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DRUG-INDUCED SURFACE MEMBRANE PHOSPHOLIPID COMPOSITION IN MURINE FIBROBLASTS

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Summary

The effects of drugs on phospholipid composition of cell surface membranes are not well understood at this time. The effects of membrane-active drugs and membrane depolarization on the phospholipid composition were determined in murine LM fibroblasts. Receptor-aggregating drugs such as concanavalin A and cytoskeleton-disrupting agents such as colchicine, vinblastine, and cytochalasin B decreased phosphatidylserine content of the plasma membrane from 5.4 ± 1.5% to as low as 1.4 ± 0.2%. In addition, concanavalin A and colchicine increased the phosphatidylglycerol content from 6.9 ± 1.6% to 13.1 ± 0.7% and 10.6 ± 1.7%, respectively, while vinblastine and cytochalasin B had no effect. Pentobarbital decreased the content of phosphatidylinositol + phosphatidylserine and of phosphatidylglycerol almost 2-fold. Propranolol, ethanol, and depolarization with 120 mM KCl had small or no effects on plasma membrane phospholipid composition. None of the above drugs or treatments significantly altered the asymmetric distribution of phosphatidylethanolamine across the LM cell plasma membrane under the conditions tested. In addition, energy inhibitors that deplete the proton-motive force of the cell (NaN_3 and KCN) and inhibitors of ATP synthesis such as $NaAsO_4$ did not affect the asymmetric distribution of phosphatidylethanolamine. It is concluded that the mechanism of action of membrane-active drugs such as concanavalin A, vinblastine, colchicine and pentobarbital may involve alterations in plasma membrane composition. It also appears that microfilaments, microtubules, β-adrenergic receptors, membrane fluidity, and membrane potential are not critical for the regulation of the asymmetric distribution of membrane phosphatidylethanolamine.

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Introduction

Phospholipids such as phosphatidylethanolamine and phosphatidylserine appear to be involved in the lectin-induced activation of lymphocytes and mast cells [1—3]. These cells can be activated by plant lectins such as concanavalin A or *Wisteria floribunda* lectin in a manner similar to the antibody anti-IgE [4,5]. The mechanism of action of the lectin appears to involve methylation and decarboxylation of the phosphatidylserine. Phospholipid methylation may alter membrane fluidity, lipid translocation, asymmetric distribution of aminophospholipid, and coupling of the β-adrenergic receptor to adenylate cyclase [6]. However, it is not known if the small alterations in incorporation of radioactively labeled methyl groups of methionine actually are concomitant with methylation of larger quantities of the membrane phospholipid. In addition it is not known if alterations in methylation reactions are the only mechanisms whereby lectins such as Con A or other membrane-active drugs may alter the phosphatidylserine contents of membranes. Several classes of drug are known to affect membrane lipid metabolism and/or surface membrane structure and properties: (1) cell surface-aggregating agents such as concanavalin A [1,2,7,8]; (2) microtubule and microfilament-disrupting agents such as colchicine, vinblastine, and cytochalasin B [9—15]; (3) anesthetic agents such as ethanol and pentobarbital [17,18]; (4) propranolol, at anesthetic concentrations, which inserts preferentially in the inner monolayer of the membrane [19—21]; (5) β-adrenergic agonists such as isoproterenol [6]; (6) depolarizing concentrations of potassium [22], and energy inhibitors [23]. In the present work we utilize the LM fibroblast system to determine if methylation of phospholipids in surface membranes is the only mechanism whereby compositional changes in phosphatidylserine, phosphatidylethanolamine, or phosphatidylcholine content can be induced by membrane-active drugs. LM cells cannot convert phosphatidylserine or phosphatidylethanolamine to phosphatidylcholine by sequential methylation [24,25].

Materials and Methods

Chemicals. Concanavalin A, colchicine, vinblastine sulfate, cytochalasin B, pentobarbital, (±)-propranolol, and trinitrobenzenesulfonic acid were purchased from Sigma Chemical Company, St. Louis, MO.

Cell culture. LM cells, a choline-requiring strain of mouse fibroblasts, were grown in suspension in a chemically defined serum- and protein-free medium. The cells were obtained from the American Type Culture Collection (CCL 1.2) and cultured at $1.0 \cdot 10^6$ cells/ml according to the procedure of Schroeder et al. [25]. The cultures were grown in the presence of [^{32}P]phosphate (New England Nuclear Inc., Boston, MA) for 5 days at 2 μCi/ml to prelabel all the phospholipids uniformly.

