Endonexin (Annexin IV)-Mediated Lateral Segregation of Phosphatidylglycerol in Phosphatidylglycerol/Phosphatidylcholine Membranes†

Matthew Junker and Carl E. Creutz*

Program in Biophysics and Department of Pharmacology, University of Virginia, Box 448 Jordan Hall, Charlottesville, Virginia 22908

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ABSTRACT: Endonexin (annexin IV) is a member of the annexin family of homologous proteins that share the ability to bind to pure lipid membranes and to aggregate vesicles in a Ca^{2+}-dependent fashion. Endonexin appears to preferentially interact with certain types of lipids such as phosphatidylglycerol (PG) in PG/phosphatidylcholine (PC) mixed lipid membranes. Such preferential binding should result in localization of PG lipids to membrane regions where endonexin is bound. This was tested using a PG derivative containing the fluorophore pyrene, which exhibits fluorescence sensitive to molecular collision frequency. Motional restriction of pyrene-PG upon endonexin–membrane binding was evident from decreased ratios of excimer-to-monomer (E/M) pyrene fluorescence with endonexin binding to 97% PG/3% pyrene-PG vesicles. A maximum decrease of 30% suggests a 30% decrease in the average diffusion constant of pyrene-PG molecules or a 53% decrease assuming that only outer-monolayer lipid molecules interact with endonexin. In vesicles containing 5% and 10% pyrene-PG in PC, segregation of lipids was evident from observed increases in E/M of 14.2 ± 1.8% and 6.8 ± 0.1%, respectively, in the presence of endonexin and either 10 mM (5% pyrene-PG) or 2 mM (10% pyrene-PG) free Ca^{2+}. At higher concentrations of Ca^{2+} (>10 mM for 5% pyrene-PG and >2 mM for 10% pyrene-PG), smaller endonexin-dependent increases in E/M are observed as endonexin molecules at high surface densities compete for the limited pool of pyrene-PG. The nature of these interactions of endonexin with mixed lipid systems has implications for the way annexins may modulate membrane structure in cells.

Endonexin (annexin IV) is a member of the annexin family of homologous Ca^{2+}-dependent membrane-binding and vesicle-aggregating proteins. These proteins may be involved in regulating or otherwise mediating biological processes involving membrane–membrane and protein–membrane interactions (Geisow & Walker, 1986; Klee, 1988; Creutz, 1992). Annexins exhibit Ca^{2+}-dependent binding to pure lipid membranes, suggesting that they may interact with the lipid component of biological membranes *in vivo*. *In vitro*, annexins typically bind with poor affinity to pure PC[1] membranes but with greater affinity (at lower Ca^{2+} concentrations) to PC membranes containing negatively charged phospholipids such as PS (Schlaepfer & Haigler, 1987; Schlaepfer et al., 1987; Shadle & Weber, 1987; Andree et al., 1990; Junker & Creutz, 1993). Such enhanced binding may result from preferential interactions between annexins and negatively charged phospholipids.

This report addresses the interactions between annexins and negatively charged phospholipids by examining the effects of endonexin on the dynamics of PG molecules in pure PG and in PG/PC mixed lipid membranes. Lipid dynamics was monitored using a derivative of PG (pyrene-PG) that contained a pyrene fluorophore incorporated into one acyl chain. Pyrene exhibits excimer fluorescence in which collision of pyrene monomers results in a unique fluorescence emission. The excimer fluorescence of pyrene-PG in membranes is thus sensitive to pyrene-PG lateral mobility and concentration. Decreases in lateral mobility resulting from binding to larger macromolecules such as proteins should result in decreased excimer fluorescence. Increases in local pyrene-PG concentration that might result from preferential localization of pyrene-PG molecules to regions where protein molecules are found should result in increased excimer fluorescence. By choosing appropriate conditions, we observed both decreased pyrene-PG lateral mobility and increased pyrene-PG segregation with endonexin–membrane binding.

METHODS

Reagent Preparation and Membrane-Binding Assay. Endonexin was purified from bovine liver and quantified as previously described (Creutz et al., 1987; Junker & Creutz, 1993). Large unilamellar vesicles (1000-Å diameter) were prepared as previously described (Junker & Creutz, 1993) according to the method of extrusion (Mayer et al., 1987). Some preliminary experiments were performed with sonicated vesicles. Phospholipid concentrations were determined by phosphate assays modified from Bartlett (1959). Binding of endonexin to membranes was monitored using fluorescence energy transfer between endonexin and dansyl-PE incorporated in lipid vesicles at 1 mol % as previously described (Bazzi & Nelsestuen, 1987; Junker & Creutz, 1993).

Measurement of Lipid Dynamics in Vesicles by Pyrene Fluorescence. Changes in the dynamics of lipid molecules in PG/PC vesicles were detected using the fluorescence of a PG derivative containing pyrene in one acyl chain (1-palmitoyl-2-(6-pyrenylhexanoyl)-3-phosphatidyl-rac′-glycerol, KSV

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* Corresponding author.

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[1] Abbreviations: dansyl-PE, *N*-(5-(dimethylamino)naphthalene-1-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LUV, large unilamellar vesicle; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazol; PC, phosphatidylcholine; PG, phosphatidylglycerol; pyrene-PG, 1-palmitoyl-2-(6-pyrenylhexanoyl)-3-phosphatidyl-rac′-glycerol; pyrene-PC, 1-palmitoyl-2-[6-pyrenylhexanoyl]-3-phosphatidylcholine; PS, phosphatidylserine.

