Kinetics of Annexin VI, Calcium, and Phospholipid Association and Dissociation†

Yuefeng Lu, Mohammad D. Bazzi, and Gary L. Nelsestuen*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Received March 29, 1995; Revised Manuscript Received June 14, 1995®

ABSTRACT: Annexins VI and V are members of the annexin family of proteins that bind to phospholipid membranes in a calcium-dependent manner. The dynamics of protein, calcium, and phospholipid assembly and dissociation were investigated by stopped-flow. At relatively low calcium levels, the kinetics of the binding reaction were sensitive to calcium concentration. However, in the presence of saturating levels of calcium and at relatively low protein/vesicle (w/w) ratios (0.4 or lower), the binding reactions were rapid and the rate constants were comparable to the collisional limit, about 1.4×10^{10} M^{-1} s^{-1} for large unilamellar vesicles (about 120 nm diameter) and about 2.7×10^9 M^{-1} s^{-1} for small unilamellar vesicles (about 31 nm in diameter). These constants are expressed on the basis of vesicle concentration. These limiting association rate constants were not sensitive to the phospholipid composition of the vesicles. In contrast, at these calcium levels, protein dissociation was so slow that the complexes could be regarded as stable. However, individual calcium ions that were bound to the complexes appeared to exchange rapidly with ions in bulk solution. EGTA-induced protein dissociation was rapid with first-order rate constants ranging from 10 to 50 s^{-1}. These were dependent on the membrane composition and on the protein type (annexin VI or V). Variations in this dissociation process were found to complement the calcium concentration needed to support annexin−membrane association; increasing the acidic phospholipid component or partially replacing phosphatidylcholine by phosphatidylethanolamine in the membrane decreased both the EGTA-induced dissociation rate and the calcium concentration needed to support binding. This correlation suggested that the rate of EGTA-induced protein dissociation was actually a function of the rate of calcium dissociation from the protein−calcium−phospholipid complexes. These behaviors were consistent with a facile interaction between the protein and membrane through a large number of calcium ions.

Annexin VI is one of a widely distributed family of proteins that bind to phospholipid membranes in a calcium-dependent manner. While its exact physiological functions are not clear (Raynal & Pollard, 1994), it may be involved in controlling the membrane−microfilament interaction (Hosoya et al., 1992), regulating sarcoplasmic reticulum Ca-release channel (Hazarika et al., 1991), and exocytotic and endocytotic events (Creutz, 1992; Goldberg et al., 1990; Lin et al., 1992). Most of these implied functions are related to calcium-dependent membrane binding.

Many *in vitro* studies of this calcium-dependent protein−membrane interaction have been devised to characterize the mechanism of this interaction. The nature of the interaction may provide insight into the *in vivo* behavior of annexins. For example, previous studies have shown the effects of calcium concentration, membrane composition, protein type, and protein/membrane ratio on the interaction at equilibrium (Andree et al., 1990; Bazzi & Nelsestuen, 1991b; Bazzi et al., 1992; Evans & Nelsestuen, 1994; Tait & Gibson, 1992). It has been proposed that the annexins bind to membranes through calcium ions (Bazzi & Nelsestuen, 1991a; Tait & Gibson, 1992). A high calcium-binding stoichiometry and the resulting multipoint interaction suggested very high affinity between the protein and membrane (Bazzi & Nelsestuen, 1991a). Dissociation constants of less than 10^{-9} M were observed for the interaction of annexin V with membranes (Andree et al., 1990; Tait & Gibson, 1992). However, other dynamic aspects of this protein−membrane association have not been fully documented.

Andree et al. (1990) reported that the kinetics of annexin V binding to large planar membranes were diffusion (collision)-limited. Because efficient capture is dependent on particle size and the number of binding sites per particle (Abbott & Nelsestuen, 1988), it is likely that most membrane-binding proteins will associate efficiently with such large planar surfaces. Collision-limited interaction with small bilayer membrane particles is a more rigorous test of binding efficiency.

The calcium required for protein−membrane binding (usually quantified as the calcium concentration for half-maximal binding, $[Ca^{2+}]_{1/2}$) is a function of both the protein and membrane. For example, increasing the acidic phospholipid decreased the calcium requirement for protein−membrane association (Andree et al., 1990; Bazzi et al., 1992; Tait & Gibson, 1992). For membranes of identical composition, annexin VI has a lower calcium requirement than annexin V and IV (Bazzi & Nelsestuen, 1991b; Edwards & Crumpton, 1991). This relationship may be applicable to all annexins so that those containing eight repeating units require less calcium than those with four repeating units (Evans & Nelsestuen, 1994). A striking finding is that

† This work was supported in part by Grant GM38819 from the National Institutes of Health.

* Address correspondence to this author at University of Minnesota, Department of Biochemistry, 1479 Gortner Ave., St. Paul, MN 55108.

® Abstract published in *Advance ACS Abstracts,* August 15, 1995.

0006-2960/95/0434-10777$09.00/0

replacement of phosphatidylcholine (PC)[1] by phosphatidylethanolamine (PE) as the neutral phospholipid also decreased the calcium requirements for several proteins including PKC, annexin VI and other annexins (Bazzi et al., 1992). However, the underlying mechanism of this effect is not clear.

Dissociation of annexin VI from membranes was slow when measured by protein exchange as long as the calcium concentration was kept constant (Bazzi & Nelsestuen, 1991a). Consequently, EGTA-induced protein dissociation is usually used to show the reversible nature of the protein−membrane interaction. The latter process is rapid, suggesting that the calcium ions in the complex exchange rapidly with free calcium ions in solution (Bazzi & Nelsestuen, 1991a). This property would enable rapid adjustment of membrane-bound annexin in response to sudden changes in cellular calcium concentration. Knowledge of parameters that influence EGTA-induced protein dissociation kinetics may improve our understanding of annexin properties and functions.

The present study used stopped-flow techniques to examine the kinetics of binding between annexin VI and vesicles of varied composition and at different calcium concentrations. At saturating levels of calcium, the binding reaction was collision-limited and independent of membrane composition. The EGTA-induced protein dissociation was always rapid but depended on the protein used and the membrane composition. Phosphatidylethanolamine had a particularly striking effect on this parameter. A direct correlation between the EGTA-induced protein dissociation rate constants and the calcium requirement for protein−membrane binding was found.

MATERIALS AND METHODS

Materials. Egg PC and dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine (dansyl-PE) were purchased from Avanti Polar Lipids, Inc. Phosphatidic acid (PA) and phosphatidylethanolamine (PE) were also purchased from the same company and were derived from egg PC. All the phospholipids were of high purity (>98%, supplier's estimate). Polycarbonate filters (100 and 600 nm in diameter) were purchased from Nucleopore Corp. (Costar Co.). Other chemicals were from Sigma Chemical Co. and were of the highest grade available. Annexin V and annexin VI were purified from bovine brain or lung by methods described elsewhere (Bazzi & Nelsestuen, 1991b).

