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Influence of phosphatidylserine on ($Na^+ + K^+$)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes

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Phosphatidylserine (PtdSer) incubated with synaptosomal plasma membranes (SPM) of dog brain is incorporated into SPM in proportion to its concentration in the incubation medium. Low PtdSer concentrations progressively activated the SPM-associated ($Na^+ + K^+$)-stimulated ATPase and acetylcholinesterase. Increasing the PtdSer concentration above that which maximally stimulated the enzyme activities effected a progressive inhibition with respect to maximal stimulation. Arrhenius plots of ($Na^+ + K^+ + Mg^{2+}$)-dependent ATPase and 5′-nucleotidase revealed a clear break at 23–24°C for both enzymes in SPM untreated with PtdSer (controls), whereas a linear relation was obtained for SPM treated with PtdSer. Changes in the allosteric properties of ($Na^+ + K^+$)-stimulated ATPase by fluoride (F^-) and/or of 5′-nucleotidase by concanavalin A (i.e. changes of Hill coefficients) indicate that PtdSer increases the membrane fluidity. These results suggest that modifications of lipid–protein interactions in SPM induced by PtdSer may have implications in the physiological processes in the central nervous system.

We have studied the effect of small amphipathic compounds (e.g. cholesterol or its glucoside, dodecanol or its glucoside, steroid hormones etc.) on the activity of some membrane-bound enzymes (Papaphilis & Deliconstantinos, 1980; Alivisatos *et al.*, 1981*a*,*b*; Deliconstantinos, 1983), suggesting that these compounds can alter the membrane fluidity, causing functional consequences in the allosteric properties of integral enzymes (Alivisatos & Deliconstantinos, 1982; Deliconstantinos & Ramantanis, 1983; Deliconstantinos *et al.*, 1983). PtdSer vesicles (liposomes) interact with biological membranes, causing changes in physicochemical properties of the membrane (Portis *et al.*, 1979). Such alterations of the membrane structure evoke several membrane-linked events, such as modifications of the activities of membrane-bound enzymes (Floreani *et al.*, 1981), increase in output of acetylcholine from rat cerebral cortex (Casamenti *et al.*, 1979), and stimulation of the release of histamine from mast-cells (Hirata *et al.*, 1979).

In the present investigation we studied the effect of PtdSer on the activity of some integral enzymes and on the membrane fluidity of dog brain SPM. A sensitive insight into protein–lipid interactions could result from the investigation of the influence of the membrane alterations on the co-operative behaviour of membrane integral enzymes (Farias, 1980; Deliconstantinos & Ramantanis, 1983). Thus the effects of PtdSer on the allosteric properties and on the temperature-dependence of the SPM-associated ($Na^+ + K^+$)-stimulated ATPase and 5′-nucleotidase activities were studied.

Abbreviations used: PtdSer, phosphatidylserine; SPM, synaptosomal plasma membranes.

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Materials and methods

SPM from dog brain were prepared and qualitatively assessed as previously described (Papaphilis & Deliconstantinos, 1980). Compared with the original homogenate from which they were prepared, they showed 7–10-fold increase in the ($Na^+ + K^+$)-stimulated ATPase (EC 3.6.1.3) activity.

PtdSer liposomes were prepared essentially as described by Deliconstantinos *et al.* (1977). Briefly, 1 ml of PtdSer solution (20 mg/ml) in chloroform (Sigma) was pipetted into 20 ml test tubes. The solvent was evaporated under N_2. The dried PtdSer was then dispersed in 2 ml of 5 mM-Tris/HCl, pH 7.4, by sonication for 20 min at 0°C under N_2 in a Branson LS-75 sonicator. The lipid dispersions were then centrifuged at 30000 *g* for 40 min, and the sediment, containing large lipo-

somes, was discarded. The supernatant was applied to a column (1 cm × 40 cm) of Sepharose-4B (Pharmacia, Uppsala, Sweden) and eluted with 5 mM-Tris/HCl, pH 7.4. Fractions (approx. 1 ml) were collected and analysed for PtdSer. Fractions from the PtdSer peak were pooled to give the required vesicle preparations. These preparations were kept at 4°C and used within 2 days.

