Highly Sequential Binding of Protein Kinase C and Related Proteins to Membranes†

Mohammad D. Bazzi and Gary L. Nelsestuen*
Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
Received February 20, 1991; Revised Manuscript Received May 15, 1991

ABSTRACT: Protein kinase C belongs to a class of proteins that displays simultaneous interaction with calcium and phospholipids. Other members of this class include two proteins (M_r 64K and 32K) isolated from bovine brain. The association of these proteins with membranes exhibited highly unusual properties that were not consistent with a simple equilibrium. Titration of protein–phospholipid binding as a function of calcium showed an apparently normal curve with a low degree of cooperativity. The binding was rapid and quickly adjusted to changes in the calcium concentration. Calcium was readily exchanged from the protein–phospholipid complex. However, at each calcium concentration, membrane-bound protein was not in rapid equilibrium with free protein in solution; the half-time for dissociation exceeded 24 h. Titration of phospholipid vesicles with proteins showed different saturation levels of bound protein at different calcium concentrations. The amount of protein bound was almost entirely determined by the concentration of calcium and was virtually unaffected by the free protein concentration. These properties suggested that protein–phospholipid binding involved a sequence of steps that were each irreversible upon completion. These binding properties were consistent with high-affinity interaction between protein and phospholipid, high cooperativity with respect to calcium ($N \geq 10$), clustering of acidic phospholipids, and negative cooperativity with respect to protein density on the membrane. A major apparent problem with the complete titration of PKC–membrane interaction was a requirement for calcium in excess of intracellular levels. However, a highly sequential binding process showed that a number of protein-binding sites on the membrane would be saturated with calcium at physiological levels. The unusual properties of sequential binding may provide other important mechanisms for calcium regulation.

Recent studies from several laboratories have identified a large number of proteins that bind phospholipids in a calcium-dependent manner (Burgoyne & Geisow, 1989; Klee, 1988; Smith et al., 1990). These proteins may constitute a new form of calcium-response element in cell. With few exceptions, the precise functions of these proteins are not known. An important exception is the calcium- and phospholipid-dependent protein kinase C (PKC).[1] PKC is an essential regulatory enzyme believed to be involved in many cell functions (Nishizuka, 1986). This enzyme, therefore, provides an important test case for understanding how these cofactors influence its activity. Biochemically, the interaction of PKC with membranes displayed certain unusual properties that were shared by a number of other proteins.

PKC belongs to a class of proteins that displays simultaneous interactions with calcium and phospholipids (Bazzi & Nelsestuen, 1990). Two additional proteins with M_r 64K and 32K had qualitatively and quantitatively similar calcium- and phospholipid-binding properties (Bazzi & Nelsestuen, 1991a). Lipocortin I (Schlaepfer & Haigler, 1987) and lipocortin II (Glenney, 1986) have some similar properties, although the latter proteins bind fewer calcium ions. These proteins could not accurately be described as calcium-binding proteins since the free protein had very low affinity for calcium. Rather, these proteins constitute portions of calcium-interacting systems. Phospholipid-dependent calcium binding, however, is not common to all proteins that bind phospholipid in a calcium-dependent manner [see Bazzi and Nelsestuen (1991a) and references cited therein].

A unique feature of PKC and the 64-kDa and 32-kDa proteins is the large number of calcium ions bound per protein–phospholipid complex. The interactions of these proteins with membranes exhibit other striking behaviors. For example, PKC and the 64-kDa and 32-kDa proteins induced extensive and reversible clustering of acidic phospholipids in membranes (Bazzi & Nelsestuen, 1991b). Thus, binding of these proteins to membranes may limit the availability of acidic phospholipids, which, in turn, may reduce the membrane's ability to bind subsequent proteins. Since a single membrane particle can bind several protein molecules, protein molecules that bind later in the titration may require higher calcium concentration.

This study examined the binding of PKC and the 64-kDa and 32-kDa proteins to phospholipid vesicles. The interaction of these proteins with membranes displayed properties that were consistent with binding by highly sequential steps. Each binding step displayed a very high protein–membrane affinity and high cooperativity ($N \geq 10$) with respect to calcium. Analysis of the overall binding curve as a single equilibrium process may lead to inexplicable or contradictory conclusions.

Experimental Procedures

Materials. Highly purified bovine brain phosphatidylserine (PS), phosphatidylethanolamine (PE), and egg yolk phosphatidylcholine (PC) were purchased from Sigma Chemical Co. or from Avanti Polar Lipids, Inc. Dipalmitoyl-*N*-dansyl-L-α-phosphotidylethanolamine (dansyl-PE) was purchased from Sigma Chemical Co. Polycarbonate filters (0.1-μm diameter) were purchased from Nucleopore Corp. Other chemicals and reagents were from Sigma Chemical Co. and were of the highest grade available. Protein kinase C, the 64-kDa protein, and the 32-kDa protein were purified from

† Supported in part by Grant GM 38819 from the National Institutes of Health.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PKC, phospholipid- and calcium-dependent protein kinase C.

0006-2960/91/0430-7970$02.50/0

bovine brain as described previously (Bazzi & Nelsestuen, 1991a).

Protein–Phospholipid Binding. The association of various proteins with phospholipid vesicles was measured by light-scattering intensity or by fluorescence energy transfer as described in detail previously (Bazzi & Nelsestuen, 1987). In both measurements, vesicles were added to 1.6 mL of buffer (20 mM Tris, pH 7.5, and 100 mM NaCl), and binding was monitored as a function of calcium concentration at a constant protein to phospholipid ratio, or as a function of protein concentration at constant calcium concentrations. Light-scattering intensity measurements provided quantitative estimates of the amount of protein bound to small unilamellar vesicles (Nelsestuen & Lim, 1977). The results are reported as a molecular weight ratio, M_2/M_1, where M_2 is the molecular weight of the protein–lipid complex and M_1 is the molecular weight of the lipid only. The relationship between the molecular weight and the light-scattering intensity has been described previously (Bazzi & Nelsestuen, 1987; Nelsestuen & Lim, 1977).

Fluorescence energy transfer measures the close proximity of proteins to phospholipids. The phospholipid vesicles used in these measurements contained 10% dansyl-PE. Excitation and emission wavelengths were 284 and 520 nm, respectively. A 500-nm cutoff filter was placed in front of the emission monochrometer. Direct excitation of the dansyl group with 284-nm light produced a fluorescence signal (I_o) that was used as an internal standard that facilitated comparisons among different experiments. The results were reported as $(I - I_o) \times 100/I_o$, where I is the fluorescence of the protein–phospholipid complex and I_o is the fluorescence of the vesicles alone.

