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ENHANCEMENT OF OPIATE BINDING BY VARIOUS MOLECULAR FORMS OF PHOSPHATIDYLSERINE AND INHIBITION BY OTHER UNSATURATED LIPIDS

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Summary

A study was undertaken on the possible involvement of phospholipids on stereospecific opiate binding to a rat brain membrane fraction comprised mainly of synaptic membranes. The addition of acidic phospholipids such as phosphatidylserine, phosphoinositides, and phosphatidic acid significantly enhanced opiate binding. With the exception of phosphatidylserine, when the acidic phospholipids contained a polyunsaturated acyl group, they were actually inhibitory, along with neutral phospholipids derived from brain. Both the $C_{18:0}$, $C_{18:1}$ form (derived from myelin) and the $C_{18:0}$, $C_{22:6}$ form of phosphatidylserine (derived from synaptic membranes) produced as much as a 45% enhancement in opiate binding. Unsaturated fatty acids were highly inhibitory, the degree of inhibition being related to the degree of unsaturation. Both phospholipase A and C were inhibitory; and the inhibitory effect of A could not be prevented by albumin or overcome with the addition of phosphatidylserine. With the use of the cross-linking agent, dinitrodifluorobenzene, it could be demonstrated that the phosphatidylserine of synaptic membranes appeared to be preferentially associated with membrane protein. The enhancement of opiate binding by phosphatidylserine diminished with increasing degree of cross-linking.

Introduction

In recent years with the demonstration of endogenous opiate-like peptides [1] it has become apparent that there exists in nervous tissue an opiate receptor, which has been identified by the use of high affinity stereospecific opiate agonists and antagonists [2—4]. Although the receptor has not yet been purified, it probably exists in the form of a membrane complex of protein and phospholipids, insofar as it is readily destroyed by proteases and phospholi-

pases [5,6]. Although stereospecific opiate binding is exhibited by cerebroside sulfates [7] as well as phosphatidyl serine [8] and other acidic phospholipids [8], the binding appears to be non-specific and has a relatively low affinity. Such observations pointed to the possibility that phosphatidylserine or other acidic lipids may be an integral component of the receptor. This hypothesis was strengthened by the finding that the addition of phosphatidylserine to neural membrane preparations greatly enhanced their high affinity stereospecific opiate binding [9].

While attempting to expand and elucidate the nature of this enhancement effect, it was observed that the degree of unsaturation in the acyl residues of the lipid was an important consideration. In the present study a wide variety of saturated and unsaturated phospholipids and fatty acids were examined for their effect on stereospecific opiate binding of synaptic membrane preparation. Among the significant findings was the observation that although polyunsaturated acyl residues were inhibitory to binding, the $C_{18:0,\ 22:6}$-diacyl phosphatidylserine, the major molecular species of synaptic membranes, was highly stimulatory.

Materials and Methods

Preparation of a neural membrane pellet from rat brain. After it had been established that a purified preparation of synaptic membranes exhibited no better opiate binding, a more easily prepared but cruder membrane preparation from rat brain was utilized [9]. The whole brains of two freshly killed rats were homogenized in 20 times their volume of 0.05 M Tris · HCl, pH 7.5, plus 10^{-3} M EDTA in a tight-fitting glass homogenizer with a teflon pestle using a mechanical stirrer at a speed of 1500 rev./min. After centrifugation the homogenate at 3000 × *g* for 20 min, the supernatant was decanted and centrifuged at 50 000 × *g* for 30 min to yield a pellet resuspended to a protein concentration of about 1.0 mg/ml. All operations were performed at 5°C. Electron microscopy revealed that this fraction was heterogeneous, consisting mainly of membranous fragments derived from nerve endings, axons and other processes; in addition it contained some fragments of smooth and rough endoplasmic reticulum, synaptic vesicles, and some ribosomes.

Preparation of phospholipids from bovine brain. With the exception of a few synthetic phospholipids, all of the lipids used in the present study were prepared from fresh calf brains utilizing the procedures of Rouser et al. [10], involving both column chromatography on DEAE-cellulose and preparative thin-layer chromatography utilizing silica gel H [8]. The source of 18 : 0, 18 : 1-enriched diacyl phosphatidylserine was from myelin prepared from bovine spinal cord by density gradient centrifugation [11]; whereas the 18 : 0, 22 : 6-enriched diacyl phosphatidylserine was derived from synaptic membranes prepared from calf's brain gray matter as described elsewhere [11]. Both preparations were over 98% pure phosphatidylserine and comprised at least 80% of monoenoic or polyunsaturated species as indicated. Phosphatidylinositol, prepared from baker's yeast by the method of Trevelyan [12] was a gift of W. Hoss. The synthetic phospholipids were from Sigma and the fatty acids and methyl esters were purchased from Supelco Inc.

