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Role of phospholipids containing docosahexaenoyl chains in modulating the activity of protein kinase C

(thermal acclimatization/phosphatidylethanolamine/docosahexaenoic acid)

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ABSTRACT **It is known that the phospholipids of the brain cells of fish are altered during cold adaptation. In particular, the 1-monounsaturated 2-polyunsaturated phosphatidylethanolamines (PEs) increase 2- to 3-fold upon adaptation to cold. One of the most striking changes is in the 18:1/22:6 species of PE. We determined how this lipid affected the bilayer-to-hexagonal-phase transition temperature of 16:1/16:1 PE. We found that it was more effective in lowering this transition temperature than were other, less unsaturated, PE species. In addition, it was not simply the presence of the 18:1/22:6 acyl chains which caused this effect, since the 18:1/22:6 species of phosphatidylcholine had the opposite effect on this transition temperature. Zwitterionic substances that lower the bilayer-to-hexagonal-phase transition temperature often cause an increase in the activity of protein kinase C (PKC). Indeed, the 18:1/22:6 PE caused an increase in the rate of histone phosphorylation by PKC which was greater than that caused by other, less unsaturated, PEs. The 18:1/22:6 phosphatidylcholine had no effect on this enzyme. The stimulation of the activity of PKC by the 18:1/22:6 PE is a consequence of this lipid's increasing the partitioning of PKC to the membrane.**

A detailed analysis of the phospholipid species in the brains of fish taken from summer (25°C) or from winter (5°C) waters revealed that the lipid species which underwent the largest change in relative amount was 1-oleoyl 2-docosahexaenoyl phosphatidylethanolamine (18:1/22:6 PE) (1). This lipid species, which is in relatively low abundance (2–5%) in warm-water fish, increases to approximately 14% (wt/wt) in cold-adapted fish (1). This occurs despite the fact that there is little change in total fatty acid composition with change in growth temperature. The finding is of particular interest because PEs with unsaturated acyl chains do not form stable bilayers, but rather form inverted hexagonal phases (H_{II}) under ordinary conditions. It has been shown (2) that microorganisms adjust the fraction of nonlamellar lipids in their membranes as a consequence of their growth temperature. It is possible that a similar modulation of the physical properties of the membranes of cells from vertebrates also occurs. This would suggest that the 18:1/22:6 PE has a particularly strong influence on lipid polymorphism.

One of the consequences of a change in the relative proportion of bilayer and non-bilayer-forming lipids is a modulation of the activity of certain membrane-bound enzymes. The activity of protein kinase C (PKC) is modulated by the physical properties of the bilayer (3, 4). PKC is also relatively abundant in brain tissue, which is the organ in which differences in phospholipid species with environmental temperature have been detected. In this work we therefore also study the role of 18:1/22:6 PE in modulating the activity of PKC.

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MATERIALS AND METHODS

Materials. Lipids were purchased from Avanti Polar Lipids. Histone H1 was from GIBCO/BRL. Bovine serum albumin fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma. [γ-^{32}P]ATP and [9,10-^{3}H]dipalmitoyl phosphatidylcholine were from NEN.

Differential Scanning Calorimetry. Lipid films were made from 16:1/16:1 PE dissolved in chloroform/methanol (2/1, vol/vol) either with or without the addition of a second phospholipid component, present at 5 mol % or less. After solvent evaporation with nitrogen, final traces of solvent were removed in a vacuum chamber for 90 min. The lipid films were suspended in 20 mM Pipes/1 mM EDTA/150 mM NaCl/0.002% NaN_3, pH 7.0, by vortex mixing at 45°C for 30 sec. The final lipid concentration was 10 mg/ml. The lipid suspension was degassed under vacuum before being loaded into an MC-2 high-sensitivity scanning calorimeter (Microcal, Amherst, MA). A heating scan rate of 39°C/h was generally employed. The observed phase transitions were independent of scan rates between 10 and 60°C/h. The bilayer-to-hexagonal-phase transition was fitted by using parameters to describe an equilibrium with a single van't Hoff enthalpy, and the transition temperature (T_H) is reported as that for the fitted curve.

