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INVESTIGATIONS OF THE MECHANISM OF SELECTIVE INHIBITION OF TYPE B MITOCHONDRIAL MONOAMINE OXIDASE BY PHOSPHATIDYLSERINE

TRENT DAVID BUCKMAN*, SAMUEL EIDUSON and ROSEANNE BOSCIA

Departments of Psychiatry and Biobehavioral Sciences and Biological Chemistry, The Neuropsychiatric and Brain Research Institutes, UCLA School of Medicine, Center for the Health Sciences, University of California, Los Angeles, Los Angeles, CA 90024, U.S.A.

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Abstract—Liposomes of phosphatidylserine (PS) were found to inhibit strongly the B-form of membrane bound monoamine oxidase (MAO) isolated from rat and bovine liver, while having no effect on the rat liver A-form. Use of ^{14}C-liposomes demonstrated high levels of PS association with the membrane, which could not be removed by extensive washing with high ionic strength buffers. The inhibition of MAO-B was not reversed on further perturbation of the membrane by chaotropic agents, sonication, or treatment with additional liposome preparations of phosphatidylcholine or phosphatidylinositol. Partial reversal of the inhibition was found when the PS-treated bovine liver membrane was solubilized with the detergent octyl glucoside. PS, however, had no effect on a solubilized preparation of bovine liver MAO. These results suggest a specific interaction between MAO and PS rather than an indirect effect of bulk changes in membrane properties, but an intact membrane was, nevertheless, required to mediate the inhibition. Comparison of the decreases in apparent levels of MAO-B in rat liver mitochondrial membranes that were calculated from changes in relative catalytic activities with A and B specific substrates or changes in sensitivity to A-form specific reversible and irreversible inhibitors, all showed good quantitative correlation. Lineweaver–Burk plots of the effect of PS incorporation into bovine liver mitochondrial membranes on MAO oxidation of phenylethylamine exhibited the expected pattern for a noncompetitive inhibitor acting on a ping-pong mechanism bireactant enzyme. On the basis of these results, a possible *in vivo* role for the acidic phospholipids in regulating apparent levels of MAO from one tissue to another and/or in response to environmental effects is proposed.

As the principal metabolic enzyme in the degradation of the biogenic amine neurotransmitters, considerable attention has been devoted to the origin of variations in substrate specificity of mitochondrial monoamine oxidase (MAO) from one tissue to another. The generally accepted explanation of this phenomenon involves the existence of two multiple forms, designated A and B, although their structural relationship remains unclear. In the past decade, there has been particular interest in the role played by lipid–peptide interactions in this intrinsic protein of the mitochondrial outer membrane (OM). A number of studies have demonstrated effects of membrane perturbations on substrate specificity, and the hypothesis has been put forward that the multiple forms may be a single peptide conformationally differentiated by alterations of membrane environment [1]. The *in vivo* significance of these studies remains questionable, however, as they generally have involved either interactions of lipids with detergent solubilized enzyme [2, 3] or an examination of the effects of membrane disruption by organic solvents or phospholipases [4–7]. Two recent reports of limited scope have claimed effects of phosphatidycholine (PC) preparations on native membrane bound MAO. In one of these [8], PC liposomes were added to a preparation of crude human brain cortex mitochondria, resulting in an inhibition of serotonin (5-HT) oxidation at low substrate concentrations and an activation at high substrate concentrations. In the second report [9], incubation of rat liver OM with a mixture of synthetic dimyristol PC and potassium cholate resulted in replacement of 95% of the membrane phospholipids by the PC without release of the MAO. The PC replacement was found to affect clorgyline inhibition patterns but not activities with β-phenylethylamine (PEA) or 5-HT. No controls for the effect of cholate alone were presented.

As an approach to a more realistic model for the effect of membrane alterations on MAO, we have undertaken an investigation of the interaction of liposomes of individual phospholipids with OM preparations, the simplest system that preserves the native membrane environment of the enzyme. Other studies have shown efficient incorporation of lipids from liposomes into both plasma [10] and mitochondrial membranes [11] of mammalian cells without the addition of phospholipid exchange proteins as intermediaries. Although the detailed mechanism of interactions of liposomes with natural membranes is not fully understood, the modes which have been observed include incorporation of individual lipid molecules, membrane fusion, endocytosis and liposome adsorption (for a review of these mechanisms, see Ref. 12). Our studies have focused on interac-

* Send correspondence and reprint requests to: Trent D. Buckman, Ph.D., Department of Psychiatry and Biobehavioural Sciences, The Neuropsychiatric Insitute, UCLA, 760 Westwood Plaza, Los Angeles, CA 90024, U.S.A.