Separation of the molecular species of phosphatidylserine. Separation of the various molecular species of phosphatidylserine were performed by argentation silica gel thin-layer chromatography as described elsewhere [13]. After the various species were extracted from the gel with chloroform/methanol/ acetic acid/ water (50 : 39 : 1 : 10, v/v) and filtered, the filtrate was extracted successively with 4 M NH_4OH, saturated NaCl, and EDTA in order to remove the Ag from the lipid. After the solvent was removed by flash evaporation, the phosphatidylserine was dissolved in benzene and stored under N_2 at $-20°C$. The purity and amounts of the various species was determined by thin-layer chromatography [13], and by infrared and nuclear magnetic resonance spectroscopy as will be presented elsewhere. The acyl composition and amount was determined by gas chromatography of the methyl esters [13]. As discussed elsewhere [13], the three major molecular species from calf's whole brain were 18 : 0, 18 : 0, 22 : 4-, and 18 : 0, 22 : 6-diacyl phosphatidylserine.

Measurement of binding of [^{3}H]dihydromorphine to membranes. The tecnique for measuring the binding of [^{3}H]dihydromorphine to various membrane preparations was essentially that of Pert and Snyder [4]. 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, spec. act. = 41 μCi/mmol, either 10^{-7} M levorphanol or dextrophan, and 1 mg membrane protein. The lipid was combined with the tissue by first introducing the lipid (dissolved in chloroform) into a glass homogenizer, evaporating off the chloroform with a stream of N_2, then adding the membrane suspension and homogenizing the mixture. After 1.2-ml aliquots of the tissue or tissue-lipid mixtures were pre-incubated for 15 min at 35°C, the [^{3}H]dihydromorphine plus either dextrophan or levorphanol were added, the incubation continued for another 15 min at 35°C, and the tubes were immersed in an ice bath. The samples were filtered under vacuum through 2.1 cm Whatman GF/B glass fiber filters; washed twice with 5 ml of ice-cold Tris buffer, and the radioactivity determined by liquid scintillation counting.

In those experiments where phospholipases were used, the membrane suspension was exposed to either 3 units/mg membrane protein of phospholipase A or 0.5 unit/mg membrane protein of phospholipase C in $1 \cdot 10^{-4}$ M cetyltrimethylammonium bromide/50 mM Tris buffer, pH 7.5, for 1 h at 35°C prior to the addition of [^{3}H]dihydromorphine. The cationic detergent was found to be as effective as Ca^{2+} which is a requirement for both phospholipase A and C [6]. The use of Ca^{2+} made it necessary to wash the treated pellet prior to measuring binding, since Ca^{2+} was inhibitory to binding, whereas the detergent was not inhibitory at the concentration used. The phospholipase A was from bee venom (10 000 units/g agarose) and the phospholipase C was Type V from *Bacillus cereus* (600 units/mg protein); both from Sigma Chemical Co.

Procedure for reaction with dinitrodifluorobenzene. Neural membranes were homogenized in 50 mM of $NaHCO_3$, pH 8.1, so that the final suspension contained 5 mg protein/ml. To the suspension was added 2,4-dinitro-1,5-difluorobenzene, dissolved in methanol, so that the final concentration of the agent was either $2 \cdot 10^{-4}$ M, while the final concentration of methanol did not exceed 0.1%. After the suspension was incubated for 1 h at 35°C, it was centrifuged at 100 100 × g for 30 min, washed with 50 mM Tris, pH 7.5, and rehomogenized in 50 mM Tris for measuring [^{3}H]dihydromorphine binding.

In order to determine the nature of the acyl composition of the lipids cross-linked to protein, the treated membranes were extracted with chloroform/methanol (2 : 1, v/v). The residue after centrifugation was treated with methanolic 5% HCl for 90 min at 60°C, a procedure which leads to the formation of both fatty acid and aldehyde derivatives. A similar analysis was made of untreated membranes. The procedures for gas chromatographic analysis of the acyl residues are described elsewhere [13]. Total *P* was determined after digestion of the residue in concentrated H_2SO_4 for treated and untreated membranes [14].