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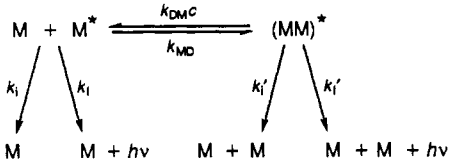
Chemical Corporation). Pyrene exhibits excimer fluorescence, in which collision of an excited-state and a ground-state monomer pyrene molecule results in a unique fluorescence emission with a maximum at a greater wavelength. A scheme for pyrene excimer fluorescence has been devised as follows (Birks et al., 1963; Galla & Sackmann, 1974): The ratio of



excimer-to-monomer fluorescence intensities (E/M) is related to the kinetic parameters by

$$E/M = k_{DM}c\{k_f'/[k_f(k_f' + k_i' + k_{MD})]\}$$

where K_{DM} is the second-order rate constant for collision of pyrene molecules, c is the concentration of pyrene, and $(k_f' + k_i' + k_{MD})^{-1}$ is the intrinsic lifetime of the excimer. When attached to an acyl chain of PG molecules, changes in pyrene E/M will reflect changes in the mobility (k_{DM}) and local concentratiton (c) of the PG-derivatized lipid (provided that the intrinsic excimer and monomer lifetimes are constant).

Measurement of E/M. Fluorescence measurements were performed on a SPEX Fluorolog 2 single-beam spectrofluorometer with excitation at 346 nm and emission typically scanned between 400 and 490 nm. Emission intensities were normalized to simultaneously monitored lamp intensity. Bandpasses of 1.8 nm for excitation and 4.6 nm for emission were typically used. The total time for each scan was approximately 20 s, with intensities measured for 0.4 s, at every 2 nm. Monomer and excimer intensities were obtained by integrating the respective emission peaks over wavelengths where there was no apparent overlap. Usually monomer fluorescence was integrated between 394 and 402 nm, while excimer emission was integrated between 474 and 482 or between 470 and 490 nm. Calculating true values of E/M requires determining the quantum yields of excimer and monomer fluorescence by integrating over their entire peaks. This was not possible because of some peak overlap. However, the measurements of monomer and excimer emissions measured here should be proportional to the respective total emissions so that calculated percent changes in E/M, $[(E/M)_{final} - (E/M)_{initial}]/(E/M)_{initial} \times 100\%$, are accurate since the proportionalities cancel out. The percent change in E/M was sufficient for analyzing the changes in pyrene-PG dynamics with endonexin and Ca^{2+} binding.

Samples were prepared as 300-μL total volume in 25 mM MOPS, 100 mM NaCl, and 50 μM EGTA at pH 7.0. The MOPS buffer was bubbled with argon for at least 1 h before being used to reduce monomer quanching by oxygen (Birks et al., 1963). Vesicles were used at 4 μM total lipid. Samples were typically mixed between additions and between repeated scans, and a separate sample was prepared for each series of additions of endonexin and Ca^{2+} to vesicles.

Scans were taken after consecutive additions of vesicles, endonexin, and Ca^{2+} to buffer. In some cases, scans were also performed after a final addition of excess EGTA with NaOH to maintain pH. Background intensities due to the vesicles themselves, evaluated using vesicles containing no pyrene-PG, fell well within experimental error and were not corrected for in calculating the E/M of pyrene-PG containing vesicles.

Excimer and monomer intensities of pyrene-PG in vesicles in the absence of endonexin and Ca^{2+} or in the presence of endonexin and EGTA exhibited decreases of 1–2% between scans, perhaps due to a slow process such as vesicle association with the cuvette walls or due to introduction of small amounts of oxygen with sample mixing. However, the net change in E/M between scans in these cases was no more than 1%. Initial changes in E/M after addition of Ca^{2+} to endonexin and vesicles or after addition of EGTA to endonexin, vesicles, and Ca^{2+} were often followed by decreases in E/M of 1–3% between scans, usually with both excimer and monomer intensities decreasing simultaneously. Only the initial E/M measurements were then used, since endonexin–membrane binding is known to be a rapid event (Junker & Creutz, 1993). In no cases did this variability affect interpretation of the results.

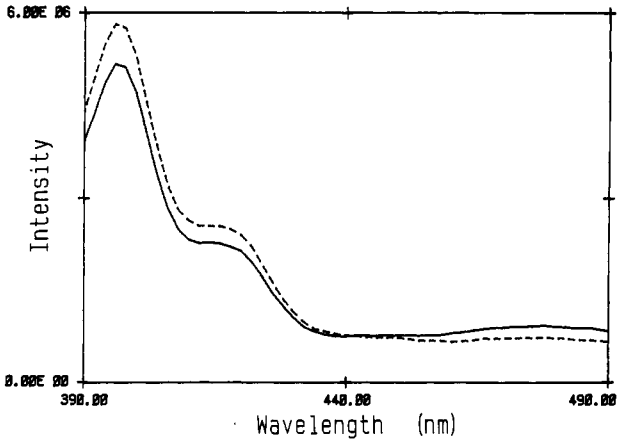


FIGURE 1: Emission spectra of PG/3% pyrene-PG vesicles (4 μM lipid) before (—) and after - - -) addition of 1 mM Ca^{2+} in the presence of 92 nM endonexin.

RESULTS

Mixing Properties of Pyrene-PG in PG/PC Membranes As Detected by E/M. The pyrene-PG probe appeared to exhibit some nonideal mixing in PG/PC membranes even in the absence of Ca^{2+}. Increasing the mol % pyrene-PG in vesicles generally resulted in slightly greater than proportional increases in E/M, suggesting phase segregation of pyrene-PG at high mol %. Vesicles containing 10% pyrene-PG/PC typically exhibited E/M values 15% greater than two times the E/M of vesicles containing 5% pyrene-PG. Changes in E/M observed when increasing the mol % PG in PC vesicles containing a constant mol % pyrene-PG were small and variable among vesicle preparations, suggesting that PG did not greatly perturb pyrene-PG mixing. Such deviations in E/M do not affect the conclusions of this study, since changes in E/M and not absolute values of E/M were analyzed.