Drug treatment. The radioactively labeled cells were centrifuged at 200 × *g*, resuspended at $2.0 \cdot 10^6$ cells/ml in fresh sterile medium with the same specific activity of ^{32}P-labeled phosphate and appropriate drug, and then incubated at 37°C for the time periods indicated. The following stock solutions of drugs were prepared in phosphate-buffered saline [25] and sterilized by Millipore

filtration: 74 μM concanavalin A, $1 \cdot 10^{-1}$ M colchicine, and $1 \cdot 10^{-3}$ M vinblastine sulfate. A cytochalasin B stock solution was prepared in dimethylsulfoxide 0.5 μM. Aliquots of the stock solution were then added to the culture medium. Specific drug concentrations, combinations, and length of incubation are presented in the results section.

Plasma membrane isolation and lipid analysis. Plasma membranes were isolated from drug-treated LM cells as described earlier [25]. Although treatment of cells with drugs such as concanavalin A may directly alter the $(Na^+ + K^+)$-ATPase activity used as the surface membrane maker enzyme herein, it might be expected that this alteration would be similar in the plasma membrane as well as the crude homogenate of the cell; thus, the ratio of the two specific activities would not be altered. In the present work neither the purification ratio, the specific activity of the enzyme, nor the ratio of sterol to phospholipid in the surface membranes was altered by drug treatment. Thus, drug treatment did not appear to affect the membrane purification. The lipids were extracted by the method of Bligh and Dyer [26], and fractionated by silicic acid chromatography to separate neutral lipids from phospholipids. The phospholipids were spotted on 250 μm Silica Gel G thin-layer chromatography plates (Analtech Inc., Newark, DL) preactivated with acetone. The plates were developed in the first direction with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and then dried under vacuum for 1 h before chromatographing in the second direction with *n*-butanol/acetic acid/H_2O (6 : 2 : 2, v/v) [25]. The spots were visualized by autoradiography and scraped into scintillation vials for determination of [^{32}P]phosphate. The plasma membrane phospholipid composition obtained in this manner was identical to that obtained by phospholipid quantitation of spots by the inorganic phosphate assay of Ames [27]. Spots were identified by comparison to standards [25]. All solvents were glass distilled, and all glassware was sulfuric acid/dichromate washed before use.

Determination of phosphatidylethanolamine asymmetry. The asymmetric distribution of phosphatidylethanolamine across the plasma membrane bilayer of LM cells was determined (see Refs. 28—33). Briefly, trinitrobenzenesulfonic acid was used as a chemical labeling reagent to determine the distribution of phosphatidylethanolamine in the plasma membrane lipid bilayers. Trinitrobenzenesulfonic acid covalently binds to all primary amine groups and reacts with amine groups not accessible to larger probe molecules. Under non-penetrating conditions it will react primarily with phosphatidylethanolamine and phosphatidylserine on the extracellular side of the lipid bilayer of whole cell plasma membranes. Penetration of trinitrobenzenesulfonic acid through the plasma membrane of whole cells was determined as described by Fontaine and Schroeder [29]. In all studies presented herein, labeling of intracellular membranes with trinitrobenzenesulfonic acid was equivalent to control values. In addition, at the drug concentrations chosen, greater than 95% of LM cells excluded trypan blue.

LM cells were exposed to drugs for the time periods and concentrations indicated. For long times of drug exposure, drug concentrations were chosen which did not decrease viable cell number more than 10%. This avoided selection for cells resistant to the drugs. Then whole LM cells were treated with trinitrobenzenesulfonic acid according to a previously published procedure [34] modified