To determine binding of PtdSer liposomes to SPM, preincubations of SPM protein with different concentrations of PtdSer specified in Fig. 1(*a*) were performed for 3 h at 25°C in an incubation mixture of 1.15% (w/v) KCl/5 mM-Tris/HCl, pH 7.4, in a final volume of 5 ml, with continuous magnetic stirring. Samples (4 ml) were withdrawn and layered over 30 ml of 20% (w/v) sucrose/5 mM-Tris/HCl, pH 7.4, and centrifuged for 60 min at 95000 ***g*** in the SW-27 rotor of a Spinco L5-75 ultracentrifuge. SPM used for measurement of PtdSer binding were extracted overnight with 20 vol. of chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (1957), and the lipid phosphorus was measured as described by Bartlett (1959) after digestion with 70% (v/v) $HClO_4$ at 180°C. The protein content was determined by the Lowry method as described by Miller (1959), with bovine serum albumin (Sigma) as standard. After incubation of SPM with PtdSer liposomes for 3 h at 25°C, the bound and free PtdSer were separated by centrifugation as described above. The bound and free PtdSer fractions were pooled separately and extracted. The pooled chloroform extracts were dried under a stream of N_2, and the residue was dissolved in 1.0 ml of chloroform. Samples of these solutions were directly spotted on a silica-gel G t.l.c. plate (Merck). Samples of chloroform extracts of a mixture of unincubated SPM with PtdSer and PtdSer alone were also chromatographed similarly. Chromatography was developed as described by Breckenridge *et al.* (1972). The migration of extracts of membrane-bound and free PtdSer was identical with those of unincubated SPM and PtdSer, suggesting that no significant metabolic conversion of membrane-bound and free PtdSer occurred under the present incubation conditions.

$(Na^+ + K^+)$-stimulated ATPase activity of SPM was assayed in an incubation medium consisting of 40 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 3 mM-disodium ATP, 80 mM-NaCl, 20 mM-KCl, 1 mM-ouabain and 0.1 mg of SPM protein, in a final volume of 1.0 ml. Incubations were performed in the reaction medium for 30 min at temperatures of 5–42°C at 3–4°C intervals. The reaction was started by the addition of ATP and stopped with 0.2 ml of 50% trichloroacetic acid. The liberated P_i was measured by the method of Fiske & Subbarow (1925). $(Na^+ + K^+)$-stimulated ATPase activity was defined as the difference between the P_i liberated during incubations in the presence and absence of ouabain. The ouabain-inhibitable component of ATPase activity is the activity that is lost when Na^+ or K^+ is omitted from the reaction mixture. For the assay of the inhibition by F^- of the $(Na^+ + K^+)$-stimulated ATPase, the reaction mixture contained increasing amounts of NaF, as indicated in Fig. 3.

Acetylcholinesterase activity was determined by measuring the hydrolysis of acetylthiocholine by the method of Ellman *et al.* (1961). The assay mixture (3 ml) contained 1 mM-acetylthiocholine iodide, 0.125 mM-5,5′-dithionitrobenzoic acid, 100 mM-NaCl, 0.24 M-sucrose and 10 mM-Tris/HCl, pH 8.0. Protein concentration was 0.07 mg/3 ml incubation mixture. The reaction was followed spectrophotometrically by the increase in A_{412} by using a Beckman Acta MVI spectrophotometer.

5′-Nucleotidase activity (EC 3.1.3.5) was assayed by the method of Bodansky & Schwartz (1963) in an incubation medium (1.0 ml final volume) containing 50 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 10 mM-AMP and 0.25 Mg of SPM protein in the presence of various concanavalin A (Sigma) concentrations as indicated in Fig. 3. Incubations were performed at 37°C for 45 min and stopped with 0.2 ml of 50% trichloroacetic acid. The liberated P_i was measured by the method of Fiske & Subbarow (1925). Lines were fitted to the data points in Arrhenius plots and in Hill plots by regression analysis. Statistical comparisons were made by regression analysis and Student's *t* tests.

