

Uptake of Exogenous Phosphatidylserine by Human Neuroblastoma Cells Stimulates the Incorporation of [methyl-¹⁴C]Choline into Phosphatidylcholine

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Abstract: The phosphatidylserine (PtdSer) content of human cholinergic neuroblastoma (LA-N-2) cells was manipulated by exposing the cells to exogenous PtdSer, and the effects on phospholipid content, membrane composition, and incorporation of choline into phosphatidylcholine (PtdCho) were investigated. The presence of liposomes containing PtdSer (10–130 μ M) in the medium caused time- and concentration-dependent increases in the PtdSer content of the cells, and smaller and slower increases in the contents of other membrane phospholipids. The PtdSer levels in plasma membrane and mitochondrial fractions prepared by discontinuous sucrose density gradient centrifugation increased by 50 and 100%, respectively, above those in control cells after 24 h of exposure to PtdSer (130 μ M). PtdSer caused a concomitant, concentration-dependent increase of up to twofold in the incorporation of [methyl-¹⁴C]choline chloride into PtdCho at a choline concentration (8.5 μ M) compatible with activation of the CDP-choline pathway, suggesting that the levels of PtdSer in membranes may serve as a stimulus to regulate overall membrane composition. PtdSer caused a mean in-

crease of 41% in PtdCho labeling, but the phorbol ester, phorbol 12-myristate 13-acetate (PMA), which stimulates PtdCho synthesis in a number of cell lines, increased [¹⁴C]PtdCho levels by only 14% in LA-N-2 cells, at a concentration (100 nM) which caused complete translocation of the calcium- and phospholipid-dependent enzyme protein kinase C to the membrane. The translocation was inhibited by prior exposure of the cells to PtdSer. Treatment with PMA for 24 h diminished protein kinase C activity by 80%, but increased the labeling of PtdCho in both untreated and PtdSer-treated cells. These data suggest that uptake of PtdSer by LA-N-2 cells alters both the phospholipid composition of the membrane and synthesis of the major membrane phospholipid PtdCho; the latter effect does not involve activation of protein kinase C. **Key Words:** Phospholipids—Phosphatidylserine—Phorbol esters—Phosphatidylcholine—Protein kinase C—Neuroblastoma. **Slack B. E. et al.** Uptake of exogenous phosphatidylserine by human neuroblastoma cells stimulates the incorporation of [methyl-¹⁴C]choline into phosphatidylcholine. *J. Neurochem.* 53, 472–481 (1989).

Phosphatidylserine (PtdSer), a major negatively-charged phospholipid component of mammalian cell membranes, influences fluidity and fusion capacity of membrane lipid bilayers (Papahadjopoulos et al., 1973) and modulates the activity of various enzymes, including cytidyltransferase (CTP: cholinephosphate cytidyltransferase; Choy and Vance, 1978) and protein kinase C (Takai et al., 1979a). A variety of techniques have been used to study the regulation and function of PtdSer in the membranes of intact cultured mammalian cells, including the development of mutants deficient in PtdSer synthesis (Kuge et al., 1985) and

the exposure of cells to exogenous PtdSer added to the medium (Nishijima et al., 1986). Using a human cholinergic neuroblastoma (LA-N-2) cell line (Seeger et al., 1977), we have attempted to manipulate the PtdSer composition of the membrane in intact cells, and to analyze the effects of such alterations on the metabolism of phosphatidylcholine (PtdCho), the major phospholipid component of cell membranes.

The rate limiting step in the synthesis of PtdCho is catalyzed by cytidyltransferase (Pelech and Vance, 1984), an enzyme of the Kennedy pathway, which is the major route of PtdCho synthesis in most cells

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Abbreviations used: AcCho, acetylcholine; GroPCho, glycerophosphocholine; LysoPtdSer, lysophosphatidylserine; PtdCho, phosphatidylcholine; PCho, phosphocholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PtdSer, phosphatidylserine; CerPCho, sphingomyelin.

(Kennedy and Weiss, 1956). In a number of different cell lines, activators of the calcium- and phospholipid-dependent enzyme protein kinase C (Nishizuka, 1984a), such as the phorbol esters and diacylglycerols, stimulate PtdCho synthesis (Kreibich et al., 1971; Suss et al., 1971; Paddon and Vance, 1980; Warden and Friedkin, 1984; Kreutter et al., 1985; Liscovitch et al., 1986, 1987b; Kolesnick and Paley, 1987). In this report, we show that exposure of LA-N-2 cells to liposomal suspensions of PtdSer alters membrane phospholipid composition and stimulates the incorporation of [14 C]choline into PtdCho. In *in vitro* mixed micellar systems, the binding of protein kinase C is dependent on the presence of PtdSer and calcium, whereas its activation requires addition of diacylglycerol (Hannun et al., 1985; Ganong et al., 1986). We investigated the possibility that the stimulation of PtdCho labeling in LA-N-2 cells by exogenous PtdSer might be due to modulation of protein kinase C activity, perhaps because of alterations in its distribution between the cytoplasm (where it is inactive) and the membrane (where it is activated in the presence of PtdSer and diacylglycerol) (Nishizuka, 1984a). To test this hypothesis, we compared the effects of PtdSer and phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C in many cell types, on PtdCho synthesis and on protein kinase C activity in these cells.

MATERIALS AND METHODS

Materials

LA-N-2 cells (passage 69) were kindly provided by Dr. R. C. Seeger (Department of Pediatrics, UCLA Medical School, Los Angeles, CA, U.S.A.). Plasticware for tissue culture was from Falcon Plastics (Los Angeles, CA, U.S.A.) or from Costar Co. (Cambridge, MA, U.S.A.). Leibovitz L-15 nutrient medium, F-12, Dulbecco's modified Eagle's medium, and sera were from GIBCO Laboratories (Grand Island, NY, U.S.A.). Serum-free N2 medium was made according to the method of Bottenstein and Sato (1979) and routinely supplemented with 100 μ M choline. When cells were labeled for short periods of time with [14 C]choline, nonradioactive choline was omitted from the medium. PMA, leupeptin, phenylmethylsulfonyl fluoride (PMSF), PtdSer, Nonidet P-40, and histone type III-S were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lysophosphatidylserine (LysoPtdSer) was kindly supplied by Fidia (Abano Terme, Italy). [γ - 32 P]Adenosine triphosphate (32 P]ATP; 10 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.) and [14 C]choline chloride (58.5 mCi/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Solutions of PMA (10 mM in dimethyl sulfoxide) were kept frozen at -20° C.

