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Effect of Calcium and Physical State of Neutral Membranes on Phosphatidylserine Requirement for Opiate Binding

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SUMMARY

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A study was undertaken to investigate the nature of the inhibitory action of phospholipase A_2 (PL-A) on stereospecific opiate binding to rat brain membrane preparations in relation to the hypothesis that phosphatidylserine (PS) is required for binding. Although the presence of albumin will prevent the inhibitory effect of PL-A, the protective action of the protein is substantially less at higher enzyme concentrations, which result in over 10% reduction of the endogenous PS. PS is able to restore opiate binding after exposure to lower, but not higher, concentrations of the enzyme. In the presence of albumin, the enhancement effect of PS is not demonstrable, presumably because the albumin conjugates the lipid. The presence of Ca or other multivalent cations is not required for the enhancement effect of PS, as evidenced by the fact that the repeated washing of the membranes by EGTA or EDTA does not alter the extent of enhancement. Opiate binding was greatly reduced by physical disruption of membranes by either freeze–thawing or sonication, while in each case the addition of PS restored binding to the control level.

INTRODUCTION

Despite the intensive efforts to elucidate the molecular nature of the opiate receptor, knowledge concerning its molecular composition and configuration is based largely on indirect evidence. The evidence that the receptor is comprised of both a protein and a phospholipid derives from the observations that various proteases, protein-modifying agents, and phospholipases can destroy stereospecific opiate binding (1). Evidence for its proteinaceous nature is complicated by the fact that the proteases as well as their autolytic peptides are inhibitory to opiate binding (2), whereas the evidence for its phospholipid requirement is confounded by the fact that the presence of bovine serum albumin during exposure of neural membranes to PL-A[1] can greatly reduce the inhibitory action of the enzyme (3).

Interest in this laboratory has focused on the attempt to demonstrate that PS is an essential component of the opiate receptor. Evidence for its role is based on the observations that PL-A and PS decarboxylase destroy opiate binding, while exogenous phosphatidylserine will restore it (4). The objectives of the present study are to examine further the nature of the membrane interaction with PS, to determine the possible requirements for Ca and other divalent cations for the lipid interaction, and to investigate further the inhibitory action of PL-A to determine the effect of physical disruption of membranes on opiate binding, as well as the protective effect of albumin against the enzyme.

MATERIALS AND METHODS

The procedures for the preparation of the neural membranes from rat brain (excluding cerebellum) are described elsewhere (4). In those experiments where EGTA or EDTA was used, the membrane pellet was homogenized in 10^{-3} mol of the chelating agent in 0.05 M Tris, pH 7.5, incubated for 30 min at 37°C, and centrifuged at 75,000*g* for 30 min. The pellet was then homogenized and diluted to 1 mg protein/ml in 0.05 M Tris, pH 7.5, prior to use. The procedure for adding PS involved the addition of 100 μg/ml homogenate of lipid dissolved in hexane to a glass homogenizer, evaporating off the hexane with a stream of N_2, adding a suspension of neural membranes to the homogenizer, and then homogenizing with a Teflon pestle for about 10 s. The fatty acid composition of the added PS was as follows: 16:0, 1.1%, 18:0, 40.3%; 18:1, 21.7%; 20:4, 2.6%, 22:6, 24.7%; other, 9.6%. Bovine serum albumin was used at a concentration of 2 mg/ml homogenate.

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[1] Abbreviations used: PL-A, phospholipase A_2; PS, phosphatidylserine.

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Membrane treatment with PL-A. PL-A (*Viper russelli*, 11 units/mg), at a concentration of 10^{-3}–10^{-4} unit/mg membrane protein, was incubated with the membrane preparation at 37°C for 15 min, which was determined to be an optimal time for PL-A hydrolysis. The membranes were then centrifuged at 75,000*g* for 20 min and the pellet was homogenized in Tris buffer. The PL-A, obtained from Sigma Chemical Co., contained less than 0.001% protease activity.

Analysis of phospholipids and fatty acids released by PL-A. The procedures for the separation of the various phospholipids and the fatty acids released by PL-A are described in detail elsewhere (5).

Preparation of phosphatidylserine from bovine brain. The procedure for the preparation of PS from bovine brain is a modification of that described by Rouser *et al.* (6). After extraction of the lipid from bovine brain by the procedure of Bligh and Dyer (7), the total phospholipids were fractionated on a silicic acid column. PS was then separated on a DEAE-cellulose column according to the procedure of Rouser *et al.* (6). Chloroform was added and the acetic acid removed by repeated extraction with water. Upon removal of the chloroform by flash evaporation and lyophilization to remove the residual acetic acid, the PS was dissolved in hexane and stored under argon at −20°C. Approximately 70 mg of PS was obtained from 20 g wet weight of cortical gray matter of bovine brain.

