Association of Protein Kinase C with Phospholipid Monolayers: Two-Stage Irreversible Binding†

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ABSTRACT: The association of protein kinase C (PKC) with phospholipid (PL) monolayers spread at the air–water interface was examined. PKC–PL binding induced surface pressure changes that were dependent on the amount of PKC, the phospholipid composition of the monolayers, the presence of Ca^{2+}, and the initial surface pressure of the monolayer (π_0). Examination of surface pressure increases induced by PKC as a function of phospholipid surface pressure, π_0, revealed that PKC–phosphatidylserine (PS) association had a critical pressure of 43 dyn/cm. Above this surface pressure, PKC cannot cause further surface pressure changes. This high critical pressure indicated that PKC should be able to penetrate many biological membranes which appear to have surface pressures of about 30 dyn/cm. PKC-induced surface pressure changes were Ca^{2+} dependent only for PL monolayers spread at a π_0 greater than 26 dyn/cm. PKC alone (in the absence of PL) formed a film at the air–water interface with a surface pressure of about 26 dyn/cm. Calcium-dependent binding was studied at the higher surface pressures which effectively excluded PKC from the air–water interface. Subphase depletion measurements suggested that association of PKC with PS monolayers consisted of two stages: a rapid Ca^{2+}-dependent interaction followed by a slower process that resulted in irreversible binding of PKC to the monolayer. The second stage appeared to involve penetration of PKC into the hydrocarbon region of the phospholipid. The commonly used in vitro substrates for PKC, histone and protamine sulfate, also associated with and penetrated PS monolayers with critical pressures of 50 and 60 dyn/cm, respectively. Despite simultaneous interaction of PKC and histone with the phospholipid monolayers, phosphorylation was minimal, even in the presence of phorbol esters. Consequently, PKC was not able to phosphorylate histones on a planar, nonaggregated membrane surface. In contrast, protamine sulfate disrupted the monolayer, dissociated PKC from the interface, and caused aggregation in the subphase. PKC was highly active in the latter circumstance. These results suggested that PKC was unable to phosphorylate these substrates when bound to planar, nonaggregated membrane surfaces or that PKC had high substrate specificity under these conditions.

Protein kinase C (PKC)[1] is believed to be a key regulatory enzyme in many cells (Nishizuka, 1986; Kikkawa & Nishizuka, 1986). In vitro, the activity of this enzyme is enhanced by phospholipid and Ca^{2+}. At low Ca^{2+} concentrations, the activity is stimulated by diacylglycerol or phorbol esters (Ashendel, 1985; Nishizuka, 1984). Recently, various other components such as protein or polyanions have been shown to substitute for phospholipid in stimulating the phosphorylation of certain substrates by PKC (Bazzi & Nelsestuen, 1987a). In fact, the cofactor requirements of PKC depended on the choice of substrate, and three categories of substrate were observed. These consisted of substrates requiring no cofactors, those requiring only phospholipid, and those requiring calcium, phospholipid, and diacylglycerol (Bazzi & Nelsestuen, 1987b).

The commonly used substrates of PKC interact strongly with phospholipid vesicles or with phospholipid–Triton mixed micelles and form extensive aggregates (Bazzi & Nelsestuen, 1987b). Protamine sulfate, which is phosphorylated by PKC in a Ca^{2+}- and phospholipid-independent manner (Takia et al., 1979; Kikkawa et al., 1983a), forms aggregates with PKC in a binary complex (Bazzi & Nelsestuen, 1987a,b). These aggregation events were proposed to be an effective means for delivery of substrate to the active site of PKC and appeared to underlie different cofactor requirements of substrate phosphorylation (Bazzi & Nelsestuen, 1987b). For phospholipid-requiring substrates, this proposal was tentative since substrate–phospholipid interaction produced two events, substrate–membrane binding and subsequent aggregation. All attempts to prevent aggregation also caused dissociation of substrate from the phospholipid as well as inhibition of phosphorylation by PKC (Bazzi & Nelsestuen, 1987b). Phosphorylation may require only substrate–membrane binding. Phospholipid monolayers provide an alternative approach for examining the activity of PKC. Unlike phospholipid vesicles or phospholipid–Triton mixed micelles, the enzyme and the substrate can both interact with the phospholipid monolayer without aggregation, and the activity of PKC can be examined in the presence of various cofactors.

The association of PKC with phospholipid appeared to be a simple step involving protein–membrane binding in the presence of Ca^{2+}. Complete dissociation of this complex, however, did not always result from calcium chelation. With phospholipid vesicles, the reversibility was dependent on the phospholipid composition. The binding was completely reversible only with membranes of low acidic phospholipid

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[1] Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PKC, protein kinase C; PC, phosphatidylcholine; PL, phospholipid(s); poly(LS), random copolymer of lysine, serine (3:1); PS, phosphatidylserine; TCA, trichloroacetic acid; π_0, initial surface pressure of phospholipid monolayer; $\Delta\pi$, surface pressure change.

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content (below 20% PS; Bazzi & Nelsestuen, 1987c). For cellular membranes, dissociation of the PKC–phospholipid complex appeared to depend on the initial conditions of binding. For example, pretreatment of platelets (Takia et al., 1985) or erythrocyte vesicles (Wolf et al., 1985) with phorbol esters rendered the association of PKC with membrane irreversible, even in the presence of high concentrations of EGTA. Subcellular distribution of PKC in brain suggests that irreversible association of PKC with membranes occurs under in vivo conditions (Kikkawa et al., 1983a,b). A possible explanation for these events is that PKC penetrated the hydrocarbon region of these membranes, thereby forming an irreversibly bound PKC–membrane complex.