Cell culture

LA-N-2 cells (passage 73–105) were maintained in L15 medium, supplemented with 10% fetal calf serum and 100 μ M choline in humidified room air at 37° C. Medium was changed once or twice weekly. Cells were subcultured at subconfluent density by brief (1 min) exposure to a solution of 0.1% Viokase (a crude extract of pancreatic enzymes; Viobin Co., Monticello, IL, U.S.A.) in phosphate-buffered saline, and plated onto 60- or 35-mm dishes. At 24 h prior to treat-

ments, the medium bathing the cells was replaced with N2 medium supplemented with 100 μ M choline.

Phospholipid assay

Cells were exposed for various periods of time to liposomal suspensions of PtdSer in N2 medium, or to N2 medium alone. To make the liposomes, PtdSer was dissolved in chloroform, dried under nitrogen, resuspended in phosphate-buffered saline, and sonicated on ice for 5 min. The suspension was boiled for 5 min to sterilize it, and diluted with N2 medium to the desired concentration. Stock concentrations of PtdSer were determined by extracting and assaying for phospholipid content. Stocks contained pure PtdSer as determined by one-dimensional thin-layer chromatography. Following exposure to PtdSer, cells were washed twice with saline, harvested with Viokase, and suspended in N2 medium. An aliquot was counted to determine cell number. The cell suspension was centrifuged briefly to pellet the cells, and the medium was discarded. The pellet was extracted in chloroform-methanol according to the method of Folch et al. (1957), and the phospholipids were separated by one-dimensional thin-layer chromatography on preadsorbent silica gel G plates (Analtech, Newark, DE, U.S.A.) using a mobile phase containing chloroform/ethanol/water/triethylamine (30:34:8:30 by volume). Under these conditions, phosphatidic acid cochromatographs with phosphatidylinositol (PtdIns), but because of the small amount normally present in the cells, its presence was neglected in our calculations. In some experiments, a different mobile phase composed of chloroform/methanol/ammmonium hydroxide (65:25:5 by volume) was used in order to separate PtdCho from LysoPtdSer. The phospholipids were measured by phosphate assay (Svanborg and Svennerholm, 1961).

Labeling of cells with [14 C]choline and measurement of lipid- and water-soluble metabolites

Following pretreatment with PtdSer or PMA, the medium was removed, and the cells were rinsed with N2 medium and then incubated for 2 h at 37° C with [14 C]choline chloride (0.5 μ Ci/ml; 8.5 μ M) in choline-free N2 medium. The medium was removed, the dishes were rinsed once with chilled N2 medium, and 1 ml of ice-cold methanol was added to each dish. [The addition of methanol to cultured cells prior to scraping has been shown to prevent cellular metabolism of lipids as effectively as immediate extraction of cells with chloroform and methanol (Van Veldhoven and Bell, 1988).] The cells were scraped from the plates and two volumes of chloroform were added. The mixture was vortexed, 1 ml of water was added, and the mixture was vortexed again. The upper phase containing the water-soluble labeled metabolites of choline [acetylcholine (AcCho), phosphocholine (PCho), and glycerophosphocholine (GroPCho)] and the lower phase containing phospholipids were separated by brief centrifugation. Both phases were dried under vacuum. The organic phase was redissolved in chloroform/methanol (1:1) and an aliquot counted by liquid scintillation spectrometry. As determined by thin layer chromatography, 90% of the label incorporated into [14 C]choline-labeled phospholipids was located in PtdCho. Labeled water-soluble choline metabolites were analyzed by a modification of a high performance liquid chromatographic assay described previously (Liscovitch et al., 1985, 1987b). Proteins were determined by the method of Lowry et al. (1951).

Protein kinase C assay

Cells previously exposed to N2 medium, or to N2 containing PtdSer, were incubated in fresh N2 medium contain-

ing various concentrations of PMA for 1 h, at which time the effect of PMA in these cells was found to be maximal. The cells were scraped from the dishes in 2 ml of Buffer A (20 mM Tris-HCl, 2 mM sodium EDTA, 0.5 mM EGTA, and 350 μ g/ml of PMSF at pH 7.5; Kraft and Anderson, 1983), to which had been added 0.33 M sucrose, and sonicated with 10 1-sec pulses. The lysate was centrifuged at 100,000 g for 1 h, and the resulting supernatant fluid kept on ice. The pellet was solubilized by homogenizing in Buffer A containing 0.1% Nonidet P-40 and centrifuged at 100,000 g for 1 h. The supernatant fluid containing the detergent-solubilized enzyme, and the supernatant fluid from the first centrifugation step containing the soluble enzyme, were applied to identical diethylaminoethyl (DEAE) cellulose columns equilibrated with Buffer A. The enzyme was eluted with 0.5 ml of 0.15 M NaCl in the same buffer. The assay tubes contained, in a volume of 0.25 ml, the following: Tris-HCl (25 mM), CaCl₂ (1 mM), MgCl₂ (10 mM), PMSF (0.4 mM), histone (500 μ g/ml), ATP (0.1 mM), 10⁶ dpm [³²P]ATP, and 1–6 μ g of the enzyme preparation. Each sample was assayed in triplicate, in both the presence and absence of PtdSer (20 μ g/ml). The assay tubes were incubated for 5 min at 37°C, and the phosphorylated histone was precipitated with 20% trichloroacetic acid and filtered using 0.45 μ m filters (Millipore Corp., Bedford, MA, U.S.A.). The amount of radioactivity on the filters was determined by liquid scintillation counting. Protein kinase C activity was calculated as the difference between the radioactivity incorporated into trichloroacetic acid-insoluble material in the presence and absence of PtdSer. The in vitro properties of protein kinase C extracted from LA-N-2 cells were similar to those reported previously for NG108-15 cells (Liscovitch et al., 1987b); in the presence of 20 μ g/ml of PtdSer and low concentrations of calcium, protein kinase activity was slightly above background, but was increased to near maximal levels by the addition of 20 nM PMA. Protein was assayed by the method of Lowry et al. (1951).