Sonication of membranes. Sonication of membranes was performed with a Branson cell disruptor using a power density of 450 W in.$^{-2}$ and an operating frequency of 50 kcycles/s. The time of sonication was 30 s, and the temperature 4°C.

Measurement of opiate binding. Opiate binding was measured by the technique of Pasternak and Snyder (1) with slight modifications as described elsewhere (4). It involved the use of ^{3}H-dihydromorphine (sp act = 80 Ci/mmol) and filtration *in vacuo* through Whatman GF/B glass-fiber filters. A typical incubation medium contained the following in a final volume of 1.2 ml: 0.05 M Tris, pH 7.5, 0.05 μCi of ^{3}H-dihydromorphine, and 10^{-7} M dextrorphan or levorphanol. Stereospecific binding is the difference in radioactivity obtained in the presence of dextrorphan from that of levorphanol.

RESULTS

Effect of PL-A on opiate binding and fatty acid with or without PS. Exposure of neural membranes to 2 × 10^{-4} units of PL-A/mg membrane protein resulted in a 44% inhibition of opiate binding, while the subsequent addition of PS resulted in an 18% increase over the control (Table 1). Under similar conditions the control value was enhanced 41% by added PS. At this concentration of PL-A, 5.0 nmol of $C_{22:6}$ fatty acid was released, as compared to 1.4 and 2.7 nmol of $C_{18:1}$ and $C_{20:4}$, respectively. In the presence of added PS, the release of $C_{22:6}$ was about threefold greater than in the absence of the lipid, as compared to a twofold increase in $C_{18:1}$ and no change in $C_{20:4}$. At a concentration of 10^{-3} units PL-A/mg protein, opiate binding was almost completely inhibited, while the subsequent addition of PS was without effect. At this concentration of PL-A, 19 nmol of $C_{22:6}$ was released in the absence of the lipid and 31 nmol in its presence. $C_{18:1}$ increased from 6 to 25 nmol after the addition of the lipid, while $C_{20:4}$ showed no change. The fact that free fatty acids are released in the absence of added PL-A may be due to endogenous PL-A activity.

Fatty acid content of membrane phospholipids after attack by PL-A. An analysis of the changes in the fatty acid content of the individual phospholipids after exposure to two concentrations of PL-A is presented in Table 2. The fatty acid showing the most significant decrease at 2 × 10^{-4} units of the enzyme was $C_{22:6}$ of PS (6%) and phosphatidylethanolamine (5.5%). At the higher enzyme concentration, $C_{22:6}$ decreased 19 and 21% from the control values of PS and phosphatidylethanolamine, respectively, while decreasing 41% in phosphatidylcholine. A decrease of 5–10% was observed in $C_{18:1}$ in all phospholipids at the higher enzyme concentration.

Effect of EGTA and $CaCl_2$ on PS enhancement of opiate binding. When a fresh or frozen EGTA-washed membrane preparation was incubated for 30 min at 37°C in 10^{-3} M Tris and washed with 0.05 M Tris, the enhancement of opiate binding by PS was comparable to that of the control (Table 3). After the addition of 10^{-4} M EGTA to the EGTA-washed preparations, PS resulted in a 41% enhancement of fresh membranes and a 115% enhancement of a day-old preparation stored at −20°C. In the presence of 10^{-5} M $CaCl_2$, the enhancement of opiate

TABLE 1

Effect of phospholipase A_2 on opiate binding and release of fatty acids in the presence and absence of added phosphatidylserine

Neural membranes were incubated for 5 min at 37°C in phospholipase A_2 (*Viper russelli*) and exposed to phosphatidylserine for another 10 min prior to the measurement of opiate binding. Equivalent samples were used for measuring the release of free fatty acids during the total incubation period. The values in parentheses indicate the increment over the control level. The data are expressed as mean ± standard deviation. All percentage changes in opiate binding are significant at $P < 0.01$–0.001 according to the *t* test.