Endonexin Decreases Pyrene-PG E/M in Pure PG Vesicles. Binding of endonexin to pure PG vesicles containing 3% pyrene-PG resulted in decreased pyrene E/M (decreased excimer, increased monomer emissions; Figure 1). As seen in Figure 2, increasing the total endonexin concentration at 1 mM Ca^{2+} caused larger decreases in E/M that saturated near 73 nM endonexin with a maximum decrease of 30% (1 mM Ca^{2+} alone caused a decrease of 7%). Protein and Ca^{2+} titrations of endonexin binding to PG-containing vesicles and PS-containing vesicles are very similar. Saturation of endonexin binding to PS vesicles at the same total lipid concentration as used here occurred with 62 nM total endonexin (Junker & Creutz, 1993). This suggests that the observed saturation of decreasing E/M at a similar 73 nM total endonexin reflected maximum binding of endonexin to the PG vesicles.

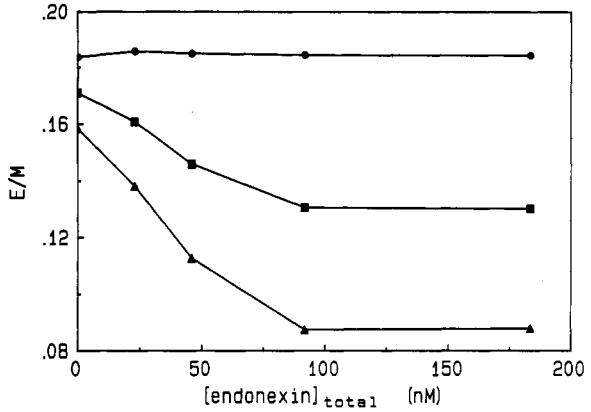


FIGURE 2: Apparent E/M of PG/3% pyrene-PG vesicles (4 μM lipid) at different concentrations of endonexin before (●) and after (■) addition of 1 mM Ca^{2+}. Also shown is calculated E/M, assuming that only outer-monolayer lipid molecules are affected by endonexin binding (▲; see text).

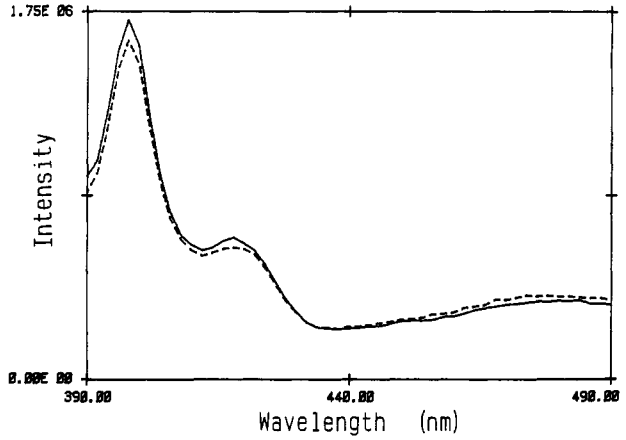


FIGURE 3: Emission spectra of 5% pyrene-PG vesicles (4 μM lipid) before (—) and after (- - -) addition of 10 mM Ca^{2+} in the presence of 92 nM endonexin.

The observed decrease in E/M is probably not due to direct interactions between endonexin proteins and pyrene fluorophores but rather due to the result of altered dynamics of excimer formation. Both decreases in excimer and increases in monomer intensities contributed to the changes in E/M: the 30% decrease in E/M observed with excess 180 nM endonexin occurred with a 20% decrease in excimer intensity and a 14% increase in monomer intensity. Also, endonexin probably does not insert into the hydrocarbon region of the bilayer to which the pyrene fluorophores are presumably constrained on PG acyl chains. The lipid head group specificity for endonexin–membrane binding and the rapid reversibility of binding upon removal of Ca^{2+} by EGTA suggest a primarily surface interaction (Meers, 1990; Junker & Creutz, 1993). In addition, studies of annexin intrinsic fluorescence quenching by membrane–lipid probes indicate that annexin proteins such as endonexin do not penetrate into the bilayer when membrane-bound (Meers, 1990). Finally, binding of the related protein annexin V (endonexin II) to PS/5% pyrene-PC membranes did not appear to significantly affect the intrinsic lifetimes for pyrene emission in pyrene-PC (Meers et al., 1991).

The decreases in E/M indicate decreases in the collision frequency of pyrene-PG molecules. Since endonexin cannot change the local concentration of pyrene-PG molecules in vesicles where all lipids contain PG head groups, the decreased collision frequency must be due to decreases in the bimolecular rate constant k_{DM} for excimer formation. This suggests that binding of endonexin, and to a lesser extent Ca^{2+}, to membranes results in decreased pyrene-PG mobility. The decrease in E/M with added endonexin may reflect an increase in the proportion of surface area and of pyrene-PG molecules that are affected by endonexin binding.

The degree of immobilization of PG molecules can be estimated from the relative change in E/M, which can be described as

$$\frac{(E/M)_{final} - (E/M)_{initial}}{(E/M)_{initial}} = \frac{k_{DM,final}c_{final} - k_{DM,initial}c_{initial}}{k_{DM,initial}c_{initial}}$$

where $c_{initial}$ and c_{final} are the local concentrations of pyrene-PG before and after endonexin binding, respectively. Since no segregation of lipid types by head group can occur in these pure PG vesicles, $c_{initial}$ and c_{final} are identical, and the relative change in k_{DM} with binding of Ca^{2+} and endonexin is equal to the relative change in E/M, $(k_{DM,final} - k_{DM,initial})/k_{DM,initial}$. Endonexin binding to PG membranes in the presence of 1 mM Ca^{2+} thus appears to have caused a 30% decrease in the average k_{DM} of pyrene-PG molecules, whereas Ca^{2+} alone caused a decrease of 7%. The decrease in k_{DM} reflects an average decrease since some lipids may be motionally restricted more than others (see Discussion).