Phospholipid monolayers spread at the air–water interface can provide valuable information of events at the protein–lipid interface. Proteins which penetrate into the hydrocarbon region of the membrane should cause large surface pressure changes while proteins that bind only to the headgroups should cause small or negligible surface pressure changes. This study examined some properties of PKC interaction with phospholipid monolayers. The results suggested that binding of PKC to phospholipids consisted of a calcium-dependent interaction followed by insertion of the protein into the membrane to form an irreversible phospholipid-bound state. Also, monolayer-bound PKC was unable to phosphorylate in vitro substrates despite simultaneous interaction of enzyme and substrate with the phospholipid in the presence of Ca^{2+} and phorbol ester.

Experimental Procedures

Materials. Bovine brain PS and egg yolk PC were purchased from Avanti Polar Lipids (Birmingham, AL) and were greater than 98% pure (manufacturer's estimate). Histone III-S, protamine sulfate, and a random copolymer of lysine and serine [poly(LS)] were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Corp. (Arlington Heights, IL). Nitrocellulose filters (pore size 0.45 μm) were purchased from Millipore Corp. (Bedford, MA). Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Services Corp. (Woburn, MA).

Protein kinase C was purified to apparent homogeneity from bovine brain as described previously (Bazzi & Nelsestuen, 1987c). PKC activity in the presence of phospholipid vesicles was assayed as described by Kikkawa et al. (1983a). Protein concentrations were determined by the method of Bradford (1970) using bovine serum albumin (BSA) as the standard.

Monolayer Techniques. The apparatus used and general methods employed have been described in detail previously (Mayer et al., 1983a). Briefly, phospholipid monolayers were formed by applying the appropriate phospholipid (2 mg/mL) dissolved in hexane/ethanol (90:10) onto 10 mL of buffer contained in a circular Teflon trough (4-cm diameter) to obtain the desired surface pressure. The trough had been etched with Teflon-treating agent (Chemplast Inc., Wayne, NJ) and also contained a small port which allowed the injection of reagents into the subphase below the monolayer. The buffer in the subphase was continuously stirred at 60 rpm with a magnetic stir bar. Surface pressure measurements were performed as described by Mayer et al. (1983a) except that a roughened platinum plate was used in place of a mica plate. Surface pressure was monitored continuously by antalog output to a recorder or digital measurement output to an Apple IIc microcomputer. The volume of an injection into the subphase was typically 100 μL and never exceeded 0.5 mL. In order to minimize the effect of buoyancy of the platinum plate when reagents were added to or removed from the subphase, an equal volume of subphase was either withdrawn or added as needed to maintain a constant volume of subphase. These injection procedures induced negligible changes in the measurements reported. Unless otherwise indicated, the buffer consisted of 20 mM HEPES (pH 7.5), 150 mM NaCl, and 10 mM $MgCl_2$, containing either 5 mM Ca^{2+} or 2.0 mM EGTA.

Subphase Depletion of PKC. If PKC that is injected into the subphase becomes bound to the monolayer, the amount of PKC activity in the subphase becomes depleted. Depletion was measured by injection of PKC (0.8–5.5 μg) followed by withdrawal of subphase (usually 100–200 μL) at various times. The sample was then assayed for PKC activity with protamine sulfate as the substrate. Protamine sulfate was chosen for these sampling assays because it is relatively insensitive to the different buffer conditions of the subphase (e.g., $\pm Ca^{2+}$, $\pm$EGTA). In the cases where small amounts of PKC were injected (0.8–2.0 μg), it was necessary to withdraw a large sample (1–3 mL) from the subphase to assay PKC activity. For these large withdrawals, only one sample was taken from each monolayer experiment.

Assay of PKC by Radioactive Product on the Monolayer. The activity of monolayer-bound PKC was assayed by monitoring the amount of phosphorylated protein in the subphase as well as that associated with the phospholipid film. In all the experiments performed to determine PKC activity, the surface pressure of the monolayers was allowed to stabilize between additions. The enzyme was first injected into the subphase which contained various activators or inhibitors (PMA and/or Ca^{2+} or EGTA), followed by addition of the substrate (50 μg/mL final concentration in the subphase). ATP was then added to start the reaction (subphase concentration equaled 20 μM). At timed intervals, the reaction was terminated by addition of unlabeled ATP (0.2 mM final concentration) to the subphase. The phospholipid film was removed by adsorption to Whatman no. 1 PS hydrophobic paper (Mayer et al., 1983a; Bhat & Brockman, 1981). The paper was extensively washed with 20% trichloroacetic acid (TCA) to remove subphase liquid, and the ^{32}P-labeled phosphoprotein associated with the paper was determined by liquid scintillation counting. The amount of phosphoprotein in the subphase was determined after precipitating the protein in the subphase with TCA solution. The precipitated protein was collected on a nitrocellulose filter, washed extensively with 20% TCA solution, and then counted.

In the case where the activity of PKC was followed as a function of time, small aliquots were withdrawn from the subphase, and proteins were precipitated with TCA, filtered, washed, and counted as describe above. At the end of the experiment, the phosphorylation on the monolayer and in the total subphase was determined as described above.

Results

Association of Protein Kinase C with Phospholipid Monolayers. Injection of PKC into the subphase below a phospholipid monolayer caused increases in surface pressure ($\Delta\pi$). The magnitude of the increase was dependent on the amount of PKC added, the phospholipid composition of the monolayer, the presence of Ca^{2+}, and the initial surface pressure of the phospholipid monolayers (π_0). Figure 1 shows changes in surface pressure of a lipid film (PS/PC, 30:70) induced by successive additions of PKC to the subphase. The maximum increase was large (>6 dyn/cm) and was saturable with respect to PKC (Figure 1, inset). The magnitude of structure perturbation by PKC and the irreversibility of PKC–phospholipid binding (see below) precluded analysis of these data by the modified Gibbs equation (Colacicco, 1970), and equilibrium

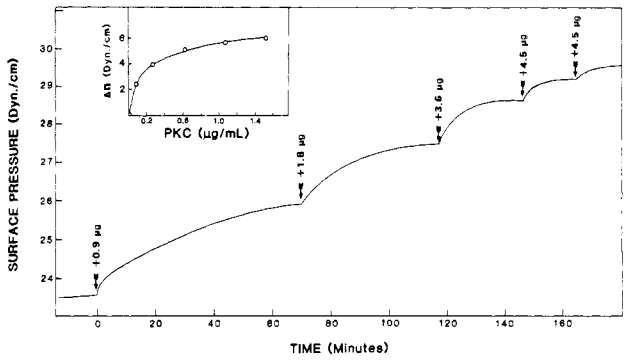

FIGURE 1: Association of PKC with phospholipid monolayers. The changes in the surface pressure of a phospholipid monolayer (30% PS and 70% PC) upon the addition of PKC were monitored as a function of time. The buffer contained 1.0 mM Ca^{2+}. The amounts of PKC added and the time of addition are shown. The inset shows a plot of the surface pressure increase versus the concentration of PKC.