Vesicle Preparation. All the phospholipids were originally stored in chloroform. The components were mixed, and the organic solvent was evaporated by a stream of nitrogen. The sample was then placed under vacuum for 1 h. The dried phospholipids were mixed with Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5) to a final concentration of 1.0 mg/mL. Large unilamellar vesicles (LUVs) were prepared by multiple freeze−thaw cycles and extrusion through polycarbonate membranes with a pore size of 100 nm (Hope et al., 1985). The vesicles were then dialyzed at least 4 h against the Tris buffer containing 100 mM NaCl before being used. Unless specified, the results presented were obtained from LUVs prepared by the freeze−thaw and extrusion method. Large vesicles were also prepared by ether injection as described previously (Deamer & Bangham, 1976). The dried phospholipids were dissolved in ether solution and slowly injected (0.4 mL/min) into Tris buffer that was heated to 60 °C. The vesicles were then extruded through a 600 nm polycarbonate filter, followed by gel-filtration chromatography on Sepharose 4B (Pharmacia Fine Chemicals). The vesicles eluting at the exclusion volume of the column were pooled. A small number of experiments were also conducted with small unilamellar vesicles (SUVs). These were prepared as described previously (Huang, 1969) by sonication followed by gel filtration chromatography on Sepharose 4B. Vesicle size was estimated by quasielastic light scattering with the method and apparatus described previously (Bloomfield & Lim, 1978). The SUVs gave average diameters of about 31 nm. The LUVs produced by freeze−thaw and extrusion gave diameters of about 120 nm, and ether injection produced LUVs with diameters of 380 nm. The molecular weights of the vesicles were estimated from the vesicle diameters, membrane thickness (3.7 nm for SUVs and 5 nm for LUVs), and the surface areas per phospholipid headgroup reported for SUVs (0.74 and 0.61 nm^2 for the outer and inner membrane leaflets, respectively; Huang & Mason, 1978) and LUVs (0.55 nm^2; Deamer & Bangham, 1976).

Steady−State Protein−Vesicle Binding. Calcium requirements for annexin interaction with phospholipids vesicles were determined by titration of a protein−vesicle solution with calcium, essentially as described elsewhere (Bazzi et al., 1992). Binding between the proteins and vesicles was monitored by fluorescence energy transfer and/or light scattering, using a Spex FluoroMax (JY/Spex Instruments SA, Inc.). Fluorescence energy transfer was measured with excitation of tryptophan residues at 287 nm and emission of the membrane-bound dansyl groups at 505 nm. Light scattering was measured with both excitation and emission wavelengths at 650 nm. In certain cases, the binding results are expressed as a change in mass or a molecular weight ratio, M_2/M_1, where M_1 is the molecular weight of the vesicles alone, and M_2 is the molecular weight of protein−lipid complex. The manner of estimating M_2/M_1 has been described previously (Nelsestuen & Lim, 1977).

Stopped-Flow Fast Kinetics. Fast kinetics of protein binding to, and dissociation from, vesicles were monitored by fluorescence energy transfer and/or light scattering using a 4800C Spectrofluorometer (SLM Aminco) with a stopped-flow attachment (Mili-Flow, SLM Aminco). Two equal volume syringes were filled with the appropriate solutions, and about 40 μL from each syringe was used per experiment. The dead time of the instrument was about 6 ms as measured by the fluorescence reaction of pyranine with bovine carbonic anhydrase as described by the manufacturer. A typical experiment for estimation of protein−membrane association used a solution of phospholipid vesicles in one syringe and annexin VI in the other. Calcium was mixed either with both the vesicle solution and the protein solution or with the protein solution only. Binding kinetics were also measured with a solution of protein and vesicles in one syringe and calcium solution in the other. The binding kinetics were not affected by the manner of calcium addition. For a typical dissociation experiment, a solution of vesicles and annexin VI in Ca^{2+}-containing buffer was placed in one syringe. EGTA was in the other. The rate of EGTA−

[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; dansyl-PE, dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; LS, light scattering; FET, fluorescence energy transfer.

calcium chelation has been studied by Harafuji and Ogawa (1980). The rate constant should provide reduction of calcium in solution that is rapid in the time scale of protein dissociation. That the rate of EGTA−calcium interaction was not rate limiting was also shown by varying the EGTA concentration (see below).

Protein binding and dissociation processes were primarily monitored by fluorescence energy transfer from tryptophan (donor) to the dansyl group attached to PE headgroup. The excitation wavelength was 287 nm, and emission was measured through a 500 ± 20 nm band-pass filter (Corion Corp.). Light at 287 nm excites the dansyl group to some degree, and this provided an initial intensity that was used as a reference for comparison (Bazzi & Nelsestuen, 1991b). The association kinetics were also measured by 90° light scattering (for SUVs, the wavelength was 320 nm, and for LUVs, 400 nm) by methods described previously (Wei et al., 1982). The entire signal at 90° was monitored. The amount of protein bound to the membrane can be estimated from M_2/M_1, the molecular weight of the protein−membrane−complex divided by the vesicle alone. This ratio is proportional to the square root of light scattering intensity changes (Nelsestuen & Lim, 1977). However, at low changes of mass (less than 40%), the progression of light scattering intensity change was similar to the progression of the mass change (M_2/M_1). Therefore, light scattering intensity change was analyzed directly as an indicator of reaction progress. Error induced by this simplification was less than signal to noise in the kinetics experiments shown.

All data shown are the average of at least five measurements with the same set of samples. The deviations for replicate experiments with same set of reagents were approximately equal to signal to noise values.

Data Analysis. Several methods of data analysis were tested including analysis as a bimolecular reaction between protein and binding sites on the vesicle surface. All methods gave similar results. Analysis as a pseudo-first-order reaction, described here, was used for simplicity.

At low protein/vesicle (w/w) ratios (0.4 or below) and high calcium concentration, the entire vesicle surface can participate in protein binding so the potential number of binding sites is in large excess over protein. Under these conditions, the binding reaction could be regarded as a pseudo-first-order reaction. Since the reverse reaction was very slow (Bazzi & Nelsestuen, 1991a), the rate of binding could be expressed by

$$v = -d[\mathrm{A}]/dt = k_{app}[\mathrm{A}] \quad (1)$$

Fluorescence intensity changes were proportional to the amount of protein bound (Bazzi & Nelsestuen, 1991b) so that fluorescence signal change was analyzed by an integrated equation:

$$\ln[(F_{max} - F)/(F_{max} - F_o)] = -k_{app}t \quad (2)$$

F_o, F, and F_{max} represent the fluorescence intensity at the beginning, at time t, and at final equilibrium for the stopped-flow experiments, respectively. Rate constants were obtained by fitting the first half of the reaction. Later portions of the binding process deviated somewhat from linearity. This became more severe at higher protein/vesicle ratios. This nonlinearity probably arose from less than strict first-order conditions. The effective concentration of the membrane may have decreased slightly as protein density on the surface increased. Generally, deviation from linearity was small, and the slope at 90% of reaction completion varied less than 2-fold. This was not considered to be of sufficient concern to warrant more complex evaluation of the results.