Endonexin Increases Pyrene-PG E/M in Pyrene-PG/PC Vesicles. Having observed a decrease in E/M with endonexin binding to PG/pyrene-PG vesicles consistent with PG and pyrene-PG immobilization, we attempted to detect endonexin-induced PG/pyrene-PG lateral segregation in vesicles that also contained PC. Unequivocal evidence of lateral segregation requires observing an increase in pyrene-PG E/M. However, immobilization that may occur simultaneously with pyrene-PG segregation can diminish increases in E/M from lateral segregation and even cause net decreases in E/M. To obviate this complication, conditions were sought to maximize detection of increases in E/M. First, low mol % totals of (pyrene and non-pyrene) PG in PG/PC vesicles were used. If PG segregation occurs, minimizing the mol % PG allows the greatest net increase in segregation of PG and the greatest net increase in pyrene-PG collision frequency. Second, high proportions of total PG that were pyrene-PG were used. This maximizes the sensitivity of E/M measurements, especially if few PG molecules are bound by each endonexin molecule or if bound PG molecules are distributed on the protein surface such that they do not make frequent collisions with each other.

Use of 92 nM endonexin caused net decreases in E/M of 3% pyrene-PG/PC vesicles containing 10%, 20%, 50%, and 97% PG. Decreases with 10% and 20% PG vesicles were smaller (7% and 10%, respectively) than decreases with 50% and 97% PG vesicles (27% and 30%, respectively). This could reflect reduced binding of endonexin to 10% and 20% PG vesicles and/or pyrene-PG segregation in 10% and 20% PG vesicles that in part compensates for the decreased pyrene-PG collision frequency resulting from immobilization. To definitively test for pyrene-PG segregation, experiments were pursued using vesicles containing only pyrene-PG and PC. As seen in Figures 3 and 4, endonexin did cause Ca^{2+}-dependent increases in E/M for 5%/95% and 10%/90% pyrene-PG/PC vesicles. Several independent measurements revealed increases of 14.2 ± 1.8% (six determinations) and 6.8 ± 0.1% (two determinations) for the 5% pyrene-PG and 10% pyrene-PG vesicles, respectively (Figure 4). Table I compares the Ca^{2+}-dependent changes in excimer and monomer intensities induced by endonexin with the variability observed between scans. For both 5% and 10% pyrene-PG vesicles, the decreases in monomer intensities upon addition of Ca^{2+} were consistently

Table I: Percent Changes in Monomer and Excimer Fluorescence Intensities between Scans of Pyrene-PG/PC Vesicles before and after Additions of Ca^{2+} and Endonexin

		% change in intensity between scans					
		before add Ca^{2+} [a]		add Ca^{2+} [b]		after add Ca^{2+} [c]	
mol % pyrene-PG[d]	[endonexin] (nM)	monomer	excimer	monomer	excimer	monomer	excimer
5	119	−1.48 ± 0.54	−2.31 ± 0.79	−4.29 ± 0.92	+0.56 ± 2.35	−1.71 ± 1.44	−2.60 ± 1.23
	0	−1.74 ± 0.51	−1.95 ± 0.78	−2.73 ± 0.58	−0.70 ± 1.52	−2.58 ± 0.18	−2.82 ± 0.85
10	119	−1.28 ± 0.47	−0.73 ± 0.50	−3.08 ± 0.65	3.36 ● 0.88	−1.21 ± 0.50	−2.93 ± 0.47
	0	−0.27 ± 0.05	−0.61 ± 0.41	−1.14 ± 0.15	−0.89 ● 0.67	−0.81 ± 0.24	−1.58 ± 0.04

[a] Before add Ca^{2+}: difference between two scans immediately before the addition of Ca^{2+}. [b] Add Ca^{2+}: difference between scan immediately before and scan immediately after the addition of Ca^{2+}. [c] After add Ca^{2+}: difference between two scans following the addition of Ca^{2+}. [d] 10 mM Ca^{2+} was added to endonexin/5% pyrene-PG mixtures, and 2 mM Ca^{2+} was added to endonexin/10% pyrene-PG mixtures. Data for 5% pyrene-PG vesicles reflect average of six determinations (five for after add Ca^{2+}) in the presence of endonexin and five determinations in the absence of endonexin (four for after add Ca^{2+}). Data for 10% pyrene-PG vesicles are average of two determinations. Percentages are relative to vesicles before addition of Ca^{2+}.

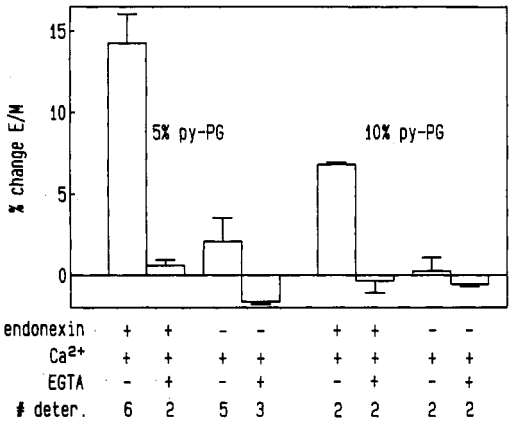


FIGURE 4: Percent changes in E/M upon additions of Ca^{2+} and then EGTA to 5% and 10% pyrene-PG/PC vesicles (4 μM lipid) in the presence and in the absence of 92 nM endonexin (for data in Table I). 10 mM Ca^{2+} followed by 20 mM EGTA was used for 5% pyrene-PG, and 2 mM Ca^{2+} followed by 5 mM EGTA was used for 10% pyrene-PG. NaOH was added with EGTA to maintain pH.