Subcellular fractionation procedure

Cells were harvested with Viokase, resuspended in serum-containing L-15 medium, and pelleted as described above. The cells were resuspended in five volumes of lysis buffer (0.5 mM dithiothreitol, 1 mM EGTA, 0.5 mM sodium bicarbonate, and 10 mM HEPES at pH 7.9; Imai and Gershengorn, 1987) and incubated at 37°C for 5 min. Ten volumes of ice-cold lysis buffer were added, and the cell sus-

pension was homogenized (five strokes with a teflon pestle in a glass homogenizer) and centrifuged for 20 min at 1,000 g to sediment the nuclei. The nuclear pellet was designated fraction no. 6. The supernatant fraction was centrifuged for 1 h at 100,000 g, and the pellet was resuspended in lysis buffer, layered on top of a discontinuous sucrose density gradient consisting of fractions of 1.2, 1.0, 0.8, 0.6, 0.4, and 0.2 M sucrose in lysis buffer, and centrifuged for 20 min at 188,000 g in a Beckman SW41Ti rotor. The resulting bands (designated fractions no. 1–5 from top to bottom of the gradient) were diluted in lysis buffer, sedimented at 70,000 g, and frozen at –70°C for later analysis. ATPase activity was determined by the method of Post and Sen (1967), and succinate dehydrogenase activity was determined by measuring tetrazolium reductase activity (Pennington, 1961). Phospholipids were extracted and assayed as described above.

Statistics

The statistical significance of differences was estimated by Student's *t* test or by two-way analysis of variance. Values quoted in the text are expressed as means \pm SEM unless otherwise stated.

RESULTS

LA-N-2 cells take up PtdSer and incorporate it into membranes

Exposure of LA-N-2 cells to PtdSer-containing liposomes suspended in N2 medium led to time- and concentration-dependent increases in cell PtdSer content (Table 1; see Fig. 2b). At low (30 μ M) PtdSer concentrations, uptake occurred progressively over a period of days and was accompanied by small but significant increases in PtdCho, PtdIns, phosphatidylethanolamine (PtdEtn), and sphingomyelin (CerPCho) contents (Table 1). After 4 days of exposure to PtdSer, when cell PtdSer contents were increased sevenfold, cell contents of both PtdCho and PtdEtn were \sim 40% higher than those of untreated time-matched controls. No further increases were observed after 6 days. Levels of PtdIns and CerPCho were approximately doubled after 2 days in PtdSer, and remained so until day 6 (Table 1). LA-N-2 cells, once transferred to N2 me-

TABLE 1. Phospholipid content of cells exposed to PtdSer (30 μ M) for 1–6 days

Day	Treatment	Phospholipid content (pmol/10,000 cells)				
		PtdSer	PtdCho	PtdIns	PtdEtn	CerPCho
1	Control	57 \pm 7 (4)	137 \pm 26 (7)	32 \pm 4 (4)	49 \pm 12 (3)	19 \pm 4 (4)
	PtdSer	143 \pm 19 (4) ^a	131 \pm 17 (7)	36 \pm 5 (4)	45 \pm 2 (3)	30 \pm 9 (4)
2	Control	42 \pm 5 (7)	151 \pm 17 (7)	27 \pm 6 (6)	73 \pm 11 (6)	22 \pm 11 (5)
	PtdSer	161 \pm 19 (7) ^a	167 \pm 17 (7)	52 \pm 10 (6) ^a	78 \pm 10 (6)	49 \pm 8 (5) ^a
4	Control	44 \pm 3 (7)	152 \pm 12 (7)	17 \pm 2 (7)	54 \pm 4 (7)	17 \pm 3 (6)
	PtdSer	331 \pm 49 (7) ^a	211 \pm 13 (7) ^a	47 \pm 6 (7) ^a	78 \pm 3 (7) ^a	33 \pm 3 (6) ^a
6	Control	58 \pm 8 (3)	175 \pm 1 (3)	38 \pm 2 (3)	63 \pm 5 (3)	29 \pm 3 (3)
	PtdSer	471 \pm 80 (3) ^a	242 \pm 1 (3) ^a	70 \pm 9 (3) ^a	86 \pm 5 (3) ^a	54 \pm 7 (3) ^a

Cells were treated with N2 medium, or N2 supplemented with 30 μ M PtdSer for 1–6 days, then harvested and assayed for phospholipid content as described in Materials and Methods. Values represent means \pm SEM. The number of individual determinations is indicated in parentheses.

^a Values are significantly different from time-matched controls, *p* < 0.05.

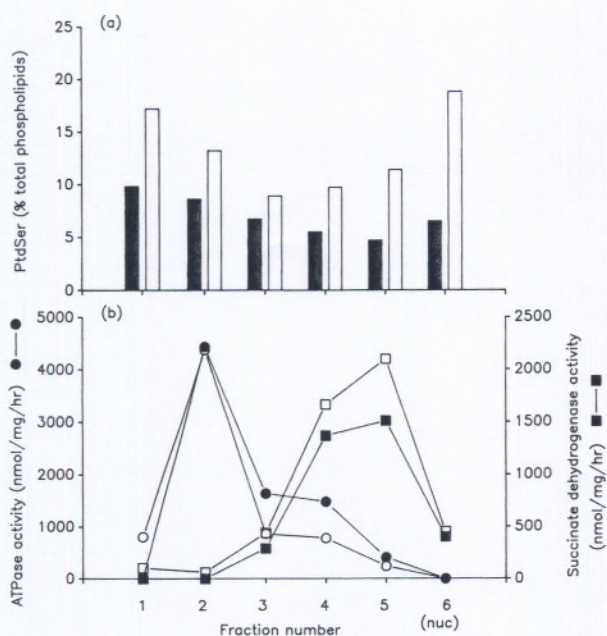


FIG. 1. PtdSer content and characterization of subcellular membrane fractions from LA-N-2 cells. Cells were exposed to N2 medium, or N2 supplemented with PtdSer (130 μ M) for 24 h. The cells were lysed and the membranes were prepared by centrifugation on a sucrose density gradient (see Materials and Methods). The fractions were collected and assayed for phospholipid content, Na⁺,K⁺-ATPase activity, and succinate dehydrogenase activity. **a:** PtdSer content of fractions 1–5 from the sucrose density gradient, and of the low-speed nuclear pellet (“nuc”, fraction 6) from control (filled bars) and PtdSer-treated (unfilled bars) cells. The graph shows values derived from two experiments. **b:** Na⁺,K⁺-ATPase (circles) and succinate dehydrogenase (squares) activity in subcellular membrane fractions from control (filled symbols) and PtdSer-treated (unfilled symbols) cells.

dium, undergo only one or two divisions before becoming quiescent. The cell number per dish after 4–6 days was not affected by the presence of 30 μ M PtdSer in the N2 medium.