according to Fontaine and Schroeder [29] as follows: Reagent A (trinitrobenzenesulfonic acid-labeling reagent) comprised 4 mM trinitrobenzenesulfonic acid, 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose and 1% delipidized bovine serum albumin. The bovine serum albumin (fraction V, Pentex, Miles Research Labs, Elkhart, IN) was delipidized by the procedure of Wilcox et al. [35]. The reagent pH was adjusted to 8.5 with NaOH. Reagent B (reaction-terminating reagent) was 0.15 μM Tris (pH 7.0) (Sigma Chemical Co., St. Louis, MO). The treatment procedure was as follows: (a) $40 \cdot 10^6$ cells (exponential phase) were washed twice with phosphate-buffered saline [25] and incubated with 10 ml of trinitrobenzenesulfonic acid reagent at 4°C for 2 h; (b) the cells were maximally labeled by shaking at 4°C on a New Brunswick Gyrotary Shaker at 140 rev./min (model G-10, New Brunswick Scientific, NJ); (c) the reaction was terminated by the addition of 40 ml of 0.15 M Tris, pH 7.0, 4°C, followed by centrifugation at 4°C, and (d) the cells were washed again with 20 ml of the Tris buffer and then once with 10 ml of phosphate-buffered saline. All washes were carried out with Tris and phosphate-buffered saline solutions at 4°C. Phosphatidylethanolamine and trinitrophenylphosphatidylethanolamine were quantitated after the phospholipids were extracted and resolved as described above.

Results

Effect of drugs on the plasma membrane phospholipid composition of LM fibroblast

Although alterations in turnover of certain phospholipids after exposure to concanavalin A or colchicine have been noted [1,2], little is known of the effects of these drugs on phospholipid composition. Both concanavalin A and colchicine decreased the phosphatidylserine content of the plasma membranes from 5.4% to 2.4% and 1.5%, respectively (Table I). The content of lysophosphosphatidylcholine was not altered. In contrast, phosphatidylglycerol content was increased from 6.9% to 13.1% by concanavalin A, and to 10.6% by colchicine. The ratios of phosphatidylcholine to phosphatidylethanolamine did not change significantly. This ratio is a rough index of potential alterations in membrane fluidity, since phosphatidylcholine is generally more fluid than phosphatidylethanolamine [36]. Concanavalin A and other lectins are known to affect lymphocyte fluidity [1]. The time course of the effects of concanavalin A and of colchicine on phosphatidylserine and phosphatidylglycerol content of LM cell plasma membranes are shown in Fig. 1. Maximal changes were noted by 4 h exposure of LM cells to these drugs, except for the effect of colchicine on phosphatidylglycerol which continued to increase until the 16th hour. These two drugs in combination were not synergistic. However, in the case of alterations in phosphatidylglycerol content they were antagonistic. Similarly, maximal effects after 4 h treatment were noted at concanavalin A and colchicine concentrations of 7.4 μM and 1 mM, respectively, as shown in Fig. 2. Vinblastine and cytochalasin B also decreased the phosphatidylserine content from 5.4 ± 1.5% to 1.4 ± 0.2% and 2.7%, respectively. However, none of the other lipid components changed significantly.

Among the anesthetic agents, pentobarbital decreased the content of phos-

TABLE I

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES FROM LM CELLS TREATED WITH DRUGS

LM cells were pretreated with the appropriate drug in fresh medium for 4 h at 37°C; Concanavalin A, 7.4 μM; colchicine, 1 mM; vinblastine, 100 μM; cytochalasin B, 10 mM; ethanol, 800 mM; pentobarbital, 0.25 mM; propranolol, 50 μM. Numbers in parentheses indicate number of observations. Values represent mean ± S.E. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; PG, phosphatidylglycerol, and CL, cardiolipin.