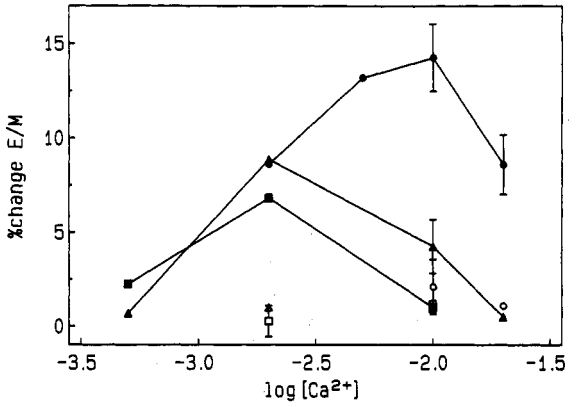


FIGURE 5: Percent change in E/M at different Ca^{2+} concentrations for endonexin binding to 5% pyrene-PG (●), 10% pyrene-PG (■), and sonicated 10% pyrene-PG (▲) vesicles. Also shown are respective data for vesicles in the absence of endonexin (○, □, △). Lipid concentration was 4 μM; endonexin concentration was 119 nM for the large vesicles and 92 nM for the sonicated vesicles.

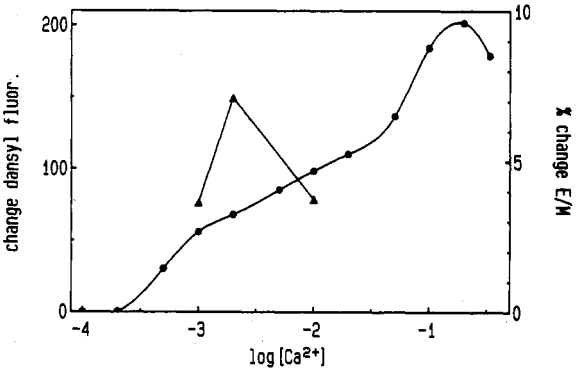


FIGURE 6: Ca^{2+} titrations of endonexin binding to 10% PG/1% dansyl-PE vesicles detected by energy transfer (●) and of the percent change in E/M by endonexin binding to 10% pyrene-PG vesicles (▲). 92 nM endonexin, 4 μM lipid.

greater than those observed between scans with no additions. Increases in excimer intensities were observed only when Ca^{2+} was added, as the excimer intensity otherwise decreased slightly between scans. The changes in E/M induced by endonexin and calcium appeared to be complete as soon as a measurement could be made after hand-mixing (10–30 s, including data collection). In all cases, the increase in E/M was greater than that seen with Ca^{2+} alone, and addition of EGTA appeared to completely reverse the increase in E/M, presumably by reversing endonexin–membrane binding (Figure 4).

Smaller Increases in E/M Seen with Very High Ca^{2+} Concentrations Result from Dilution of Pyrene-PG among Competing Endonexin Molecules. The increases in E/M induced by endonexin in 5% and 10% pyrene-PG/PC vesicles titrate with Ca^{2+} at low Ca^{2+} concentrations. However, at greater Ca^{2+} concentrations, these increases in E/M actually become smaller with increased Ca^{2+} (Figure 5). The diminished ability of endonexin to increase E/M at high Ca^{2+} could result from reduced endonexin binding, Ca^{2+} immobilization of lipids, or dilution of pyrene-PG molecules among excess endonexin molecules present at high surface concentrations. These three possibilities were investigated.

The possible contribution of reduced endonexin–membrane binding at high Ca^{2+} concentrations was tested by monitoring endonexin binding to 10% PG/1% dansyl-PE vesicles detected by fluorescence energy transfer between endonexin and dansyl-PE (Bazzi & Nelsestuen, 1987; Junker & Creutz, 1993). Ca^{2+} titrations of endonexin binding to 10% PG vesicles revealed that protein binding increased even above 2 mM Ca^{2+}, where the E/M of 10% pyrene-PG vesicles was observed to decrease (Figure 6). This suggests that the decrease in E/M observed at high Ca^{2+} concentrations was not due to reduced binding of endonexin, as would be the case, for example, if vesicle aggregation were reducing available surface area for endonexin binding.

As seen in Figure 5, Ca^{2+} alone caused little, if any, change in the E/M of 5% and 10% pyrene-PG vesicles in the absence of endonexin, suggesting that Ca^{2+} alone does not significantly immobilize pyrene-PG. However, lateral pyrene-PG segregation concomitant with immobilization could result in a coincident no net change in E/M. The small increases in E/M at 10 mM Ca^{2+} and above may, indeed, reflect some