tions between the acidic phospholipids which are found in significant amounts in the mitochondria [phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipid (CL)] and OM preparations containing the MAO-A and/or -B forms. The scope of effects of these three phospholipids on the individual multiple forms is detailed in a separate publication [13]. Although lipids of this class are minor structural components of biological membranes, numerous studies have shown that they play a unique role in binding and regulation of membrane enzymes. Of particular relevance to the present work are reports of effects on a number of other enzymes involved in neurotransmission. A 2- to 3-fold stimulation of purified bovine caudate tyrosine hydroxylase by PS, which appeared to be the result of lower K_m for cofactor binding, has been reported [14]. The $(Na^+–K^+)$ATPase of cerebral cortex was shown in another study to be specifically activated by the acidic phospholipids and most strongly by PS [15]. In the myocardial catecholamine receptor–adenylcyclase system, PI was found uniquely to stimulate catecholamine binding while PS stimulated binding only to the second messenger hormone binding site for glucagon and histamine [16]. Several mitochondrial membrane enzymes including cytochrome oxidase [17] and the electron transport coupled ATPase [18], have been found to be subject to acidic phospholipid activation. The mitochondrial matrix enzyme, glutamate dehydrogenase, is inhibited by CL and PS [19]. This inhibition appears to involve binding of the enzyme specifically to these lipids as components of the inner membrane. There have also been several reports implicating the acidic phospholipids in the binding of MAO to the mitochondrial OM [4, 7].

In the course of our survey of the effects of the acidic phospholipids on membrane bound MAO, we have found a strong and highly specific inhibition of the B-form of the enzyme by PS. The present report concerns further studies of this phenomenon to probe the nature of the interaction of PS with the membrane and the mechanism of its inhibition of MAO-B. These experiments include various attempts to reverse the inhibition by disruption of the membrane or displacement of the PS, a study of the effect of incubation temperature on PS incorporation and MAO inhibition, and an examination of the effects of PS on inhibitor specificity and kinetics with the B-substrate, phenylethylamine (PEA). As inhibition of the B-form of MAO is the focus of the work described here, experiments were carried out primarily with OM isolated from a tissue with only B-type activity, bovine liver. Specificity of PS inhibition for the B-form and sensitivity to A-form selective inhibitors were demonstrated using OM from rat liver with comparable levels of MAO-A and -B.

MATERIALS AND METHODS

Chemicals. Phosphatidylserine and phosphatidylcholine from bovine brain, soybean phosphatidylinositol and harmaline were obtained from the Sigma Chemical Co., St. Louis, MO. The phospholipids were the highest purity available (approximately 98%) and used without further purification. Each ran as a single spot in standard TLC systems for phospholipid separation. Dioleoyl phosphatidylserine, ^{14}C-labeled (60 mCi/mmole), was from the Amersham Corp., Arlington Heights, IL. Octyl-β-D-glucopyranoside was from CalBiochem, La Jolla, CA. ^{14}C-Labeled MAO substrates, 5-HT, PEA and tryptamine, for radioassays, were obtained from the New England Nuclear Corp., Boston, MA. Cold PEA for radioisotope dilution and oxygen electrode assays was from Sigma; clorgyline was a gift of Dr. Jean Shih, University of Southern California.

Mitochondrial OM preparations. Fresh mitochondria isolated by differential centrifugation in 0.25 M sucrose were used for OM preparations. The OM was separated from inner membrane (IM) and matrix material by hypotonic swelling of the mitochondria followed by shrinkage of the IM with ATP-MG^{2+} and brief sonication, as described by Kearney *et al.* [20]. Purity of the OM preparations was ascertained from increases in the ratio of MAO activity with PEA as a substrate relative to the inner membrane marker succinate dehydrogenase (SDH), assayed according to the colorimetric method described by Singer [21].

Rat livers were obtained from young Sprague–Dawley rats within 10 min of decapitation. After removal of blood clots and connective tissue, livers were minced and a 25% homogenate was prepared in pH 7.2, 10 mM potassium phosphate, 0.25 M sucrose, using a glass–teflon homogenizer. The OM preparations from this tissue showed 1.4- to 3.8-fold purifications with respect to MAO (PEA) (specific activity = 5.7 to 12.6 nmoles per mg per min) and a 9.5- to 10-fold increase in the ratio of MAO to SDH.

Beef liver was obtained from a packing house within 30 min of death and immediately placed on ice. Mitochondria preparation was begun within 2 hr. Procedures were the same as for rat liver except that tissue was homogenized using a Waring blender (60 sec). The OM isolation gave a 5.5-fold purification with respect to MAO (PEA) (specific activity = 28 nmoles per mg per min) and a 21-fold increase in MAO/SDH. Membranes were stored at 10 mg/ml protein in 0.05 M potassium phosphate (pH 7.4) at −80°, in aliquots sufficient for individual experiments.

Liposome preparation. Phospholipid solutions in $CHCl_3$-methanol or ethanol were dried under a stream of N_2 or at high vacuum in a rotary evaporator to give a uniform thin film in a conical vessel. Residual solvent was removed at high vacuum for 30 min at room temperature. N_2-purged, pH 7.4, 50 mM potassium phosphate was added to give a final liposome concentration of 5 mg/ml. The vessel was sealed under N_2 and sonicated until clear (10–15 min) with a Laboratory Supply Co. high energy ultrasonic bath at a bath temperature of 50°.

For determination of levels of PS association with the membrane, liposomes were dosed with the synthetic ^{14}C-labeled dioleoyl PS by adding this material to the initial $CHCl_3$-methanol solution of bovine brain PS to give a final specific activity of approximately 0.3 μCi/mg total lipid.