Overnight treatment with higher concentrations of PtdSer also increased the total PtdCho content of the cells (Fig. 2b). The increase in cell PtdSer content (with 100 μ M PtdSer in the medium) was rapid, approaching half-saturation after 3 h (not shown). In four of five experiments, PtdCho content was elevated (to 143 \pm 6% of control) by 24-h treatment with 100 μ M PtdSer. Concomitant increases in PtdEtn, PtdIns, and CerPCho to 164 \pm 7, 173 \pm 39, and 144 \pm 32% of control, respectively, were also observed. The most consistently affected phospholipids, other than PtdSer itself, were PtdCho and PtdEtn. The increase in PtdCho content was observed irrespective of the thin-layer chromatographic system used for its purification (see Materials and Methods). Incubation of cells in the presence of elevated serine concentrations (up to 50 mM) or in 10 μ M LysoPtdSer (higher concentrations were toxic to the cells) did not increase their PtdSer content, suggesting that uptake of PtdSer did not require its prior metabolism.

Subcellular membrane fractions prepared from cultures of LA-N-2 cells exposed to PtdSer (130 μ M) for 24 h contained increased amounts of PtdSer (expressed as a percentage of total phospholipid content) (Fig. 1a). The observed increases ranged from approximately 30% in fraction 3, and 50% in fraction 2, to twofold increases (on average) in fractions 4 and 5. A threefold elevation of PtdSer was observed in the crude nuclear pellet (which should also contain unbroken cells and large cell fragments) (Fig. 1a). Fraction 2 was enriched with the plasma membrane marker Na⁺,K⁺-ATPase, and fractions 4 and 5 contained the mitochondrial marker succinate dehydrogenase (Fig. 1b). (No attempt was made to determine the extent to which the mitochondrial fraction was contaminated with microsomal membranes.) The results are consistent with the possibility that the contents of PtdSer in both intracellular and plasma membranes were elevated by pretreatment with PtdSer. Small elevations in PtdEtn and PtdCho content (nanomoles per milligram of protein) also occurred in some fractions (data not shown). The activities of Na⁺,K⁺-ATPase and succinate dehydrogenase and their distribution among various membrane fractions were similar in PtdSer-treated and untreated cells (Fig. 1b).

Exposure to PtdSer stimulates incorporation of [¹⁴C]choline into PtdCho

LA-N-2 cells treated overnight with various concentrations of PtdSer (0–130 μ M) and then labeled for 2 h with [¹⁴C]choline showed a concentration-dependent stimulation of PtdCho labeling by PtdSer of up to twofold (Fig. 2a). The degree of stimulation was correlated with the amount of PtdSer taken up by the cells (Fig. 2b) and paralleled the increase in PtdCho content (Fig. 2b). PtdCho labeling was also stimulated by PtdSer in [¹⁴C]choline-prelabeled cells (not shown), suggesting that this effect was not due to a change in the specific activity of the precursor pool. The mean increase of [¹⁴C]choline incorporation into PtdCho by 100 μ M PtdSer in 11 experiments was 141 \pm 6% (mean \pm SEM); no significant change in the protein content per dish was observed (control, 0.523 \pm 0.06; PtdSer, 0.525 \pm 0.056 mg per dish). Table 2 shows a typical experiment in which the increase in [¹⁴C]PtdCho (after 2-h labeling with [¹⁴C]choline) elicited by 24-h pretreatment with 100 μ M PtdSer was accompanied by a decrease in [¹⁴C]PCho levels. When the results from three such experiments were combined and expressed as a percentage of control values, cells pretreated with PtdSer showed a mean significant increase in [¹⁴C]PtdCho to 144 \pm 9% of control, and a mean decrease in [¹⁴C]PCho to 90 \pm 4% of control. (The average decrease did not achieve significance.) [¹⁴C]AcCho levels did not change (101 \pm 9% of control in PtdSer-treated cells) (Table 2), again suggesting that PtdSer did not affect choline specific activity. In two of three experiments, the amount of label lost from [¹⁴C]PCho was greater than that transferred to [¹⁴C]PtdCho (Table 2).

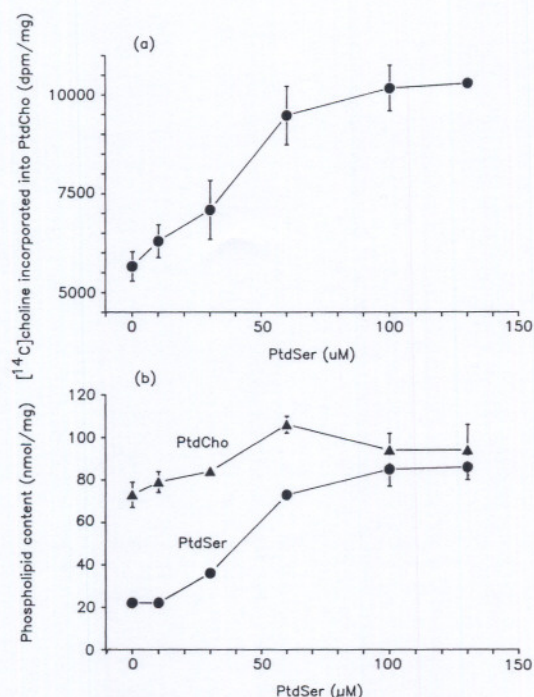


FIG. 2. PtdSer causes concentration-dependent increases in PtdCho labeling and content. Cells were treated overnight with various concentrations of PtdSer in N2 medium. **a:** Cells were washed once, and labeled for 2 h with [^{14}C]choline. The incorporation of radioactivity into PtdCho was determined as described in Materials and Methods. Values represent means \pm SEM of replicate determinations from two experiments. **b:** Cells were extracted and assayed for PtdSer (filled circles) and PtdCho (filled triangles). Values represent means \pm SEM from a representative experiment.