Protein–Phospholipid Dissociation. The rate of dissociation of PKC and the 64-kDa and 32-kDa proteins from phospholipid was examined by redistribution of protein between fluorescent vesicles and an excess of unlabeled vesicles. Protein (2–16 μg, see below) was initially bound to phospholipid vesicles containing dansyl-PE [12 μg of PS/PC/dansyl-PE (20:70:10)], and the fluorescence energy transfer was determined (ΔI_m). Unlabeled vesicles (20–40-fold excess) were added at zero time; the signal due to fluorescence energy transfer (ΔI_i) was monitored and is reported as a percentage change [fluorescence change (%) = $\Delta I_i \times 100/\Delta I_m$]. In all cases, control experiments were conducted simultaneously in which all reagents were added except protein. The control allowed correction for small changes in the signal that arose from the excess phospholipids or from other events such as photobleaching or lamp fluctuations that may occur over the extended time of the experiment. Additional experiments examined the distribution of protein on the various phospholipids. These experiments were conducted by mixing both types of vesicles prior to the addition of proteins. In all experiments, the reversibility of protein–phospholipid binding was examined by adding excess EGTA at the end of the incubation.

Calcium Exchange. Dissociation of calcium from protein–phospholipid complex was monitored with two steps of gel-filtration chromatography (Hummel & Dryer, 1962). In these experiments, Sephacryl S300 columns (1.0 × 30 cm) were equilibrated with a buffer containing 20 mM Tris, pH 7.9, 100 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, and 50 μM calcium or radiolabeled calcium (^{45}Ca). In the first step, small unilamellar vesicles (180 μg) composed of PS/PC (25:75) were mixed with either the 64-kDa protein (140 μg), the 32-kDa protein (700 μg), or buffer in the presence of 50 μM ^{45}Ca. These samples (0.4 mL each) were incubated for 20 min, applied on Sephacryl S300 columns that had been equilibrated with a buffer containing ^{45}Ca and eluted (0.72 mL/fraction) with the same buffer. The ^{45}Ca and the protein content of each fraction were determined separately. This step resulted in binding of protein to phospholipid vesicles in the presence of ^{45}Ca and the separation of membrane-bound protein from free protein.

In each elution profile, the two column fractions containing the highest amount of membrane-bound protein were combined. One milliliter of this sample was applied on Sephacryl S300 columns that were equilibrated with a buffer containing 50 μM unlabeled calcium, and the columns were eluted with the same buffer. The ^{45}Ca and the protein content of each fraction were determined separately. Nonspecific binding of ^{45}Ca to phospholipid vesicles as well as the contribution of free ^{45}Ca in the elution buffer were estimated from samples that contained phospholipid vesicles but no protein.

Modeling of Sequential Binding of Proteins to Phospholipid Vesicles. Light-scattering measurements indicated that a maximum of 0.85 g of the 64-kDa protein could bind to 1 g of small unilamellar vesicles [M_2/M_1 = 1.85, Bazzi and Nelsestuen (1991b)]. This corresponded to 53 proteins per vesicle of M_r 4 × 10^6. Consequently, the binding process was modeled as a sequence of 53 steps, each involving addition of one protein to each vesicle. Since each step also involved a large number of calcium ions, eq 1 (the Hill equation) would apply.

$$\bar{Y}_i = \frac{(K_i Ca)^N (K_p \mathrm{ProPL}_i)}{1 + (K_i \mathrm{Ca})^N (K_p \mathrm{ProPL}_i)} \quad (1)$$

In this equation Y_i is the fractional binding of protein i (or the fractional saturation of site i on the vesicle), K_i is the calcium association constant of protein i (the reciprocal of the calcium concentration at the midpoint of binding the ith protein), K_p is the protein–phospholipid association constant, N is the cooperativity of binding with respect to calcium, Ca and Pro are the concentrations of free calcium and protein, respectively. The term PL_i refers to the concentration of the ith protein-binding site.

For modeling, the values of K_i were estimated from the experimental measurements shown in Figure 1. The total binding curve was divided into 53 equal steps, each corresponding to the saturation of one binding site. The calcium concentration at each step was used as an estimate of K_i. The minimum value for K_p was estimated to be 10^{14} M^{-1} (see Results) so that, under the conditions used in Figure 1, the product of the term (K_pProPL) approached unity for the first binding event (see below). This allowed treatment of N as a variable and was assigned a constant value of 1–13. These various parameters were used to generate the individual binding curves and the total binding curve.

Other Methods. Small unilamellar vesicles were prepared by sonication and gel filtration as described previously (Bazzi & Nelsestuen, 1987; Huang, 1969). Large unilamellar vesicles were prepared by the extrusion method (Hope et al., 1985) using 0.1-μm polycarbonate filters. Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) with a phosphorus to phospholipid weight ratio of 1:25. Protein concentration was determined according to Bradford by using BSA as a standard (Bradford, 1976). Unless indicated, the buffer consisted of 20.0 mM Tris, pH 7.5, containing 0.1 M NaCl plus calcium or EDTA at the indicated concentrations.

Results

The association of the 64-kDa protein with phospholipid vesicles was examined by using fluorescence energy transfer

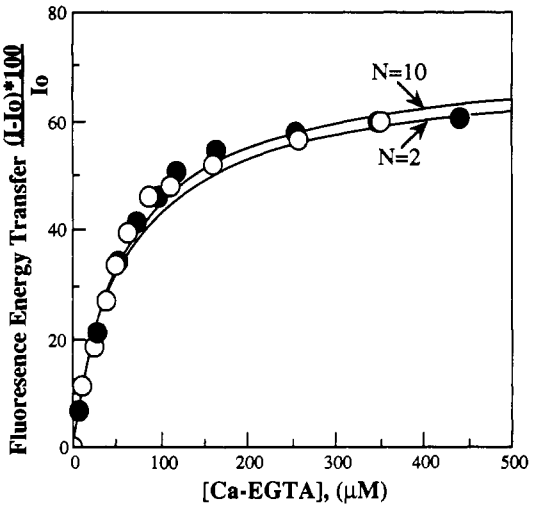

FIGURE 1: Binding of the 64-kDa protein to phospholipid vesicles. Fluorescence energy transfer was used to measure the association and dissociation of the protein–phospholipid complex as a function of calcium. Protein (10 μg) was mixed with 17 μg of phospholipid vesicles in 1.6 mL of buffer. The calcium concentration in the medium was first increased (○) by successive additions of calcium and then decreased by successive additions of EGTA (●). The phospholipid vesicles were composed of 25% PS, 65% PC, and 10% dansyl-PE. The two solid lines represent the overall binding curves calculated by the model given under Experimental Procedures with cooperativity coefficients for individual binding events of $N = 2$ and $N = 10$.