Results

Effect of phospholipids with varying unsaturation on opiate binding

A variety of phospholipids with known fatty acid composition were examined for their effect on opiate binding to neural membranes (Table I). With 18 : 0, 18 : 1-, and 18 : 0, 22 : 6-phosphatidylserine a 39 and 38% increase, respectively, was observed in opiate binding. Phosphatidylserine derived from white matter and gray matter produced a 36 and 37% enhancement, respectively. Fatty acid analysis of these phosphatidylserine samples indicates that the 18 : 0, 18 : 1 species comprises about 26% of the synaptic membrane phosphatidylserine, 76% of the white matter phosphatidylserine and 30% of the gray matter phosphatidylserine. The 18 : 0, 22 : 6 species comprises 52% of the synaptic membrane phosphatidylserine, 15% of the white matter phosphatidylserine, and 57% of the gray matter phosphatidylserine (Table I).

Both the saturated forms of phosphatidic acid and phosphoinositides produced an enhancement in opiate binding, the effect being 30 and 28%, respectively, (Table I). With phosphatidic acid derived from egg phosphatidylcholine containing about 17% $C_{18:2}$, there occurred a 25% inhibition in opiate binding. With brain phosphoinositides, which contained about 40% $C_{20:4}$, a 37% inhibition was obtained. Synthetic (—)-α-dipalmitoyl phosphatidylcholine and whole brain phosphatidylcholine were without effect on opiate binding. Synthetic (—)-α-dipalmitoyl phosphatidylethanolamine produced a 10% inhibition on binding; and whole brain ethanolamine phosphatides, which contained both plasmalogens and phosphatidylethanolamine [15] in a largely unsaturated form, resulted in a 20% inhibition of opiate binding.

Effect of various fatty acids on opiate binding

A plot of the number of unsaturated groups in various fatty acids against opiate binding showed a precipitous drop with one unsaturated group with a gradual decrease thereafter (Fig. 1). If the data is plotted semilogarithmically, an essentially linear curve is obtained (not shown). As the number of unsaturated bonds reach 4 (20 : 4), the inhibition of opiate binding was complete. The concentration of lipid used was $2 \cdot 10^{-4}$ M. When the concentration of the 18 : 1 acid was plotted against the binding activity, the decrease was almost linear; whereas with 22 : 6 an exponential-type curve was obtained (Fig. 2). Virtually complete inhibition occurred at a concentration of about $7 \cdot 10^{-5}$ M with the 22 : 6 acid and $2.5 \cdot 10^{-4}$ M with 18 : 1.

Various methyl esters of fatty acids were tested for their ability to inhibit

TABLE I

EFFECT OF PHOSPHOLIPIDS WITH VARYING UNSATURATION ON STEREOSPECIFIC OPIATE BINDING

Percent of principal acyl groups given in parentheses. The control value for [^{3}H]dihydromorphine was 720 counts/min; and the values represent an average of three separate experiments agreeing with 6% of the mean. Last column lists references to fatty acid composition of brain lipids; all other data being obtained by us. [^{3}H]dihydromorphine.

Phospholipid	Source	Acyl composition	[^{3}H]Dihydromorphine bound (percent control)	Ref.
None	—	—	100	—
Phosphatidylserine	Myelin	18 : 0 (40), 18 : 1 (43.3)	139	15
	Synaptic membrane	18 : 0 (39.1), 18 : 1 (13.1) 22 un (14.0), 22 : 6 (25.8)	138	—
	White matter	18 : 0 (37.6), 18 : 1 (37.6) 22 un (4.5), 22 : 6 (7.6)	136	—
	Gray matter	18 : 0 (41.6), 18 : 1 (15.3) 22 un (8.6), 22 : 6 (28.7)	137	—
Phosphatidic acid (from egg phosphatidylcholine)	Synthetic	16 : 0, 16 : 0	130	—
	Egg	16 : 0 (37 : 7), 18 : 0 (9.2) 16 : 1 (3.1), 18 : 1 (32.9) 18 : 2 (17.0)	75	16
Phosphoinositides	Yeast	16 : 0 (23), 16 : 1 (32) 18 : 0 (8), 18 : 1 (33)	128	—
	Brain	18 : 0 (42), 20 : 4 (40)	63	—
Phosphatidylcholine	Synthetic	18,: 0, 18 : 0	102	—
	Brain	16 : 0 (35.3), 18 : 0 (16 : 3) 18 : 1 (34.6), 20 : 4 (4.4) 22 : 6 (7.4)	95	17
Ethanolamine phosphatides	Synthetic	16 : 0, 16 : 0	90	—
	Brain	16 : 0 (8.3), 18 : 0 (24.4) 18 : 1 (12.5), 20.4 (13.6) 22.6 (29.2)	80	17