Results

The incorporation of PtdSer into SPM as a function of PtdSer concentrations is shown in Fig. 1(*a*). The amounts of PtdSer incorporated per mg of SPM protein considerably increase, in a relatively short (up to 3 h) incubation period. Within the range of PtdSer concentrations used (up to 5 μmol/mg of SPM protein) the total P content of SPM was increased from 0.825 ± 0.020 to 1.010 ± 0.030 μmol/mg of SPM protein. An initial (zero-time) absorptive binding which does not lend to any functional changes was subtracted from all measurements at all concentrations used. This initial binding may well arise from the simple adherence of PtdSer liposomes to the SPM. To determine whether or not the incubation procedure for the incorporation assay caused a metabolic conversion of the PtdSer, samples of the PtdSer-enriched SPM and free PtdSer were analysed by t.l.c. as described in the Materials and methods section. No significant degradation of incorporated or free PtdSer occurred under the present incubation conditions.

The effects of PtdSer incorporation on the main

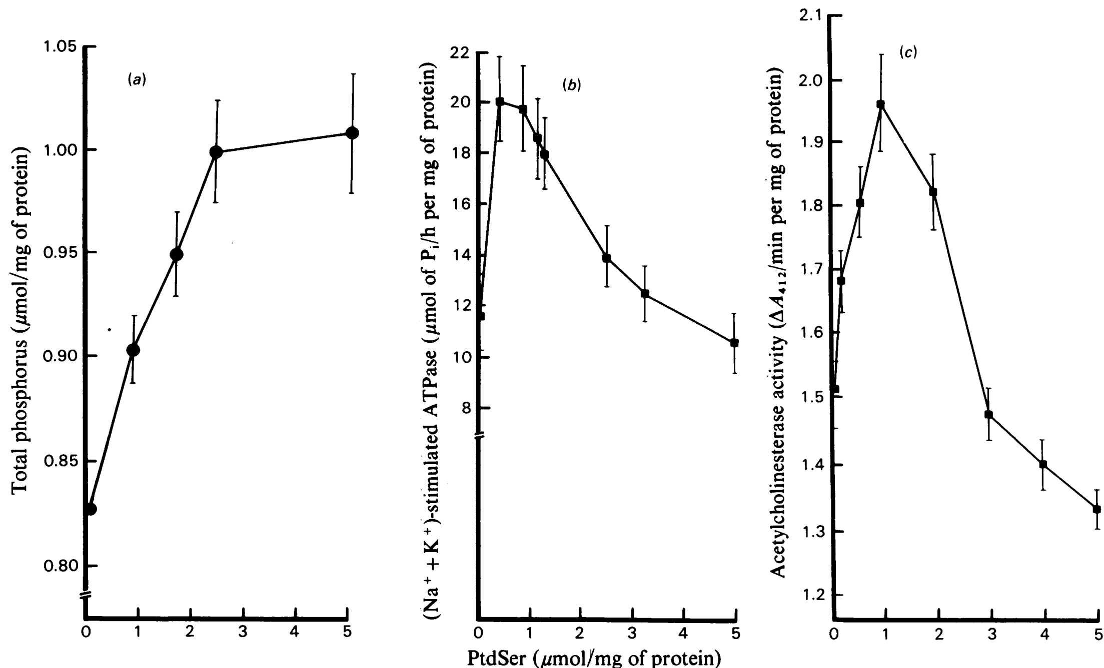

Fig. 1. *Incorporation of PtdSer into dog brain SPM (a) and its effects on membrane-bound ($Na^+ + K^+$)-stimulated ATPase activity (b) and on acetylcholinesterase activity (c)*

In (*a*) PtdSer incorporation up to 3h at 25°C was estimated by measuring the total phosphorus content of SPM after pretreatment with PtdSer liposomes. In (*c*) Acetylcholinesterase activity was estimated spectrophotometrically by measuring the increase in A_{412} with a Beckman Acta MVI spectrophotometer. Points and bars represent means ±S.D. from three different experiments.