	Added PS	Opiate binding		Free fatty acids (nmol/mg)		
		mol/mg × 10^{14}	% Change	18:1	22:6	20:4
Control	−	4.6 ± 0.32	—	3.7 ± 0.2	0.5 ± 0.04	2.5 ± 0.03
	+	6.5 ± 0.50	41	3.2 ± 0.2	0.5 ± 0.05	2.4 ± 0.02
2 × 10^{-4} units PL-A	−	2.6 ± 0.30	−44	5.1 ± 0.5 (1.4)	5.5 ± 0.5 (5.0)	5.2 ± 0.05 (2.7)
	+	5.0 ± 0.40	18	10.4 ± 0.9 (7.2)	15.8 ± 1.8 (15.3)	5.1 ± 0.4 (2.7)
1 × 10^{-3} units PL-A	−	0.3 ± 0.02	−93	9.7 ± 0.9 (6.0)	19.4 ± 1.5 (18.8)	11.1 ± 0.9 (8.5)
	+	0.4 ± 0.04	−86	28.6 ± 2.0 (25.4)	31.8 ± 3.0 (31.3)	10.0 ± 0.8 (7.5)

TABLE 2

Fatty acid content of membrane phospholipids after attack by phospholipase A_2

Values are expressed in nmol/mg membrane protein and represent an average of two separate experiments agreeing with 10% of the mean. PL-A concentration = 2×10^{-4} and 1×10^{-3} units/mg protein.

	Phosphatidylserine			Phosphatidylethanolamine			Phosphatidylcholine		
	C	0.0002	0.001	C	0.0002	0.001	C	0.0002	0.001
16:0	3.0	3.0	2.4	22.0	21.6	20.2	150.5	148.0	134.8
18:0	61.5	58.2	55.8	72.5	69.1	65.5	43.9	43.2	40.8
18:1	23.4	23.4	22.3	49.2	48.7	46.3	84.9	83.9	79.0
20:4	4.1	3.9	2.9	34.7	33.6	30.0	16.1	14.7	11.3
22:6	30.0	28.2	24.4	69.3	65.5	54.5	7.3	7.0	4.0
Other	7.1	8.1	7.8	97.8	97.5	89.6	13.6	13.3	13.1
Total nmol	129.1	124.7	115.6	346.5	336.0	306.1	316.3	310.1	283.0

binding was 47% with the fresh and 69% with the frozen membranes. The enhancement also occurred in both preparations with 10^{-4} M $CaCl_2$, although the degree of opiate binding was diminished by this concentration of $CaCl_2$. In the presence of 10^{-4} M $MnCl_2$, a 29% enhancement was observed in the EGTA-washed fresh membranes, and an 84% enhancement in the frozen preparation. Similar results were obtained with EDTA (data not shown).

Effect of repeated EGTA washing on Ca levels and opiate binding in neural membranes. When neural membranes were prepared in and exposed repeatedly to 10^{-3} M EGTA, the degree of opiate binding was not significantly decreased until the third washing with EGTA (Table 4). After the fourth wash, opiate binding decreased about 30%. On the other hand, the ability of PS to enhance opiate binding increased from 40% initially to 69% after the fourth wash with EGTA. The presence of 10^{-4} M EGTA in the incubation medium after the fourth wash slightly decreased opiate binding and the enhancement by PS. With repeated washing in EGTA, the level of ^{45}Ca remaining in the membranes decreased progressively, attaining a value of 1–2% of the original after the fourth wash with EGTA.

Effect of albumin on protection of opiate binding to PL-A treatment. In the presence of bovine serum albumin, the addition of PS to neural membranes had no enhancement effect on opiate binding (Table 5). After exposure of membranes to 2×10^{-4} units PL-A/mg membrane protein, opiate binding was reduced to 42% of the control, while the subsequent addition of the lipid restored binding to the control level. The presence of albumin greatly reduced the inhibitory effect of the li-

TABLE 3

Influence of EGTA and $CaCl_2$ on PS enhancement of opiate binding of neural membranes

Neural membranes were incubated in 0.05 M Tris containing 10^{-3} M EGTA at 37°C for 30 min and washed once with 0.05 M Tris. Membranes were used immediately or frozen at −20°C and used 24 h later. Control = membranes prepared without EGTA. 10^{-4} M EGTA and 10^{-4} or 10^{-5} M $CaCl_2$ were added to the incubation medium of EGTA-washed preparations. DHM bound is in mol $\times 10^{14}$/mg protein. PS = phosphatidylserine. The data are presented as mean ± standard deviation and are significant at $P < 0.01$–0.001.