opiate binding to neural membranes (Table II). The methyl ester of the two saturated fatty acids, C_{18} and C_{20}, inhibited 12 and 19%, respectively; whereas the methyl ester of $C_{20:4}$ inhibited binding 57%. A mixture of saturated fatty acids produced only a slight inhibition in opiate binding: while a mixture of $2 \cdot 10^{-5}$ M each of various unsaturated fatty acids was almost completely inhibitory. When a comparable mixture of unsaturated fatty acids contained in addition $1 \cdot 10^{-4}$ M C_{18}, the inhibition was only 65%.

Effect of phospholipase treatment on binding

In an attempt to determine whether the unsaturated acids released by the

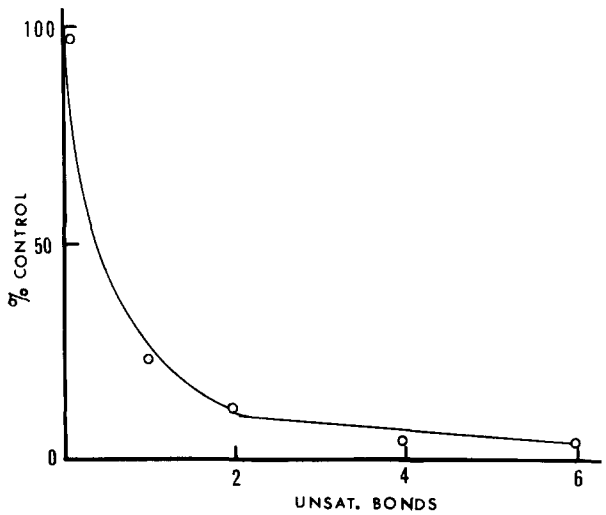

Fig. 1. Effect on opiate binding of degree of unsaturation in various fatty acids. Ordinate is percent control value of opiate binding. Abcissa is expressed as number of unsaturated bonds of following acids: $C_{18:1}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$. Concentration of fatty acids was $2 \cdot 10^{-4}$ M. Each point is an average of three determinations agreeing with 5%.

action of phospholipase A on neural membranes was responsible for the inhibitory action of the enzyme, various procedures intended to remove the fatty acids were employed (Table III). Upon exposure to 2 units of phospholipase A/mg membrane protein, stereospecific opiate binding was inhibited 84%. If either fatty acid-free bovine serum albumin or cetyltrimethylammonium bromide were present with the enzyme there was no alteration of the enzymic inhibition. Repeated washing of the membrane with 50 mM Tris buffer also failed to alter the inhibitory action. If phosphatidylserine were added after treatment with phospholipase A and removal of the enzyme by washing the membrane pellet, some restoration of opiate binding occurred. Treatment with phospholipase C resulted in a 60% inhibition of opiate binding; and upon the

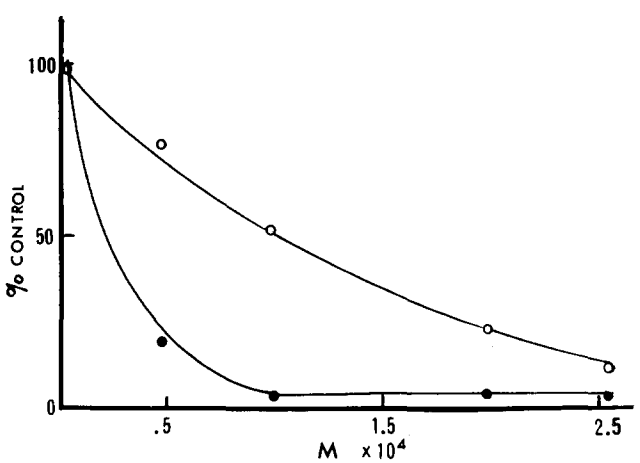

Fig. 2. Opiate binding vs. concentration of $C_{18:1}$ and $C_{22:6}$ fatty acids. The concentration of fatty acid was $2 \cdot 10^{-4}$ M. Each point is an average of three experiments agreeing within 5%.