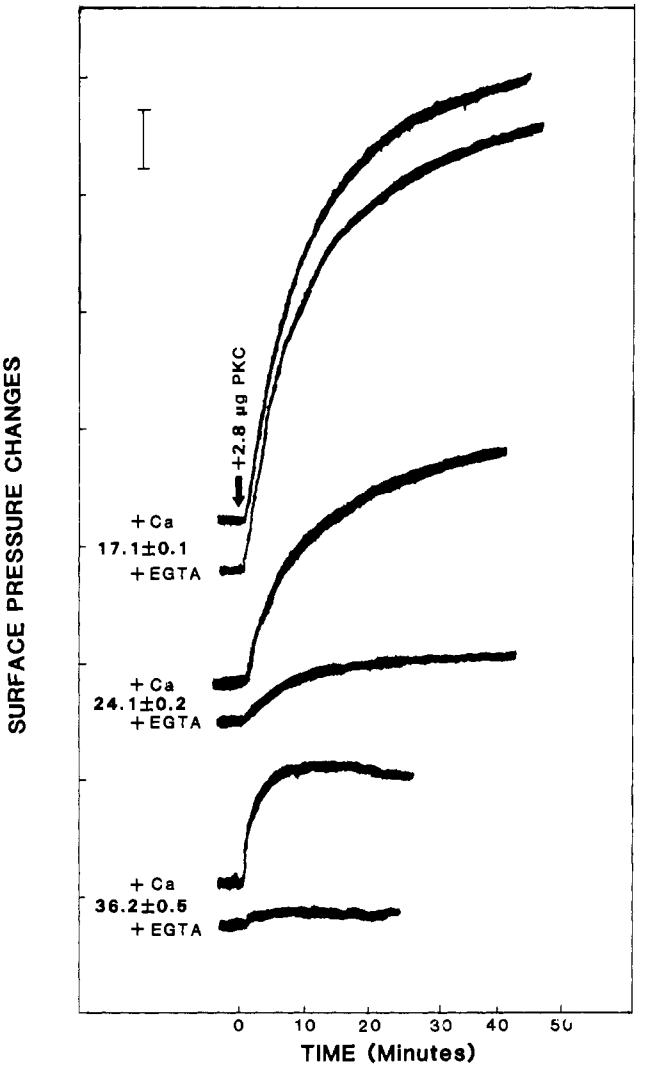

FIGURE 2: Ca^{2+} requirement of association of PKC with PS monolayers. PS monolayers were spread at the indicated surface pressure in the presence of either 5.0 mM Ca^{2+} or 2.0 mM EGTA. After stabilization of the surface pressure of the monolayer, 2.8 μg of PKC was added to the subphase, and the surface pressure was monitored as a function of time. The traces for various experiments were offset for clarity. Each division on the ordinate axis equals 2 dyn/cm, and the bar in the upper left-hand corner represents 1 dyn/cm.

binding constants could not be determined.

The Ca^{2+} requirement for PKC–phospholipid interaction was examined, and typical results are shown in Figure 2. At

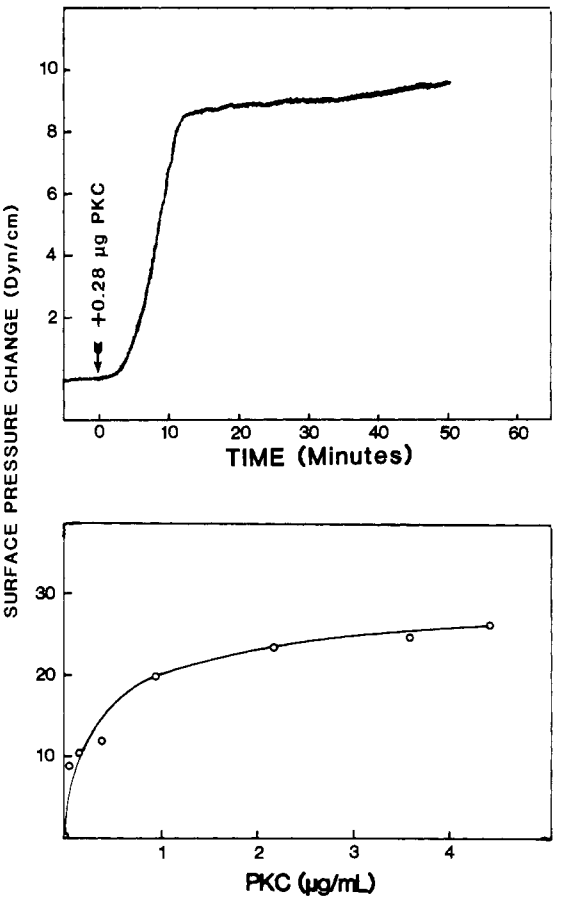

FIGURE 3: Surface pressure of PKC films at the air–water interface. The upper panel shows the effect of a single addition of PKC (0.28 μg) on the surface pressure of the buffer. The lower panel shows the effect of PKC concentration on the magnitude of the surface pressure change at the air–water interface.

low surface pressures ($\pi_0 < 17$ dyn/cm), PKC induced large increases in the surface pressure that were virtually independent of Ca^{2+}. At intermediate surface pressures ($15 < \pi_0 < 25$ dyn/cm), Ca^{2+} enhanced the surface pressure changes induced by PKC. Finally, at high surface pressures ($\pi_0 \geq 26$ dyn/cm), PKC-induced surface pressure changes showed an absolute requirement for Ca^{2+}.