Drug treatment	Phospholipid composition (%)								
	PC	PE	PI	PS	SP	PG	CL	Other	PC/PE
Glycoprotein-aggregation agent									
None (3)	36.6 ± 0.7	32.4 ± 6.4	5.1 ± 1.4	5.4 ± 1.5	4.4 ± 1.0	6.9 ± 1.6	0.3 ± 0.1	9.1 ± 0.8	1.13
Concanavalin A (4)	36.0 ± 1.1	29.3 ± 0.5	6.6 ± 0.3	2.4 ± 0.5 *	5.4 ± 0.4	13.1 ± 0.7 *	0.4 ± 0.1	7.0 ± 2.2	1.23
Microtubule and microfilament-disrupting agents									
None (3)	36.6 ± 0.7	32.4 ± 1.4	5.1 ± 1.4	5.4 ± 1.5	4.4 ± 1.0	6.9 ± 1.6	0.3 ± 0.1	9.1 ± 0.8	1.13
Colchicine (5)	36.1 ± 1.9	30.0 ± 1.7	6.7 ± 1.0	1.5 ± 0.3 *	4.5 ± 0.6	10.6 ± 1.7 *	0.4 ± 0.1	10.4 ± 2.3	1.20
Vinblastine (3)	38.5 ± 1.4	33.5 ± 1.3	8.0 ± 0.7	1.4 ± 0.2 *	4.7 ± 0.3	5.5 ± 0.6	0.4 ± 0.1	7.7 ± 0.4	1.15
Cytochalasin B (1)	37.5	32.4	7.8	2.7	5.8	6.1	0.4	7.2	1.16
Anesthetic agents									
None (3)	38.7 ± 1.2	25.9 ± 0.6	11.9 ± 1.0		5.0 ± 0.4	8.2 ± 0.8	<0.01	8.4 ± 1.3	1.49
Ethanol (3)	39.5 ± 1.2	28.4 ± 0.2	10.7 ± 0.2		5.1 ± 0.8	8.6 ± 0.7	<0.01	7.3 ± 1.0	1.39
Pentobarbital (3)	40.3 ± 3.0 *	26.0 ± 0.4	6.4 ± 0.3 *		6.0 ± 1.7	4.5 ± 1.5 *	<0.01	14.8 ± 4.0 *	1.55
β-Receptor agents									
None (3)	38.5 ± 0.6	31.7 ± 0.6	4.0 ± 0.8	3.9 ± 0.5	3.3 ± 0.2	8.4 ± 2.3	<0.01	10.3 ± 1.0	1.21
Propranolol (6)	36.6 ± 0.7 *	28.8 ± 0.6 *	6.1 ± 0.3 *	5.7 ± 0.5 *	3.0 ± 0.2	8.3 ± 0.9	<0.01	11.4 ± 0.6	1.27

* $P \leq 0.01$.

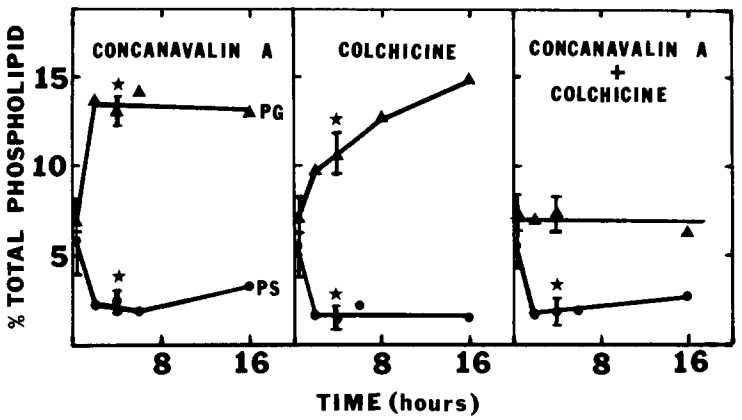

Fig. 1. Phospholipid composition of plasma membranes after exposure to concanavalin A and colchicine for increasing time. LM cells were treated for increasing time with either 7.4 μM concanavalin A/ml and/or $1 \cdot 10^{-6}$ M colchicine at 37°C. Plasma membranes were isolated and lipid composition was determined as described in Materials and Methods. Values with error bars represent the mean ± S.E. (n = 3). ▲———▲, phosphatidylglycerol (PG); ●———●, phosphatidylserine (PS).

phatidylinositol + phosphatidylserine from 11.9 ± 1.0% to 6.4 ± 0.3% and of phosphatidylglycerol from 8.2 ± 0.8% to 4.5 ± 1.5%, respectively, while ethanol produced no significant alterations. Pentobarbital also increased the lysophosphatidylcholine content (data not shown). Propranolol, a β-adrenergic antagonist, may act as a weak anesthetic at the concentration used herein [21]. It decreased the phosphatidylcholine and phosphatidylethanolamine content of LM cell plasma membranes slightly from 38.5 ± 0.6% to 36.6 ± 0.6% and from 31.7 ± 0.6% to 28.8 ± 0.6%, respectively. All of these agents increased the ratio