TABLE II

EFFECT OF METHYL ESTERS OF FATTY ACIDS AND FATTY ACID MIXTURES ON OPIATE BINDING

Concentration of methyl esters of fatty acids was $2 \cdot 10^{-4}$ M. Fatty acid mixture 1: $4 \cdot 10^{-5}$ M each of 16 : 0, 18 : 0, 18 : 1 fatty acids. Fatty acid mixture 2: $2 \cdot 10^{-5}$ M each of 18 : 1, 18 : 2, 20 : 4, 22 : 6 fatty acids. Fatty acid mixture 3: $2 \cdot 10^{-5}$ M each of 18 : 1, 18 : 2, 20 : 4, 22 : 6 plus $1 \cdot 10^{-4}$ M 18 : 0 fatty acids.

	[^{3}H]Dihydromorphine bound	Inhibition (%)
Control	550	—
C_{18} ester	480	12
C_{20} ester	450	19
$C_{20:4}$ ester	238	57
Fatty acid mixture 1	493	11
Fatty acid mixture 2	55	90
Fatty acid mixture 3	183	65

addition of phosphatidylserine to the treated membranes, the inhibition was only 32%.

Effect of dinitrodifluorobenzene on opiate binding

The effect of dinitrodifluorobenzene, an agent which cross-links amino groups of phospholipids and proteins, was examined on opiate binding. Exposure of the membrane to a concentration of $4 \cdot 10^{-5}$ M of the agent resulted in a 13% inhibition of opiate binding; while the enhancement effect of phosphatidylserine was only 20%, as compared to 35% for the untreated membranes (Table IV). At a concentration of 10^{-4} M, the agent produced a 38% inhibition in opiate binding, while the addition of the lipid was without an enhancing effect.

In an effort to determine the nature of the phospholipid associated with membrane proteins, an analysis was carried out on the acyl composition of the

TABLE III

EFFECT OF PHOSPHATIDYLSERINE ON OPIATE BINDING AFTER TREATMENT WITH PHOSPHOLIPASES

Membranes were incubated for 1 h with either phospholipase A or C, washed, and then exposed to gray matter phosphatidylserine (see text). Bovine serum albumin (fatty acid free) was incubated with phospholipase A at a concentration of 5 mg/mg membrane protein. The results are an average of three separate experiments and agreed within 7% of the mean. CTAB, cetyltrimethylamonium bromide.

Treatment	[^{3}H]Dihydromorphine bound	Change (%)
Control	750	—
Phospholipase C	305	—60
Phospholipase C + phosphatidylserine	520	—32
Phospholipase A	120	—84
Phospholipase A + borine serum albumin	145	—81
Phospholipase A + CTAB	135	—82
Phospholipase A + washing	110	—85
Phospholipase A + phosphatidylserise	340	—55

TABLE IV

EFFECT OF DINITRODIFLUOROBENZENE ON OPIATE BINDING WITH AND WITHOUT PHOSPHATIDYLSERINE

Membranes were incubated 1 h at 35°C in dinitrodifluorobenzene (DNDFB) and washed prior to the measurement of stereospecific binding of [^{3}H]dihydromorphine. Last column refers to the percent enhancement for each experimental condition upon the addition of phosphatidylserine. Results are an average of three separate experiments agreeing within 6% of the mean.