functional parameters measured in this study, i.e. the specific activities of the ($Na^+ + K^+$)-stimulated ATPase and of acetylcholinesterase are shown in Figs. 1(*b*) and 1(*c*). Up to a concentration of 1 μmol of PtdSer/mg of SPM protein, a dramatic increase in the specific activities of both enzymes was induced, with maximal effects of approx. 80% and 40% above their original values respectively. Further increases in PtdSer concentration above this point, however, led to inhibition of the activities of the enzymes with respect to the maximal percentage of stimulation. The activity of two other SPM-bound enzymes tested, namely 5′-nucleotidase and Mg^{2+}-dependent ATPase, showed a slight but not statistically significant increase ($P > 0.05$) at different PtdSer concentrations. To examine the relative specificity of the functional effects of PtdSer on SPM, liposomes prepared from the uncharged phospholipids phosphatidylcholine and dipalmitoyl phosphatidylcholine and from the negatively charged phosphatidylglycerol and phosphatidic acid were incubated with SPM under the same conditions as those described for PtdSer. No statistically significant difference ($P > 0.05$) in the ($Na^+ + K^+$)-stimulated ATPase activity was observed in SPM treated with these phospholipids compared with untreated SPM.

Arrhenius plots of the activities of ($Na^+ + K^+ + Mg^{2+}$)-dependent ATPase and 5′-nucleotidase were clearly biphasic, exhibiting one break point at around 23–24°C for both enzymes (Figs. 2*a* and 2*b*). The biphasic nature of the Arrhenius plots for both enzymes was abolished and a linear relation was obtained after preincubation of SPM with 1 μmol of PtdSer/mg of SPM protein at 25°C for 3h. The Arrhenius activation energy (E_a) of 44.17±14.30 kJ/mol for the ($Na^+ + K^+ + Mg^{2+}$)-dependent ATPase in PtdSer-treated SPM was intermediate between the E_a obtained above (31.17±9.10 kJ/mol) and below (60.04±11.13 kJ/mol) the break point for the untreated SPM. For the 5′-nucleotidase activity, the E_a value of 41±12.80 kJ/mol in the PtdSer-treated SPM was also intermediate between those obtained above (28.49±3.91 kJ/mol) and below (59.85±9.00 kJ/mol) the break point for the untreated SPM. Thus from the data in Fig 1 it is obvious that in PtdSer-treated SPM the E_a below the break point was decreased for both

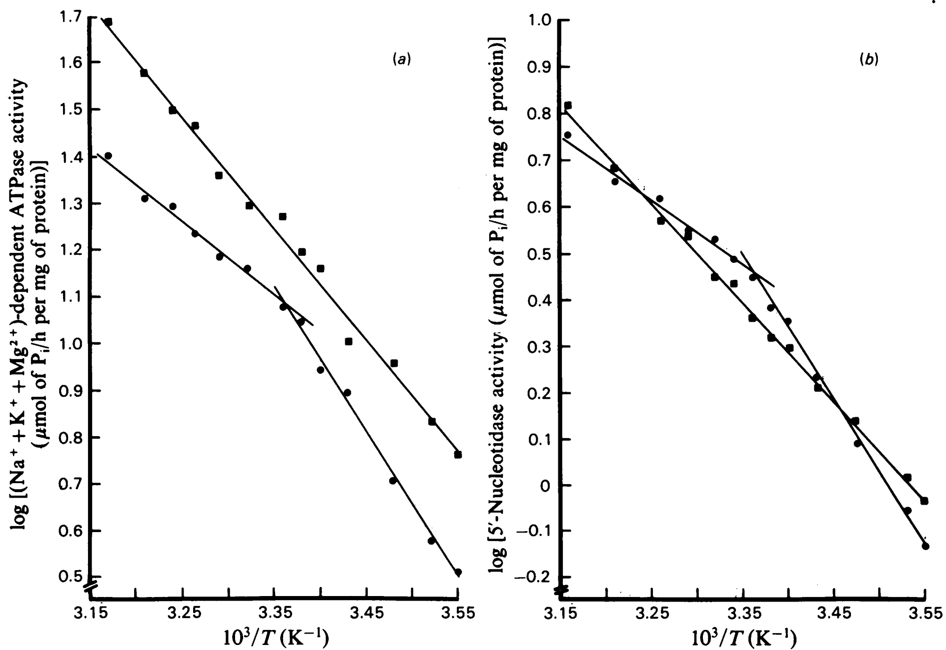

Fig. 2. *Effect of temperature on the activity of* $(Na^+ + K^+ + Mg^{2+})$*-dependent ATPase (a) and 5'-nucleotidase (b) in native (●) and PtdSer-treated (■) SPM*

Arrhenius plots of both enzyme activities were performed as described in the Materials and methods section. Each point represents the average value of duplicate determinations from a typical experiment which has been repeated three times. Units of both activities are μmol of P_i/h per mg of protein. The straight lines were fitted by the method of least squares.