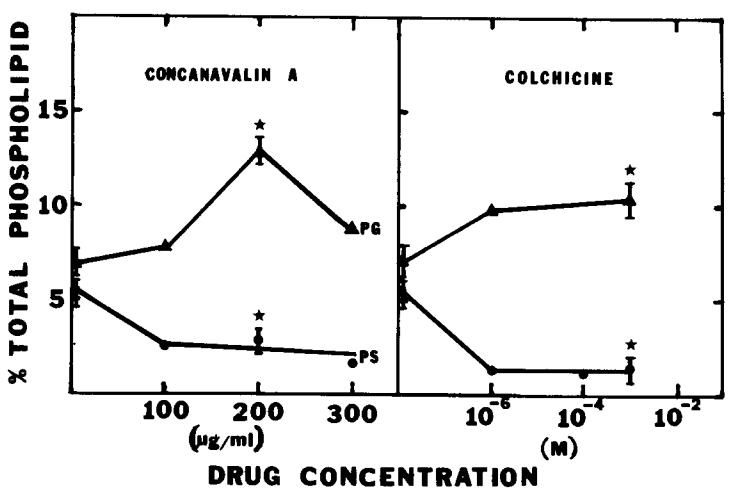

Fig. 2. Phospholipid composition of plasma membranes after exposure to increasing concentrations of concanavalin A and colchicine. All conditions were as described in legend of Fig. 1 except that treatment time was for 4 h with variable drug concentrations. ▲———▲, phosphatidylglycerol (PG); ●———●, phosphatidylserine (PS).

TABLE II

EFFECT OF DIFFERENT COMBINATIONS OF DRUGS ON THE PHOSPHOLIPID COMPOSITION OF LM CELL PLASMA MEMBRANE

LM cells were pretreated with drug combinations in fresh medium for 4 h at 37°C. Concanavalin A, 7.4 μM; colchicine, 1 mM; cytochalasin B, 10 mM, and vinblastine, 100 μM. Values represent the mean ± S.E. Numbers in parentheses refer to number of observations. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; PG, phosphatidylglycerol, and CL, cardiolipin.

Drug combination	Phospholipid composition (%)								
	PC	PE	PI	PS	SP	PG	CL	Other	PC/PE
None (3)	36.6 ± 0.7	32.4 ± 1.4	5.1 ± 1.5	5.4 ± 1.5	4.4 ± 1.0	6.9 ± 1.6	0.3 ± 0.1	9.1 ± 0.8	1.13
Concanavalin A + colchicine (4)	37.9 ± 0.6	32.3 ± 1.2	6.9 ± 0.6	2.0 ± 0.4 *	5.0 ± 0.3	7.3 ± 2.4	0.3 ± 0.1	7.7 ± 0.7	1.17
Concanavalin A + vinblastine (3)	39.8 ± 1.5	32.6 ± 1.1	7.3 ± 0.7	1.1 ± 0.2 *	5.0 ± 0.4	6.5 ± 0.6	0.4 ± 0.1	6.7 ± 0.8	1.22
Cytochalasin B + colchicine	37.4	33.6	9.7	1.5	4.9	5.9	0.4	6.5	1.11
Cytochalasin B + vinblastine	40.0	30.6	9.5	1.5	5.9	5.4	0.4	6.8	1.31
Cytochalasin B + colchicine + concanavalin A	38.6	30.6	8.8	1.4	6.2	6.8	0.5	7.1	1.26

* $P \leq 0.01$.

TABLE III

EFFECT OF DEPOLARIZATION ON THE PHOSPHOLIPID COMPOSITION OF LM CELL PLASMA MEMBRANES

LM cells were grown as described in Materials and Methods and resuspended in 120 mM KCl for 5 min at 37°C followed by trinitrobenzenesulfonic acid treatment and plasma membrane isolation as described in Materials and Methods. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; PG, phosphatidylglycerol, and CL, cardiolipin (not detected).

Treatment	Supplement	PC	PE	PI	PS	SP	PG	Other
None	Choline (3)	40.6 ± 1.5	24.9 ± 1.4	8.1 ± 1.4	3.0 ± 1.5	4.2 ± 1.0	10.0 ± 1.6	9.2 ± 1.8
120 mM KCl	Choline (2)	37.1 ± 2.9	25.1 ± 1.2	11.5 ± 2.4		3.5 ± 1.4	9.6 ± 3.0	13.2 ± 3.5

of phosphatidylcholine to phosphatidylethanolamine, a finding consistent with the increased fluidization of membranes by these drugs. This action appears apart from direct fluidization by anesthetic agents. Propranolol increased phosphatidylinositol and phosphatidylserine from 4.0 ± 0.6% to 6.1 ± 0.3% and 3.9 ± 0.5% to 5.7 ± 0.5%, respectively. Yavin and Zutra [19] also showed alterations in phospholipid composition of cultured neuroblastoma cells after propranolol treatment.