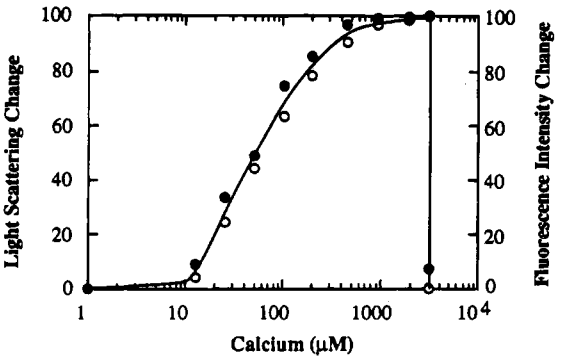

FIGURE 1: Equilibrium binding of annexin VI to vesicles. Calcium titrations are shown for annexin VI (10 μg/mL) association with LUVs (25 μg/mL, PA/dansyl-PE/PE/PC, 12.5:3:10:74.5). The interaction was measured by both light scattering at 400 nm (●) and fluorescence energy transfer (○). The maximum light scattering intensity was 1.7 times the intensity of the vesicles alone. The maximum fluorescence intensity (○) was 1.52 times the intensity of the vesicles alone. Addition of EGTA returned the signals to their initial values (final data points on the right).

The second-order rate constant per vesicle (k_{2exp}) was obtained from

$$k_{2exp} = k_{app}/[\mathrm{Ves}] \quad (3)$$

The collisional efficiency was estimated by comparing k_{2exp} with the theoretical rate constants for collision of protein with vesicles, assuming spherical shape [k_{coll} (Smoluchowski, 1917)]:

$$k_{coll} = 4\pi N_A DR/1000 \quad (4)$$

N_A is Avagadro's number, D is the sum of the vesicle and protein diffusional coefficients (effectively equal to that of the protein), and R is the sum of the radii of the vesicle particles and the protein particles (effectively that of the vesicle). The diffusion coefficient for annexin VI (6.1×10^{-7} cm^2/s) was estimated from its molecular weight, assuming a spherical shape (Zubay, 1988).

EGTA-induced annexin dissociation from the membrane was also fitted by a first-order expression:

$$F = F_o - (F_o - F_{max})\, e^{-k_{app}t} \quad (5)$$

Other Methods. Protein concentrations were determined according to Bradford (1976), and phospholipid concentration was determined according to Chen et al. (1956), assuming a 25:1 phospholipid to phosphorus weight ratio. Unless indicated, the buffer system used throughout this study was 50 mM Tris and 100 mM NaCl, pH 7.5. The temperature was 20 ± 1 °C.

RESULTS

Figure 1 illustrates the use of steady-state light scattering and fluorescence energy transfer to measure the interaction of annexin VI with vesicles. Fluorescence energy transfer detected proximity between protein and membrane and light

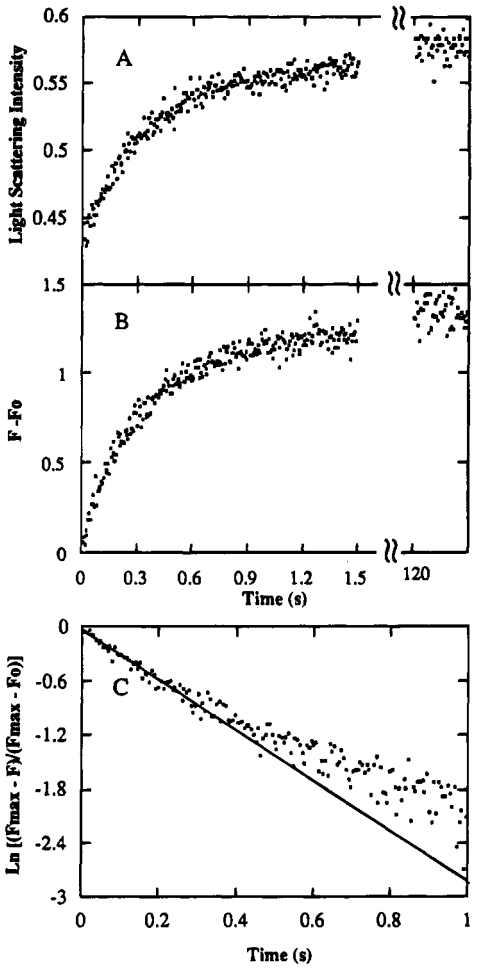

FIGURE 2: Rates of annexin VI association with LUVs. Reactions were initialized by rapid mixing of an equal volume of annexin VI solution (20 μg/mL) with vesicles (50 μg/mL, PA/dansyl-PE/PE/PC, 10:3:40:57) in the stopped-flow machine. Both solutions contained 1 mM Ca^{2+}. The light scattering intensity of the sample is shown in panel A. The fluorescence signal (excitation at 287 nm, emission at 500 ± 20 nm) is shown in panel B. F_o, F, and F_{max} represent the fluorescence intensity at the beginning, at time t, and at the end of the reaction, respectively. The kinetic curve in panel B was fit to a pseudo-first-order expression (eq 2, Materials and Methods). The rate constant obtained from the line drawn in panel C was 3.2 s^{-1}.

scattering detected change in the mass of the vesicles (Bazzi & Nelsestuen, 1991b). The close correlation of these two measurements supported previous observations regarding the utility of fluorescence or light scattering to measure these reactions (Bazzi & Nelsestuen, 1991b). The calcium concentration at half-maximum reaction ($[Ca]_{1/2}$) was estimated from these plots.

Annexin VI–Membrane Association Kinetics at Saturating Calcium. The dynamics of annexin VI binding to vesicles was examined by both light scattering (Figure 2A) and fluorescence energy transfer (Figure 2B). The binding reaction was fast and was completed within several seconds. The first half of the binding reaction was fitted by a pseudo-first-order kinetic expression (Figure 2C). Later portions of the reaction deviated from linearity suggesting that pseudo-first-order conditions did not apply to the entire reaction. This might result from protein crowding as its concentration on the membrane was increased. Therefore, the rate constants reported in this study represent values obtained from the first half of each reaction.