PKC Purification. Insect cells overexpressing PKC-α in the baculovirus system were kindly supplied by Robert Burns and Nancy Rankl of Sphinx Pharmaceutical (Durham, NC). The isolation procedure was a modification of that previously described by Stabel *et al.* (5). Briefly, insect cell pellets were lysed in 2 mM benzamidine/1% Triton X-100/50 mM Tris·HCl, pH 7.5/2 mM EDTA/10 mM EGTA/1 mM dithiothreitol/40 μg of leupeptin per ml/200 μM phenylmethylsulfonyl fluoride (PMSF). After homogenization and centrifugation at 200,000 × *g*, the supernatant was applied to a Q-Sepharose column (Pharmacia; 2.5 × 20 cm) equilibrated with 1 mM EGTA/20 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol. Elution was carried out with 140 ml of 20 mM, pH 7.5, Tris buffer alone, then 140 ml each of this Tris buffer containing 0.05 M, 0.15 M, 0.45 M, and 1.0 M KCl. PKC-α was eluted with the 0.15 M KCl. Fractions were collected and analyzed for PKC activity in a previously described micellar assay (6). Fractions with the highest activity were pooled, adjusted to 1.5 M KCl, and loaded onto a phenyl-Sepharose column (Pharmacia; 1.0 × 10 cm) equilibrated with 1.5 M KCl/1 mM EDTA/1 mM EGTA/20 mM Tris·HCl, pH 7.5/10% glycerol. A reverse salt gradient from 1.5 M to 0 M KCl

Abbreviations: PE, phosphatidylethanolamine; 18:1/22:6 PE, 1-oleoyl 2-docosahexaenoyl PE (other PEs used are also designated by the nature of the acyl chains—i.e., length:number of *cis* double bonds); H_{II}, inverted hexagonal phase; PKC, protein kinase C; T_H, bilayer-to-hexagonal-phase transition temperature; DAG, 1,2-dioleoyl-*sn*-glycerol (diacylglycerol); POPS, 1-palmitoyl 2-oleoyl phosphatidylserine; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; PC, phosphatidylcholine; SLV, sucrose-loaded large unilamellar vesicle.

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was used to elute the enzyme. Collected fractions were tested for activity as above. Fractions containing the highest activity were pooled and concentrated to ≈0.40 mg of protein per ml and stored at −70°C in 50% glycerol. Purified PKC-α had a specific activity towards histone in the micelle assay (6) of 1–2 μmol/min per mg of protein, with the phospholipid-independent activity not exceeding 4% of the total kinase activity. The enzyme displayed a single band on a silver-stained electrophoresis gel.

PKC Binding Assays. A modification of the procedure of Rebecchi *et al.* (7) was used as previously described (8). Briefly, PKC-α was incubated with sucrose-loaded large unilamellar vesicles (SLVs), then centrifuged at 100,000 × *g* for 30 min at 25°C to separate the membrane-bound enzyme. The pellet and supernatant fractions were assayed under identical conditions for activity towards protamine sulfate, in a buffer of 100 mM KCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.0/1–100 μM $CaCl_2$ (depending on the experiment) containing bovine serum albumin at 0.3 mg/ml.

PKC Activity Assays. The activity assay towards histone was performed as previously described (8, 9). Histone was used at a final concentration of 0.2 mg/ml, phospholipid in the form of SLVs was 85 μM, [γ-^{32}P]ATP (0.2 mCi/ml; 1 mCi = 37 MBq) was 20 μM, and PKC was 100 ng/ml. The total volume was 250 μl, in the same buffer as in the binding assays. The results are expressed as fold activation. Fold activation is defined as the ratio of the activity in the presence of additive [1-palmitoyl 2-oleoyl phosphatidylserine (POPS)/1-palmitoyl 2-oleoyl phosphatidylcholine (POPC), 7:3 (mol/mol)] to that in its absence after correcting for the nonspecific activity measured in the absence of 1,2-dioleoyl-*sn*-glycerol (DAG) and Ca^{2+}. An analogous expression for binding was used to calculate the fold binding.

Phospholipid Vesicles. Lipid films were made by dissolved phospholipids in 2/1 (vol/vol) chloroform/methanol and drying under a stream nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in sucrose buffer (0.170 M sucrose/5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.0) and subjected to five freeze–thaw cycles. Vesicles were extruded through two 0.1-μm-pore polycarbonate filters in a microextruder. The vesicles were suspended in the buffer used for binding assays and centrifuged at 100,000 × *g* to dilute out the sucrose. Argon was bubbled through all solutions and vesicle preparations to protect lipids from oxidation.