(Figure 1). Previous studies have shown that fluorescence intensity due to energy transfer was directly proportional to the amount of membrane-bound protein for the 64-kDa protein (Bazzi & Nelsestuen, 1991a) as well as for protein kinase C (Bazzi & Nelsestuen, 1987a). Fluorescence intensity due to energy transfer increased with increasing calcium concentrations (open circles in Figure 1) and decreased upon the addition of EGTA (closed circles). Binding was rapid, and the protein–membrane complex reached a steady-state value within seconds after addition of either calcium or EGTA. The curves for association and dissociation were virtually superimposible, a property that was consistent with a simple equilibrium process. Analysis of the data by the Hill equation gave an apparent Hill coefficient of approximately 2 with a calcium concentration at midpoint binding of approximately 40 μM. The binding of PKC (Bazzi & Nelsestuen, 1987) and the 32-kDa protein (data not shown) displayed similar association and dissociation curves.

Simple equilibrium events should exhibit many characteristics. For example, there should be a predictable balance between materials in the complex and those free in solution. In addition, the binding should display association and dissociation rate constants that are consistent with the overall equilibrium constant. However, several properties of the protein–phospholipid binding (see below) were at variance with these characteristics. In fact, application of the Hill equation to the overall titration shown in Figure 1 may be inappropriate.

Effect of Calcium on the Saturation Level of Membrane-Bound Protein. Titration of the vesicles with the 64-kDa protein at constant calcium concentrations showed that the initial portions of the binding curves were the same at 0.1 and 1.0 mM calcium (Figure 2A). This property was consistent with a high-affinity interaction that was at saturating levels of calcium. However, at higher protein concentrations, both titrations abruptly reached apparent saturation at different levels of membrane-bound protein. Within reasonable limits, the amount of membrane-bound protein could not be increased further by increasing the concentration of free protein. Thus,

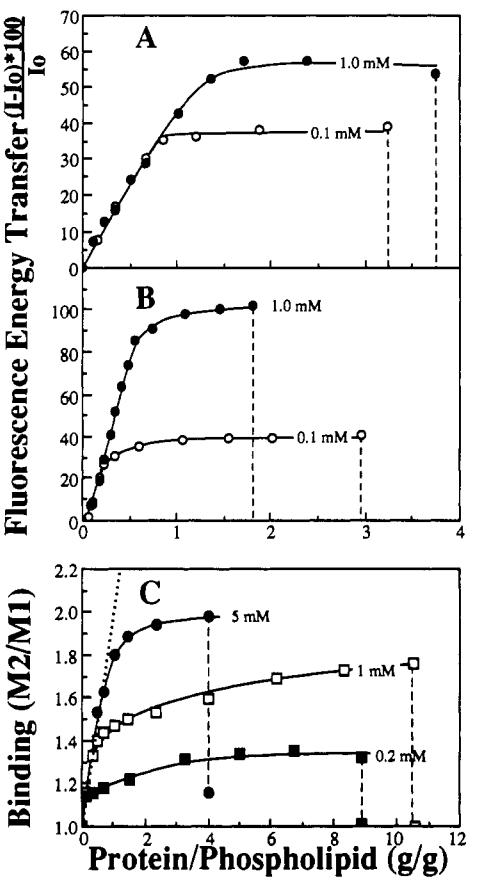

FIGURE 2: Effect of calcium on the apparent saturation point for protein–membrane binding. Protein–phospholipid binding was measured by either fluorescence energy transfer (panels A and B) or light scattering intensity (panel C). Panel A shows titrations of phospholipid vesicles (25 μg) with the 64-kDa protein in the presence of either 0.1 mM (○) or 1.0 mM (●) calcium. Panel B shows similar titrations conducted with the 32-kDa protein. Panel C shows binding of the 32-kDa protein, as determined by light-scattering intensity, to phospholipid vesicles in the presence of either 0.2 (■), 1.0 (□), or 5.0 mM (●) calcium. The dotted line in panel C shows the theoretical result if all of the protein added to the solution were bound to the vesicles. In all panels, the binding measurements were conducted with 25 μg of phospholipid vesicles in 1.6 mL of buffer. The dashed lines at the end of the titration show the effect of adding excess EGTA. Fluorescence energy transfer measurements (panels A and B) were conducted with large (100 nm in diameter) unilamellar vesicles composed of 20% PS, 70% PC, and 10% dansyl-PE. Light-scattering intensity measurements (panel C) were conducted with small unilamellar vesicles composed of 25% PS and 75% PC.

the protein to phospholipid ratio at half-maximum binding was dependent on the calcium concentration. This unusual property was not anticipated by an equilibrium binding event. The protein–phospholipid binding was entirely reversed by excess EGTA (dashed lines in Figure 2A).

The 32-kDa protein exhibited the same unusual property (Figure 2B). Protein–phospholipid binding appeared to reach a saturation level that was dependent on calcium and could not be increased by addition of more protein. Binding of the 32-kDa protein required higher concentrations of calcium than the 64-kDa protein (Bazzi & Nelsestuen, 1991a) as was indicated by a lower level of binding at 0.1 mM calcium (Figure 2B).

PKC as well as the 64-kDa and the 32-kDa proteins displayed high affinity for phospholipids in the presence of calcium. For example, the PKC–phospholipid complex was reported to have a dissociation constant of less than 5 nM (Bazzi & Nelsestuen, 1987). In order to determine if protein–phospholipid binding affinity was regulated by calcium,

the interaction of the 32-kDa protein with phospholipid vesicles was measured by light-scattering intensity. This technique allows quantitative estimation of the amount of membrane-bound protein (Nelsestuen & Lim, 1977). The results (Figure 2C) were qualitatively similar to those obtained by fluorescence energy transfer measurements. Protein–phospholipid binding appeared to reach different saturation levels at different calcium concentrations. Again, the saturation level was not greatly influenced by excess free protein.

An important observation was that the initial portions of the binding curves at all calcium concentration appeared to coincide with the theoretical limit for membrane binding (dotted line in Figure 2C). This line depicted the light-scattering changes expected if all the added protein were to bind to the vesicles. This suggested that the protein–phospholipid interaction was of high affinity at all calcium concentrations. Variations in calcium seemed to influence only the saturation point.

