Interfacial Membrane Properties Modulate Protein Kinase C Activation: Role of the Position of Acyl Chain Unsaturation†

Jennifer R. Giorgione,‡ Ruud Kraayenhof,§ and Richard M. Epand*,‡

Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada, and Institute of Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Received January 26, 1998; Revised Manuscript Received May 26, 1998

ABSTRACT: We studied the effects of the addition of a series of 1,2-dioctadecenoyl-*sn*-glycerol-3-phosphoethanolamines to vesicles composed of 1-palmitoyl-2-oleoylphosphatidylserine and 1-palmitoyl-2-oleoylphosphatidylcholine on the activity and membrane binding of protein kinase C (PKC). The three phosphatidylethanolamines (PEs) were dipetroselinoyl-PE, dioleoyl-PE, and divaccenoyl-PE, which have double bonds in positions 6, 9, and 11, respectively. These lipids represent a group of structurally homologous compounds whose physical properties have been compared. We also used a fluorescent probe, 4-[(*n*-dodecylthio)methyl]-7-(*N*,*N*-dimethylamino)coumarin to measure the relative interfacial polarities of LUVs containing each of the three PEs. We find dipetroselinoyl-PE allows the least access of the fluorescent probe to the membrane. This is also the lipid that shows the lowest activation of PKC. The activity of PKC was found to correlate best with the interfacial properties of the three PEs rather than with the curvature energy of the membrane. The results show the sensitivity of the activity of PKC to small changes in lipid structure.

Protein kinase C (PKC)[1] is a family of Ser/Thr protein kinases (*1*) which play an important role in signal transduction (*2*). The principle isoforms of rat brain PKC are the Ca^{2+}-dependent, c-PKC isoforms (*3*). Aspects of the structural and regulatory properties of PKC have recently been reviewed (*4*−*7*).

Protein kinase C can be activated by binding to membranes. The enzyme activity is modulated, in part, by the physical properties of the membrane to which it binds (*8*, *9*). It has been shown that uncharged or zwitterionic additives which lower the bilayer-to-hexagonal phase transition temperature of model PE bilayers are activators of PKC (*10*). In addition, replacement of phosphatidylcholine (PC), a lipid that forms stable bilayers, with phosphatidylethanolamine (PE), a lipid which is prone to forming inverted phases, has been shown to increase the activation of PKC (*11*−*14*). It has been suggested that the relative activation seen with different PEs correlates with their bilayer-to-hexagonal phase transition temperature (T_H), with the strongest activators having the lowest T_Hs (*11*, *12*). There are two aspects by which agents that lower T_H increase the activity of PKC. One aspect is a lowering of the mole fraction of PS required for activation, and the other is the maximal activity observed with a high PS content (*12*, *15*). The exact physical property responsible for these correlations are not known.

To further investigate this phenomenon, a series of 1,2-dioctadecenoyl-*sn*-glycerol-3-phosphoethanolamines (di-18:1 PEs), differing only in the position of the double bond, was used. Double bonds were at position 6, 9, or 11. This series of structurally homologous PEs has been well characterized with regard to the T_H, the intrinsic radius of curvature, and the monolayer bending modulus (*16*). Determining the effects of these homologous phospholipids on PKC binding and activation can lead to a better understanding of the physical properties responsible for the observed correlation between "hexagonal phase propensity" and enzyme activity (*8*).

EXPERIMENTAL PROCEDURES

Materials. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL); histone H1 was from GIBCO/BRL (Grand Island, NY). Bovine serum albumin fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma (St. Louis, MO). [γ-^{32}P]ATP was from ICN, and [9,10-^{3}H]dipalmitoylphosphatidylcholine was from NEN. $CaCl_2$ was from Fisher, and Tris was from Boehringer Mannheim. The synthesis of 4-[(*n*-dodecylthio)methyl]-7-(*N*,*N*-dimethylamino)coumarin (DTMAC) has been described previously (*17*).