The effects of PtdSer on [^{14}C]PtdCho degradation were examined in pulse-chase experiments in which cells were pre-labeled for 24 h in the absence of PtdSer, then exposed to various concentrations of PtdSer for the first 24 h of a 72-h chase period in N2 medium supplemented with 100 μM choline (Fig. 3). The relatively long chase period was chosen in order to allow

TABLE 2. Effect of PtdSer on labeling of PtdCho, AcCho, and PCho

Treatment	Radioactivity (dpm/mg of protein)		
	PtdCho	AcCho	PCho
Control	3,560 \pm 48	64,024 \pm 2,889	93,421 \pm 2,696
PtdSer	5,151 \pm 202 ^a	66,010 \pm 2,601	78,798 \pm 1,515 ^a

Cells were treated for 24 h with 100 μM PtdSer or N2 medium alone, then washed and labeled with [^{14}C]choline for 2 h. The cells were extracted and the metabolites in the aqueous and lipid-soluble phases were measured as described in Materials and Methods. Values represent means \pm SEM of triplicate determinations from a representative experiment which was repeated three times.

^a Values are significantly different from controls, $p < 0.01$ by Student's t test.

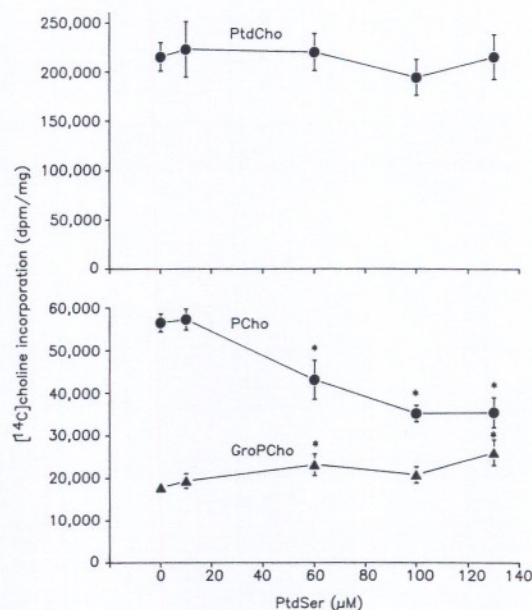


FIG. 3. PtdSer stimulates PtdCho degradation in prelabeled cells. Cells were labeled overnight with [^{14}C]choline in N2 medium containing 5% dialyzed fetal calf serum, then chased with N2 containing 100 μM choline and various concentrations of PtdSer. After 24 h, the medium was replaced with control N2 medium. After an additional 48 h, the cells were extracted and assayed for [^{14}C]PtdCho (upper panel), and for [^{14}C]PCho (filled circles) and [^{14}C]GroPCho (filled triangles) (lower panel). Values represent means \pm SD of replicate determinations from a representative experiment that was repeated twice. * $p < 0.05$; values are significantly different from corresponding control values.

detectable amounts of the degradation product [^{14}C]GroPCho to accumulate. PtdSer added during the chase period resulted in a concentration-dependent decrease in [^{14}C]PCho and a concomitant significant increase in the production of [^{14}C]GroPCho (Fig. 3), the product of sequential degradation of PtdCho by phospholipase A and lysophospholipase. The levels of [^{14}C]choline and [^{14}C]AcCho in these cells were very low (the two compounds combined contained $< 5,000$ dpm/mg of protein), and were not altered by PtdSer treatment. Treatment of cells for 24 h with various concentrations of PtdCho did not alter the incorporation of [^{14}C]choline into PtdCho in cells in which PtdSer treatment increased labeling by 50% (Fig. 4).

PMA activates protein kinase C but has little effect on PtdCho labeling

It is known that phorbol esters stimulate choline incorporation into PtdCho in many cell lines, an effect which may be partially attributable to activation of protein kinase C (Liscovitch et al., 1987b). Because the PtdSer concentration can influence the binding of protein kinase C in vitro to mixed micelles composed of Triton X-100, phospholipids, and diacylglycerols (Hannun et al., 1985; Ganong et al., 1986), we hypothesized that increased levels of PtdSer in LA-N-2 cell membranes might stimulate PtdCho labeling by

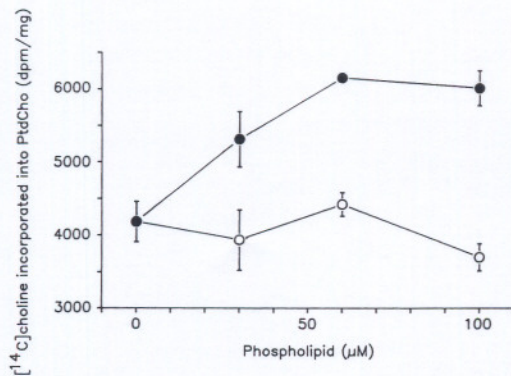


FIG. 4. PtdSer, but not PtdCho, stimulates [¹⁴C]PtdCho synthesis. Cells were treated for 24 h with various concentrations of PtdSer (filled circles) or PtdCho (unfilled circles) in N2 medium. The cells were then washed once, and labeled for 2 h with [¹⁴C]choline. Incorporation of radioactivity into PtdCho was determined as described in Materials and Methods. Values represent means ± SEM of triplicate determinations from a single experiment.

enhancing the binding of protein kinase C to membranes, thereby stimulating its activity. Therefore, the effects of PtdSer and the phorbol ester PMA on PtdCho synthesis in LA-N-2 cells were compared. In experiments in which cells were treated with 100 µM PtdSer or N2 medium alone for 24 h, then washed and labeled for 2 h in the presence of vehicle (dimethyl sulfoxide) or PMA (100 nM), respectively, PtdSer increased [¹⁴C]PtdCho synthesis to 140 ± 8% of control values (mean ± SEM of six experiments), whereas, in the same experiments, PMA increased labeling to only 114 ± 4% of control values (data not shown). However, in separate experiments, this concentration of PMA caused maximal activation of protein kinase C [seen as the translocation of the enzyme from the cytosol to the membrane (Fig. 5)] within 1 h of administration in LA-N-2 cells. Prolonged treatment with phorbol es-