Dissociation of Protein–Phospholipid Complexes. Another method for assessing an equilibrium is the analysis of rate constants. Initially, the 64-kDa protein was added to phospholipid vesicles containing dansyl-PE. Dissociation of the complex was then monitored by decrease in the fluorescence signal after addition of a 40-fold excess of vesicles that did not contain dansyl-PE. The unlabeled vesicles would capture all free protein as well as any protein that may dissociate from the labeled vesicles. However, the results showed little or no dissociation of the original protein–phospholipid complex (Figure 3A). This was true at 1.0 or 0.1 mM calcium. The lower concentration was below saturating calcium levels (see Figure 1) and should favor dissociation of the protein–phospholipid complex. In fact, more than 80% of the initial protein–phospholipid complex remained intact after nearly 20 h. Failure to exchange on this time scale seemed inconsistent with the properties described for the titrations in Figure 1. Once again, the protein–membrane complexes dissociated rapidly upon addition of excess EGTA.

The calcium requirement for binding of the 64-kDa protein was highly dependent on the PS composition of the vesicles (Bazzi & Nelsestuen, 1991a). This class of proteins induced extensive segregation of acidic phospholipids in the membranes (Bazzi & Nelsestuen, 1991b). Consequently, it was possible that the 64-kDa protein formed PS-enriched regions in the first vesicle population that altered the binding parameters. The competing vesicles may be unable to provide such PS-rich regions to successfully compete for the protein. To examine this possibility, dissociation of the 64-kDa protein from vesicles containing 20% PS was tested with a 20-fold excess of unlabeled vesicles containing 90% PS. The results (Figure 3B) showed that dissociation was minimal under these conditions as well. Overall, these properties suggested that these protein–phospholipid binding events consisted of several sequential steps that, upon completion, were essentially irreversible, except by calcium manipulation.

Dansyl-PE did not have a significant effect on the ability of vesicles to bind this class of proteins. Experiments similar to those described in Figure 3 were conducted under the same conditions, except that both forms of phospholipid vesicles were mixed prior to the addition of proteins. These experiments gave very low levels of fluorescence energy transfer, consistent with random distribution of protein between labeled and unlabeled vesicles. Thus, the lack of redistribution of protein seen in Figure 3 was not a result of a selective interaction between protein and labeled vesicles but rather was the result of slow dissociation of the protein–phospholipid complex.

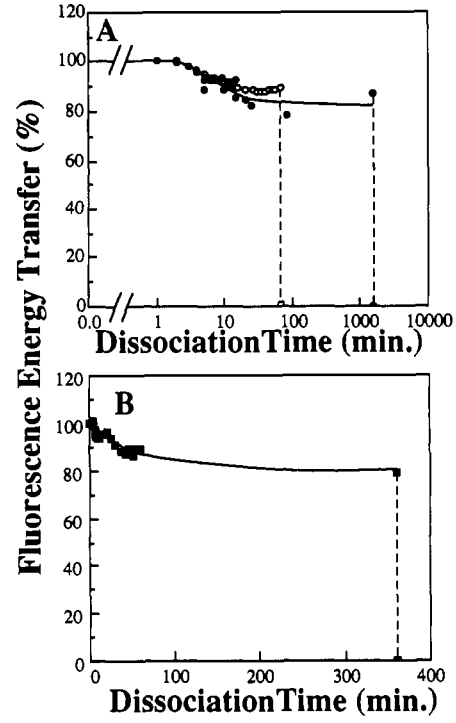

FIGURE 3: Dissociation of the 64-kDa protein–phospholipid complex. Panel A shows two experiments. In the first experiment (○), 16 μg of protein were mixed with 12.1 μg of phospholipid vesicles (PS/PC/dansyl-PE, 20:70:10) in 1.6 mL of buffer containing 1.0 mM calcium. Dissociation of the complex was monitored with time after the addition of 484 μg of phospholipid vesicles composed of PS/PC/PE (20:70:10). In the second experiment (●), 7.3 μg of protein were mixed with 12.1 μg of vesicles (PS/PC/dansyl-PE, 20:70:10) in the presence of 0.1 mM calcium. Dissociation of this complex was monitored after the addition of 242 μg of the unlabeled vesicles. Panel B shows a dissociation experiment conducted under conditions similar to those of second experiment (above), except that the competing vesicles were composed of PS/PE (90:10). In both panels, the dashed lines show the effect of adding excess EGTA.

The dissociation of the PKC–phospholipid complex was also examined (Figure 4A). Binding of PKC to phospholipid vesicles was performed in the presence of either 0.1 or 5.0 mM calcium. The lower concentration produced about half-saturation of protein–membrane binding with these vesicles (Bazzi & Nelsestuen, 1987). The results showed that addition of a 20-fold excess of unlabeled phospholipid vesicles produced only a slight dissociation of the PKC–phospholipid complex (Figure 4A). After 20 h, more than 80% of the initial PKC–phospholipid complex remained intact. Like the 64-kDa protein, PKC appeared to form a membrane-bound complex that was not in rapid equilibrium with free protein but was rapidly reponsive when the calcium concentration of the medium was manipulated. Once again, the PKC–phospholipid complex was rapidly dissociated by excess EGTA (dashed line in Figure 4A).

The 32-kDa protein displayed only slightly different properties (Figure 4B). The rate of redistribution of the 32-kDa protein from the labeled to the unlabeled vesicles showed a small dependence on the calcium concentration. Relatively higher rates of redistribution were observed at lower calcium concentrations. Qualitatively, however, the rate of dissociation of this complex remained extremely slow. For example, at any calcium concentration, more than 50% of the initial protein–phospholipid complex remained unexchanged after almost 24 h.