† This work was supported by the Medical Research Council of Canada (Grant MT-7654).

* Address correspondence to Dr. Richard M. Epand. Telephone: (905) 525-9140, ext. 22073. Fax: (905) 522-9033. E-mail: epand@fhs.mcmaster.ca.

‡ McMaster University Health Sciences Centre.

§ Vrije Universiteit.

[1] Abbreviations: PKC, protein kinase C; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; DPLPE, dipetroselinoyl-PE (Δ6); DOPE, dioleoyl-PE (Δ9); DVPE, divaccenoyl-PE (Δ11); DTMAC, 4-[(*n*-dodecylthio)methyl]-7-(*N*,*N*-dimethylamino)-coumarin; 5-doxyl-PC, 1-palmitoyl-2-stearoyl-(5-doxyl)phosphatidylcholine; T_H, bilayer-to-hexagonal phase transition temperature; LUVs, large unilamellar vesicles; SLVs, sucrose-loaded vesicles; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; R_o, intrinsic radius of curvature; K_c, elastic bending modulus; DAG, dioleoylglycerol.

S0006-2960(98)00185-8 CCC: $15.00
Published on Web 07/15/1998

PKC Purification. Rat brain PKC was purified with a modified procedure of Huang et al. (*18*) described previously (*15*).

Lipid Vesicles. Lipid films were made by dissolving phospholipids, with or without DTMAC in 2:1 (v:v) chloroform/methanol, and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in standard buffer (100 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris at pH 7.0) and subjected to five freeze−thaw cycles. These films were extruded through two 0.1 μm pore polycarbonate filters in a microextruder (Avanti Mini Extruder, Avanti Polar Lipids) to form LUVs. Sucrose-loaded vesicles (SLVs) were prepared by hydrating in a buffer of 0.170 M sucrose, 5 mM $MgCl_2$, and 20 mM Tris at pH 7.0. The vesicles were sedimented in standard buffer for 30 min at 100000*g* to flush out the excess sucrose.

PKC Activity Assays. The activity of PKC toward histone was determined as previously described (*15*). Histone was added to a final concentration of 0.2 mg/mL, in a 150 μL total volume. The phospholipid was 100 μM in the form of either LUVs or sucrose-loaded vesicles (SLVs) (as noted in the figure legends). [γ-^{32}P]ATP (0.2 mCi/mL) was 20 μM, and PKC was 575 ng/mL. Ca^{2+} was 200 μM unless otherwise stated. The reaction temperature was 25 °C.

PKC Binding Assays. The sucrose-loaded vesicle (SLV) assay was modified from the procedure of Rebecci et al. (*19*) as described previously (*15*). PKC was incubated with SLVs and then centrifuged for 30 min at 100000*g* at 25 °C to separate the membrane-bound enzyme. The pellet and supernatant were assayed under identical conditions for activity toward protamine sulfate.

Fluorescence Measurements. LUVs (100 nm in diameter) contained 20 mol % POPS, one of the di-18:1 PEs at 50 mol %, 0.5 mol % DTMAC, and either 15 mol % 1-palmitoyl-2-stearoyl-(5-doxyl)-PC (5-doxyl-PC) and 14.5 mol % POPC or 29.5 mol % POPC. The final concentration of LUVs was 3.5 mM in standard buffer. The synthesis and initial characterization of DTMAC were reported in ref *17*. Fluorescence was measured in glass cuvettes at 25 °C with an SLM Aminco Series II spectrofluorimeter. The temperature was regulated with a constant-temperature bath which circulated fluid through the cell holder. A filter which blocked wavelengths below 420 nm was used between the sample and photomultiplier. The excitation wavelength for all LUVs containing DTMAC was 397 nm. An emission scan was taken to determine the wavelength of maximum emission specific for LUVs containing each of the three PEs. There was little difference in the emission maxima in the presence of each of the three PEs, with the maximum being 475 ± 1 nm.