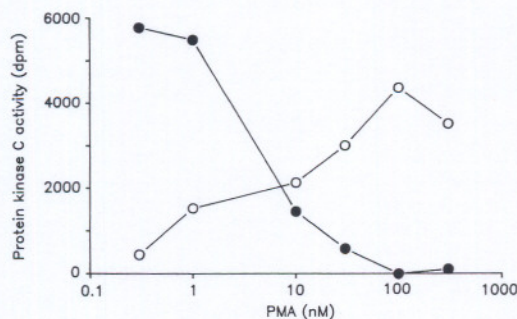


FIG. 5. Translocation of protein kinase C activity from the cytoplasm to the membrane of LA-N-2 cells by PMA. Cells were pretreated with N2 medium for 24 h, then exposed to various concentrations of PMA in N2 medium for 1 h. Cells were sonicated and separated into soluble and particulate fractions which were assayed for protein kinase C activity as described in Materials and Methods. Filled symbols represent cytosolic fraction; unfilled symbols represent membrane fraction.

TABLE 3. Effect of 24-h exposure to PMA on basal and PtdSer-stimulated accumulation of [¹⁴C]PtdCho

Pretreatment	Radioactivity in PtdCho (dpm/mg of protein)
None	7,390 ± 248
PMA (24 h)	14,750 ± 951 ^a
PtdSer (24 h)	14,687 ± 826 ^a
PtdSer + PMA (24 h)	19,558 ± 2,230 ^a

Cells were treated overnight with PtdSer (100 µM), PMA (100 nM), both PtdSer and PMA, or N2 medium alone. The cells were washed once with N2, labelled for 2 hours with [¹⁴C]choline, and extracted as described in Materials and Methods, and the radioactivity in the lipid fraction was determined. Values represent means ± SEM of triplicate determinations from a representative experiment.
^a Values are significantly different from control, *p* < 0.05.

ters has been reported to desensitize cells to subsequent treatment with these agents (Solanki and Slaga, 1981; Collins and Rozengurt, 1982), probably by depleting cells of protein kinase C (Rodriguez-Pena and Rozengurt, 1984). In LA-N-2 cells, 24 h of exposure to 100 nM PMA reduced protein kinase C activity by 80% (from 1,316 to 252 pmol/mg of protein in one experiment). However, following this treatment, incorporation of [¹⁴C]choline into [¹⁴C]PtdCho was significantly increased (Table 3), suggesting that in LA-N-2 cells, protein kinase C may actually inhibit PtdCho accumulation. Stimulation of PtdCho labeling by PtdSer was not inhibited by coincubation for 24 h with PMA (Table 3); in fact, the effect of PtdSer appeared to be further increased (although not significantly) under these conditions.

PtdSer inhibits translocation of protein kinase C to membranes by PMA

The tumor-promoting phorbol ester PMA stimulated in a concentration-dependent fashion the redistribution of protein kinase C from the cytosol to the membrane in intact LA-N-2 cells (Fig. 5), and increased the activity of the enzyme *in vitro* in the presence of suboptimal concentrations of calcium (see Materials and Methods); both actions have been observed in many cell types. PtdSer alone caused no significant change in the distribution of protein kinase C in the absence of PMA (Fig. 6). However, treatment for 3 h or longer with PtdSer (130 µM) shifted the PMA dose-response curve to the right (Fig. 6). This shift was maximal after 3 h of exposure to PtdSer, at which time the PtdSer content of the cells was approximately half that achieved at saturation. At this time, the EC₅₀ for PMA was increased from about 20 nM in controls to 140 nM in treated cells (Fig. 6b). A shift of similar magnitude was seen after 24 h of PtdSer treatment.

DISCUSSION

A number of cell lines have the ability to accumulate exogenous phospholipids. Chinese hamster fibroblasts

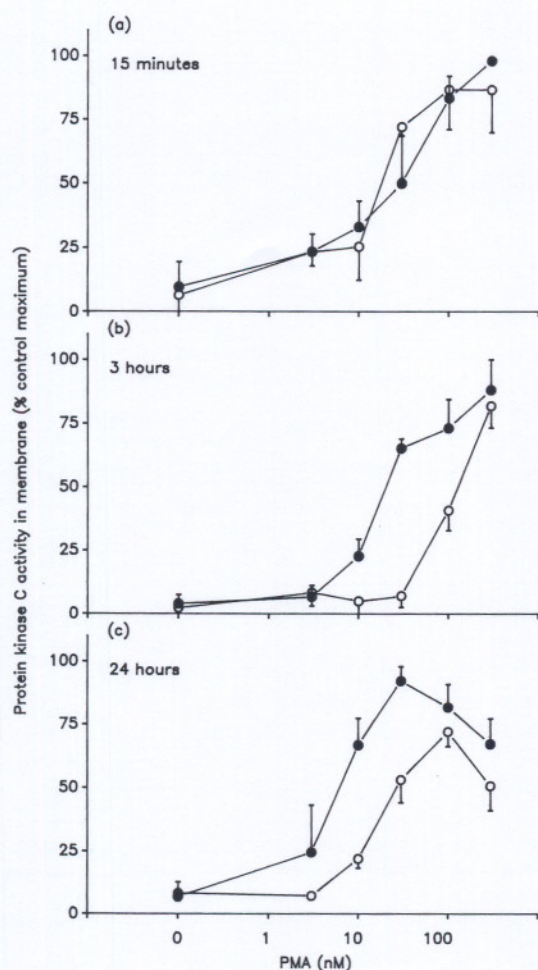


FIG. 6. Inhibition of PMA-induced translocation of protein kinase C by prior treatment with PtdSer. Cells were treated with N2 medium (filled symbols) or 130 μ M PtdSer in N2 medium (unfilled symbols) for (a) 15 min, (b) 3 h, or (c) 24 h. The medium was replaced with N2 containing various concentrations of PMA for 1 h. Then the cells were collected, extracted, and assayed for protein kinase C activity (see Materials and Methods). Data were normalized to express protein kinase C activity as a percentage of the maximum membrane-associated activity observed in the presence of PMA. Values represent means \pm SEM of at least three separate experiments.

incubated with liposomes containing a fluorescent analog of PtdSer were able to incorporate the analog into the plasma membrane and various intracellular membranes (Martin and Pagano, 1987). In Chinese hamster ovary cells treated with exogenous PtdSer, synthesis of PtdSer was depressed and total PtdSer content was unchanged; addition of PtdEtn to the medium, however, did not suppress PtdEtn synthesis, and PtdEtn content was increased (Nishijima et al., 1986).