To understand the basis of the conditional Ca^{2+} requirement, the behavior of PKC itself at the air–water interface was examined. In the absence of phospholipid monolayers, PKC increased the surface pressure of the buffer (Figure 3, top panel). This increase was rapid and large even with small amounts of PKC. The final surface pressure was not entirely stable and showed a slow upward drift which could be due to slow denaturation of protein in the air–water interface (Macrichtie, 1978). The magnitude of the induced surface pressure change was dependent on the amount of PKC and reached a maximum of approximately 26 dyn/cm (Figure 3, lower panel). Similar results were obtained with several different preparations of PKC.

These results provided a potential explanation for the conditional Ca^{2+} dependence of PKC-induced surface pressure changes in the presence of PS monolayers (Figure 2). Since PKC itself interacted with the air–water interface with a surface pressure of 26 dyn/cm, PKC may penetrate phospholipid films spread at lower pressures and interact directly with the air–water interface. This was calcium independent since it did not depend on the PKC–phospholipid interaction. Above a π_0 of 26 dyn/cm, PKC was excluded from the air–water interface so that surface pressure changes depended on

Table I: Subphase Depletion of PKC Activity

experimental conditions[a]						
expt	[EGTA] (mM)	[Ca] (mM)	PS[b]	[BSA] (μg/mL)	π_0 (dyn/cm)	subphase act. (%)[c]
1	2.0	0.0	+	0	29.0	107.5
2	2.0	0.0	+	0	18.8	12.4
3	0.0	5.0	+	0	21.1	0.0
4	0.0	5.0	+	0	26.1	0.0
5	0.0	0.5	+	0	26.6	6.2
6	0.0	0.5	-	0	0	0.0
7	0.0	0.5	-	150	15.3[d]	78.8
8	0.8	1.2	+	0	33.0	100/9[e]
9	0.4	2.5	+	0	30.5	6/5[f]

[a] PKC (2.7 μg) was injected into the subphase below a PS monolayer spread at the surface pressure indicated. After a 30-min incubation, an appropriate volume (50–3000 μL depending on the anticipated activity) was taken from the subphase and assayed for PKC activity. [b] The depletion measurements were performed in the presence (+) or in the absence (-) of a PS monolayer. [c] The measured activity was expressed as a percentage of that expected from the known amount of the injected PKC. [d] BSA, injected into the subphase prior to PKC addition, induced the observed surface pressure. [e] PKC was injected into the subphase in the presence of 0.8 mM EGTA. After a 30-min incubation, Ca^{2+} was added to a final concentration of 1.2 mM. The activity of PKC was measured several times before and after the addition of Ca^{2+} and is expressed as a percentage of that measured before Ca^{2+} addition in the following form: activity before Ca^{2+} addition/activity after Ca^{2+} addition. [f] In this case, PKC was injected into the subphase in the presence of 0.4 mM Ca^{2+}. The sample was incubated for 30 min, and EGTA was added to a final concentration of 2.5 mM. The activity of PKC in the subphase was measured before and 30 min after the EGTA addition in the following form: activity before EGTA addition/activity after EGTA addition.

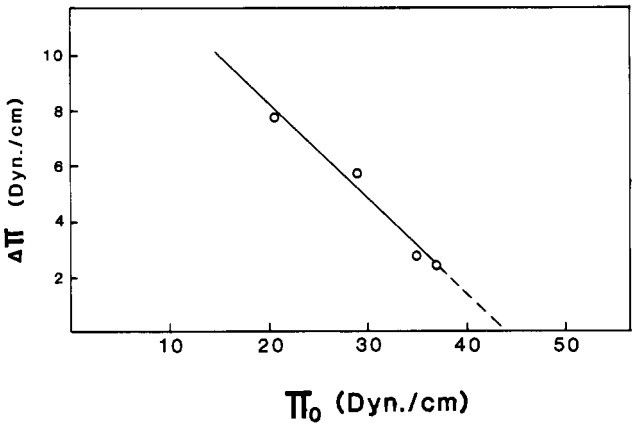

FIGURE 4: Critical surface pressure of PKC–PS association. PS monolayers were spread at different initial surface pressures in the presence of 5.0 mM Ca^{2+}. After stabilization of surface pressure, 9 μg of PKC was injected into the subphase, and the increase in the surface pressure ($\Delta\pi$) was plotted versus the initial surface pressure of the monolayer. Each point represents a different experiment.

association of PKC with phospholipid, a Ca^{2+}-dependent event.

Critical Pressure for PKC–PS Association. The critical pressure is the surface pressure above which a protein cannot induce a surface pressure change (Bougis et al., 1981). As expected, the increase in surface pressure induced by PKC was inversely proportional to the initial surface pressure of the monolayer (Figure 4). Extrapolation to zero surface pressure change gave a critical pressure of about 43 dyn/cm. The collapse pressure of PS monolayers, determined separately by using the apparatus and phospholipid described above, was 46 dyn/cm. Since the critical pressure for the protein was less than the collpase pressure of the phospholipid, PKC should not be capable of disrupting a membrane (Pethica, 1955).

The critical surface pressure of the PKC–PS interaction (43 dyn/cm) was considerably higher than the pressure of PKC obtained in the absence of phospholipid (26 dyn/cm). This implied that PKC either penetrated into the hydrocarbon region of the monolayer or deformed the acyl groups of monolayers (Kimelberg & Papahadjopoulos, 1971). Recent studies suggested that PKC penetrated phospholipid vesicles under conditions required for development of PKC activity (Bazzi & Nelsestuen, 1988). The surface pressure of red blood cells is estimated at 31 dyn/cm (Demel et al., 1975). It is therefore possible that PKC could penetrate into the hydrocarbon region of most biological membranes.