$(Na^+ + K^+ + Mg^{2+})$-dependent ATPase and 5'-nucleotidase, consistent with a general increase in bilayer fluidity.

The co-operative behaviour of several membrane-bound enzymes could be used as a tool to detect modifications at the cell membrane level, since variations in the Hill coefficient in co-operative membrane enzymes depend on their relationship to lipids and on the fluidity of the latter (Farias, 1980). The allosteric inhibition of $(Na^+ + K^+)$-stimulated ATPase by fluoride (F^-) and/or of 5'-nucleotidase by concanavalin A was studied to detect possible influence of PtdSer on membrane fluidity. The enzyme 5'-nucleotidase offers a potentially interesting model for the study of the interactions between a membrane-bound enzyme and its lipid environment (Merisko *et al.*, 1981). Fig. 3 shows the curves obtained when the relative rates of the enzymic activities were plotted against different concentrations of F^- for $(Na^+ + K^+)$-stimulated ATPase and of concanavalin A for 5'-nucleotidase in SPM preincubated with PtdSer (1 μmol of PtdSer/mg of SPM protein) at 25°C for 3h. The Hill coefficient *h* (slope of the plot) for the F^- inhibition of the $(Na^+ + K^+)$-stimulated ATPase activity for the control (untreated SPM) was 1.76 ± 0.20, indicating the presence of co-operativity, which was abolished in the PtdSer-treated SPM ($h = 0.88 \pm 0.10$) (Fig. 3*a*). Likewise, the Hill coefficient *h* (1.85 ± 0.23) for the concanavalin A inhibition of the 5'-nucleotidase activity for the control was decreased to 0.93 ± 0.11 in the PtdSer-treated SPM (Fig. 3*b*). The effects of other charged phospholipids (phosphatidylglycerol and phosphatidic acid) on the allosteric properties of $(Na^+ + K^+)$-stimulated ATPase and 5'-nucleotidase were also evaluated. There was no statistically significant difference ($P > 0.005$) in Hill coefficient of both enzyme activities assayed in the presence or absence of these phospholipids. These results may suggest that PtdSer increases the membrane fluidity and subsequently changes the allosteric behaviour of the SPM-bound $(Na^+ + K^+)$-stimulated ATPase and 5'-nucleotidase.

Discussion

After injection of PtdSer liposomes, modification of glucose distribution in the body and increase in the metabolism of catecholamines in the brain of animals, with release of acetylcholine

