}" (PS [□], PL-Mix [○]). "Low Ca^{++} refers to the conditions of excess chelators over Ca^{++}, as described in Methods. For the experiments with "high Ca^{++}" the chelators were removed from the receptor preparation by gel filtration (see Methods), and the binding of [^{3}H]PDBu was measured in the presence of 0.5 mM EGTA and 0.6 mM Ca^{++}. Given are percent of B_{max} values derived from the measured values of bound [^{3}H]PDBu according to the relationship $B_{max} = B\ (K_D + L)/L$, where L = concentration of free ligand, and B = concentration of bound ligand measured at that ligand concentration. The K_D values used for the receptor complex with PS or PL-Mix were 0.8 nM and 22 nM, respectively. L was between 11 and 16 nM. Each point is the mean ± SE determined for quadruplicate samples. Results are representatives of at least two independent experiments.

DISCUSSION

Protein kinase C has been found to distribute between the particulate and cytosolic fraction. Phorbol ester treatment of cells reportedly altered the distribution of kinase C between those fractions. The finding of the present study that the apo-receptor in the cytosol is already partially reconstituted suggested caution in the quantitative interpretation of such experiments. The results show, moreover, the advantage of measurements of binding activity as compared to enzymatic activity for quantitation of the apo-receptor/protein kinase C. The enzymatic measurements depend on the difference in activities with and without the exogenously added co-factors Ca^{++} and phospholipid and do not take into account the presence of endogenous co-factors.

Conceptually, a critical finding of the present studies is that phospholipids play a major role in determining the binding properties of the reconstituted apo-receptor. The active phorbol ester receptor is thus the phospholipid/apo-receptor complex rather than simply the protein moiety. This concept clarifies a number of issues in the analysis of phorbol ester binding. The average binding affinity of [^{3}H]PDBu in intact

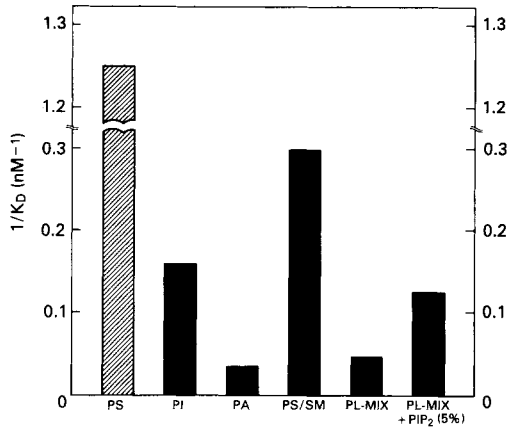

Fig. 6. Effects of phospholipids on the [^{3}H]PDBu binding affinity of the reconstituted apo-receptor. The partially purified apo-receptor was reconstituted with saturating amounts of phosphatidylserine (100 μg/ml), phosphatidylinositol (100 μg/ml), phosphatidic acid (30–100 μg/ml), a 1:1 (w/w) mixture of phosphatidylserine and sphingomyelin (total phospholipid: 100 μg/ml), PL-Mix (1–2 mg/ml) and PL-Mix including 5% (w/w) phosphatidylinositol-4,5-bisphosphate (0.3–1 mg/ml). The association constants $1/K_D$ were determined by Scatchard analysis. Values represent the mean of at least two independent experiments.

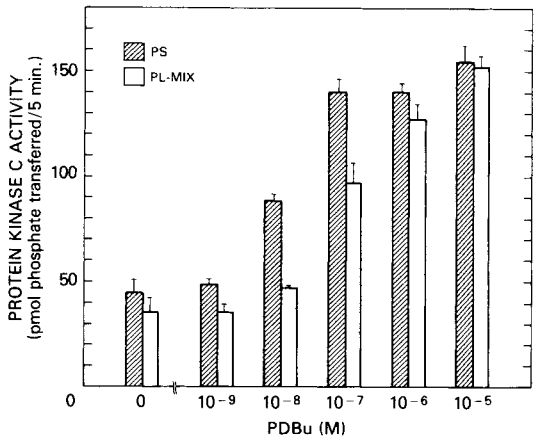

Fig. 7. Stimulation by PDBu of protein kinase C reconstituted with different phospholipids. Protein kinase C activity was assayed in the presence of 1 μg/ml of phosphatidylserine (■) or 3 μg/ml of PL-Mix (□) as described in Methods. The mean values ± SE from triplicate samples are shown. Results are representative of several similar experiments.

cells is 23 nM [16], and in no case has an intact cell system yielded a binding affinity as high as that (0.8 nM) measured for the apo-receptor reconstituted into phosphatidylserine. The finding that the binding affinity of the apo-receptor reconstituted into the red blood cell phospholipid mixture is decreased to 22 nM (Fig. 6) resolves this discrepancy.

[^{3}H]PDBu binds in a heterogenous fashion, yielding curved Scatchard plots, to mouse skin particulate preparations [17], to rat fibroblasts [18], and intact mouse keratinocytes [19]. Although such findings could reflect the existence of multiple receptor proteins, an attractive alternative is that they represent heterogeneity in the lipids associated with the same apo-receptor so as to yield different binding affinities. The demonstration that appropriate manipulation of the lipids used for reconstitution can generate curved Scatchard plots (Fig. 2) supports this possibility. A further implication is that curved Scatchard plots may reflect a continuum of binding affinities corresponding to a continuum of phospholipid microdomains and that analysis in terms of a small number of unique subclasses of bindings sites may in some cases be simplistic.

The important contribution of phospholipids in determining the recognition properties of the phospholipid/apo-receptor complex suggests that the lipid domain may help regulate protein kinase C activity. It is thus of particular interest that inclusion of 5% PIP_2 in the red blood cell phospholipid mixture caused a two- to threefold increase in receptor binding affinity (Fig. 6). Breakdown of PIP_2 is thought to be the endogenous pathway for activation of protein kinase C through generation of diacylglycerol. The decrease in PIP_2 could act as a negative feedback control in this pathway. Similarly, diacylglycerol is further metabolized to form phosphatidic acid, which dramatically decreases the binding affinity of the receptor complex relative to phosphatidylserine (Fig. 6). The availability of photoactivatable phorbol esters sensitive for the lipid domain of the phospholipid/apo-receptor complex [20] may provide a probe to assess the importance of such effects *in vivo*.

The phorbol ester aporeceptor provides a very attractive system for studying lipid-enzyme interactions. Most of the lipid-dependent enzymes are tightly bound membrane proteins that usually are solubilized with the aid of detergents [21]. These extracts may contain lipid that remains bound to the protein. To isolate and purify membrane proteins in lipid-free form, organic solvents have been applied successfully in some cases [22]. The phorbol ester receptors appear to be only loosely associated with the membrane, as they can be, at least partially, solubilized from the membrane by use of chelating agents. Further purification of the receptor results in an apparently lipid-free preparation which is required to study specific effects of phospholipids. Among the lipid-dependent membrane associated proteins, only a small number require the presence of specific phospholipids for reconstitution of activity [23]. For the phorbol ester receptor, however, it could be demonstrated that specific phospholipids are required to reconstitute binding activity. Furthermore, the results of the present study showed that the phospholipids determine the properties of the phospholipid/apo-receptor complex.

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