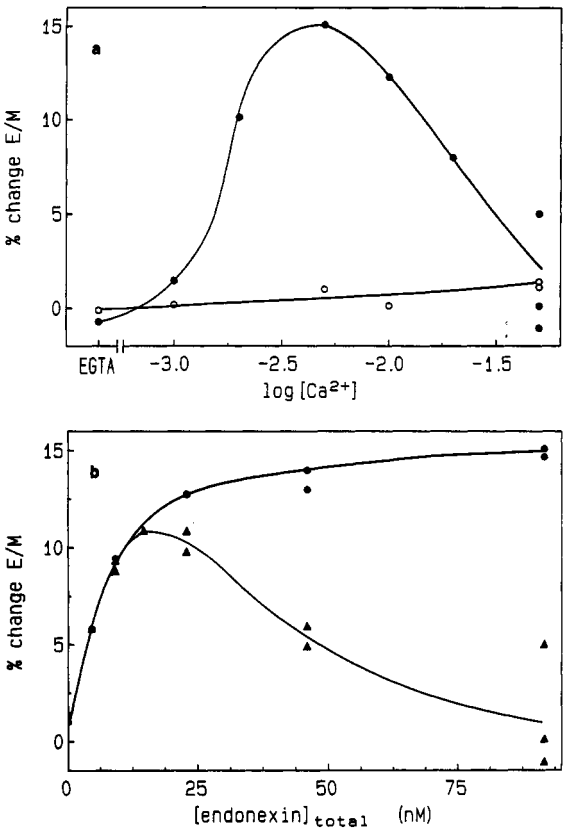


FIGURE 7: (a) Ca^{2+} titrations of percent change in E/M for 5% pyrene-PG/PC vesicles (4 μM lipid) in the presence (●) and in the absence (○) of 92 nM endonexin. (b) Protein titrations of endonexin-induced changes in E/M of 5% pyrene-PG/PC vesicles (4 μM lipid) at $\log[Ca^{2+}] = -2.30$ (5 mM Ca^{2+}; ●) and at $\log[Ca^{2+}] = -1.30$ (50 mM Ca^{2+}; ▲).

Ca^{2+}-induced pyrene-PG lateral segregation.

A more thorough examination of the diminished increases in E/M at high Ca^{2+} concentrations was achieved by comparing Ca^{2+} and protein titrations of endonexin-induced increases in E/M with the same preparation of vesicles. If Ca^{2+}–lipid immobilization does impede endonexin-mediated segregation of pyrene-PG at high Ca^{2+}, the endonexin-induced increases in E/M at high Ca^{2+} should be relatively insensitive to the concentration of membrane-bound endonexin. On the other hand, if pyrene-PG molecules become diluted among excess competing endonexin molecules bound at high Ca^{2+}, decreasing the concentration of membrane-bound endonexin should result in larger increases in E/M. This is because pyrene-PG molecules will be distributed among fewer endonexin molecules, resulting in a greater net segregation of pyrene-PG (a greater average number of pyrene-PG molecules bound per endonexin molecule). Experimentally, the surface concentration of membrane-bound endonexin at constant Ca^{2+} can be varied by titrating the total endonexin present.

Figure 7a illustrates a Ca^{2+} titration of endonexin-induced increases in E/M for 5%/95% pyrene-PG/PC vesicles and 92 nM endonexin. As with the titrations in Figure 5, the titration is biphasic, with a maximum at 5 mM Ca^{2+} ($\log[Ca^{2+}] = -2.3$), and returning to near-zero at 50 mM Ca^{2+} ($\log[Ca^{2+}] = -1.3$). As also seen with titrations in Figure 5, Ca^{2+} alone caused only a slight increase in E/M that may not be significant. Protein titrations were then performed on this same preparation of vesicles at 5 and 50 mM Ca^{2+} to determine the increases in E/M at lower concentrations of membrane-bound endonexin (only the total concentration of endonexin and not the membrane-bound concentration of endonexin is quantitatively known). As seen in Figure 7b, decreasing the total endonexin concentration at 5 mM Ca^{2+} resulted in diminished E/M. However, decreasing the total endonexin concentration at 50 mM Ca^{2+} actually resulted in increased E/M down to 15 nM endonexin, below which increases in E/M became smaller again (data for 0 and 92 nM endonexin in Figure 7b are also in 7a).

The protein titrations of E/M at 5 and 50 mM Ca^{2+} suggest that endonexin-induced changes in E/M depend on the surface density of endonexin molecules. At high total endonexin concentrations, endonexin binding to vesicles at 5 mM Ca^{2+} may be limited because most of the pyrene-PG is segregated into endonexin–membrane complexes, leaving PC–membrane regions depleted of pyrene-PG. Endonexin does not bind pure PC membranes below 10 mM Ca^{2+} (Junker & Creutz, 1993). Endonexin does bind to pure PC membranes between 10 and 100 mM Ca^{2+} (Junker & Creutz, 1993) and so should be able to achieve greater surface densities on the 5% pyrene-PG at high total endonexin concentrations. Such additional binding above approximately 23 nM total endonexin could result in the smaller increases in E/M at 50 mM Ca^{2+} (see Discussion). The coincidence of the two protein titrations below approximately 15 nM endonexin probably reflects equal concentrations of membrane-bound endonexin at the low total endonexin concentrations, since 5 mM Ca^{2+} may be sufficient to promote maximum binding of endonexin to 5% pyrene-PG/PC vesicles when little pyrene PG is depleted.

Endonexin Binds PG and Pyrene-PG Equivalently. Since endonexin may primarily bind to membrane surfaces without insertion (discussed above), endonexin may not significantly discriminate between PG and pyrene-PG molecules. This appears to be borne out by the data. At 2 mM Ca^{2+}, 90 nM endonexin caused a 10% increase in the E/M of 5% pyrene-PG vesicles but only a 2% increase in the E/M of 5% PG/5% pyrene-PG vesicles. If PG and pyrene-PG bind to different sites on the endonexin protein, similar increases in E/M would have been expected for the two types of vesicles. The smaller increase in E/M observed with vesicles containing an additional 5% PG suggests less net segregation of PG and pyrene-PG, consistent with PG and pyrene-PG binding to the same sites on endonexin.