	Added PS	Fresh membranes		Frozen membranes	
		DHM bound	% Increase by +PS	DHM bound	% Increase by +PS
Control	−	4.2 ± 0.30	—	2.2	—
	+	6.0 ± 0.45	44	3.7	67
EGTA washed	−	4.0 ± 0.30	—	2.1	—
	+	5.7 ± 0.45	42	3.6	67
EGTA washed + 10^{-4} M EGTA	−	4.3 ± 0.30	—	1.6	—
	+	6.1 ± 0.45	41	3.4	115
EGTA washed + 10^{-5} M $CaCl_2$	−	4.2 ± 0.30	—	2.3	—
	+	6.2 ± 0.40	47	3.9	69
EGTA washed + 10^{-4} M $CaCl_2$	−	2.7 ± 0.20	—	2.2	—
	+	3.7 ± 0.25	37	3.6	64
EGTA washed + 10^{-4} M $MnCl_2$	−	4.5 ± 0.30	—	2.3	—
	+	5.8 ± 0.40	29	4.2	84

TABLE 4

Effect of repeated EGTA washing on Ca level and opiate binding of neural membranes

Rat brain was homogenized in 10^{-3} M EGTA + 0.05 M Tris, incubated for 30 min, and recentrifuged (1st wash). The procedure was repeated three times. To measure Ca, 0.1 μCi of ^{45}Ca (10 Ci/g) was incubated with membranes (prepared without EGTA) for 1 h, centrifuged, and resuspended in 10^{-3} M EGTA. The subsequent procedure was then identical as described. The results are an average of three separate experiments agreeing within 6% of the mean.

	Bound ^{45}Ca		Opiate binding (mol × 10/mg protein)		
	Radioactivity	% Remaining	Control	+PS	% Change
	dpm				
Initial pellet	13,690	100	4.3	6.0	40
10^{-3} M EGTA (1st wash)	5,060	35	4.2	6.3	50
10^{-3} M EGTA (2nd wash)	2,590	19	4.0	6.1	52
10^{-3} M EGTA (3rd wash)	1,130	8	3.7	5.8	57
10^{-3} M EGTA (4th wash)	220	1.5	3.2	5.4	69
4th wash + 10^{-4} M EGTA[a]	220	1.5	3.0	4.9	63

[a] 10^{-4} M EGTA added to incubation medium.

TABLE 5

Effect of bovine albumin on protection of opiate binding to phospholipase treatment in the presence and absence of phosphatidylserine

Bovine serum albumin (BSA) at a concentration of 2 mg/ml was added to membranes 2 min prior to the addition of phospholipase A_2 and the mixture incubated for 5 min at 37°C. A final concentration of 10^{-4} M EGTA or $CaCl_2$ was added 2 min prior to PS, the mixture briefly homogenized and incubated for 15 min at 37°C. Opiate binding was then measured. The results are presented as mean ± standard deviation and are significant at $P < 0.01$.

	Added BSA	Added PS	Opiate binding	% Control
			mol × 10^{14}/*mg*	
Control	−	−	4.3 ± 0.30	—
		+	6.0 ± 0.45	140
	+	−	4.3 ± 0.35	100
		+	4.4 ± 0.35	102
PL-A (2×10^{-4} units)	−	−	2.1 ± 0.15	48
		+	4.4 ± 0.30	102
	+	−	3.8 ± 0.25	85
		+	3.8 ± 0.30	85
PL-A (2×10^{-3} units)	−	−	0.2 ± 0.01	5
		+	0.2 ± 0.01	5
	+	−	2.3 ± 0.20	52
		+	2.2 ± 0.20	50
PL-A (2×10^{-3} units) + 10^{-4} M EGTA	−	−	4.0 ± 0.30	93
		+	4.0 ± 0.30	93
PL-A (2×10^{-3} units) + 10^{-4} M $CaCl_2$	−	−	2.6 ± 0.25	56
		+	2.6 ± 0.25	56

pase, while adding PS failed to produce any enhancement. At a 10-fold higher concentration, the lipase inhibited opiate binding completely and about 50% in the absence and presence of albumin, respectively, while the lipid had no effect with or without albumin present. In the presence of 10^{-4} M EGTA, the inhibitory effect of PL-A and the enhancement effect of PS on opiate binding were abolished. The presence of 10^{-4} M $CaCl_2$ did not alter the inhibitory effect of the enzyme, but prevented the enhancement of opiate binding.

Effect of sonication on opiate binding. Sonication of rat brain membranes resulted in an over 50% loss of opiate binding, while the addition of PS increased binding to 80% of the control level without added lipid (Table 6).