These results were used to estimate an upper limit for the protein–phospholipid dissociation constant (K_D). The half-time

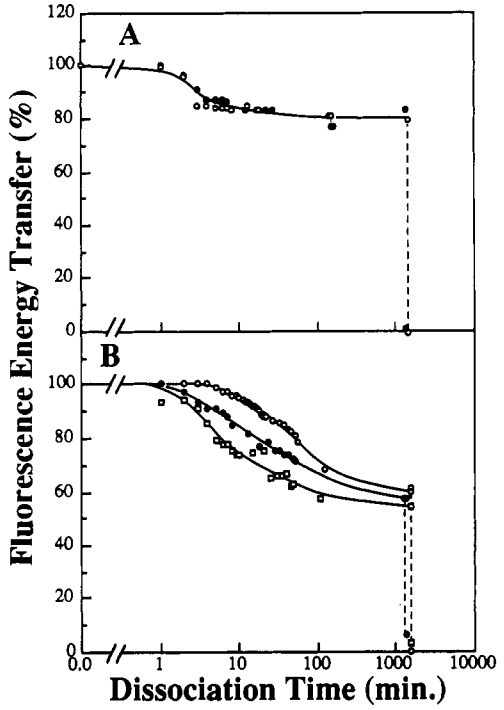

FIGURE 4: Dissociation of PKC and the 32-kDa protein from the membrane. Panel A shows dissociation of the PKC–phospholipid complex. PKC (7.3 μg) was mixed with 12.1 μg of phospholipid vesicles (PS/PC/dansyl-PE, 20:70:10) in the presence of either 0.1 (●) or 5.0 mM calcium (○). In each case, dissociation of the complex was monitored after the addition of 242 μg of unlabeled phospholipid vesicles (PS/PC/PE, 20:70:10). Panel B shows dissociation of the 32-kDa protein at various calcium concentrations. In all cases, 12.1 μg of phospholipid vesicles (PS/PC/dansyl-PE, 20:70:10) were mixed with protein and calcium in 1.6 mL of buffer. Dissociation was monitored after the addition of 242 μg of unlabeled vesicles (PS/PC/PE, 20:70:10). The protein and the calcium concentrations used in these experiments were (○) 5.5 μg of protein and 1.0 mM Ca; (●) 2.4 μg of protein and 0.2 mM Ca; (□) 2.4 μg of protein and 0.1 mM Ca. In both panels, the dashed lines show the effect of excess EGTA.

for dissociation of protein–phospholipid complex (≥24 h, see Figures 3 and 4) corresponds to a dissociation rate constant (k_{dissn}) of $\leq 1 \times 10^{-6}$ s^{-1}. Rapid association ($T_{1/2} \leq 10$ s) at 10^{-9} M protein and 10^{-9} M total protein-binding sites (Bazzi & Nelsestuen, 1987) corresponded to an association rate constant for filling a single site on a vesicle (k_{assn}) of $\geq 1 \times 10^{8}$ M^{-1} s^{-1}. These values corresponded to an overall dissociation equilibrium constant of $K_D = k_{dissn}/k_{assn} \leq 10^{-14}$ M. The actual value was probably much lower, especially for PKC and the 64-kDa protein.

As indicated above, upon addition or removal of free calcium to/from the solution, the proteins rapidly reached new and constant levels of membrane-associated protein. Other experiments demonstrated that the rapidly attained level of membrane-bound protein was a true steady-state level. Experiments such as those in Figure 1 were conducted. At the calcium concentration that produced half-maximum protein binding, the mixture was incubated for 4–14 h before continuing the titration. No change in the amount of membrane-bound protein was detected during these extended incubations at 22 or 37 °C (data not shown). Thus, rapid adjustment of membrane-bound protein to changes in free calcium, as observed during titrations such as those in Figure 1, represented a steady state or equilibrium, unaccompanied by secondary or slow binding events.

Calcium Exchange from the Protein–Phospholipid Complex. The ability of calcium to dissociate from the complex was examined by using two steps of gel-filtration chromatography (Figure 5). The 64-kDa and the 32-kDa proteins were bound to phospholipid vesicles in the presence of radiolabeled calcium, and the membrane-bound proteins were separated (Hummel & Dreyer, 1962). Figure 5A shows typical results obtained with the 64-kDa protein. At 50 μM calcium, the majority of the 64-kDa protein was associated with the phospholipid vesicles and eluted with the vesicles at the exclusion volume of the column. Fractions containing membrane-associated 64-kDa protein also had much higher total calcium concentration as a consequence of calcium binding to the protein–phospholipid complex. Similar results were obtained with the 32-kDa protein, although the protein profile contained two peaks, free protein and membrane-associated protein (data not shown). Nonspecific binding of calcium to vesicles was minimal as determined independently with samples containing phospholipid vesicles only. These results were consistent with an earlier study of protein–calcium binding (Bazzi & Nelsestuen, 1991a).

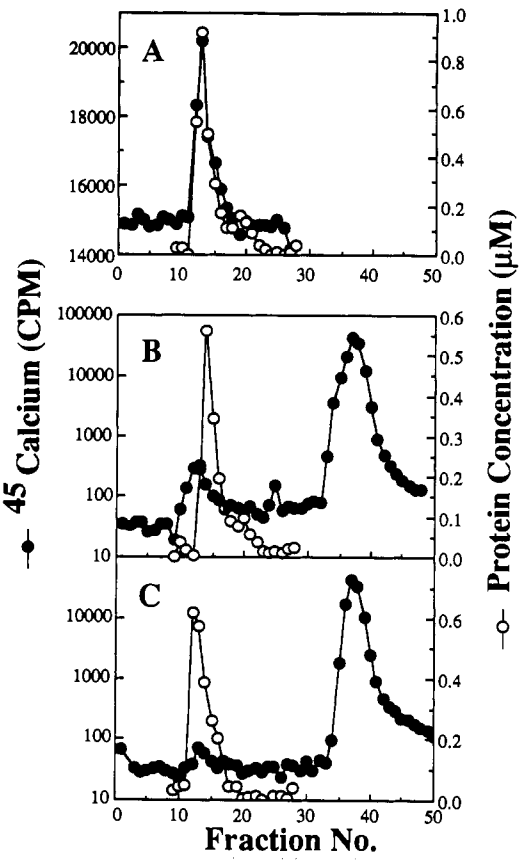

FIGURE 5: Calcium exchange from the protein–phospholipid complex. Two steps were used. First, phospholipid vesicles (182 μg) were mixed with either the 64-kDa protein (138 μg), the 32-kDa protein (720 μg) or buffer in the presence of 50 μM radiolabeled calcium (^{45}Ca). The samples were applied and eluted on Sephacryl S300 columns equilibrated with buffer containing the same amount of ^{45}Ca. Panel A shows representative results obtained with the 64-kDa protein. 1.0 mL of the two peak fractions containing the membrane-bound proteins was applied and eluted on a similar column that was equilibrated with 50 μM unlabeled $CaCl_2$. Results for the 64-kDa protein (panel B) or the 32-kDa protein (panel C) are shown. In all panels, the elution of protein (○) and ^{45}Ca (●) are shown. The phospholipid vesicles used in these experiments were small unilamellar vesicles composed of PS/PC (25:75).