Subphase Depletion Measurements. The ability of PKC to cause surface pressure changes correlated qualitatively with loss of PKC from the subphase. PKC was injected into the subphase below PS monolayers spread at different π_0's and in the presence or absence of Ca^{2+}. After a 30-min incubation, an aliquot of the subphase was withdrawn and assayed for PKC activity. A summary of some of the experimental results and conditions is shown in Table I.

In the presence of 2.0 mM EGTA and at a π_0 of 29 dyn/cm (experiment 1, Table I), PKC remained entirely in the subphase, indicating a lack of PKC–PS association. At a π_0 of about 19 dyn/cm and in the presence of EGTA (experiment 2, Table I), only 17% of the PKC activity was recovered in the subphase which indicated a migration of PKC to the surface. This condition allowed calcium-independent surface pressure changes by PKC (π_0 = 17 dyn/cm, Figure 2). In the presence of Ca^{2+} and at $\pi_0 \leq 26$ dyn/cm (experiments 3–5; Table I), the subphase was depleted of essentially all PKC activity when 2.7 μg was injected. Under these conditions, significant PKC activity remained in the subphase only when more than 6 μg of PKC was injected (data not shown). This activity was then stable for at least 2 h (the time of assay). This long-term stability indicated that the remaining PKC represented an access over that needed to saturate the monolayer. Other explanations for PKC disappearance, such as denaturation, should not be saturable. Therefore, like the surface pressure changes (Figure 1), subphase depletion was a saturable phenomenon. Furthermore, at high surface pressures ($\pi_0 > 26$ dyn/cm), subphase depletion of PKC activity required the presence of Ca^{2+} and monolayers containing PS; in the absence of calcium or in the presence of Ca^{2+} plus monolayers composed of PC only, PKC activity was recovered in the subphase (see below).

These results indicated that, under conditions where large increases in surface pressure were observed upon addition of PKC, the subphase was depleted of PKC activity regardless of the presence of Ca^{2+} or EGTA (compare Figure 2 and Table I, experiments 2–5). However, under conditions where no significant surface pressure changes were induced by PKC, the subphase showed no depletion of PKC activity (compare Figure 2 and Table I, experiment 1). The increase in surface pressure was therefore associated with loss of PKC from the subphase. Experiments outlined below showed that monolayer-associated PKC was still active in that it could phosphorylate substrate when released from the monolayer.

Qualitative correlation between induced surface pressure changes and loss of PKC activity from the subphase was also observed in the absence of a phospholipid monolayer. Injection of PKC into the subphase, which resulted in large increases in the surface pressure of the solution (Figure 3, top panel), also induced rapid loss of PKC activity from the subphase (experiment 6, Table I). Under the same conditions, bovine serum albumin, injected prior to PKC addition, occupied the air–water interface so that nearly 80% of the PKC remained in the substrate (experiment 7, Table I). These experiments again showed that loss of PKC activity from the subphase required exposure of the protein to the air–water interface and correlated with large surface pressure changes. While no attempt was made to recover PKC activity from the air–water interface, it is possible that this protein could become denatured. If this occurred, dilute solutions of purified PKC would appear unstable. Whether or not denaturation occurs at the air–water interface, simple migration of PKC to the air–water interface will deplete the enzyme in bulk solution. Since pure preparations of PKC are often very dilute, they could appear unstable due to this process.

Addition of calcium to a subphase containing PKC resulted in rapid PKC–PS association and subsequent depletion of PKC activity from the subphase (experiment 8, Table I). However, subsequent addition of excess EGTA to monolayer-associated PKC did not release PKC into the subphase (experiment 9, Table I). Monolayer-associated PKC was still active when released from the monolayer by protamine sulfate (see below). These results suggested that PKC–PS binding was a calcium-dependent event but calcium was not needed to sustain the complex. Formation of irreversibly bound PKC suggested penetration into the hydrocarbon region of the monolayer.

Despite the strong qualitative correlation between PKC–PS binding and induced surface pressure changes, other properties showed a lack of correlation. For example, depletion of PKC from the subphase was usually faster than the surface pressure change. Loss of kinase activity was essentially complete within the mixing time (a few minutes, data not shown) while surface pressure changes required up to 30 min to reach the maximum. Rapid depletion of PKC from the subphase was also observed upon the injection of Ca^{2+} (data from experiment 8, Table I). This suggested that association of PKC with PS monolayers might be composed of two steps: a rapid PKC–PS binding step which resulted in depletion of PKC from the subphase but which gave small or negligible surface pressure changes. This was followed by a slower process, possibly insertion of PKC into the hydrocarbon region of the phospholipid, which induced the large surface pressure changes.

Association of Substrates with Phospholipid Monolayers. Previous studies have established that substrate–phospholipid interaction is an essential component of PKC activity (Bazzi & Nelsestuen, 1987b,d). Therefore, we examined the interaction of histone III-S, the commonly used substrate of PKC, with phospholipid monolayers. Histone, in the absence of phospholipid monolayers, caused an increase in the surface pressure of the buffer which saturated at about 29 dyn/cm. Injecting histone into the subphase of PS monolayers also caused an increase in the surface pressure that was dependent on the amount of histone added and on the initial surface pressure of the phospholipid. Experiments similar to those shown for PKC (Figure 4) showed that the critical pressure for histone interaction with PS monolayers was about 51 dyn/cm (Figure 5). The high critical pressure suggested that histones may be capable of disrupting the phospholipid monolayer.