Effect of PL-A on opiate binding of membranes from spinal cord. Opiate binding to rat spinal cord membranes was enhanced 60% by the addition of PS (Table 7). Exposure of the membranes to 0.02 μg of PL-A/mg protein resulted in a 32% reduction in opiate binding, while the subsequent addition of PS increased binding

TABLE 6

Effect of sonication on opiate binding to rat brain membranes

Membranes were sonicated in the presence and absence of added phosphatidylserine prior to measurement of opiate binding. The results are presented as mean ± standard deviation and are significant at $P < 0.01$.

Membranes	Added PS	DHM bound	% Control
		× 10^{14} *mol/mg*	
Control	−	4.2 ± 0.30	—
	+	5.7 ± 0.40	135
Sonicated	−	2.0 ± 0.20	53
	+	3.4 ± 0.25	81

TABLE 7

Effect of phospholipase A_2 on opiate binding to rat spinal cord membranes in the presence and absence of added phosphatidylserine

Spinal cord membranes were incubated with phospholipase A_2 (PL-A) for 5 min at 37°C. After the addition of 100 μg phosphatidylserine/mg protein, tubes were incubated for another 10 min at 37°C, prior to the addition of ^{3}H-DHM and either levorphanol or dextorphan. The results are based on three separate experiments agreeing within 6% of the mean.

	DHM bound	% Change
	× 10^{14} *mol/mg*	
Control	0.70	—
Control + PS	1.12	60
2×10^{-4} units PL-A	0.49	−32
2×10^{-4} units PL-A + PS	0.80	14
5×10^{-4} units PL-A	0.40	−43
5×10^{-4} units PL-A + PS	0.32	−54

14% over the control level. At a concentration of 0.4 μg of PL-A/mg protein, binding was reduced 43% in the absence and 54% in the presence of PS.

DISCUSSION

One of the objectives of the present study was to determine whether Ca or another multivalent cation was essential for the ability of PS to enhance stereospecific opiate binding. The failure to alter the lipid enhancement appreciably by repeated washing of membranes with EGTA or EDTA indicates that Ca or related cations are not required. Although it is difficult to remove all Ca from the membranes even after extensive washing with EGTA, the enhancement effect is only marginally reduced when only 2% of the original bound Ca remains. Even the presence of EGTA, Ca, or Mn in the incubation medium of the EGTA-washed membranes failed to modify the lipid enhancement of opiate binding. It can be concluded that multivalent cations are not required for the enhancement effect. It had been previously demonstrated that PS in the Ca form is no longer effective (8).

It should be mentioned that the purity of the PS is essential for obtaining the results described in the present study. Slight contaminants, particularly those obtained from the DEAE-cellulose used in the purification, greatly reduce or abolish the enhancement effect on opiate binding. Other factors which reduce the enhancement are the oxidation and hydrolysis of the lipid, the latter effect resulting in the formation of the highly inhibitory unsaturated fatty acids (8). When working with such suboptimal preparations of lipid, the enhancement effect was not demonstrable in the EGTA-washed membrane preparations, but returned upon the subsequent addition of 10^{-5} M Ca. The explanation for such results is unknown.

To establish the validity of the argument that PS is a requirement for the opiate receptor (2, 4), an explanation must be offered for the fact that albumin is able to prevent the inhibitory effects of PL-A treatment on opiate binding. One possibility is that the albumin alters the pattern of the enzymic attack of the membrane PS which is believed to be asymmetrically distributed (9, 10). The similarity in the pattern of fatty acid hydrolysis by the enzyme in the presence and absence of albumin

does not lend support to this argument. Another possibility is that, following enzymic cleavage of the lipid associated with the receptor, the PS may redistribute and replenish the depleted lipid at the receptor site. The fact that lateral diffusion of membrane phospholipids (11, 12) and phospholipid exchange (13) between membrane components are relatively rapid processes lends support to this argument. It may also be argued that the protective effect of albumin against PL-A supports the argument that phospholipids are not required for opiate binding. Although the protective effect of albumin is evident at lower concentrations of PL-A, it is less evident at higher enzyme concentrations when the levels of PS are reduced more than 10% of normal. At concentrations of the enzyme exceeding 0.01 unit/mg membrane, albumin is ineffective in preventing the inhibition of opiate binding. In previous studies where albumin was reported to be ineffective, only the higher range of enzyme concentrations was used (4).