Endonexin-induced changes in E/M for vesicles containing the same mol % total PG but different proportions of pyrene-PG can also be compared. In the presence of 46 nM endonexin, 2 mM Ca^{2+} caused a 7% increase in E/M, to 1.4, for 10% pyrene-PG vesicles but only a 4% increase in E/M, to 0.6, for 5% PG/5% pyrene-PG vesicles. The smaller E/M and smaller percent increase in E/M for 5% PG/5% pyrene-PG vesicles may reflect pyrene-PG molecules becoming so diluted among competing nonderivatized PG lipids when bound to endonexin that monomer collision frequency is greatly diminished. Alternatively, PG molecules may bind to the same sites on endonexin as pyrene-PG molecules but with greater affinity. In either case, PG and pyrene-PG would be binding to the same sites on endonexin. If endonexin was only binding pyrene-PG and not PG molecules, the same final E/M would have been expected for 10% pyrene-PG and 5% PG/5% pyrene-PG vesicles, since lipids sequestered by endonexin should experience the same local concentration.

DISCUSSION

Endonexin appears to dramatically affect the dynamics of PG lipid molecules when bound to PG/PC lipid membranes. Both restricted lateral mobility and induced lateral segregation of PG molecules were apparent from changes in pyrene-PG

E/M. The extent of PG mobility restriction was most evident in pure PG vesicles, where changes in pyrene-PG collision frequency and E/M could only occur with changes in lipid mobility and not by segregation of lipids by head group type. The 30% maximum decrease in E/M observed with maximum binding of endonexin to these vesicles suggests a corresponding 30% decrease in the average bimolecular rate constant k_{DM} for excimer formation. This may correspond to a 30% decrease in the lateral diffusion constant for pyrene-PG molecules, which is proportional to the k_{DM} (Galla & Sackmann, 1974).

The actual decreases in k_{DM} and the diffusion constant for some pyrene-PG molecules when bound to endonexin may be even greater than 30%, since the measured E/M reflects an average of all pyrene-PG lipid molecules. Lipid molecules may experience different degrees of restriction by endonexin, depending where lipids make contact on the protein. A lipid may even be in a relatively unrestricted area between membrane-bound protein molecules. In fact, half the lipid molecules may not experience any effect of endonexin binding because they are located on the inner leaflet of vesicle bilayers. To calculate the change in E/M for outer-leaflet pyrene-PG molecules only, the contribution of the unaffected inner-leaflet pyrene-PG molecules to the observed excimer and monomer intensities after addition of Ca^{2+} and endonexin can be corrected by subtracting half the observed monomer and excimer emission intensities in the absence of Ca^{2+} and endonexin from the respective values in the presence of Ca^{2+} and endonexin. This yields decreases in E/M and k_{DM} of 53.1% for outer-leaflet pyrene-PG molecules with endonexin binding and of 13.8% with Ca^{2+} alone. Endonexin-induced decreases in lipid mobility inferred here from changes in E/M are consistent with measured high-affinity binding ($K_b > 2.5 \times 10^9$ M^{-1} for 95% PS lipid membranes) and slow rates of dissociation for endonexin–membrane binding (Junker & Creutz, 1993).

Increases in E/M observed with endonexin binding to 5 and 10 mol % pyrene-PG/PC vesicles indicate that endonexin can increase the collision frequency of pyrene-PG molecules when bound to these membranes by increasing pyrene-PG local concentration, consistent with formation of PG–endonexin complexes. This lateral segregation of pyrene-PG was extensive enough to cause a net increase in pyrene-PG monomer collision frequency despite the simultaneous restriction of lipid mobility. Maximum increases for outer-monolayer pyrene-PG molecules, assuming that only these lipids were affected by endonexin–membrane binding, were 32% for 5%/95% pyrene-PG/PC vesicles and 15% for 10%/90% pyrene-PG/PC vesicles. The net segregation of pyrene-PG observed with 10 mol % pyrene-PG vesicles indicates that maximum binding of endonexin to these vesicles requires greater local concentrations of PG in regions bound by endonexin than provided by the average distribution of PG in 10 mol % PG vesicles. In other words, the local mole fraction of PG in areas bound by endonexin is greater than 0.1. Since a membrane-bound endonexin molecule appears to occupy an area equivalent to 32 outer-monolayer phospholipid molecules (Junker & Creutz, 1993), this suggests that the number of PG molecules bound by endonexin within the 32 lipid site is greater than 3.2.

Lateral segregation of PG and pyrene-PG molecules may occur in vesicles containing larger mole fractions of total (pyrene and non-pyrene) PG but still result in net decreases in E/M, since the segregation may not be great enough to fully compensate for the decrease in collision frequency by lipid immobilization. While endonexin caused decreases in

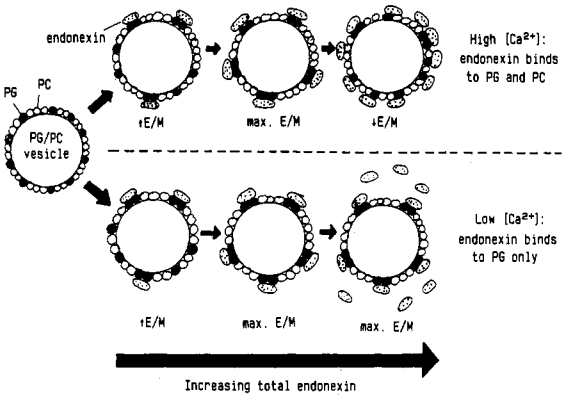


FIGURE 8: Proposed mechanism for endonexin binding to PG/PC membranes in which endonexin binds only PG lipids at low Ca^{2+} concentrations but binds both PG and PC lipids at high Ca^{2+} concentrations. In this cartoon, each endonexin molecule binds two membrane lipids, and both lipids must be PG to maintain a stable protein–membrane complex at low Ca^{2+} concentration.