RESULTS

We have compared PKC activity at various Ca^{2+} concentrations, in the presence of LUVs composed of 30% POPS with or without the addition of one of the three PEs at 20%, the remaining lipid being POPC (Figure 1). It is clear that LUVs containing 20 mol % PE significantly increased the PKC-catalyzed phosphorylation of histone compared to LUVs containing only 30 mol % PS and 70 mol % PC. DOPE supported the highest level of PKC activity under differing Ca^{2+} concentrations. DPLPE and DVPE generally supported similar levels of activity, which were lower than that of the DOPE. A similar order for the three PEs was seen when the mole fraction of PE was varied from 25 to 75 mol % at a constant 20 mol % POPS and 200 μM Ca^{2+} (Figure 2).

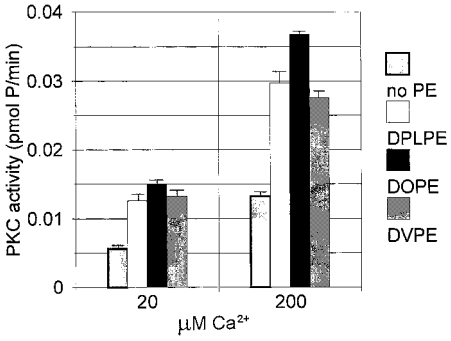

FIGURE 1: Effect of DPLPE, DOPE, or DVPE on the PKC-catalyzed phosphorylation of histone with various Ca^{2+} concentrations. LUVs were composed of 30 mol % POPS and 70 mol % POPC, with 20 mol % PE when present replacing POPC. Activity is expressed in picomoles of phosphate incorporated per minute. Data are expressed as the mean of triplicate determinations ± standard error (SE). The assay temperature was 25 °C.

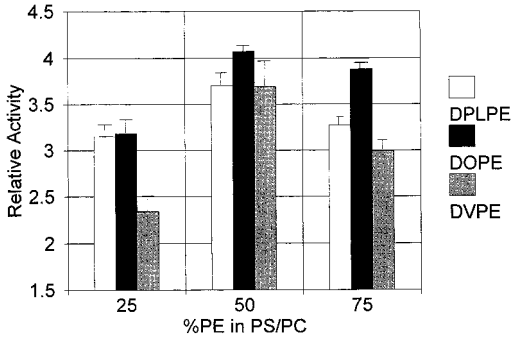

FIGURE 2: PKC-catalyzed phosphorylation of histone in the presence of various mole fractions of DPLPE, DOPE, or DVPE. SLVs were composed of 20 mol % POPS, with the indicated percentage of PE, and the remainder being POPC. Activity is expressed in picomoles of phosphate incorporated per minute. Data are expressed as the mean of triplicate determinations ± SE. Ca^{2+} was 200 μM, and the assay temperature was 25 °C.

Binding was determined as a function of PS, at a constant 50 mol % for each PE (not shown). The binding shows sigmoidal behavior, typical for PKC (*15*). However, there is more error in the binding curves than is usually obtained. It may be that these vesicles, with their particular lipid compositions, are not stable to centrifugation and resuspension. Therefore, two PS concentrations were chosen for a more detailed study with a higher number of replicates (see below).

Activity curves revealed half-maximal activity at 16 ± 2, 14 ± 2, and 11 ± 2 mol % PS for DPLPE, DOPE, and DVPE, respectively (Figure 3). Similar results were obtained when LUVs were used instead of SLVs (data not shown). Because the differences in activity were small for the curves for the three different PEs, to further test whether there was a difference among these PEs, we did multiple assays at a fixed PS concentration, either near half-maximal activity (20% PS, Figure 4) or at maximal activation (40% PS, Figure 5). At 40% PS, the activity is greatest in the presence of DOPE, while the activity is similar for both DOPE and DVPE at 20% PS. In both cases, the activity is least with