Calcium exchange from this membrane–protein complex was examined by a second cycle of gel-filtration chromatography on columns containing unlabeled calcium (Figure 5B,C). The results showed virtually complete exchange of ^{45}Ca from the protein–membrane complex for both the 64-kDa protein (Figure 5B) and the 32-kDa protein (Figure 5C). There

appeared to be no leading edge for the peak of free ^{45}Ca (Figure 4B,C), indicating that exchange had occurred rapidly. The peak of radioactivity eluting with the protein–phospholipid complex at the exclusion volume of the column was less than 3% of the ^{45}Ca that was bound to the protein–phospholipid complex in the first chromatography. Thus, on the time scale of these experiments, the exchange rate for ^{45}Ca was rapid and could not be determined accurately.

While calcium had exchanged from the complex, the protein remained membrane bound throughout the experiment with no detectable levels of free 64-kDa protein (Figure 5B) or 32-kDa protein (Figure 5C). Other properties also indicated a slow exchange of protein from the membrane. For example, at 50 μM calcium the 32-kDa protein gave two discrete peaks corresponding to free- and membrane-associated protein. There was virtually base-line separation of the two peaks. Similar results were obtained for PKC (Bazzi & Nelsestuen, 1990) and the 64-kDa protein at 20 μM calcium (Bazzi & Nelsestuen, 1991a). If dissociation of membrane-bound protein occurred on the time scale of these gel-filtration experiments (usually several hours), these two peaks would not have been well resolved.

Effect of Protein on the Calcium Requirement for Binding. The calcium concentration required for protein-membrane binding was examined at different protein to phospholipid ratios (Figure 6). As expected for conditions with limiting proteins, different protein to phospholipid ratios gave different maximum levels of protein–membrane complex (Figure 6A). However, the initial portions of all these binding curves were virtually identical. Consequently, the calcium concentration at half-maximum binding was dependent on the protein to phospholipid ratio (inset in Figure 6A).

A possible explanation for these different calcium requirements for binding was the ability of these proteins to induce extensive segregation of acidic phospholipids in membranes (Bazzi & Nelsestuen, 1991b). Since the calcium requirement for binding was strongly influenced by the composition of the vesicles (Bazzi & Nelsestuen, 1991a), proteins binding later in the titration may detect reduced acidic phospholipid and, therefore, have higher calcium requirements. However, other experiments suggested that phospholipid segregation was not the only factor that influenced the calcium concentration needed for protein–membrane binding. Figure 6B shows the calcium requirement for protein binding to vesicles containing 90% PS. This composition assured that sequestering of acidic phospholipid would have minimal effect. Typical binding curves showed a pattern similar to that obtained with vesicles containing 20% PS; the initial portions of the binding curves were nearly identical and independent of protein concentration. Thus, it appeared that increased calcium requirement for proteins binding later in the titration curve arose from several factors including possible sequestering of acidic phospholipids plus factors such as structural changes in the bilayer caused by the first protein–membrane association events.

The midpoints of titrations for membranes containing 20 and 90% PS showed expected behavior. That is, variation in the midpoint of the calcium requirement was more pronounced for vesicles containing 20% PS than for vesicles of 90% PS (compare insets in Figure 6, panels A and B). This agreed with changes caused by both phospholipid segregation and other structural changes for the membranes of 20% PS. Only the other structural factors could influence membranes of 90% PS.

Sequential Binding. The results suggested that the binding of PKC and the 64-kDa and 32-kDa proteins to membranes

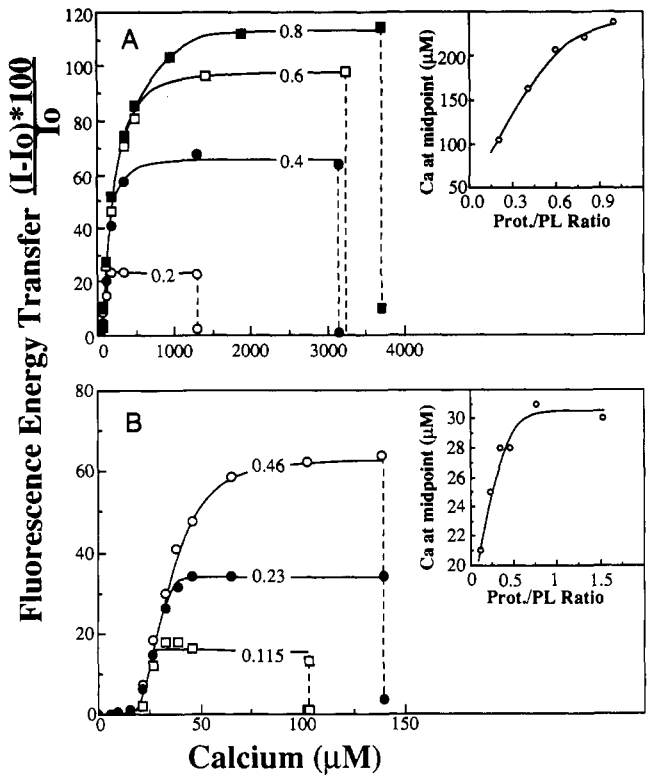

FIGURE 6: Effect of protein concentrations on the apparent calcium requirements for binding. Large unilamellar vesicles composed on PS/PC/dansyl-PE (20:70:10, panel A; or 90:0:10, panel B) were used. In both panels, phospholipid vesicles (20 μg) were mixed with appropriate amounts of the 32-kDa protein to give the indicated protein to phospholipid (w/w) ratios. In each case, protein–phospholipid binding was monitored as a function of calcium concentration. The dashed lines show the effect of adding excess EGTA. The inset in each panel shows the corresponding calcium concentrations at half maximum binding plotted as a function of protein to phospholipid ratio.

consisted of steps that were each essentially irreversible, except by calcium manipulation. To estimate the parameters needed to generate the unusual behaviors shown in Figures 1–3, a sequential-binding model was developed. Briefly, the titration curve in Figure 1 was treated as the sum of separate binding steps, each described by the equilibrium expression given under Experimental Procedures.

Total binding curves were not very revealing; virtually any value of $N \geq 2$ could be applied to obtain a satisfactory fit to the overall titration curve. Figure 1 shows two curves generated with $N = 2$ and $N = 10$. Thus, other comparisons were needed to estimate the magnitude of N that could produce the binding properties shown in Figures 2 and 3.