The binding kinetics were measured for membrane vesicles of different size and widely varied composition. The first-order rate constants obtained by the method shown in Figure 2 were divided by the vesicle concentrations to obtain the second-order rate constants (Table 1). The second-order rate constants for annexin VI binding to LUVs with diameters about 120 nm were about 1.4×10^{10} M^{-1} s^{-1} (Table 1). This value corresponded to 47% of k_{coll} for protein and vesicles, suggesting that annexin binding approached the collisional limit. For SUVs (31 nm in diameter), this value was about 2.7×10^9 M^{-1} s^{-1} (Table 1). This corresponded to 37% of k_{coll} (Table 1). Somewhat lower collisional efficiencies for smaller vesicles was consistent with the number of potential binding sites (Stankowski, 1983). That is, larger vesicles with more binding sites have a greater probability of capture. However, all of these values may be within the error associated with estimation of k_{coll}. A high efficiency of capture was also supported by the fact that the rate constant was independent of the protein concentration but varied with the vesicle concentration (Figure 3). Furthermore, as predicted from eq 4, rate constants gave nearly linear correlation with vesicle radius (Figure 4B). Thus, a major property of these interactions was a high collisional efficiency for all membrane compositions. This conclusion did not imply that the binding of annexin VI lacked preference for membrane composition. Preference was observed at equilibrium and in the dissociation process (see below).

Protein–Membrane Binding Kinetics at Low Calcium Concentration. The extent of protein–membrane binding is known to be influenced by calcium levels (Bazzi & Nelsestuen, 1991a). This study examined the impact of calcium concentration on the association rate behavior, a property that could determine the responsiveness of this protein to changes in intracellular calcium. Both the protein and the phospholipid provide relatively low affinity calcium binding sites (Bazzi & Nelsestuen, 1991b; Plager & Nelsestuen, 1994), while the complex binds a large number of calcium ions and with higher affinity. The kinetic behavior at subsaturating calcium concentration should reveal aspects of the mechanism of protein–calcium–phospholipid interaction.

Both the rate of binding and the amount of protein bound declined at low calcium concentrations (Figure 5A). The initial slope of the fluorescence change provided an estimate of the initial velocity for the reaction and is plotted as a function of calcium concentration (Figure 5B). The half-maximum rate occurred at 0.3 mM calcium.

No significant difference was found in the calcium required for protein binding to LUVs and SUVs (not shown). The initial velocities showed an approximately linear relationship with calcium concentration (Figure 5B) without significant cooperative behavior (i.e., curves were not sigmoidal). A similar pattern of behavior was observed for SUVs (not shown). These results suggested that the rate-limiting step for protein–membrane interaction involved a small number of calcium ions, perhaps even a single ion. Other calcium ions may be added to stabilize the complex. In agreement with the reaction at equilibrium (Bazzi et al., 1992), adding PE to the membrane lowered the calcium required to reach half-maximum binding rate (compare Figure 5 panels B and C).

Table 1: Characteristics of Annexin VI−Membrane Association Kinetics

vesicle composition[a]	size (nm)	MW[d] (D)	P/V[e] (w/w)	[Ves] (M)	[Ca] (mM)	k_{app} (s^{-1})	k_{2exp}[f] (M^{-1} s^{-1})	k_{2coll}[g] ($M^{-1}s^{-1}$)	collision efficiency (%)
PA/PC (12.5:84.5)	31[b]	5.5×10^6	10/25	4.6×10^{-9}	1.0	12.5	2.7×10^9	7.3×10^9	37
PA/PC (12.5:84.5)	120	1.2×10^8	10/25	2.1×10^{-10}	1.0	3.0	1.4×10^{10}	2.8×10^{10}	51
PA/PC (12.5:84.5)	380[c]	1.3×10^9	10/25	1.9×10^{-11}	1.0	1.3	6.8×10^{10}	9.1×10^{10}	74
PA/PC (22.5:74.5)	120	1.2×10^8	15/75	6.1×10^{-10}	1.0	8.4	1.4×10^{10}	2.9×10^{10}	48
PA/PC (62.5:34.5)	120	1.2×10^8	15/38	3.1×10^{-10}	1.0	4.1	1.4×10^{10}	2.9×10^{10}	47
PA/PE/PC (12.5:20:64.5)	115	1.1×10^8	20/50	4.6×10^{-10}	2.0	5.6	1.2×10^{10}	2.7×10^{10}	45
PA/PE/PC (10:40:57)	150	1.9×10^8	10/25	1.3×10^{-10}	1.0	3.2	2.4×10^{10}	3.5×10^{10}	69
PA/PE/PC (12.5:50:34.5)	140	1.7×10^8	10/25	1.5×10^{-10}	0.5	3.6	2.5×10^{10}	3.4×10^{10}	73

[a] All the vesicles also contained 3% dansyl-PE. [b,c] These were prepared by sonication and ether injection, respectively. All the other vesicles were prepared by freeze−thaw and extrusion. The values for the vesicle sizes are the average of at least three measurements with a standard deviation (not shown) of less than 5%, except for the SUVs which gave a standard deviation of ±30%. [d] The molecular weights for the vesicles were estimated as described in Materials and Methods. [e] P/V represents protein to vesicle ratio. [f,g] k_{2exp} and k_{coll} represent the experimentally determined and theoretically calculated binding rate constants, respectively.

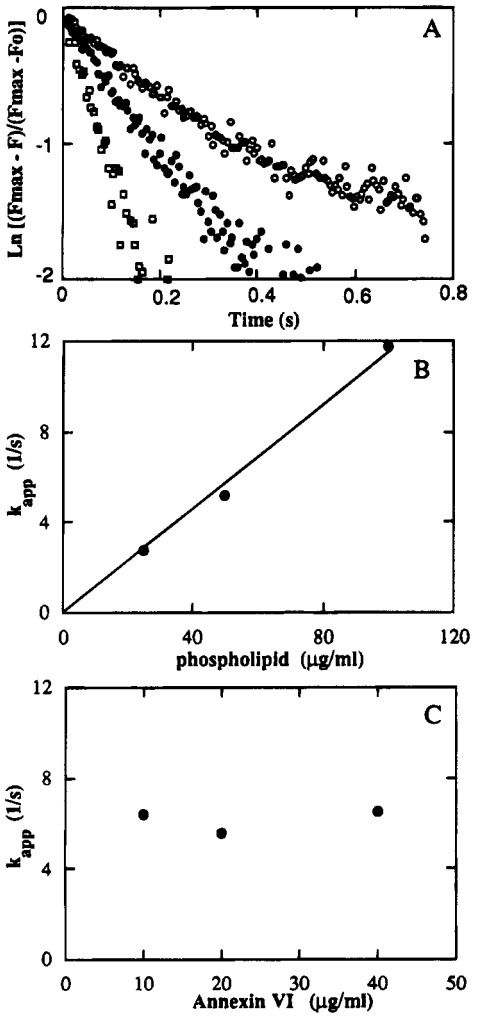

FIGURE 3: Dependence of association rate constants on protein and vesicle concentration. (Panel A) The binding reactions were measured at fixed protein concentration (20 μg/mL after mixing) and varied vesicle (PA/dansyl-PE/PE/PC, 12.5:3:20:64.5) concentrations. Fluorescence energy transfer was used to detect the protein−membrane association. (Panel B) The pseudo-first-order rate constants shown were obtained from the reactions shown in panel A. (Panel C) The pseudo-first-order rate constants are shown for reactions with constant vesicle concentration (50 μg/mL after mixing, composition as in panel A) and varied protein concentrations. In all cases, the calcium concentration was 2 mM after mixing.