To obtain optimum results with respect to effects

on MAO activity, it was critical to use fresh lipid, stored at −20° under nitrogen for preparation of the liposomes. Also liposomes had to be obtained in a highly dispersed state (i.e. a high degree of clarification in the sonicated suspension) and had to be used within a day of preparation.

Incubation of OM preparations with PS. Aliquots of stock 5 mg/ml liposome were added to 10 mg/ml OM suspension in 0.05 M potassium phosphate buffer, pH 7.4. Mixtures were diluted to a constant volume corresponding to the largest aliquot of liposome used and incubated at 37° for 30 min. Incubation mixtures contained 1 to 2.7 mg OM protein/PS aliquot. Excess lipid was removed by centrifugation at 275,000 *g* for 30 min in a Beckman 75Ti, fixed angle rotor to pellet the membrane. The membrane was resuspended in phosphate buffer to approximately 1 mg/ml with the aid of a glass–teflon homogenizer cooled in ice. Controls used in the measurement of effects of PS on MAO activity were membranes handled in exactly the same manner except for addition of the liposomes. Results were plotted as normalized specific activities relative to these controls.

Measurement of [^{14}C]*PS incorporation.* Aliquots of resuspended OM (~0.5 mg protein) treated with [^{14}C]PS containing liposomes as described above were dissolved by heating to 37° for 60 min with an equal volume of hyamine hydroxide. The samples were counted in 10 ml Aquasol II after overnight dark adaptation in a Beckman LS 8000 scintillation counter.

KCl Washes of [^{14}C]*PS-treated bovine liver OM.* The efficacy of additional washes of the OM pellet with buffer at high ionic strength in removing the associated lipid was ascertained for a preparation of bovine liver OM treated with radiolabeled PS liposomes (1 mg/mg protein), pelleted, and resuspended in 0.05 M, pH 7.4 phosphate according to our usual procedures (see above). After assay for PEA activity and measurement of [^{14}C]PS association, the material was pelleted again and resuspended in the same volume of phosphate buffer containing 0.1 M KCl. This procedure was repeated twice more, and the final pellet was suspended in the buffer without KCl, reassayed, and recounted for associated [^{14}C]PS.

Detergent treatment of bovine liver OM before and after incubation with PS. To solubilize the membrane preparation prior to PS treatment, 5 mg of membrane protein (10 mg/ml) was made 1% in octyl glucoside from a 10% stock solution. Opacity of the membrane suspension immediately cleared up and, after standing for 10 min, the material was passed through a 1.0 × 18 cm Sephadex G25 column to remove excess detergent [22]. Aliquots of pooled protein-containing fractions were then mixed with PS liposomes at lipid/protein ratios of 0.1, 0.2, 0.5 and 1, incubated 30 min at 37°, and assayed for MAO activity. OM treated first with PS was centrifuged and resuspended in phosphate buffer as usual and then octyl glucoside added to 1%. Excess detergent was removed on a G25 column, and protein-containing fractions were pooled and assayed for MAO. MAO in OM preparations, solubilized with octyl glucoside and passed through Sephadex G-25 to remove excess detergent, has been found to show a negligible loss of activity relative to the membrane bound enzyme, and is stable for a period of weeks when stored in ice. On the other hand, if the solubilized enzyme was not treated with the G-25, substantial losses of activity were observed in less than 1 day.

MAO and protein assays. Protein in membrane preparations was quantitated using the method of Lowry *et al.* [23]. MAO radioassays were conducted at 30° for 15 min using the procedure of Wurtman and Axelrod [24]. A lower concentration of PEA (50 μM as compared to 200 μM) was used, as specificity of this substrate for MAO-B has been shown to decrease at higher concentrations [25]. Under these conditions assays have been found to be linear with respect to product formation for a minimum of 20–30 min. Toluene was used as the extraction solvent for PEA and tryptamine products while benzene–ethyl acetate (1:1) was used for 5-HT. Extracts were counted in 10 ml of Betafluor scintillation counting solution (National Diagnostics) or Biocount (Research Products International) in the case of 5-HT. All assays were in triplicate. Oxygen uptake assays were carried out using a Clark oxygen electrode apparatus from the Yellow Springs Instrument Co. (Yellow Springs, OH), in a 1.2 ml volume of 0.05 M potassium phosphate, pH 7.4, at an assay temperature of 30°. Assay mixtures contained 0.5 mg protein. Rates were linear for a minimum of 5 min. All MAO assays were run at atmospheric oxygen concentration. MAO activities, except in Fig. 6, are presented as normalized specific activities relative to control membrane preparations (no PS) treated in exactly the same manner, including incubation and centrifugation steps. For those experiments where the PS perturbations shown were small (Figs. 4 and 5) data are given for the mean of three independent experiments ± S.E.M.