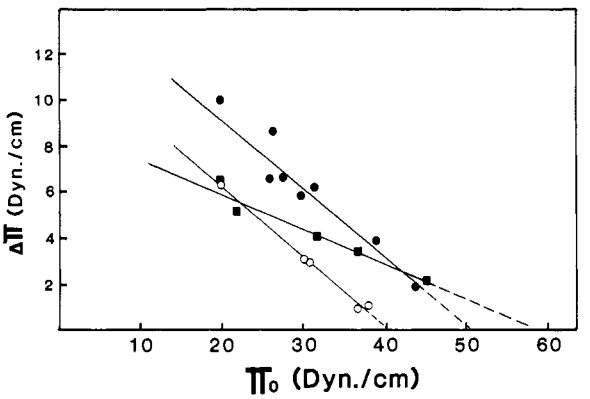

FIGURE 5: Association of histone and protamine sulfate with phospholipid monolayers. The increase in the surface pressure of PS (●, ■) or PC (○) monolayers upon the addition of 0.5 mg of either histone (●, ○) or protamine sulfate (■) was plotted as a function of the initial surface pressure of the monolayers. Each data point represents a different experiment.

The magnitude of the surface pressure change suggested that histone bound to PS by means other than just electrostatic interaction. Interaction of histone with PC monolayers was therefore investigated. The results showed that histone induced surface pressure changes in PC monolayers with a critical pressure of about 40 dyn/cm (Figure 5). This was larger than the surface pressure change induced by histone in the absence of phospholipid (29 dyn/cm), suggesting a histone–PC interaction. Light-scattering intensity measurements, performed with histone and PC vesicles [as in Bazzi and Nelsestuen (1987c)], indicated that histone did not bind to phospholipid vesicles composed of PC only. Consequently, histones appeared capable of interaction with PC but at a surface pressure lower than that of bilayer vesicles.

Protamine sulfate also interacted with and penetrated PS monolayers with a critical surface pressure of about 60 dyn/cm (Figure 5). This pressure was much greater than the collapse pressure of the phospholipid (46 dyn/cm) and implied that protamine sulfate would disrupt the monolayer and actually displace lipid from the air–water interface (Pethica, 1955).

Activation of PKC on Phospholipid Monolayers. Previous studies have shown that substrate–phospholipid interaction was a required component of PKC activity toward most substrates. This interaction was complicated by aggregation of substrate–lipid complexes (Bazzi & Nelsestuen, 1987b,d). Monolayers provide a system for testing phosphorylation without the aggregation event. The other cofactor of PKC activation, phorbol esters, has been shown to interact with phospholipid monolayers (Jacobson et al., 1975). Surface pressure changes induced by these reagents were measured in the course of these investigations (data not shown) and showed that this interaction did occur in these experiments.

Phosphorylation of histone was monitored by injecting PKC into the subphase in the presence or the absence of Ca^{2+} and/or phorbol ester, followed by the addition of histone. The surface pressure changes were monitored continuously to assure binding of the various reagents to the monolayer. After the surface pressure had stabilized, ATP was injected to start the reaction. The phosphorylation reaction proceeded for 30 min and was terminated by addition of cold ATP as described under Experimental Procedures. Phosphorylated proteins associated with the phospholipid film were determined by recovering the film, while those in the subphase were determined by TCA precipitation. Table II shows the results for histone phosphorylation under several conditions. A low level of phosphorylated was observed (<5% of the activity observed

Table II: Activity of Monolayer-Bound PKC toward Histone

experimental conditions[a]				PKC act. (cpm)		
mM Ca^{2+}	mM EGTA	μM PMA	[histone] (mg/mL)	film	subphase	sum
1.0	0.0	0.0	0[b]	5466	5295	10761
1.0	0.0	0.0	0.1	9550	4631	14181
0.0	1.0	0.0	0.1	10048	4526	14574
0.2	0.0	0.3	0.1	8468	6703	15171

[a] The activity of PKC was measured by injecting PKC (2.7 μg) into the subphase below a PS monolayer spread at an initial pressure of 28 ($\pm$3) dyn/cm, followed by the addition of histone (if any) and PMA (if any). The phosphorylation reaction was initiated by the addition of ATP and proceeded for 20 min. The film and the subphase were recovered as described under Experimental Procedures. The concentration of the reagents shown refers to the final concentration in the subphase. No attempts were made to quantitate the partition of these reagents between the subphase and the film. [b] The substrate was omitted in this experiment to determine the background and the autophosphorylation levels of PKC.

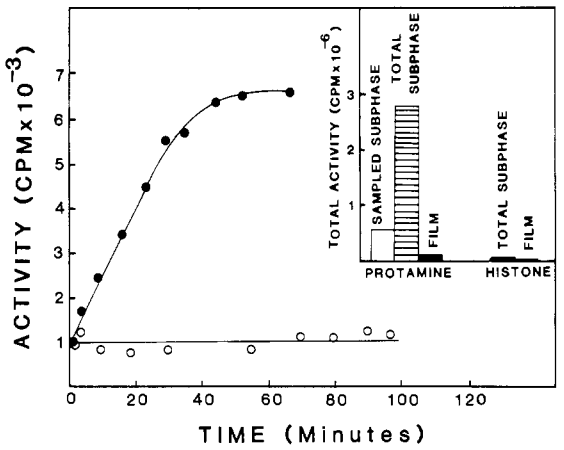

FIGURE 6: Activity of PKC on a phospholipid monolayer. PS monolayers were spread at an initial surface pressure of 27 dyn/cm in the presence of 0.2 mM Ca^{2+}, 5.4 μg of PKC was injected into the subphase followed by 1.0 mg of either protamine sulfate (●) or histone III-S (○). The surface pressure was allowed to stabilize between additions. After the addition of ATP to start the phosphorylation reaction, 100-μL aliquots were removed from the subphase at timed intervals and precipitated by TCA addition. The inset shows the radiolabeled protein obtained by recovering the film (film, inset) and precipitating the entire subphase (total subphase, inset) as described under Experimental Procedures. The total amount of phosphoprotein calculated from the withdrawn sample (sampled subphase, inset) is shown for comparison.

with a similar amount of PKC and phospholipid vesicles). This activity was not influenced by Ca^{2+} and/or phorbol esters and was therefore considered to be a background level. Studies at a phospholipid surface pressure of 35 dyn/cm gave similar results (data not shown). This showed that PKC bound to the monolayer was not capable of avid phosphorylation of histone substrate.