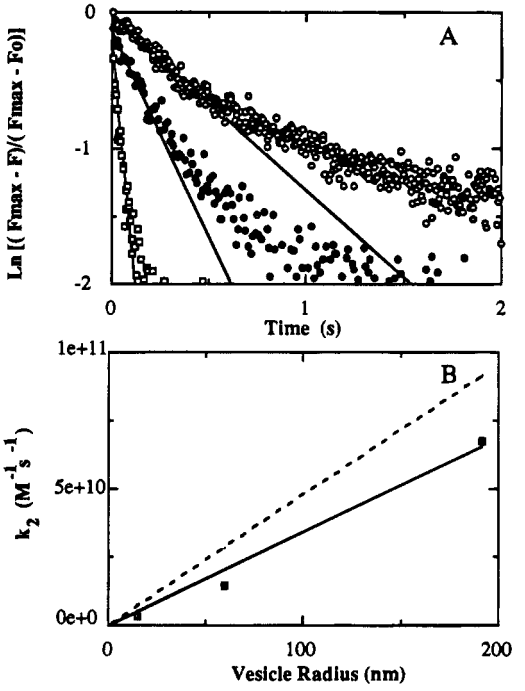

FIGURE 4: Dependence of association rate constants on vesicle radius. (Panel A) The binding reactions were measured with annexin VI (10 μg/mL after mixing) and vesicles (25 μg/mL after mixing, PA/dansyl-PE/PC, 12.5:3:84.5) of different diameters (□, 31 nm; ●, 120 nm, and ○, 380 nm). The pseudo-first-order rate constants were obtained from the solid lines drawn. The calcium concentration was 2 mM after mixing. (Panel B) Second-order rate constants, obtained from the pseudo-first-order rate constants in panel A and eq 3, are shown. The behavior for a collisional-limited reaction is shown by the dashed lines.

Other variables were tested and gave results that were consistent with the properties of a reaction that was near the collision limit. For example, at 0.2 mM calcium, k_{app} was highly dependent on vesicle size (not shown). Decreasing the protein/vesicle ratio also lowered the calcium required to reach maximum velocity (results not shown). Thus, despite some reduction in capture efficiency, the rate of binding always displayed evidence of some limitation by the rate of collision.

EGTA-Induced Protein Dissociation. In the presence of calcium ions, annexin VI binds to phospholipid membranes with very high affinity and displays a very slow dissociation rate (Bazzi & Nelsestuen, 1991a). However, the complex was able to rapidly adjust to variations in calcium level (Bazzi & Nelsestuen, 1991a). It was suggested that the EGTA-induced dissociation of prothrombin from membranes was a function of the exchange rate of individual calcium ions from the complex (Wei et al., 1982). Rapid release of

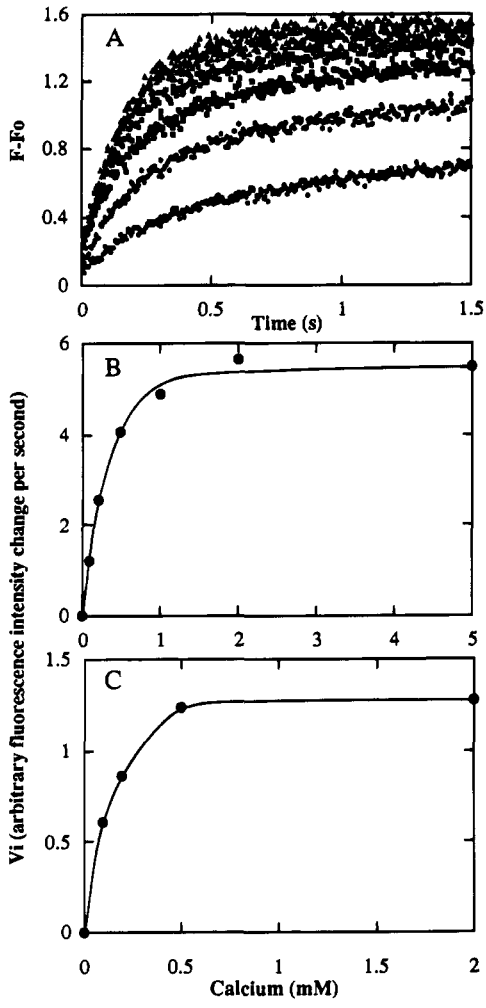

FIGURE 5: Effect of calcium concentration on annexin VI–membrane association rates. (Panel A) The binding reactions for annexin VI (20 μg/mL after mixing) and LUVs (50 μg/mL after mixing) of different composition were measured at different calcium concentrations by fluorescence energy transfer. (Panel A) The phospholipid composition was PA/dansyl-PE/PC, 12.5:3:84.5. The data shown, beginning with the lower-most curve, were obtained at 0.1, 0.25, 0.5, 1.0, 2.0, and 5.0 mM calcium, respectively. The data at higher calcium concentrations became indistinguishable. (Panel B) The initial slopes of the reactions shown in panel A are plotted as a function of calcium concentration. (Panel C) Initial slopes of binding reactions with annexin VI (10 μg/mL after mixing) and LUVs (25 μg/mL after mixing, PA/dansyl-PE/PE/PC, 12.5:3:50:34.5) were obtained as outlined in panels A and B. These are plotted versus calcium concentration.

annexin from the membrane upon lowering the calcium concentration may depend on a similar mechanism. Factors such as partial replacement of PC by PE in the membrane, the presence of a higher percentage of acidic phospholipids in vesicles or lower protein/vesicle ratios all reduce the calcium required to support protein–membrane binding (Bazzi & Nelsestuen, 1991a; see below). These should slow the rate of calcium dissociation from the complex and the related phenomenon, EGTA-induced protein dissociation.