Our data show that human LA-N-2 neuroblastoma cells take up PtdSer from the medium in a time- and concentration-dependent manner (Fig. 2b and Table 1). Measurement of phospholipids following subcellular

fractionation of PtdSer-treated cells showed increased levels of PtdSer in intracellular and plasma membranes (Fig. 1). The increased PtdSer content was accompanied by an increase in cell content of other phospholipids and by a concentration-dependent increase in the incorporation of [14 C]choline into [14 C]PtdCho, a response which does not appear to be related to activation of protein kinase C. [Although high concentrations (100 μ M) of PtdSer were found to be somewhat toxic to mutant Chinese hamster ovary cells (Voelker and Frazier, 1986), we found no significant alterations in protein content or in AcCho content in cells exposed to 100 or 130 μ M PtdSer for 24 h, whereas alterations in protein kinase C activation were detectable within 3 h and increases in PtdCho labeling within 24 h. Furthermore, no alteration in cell number relative to controls was elicited by exposure to 30 μ M PtdSer for up to 6 days (data not shown).]

Although the cell content of PtdSer increased several-fold after 24 h of exposure to 130 μ M PtdSer, the ratio of PtdSer to total phospholipids in subcellular membrane fractions was increased by 50–140% above control, indicating that not all of the PtdSer taken up by the cells ultimately became associated with membranes. The balance presumably remained in the cytoplasm, or may have been taken up by lysosomes. The distribution of Na⁺,K⁺-ATPase and succinate dehydrogenase among the various membrane fractions obtained from the sucrose density gradient was not altered by treatment of the cells with PtdSer (Fig. 2), suggesting that the observed increase in PtdSer associated with plasma or mitochondrial membranes did not affect ATPase activity, and did not interfere with the separation of plasma membrane fragments on the gradient.

The subcellular fractionation data, although suggestive, do not demonstrate unequivocally that exogenous PtdSer is actually incorporated into the cell membranes. However, this possibility seems more likely in view of the evidence that the amounts of other membrane phospholipid species also increased. PtdSer may serve as a precursor for both PtdEtn and PtdCho via decarboxylation (Borkenhagen et al., 1961) to PtdEtn and subsequent stepwise methylation to PtdCho (Bremer et al., 1960). The latter synthetic pathway is present in LA-N-2 cells (Blusztajn et al., 1987) and, coupled with decarboxylation of excess PtdSer, could lead to enhanced levels of PtdEtn and PtdCho content in PtdSer-treated cells. Since the enzymes catalyzing these reactions are membrane-bound, exogenous PtdSer must become rather closely associated with the membrane in order to act as a substrate. (It is worth noting that ethanolamine is not present in the medium; therefore, decarboxylation of PtdSer is the only route of PtdEtn synthesis available to these cells.) Alternatively, metabolism of excess PtdSer might release diglyceride metabolites, which could increase the synthesis of other phospholipid species by acting as precursors or as regulators of synthetic enzyme activity (e.g., cytidyltransferase; Choy et al., 1979). Although the mechanism of

the PtdSer-related elevations in other phospholipids in LA-N-2 cells is as yet unclear, this process partially offsets the increase in PtdSer relative to other membrane phospholipids that results from PtdSer uptake.

The uptake of PtdSer by LA-N-2 cells was associated with a concentration-dependent increase in the incorporation of [14 C]choline into [14 C]PtdCho. The lack of effect of PtdSer on formation of [14 C]AcCho (Table 2) suggests that the specific activity of the choline pool was unchanged by this treatment, and supports the conclusion that PtdCho synthesis was increased by PtdSer. This interpretation is further supported by the observation that PtdSer enhanced PtdCho labeling within 1 h in cells prelabeled with [14 C]choline (not shown). Concomitant with the increase in PtdCho labeling was a variable decrease in the radioactivity of the PCho pool in PtdSer-treated cells (Table 2), consistent with increased conversion of PCho to PtdCho, again suggesting stimulation of the synthetic pathway. The loss of label from PCho was greater than the increase in PtdCho, possibly reflecting increased degradation of PtdCho in PtdSer-treated cells. [Activation of phospholipase D in NG108-15 cells (Liscovitch et al., 1987a) released choline into the medium; however, in the present study, released metabolites were not quantitated.] Pulse-chase experiments (in which cells were exposed to PtdSer during a 72-h chase period) show that the formation of the PtdCho metabolite [14 C]GroPCho (which was not detectable after short labeling periods) was increased in cells exposed to PtdSer (Fig. 3). This, together with the observed decrease in [14 C]PCho, suggests that both synthesis and degradation of PtdCho may be increased by PtdSer, and that the enhancement of PtdCho labeling is not due to decreased degradation. The levels of labeled choline and AcCho were very low in these cells, and were not affected by PtdSer treatment, indicating that the decrease in PCho was probably not due to accelerated dephosphorylation. The unchanged levels of [14 C]PtdCho after a prolonged chase period (Fig. 3) may reflect a compensatory increase in PtdCho degradation with increasing concentrations of PtdSer. The stability of the labeled PtdCho pool in these experiments contrasts with the net increase in labeling of PtdCho seen after short (2 h) labeling periods in PtdSer-treated cells (Fig. 2), and may indicate that recently synthesized PtdCho in these cells is not subject to degradation.