Phosphorylation of protamine sulfate was also examined. PKC was injected into the subphase below a PS monolayer (π_0 = 31 dyn/cm) in the presence of 0.2 mM Ca^{2+}. Binding was completed before addition of protamine sulfate. After protamine sulfate had been added and the surface pressure had stabilized, ATP was injected, and phosphorylation was monitored by sampling the subphase at time intervals. Phosphorylation of protamine sulfate occurred, and it appeared to reach a maximum after approximately 40 min (Figure 6). Under the same conditions, there was little or no phosphorylation of histone (Figure 6).

After 2 h, the entire phosphorylated protein in the subphase and on the monolayer film was measured. The total phosphorylation of protamine sulfate (Figure 6, inset, hatched bar) was about 4 times the amount predicted by the sampling procedure (Figure 6, inset, open bar). This discrepancy could arise if protamine sulfate removed PKC from the monolayer and formed aggregates in the subphase that sedimented and were not detected by the sampling procedure. Formation of such large aggregates was predicted by previous studies which showed formation of large PKC–protamine sulfate aggregates that could be sedimented by low-speed centrifugation (Bazzi & Nelsestuen, 1987b).

Table III: Activity of Monolayer-Bound PKC toward Poly(LS)

experimental conditions[a]		PKC act. (cpm)	
phospholipid	π_0	film	subphase
PS	31.0	21 512	167 522
PC	32.6	22 452	417 062

[a] Phospholipid monolayers were spread at the indicated surface pressure in the presence of 0.5 mM Ca^{2+}. 2.7 μg of PKC was injected into the subphase and the surface pressure allowed to stabilize (about 15 min). This was followed by addition of 0.5 mg of poly(LS). The phosphorylation reaction was initiated by the addition of ATP and proceeded for 20 min. The film and the subphase were recovered and counted as described under Experimental Procedures.

To further test the activity of the monolayer-bound PKC, poly(LS) was used as the substrate. This substrate binds PKC but does not form aggregates, and its phosphorylation by PKC is greatly stimulated by acidic phospholipids (Bazzi & Nelsestuen, 1987b). Monolayers composed of either PC or PS were used to determine phospholipid-dependent and -independent phosphorylation of poly(LS). The activity of PKC in the presence of a PC monolayer was considered to be phospholipid-independent activity, since PC vesicles do not support the phosphorylation of poly(LS). Phospholipid monolayers were spread at an initial pressure of 32 dyn/cm to prevent nonspecific adsorption of PKC or poly(LS) to the air–water interface. At this surface pressure, the addition of PKC or poly(LS) to PC monolayers did not alter the surface pressure. With PS monolayers (plus Ca^{2+}), both PKC and poly(LS) induced changes in the surface pressure, indicating binding or insertion of these proteins into the monolayer. Surface pressure changes induced by poly(LS) were independent of Ca^{2+}. The phosphorylation results (Table III) showed that the majority of phosphorylated poly(LS) was in the subphase with a small amount recovered on the film. Total phosphorylation was greater than that observed for histone but was much less than that observed for protamine sulfate (Table III, Figure 6). More phosphorylation occurred with the PC monolayer than with the PS monolayer (Table III). Therefore, binding of PKC to the monolayer actually inhibited phosphorylation. Overall, PKC did not appear to be able to phosphorylate any of these substrates on a planar phospholipid surface.

Discussion

Like many phospholipid binding proteins, PKC, added to the subphase below a phospholipid monolayer, caused increases in surface pressure [for reviews, see Verger and Pattus (1982), Macrichtie (1978), and Phillips et al. (1975)]. PKC also

interacted with the air–water interface, and it was essential to monitor PKC–phospholipid interactions where the phospholipid monolayer effectively excluded PKC from the air–water interface. The minimum phospholipid pressure needed was about 26 dyn/cm. Above this phospholipid surface pressure, PKC–monolayer interaction was dependent on calcium and showed characteristics of PKC interaction with phospholipids. The results presented in this study showed two properties of PKC that may suggest events related to the function of PKC. First, PKC formed a phospholipid-bound structure that was not dissociable by EGTA. Second, monolayer-bound PKC did not avidly phosphorylate substrate in the absence of substrate–enzyme–phospholipid aggregation.

Surface pressure changes induced by PKC correlated qualitatively with depletion of the subphase from PKC activity. Both the surface pressure changes and the depletion of PKC activity from the subphase were the result of a specific PKC–PS association, as evidenced by the Ca^{2+} requirements for both events. However, the disappearance of PKC from the subphase appeared to be substantially faster than the maximum surface pressure change. This suggested that interaction of PKC with the monolayers consisted of two steps: calcium-dependent binding followed by an event that produced a large change in the surface pressure. The simplest explanation for the latter behavior is protein penetration into the hydrocarbon region of the monolayer. Such binding-mediated penetrations have been described for other proteins (Colacicco, 1970; Kimelberg & Paphadjopoulos, 1971). Penetration into the hydrocarbon region of the monolayer is characterized by large changes in surface pressure and by an initial rapid loss of film pressure. In the case of PKC, however, the rapid loss of film pressure was not observed because of prior addition of Ca^{2+}. Precondensing phospholipid monolayers with Ca^{2+} abolishes the effect of charge neutralization (Verger & Pattus, 1982) which is the basis for the initial loss of film pressure.