RESULTS

Figure 1 shows the selective effects of PS liposomes containing ^{14}C-labeled lipid on MAO catalytic activities toward 5-HT (200 μM) and PEA (50 μM) in rat liver OM after centrifugation at 275,000 *g* to remove excess liposome and resuspension to approximately 1 mg/ml in phosphate buffer. Also plotted are levels of PS associated with the resuspended membrane. Over a range of added PS concentrations between 0.2 and 5.0 mg/mg OM protein, membrane bound PS increased at almost a linear rate from 0.12 to 2.55 mg/mg protein while the effect on MAO-B activity (PEA) leveled off above 1.28 mg bound PS/mg protein at about 55% inhibition. At the lowest level of associated PS shown (0.12 mg/mg protein), which corresponds to 14–40% of reported values for total phospholipid in liver mitochondria [26, 27], there was a 20% inhibition of MAO-B. No significant effect on MAO-A (5-HT) was observed throughout the range of added PS liposome concentrations.

To determine whether co-sedimentation of the liposomes contributes significantly to apparent levels of membrane bound PS after centrifugation to remove free lipid, ^{14}C-labeled liposomes were sedimented alone (2.5 mg/ml) under the same conditions. Loss of radioactivity from the supernatant

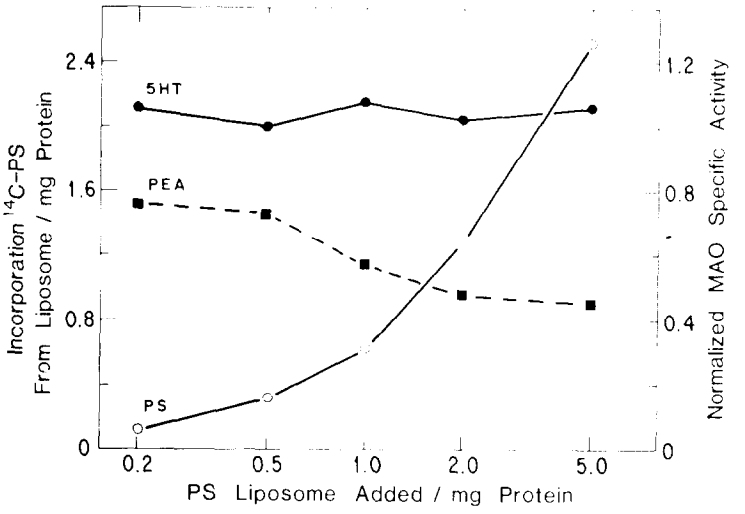

Fig. 1. PS incorporation into rat liver mitochondrial OM and inhibition of MAO oxidation of PEA and 5-HT. Key: PS incorporation from ^{14}C-labeled dioleoyl-PS (○——○); and normalized MAO specific activities in the same OM preparation as a function of added PS/OM protein ratios: PEA (■- - -■), 5-HT (●——●). PS incubation conditions: 30 min, 37°. Control MAO specific activity (PEA) = 12.6 nmoles per mg per min.

fraction was only 12% as compared to 80–90% in the presence of the membrane suspension. This small decrease in supernatant activity may actually have represented absorption of lipid to the wall of the plastic centrifuge tube rather than sedimentation, as no detectable pellet was observed.

KCl washes of [^{14}C]PS treated bovine liver OM. The least complex form of interaction of the liposomes with OM vesicles would be adsorption to the OM surface, although this would be unlikely to provide a mechanism for the observed highly specific effects on MAO. To test this possibility, a preparation of PS-treated bovine liver OM, which contained 0.493 mg [^{14}C]PS/mg protein and inhibited MAO activity (PEA) by 71.6% after removal of unbound lipid by our usual centrifugation procedure, was washed repeatedly with a large excess of 0.1 M KCl–0.05 M potassium phosphate, pH 7.4, to dissociated adsorbed PS vesicles. The decrease in radioactivity/mg protein after three such washes, using high speed centrifugation to re-isolate the membrane each time, indicated a loss of only 2% of the associated PS. No recovery of MAO activity was observed after this treatment.

Effect of chaotropic agents and sonication on MAO inhibition. On the basis of the alternative assumption that PS inhibition of MAO may involve lipid actually incorporated into the membrane lipid bilayer, the effects of a number of agents that were expected to disrupt lipid-protein interactions to varying degrees were examined. In one such set of experiments, bovine liver OM treated with PS and control membranes were incubated with two different chaotropic agents, a salt commonly used for this purpose, potassium thiocyanate (KSCN), and ammonium sulfate. Oreland and Oliverona [28] have shown that the latter compound, in conjunction with butanone extraction, is effective in solubilizing MAO from pig liver mitochondria, in a process that appears to involve release of the acidic phospholipids. The effects of these two salts on inhibition of MAO by PS were investigated by first treating a suspension of bovine liver OM with PS (1 mg/mg protein) at 37° for 30 min (as described in Materials and Methods) and then incubating the PS-treated and control membranes with the two salts at a concentration of 0.1 M for 10 min at room temperature. The results of this experiment with respect to changes in MAO activity (PEA) are summarized in Table 1.

The chaotropic agents were not effective in reversing the MAO inhibition and actually brought about an additional loss of activity in both control and PS-treated membranes. This effect was not reversible when the salts were removed by pelleting the membranes and resuspending them in phosphate buffer.

Membrane disruption to perturb the MAO inhibition in PS-treated bovine liver OM was also attempted by 10-min sonication at room temperature in the bath sonicator used for liposome preparation. This procedure, however, only resulted in a further 34–37% inactivation of MAO in both control and PS containing membranes.