The effects of several combinations of receptor-aggregating, microtubular-disrupting, and microfilament-disrupting drugs are shown in Fig. 2 and Table II. In all cases, the phosphatidylserine content decreased significantly. In contrast, combinations of these drugs were antagonistic in their effects on phosphatidylglycerol content. Lastly, the effect of membrane depolarization on the phospholipid composition is shown in Table III. Exposure of the cells to 120—140 mM KCl for 5 min (Table III) or 10, 15 or 20 min (data not shown) did not significantly alter the phospholipid composition. In summary, the above drugs altered the phospholipid composition of the LM cell surface membranes while membrane depolarization had no effect. The effects were primarily on phosphatidylserine and phosphatidylglycerol content. Phosphatidylethanolamine content, however, was not significantly altered.

Effect of drug agents on the asymmetric distribution of phosphatidylethanolamine

The investigations reported below were designed to determine the potential role of cell surface-aggregating agents, cytoskeletal-disrupting drugs, cationic amphipathic drugs, anesthetic agents, membrane depolarization, and energy inhibitors in the regulation of lipid asymmetry found in the mammalian cell plasma membrane. The distribution of phospholipids in membranes has been reviewed elsewhere [37]; Zwitterionic phospholipids such as phosphatidylcholine are located in the outer monolayer of plasma membrane bilayers while the acidic phospholipids, such as phosphatidylserine and phosphatidylethanolamine, are present in the inner (cytoplasmic) monolayer. Changes in the fluidity of the membrane may affect transbilayer migration of phospholipids [38]. The membrane also acts as a bilayer couple, and cationic amphipathic compounds such as propranolol can insert into the inner monolayer and disrupt the membrane structure [20].

In the experiments reported herein the degree of leakage of trinitrobenzenesulfonic acid into the cell under non-penetrating conditions [24], as determined by labeling of phospholipids in the intracellular organelles (microsomes and mitochrondia) was equal to or less than control values. In the absence of drug treatment, approx. 4—6% of LM cell plasma membrane phosphatidylethanolamine appears to reside in the outer monolayer (Table IV). Similar results have recently been reported when the chemical labeling reagents trinitrobenzenesulfonic acid and isethionyl acetimidate were used under non-penetrating conditions as described herein [29]. However, under penetrating conditions trinitrobenzenesulfonic acid has been shown to trinitrophenylate approx. 80—90% of LM cell plasma membrane and synaptosomal plasma membrane phosphatidylethanolamine [28—33]. The location of the remaining 10—20% is not known.

TABLE IV

ASYMMETRIC DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE IN PLASMA MEMBRANES FROM LM CELLS TREATED WITH DRUGS

All drug treatments were as described in Tables I–III. LM cells were exposed to $NaAsO_4$, NaN_3, and KCN for 20 min at 37°C. Exposure for 5 or 10 min gave similar values. Values represent mean ± % S.E. Figures in parentheses refer to number of observations.

Drug treatment	% labeling of phosphatidylethanolamine
None (3)	5.8 ± 2.0
Concanavalin A (3)	7.5 ± 1.0
Colchicine (3)	7.2 ± 1.2
Vinblastine (3)	7.4 = 2.2
Cytochalasin B (1)	7.0
Cytochalasin B + colchicine (1)	5.7
Cytochalasin B + vinblastine (1)	6.8
Ethanol (3)	5.1 ± 1.4
Pentobarbital (2)	3.4 ± 1.3
Propranolol (3)	2.9 ± 1.8
120 mM KCl (3)	3.6 ± 1.3
1 mM $NaAsO_4$ (1)	5.2
50 mM NaN_3 (1)	4.2
10 mM KCN (1)	4.6

To test the possibility that concanavalin A can affect the asymmetric distribution of phosphatidylethanolamine, LM cells were incubated in the presence of this drug at concentrations able to agglutinate LM cells. The percent of trinitrophenylphosphatidylethanolamine in cells treated with concanavalin A was 7.5 ± 1% indicating no significant increase in phosphatidylethanolamine exposure to the trinitrobenzenesulfonic acid. Internal labeling of both microsomes and mitochondria did not exceed control values.