Figure 6A shows the kinetics of EGTA-induced dissociation of annexin VI from LUVs. The presence of PE decreased the EGTA-induced dissociation rate (Figure 6). EGTA-induced dissociation of annexin V from membranes of different PE content showed a similar trend (results not shown).

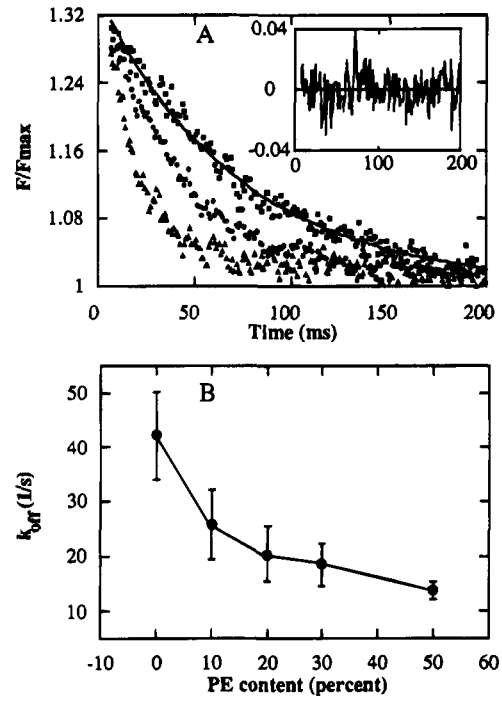

FIGURE 6: EGTA-induced dissociation of annexin VI from LUVs. (Panel A) A solution of annexin VI (20 μg/mL), Ca^{2+} (1 mM), and vesicles (50 μg/mL) was mixed with an EGTA solution (5 mM) at zero time, and the fluorescence signal was monitored. From left to right, the results were from the following vesicles containing PA/dansyl-PE/PC/PE at ratios of 12.5:3:84.5:0, 12.5:3:64.5:20, and 12.5:3:34.5:50, respectively. These curves were fit to eq 5. The solid line shows the fit of the uppermost curve, and the inset shows residuals between the experimental data and the fitted curve. (Panel B) The first-order dissociation rate constants from experiments such as those in panel A are plotted as a function of PE composition of the membranes. The concentration of PC was varied to accommodate the PE composition. The results represent the averages and standard deviations from at least three independent measurements.

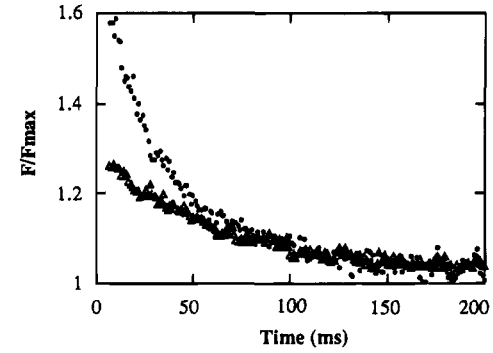

FIGURE 7: Impact of protein density on EGTA-induced dissociation of annexin VI from LUVs. A solution of annexin VI, Ca^{2+} (0.2 mM), and vesicles (PA/dansyl-PE/PC, 42.5:3:44.5, 50 μg/mL) was mixed with an EGTA solution (5 mM), and the fluorescence change was monitored. The results show experiments for annexin concentrations of 20 μg/mL (△) and 40 μg/mL (○).

The calcium required for protein–membrane association increased as a function of the protein/vesicle ratio (Bazzi & Nelsestuen, 1991a). This parameter also influenced EGTA-induced protein dissociation (Figure 7). Doubling the protein/vesicle ratio nearly doubled the protein dissociation rate constant, from about 14.0 s^{-1} for a ratio of 2:5 to 25.1 s^{-1} for a ratio of 4:5 (Figure 7). Once again, it appeared that different calcium dissociation rate behavior was largely responsible for different equilibrium behavior.

Table 2: Effects of Acidic Phospholipid Content and Protein Type on the Calcium Required for Annexin−Membrane Association and EGTA-Induced Protein Dissociation

vesicle[a] composition	protein type	$[Ca]_{1/2}$ (μM)	k_{off} (1/s)
PA/dansyl-PE/PC (12.5:3:84.5)	annexin VI	128[b]	45
PA/dansyl-PE/PC (22.5:3:74.5)	annexin VI	27	24
PA/dansyl-PE/PC (22.5:3:74.5)	annexin V	140	73

[a] The vesicles were LUVs prepared by freeze−thaw and extrusion. [b] All values are the average of two independent measurements.

As expected, EGTA-induced protein dissociation was slower from membranes with higher percentages of acidic phospholipids (Table 2). Dissociation of annexin VI was slower than that of annexin V from the same membrane (Table 2). This latter property was consistent with the fact that annexin VI−membrane binding required lower calcium concentration than annexin V (Bazzi & Nelsestuen, 1991b).

That EGTA was not directly involved in the rate-determining step was shown by control experiments which varied EGTA or calcium concentrations. The dissociation was independent of EGTA concentration over a 40-fold range (from 0.5−20 mM) as long as it was kept in large excess over calcium. A 5-fold variation of calcium concentration (from 0.2 to 1 mM) before EGTA addition (with LUVs consisted of PA/dansyl-PE/PE/PC, 12.5:3:50:34.5) did not influence the dissociation kinetics to a detectable extent, either.

Correlation between the Calcium Required for Protein−Membrane Association and EGTA-Induced Protein Dissociation. The calcium required to support protein−membrane interaction is usually characterized by the calcium concentration for half-maximal binding ($[Ca^{2+}]_{1/2}$) at a constant protein/membrane ratio. This value should be related to some aspect of equilibrium calcium binding. Actually, it has been previously used to describe affinities of annexins for calcium (Jost et al., 1994). In agreement with previous studies (Andree et al., 1990; Bazzi & Nelsestuen, 1991b; Bazzi et al., 1992; Evans & Nelsestuen, 1994; Tait & Gibson, 1992), this parameter was found to be highly dependent on the membrane composition and the type of protein used (Table 2). Increasing either the PE content or the percentage of negatively charged phospholipid, or both, decreased the calcium required for binding.

The values of $[Ca^{2+}]_{1/2}$ were determined for membranes of various PE contents by the manner illustrated in Figure 1. These values were compared with the rate constants for EGTA-induced protein dissociation. The results showed a nearly linear relationship between these two parameters (Figure 8). Thus, the rate of EGTA-induced protein dissociation from the membrane appeared to be the major contributor to the different overall equilibrium calcium-binding properties of various annexin−membrane complexes.