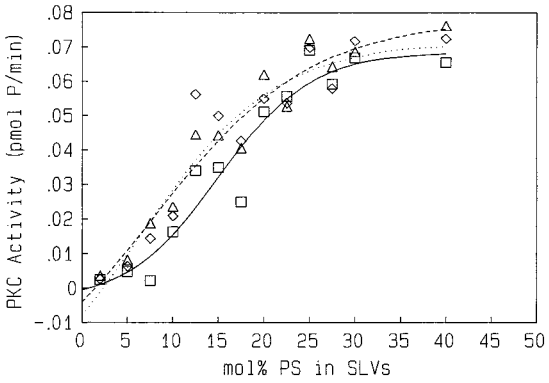

FIGURE 3: PKC-catalyzed phosphorylation of histone in the presence of PE-containing SLVs as a function of the mole fraction of POPS. SLVs contained 50 mol % PE, and the indicated POPS, with the remainder being POPC. Activity is expressed as picomoles of histone phosphorylated per minute and is the mean of triplicate determinations. Curves were fitted to the nonlinear Hill equation: DPLPE (□ with the solid curve), DOPE (△ with the dashed curve), and DVPE (◇ with the dotted curve).

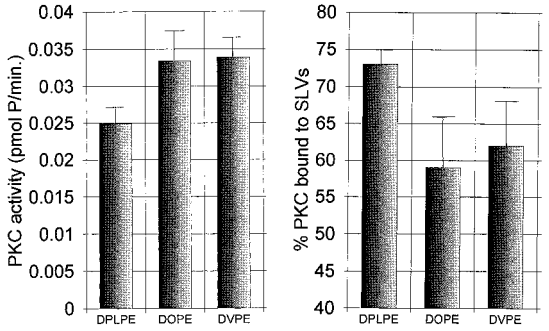

FIGURE 4: Comparisons of PKC-catalyzed phosphorylation of histone and the percentage of PKC bound to LUVs or SLVs containing 50 mol % PE. Vesicles were composed of 20 mol % POPS, 50 mol % PE, and 30 mol % POPC. Six samples of each vesicle composition were made up and extruded separately. Activity or binding was then assayed in triplicate for each sample. Activity is expressed as picomoles of phosphate incorporated per minute, and binding is expressed as a percentage of the total enzyme added. Data are expressed as the mean of 18 determinations ± SE. Ca^{2+} and temperature were as above. The differences between the results for DPLPE and DOPE, as well as those for DPLPE and DVPE, are significant ($p < 0.01$) as determined by the two-tailed Student's *t* test using Quatro Pro.

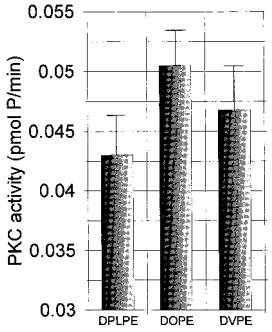

FIGURE 5: Comparisons of PKC-catalyzed phosphorylation of histone with LUVs containing 50 mol % PE. Vesicles were composed of 40 mol % POPS, 50 mol % PE, and 10 mol % POPC. Six samples of each vesicle composition were made up and extruded separately. Activity was then assayed in triplicate for each sample. Activity is expressed as picomoles of phosphate incorporated per minute. Data are expressed as the mean of 18 determinations ± SE. Ca^{2+} and temperature were as above. Differences between all three values are significant. For DPLPE and DOPE, $p < 0.01$. For DPLPE and DVPE, as well as for DOPE and DVPE, $0.01 < p < 0.05$. Calculations were carried out as described in the legend of Figure 4.