	[^{3}H]Dihydromorphine bound	Control (%)	Phosphatidylserine enhancement (%)
Control	740	—	—
Control + phosphatidylserine	1030	135	35
4×10^{-5} M DNDFB	645	87	—
4×10^{-5} N DNDFB + phosphatidylserine	790	107	20
10^{-4} M DNDFB	460	62	—
10^{-4} M DNDFB + phosphatidylserine	475	63	1

lipids cross-linked to proteins following treatment with dinitrofluorobenzene. Advantage was taken of the characteristic fatty acid composition of individual brain phospholipids. The major saturated fatty acids were $C_{16:0}$ and $C_{18:0}$, while the major unsaturated acids were $C_{20:4}$ and $C_{22:6}$, with a small amount of $C_{22:4}$. Relatively small amounts of the $C_{16:0}$ and $C_{18:0}$ aldehydes were present (Table V). An analysis of the acyl composition of the untreated protein fraction yielded a somewhat different spectrum of acyl residues, with the $C_{16:0}$ fatty acid being the major component and $C_{22:6}$ and $C_{22:4}$ being completely absent. The difference in total *P* between the treated and untreated

TABLE V

ACYL COMPOSITION OF AMINOPHOSPHOLIPIDS CROSS-LINKED TO PROTEIN AFTER TREATMENT WITH DINITRODIFLUOROBENZENE AND CHLOROFORM/METHANOL EXTRACTION

Data expressed as average of two experiments agreeing within 6%.

Fatty acid and aldehyde	DNDFB (percent total)	Control (percent total)
16 : 0 dimethylacetal derivative	2.0	—
16 : 0	16.7	35.0
16 : 1	0.9	1.5
18 : 0 dimethylacetal derivative [a]	2.4	—
18 : 0	27.4	24.5
18 : 1	11.1	10.0
20 : 4 [b]	13.9	15.5
22 : un [c]	3.0	12.9
22 : 4	3.0	—
22 : 6	16.9	—
Other	2.7	0.6
Total *P* *	0.109	0.084

[a] A small amount of 17 : 0 is included.
[b] A small amount of 22 : 1 is included.
[c] Tentatively identified as 22 : 3 [25].
* Total *P* expressed as μmol per 4.0 mg membrane protein.

membranes was found to be 0.025 μmol; and this value represents the total aminophospholipids conjugated to the protein by the cross-linking agent (Table V).

Discussion

In attempting to interpret the present findings in relationship to the membraneous site for opiate binding, there are a number of questions to consider. The most germane is whether phosphatidylserine and/or other acidic membrane lipids are normally associated with the site. Insofar as the stereospecific binding site seems to be located mainly in synaptic membranes [9], where the major acidic lipid is phosphatidylserine [18], it is most likely that this lipid would be associated with the site. Although there are sufficient amounts of other acidic membrane lipids to account for opiate binding, these lipids are present in the unsaturated form which is inhibitory to binding. On the other hand, the $C_{22:6}$ form of phosphatidylserine, which is the principal molecular species of this lipid present, has a marked enhancing effect on the opiate binding of neural membranes.

Another question to consider is whether the acidic lipid is involved directly in binding or is needed to maintain a membrane protein in the appropriate molmolecular configuration. Since the K_D values for opiate binding to phosphatidylserine alone is 2—3 orders of magnitude greater than that for neural membranes [9], the lipid alone cannot account for the binding. Since opiate binding is abolished by treatment with phospholipase A, it would seem that the phospholipid was an essential component of the site. It has not been possible, however, to restore opiate binding after exposure of neural membranes to phospholipase A. Evidently, the membrane configuration was altered by the enzymic treatment and could not be restored by exposure to exogenous phosphatidylserine. It has been shown that exposure of giant squid axons to phospholipase A or C destroys excitability, evidently due to the destruction of the axolemmal membrane [19]. Since bovine albumin, which is known to complex with fatty acids and lysophospholipids [20], does not prevent or reverse the inhibitory effects of phospholipase A, it appears that the binding site had been altered by removal of the lipid. Insofar as exogenous phosphatidylserine does not restore binding activity after treatment with phospholipase A, the normal molecular configuration of the site cannot be restored by the lipid. In an effort to determine whether phospholipase A treatment solubilized the receptor, opiate binding was measured by equilibrium dialysis; but the results were similar to those obtained by filtration. It is possible, however, that solubilization may irreversibly destroy opiate binding [6]. On the other hand, with phospholipase C where the inhibition was not complete, the addition of phosphatidylserine resulted in a significant enhancement of opiate binding. Nevertheless, it was not possible to restore the binding to the normal level by addition of the lipid. The inhibitory action of phospholipase C, probably due to the removal of the phosphate group which is evidently a requirement for the lipid enhancement [6], may be due to the fact that the diglyceride end product cannot be displaced by the exogenous phosphatidylserine.