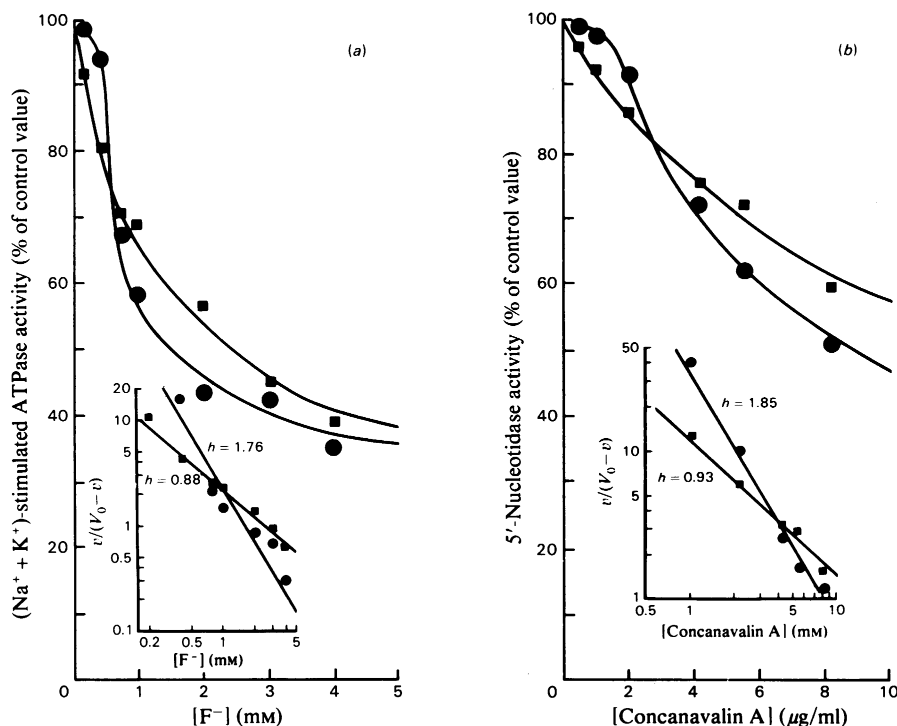

Fig. 3. (*a*) *Effect of* F^- *on the reaction rate of the* $(Na^+ + K^+)$*-stimulated ATPase, and* (*b*) *effect of concanavalin A on the reaction rate of 5′-nucleotidase for PtdSer-treated SPM* (■) *and untreated SPM* (*control*; ●)

The inserts show Hill plots of the same data. Corresponding Hill coefficients (*h*) are as indicated. The correlation coefficients (r^2) for the straight lines in the inserts are >0.95. v is the reaction velocity, and V_0 is the rate of the reaction in the absence of F^- or concanavalin A. The specific activity of 5′-nucleotidase at 37°C was 4.6 μmol of P_i/h per mg of protein. Points in the curves drawn are mean values of duplicate determinations from a typical experiment which has been repeated three times.

from the brain cortex, is observed (Mantovani *et al.*, 1982; Bruni *et al.*, 1976). Such liposomes also increased both the dopamine-sensitive adenylate cyclase activity and the cyclic AMP content of mouse brain (Leon *et al.*, 1978). PtdSer liposomes injected into human subjects affected pituitary function, possibly at the level of dopamine-mediated prolactin regulation (Masturzo *et al.*, 1977).

The ability of PtdSer to be incorporated *in vitro* into SPM isolated from dog brain, causing significant changes of the SPM-associated $(Na^+ + K^+)$-stimulated ATPase activity by altering the membrane fluidity, was investigated in the present study. Discontinuities in Arrhenius plots of membrane-bound enzymes have widely been considered to reflect a lipid-phase transition and therefore to indicate a lipid-dependence of the enzymes (Shinitzky & Barenholz, 1978). Abrupt changes in slope at a particular temperature have been taken to represent a phase transition in the lipid environment of the $(Na^+ + K^+)$-stimulated ATPase (Lenaz *et al.*, 1975). In the present study the $(Na^+ + K^+ + Mg^{2+})$-dependent ATPase and 5′-nucleotidase activities showed a break point around 23–24°C, which was abolished to give a linear relation in the Arrhenius plots in PtdSer-treated SPM (Figs. 2*a* and 2*b*). The observed intermediate values of the Arrhenius activation energies (E_a) for both enzymes in PtdSer-treated SPM as compared with untreated SPM (controls) would be expected if it is assumed that PtdSer 'fluidizes' the lipid bilayer of the SPM.

It has been reported that in the liver concanavalin A is an allosteric effector of 5′-nucleotidase, and changes of the Hill coefficient were used to detect alterations of membrane fluidity (Bruscalupi *et al.*, 1980). We have previously reported investigations of perturbations of $(Na^+ + K^+)$-stimulated ATPase of liver plasma membranes resulting from changes in bilayer fluidity during liver regeneration, based on the estimate that this enzyme of liver plasma membranes from normal animals exhibits a substantial allosteric inhibition