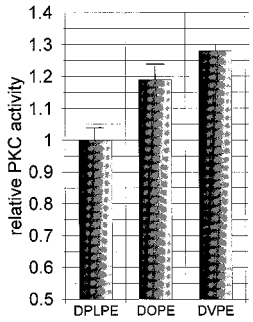

FIGURE 6: Comparisons of relative PKC-catalyzed phosphorylation of histone with LUVs containing 50 mol % PE and 1 mol % DAG. Vesicles were composed of 20 mol % POPS, 50 mol % PE, 1 mol % DAG, and 29 mol % POPC. Activity was assayed in triplicate for each sample. Activity is plotted relative to that obtained in the presence of DPLPE. Activity is shown as relative activity since a new batch of PKC was used, and comparison of absolute activity levels is not possible. Differences between DPLPE and DOPE, as well as between DPLPE and DVPE, are significant. Calculations were carried out as described in the legend of Figure 4.

DPLPE. This is also the case in the presence of 1% DAG (Figure 6). Binding assays under these conditions revealed that there was significantly more PKC bound to DPLPE-containing membranes than either DOPE or DVPE. Therefore, the specific activity of membrane-bound PKC is much lower with DPLPE.

DTMAC is a fluorescent probe whose depth of burial in the membrane has been found to be sensitive to the nature of the lipid headgroup (*17*, *20*). DTMAC fluorescence can be quenched by the 5-doxyl-PC quencher, which has a nitroxide group at the 5 position of the *sn*-2 acyl chain. The position of the quencher is more or less fixed by the phospholipid headgroup. DTMAC is more free to move along the bilayer normal. Therefore, a greater amount of quenching of DTMAC fluorescence is expected when the interfacial fluorescence probe is more deeply inserted into the membrane, since the 5-doxyl group will be embedded at a fixed position in the membrane. The fluorescence intensity ratio was calculated by measuring the fluorescence of 0.5 mol % DTMAC, at the wavelength of maximum emission, in the presence and the absence of 15 mol % 5-doxyl-PC (Figure 7). Since the intensity ratio is significantly higher for DPLPE than for the other two PEs, there is less quenching of DTMAC in DPLPE-containing membranes, and therefore, the depth of insertion of DTMAC is greater in LUVs containing 50 mol % DOPE or DVPE than in those containing the same amount of DPLPE. This suggests a difference in the interfacial polarity of these membranes, since DTMAC has been suggested to partition into a membrane with its fluorophore group in a region with a particular dielectric constant (*20*).

DISCUSSION

It has been shown that phospholipids or hydrophobic substances that favor the formation of the hexagonal phase are generally activators of PKC. Some studies have shown that the level of activation correlates with the bilayer-to-hexagonal phase transition temperature (T_H) of the phospholipid (*11*, *12*). The results from this study are consistent

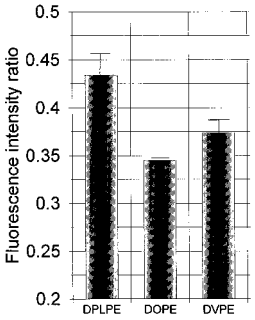

FIGURE 7: Fluorescence quenching of DTMAC by 5-doxyl-PC in PE-containing LUVs. The fluorescence intensity of 0.5 mol % DTMAC in LUVs composed of 20 mol % POPS, with 50 mol % PE and the remainder being POPC, was compared to that of LUVs containing 15 mol % 5-doxyl PC as a quencher. The ratio of fluorescence intensity with the quencher vs that without the quencher is shown. The smaller the ratio, the more quenching. Data are the mean of three separate experiments ± SE. Differences between all three measurements were significant and were determined as described in the legend of Figure 4. For DPLPE and DOPE, $p < 0.01$. For DPCPE and DVPE, as well as for DOPE and DVPE, $0.01 < p < 0.05$.

with this generalization. DOPE (Δ9) exhibits the greatest activation, although in some cases the activity in the presence of DVPE (Δ11) is comparable. DOPE and DVPE have the lowest values of T_H, of 8 and 28 °C, respectively. DPLPE (Δ6) almost always has the lowest activity of the three PEs, and it has the highest T_H of 37 °C. The relative activities also correlate with the L_β-to-L_α phase transition temperature: −1, −8, and 16 °C for DVPE, DOPE, and DPLPE, respectively. However, there is no other evidence for a correlation between this transition temperature and PKC activation. In fact, Senisterra and Epand (*12*) have shown that there is little change in activity in going from the L_β to L_α phase.