Under control conditions, the amount of radioactivity located in PCho in LA-N-2 cells following short labeling periods was 10–30 times greater than that in PtdCho; in contrast, about threefold (Liscovitch et al., 1986) and fivefold (Warden and Friedkin, 1984) greater amounts were observed in NG108-15 neuroblastoma cells and 3T3 fibroblasts, respectively. This suggests that the rate of conversion of PCho to PtdCho in LA-N-2 cells is relatively slow, and may account for the long doubling time (about 56 h) of these cells and the delayed appearance of the metabolite [14 C]GroPCho.

In many cell lines, the incorporation of [14 C]choline into [14 C]PtdCho is stimulated by the tumor promoter PMA (Kreibich et al., 1971; Suss et al., 1971; Paddon and Vance, 1980; Warden and Friedkin, 1984; Liscovitch et al., 1986, 1987b; Kolesnick and Paley, 1987) and by diacylglycerol (Kreutter et al., 1985; Muir and Murray, 1986; Kolesnick and Paley, 1987; Liscovitch et al., 1987b). Both compounds can activate cytidyltransferase (Choy et al., 1979; Paddon and Vance, 1980; Pelech et al., 1984b), the rate limiting enzyme in the PtdCho synthetic pathway, as well as protein kinase C (Nishizuka, 1984a,b). There is evidence that in NG108-15 cells the stimulation of cytidyltransferase activity by PMA is due to activation of protein kinase C (Liscovitch et al., 1987b). However, PtdCho synthesis also can be regulated via protein kinase C-independent mechanisms (Kolesnick and Paley, 1987; Liscovitch et al., 1987b).

Because protein kinase C may regulate PtdCho synthesis in some cell lines, and because this enzyme is dependent on phospholipids (particularly PtdSer) for activation, we assessed the involvement of protein kinase C in the stimulation of PtdCho labeling in cells exposed to PtdSer. Three observations suggest that protein kinase C does not mediate this response to PtdSer in LA-N-2 cells. First, the effect of PtdSer was not mimicked by concentrations of PMA that maximally activated protein kinase C. Acute exposure to PMA increased PtdCho synthesis in LA-N-2 cells by only 14%, although it caused a complete, concentration-dependent translocation of protein kinase C to the membrane (Fig. 5). [Activation of protein kinase C by hormones and neurotransmitters that generate diacylglycerol (Fearon and Tashjian, 1985; Hirota et al., 1985; Sugden et al., 1985), or by the action of diacylglycerol analogs such as the phorbol ester PMA (Kraft and Anderson, 1983), is accompanied by translocation of the enzyme's activity from the cytosol to cell membranes, and an increase in the apparent affinity of the enzyme for both PtdSer and calcium (Takai et al., 1979b; Kishimoto et al., 1980).] Second, the effect of PtdSer was not inhibited by prolonged treatment with PMA. Although in NG108-15 cells (Liscovitch et al., 1987b) and in GH₃ pituitary cells (Kolesnick and Paley, 1987) prolonged treatment with PMA reduced protein kinase C levels and abolished the stimulation of PtdCho synthesis by acute administration of PMA, prolonged treatment of LA-N-2 cells with PMA (which reduced protein kinase C activity by 80%) increased basal synthesis of PtdCho and did not inhibit the action of PtdSer (Table 3). Third, PtdSer treatment alone did not affect the distribution of protein kinase C, and inhibited translocation of this enzyme to the membrane in LA-N-2 cells by PMA. The increase in [14 C]PtdCho levels following prolonged exposure to PMA (Table 3) may indicate that protein kinase C inhibits PtdCho synthesis, or stimulates its degradation in LA-N-2 cells. If this is the case, then depletion of the enzyme might be expected to result in accumulation of PtdCho.

Alternative mechanisms which might underlie activation of PtdCho synthesis by PtdSer include direct activation of cytidyltransferase by the phospholipid (Choy and Vance, 1978), by its diglyceride metabolites (Choy et al., 1979), or by its fatty acid constituents (Pelech et al., 1984a). The levels of the latter might be increased by metabolism of some of the PtdSer accumulated by LA-N-2 cells. Hence, although PMA stimulates PtdCho synthesis via activation of protein kinase C in some cell lines, there is evidence that an alternative, protein kinase C-independent pathway, which is stimulated by diacylglycerol, also exists (Kolesnick and Paley, 1987; Liscovitch et al., 1987b).

PtdSer treatment caused a rightward shift in the dose-response curve to PMA, but did not alter the total protein kinase activity associated with the membrane in the presence of supramaximal PMA concentrations (Fig. 6). This is consistent with a decreased affinity of protein kinase C for the membrane-phorbol ester complex to which it is believed to bind. It is significant that the inhibitory effect of PtdSer observed in our study was seen after 3 h of exposure to PtdSer, but not after 15 min (Fig. 6), suggesting that this effect requires incorporation of PtdSer into the cell membrane. It has been shown that the ratio of PtdEtn to PtdSer influences protein kinase C activity *in vitro* (Kaibuchi et al., 1981); lowering this ratio by elevating membrane PtdSer in LA-N-2 cells might inhibit binding of the enzyme to the membrane. Cell-specific variations in PtdSer composition might partially explain the wide range of EC₅₀ values for PMA which have been observed in various cell types. Alternatively, we have not ruled out the possibility that PtdSer accumulation in the cell cytoplasm may impede translocation of protein kinase C to the membrane by PMA.

Elsewhere, it has been shown that the CerPCho precursor and metabolite, sphingosine, inhibited phorbol ester binding and protein kinase C activity in platelets (Hannun et al., 1986), whereas sphinganine inhibited the differentiation of human promyelocytic leukemic cells by phorbol esters (Merrill et al., 1986). In contrast, alterations in the phospholipid head group composition of human promyelocytic leukemic cells enhanced binding of phorbol esters (Cabot, 1983). It remains to be determined whether other phospholipids will also modify protein kinase C activity in intact LA-N-2 cells.

The data presented in this report suggest that changes in the PtdSer composition of cell membranes may modify the synthesis and content of PtdCho, the major phospholipid component of mammalian cell membranes. It will be of interest to determine whether this effect is confined to PtdSer, or shared by other phospholipids. The ease with which the phospholipid composition of LA-N-2 neuroblastoma cells can be manipulated to produce measurable physiological consequences demonstrates the potential value of this system in elucidating relationships between cell membrane phospholipid composition, membrane synthesis, and neuronal cell function.

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