by F^-, which was significantly decreased in hepatectomized animals (Deliconstantinos & Ramantanis, 1983). Several preliminary experiments were conducted here to indicate that concanavalin A allosterically inhibits the SPM-associated 5′-nucleotidase and that this co-operative behaviour of the enzyme could be used as a tool to detect possible modulations of the membrane fluidity. Thus in the present study, further evidence for the increase in the bilayer fluidity in PtdSer-treated SPM was obtained from the alterations in the co-operative behaviour of the SPM-bound enzymes $(Na^+ + K^+)$-stimulated ATPase and 5′-nucleotidase. As shown in Fig. 3, the values of the Hill coefficient, *h*, for the inhibition of $(Na^+ + K^+)$-stimulated ATPase by F^- and of 5′-nucleotidase by concanavalin A were decreased in PtdSer-treated SPM, indicating a loss of the co-operativity of the enzymes consistent with a general increase in lipid fluidity. It has been reported that PtdSer is one of the acidic phospholipids that restores the activity of $(Na^+ + K^+)$-activated ATPase in delipidated membrane systems (Wheeler & Whittam, 1970), and that PtdSer, which has the highest affinity for Ca^{2+} in comparison with other phospholipids, can function as a calcium ionophore (Green *et al.*, 1979). Wheeler (1971) demonstrated that the activating effects of chelating agents, such as EGTA, on particulate as well as delipidated $(Na^+ + K^+)$-activated ATPase from brain microsomal functions was due to the removal of inhibitory Ca^{2+} ions by the chelator, and that this may also be accomplished by PtdSer. It has also been reported that Ca^{2+} ions decrease the fluidity of biological membranes, and this effect can be readily reversed on addition of excess of EGTA (Livingstone & Schachter, 1980; Gordon *et al.*, 1983). The lack of effect of phosphatidylcholine, dipalmitoyl phosphatidylcholine, phosphatidylglycerol and phosphatidic acid on the SPM-associated $(Na^+ + K^+)$-stimulated ATPase activity and their inability to alter the membrane fluidity suggest that the PtdSer-dependent fluidization of SPM may not exclusively reflect the removal of endogenous Ca^{2+} from the membrane. The activation of the endo-enzyme $(Na^+ + K^+)$-stimulated ATPase and of the ecto-enzyme acetylcholinesterase by PtdSer in intact membranes is probably due to the phase properties of this phospholipid, and this suggestion is in agreement with the view that SPM-associated $(Na^+ + K^+)$-stimulated ATPase activity requires a fluid state of the membrane lipid bilayer to be fully active (Levental & Tabakoff, 1980). Such a presumed increase in bilayer fluidity caused by PtdSer has different effects on the membrane enzymes tested. Thus 5′-nucleotidase and Mg^{2+}-activated ATPase, both of which are ecto-enzymes, are relatively insensitive to changes in bilayer fluidity, although Arrhenius plots of their activity clearly indicate lipid phase separations occurring in the bilayer. In the present study, the curves representing the changes in $(Na^+ + K^+)$-stimulated ATPase and acetylcholinesterase activities at different concentrations of PtdSer have biphasic character (Figs. 1*b* and 1*c*). The increase in the activity of the enzymes at low concentrations of PtdSer is probably due to the increase in lipid fluidity of the enzymes' immediate environment, which would relieve a constraint on the protein molecule and increase its conformational flexibility and hence its activity. Above that turning point (1 μmol of PtdSer/mg of SPM protein), the decrease in the activities of the enzymes with respect to the maximal stimulation is probably due to fluidizing effects of PtdSer on bulk lipids of SPM (see, e.g., Houslay *et al.*, 1981).

In addition to altering $(Na^+ + K^+)$-stimulated ATPase and acetylcholinesterase activities, changes in membrane characteristics by PtdSer may also affect the function of other integral enzymes or even receptors on neuronal membranes. For instance, we have shown that changes in SPM microenvironment resulted in a considerable decrease in both neurotransmitter binding and the activity of adenylate cyclase (Papaphilis & Deliconstantinos, 1980). Modulation of SPM architecture caused by changes in lipid fluidity by PtdSer could be associated with changes in enzyme activity, binding of transmitters, agonists and antagonists, as well as in synaptic plasticity, which may account for the pharmacological effects of PtdSer in the brain.