RESULTS

The T_H of PE is particularly sensitive to the presence of certain additives (10). In the present work we use 16:1/16:1 PE as the host lipid, which has a relatively low T_H, so as to avoid decomposition of the polyunsaturated acyl chains. For one PE, at a low mole fraction, to affect the T_H of another PE, it must have a markedly different intrinsic radius of curvature (11). The extent to which the added lipid shifts the T_H of the host 16:1/16:1 PE can be evaluated from the slope of a plot of T_H versus mole fraction of the added lipid. Various mole fractions of added lipid between 0 and 0.05 were used. Addition of several unsaturated PEs to the 16:1/16:1 PE showed that they can all lower T_H. The 18:1/22:6 PE is the most effective among them (Table 1). In contrast, the 18:1/22:6 phosphatidylcholine (PC) has the opposite effect and raises T_H.

Table 1. Effect of several phospholipids on the T_H of 16:1/16:1 PE

Lipid additive	Shift of T_H, °C/mole fraction of additive
18:0/22:6 PE	−84 ± 6
18:1/18:1 PE	−72 ± 19
18:1/22:6 PE	−143 ± 8
18:1/22:6 PC	+265 ± 8

SLVs consisting of 30 mol % POPS, 68 mol % POPC, and 2 mol % of DAG, 1,2-dioleoyl PE (18:1/18:1 PE), 1-stearoyl 2-docosahexaenoyl PE (18:0/22:6 PE), or 1-oleoyl 2-docosahexaenoyl PE (18:1/22:6 PE) caused an activation of the PKC-α-catalyzed phosphorylation of histone at 2 μM Ca^{2+} compared to SLVs consisting of 30 mol % POPS and 70 mol % POPC (Fig. 1). The greatest activation in POPS/POPC vesicles was observed with DAG (14.6-fold), followed by 18:1/22:6 PE (1.49-fold). 18:0/22:6 PE and 18:1/18:1 PE were equally effective, while 2 mol % 1-oleoyl 2-docosahexaenoyl PC (18:1/22:6 PC) had no significant effect. Note that 2 mol % 18:1/22:6 PE appears weakly synergistic with 2 mol % DAG.

The activation of PKC-α-catalyzed phosphorylation of histone by 18:1/22:6 PE was dependent on the concentration of calcium (Fig. 2). Replacement of a portion of POPC in SLVs with 2 mol % or 10 mol % PE showed the largest enhancement of activity at 1 μM Ca^{2+} (1.78-fold and 3.54-fold increase in