Table 1. Effect of chaotropic agents on MAO-B activity (PEA) in PS-treated bovine liver OM

Agent	MAO activity (% of control)	
	No PS	+PS (1:1)
None	100.0*	49.8
0.1 M $(NH_4)_2SO_4$	84.0	38.1
0.1 M KSCN	90.4	41.9

* Control membrane preparation contained no added PS but otherwise was handled identically to material to which PS liposomes were added. MAO specific activity of the OM preparation was 28 nmoles per mg per min (PEA).

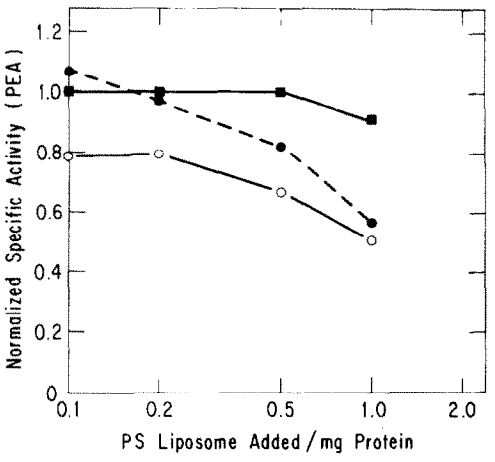

Fig. 2. Effect of octyl glucoside solubilization on inhibition by PS of MAO in bovine liver OM. The mitochondrial OM preparation was treated with 1% octyl glucoside before (■——■) or after (●– –●) incubation with PS liposomes (30 min, 37°). PS inhibition of membrane bound MAO in the absence of detergent: (○——○). MAO substrate was 50 μM PEA. Activities shown are specific activities normalized relative to the appropriate membrane bound and solubilized controls respectively. Specific activity of the membrane bound bovine liver MAO (PEA) = 28 nmoles per mg per min.

Detergent solubilization of PS-treated bovine liver OM. A more drastic disruption of the PS-treated bovine liver OM was achieved by solubilization with 1% octyl glucoside after PS incorporation. Liberated membrane lipid and excess detergent were removed following this treatment by passing membrane–detergent mixtures through small columns of Sephadex G25. In this manner artifacts due to direct inhibition of MAO by the detergent could be eliminated. The effect of detergent on the PS inhibition was studied over a range of liposome concentrations from 0.1 to 1.0 mg/ml protein. As a second set of controls, bovine liver OM was treated first with the octyl glucoside, excess detergent was removed on a Sephadex G25 column, and then the solubilized membrane was incubated with the same concentrations of PS liposomes. Figure 2 shows that the octyl glucoside treatment was partially successful in reversing PS inhibition of the membrane bound MAO. This figure is a plot of normalized MAO specific activities as a function of added liposome for PS-treated OM alone and in conjunction with the two methods of detergent solubilization described above. The lower plot (solid line and open circles) shows PS inhibition of MAO in the intact membrane as normalized specific activities relative to the unsolubilized control. The middle plot (dashed line and closed circles) is the same set of preparations after the detergent-G25 treatment with activities shown relative to the octyl glucoside-treated control. The detergent appears to have brought about some reversal of the PS inhibition at each concentration, with the effect being more pronounced at the lower lipid concentrations. The uppermost curve (solid line and closed squares) shows the effect of detergent solubilization prior to the PS treatment. In this case, there was no PS inhibition of the solubilized MAO except for a small effect at the highest concentration. As mentioned in Materials and Methods, the detergent treatment itself did not have a significant effect on the bovine liver OM MAO activity if excess detergent was removed with Sephedex G25 using the method described by Baron and Thompson [22].

Incubation of PS-treated bovine liver OM with PC and PI liposomes. Reversal of the PS inhibition was also attempted by incubation of a PS-treated bovine

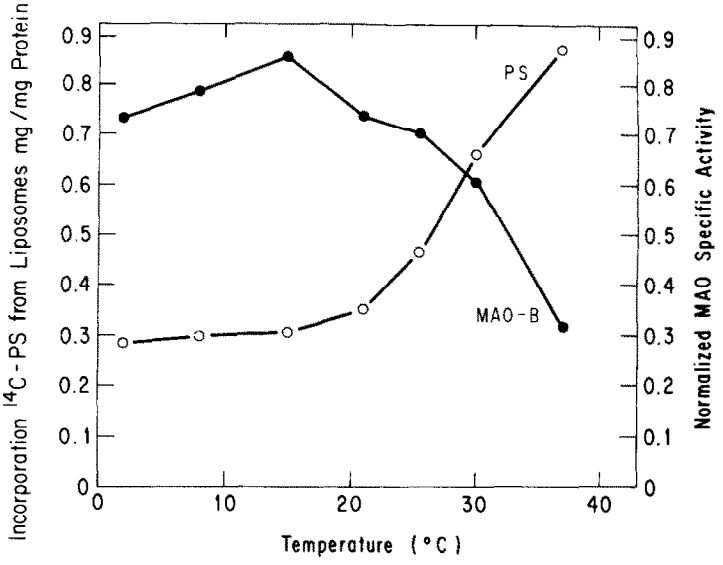

Fig. 3. Effect of incubation temperature on incorporation of [^{14}C]PS into bovine liver OM and inhibition of MAO by PS. Incubation was for 30 min at the indicated temperatures. Control for calculation of MAO normalized specific activities was OM incubated 30 min at 37° in the absence of PS, centrifuged, and resuspended as described in the text. Substrate was 50 μM PEA. Key: MAO activity (●——●), and [^{14}C]PS incorporation in OM (○——○). MAO specific activity was the same as in Fig. 2.