E/M for all PC vesicles containing 3% pyrene-PG and 10–97% PG, the smaller decreases in E/M observed with the 10% and 20% PG vesicles may result from lateral segregation of PG and pyrene-PG that compensates for PG immobilization. For the larger mol % PG vesicles, no reorganization of PG may be required to support maximum endonexin–membrane binding. Compensating segregation of PG molecules may not occur above 53% total PG, since the endonexin-induced decreases in E/M for 53% and 100% total PG vesicles were so similar (27% and 30%, respectively). Using 53% as an upper limit for the mol % total PG in vesicles that supports maximum binding of endonexin without lipid rearrangement suggests a maximum 17 PG molecules bound in each endonexin–membrane complex.

The biphasic Ca^{2+} titrations of E/M observed with endonexin binding to 5% and 10% pyrene-PG vesicles may reflect diminished segregation of pyrene-PG at high Ca^{2+}. The increase and subsequent decrease in E/M with increasing Ca^{2+} occurred over a Ca^{2+} range where endonexin–membrane binding increased, suggesting that net segregation of pyrene-PG reached a maximum at some surface density of endonexin. Above this surface density, the average number of pyrene-PG molecules bound per endonexin protein may have decreased as the limited pool of pyrene-PG became distributed among competing endonexin molecules. That high Ca^{2+} caused this dilution of pyrene-PG by promoting increased binding of endonexin was confirmed by changing the surface density of membrane-bound endonexin at a Ca^{2+} concentration where increases in E/M were greatly diminished. Decreasing the surface density of endonexin at 50 mM Ca^{2+} by decreasing the total endonexin concentration did result in increased E/M, with a maximum increase at 15 nM total endonexin.

Decreased segregation of pyrene-PG at high Ca^{2+} concentrations may result from the ability of endonexin to bind to pure PC vesicles at high Ca^{2+} concentrations. Endonexin binds to pure PC vesicles at 100 mM Ca^{2+}, with a Ca^{2+} threshold greater than 10 mM (Junker & Creutz, 1993). Such binding to PC may explain the apparent biphasic Ca^{2+} titration of protein binding to 10% PG vesicles with inflection points near 0.5 mM and at greater than 10 mM Ca^{2+}. The binding phase below 1 mM Ca^{2+} may represent endonexin binding to PG and PC, whereas the phase above 1 mM Ca^{2+} may represent endonexin binding primarily to PC.

A mechanism by which endonexin binding to PC can result in decreased E/M is illustrated schematically in Figure 8. Increasing the Ca^{2+} concentration above the level needed to

support endonexin binding to pure PC membranes allows more protein to bind to membrane surfaces, since PC regions depleted of PG can support endonexin binding. Endonexin may still prefer to bind PG molecules over PC molecules at high Ca^{2+}, but all membrane-bound endonexin molecules will compete equally for the PG. This leads to a random distribution and effective dilution of PG molecules among membrane-bound endonexin molecules. At 5 mM Ca^{2+}, where stable endonexin–PC membrane association requires PG, the protein titration of endonexin-induced changes in E/M in 5% pyrene-PG vesicles saturated near 15 nM total endonexin. At 50 mM Ca^{2+}, the maximum increase in E/M also occurred at 15 nM total endonexin. Thus, in the presence of high Ca^{2+} concentrations, where endonexin can bind pure PC membrane surfaces, PG molecules are still preferentially segregated when few endonexin molecules are bound to vesicles.

The levels of Ca^{2+} used in this study to observe the interaction of endonexin with 5% and 10% pyrene-PG vesicles far exceed the intracellular physiological range in which endonexin presumably functions. Experimentally, the high levels of Ca^{2+} used here were necessary for understanding the mechanism for the changes in lipid dynamics mediated by endonexin. These changes in lipid dynamics are likely to be relevant at lower levels of Ca^{2+} (<1 mM) with mixed lipid membranes occurring in the cell, e.g., membranes containing mixtures of different acidic phospholipids such as PS and PG, since the principles of the endonexin–lipid interactions should be the same.

The estimated segregation of 3–17 PG molecules per membrane-bound endonexin molecule is far fewer than the 42 (Andree et al., 1990) to 59 (Meers et al., 1991) outer-monolayer lipids bound by annexin V and the 32 outer-monolayer lipids bound by endonexin (Junker & Creutz, 1993). This suggests that endonexin causes a local enriching of PG and not formation of a pure PG domain. Similar local enriching without pure domain formation of phosphatidic acid (PA) and PG in PA/PC (or PG/PC) membranes has been inferred with the binding of protein kinase C (PKC) and two other Ca^{2+}-dependent membrane-binding proteins using fluorescence quenching of PA and PG NBD derivatives (Bazzi & Nelsestuen, 1991). Ten or greater NBD-PA molecules were estimated to be "clustered" by each of these proteins when bound to NBD-PA/PC membranes. While the two unidentified proteins may be annexins, PKC is not and exhibits no sequence similarity with annexins. A comparison between the membrane-binding site of PKC with that of annexins, typified by annexin V (Huber et al., 1990; Brisson et al., 1991), will have to await a structure determination of PKC.

Another study (Meers et al., 1991) concluded that binding of some annexins, including endonexin, to PS/5% pyrene-PC vesicles resulted in decreased lipid mobility but no segregation. However, for vesicles containing 95% PS, probably no lateral segregation of PS would be required for maximum binding of annexin proteins. In preliminary studies with sonicated vesicles, we found that endonexin caused a small (<8%) decrease in E/M for vesicles containing 5% pyrene-PC and either 10% or 20% PG. Since endonexin does not appear to interact with pure domains of PG (i.e., not all lipids covered by membrane-bound endonexin molecules are PG), the immobilization of PC seen in the study of Meers et al. (1991) could have resulted from weaker interactions of PC with annexins or from immobilization of neighboring PG molecules.

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