However, the specific physical property which is responsible for the correlation between T_H and the activation of PKC is not known. When hexagonal phase-promoting lipids are added, there is an increase in headgroup spacing which may make the acyl chains more accessible and allow greater interaction of PKC with the membrane bilayer (*21*, *22*). It should be noted, however, that even under conditions which promote strong interaction of PKC with membranes, the bnding of the enzyme is reversible, indicating that the protein does not penetrate deeply into the hydrohobic core of the membrane (*23*). There are several physical parameters that determine the relative stability of lamellar and hexagonal phases. The differences among phospholipids with regard to lipid polymorphism result from differences in their intrinsic radius of curvature (R_o) and from their elastic bending modulus (K_c). The intrinsic radius of curvature was found to be similar for DOPE and for DPLPE over a range of temperatures (see Figure 5 in ref *16*). Since these two PEs show the highest and the lowest extent of activation of PKC, it is not likely that R_o determines the extent of activation of PKC. The bending modulus K_c correlates better with activation in that DOPE, the best activator, has the highest K_c. However, DPLPE and DVPE have similar K_cs, yet DVPE is a better activator. We can also assess the curvature strain energy of a monolayer of each of the PEs, constrained to be a flat bilayer. This energy is given by $0.5K_c/R_o^2$ (*24*). Using the values of K_c and R_o at 25 °C (*16*), we calculate the curvature energy to be 3.08, 3.91, and 2.04 erg/cm^2 for DPLPE (Δ6), DOPE (Δ9), and DVPE (Δ11), respectively. The lipid that supports the least activity is DPLPE, but the curvature energy for this lipid is intermediate between those of the other two. The addition of 1 mol % DAG to LUVs containing 20% PS and 50% PE did not alter the relative activity levels (compare Figure 6 with Figure 4). Activation was similar for DOPE and DVPE and lowest for DPLPE. Therefore, none of the parameters directly associated with membrane curvature correlates well with PKC activation. This is consistent with recent evidence showing that cubic phase membranes with lowered curvature strain can support a greater activity of membrane-bound PKC than a lamellar lipid with a similar composition (*25*).

The quenching of the fluorescence of DTMAC by the nitroxide-labeled PC is indicative of the facility with which substances can enter the membrane bilayer. The results show the sensitivity of the extent of quenching on lipid structure (Figure 6). There is also an excellent correlation between the extent of quenching in the presence of the three lipids and the activation of PKC. It should be noted that the PKC assays were performed at several different calcium concentrations. Calcium would also affect the interfacial properties of membranes rich in PS, although in this case there is only 20% PS in the membrane. The fluorescence measurements were taken to compare the properties of membranes containing each of the three PEs at a single fixed condition. Calcium was omitted from these experiments to prevent any complication due to differing effects of this ion on the fluorescent probe. In most activity assays, the lipid that shows the least activation of PKC is DPLPE which stands out as the lipid allowing less entry into the membrane (Figure 7). This lipid has the double bond position closest to the headgroup. Because of the rigidity of the double bond and the fact that it will cause disordering at positions further into the bilayer center, the accessibility of DTMAC to DPLPE is smallest. DOPE and DVPE generally show similar extents of activation, and they exhibit similar behavior in this quenching assay. Thus, this quenching assay appears to be an even better predictor of PKC activation than T_H, since the activities and fluorescence quenching of DVPE and DOPE are similar but the T_H value of DVPE is intermediate between those of the other two PEs. The penetration of DTMAC into the bilayer may be related to the ability of PKC to enter the membrane. This property has previously been suggested to be important for PKC activation (*26*).