liver OM preparation with second liposomes of PI or phosphatidylcholine (PC). We had previously found that both of these lipids have no effect on MAO-B when incubated with OM preparations using our standard procedures. In the case of PI, we had also found levels of lipid incorporation comparable to that for PS [13]. By treating with these inactive liposomes, it was hoped to reduce PS inhibition of MAO by diluting the PS within the lipid bilayer or exchanging it back out of the membrane, depending on the incorporation mechanism. When the bovine liver OM preparation, previously treated with a 1:1 ratio of PS, was incubated with the PC or PI liposomes at the same concentration and the OM isolated as previously, inhibition of MAO-B was actually found to increase from 50.2 to 61.6% with PC and to 64.6% with PI.

MAO-B inhibition and PS incorporation as a function of temperature in bovine liver OM. Studies have shown both the mechanism and extent of liposome association with cell membranes to be temperature dependent [29]. Therefore, we have looked at changes in MAO inhibition and PS incorporation as a function of the temperature at which the liposomes and OM preparation were incubated to obtain additional information as to their mode of interaction. The 30-min incubations were carried out with bovine liver OM and [^{14}C]PS liposomes at 1 mg/mg protein at seven different temperatures between 2 and 37°. In Fig. 3 inhibition of MAO with PEA as substrate (normalized specific activity) and PS incorporation are plotted with respect to the incubation temperature. MAO-B inhibition appeared to decrease somewhat between 2 and 15° and then increase above this temperature with the rate of change becoming more pronounced above 25°. PS incorporation from the radiolabeled liposomes exhibited a fairly close correlation with little change in association of the lipid below 20° and then a rapid increase beginning between 20 and 25°.

Effect of PS association on the concentration dependence of clorgyline and harmaline inhibition of MAO in rat liver OM. As was shown in Fig. 1, a liposome concentration of 1 mg/mg protein resulted in a 44% inhibition of MAO-B in rat liver OM while the A-form was not affected significantly. Calculated on the basis of the change in relative specific activities with the two substrates, this corresponded to a decrease of approximately 25% in the fraction of MAO-B in the membrane preparation.

Relative proportions of the two forms in a particular tissue can be alternatively estimated from concentration dependence of inhibition by selective, irreversible inhibitors using a nonselective substrate. Plots of MAO inhibition as a function of inhibitor concentration obtained using this protocol are biphasic with a plateau region separating portions of MAO with high and low sensitivity to the inhibitor. The fractions of enzyme inhibited above and below the inflection point have, in turn, been taken as a measure of the relative amounts of MAO-A and -B

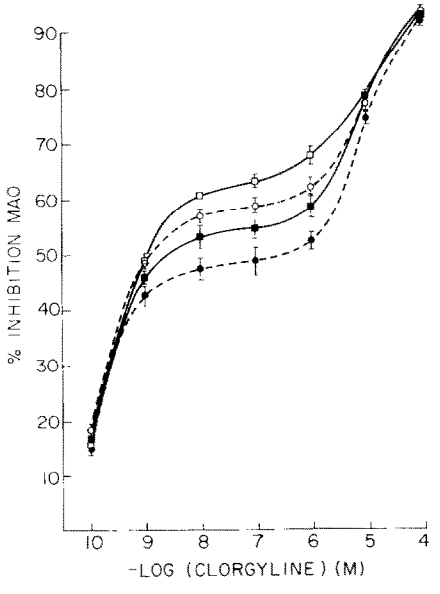

Fig. 4. Effect of preincubation with PS on inhibition of MAO by clorgyline in rat liver OM. Membrane preparations were incubated with PS as described in the text for 30 min at 37°. Aliquots of PS-treated membranes were added to MAO assay mixtures containing clorgyline at the indicated concentrations and incubated for 30 min at room temperature before addition of substrate (200 μM tryptamine). Controls for clorgyline inhibition were membranes pretreated with the same amount of PS. The curves represent PS liposome/membrane protein ratios of: 0.0 (●----●), 0.1 (■——■), 0.5 (○- - - -○) and 1.0 (□——□). Control MAO specific activity (PEA) in the absence of PS = 7.0 nmoles per mg per min. The data represent the mean of three experiments ± S.E.M.