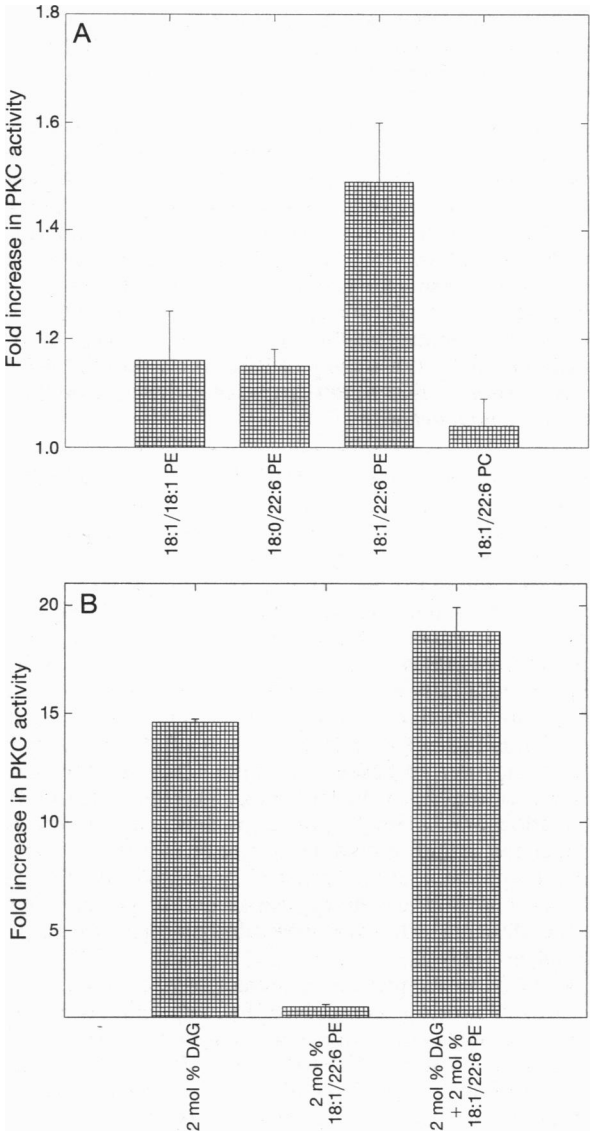

FIG. 1. Fold increase in activity of PKC-α towards histone for SLVs containing 2 mol % various phospholipids and/or DAG. The SLVs were composed of 30 mol % POPS, 2 mol % of each additive, with the remainder POPC. The final Ca^{2+} concentration was 2 μM. (*A*) PEs or PC. (*B*) DAG and/or PE. The activity is expressed as a fold increase obtained with SLVs containing no additive. Data are expressed as the mean of triplicates ± SD.

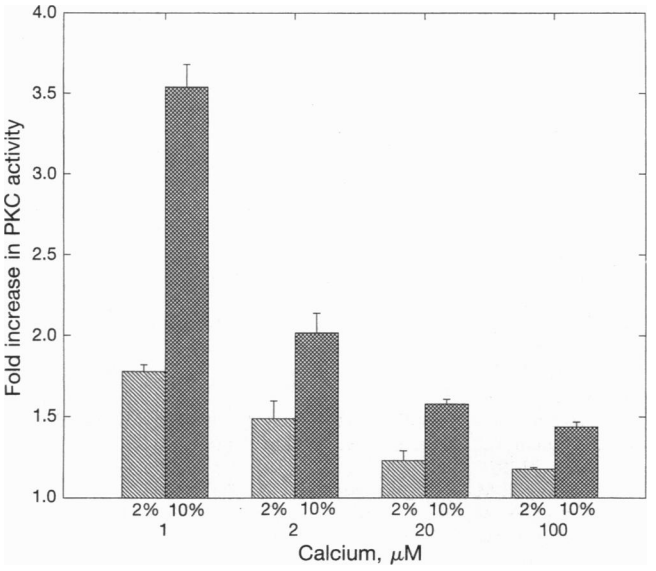

FIG. 2. Fold increase in activity of PKC-α towards histone for SLVs containing 2 mol % or 10 mol % 18:1/22:6 PE at various calcium concentrations. The SLVs were composed of 30 mol % POPS with 2 or 10 mol % 18:1/22:6 PE, the remainder being POPC. The activity is expressed as a fold increase in the activity obtained with SLVs containing no additive. Data are expressed as the mean of triplicates ± SD.

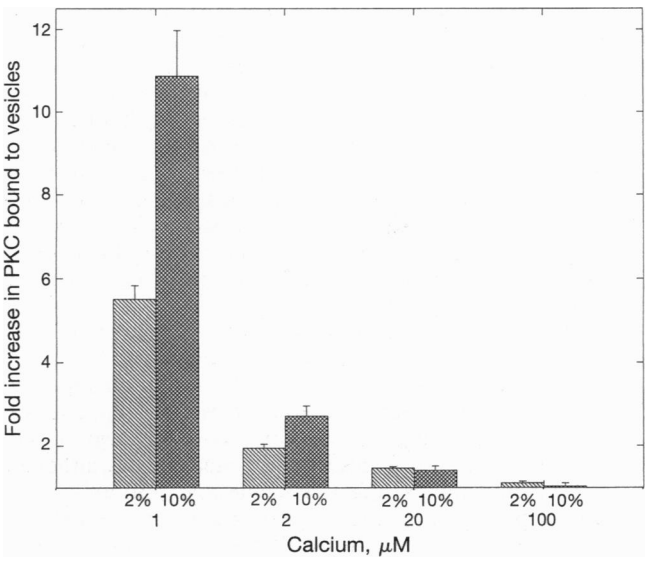

FIG. 3. Fold increase in binding of PKC-α to SLVs containing 2 mol % or 10 mol % 18:1/22:6 PE at various calcium concentrations. The SLVs were composed of 30 mol % POPS, with 2 or 10 mol % 18:1/22:6 PE, the remainder POPC. The binding is expressed as a fold increase in % of PKC-α bound to SLVs containing no additive. Data are expressed as the mean of triplicates ± SD.

activity, respectively), with the effect decreasing as the Ca^{2+} concentration was increased to 100 μM (1.18-fold and 1.44-fold increase, respectively). This effect correlated well with binding assay results (Fig. 3). Two mol % and 10 mol % 18:1/22:6 PE increased the percentage of PKC-α that was bound to SLVs of 30/70 mol % POPS/POPC by 5.5-fold and 10.9-fold, respectively, at 1 μM Ca^{2+}. This effect dropped to a 1.11-fold and 1.03-fold increase at 100 μM Ca^{2+}.