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References

Alivisatos, S. G. A. & Deliconstantinos, G. (1982) in *On Cell Function and Differentiation* (Evangelopoulos, A., ed.), part B, pp. 479–489, Alan R. Liss, New York

Alivisatos, S. G. A., Deliconstantinos, G., Papaphilis, A. & Theodosiadis, G. (1981*a*) *Biochim. Biophys. Acta* **643**, 642–649

Alivisatos, S. G. A., Deliconstantinos, G. & Theodosiadis, G. (1981*b*) *Biochim. Biophys. Acta* **643**, 650–658

Bartlett, S. R. (1959) *J. Biol. Chem.* **234**, 466–468

Bodansky, O. & Schwartz, M. K. (1963) *J. Biol. Chem.* **238**, 3420–3427

Breckenridge, W. C., Gombos, G. & Morgan, I. G. (1972) *Biochim. Biophys. Acta* **266**, 695–707

Bruni, A., Toffano, G., Leon, A. & Boarato, E. (1976) *Nature (London)* **260**, 331–333

Bruscalupi, G., Curatola, G., Lenaz, G., Leoni, S., Mangiantini, M. T., Mazzanti, L., Spagnualo, S. &

Trentalance, A. (1980) *Biochim. Biophys. Acta* **597**, 263–273
Casamenti, F., Mantovani, P., Amaducci, L. & Pepeu, G. (1979) *J. Neurochem.* **32**, 529–533
Deliconstantinos, G. (1983) *Neurochem. Res.* **8**, 1143–1152
Deliconstantinos, G. & Ramantanis, G. (1983) *Biochem. J.* **212**, 445–452
Deliconstantinos, G., Gregoriadis, G., Abel, G., Marvyn, J. & Robertson, D. (1977) *Biochem. Soc. Trans.* **5**, 1327–1329
Deliconstantinos, G., Anastasopoulou, K. & Karayiannakos, P. (1983) *Biochem. Pharmacol* **32**, 1309–1312
Ellman, G. L., Courtney, D., Andres, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95
Farias, R. N. (1980) *Adv. Lipid Res.* **17**, 251–283
Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
Floreani, M., Bonetti, A. C. & Carpenedo, F. (1981) *Biochem. Biophys. Res. Commun.* **31**, 1337–1344
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
Gordon, L. M., Whetton, A. D., Rawal, S., Esgate, J. A. & Houslay, M. D. (1983) *Biochim. Biophys. Acta* **729**, 104–114
Green, D. E., Fry, M. & Blondin, G. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 257–261
Hirata, F., Axelrod, J. & Crews, F. T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4813–4816
Houslay, M. D., Dipple, I. & Gordon, L. M. (1981) *Biochem. J.* **197**, 675–681
Lenaz, G., Curatola, G. & Masotti, L. (1975) *J. Bioenerg.* **7**, 223–229
Leon, A., Benvegnu, D., Doffano, G., Orlando, P. & Mascori, P. (1978) *J. Neurochem.* **30**, 23–26
Levental, M. & Tabakoff, B. (1980) *J. Pharmacol. Exp. Ther.* **212**, 315–319
Livingstone, C. J. & Schachter, D. (1980) *Biochemistry* **19**, 4823–4827
Mantovani, P., Aporti, F., Bonetti, A. C. & Pepeu, G. (1982) in *Phospholipids in the Nervous System* (Horrocks, L., Ansell, G. B. & Procellati, G., eds.), vol. 1, pp. 165–172, Raven Press, New York
Masturzo, P., Galamini, A., Murialdo, G., Nizzo, M. C. & Toffano, G. (1977) *N. Engl. J. Med.* **297**, 338–339
Merisko, E. M., Ojakian, G. K. & Widnell, C. C. (1981) *J. Biol. Chem.* **256**, 1983–1993
Miller, G. L. (1959) *Anal. Chem.* **31**, 964–968
Papaphilis, A. & Deliconstantinos, G. (1980) *Biochem. Pharmacol.* **29**, 3325–3327
Portis, A., Newton, C., Pangborn, W. & Papahadjopoulos, D. (1979) *Biochemistry* **18**, 780–790
Shinitzky, M. & Barenholz, Y. (1978) *Biochim. Biophys. Acta* **515**, 367–394
Wheeler, K. P. (1971) *Biochem. J.* **125**, 71P
Wheeler, K. P. & Whittam, R. (1970) *J. Physiol. (London)* **207**, 303–328