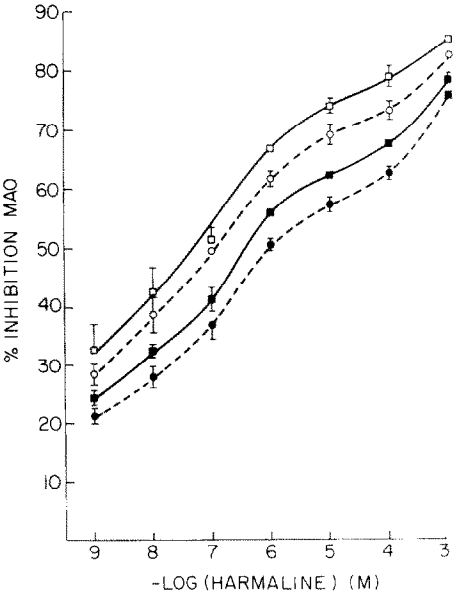

Fig. 5. Effect of preincubation with PS on inhibition of MAO by harmaline in rat liver OM. PS incubations and assay conditions were as indicated in the legend of Fig. 4. Substrate was 200 μM tryptamine. Controls for harmaline inhibition were membranes treated with the same amount of PS. Curves represent PS liposome/membrane protein ratios of: 0.0 (●----●), 0.1 (■——■), 0.5 (○- - -○) and 1.0 (□——□). Control MAO specific activity (PEA) in the absence of PS = 6.1 nmoles per mg per min. The data represent the mean of three experiments ± S.E.M.

in the tissue. We have examined the effect of PS on the apparent relative amounts of the two forms in rat liver by this criteria using the specific A-form, irreversible inhibitor clorgyline with tryptamine as a nonselective substrate. Figure 4 shows a plot of inhibition patterns for clorgyline concentrations from 10^{-10} to 10^{-5} M. The four curves represent PS-treated rat liver OM at liposome/membrane protein ratios of 0.1, 0.5 and 1.0, as well as a non-PS-treated control. The upward displacement of the plateau region of the curves with increasing PS incorporation is consistent with the decrease in B-form activity seen in terms of the inhibition of PEA oxidation. The decrease in the fraction of MAO-B calculated from the displacement of the inflection point at the maximum PS concentration, 27%, is also in reasonable agreement with the value calculated from the change in PEA and 5-HT specific activities.

Effects of PS incorporation on rat liver MAO inhibition patterns were also examined using the MAO-A specific competitive inhibitor, harmaline, at concentrations from 10^{-9} to 10^{-3} M. Tryptamine was again used as the MAO substrate and membrane aliquots were pretreated with the same concentrations of PS. Figure 5 shows curves of harmaline inhibition obtained for the same three levels of PS incorporation and control membranes. The apparent increase in sensitivity to the A-form inhibitor with increasing PS is again consistent with the catalytic inhibition of the B-form. The family of curves shows a slight inflection centered at approximately 10^{-5} M harmaline and corresponding to 57% inhibition of tryptamine oxidation by MAO for the control (no PS). The incremental shift in the curves with increasing PS at this harmaline concentration was very similar to the pattern seen with clorgyline. A larger spread was seen at highest and lowest harmaline concentrations, however.

Kinetic aspects of PS inhibition of MAO in bovine liver OM. We have examined the mechanism of the PS inhibition of MAO with PEA as substrate in a bovine liver OM preparation from the standpoint of effects on kinetic parameters. An oxygen electrode was used to measure activity rather than the radioassay procedure for this study to obtain more accurate initial rates. In Fig. 6, MAO activity at various levels of PS incorporation, as a function of amine substrate concentration, is presented in the form of a Lineweaver–Burk plot. Data were plotted for OM preincubated with four different concentrations of PS between 0.1 and 1.0 mg/mg protein, and a non-PS-treated control. The plots show a mixed-type inhibition pattern with effects on both K_m and V_{max}.

DISCUSSION

Among the possible modes of liposome–membrane interaction listed at the beginning of the paper, fusion between the PS liposomes and mitochondrial OM vesicles appears most consistent with the nature of the system and observations presented here. The small loss of [^{14}C]PS from the supernatant fraction, when the liposomes were centrifuged at 275,000 *g* alone, demonstrated that co-sedimentation did not contribute significantly to observed levels of lipid association after the OM was separated from excess lipid by centrifugation. The highly specific effects of PS on the multiple forms on MAO in the membrane and the failure of extensive washing with high ionic strength buffer to reverse MAO-B inhibition or remove associated [^{14}C]PS make adsorption of the liposomes to the membrane surface an unlikely mechanism to explain our observations. Exchange of individual phospholipid molecules and endocytosis are mechanisms that require agents not likely to be present in the purified membrane preparations. Phospholipid exchange has been shown to be mediated by specific carrier proteins [30]. In the association of liposomes with plasma membranes, endocytosis appears to require active respiration and