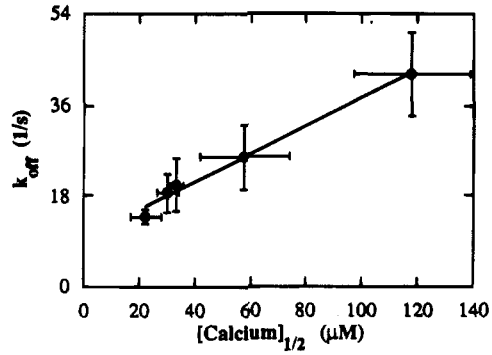

FIGURE 8: Correlation between the calcium required for annexin−membrane binding ($[Ca^{2+}]_{1/2}$) and the rate constants for EGTA-induced protein dissociation. All vesicles contained PA as the acidic phospholipid plus 3% dansyl-PE and varied levels of PE and PC to produce the range of calcium requirement shown. Rate constants were obtained as described in Figure 6A, while $[Ca^{2+}]_{1/2}$ values were determined by calcium titration in the manner illustrated in Figure 1. The data represent the averages of at least three independent determinations, and the error bars show the standard deviations for the results.

DISCUSSION

Annexins are widespread in nature and occur in mammals as well as molds and plants. Most annexins are abundant intracellular proteins and can comprise more than 2% of the total cell protein (Raynal & Pollard, 1994). The concentration of annexins (M_r = 32 000) could be as high as 100 μM in these cells and even higher at some locations. One annexin molecule can bind 10 calcium ions (Evans & Nelsestuen, 1994) for a total calcium-binding capacity of as much as 1 mM. This suggests that annexins could serve to buffer intracellular calcium levels in addition to other functions. The dynamics of annexin−calcium−membrane association may have important implications for calcium regulation.

This study found that, at saturating levels of calcium and relatively low protein/vesicle ratios, the rate of binding of annexin VI to membranes was essentially equal to the collisional limit. In agreement with this property, the association rate was sensitive to the radius of the vesicle (compare results with SUVs and LUVs, Table 1) and the protein/phospholipid ratio (Figure 3). This behavior applied to membranes of widely varied composition (Table 1). Binding of annexin V to planar membranes may also be limited by diffusion of the protein (Andree et al., 1990). Thus, at these calcium levels, the membrane surface may contain adequate numbers of protein binding sites to create an interaction that is limited only by protein proximity.

Efficient capture may apply to nearly all conditions inside the cell. While capture efficiency was reduced at lower calcium concentrations (Figure 5), it is a function of membrane particle size with larger particles having greater capture potential (Abbott & Nelsestuen, 1988). This study utilized membranes of 120 nm. Larger surfaces such as the cell membrane may provide highly efficient capture of annexins, even at the calcium concentrations found in the cell.

At low calcium concentrations, the relationship between association rate and calcium concentration may suggest properties of the annexin−membrane interaction. For example, the association rate increased in a linear or hyperbolic fashion with respect to calcium concentration. There was little evidence of cooperativity. This result would be consistent with a rate-limiting step consisting of simultaneous interaction of annexin VI, phospholipid, and a small number, perhaps even one, calcium ion. This would be followed by recruitment of other calcium ions to reach the final stoichiometry of 8 to 15 (Evans & Nelsestuen, 1994). The starting point for calcium binding involved very few ions since the free protein bound very little calcium at concentrations adequate to support protein−membrane interaction. Calcium identified as "membrane-associated" (either bound or se-

questered by electrostatic attraction) by equilibrium binding measurements was also very low (Plager & Nelsestuen, 1994).

Many properties of the overall interaction suggested that, under many conditions, dissociation rate behavior is the dominant contribution to the different equilibrium behaviors of various membranes. For example, the high cooperativity and high affinity of equilibrium binding may depend on dissociation rate constants. Protein dissociation from the membrane was very slow at constant calcium concentration (Bazzi & Nelsestuen, 1991a), a property that appeared to be responsible for the very high affinity of annexins for membranes. Reports of dissociation constants have varied from 10^{-9} M to 10^{-11} M for annexin V (Andree et al., 1990; Tait & Gibson, 1992) and are suggested to be less than 10^{-14} for annexin VI (Bazzi & Nelsestuen, 1991a). Such high affinity could be created by a large number of calcium-contact sites between the protein and the membrane.

Dissociation rates also appeared to play a dominant role in determining the calcium required to support protein–membrane interaction. For example, there was a nearly linear relationship between apparent calcium-binding affinity, $[Ca^{2+}]_{1/2}$, and the protein dissociation rate constant obtained upon EGTA addition (Figure 8). The latter was probably closely related to the rate of calcium exchange from the protein–membrane complex. That this rate was affected by membrane composition, annexin type, and protein/vesicle ratio suggested that these factors all impacted on calcium affinity. Despite rapid exchange and transient release of individual calcium ions (Bazzi & Nelsestuen, 1991a) and phospholipids (Meers & Mealy, 1993a) from the complex, annexins would remain bound to the membrane through the large number of remaining contact points. However, calcium exchange becomes irreversible when excess EGTA is added to the medium, and protein dissociates at the rate at which calcium is lost.

The complex nature of these protein–membrane interactions will impact on detailed interpretation of the results. For example, while the binding curve from which $[Ca^{2+}]_{1/2}$ was obtained (e.g., Figure 1) can be fit to simple relationships such as the Hill equation, the binding process actually consists of more complex events such as a series of equilibria, one for each protein–membrane binding event (Bazzi & Nelsestuen, 1991a). The midpoint of the calcium titration was therefore the value for the particular protein–membrane binding event that occurred at half saturation.

Complex behavior was also implied by the EGTA-induced dissociation kinetics. Although the data fit a simple first-order dissociation process, the rate constant varied with protein density on the membrane (Figure 7). This "negative cooperativity" could arise in several ways. First of all, dissociation may be truly first order and involve a homogeneous population of membrane-bound proteins. This explanation would require that the calcium-binding affinity of all sites would decrease as protein density on the membrane increased. Alternatively, proteins bound to the membrane may have heterogeneous affinities, and the EGTA-induced protein dissociation from the membrane may consist of a series of nonidentical steps, the sum of which fit a first-order expression. Another possibility would be a combination of these two mechanisms where membrane-bound protein represented a homogeneous population but where individual binding interactions underwent constant change as other protein molecules dissociated from the membrane. This would produce heterogeneous dissociation events from an initial population of homogeneous interactions. The models are compatible with a highly sequential protein–membrane binding behavior (Bazzi & Nelsestuen, 1991a) and with a great variation in enthalpy and entropy of protein binding at different protein densities (Plager & Nelsestuen, 1994).