DISCUSSION

We have measured the effects of several forms of PE, containing unsaturated fatty acids, as well as 18:1/22:6 PC in affecting T_H of 16:1/16:1 PE. The 18:1/22:6 PE is moderately effective in lowering T_H. More hydrophobic substances such as DAGs or alkanes are about 5–7 times more effective in this regard (10). Nevertheless, considering that the 18:1/22:6 PE is being added to another unsaturated PE, the finding that there is still a moderate lowering of T_H illustrates the greater H_{II}-forming tendency of 18:1/22:6 PE compared with 18:0/22:6 PE, 16:1/16:1 PE, or 18:1/18:1 PE. The effect on T_H is a consequence of the properties of the added phospholipid and not just on the presence of certain acyl chains as illustrated by the opposite effects of 18:1/22:6 PE and 18:1/22:6 PC on T_H (Table 1). Therefore, the 9–12% increase in the 18:1/22:6 PE content of neuronal membranes of cold-adapted fish (1) would be expected to have a significant effect on the physical properties of these membranes. One possible consequence of this change in phospholipid composition is to alter the functioning of membrane-bound enzymes such as PKC.

As stated earlier, the activity of PKC is modulated by the bulk biophysical properties of the membrane bilayer (3, 4). Uncharged or zwitterionic additives which lower the bilayer-to-hexagonal-phase transition temperature (therefore destabilize) model PE bilayers are activators of PKC (12). It is possible that the destabilized bilayer facilitates binding of PKC and rearrangement to an active form (13). It has also been suggested that there is an optimal intrinsic bilayer curvature for the activation of PKC (14). The effect of the various species of PE on PKC activity (Fig. 1*A*) is in close agreement with their effects on T_H (Table 1). The 18:1/22:6 PC, which raises T_H, has essentially no effect on the activity of PKC. This is also expected, since the added lipid is replacing POPC in the PKC assay. Both the POPC and the 18:1/22:6 PC would form stable bilayers, having little negative curvature strain. It has previously been shown that the stimulatory effect of PEs on PKC is related to their T_H values (15). This would suggest that, as expected, the 18:1/22:6 PE, which has a particularly low T_H, activates PKC more than the other phospholipids tested. However, in comparison to DAG, the effect of 18:1/22:6 PE is small (Fig. 1*B*).

DAG is likely to have a much larger effect on PKC because it is a better hexagonal phase promoter (10) and it appears to bind specifically to a site on PKC (16). There appears to be a small degree of synergism between the effects of DAG and of 18:1/22:6 PE (Fig. 1*B*). A synergism between DAG and unsaturated fatty acids is also known to occur (17, 18).

A number of factors, including the total lipid concentration, mole fraction of phosphatidylserine, Ca^{2+} concentration, and the presence of DAG promote the activation of PKC. To the extent it is known, these factors act largely independently of one another (8). In most physiological circumstances all three cofactors—i.e., phosphatidylserine, Ca^{2+}, and DAG—are required for activation of PKC. However, this enzyme can also be activated in the absence of phosphatidylserine with PE at high concentrations of Ca^{2+} (19). It is also well known that, in the presence of DAG, lower concentrations of Ca^{2+} are required to activate PKC (20). Similarly, lower concentrations of Ca^{2+} are required in the presence of 18:1/22:6 PE (Fig. 2). Thus 18:1/22:6 PE, like other activating substances, can lower the requirements for other cofactors such as Ca^{2+}. Similarly, high Ca^{2+} concentration can conversely lower the activation of PKC by 18:1/22:6 PE.

While both DAG and 18:1/22:6 PE lower the Ca^{2+} requirement for PKC activation, the effect of DAG is more than an order of magnitude greater. However, the potency of these two activators should not be compared directly, as they are likely to serve quite different functions physiologically. DAG can act as a signal to turn on PKC activity. This occurs at low concentrations of DAG and the DAG itself is rapidly metabolized, mostly to phosphatidic acid, so that the activation of

PKC is transient, as should occur for a signaling mechanism. In contrast, 18:1/22:6 PE can build up to substantial concentrations in the membrane, about an order of magnitude greater than the concentration of DAG. Furthermore, once a steady-state level of 18:1/22:6 PE is established, as a consequence of the environmental conditions, this level is maintained over a long period of time. The 18:1/22:6 PE does not act as a signal to activate PKC to maximal or close to maximal levels, but rather modestly increases PKC activity both in the presence and in the absence of signals. That this modest increase in activity occurs over a long period of time likely has important physiological consequences.