The cell cytoskeleton may also have a role in plasma membrane lipid distribution. The negatively charged cytoskeletal structures may constrain negatively charged phospholipids to reside in the cytoplasmic half of the bilayer, possibly via Ca^{2+} bridges. Alternately they may impose a higher viscosity on the inner monolayer thereby decreasing transbilayer diffusion of phosphatidylethanolamine [28,33]. To test this hypothesis, cells were treated with colchicine and vinblastine, microtubule-disrupting agents, and cytochalasin B, a microfilament-disrupting agent at several concentrations. These drugs dissociate the cytoskeletal assemblies and may therefore allow the spontaneous transbilayer diffusion rate to give rise to a different distribution of phosphatidylethanolamine. As shown in Table IV, treatment of LM cells with these disrupting agents did not significantly alter the distribution of phosphatidylethanolamine across the bilayer. After 4 h of exposure to these drugs the percent of phosphatidylethanolamine labeled by trinitrobenzenesulfonic acid was not significantly different from control values of 5.8 ± 2%. Drug treatment over a 100-fold range in concentration for up to 26 h did not alter the phosphatidylethanolamine distribution (data not shown). If the regulation of aminophospholipid asymmetry requires the presence of both the microtubules and microfilaments, then one would expect the degeneration of bilayer asymmetry to be greater than with either drug alone. After the administration of cytochalasin B plus either colchicine or vinblastine, the labeling of plasma membrane phosphatidylethanolamine

was 5.7 and 6.8%, respectively (Table IV). Intracellular labeling did not exceed control values. Thus, the microtubular and microfilament-disrupting drugs did not alter the exposure of phosphatidylethanolamine to trinitrobenzenesulfonic acid.

Membrane-expanding agents such as ethanol or pentobarbital can fluidize membranes by their anesthetic action. This could lead to increased transbilayer migration of phosphatidylethanolamine and possibly degeneration of the asymmetric distribution of the aminophospholipids. However, as shown in Table IV, neither ethanol nor pentobarbital significantly altered the asymmetric distribution.

It has been shown that binding of drugs to β-receptors can increase methylation of phosphatidylethanolamine in red blood cell membranes [6]. However, as shown in Table IV, although propranolol did not alter the phosphatidylethanolamine asymmetry, it did lower the content of phosphatidylethanolamine slightly (Table I). Treatment with 10—100 M propranolol for up to 24 h gave essentially the same results.

It is possible that membrane depolarization may alter aminophospholipid asymmetry by altering charge restraints across the membrane. It has been shown that 120—140 mM KCl in the extracellular fluid can depolarize Ehrlich ascites tumor cells, which are non-neural cells [22]. As shown in Table IV, depolarization of LM cells with 120 mM KCl did not alter the phosphatidylethanolamine distribution across the membrane. Similar results were obtained with 5, 10, 15 and 20 min of depolarization with 120 mM KCl.

Lastly, it was recently reported that energy inhibitors such as NaN_3 and KCN that deplete the proton-motive force (but not ATP inhibitors such as $NaAsO_4$) markedly decrease the translocation of phosphatidylcholine between the inner and outer membrane of *Escherichia coli.* As shown in Table IV, none of these agents affected the asymmetric distribution of phosphatidylethanolamine in the LM cell plasma membrane.

Discussion

The data presented above indicate that concanavalin A, colchicine, vinblastine and cytochalasin B may have, as part of their mechanism of action, effects on surface phospholipid composition of transformed murine fibroblasts. Both the content of phosphatidylserine and phosphatidylglycerol were significantly affected by these drugs, especially by concanavalin A and colchicine. The mechanism whereby receptor-aggregating agents and cytoskeleton-disrupting drugs may affect phospholipid metabolism is not known. However, small alterations in membrane phospholipid compositions may elicit considerable alteration in membrane-bound protein activity. For example, bovine caudate tyrosine hydroxylase is stimulated by phosphatidylserine [39]. Phosphatidylserine is involved in the hormonal control of rat liver plasma membrane adenylate cyclase. Phosphatidylserine enhances opiate binding and immobilizes opiates in membrane lipids [41,42]. Lastly, phosphatidylserine is intimately involved in divalent ion binding to membranes and thereby may partially regulate lipid phase alterations and/or activities of membrane-bound enzymes [43,44]. Other enzymes such as $(Na^+ + K^+)$-ATPase also require acidic phospholipids for activity [45].