Opiate binding was reduced about 50% when the fatty acids released by PL-A—mostly $C_{22:6}$—were about 5 nmol/mg membrane protein. The PS content of the membranes was 0.060 mg/mg protein, which represents about 200 nmol of fatty acid at the C_2 position, so that less than 3% of the endogenous PS was depleted by PL-A. At greater concentrations of PL-A, when the released $C_{22:6}$ approached 20 nmol/mg protein, the inhibition of opiate binding was complete. It is conceivable that the PS associated with the receptor may be more readily accessible to the enzyme.

It has been reported that the binding of opiate agonists, but not antagonists, to neural membranes is enhanced by Mn and other divalent cations (14). Since opiate binding was decreased by EDTA and could be subsequently restored by the addition of Mn or Ni, it was inferred that a divalent cation served as a modulator of receptor function (14). The present studies do not indicate that washing with either EDTA or EGTA significantly diminishes opiate binding or that the addition of either Mn or Ca to EGTA-washed membranes significantly increases opiate binding. In frozen membrane preparations there occurs a marked reduction of opiate binding, and the degree of enhancement resulting from PS is twice that obtained with the unfrozen membranes. The addition of 10^{-4} M $MnCl_2$ to EGTA-washed frozen membranes does appear to augment the enhancement effect of PS. No explanation is evident for this binding. Nevertheless, the possibility that an extremely low concentration of Ca or some other multivalent cation is required for opiate binding cannot be ruled out.

Although the lipid concentration of membrane preparations from rat spinal cord are somewhat greater than that found in rat brain, the addition of PS produced an even greater enhancement than that generally found in membranes from brain. It is conceivable that the increased enhancement is related to the fact that the concentration of binding sites in spinal cord is only 16% of that bound in brain. The enhancement by PS appears to be greater when the degree of opiate binding is decreased, as exemplified by the studies with frozen and sonicated membranes.

REFERENCES

1. Pasternak, G. W. and S. H. Snyder. Opiate receptor binding: effects of enzymatic treatments. *Mol. Pharmacol.* **10:** 183–193 (1974).
2. Abood, L. G. and M. MacNeil. Phospholipid involvement in the interaction of abused drugs with neural membranes, in *Membrane Mechanisms of Drugs of Abuse* (C. Sharp and L. G. Abood, eds). Alan Liss, New York, 1–26 (1979).
3. Lin, H. K. and E. J. Simon. Phospholipase A inhibition of opiate binding can be reversed by albumin. *Nature (Lond.)* **271:** 383–384 (1978).
4. Abood, L. G., N. Salem, M. MacNeil and M. Butler. Phospholipid changes in synaptic membranes by lipolytic enzymes and subsequent restoration of opiate binding with phosphatidylserine. *Biochim. Biophys. Acta* **530:** 35–46 (1978).
5. Salem, N., L. G. Abood and W. Hoss. Separation of brain phosphatidylserine according to degree of unsaturation by thin layer chromatography. *Anal. Biochem.* **76:** 407–415 (1976).
6. Rouser, G., G. Kutchevsky and A. Yamamoto. Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids, in *Lipid Chromatographic Analysis* (G. V. Marinette, ed.). M. Dekker, New York, Vol. 1, 99–162 (1967).
7. Bligh, E. G. and W. J. Dyer. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917 (1959).
8. Abood, L. G., N. Salem, Jr., M. MacNeil, L. Bloom and M. E. Abood. Enhancement of opiate binding by various molecular forms of phosphatidylserine and inhibition by other unsaturated lipids. *Biochim. Biophys. Acta* **468:** 51–62 (1977).
9. Verkleij, A. J., R. F. A. Zwaal, B. Roelofson, P. Comfurius, D. Kastchyn and L. L. M. van Deenen. The asymmetric distribution of phospholipids in the human red cell membrane. *Biochim. Biophys. Acta* **323:** 178–193 (1973).
10. Marinetti, G. V. Arrangement of phosphatidylserine and phosphatidylethanolamine in erythrocyte membranes. *Biochim. Biophys. Acta* **465:** 198–209 (1977).
11. Edden, M. Rotational and translational movements in membranes. *Annu. Rev. Biophys. Bioeng.* **3:** 179–211 (1974).
12. Cherry, R. J. Protein motility in membranes. *FEBS Lett.* **55:** 1–7 (1975).
13. Bloj, B. and D. B. Zilversmith. Asymmetry and transposition rates of phosphatidylcholine in rat erythrocyte ghosts. *Biochemistry* **15:**1277–1283 (1976).
14. Pasternak, G. W., A. M. Snowman and S. H. Snyder. Selective enhancement of [^{3}H] opiate agonist binding by divalent cations. *Mol. Pharmacol.* **11:** 735–744 (1974).

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