Theoretical binding curves were generated as a function of protein concentration at constant calcium (Figure 7A). With low values of N (2–6), the binding curves were hyperbolic and showed a considerable dependence on free protein concentration. This differed from the experimental results where the binding curves appeared to reach saturation abruptly (Figure 2). Thus, qualitative comparison between experimental results (Figure 2) and theoretical curves (Figure 7A) suggested that an N value of ≥ 10 was needed to produce the observed pattern of behavior. Figure 7B shows the calculated titration curves, at different calcium concentrations, for a cooperativity coefficient of 10. The binding curves appeared to reach saturation abruptly, in a manner consistent with the experimental data (Figure 2).

A high degree of cooperativity ($N = 10$) could also account for other unusual features of this protein–membrane inter-

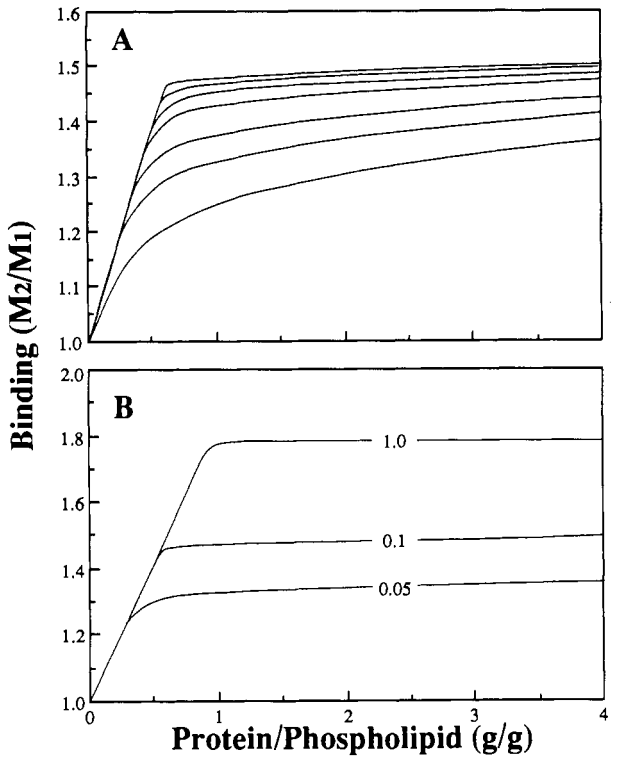

FIGURE 7: Theoretical plots for protein titration of phospholipid vesicles. Panel A shows the calculated binding curves at 100 μM calcium for several cooperativity coefficient values. The plots shown were calculated with N value of (from the top to bottom) 12, 10, 8, 6, 4, 3, and 2. Panel B shows theoretical plots for protein titration at the indicated calcium concentrations, assuming a cooperativity value of $N = 10$. In both panels, parameters for the binding of the 64-kDa protein to phospholipid vesicles containing 25% PS were calculated as outlined under Experimental Procedures.

action. For example, with $N = 10$, the binding curves for individual proteins were extremely steep (Figure 8), with little overlap between sequential curves. Thus, at any calcium concentration, the individual binding curves were either saturated with calcium or were below the calcium concentration needed to initiate a significant interaction. If each calcium-saturated interaction was of very high affinity ($K_D \leq 10^{-14}$ M), dissociation would be exceedingly slow and the titration could appear as a sequence of irreversible steps. Furthermore, with high N, the calcium term in eq 1 would dominate the equilibrium expression so that the amount of bound protein would almost be determined by the concentration of calcium alone. Large variations in the concentrations of free protein or phospholipids would have only a minor impact. Once again, these properties all correlated with the observed behavior.

Finally, it is important to note that a series of sequential binding events, as proposed, would not contradict an equilibrium process. The appearance of nonequilibrium behavior would arise from attempts to solve the overall binding curve as a single equilibrium. Some of the data in Figure 2 were obtained with large vesicles that should bind many more than 53 protein molecules. However, the general approach would apply to membrane particles of any size. The model simply divides the total binding curve into its individual steps as determined by light-scattering measurements. A large membrane particle simply involves a larger number of binding events.

Discussion

Protein kinase C is the most well known member of a class of proteins that also includes 64- and 32-kDa proteins. A striking feature of this class of proteins is their simultaneous

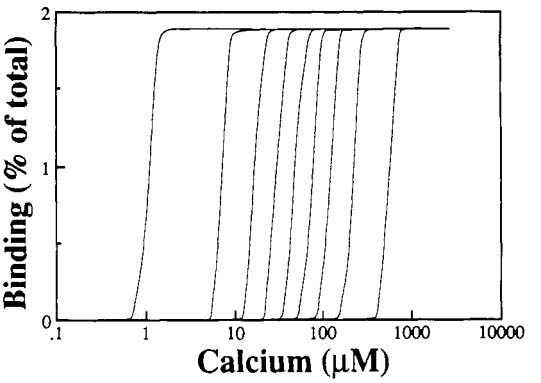

FIGURE 8: Binding parameters of the individual protein molecules. Binding curves for individual 64-kDa protein molecules, calculated as outlined under Experimental Procedures, are shown for the model with $N = 10$. Each vesicle was able to bind 53 protein molecules. The various curves show the binding parameters (from left to right) for the 1st, 6th, 12th, 18th, 24th, 30th, 36th, 42nd, and 48th protein molecules, respectively. The plots reach saturation at a value of 1.89 or 100/53. This is the percentage contribution of a single protein to the overall binding curve (53 binding sites per vesicle).

interaction with calcium and phospholipids (Bazzi & Nelsestuen, 1990, 1991a). The large number of calcium ions involved in this interaction appeared to underlie a number of unusual properties. For example, calcium titration of protein–membrane binding was extremely sensitive to membrane composition (Bazzi & Nelsestuen, 1991a). In addition, these proteins were able to induce extensive clustering of acidic phospholipids in the membrane (Bazzi & Nelsestuen, 1991b). This study described additional unusual properties.

The membrane–protein binding process seemed to display properties that were incompatible with a simple equilibrium process. For example, binding appeared rapid, dependent on calcium with modest cooperativity, and showed rapid adjustment to increases or decreases in calcium concentration. However, at any point in the calcium titration curve, binding was not influenced by large variations in the concentration of free protein. Also, at constant calcium, binding appeared virtually irreversible as indicated by extremely slow dissociation rates. Although unusual, these properties were consistent with a series of highly sequential binding events. In each binding event, protein–membrane interaction was of high affinity and displayed a high degree of cooperativity with respect to calcium and negative cooperactivity with respect to protein. Each binding event had a unique equilibrium constant. Although somewhat smaller values may also work, a cooperativity coefficient of ≥ 10 seemed to fit the data most adequately.