In contrast to results presented elsewhere [2], concanavalin significantly decreased the content of phosphatidylserine in the LM cell plasma membrane. This mechanism did not require the decarboxylation of phosphatidylserine and subsequent methylation of phosphatidylethanolamine as previously suggested [2]. Decarboxylation to phosphatidylethanolamine or production of lysophosphatidylserine could have occurred, however. Lysophosphatidylserine was approx. 1000-fold more effective in stimulating histamine secretion by blast cells than phosphatidylserine alone [3]. In addition, direct effects of these drugs on enzymes active in lipid metabolism have also been postulated [7,8]. The significance of these alterations may lie in altered protein-lipid interactions that may affect systems other than the cytoskeleton. Furthermore, colchicine, vinblastine and cytochalasin B, although affecting phospholipid composition, did not affect the asymmetric distribution of phosphatidylethanolamine. Combinations of these drugs did not change the bilayer distribution of phosphatidylethanolamine.

Chronic pentobarbital treatment of rats led to an increased rate of incorporation of [^{32}P]phosphate into phosphatidylserine and phosphatidylinositol in one fraction of synaptosomal plasma membranes, the SPM-H. While in the other fraction, the SPM-L, there was a decreased rate of [^{32}P]phosphate incorporation into these acidic phospholipids [17]. The data reported herein, using acute pentobarbital treatment, indicated a decreased content of these acidic phospholipids. These differences may be due to variations in treatment, to the isolation of different plasma membrane fractions, or to methodological differences. Hitzemann and Loh [17] determined the turnover of phosphatidylserine and phosphosphatidylinositol, while the data reported herein are based on total relative content of these phospholipids. Yavin and Zutra [19] reported a large increase in the rate of incorporation of [^{32}P]phosphate into phosphatidylglycerol of cultured neuroblastoma cells after 3 h incubation in the presence of 0.25 mM (±)-propranolol. However, the data reported herein indicate that the relative amount of phosphatidylglycerol in the plasma membrane of LM cells was not altered by 50 μM (±)-propranolol. Several differences between the former investigation [19] and the one reported herein include: (1) different cell types; (2) different propranolol concentration; (3) whole cell vs. plasma membrane investigations, and (4) determination of turnover or incorporation rates vs. relative content of phosphatidylglycerol.

It has been reported that in rat reticulocytes β-adrenergic agonists such as (—)-isoproterenol stimulated the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine [6]. The β-adrenergic antagonist, propranolol, reduced the (−)-isoproterenol-stimulated methylation of phosphatidylethanolamine. However, we were unable to detect specific binding of [^{3}H]dihydroalprenolol to LM cell plasma membranes, suggesting the absence of specific β-receptors (Bylund, D.B. and Schroeder, F., unpublished results). Nevertheless, we were able to determine if propranolol, a cationic amphipathic compound, could by virtue of its ability to insert into the inner monolayer of mammalian membranes force phosphatidylethanolamine to the outer monolayer of the bilayer membrane [20]. We have shown herein that although treatment of LM cells with propranolol decreased phosphatidylethanolamine content slightly, no effect on phosphatidylethanolamine asymmetry was noted. In addition to pos-

sibly affecting lipid composition, propranolol can also act as an anesthetic agent at a concentration of 50 μM, thereby fluidizing the membrane [21].

It should be noted that the drug dosage ranges chosen for treatments described herein were selected from concentrations that elicited receptor aggregation or cytoskeletal disruption as reported in the literature [46—50]. The effects of these drugs on agglutination of LM fibroblasts were tested herein and found to be consistent with those reported elsewhere [12,13,46,50]. However, our free drug concentrations may have been higher than those utilized by other investigators who incubated their cells with drugs in the presence of serum-containing medium. Serum albumin could bind drug, in this way lowering the free drug concentration.

In conclusion, one action of the four classes of drugs tested in LM fibroblasts involves significant changes in the plasma membrane lipid composition but not in aminophospholipid distribution. The data are also consistent with the conclusion that the microtubules and microfilaments need not be involved in maintenance of phosphatidylethanolamine asymmetry. Despite exposure of LM cells to agents known to alter cellular function (microfilaments, microtubules, membrane potential, β-adrenergic receptors, fluidity, and energy state) at several concentrations for up to 26 h, there was no significant alteration in the asymmetric distribution of phosphatidylethanolamine.

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