The differences in activity of PKC in membranes composed of each of these three similar lipids are not very great. However, in the assays carried out with many replicates, it is clear that DPLPE has the lowest extent of activation. The fact that the activity shows a sigmoidal dependence on PS means that, at a particular PS concentration that is found in vivo, the activity of PKC may be quite different depending on subtle factors of lipid structure, such as the position of a double bond in the acyl chain in a mixed lipid system.

REFERENCES

1. Dekker, L. V., and Parker, P. J. (1994) *Trends Biochem. Sci. 19*, 73−77.
2. Nishizuka, Y. (1995) *FASEB J. 9*, 484−496.
3. Burns, D. J., Bloomenthal, J., Lee, M.-H., and Bell, R. M. (1990) *J. Biol. Chem. 256*, 12044−12051.
4. Newton, A. C. (1997) *Curr.Opin. Cell Biol. 9*, 161−167.

5. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) *Protein Sci. 6*, 477−480.
6. Mosior, M., and Epand, R. M. (1997) *Membr. Mol. Biol. 14*, 65−70.
7. Hurley, J. H., and Grobler, J. A. (1997) *Curr. Opin. Struct. Biol. 7*, 557−565.
8. Epand, R. M. (1992) in *Protein Kinase C: Current Concepts and Future Perspectives* (Lester, D. S., and Epand, R. M., Eds.) pp 135−156, Ellis Horwood, Chichester, U.K.
9. Goldberg, E. M., and Zidovetzki, R. (1998) *Biochemistry 37*, 5623−5632.
10. Epand, R. M., and Lester, D. A. (1990) *Trends Pharmacol. Sci. 11*, 317−320.
11. Giorgione, J., Epand, R. M., Buda, C., and Farkas, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9767−9770.
12. Senisterra, G., and Epand, R. M. (1993) *Arch. Biochem. Biophys. 300*, 378−383.
13. Mosior, M., Golini, E., and Epand, R. M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1907−1912.
14. Slater, S. J., Kelly, M. B., Taddeo, F. J., Ho, C., Rubin, E., and Stubbs, C. D. (1994) *J. Biol. Chem. 269*, 4866−4871.
15. Mosior, M., and Epand, R. M. (1993) *Biochemistry 32*, 66−74.
16. Epand, R. M., Fuller, N., and Rand, R. P. (1996) *Biophys. J. 71*, 1806−1810.
17. Sterk, G. J., Thijsse, P. A., Epand, R. F., Wong Fang Sang, H. W., Kraayenhof, R., and Epand, R. M. (1997) *J. Fluoresc. 7*, 115S−118S.
18. Huang, K. P., Chan, K. F., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986) *J. Biol. Chem. 261*, 12134−12140.
19. Rebecchi, M., Peterson, A., and McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
20. Epand, R. F., Kraayenhof, R., Sterk, G. J., Wong Fong Sang, H. W., and Epand, R. M. (1996) *Biochim. Biophys. Acta 1284*, 191−195.
21. Snoek, G. T., Rosenburg, I., de Laat, S. W., and Gitler, C. (1986) *Biochim. Biophys. Acta 860*, 336−344.
22. Snoek, G. T., Feijen, A., Hage, W. J., Van Rotterdam, W., and de Laat, S. W. (1988) *Biochem. J. 255*, 629−637.
23. Mosior, M., and Newton, A. C. (1995) *J. Biol. Chem. 270*, 25526−25533.
24. Helfrich, W. (1973) *Z. Naturforsch. 28C*, 693−703.
25. Giorgione, J. R., Huang, Z., and Epand, R. M. (1998) *Biochemistry 37*, 2384−2392.
26. Epand, R. M., Stafford, A. R., and Lester, D. S. (1992) *Eur. J. Biochem. 208*, 327−332.

BI980185A