There are two mechanisms by which changes in membrane physical properties can affect the activity of PKC. One is by promoting the translocation of the enzyme from the aqueous phase to the membrane, where it becomes activated, and the second is by activating the membrane-bound form of the enzyme. DAG can activate PKC by both mechanisms, although it acts predominantly by affecting membrane partitioning (8). The tumor promoter pristane is also able to activate PKC by increasing the proportion of membrane-bound enzyme (21). The authors of ref. 21 conclude that this is a result of the effects of pristane on the biophysical properties of the membrane. The activation caused by 18:1/22:6 PE (Fig. 2) is also largely accounted for by increased partitioning of the enzyme to the membrane (Fig. 3).

Another amphitropic enzyme whose activity is stimulated by the presence of non-lamella-forming lipids is CTP:phosphocholine cytidylyltransferase (22). It has recently been shown that incorporation of docosahexaenoic free acid into fetal lung tissue results in the stimulation of this enzyme (23). It may thus be a general phenomenon that incorporation of 22:6 into certain phospholipids increases the negative curvature strain of the membrane and leads to an activation of specific enzymes.

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1. Buda, C., Dey, I., Balogh, N., Norvath, L. I., Maderspach, K., Juhasz, M., Leo, Y. K. & Farkas, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8234–8238.
2. Rilfors, L., Hauksson, J. B. & Lindblom, G. (1994) *Biochemistry* **33,** 6110–6120.
3. Epand, R. M. (1992) in *Protein Kinase C: Current Concepts and Future Perspectives*, eds. Lester, D. S. & Epand, R. M. (Horwood, Chichester, England), pp. 135–156.
4. Zidovetzki, R. (1995) in *Structural and Biological Roles of Lipids Forming Non-Lamellar Structures*, ed. Epand, R. M. (JAI, Stanford, CT), in press.
5. Stabel, S., Schaap, D. & Parker, P. J. (1991) *Methods Enzymol.* **200,** 670–763.
6. Hannun, Y. A., Loomis, C. R. & Bell, R. M. (1995) *J. Biol. Chem.* **260,** 10039–10043.
7. Rebecchi, M., Peterson, A. & McLaughlin, S. (1992) *Biochemistry* **31,** 12742–12747.
8. Mosior, M. & Epand, R. M. (1993) *Biochemistry* **32,** 66–74.
9. Kaibuchi, K., Takai, Y. & Nishizuka, Y. (1981) *J. Biol. Chem.* **256,** 7146–7149.
10. Epand, R. M. (1985) *Biochemistry* **24,** 7092–7095.
11. Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. USA* **71,** 3036–3040.
12. Epand, R. M. & Lester, D. A. (1990) *Trends Pharmacol. Sci.* **11,** 317–320.
13. Mosior, M. & Epand, R. M. (1995) SUBMITTED.
14. Slater, S. J., Kelley, M. B., Taddeo, F. J., Ho, C., Rubin, E. & Stubbs, C. D. (1994) *J. Biol. Chem.* **269,** 4866–4871.
15. Senisterra, G. & Epand, R. M. (1993) *Arch. Biochem. Biophys.* **300,** 378–383.
16. Young, N. & Rando, R. R. (1984) *Biochem. Biophys. Res. Commun.* **122,** 818–823.
17. Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5149–5153.
18. Lester, D. S., Collin, C., Etcheberrigaray, R. & Alkon, D. L. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1522–1528.
19. Bazzi, M. D., Youakim, A. & Nelsestuen, G. L. (1992) *Biochemistry* **31,** 1125–1134.
20. Nishizuka, Y. (1986) *Science* **233,** 305–312.
21. Janz, S., Gawrich, K. & Lester, D. S. (1995) *Cancer Res.* **55,** 518–524.
22. Cornell, R. B. (1991) *Biochemistry* **30,** 5881–5888.
23. Mallampalli, R. K., Salome, R. G. & Spector, A. A. (1994) *Am. J. Physiol.* **267,** L641–L648.