The complex behaviors outlined above could arise in various ways. Two factors that contribute to calcium interaction with membranes are intrinsic binding properties and electrostatic attraction of calcium to the polyvalent membrane surface. The electrostatic forces can concentrate calcium near the surface, thereby enhancing protein–membrane–calcium interaction. Sequential interaction of proteins with the surface may lower charge density, thereby altering the electrostatic attraction and changing the calcium required for subsequent protein-binding events. This could produce an apparent negative cooperativity with respect to protein density on the membrane surface, a phenomenon that is keenly observed (Bazzi & Nelsestuen, 1991a). The effect of PE on the membrane may be partially due to these electrostatic properties. Early studies showed that PE membranes may contain a charge density comparable to membranes with 10% anionic lipid (Papahadjopoulos, 1968). Partial ionization of PE may enhance electrostatic attraction of calcium to the membrane surface. However, it seems unlikely that this mechanism would account for all observed results. For example, inclusion of 20% PE in membranes of 12.5% PA reduced the dissociation rate by about one-half (Figure 6B). This was similar to the impact of nearly doubling the PA content from 12.5% to 22.5% (Table 2). If based entirely on electrostatic parameters, the PE would have to be about half ionized. In addition, histone and cytochrome *c* protein that interact with membranes electrostatically failed to associate with membrane containing PE but no anionic phospholipids.[2] Thus, it appeared that the PE effect may stem from a combination of several factors that contribute to enhance the ability of membranes to interact with proteins.

The descriptions outlined above suggest protein–phospholipid interaction via simultaneous chelation of a large number of calcium ions. However, involvement of other types of forces in the protein–membrane contact is possible, provided that they dissipate concomitant with calcium dissociation. For example, conformational changes of annexin V have been found at high calcium concentration (Concha et al., 1993; Meers & Mealy, 1993b). Direct interaction between the tryptophan residue of annexin V and membrane has also been found (Concha et al., 1993; Meers, 1990). This kind of interaction may largely be hydrophobic and could stabilize the protein on membrane surface (Concha et al., 1993; Meers & Mealy, 1993b). Such interaction would not appear sufficient to support annexin binding to the membrane. For example, a calcium level which was high enough to induce the conformational change was not sufficient to induce the protein to bind to pure PC membranes (Meers & Mealy, 1993b). Thus, it appeared that other forces involved in annexin–membrane binding were not sufficient to support protein–membrane binding after calcium dissociation.

[2] M.D.B. and G.L.N., unpublished data.

Overall, a general model of annexin interaction with membrane via a large number of calcium ions appears an attractive explanation for the observed equilibrium and dynamic binding properties. The current study shows that this system would provide rapid response as calcium levels are either raised or lowered in the cell. It may also produce changes in membrane organization (Bazzi & Nelsestuen, 1991c). The extremely large calcium stoichiometry could provide a mechanism for calcium buffering without changing the amount of protein bound to the membrane. For example, the sequential binding process described earlier (Bazzi & Nelsestuen, 1991a) required that the cooperativity coefficient for calcium be high. Actual stoichiometries of up to 15 calcium ions (Evans & Nelsestuen, 1994) may exceed the actual cooperativity coefficient and allow binding of a variable number of calcium ions (e.g., 8−15) per protein, without changing the amount of protein bound to the membrane. In any event, these proteins may absorb a considerable portion of the total calcium that is mobilized in response to cell signaling events, and this may constitute a facet of calcium regulation.

REFERENCES

Abbott, A. J., & Nelsestuen, G. L. (1988) *FASEB J. 2,* 2858.

Andree, H. A., Reutelingsperger, C. P., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265,* 4923.

Barton, G. J., Newman, R. H., Freemont, P. S., & Crumpton, M. J. (1991) *Eur. J. Biochem. 198,* 749.

Bazzi, M. D., & Nelsestuen, G. L. (1991a) *Biochemistry 30,* 7970.

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

Bazzi, M. D., & Nelsestuen, G. L. (1991c) *Biochemistry 30,* 7961.

Bazzi, M. D., Youakim, A., & Nelsestuen, G. L. (1992) *Biochemistry 31,* 1125.

Bloomfield, V. A., & Lim, T. K. (1978) *Methods Enzymol. 48,* 415.

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

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

Concha, N. O., Head, J. F., Kaetzel, M. A., Dedman, J. R., & Seaton, B. A. (1993) *Science 261,* 1321.

Creutz, C. E. (1992) *Science 258,* 924.

Deamer, D., & Bangham, A. D. (1976) *Biochim. Biophys. Acta 443,* 629.

Edwards, H. C., & Crumpton, M. J. (1991) *Eur. J. Biochem. 198,* 121.

Evans, T. C., & Nelsestuen, G. L. (1994) *Biochemistry 33,* 13231.

Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature 320,* 636.

Goldberg, M., Feinberg, J., Rainteau, D., Lecolle, S., Kaetzel, M. A., Dedman, J. R., & Weinman, S. (1990) *J. Biol. Buccale 18,* 289.

Harafuji, H., & Ogawa, Y. (1980) *J. Biochem. (Tokyo) 87,* 1305.

Hazarika, P., Sheldon, A., Kaetzel, M. A., Diaz, M. M., Hamilton, S. L., & Dedman, J. R. (1991) *J. Cell Biochem. 46,* 86.

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

Hosoya, H., Kobayashi, R., Tsukita, S., & Matsumura, F. (1992) *Cell Motil. Cytoskel. 22,* 200.

Huang, C., & Mason, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 308.

Jost, M., Weber, K., & Gerke, V. (1994) *Biochem. J. 298,* 553.

Lin, H. C., Sudhof, T. C., & Anderson, R. G. W. (1992) *Cell 70,* 283.

Meers, P. (1990) *Biochemistry 29,* 3325.

Meers, P., & Mealy, T. (1993a) *Biochemistry 32,* 11711.

Meers, P., & Mealy, T. (1993b) *Biochemistry 32,* 5411.

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

Papahadjopoulos, D. (1968) *Biochim. Biophys. Acta 163,* 240.

Plager, D. A., & Nelsestuen, G. L. (1994) *Biochemistry 33,* 13239.

Raynal, P., & Pollard, H. B. (1994) *Biochim. Biophys. Acta 1197,* 63.

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

Smoluchowski, M. (1917) *Z. Phys. Chem. 92,* 129.

Stankowski, S. (1983) *Biochim. Biophys. Acta 777,* 167.

Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58,* 951.

Tait, J. F., & Gibson, D. (1992) *Arch. Biochem. Biophys. 298,* 187.

Wei, G. J., Bloomfield, V. A., Resnick, R. M., & Nelsestuen, G. L. (1982) *Biochemistry 21,* 1949.

Zubay, G. (1988) in *Biochemistry* (2nd ed.) p 106, Collier Macmillan Canada, Inc., New York.

BI9507084