Two-stage association of PKC with the monolayer was further supported by the fact that binding was irreversible. The initial binding to PS monolayers was calcium dependent above a π_0 of 26 dyn/cm (Table I). However, subsequent addition of EGTA did not increase the level of PKC activity in the subphase. This property also indicated that binding of PKC was followed by an irreversible event. Although protein denaturation is an irreversible event, experiments with protamine sulfate (Figure 6) established that monolayer-bound PKC retained activity which was expressed when PKC was displaced from the monolayer. Another type of secondary, irreversible process consists of protein insertion into the hydrocarbon region of the monolayer to produce a calcium-independent PKC–monolayer complex. The existence of this process was suggested by earlier studies with vesicles which showed partial irreversibility under certain conditions (Bazzi & Nelsestuen, 1987c). The latter has been corroborated by further studies with phospholipid vesicles (Bazzi & Nelsestuen, 1988) and appears to correlate with events observed in whole cells and cell membranes (Gopalakrishna et al., 1986).

Despite the fact that most of the experiments in this study were performed with an amount of PKC that was substantially below saturation of the monolayer, the magnitudes of the surface pressure increases were relatively large. At 30 dyn/cm and near saturation with PKC (9 μg), the increase in surface pressure was about 4 dyn/cm (Figure 4). This was larger than changes obtained under similar conditions with other proteins such as myelin basic protein, prothrombin, blood coagulation factor V or X, polylysine [see Mayer et al. (1983b) and references cited therein], or with several cardiotoxins (Bougis et al., 1982). In fact, membrane perturbation induced by PKC at saturating concentrations might approach that of melittin or δ-lysin (Bhakoo et al., 1982). Interestingly, melittin, δ-lysin, and PKC formed films at the air-water interface (in the absence of phospholipid) with a collapse pressures of 24.5, 25.5, and 26 dyn/cm, respectively. It is possible that all of these proteins penetrated into the hydrocarbon region of the monolayer and formed structures with similar surface areas.

Kimelberg and Papahadjopoulos (1971) described two possible mechanisms for the increase in surface pressure of phospholipid monolayers upon protein binding; protein penetration and phospholipid acyl chain deformation. The former involves protein conformational changes that allow penetration, and the latter involves changes in the arrangement of phospholipid acyl chains that allow interaction with hydrophobic regions of the protein. While it is possible that both mechanism could function simultaneously, the irreversible nature of binding and the large changes observed with PKC made protein penetration the more attractive explanation.

The critical surface pressure for PKC interaction with PS monolayers was 43 dyn/cm. The surface pressure of an erythrocyte membrane has been estimated to be about 31 dyn/cm (Demel et al., 1975), and many membranes may exhibit comparable surface pressures (Blume, 1979). This indicated that PKC should be able to penetrate many biological membranes. In fact, subcellular distribution of PKC in brain implied that PKC penetrated membranes under in vivo conditions; tissue fractionations in the presence of EGTA revealed that only about one-third of the PKC activity was recovered in the soluble fraction (Kikkawa et al., 1983a,b). The monolayer studies shown here indicated that virtually all of the PKC was capable of forming an irreversible membrane-bound state. Previous studies with phospholipid vesicles indicated the formation of a significant population of irreversible PKC–membrane complex (Bazzi and Nelsestuen, 1987c). More recent studies have investigated this irreversibility further and indicate that PKC binding to bilayers can also produce a PKC–membrane insertion which appears to be similar to the two-stage binding of PKC to monolayers. The distribution of PKC between its soluble, membrane-bound, and irreversible membrane-bound states may be a regulated function in vivo (Bazzi & Nelsestuen, 1988).

Activation of PKC by phospholipids has been the subject of many investigations which have sought to identify conditions that generate maximum activity (Kaibuchi et al., 1981; Wise et al., 1982; Schatzman et al., 1983; Parker et al., 1984; Boni & Rando, 1985; Hannun et al., 1985). Recent studies showed a correlation between a substrate's ability to aggregate phospholipid vesicles or Triton–phospholipid mixed micelles and the extent of its phosphorylation by PKC (Bazzi & Nelsestuen, 1987b). All of the "good" in vitro substrates of PKC caused extensive aggregation of either the phospholipid component or the enzyme itself. One objective of the present study was to examine the activity of phospholipid-bound PKC in the absence of aggregation. The monolayer satisfied the other known requirements for PKC activity including formation of enzyme, substrate, Ca^{2+}, and phorbol ester complexes with phospholipid. Failure to phosphorylate histone or poly(LS) in this system indicate that aggregation was required for phosphorylation of these in vitro substrates.

The association of PKC with phospholipid monolayers, including the formation of irreversibly bound PKC, did not denature the enzyme. The results with protamine sulfate showed that phosphorylation occurred when PKC was displaced from the monolayer and formed aggregates in the

subphase (Figure 6). Such uncontrolled aggregation is unlikely to be a physiological event, and PKC must function by other mechanisms. It is possible that PKC, bound to a planar nonaggregated surface, has a very high substrate specificity and that histone and poly(LS) do not meet this specificity. An alternative mechanism might consist of substrates residing on a surface apposed to the one bearing PKC. This situation would occur during membrane fusion events.

While phosphorylation of substrate in the monolayer system was not observed, the studies reported here have helped to establish that two-stage binding of PKC to phospholipids does exist. The formation of irreversibly membrane-bound PKC represents a new parameter that might be considered in other ways. That is, the relationship between the two states of membrane-bound PKC and phosphorylation activity can be tested. In fact, studies with phospholipid vesicles, completed subsequent to the work reported here, have shown that phorbol esters appear to exert their effect by promoting insertion of PKC into phospholipid bilayers. The resulting membrane-inserted PKC shows constitutive activity toward the substrate, histone (Bazzi & Nelsestuen, 1988). This property suggests that PKC is a long-term regulator which becomes irreversibly inserted into the membrane in response to transient exposure to second messengers. Repeated exposure to second messengers should cause accumulation of PKC in the membrane and major cell changes due to an altered kinase activity in the cell. Thus, the two stages of PKC–membrane interaction may constitute an important regulatory function that accounts for many properties of PKC in vivo.

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