It is apparent from the studies with the cross-linking agent, dinitrodifluoro-

benzene, that conjugation of membrane proteins and aminophospholipids together or with one another prevents the lipid enhancement of opiate binding; the degree of prevention being dependent upon the concentration of the agent used. One explanation for this observation is that the cross-linking of proteins and aminophospholipids contributes to a less fluid membrane, so that the added phosphatidylserine is less likely to gain access to the opiate binding sites. Marinetti and Love [21] have demonstrated that as much as 84% of the phospholipid cross-linked to protein is phosphatidylserine. Since it is well-established that only the phosphatidylethanolamine contains a large proportion of $C_{18:0}$ and $C_{16:0}$ ethers [22], the ratio of $C_{22:6}$ to $C_{18:0}$ aldehyde (Table V) can be used as an index of the ratio of phosphatidylserine to phosphatidylethanolamine. In whole rat brain 55% of the phosphatidylethanolamine is comprised of plasmalogens, whereas phosphatidylserine exists only in the diacyl form [23]. Since the $C_{22:6}$ was found associated with the protein fraction, it can be inferred that phosphatidylserine of neural membranes is preferentially associated with proteins. It also appears as if a small amount of phosphatidylethanolamine is associated with protein. It is of interest to note that the $C_{20:4}$ is also associated with the untreated membranes (Table V); a finding which suggests that phosphoinositides are also associated with proteins from neural membranes [24,25]. It is well-known that chloroform/methanol, unless acidified with HCL, does not extract phosphoinositides along with other lipids; and a plausible explanation for this phenomenon may be that this lipid is closely associated with protein. The fact that phosphatidylserine appears to be closely associated with the membrane, while having a marked enhancement effect on opiate binding, is additional evidence in support of the argument that it may be a component of the opiate receptor. It is also tempting to speculate that since the phosphoinositides are inhibitory to binding, while appearing to be associated with proteins, they may be exerting a modulatory effect on opiate binding.

There are numerous studies demonstrating the deleterious effect of fatty acids and lysophospholipids on various biological preparations. Unsaturated fatty acids, liberated by the action of phospholipase A on rat mitochondria, result in an uncoupling of oxidative phosphorylation [26]. Mitochondria isolated from the brown adipose tissue of rats shows a decreased level of oxidative phosphorylation; and the effect, which is due to the presence of fatty acids, is reversible by 2% albumin plus GTP plus carnitine [26]. Both the K^+- and Na^+-stimulated component of brain ($Na^+ + K^+$)-ATPase are reversibly inhibited by fatty acids at concentrations as low as $5 \cdot 10^{-5}$ M; while the inhibition is far greater with unsaturated than with saturated fatty acids and increases with increasing chain length of fatty acid [27]. The $Na^+ + K^+$)-ATPase of synaptosomal membranes has been reported to increase after exposing mice to a diet deficient in essential fatty acids [28]. The effect may be related to the resulting decrease in the $C_{22:6}$ and $C_{22:4}$ acyl groups of the membrane phospholipids, which appear to be part of the enzyme complex. It is now well-established that the acyl composition of the membrane lipids is essential to the maintenance of the physical and biochemical characteristics of membranes; and that alterations either in their composition or molecular interrelationships can affect such properties as the phase transition [29], fluidity [30], and membrane fusion [31].

Phosphatidylserine has been shown to potentiate the chemically induced contractile responses of guinea pig ileum [32] and aortic smooth muscle [33] in addition to enhancing the antigen-induced release of histamine from mast cells [34]. It has also been suggested that phosphatidylserine might be involved in regulation of Ca^{2+} binding to membranes, and that various drugs, including the opiates, may compete with Ca^{2+} for such sites [35,36]. Since the enhancement effect on the contraction of smooth muscle did not occur in the absence of Ca^{2+}, it was inferred that the exogenous phosphatidylserine was influencing the Ca^{2+}-dependent contractile system [21]. Insofar as the removal of membrane Ca^{2+} by EDTA or ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) does not prevent the enhancement of opiate binding by phosphatidylserine but rather facilitates it [9], it does not appear as if the lipid exerts its action by sequestering endogenous Ca^{2+} which may be inhibitory to the receptor. Other evidence excluding the possibility that the lipid enhances by complexing Ca^{2+} is derived from the fact that the ethyl glycolate ester of phosphatidylserine, which does not complex Ca^{2+}, is also enhancing to opiate binding [6].

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