A number of properties that have been observed for these proteins provide a mutually consistent pattern of behavior. For example, high affinity of protein–phospholipid interaction indicated a large number of contact points between these two components [possibly 8–10 calcium ions (Bazzi & Nelsestuen, 1990, 1991a)]. This was also consistent with a high degree of cooperactivity with respect to calcium and with extensive clustering of acidic phospholipids (Bazzi & Nelsestuen, 1991b). Despite this high-affinity interaction, rapid exchange of individual calcium ions from the complex could still allow rapid adjustment of membrane-bound protein to changes in external calcium concentrations. Rapid exchange of individual calcium ions was consistent with the proposed model. For example, dissociation of a protein molecule would require the simultaneous exchange of all calcium ions in the complex. Since each complex involved 8–10 calciums, the probability of such an exchange was very low, even though single calcium ions were able to exchange rapidly.

A highly sequential binding process may have significant impact on our understanding of protein–phospholipid interaction under physiological conditions. For example, protein–membrane binding events (as in Figure 1) appeared to require calcium concentrations that are higher than intracellular levels. It was proposed that other factors or mechanisms, such as special membrane composition or covalent protein modifications, might serve to lower the calcium requirement under physiological conditions (Bazzi & Nelsestuen, 1990, 1991a). However, the sequential binding model, as presented in Figure 8, gives a better representation. These curves show that the first few protein-binding sites on a membrane will be fully saturated at intracellular calcium levels. Thus, sequential binding suggested that a number of protein-binding sites on a membrane will become saturated at physiological calcium levels. Of course, the number of binding sites filled at a given calcium concentration will be highly dependent on the membrane composition (Bazzi & Nelsestuen, 1991a). Complete titrations (as in Figure 1) described a membrane that becomes completely covered with protein, a situation that may not be physiologically relevant.

The influence of phospholipid on the activity of PKC has been the subject of many studies (Rando, 1988; Nelsestuen & Bazzi, 1991). Several attempts have been made to determine the number of phospholipid molecules required for the activation of PKC. Most of these studies utilized acidic phospholipids dispersed in detergent micelles. Quantitatively, the various studies suggested that a minimum of four PS molecules are required (Hannun et al., 1985, 1986), but full activation may require 8–11 PS molecules (Newton & Koshland, 1989, 1990). Newton and Koshland (1989) recently proposed that the activation of PKC involved the sequestering and cooperative interaction among 12 or more PS molecules. While this value is very similar to that obtained in our study, the two situations are not equivalent. The activation of PKC does not always correlate with the binding of the enzyme to membrane (Bazzi & Nelsestuen, 1987). The activity of PKC is also sensitive to other components in the assay, such as diacylglycerols or phorbol esters, that do not appear to influence the PKC–membrane binding event investigated in this study (Bazzi & Nelsestuen, 1987, 1990). In addition, the apparent cooperativity of PKC activation is dependent on the choice of substrate and on the physical nature (vesicles verses micelles) of the phospholipid component (Newton & Koshland, 1990). While the activity of PKC may require interaction with phospholipid, the relationship between apparent cooperativity observed in PKC activity assays and the cooperativity observed in direct protein–membrane binding is not clear at the present time.

An important consideration was the possible role that these proteins might play in calcium regulation. While most studies have focused on the effects of calcium and membrane on protein properties, it is possible that these proteins have important reciprocal effects on the membrane. For example, the clustering of acidic phospholipids may decrease their availability to other proteins that require acidic phospholipids. This may limit the binding or force the dissociation of other proteins. Clustering of phosphatidylserine might also create an annulus of protein-bound phospholipids that alters membrane structure or permeability. Recent studies suggest that annexin VII (Pollard & Rojas, 1988; Burns et al., 1989) and annexin V (Rojas et al., 1990) form ion channels. Whether these annexins interact with membranes in a manner that is consistent with the model proposed here is not known. However, it is possible that the channel-forming ability of these proteins results from specific rearrangements of the phospholipids.

The interaction of these proteins with membranes may also involve mechanisms other than the calcium-dependent process described here. For example, PKC is capable of forming two membrane-associated forms, a reversible calcium-dependent structure and an irreversible membrane-bound form (Bazzi & Nelsestuen, 1988). To date, the 64-kDa and the 32-kDa proteins have not been observed to form irreversible complexes. However, annexin V also appeared to interact with membrane reversibly, but its channel-forming structure may have been the result of irreversible interaction with membranes (Rojas et al., 1990). Other features of protein–membrane interaction as well as their role in cell regulation remain to be determined. The highly unusual properties of these proteins suggests a plethora of potential roles that need to be examined.

ACKNOWLEDGMENTS

We are indebted to Angelique Youakim for her excellent technical assistance.

REFERENCES

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115.

Bazzi, M. D., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 7589.

Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29*, 7624.

Bazzi, M. D., & Nelsestuen, G. L. (1991a) *Biochemistry 30*, 971.

Bazzi, M. D., & Nelsestuen, G. L. (1991b) *Biochemistry* (preceding paper in this issue).

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Burgoyne, R. D., & Geisow, M. J. (1989) *Cell Calcium 10*, 1.

Burns, L. A., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M. R., & Pollard, H. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3798.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Glenney, J. (1986) *J. Biol. Chem. 261*, 7247.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 7184.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55.

Huang, C. (1969) *Biochemistry 8*, 344.

Klee, C. B. (1988) *Biochemistry 27*, 6645.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164.

Nelsestuen, G. L., & Bazzi, M. B. (1991) *J. Bioenerg. Biomembr. 23*, 43.

Newton, A. C., & Koshland, D. E., Jr. (1989) *J. Biol. Chem. 264*, 14909.

Newton, A. C., & Koshland, D. E., Jr. (1990) *Biochemistry 29*, 6656.

Nishizuka, Y. (1986) *Science (Washington D.C.) 233*, 305.

Pollard, H. B., & Rojas, E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2974.

Rando, R. R. (1988) *FASEB J. 2*, 2348.

Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., & Burns, A. L. (1990) *J. Biol. Chem. 265*, 21207.

Schlaepfer, D. D., & Haigler, H. T. (1987) *J. Biol. Chem. 262*, 6931.

Smith, V. L., Kaetzel, M. A., & Dedman, J. R. (1990) *Cell Regul. 1*, 165.