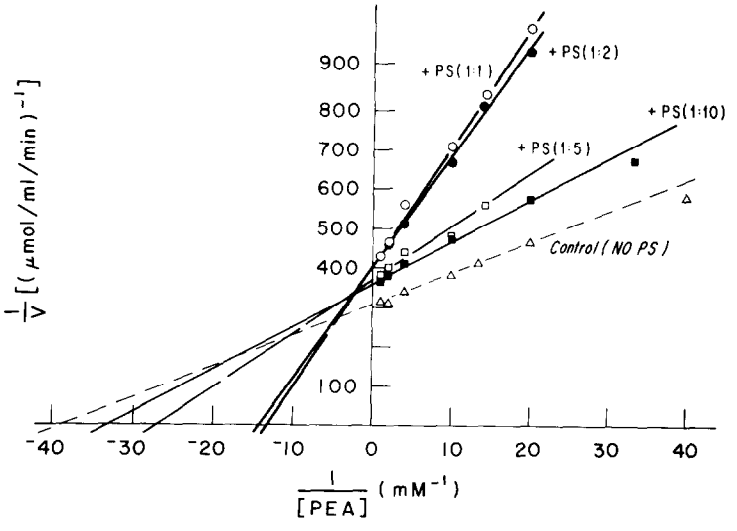

Fig. 6. Lineweaver–Burk plot of the effect of PS on PEA oxidation by MAO in bovine liver OM. MAO was assayed with respect to PEA concentration at 30° at atmospheric oxygen concentration by means of oxygen uptake as described in the text. Aliquots of OM were pretreated with PS liposome/membrane protein ratios of: 0.0 (△---△), 0.1 (■), 0.5 (□), 0.2 (●) and 1.0 (○).

glycolysis [29, 31]. Temperature dependence of incorporation has also been found to differ for endocytosis and fusion. Poste and Papahadjopoulous [29] demonstrated that fluid liposomes (above the solid–liquid crystal phase transition) of the acidic phospholipids are incoporated into cell membranes by fusion, while for solid liposomes of acidic phospholipids, as well as both solid and fluid liposomes of zwitterionic phospholipids, the mechanism is endocytosis. Endocytosis exhibits a linear temperature dependence of lipid incorporation while for fusion there is a characteristic, pronounced increase in slope at about 18° [29]. Incorporation curves for fusion, shown by these authors, bear a strong resemblance to that for the uptake of [^{14}C]PS liposomes by the bovine liver OM in our studies (Fig. 3). Bovine brain PS with a transition temperature of 5° [32] satisfies the criteria for a fluid anionic liposome. The temperature above which the slope of the [^{14}C]PS association curve increases (~20°) could, on the basis of this mechanism, be related to the phase transition reported for liver mitochondria, which is believed to correspond to the completion of lateral phase separation in the lipid bilayer [33]. Studies of effects of PS on various physical parameters of the OM are presently in progress to obtain a more precise understanding of the mode of PS binding to the membrane and its relationship to MAO inhibition.

Failure to reverse the PS inhibition of MAO on disruption of the membrane by chaotropic agents or sonication, together with the partial inhibition still remaining at high PS levels after complete solubilization by octyl glucoside are more suggestive of a specific interaction between PS and the membrane bound enzyme than of indirect effects of bulk changes in membrane fluidity or surface charge. Our findings, reported elsewhere [13], that at comparable levels of association with the membrane the other acidic phospholipids, PI and CL, show very different effects on the MAO multiple forms also argue against bulk membrane effects. The inability of PS to inhibit solubilized bovine liver MAO indicates that association with the native lipid bilayer is, nevertheless, a first step in the process. If membrane fusion were the dominant mechanism by which the PS liposomes interact with the OM, some lateral diffusion of the PS would be required to bring the lipid in contact with MAO. This would also mean that only a small portion of the bound PS may actually be responsible for inhibition of the enzyme.

In the PS-treated bovine liver OM, the failure of subsequent incubation with PC or PI liposomes to reverse the inhibition of MAO could be a reflection either of their inability to displace excess PS from the membrane (as a fusion process for liposome association would suggest) or of the specific nature of the PS-MAO interaction. The increased inhibition after treatment with the second liposomes, which by themselves are inactive, could be the result of increased diffusion of PS to regions of MAO binding.

One of the studies of the interaction of phospholipids with MAO mentioned at the beginning of the paper, that of Huang and Faulkner [7], which used methodologies somewhat similar to ours, demonstrated different effects on the multiple forms of the enzyme. In this work, whole rat brain mitochondria were delipidated with phospholipase A and incubated with liposomes of the acidic phospholipids. Liposomes of PS and CL produced comparable recoveries of activity lost as a result of the phospholipase treatment while PI uniquely activated MAO-A to 80% above its original level. However, as reported in detail elsewhere [13], we reexamined this methodology using PI liposomes and found it to involve complete solubilization of the enzyme from the mitochondria, and thus these observations are not applicable to effects on native membrane bound MAO.

Although the higher concentrations of PS used in the present work, at which maximum MAO-B inhibition was achieved, represent large excesses over natural PS levels in the mitochondrial OM, a significant inhibition (20%) was found at levels low enough to be a physiologically reasonable perturbation (0.12 mg/mg protein). A number of studies in recent years have produced credible evidence for the existence of unique MAO-A and -B proteins [34–37,*], contrary to the hypothesis of Houslay and Tipton [1] of a single lipid environment-modulated peptide. Even if MAO substrate specificity is primarily related to the presence of two different A and B peptides, the present observations of inhibitory effects of PS on native membrane bound MAO suggest the possibility of a second level of regulation of the relative activities of the two forms from one tissue to another or in response to environmental perturbations.

An example of particular clinical relevance where such considerations might prove applicable is the recent report of higher PS levels in schizophrenic blood platelets [38]. Inhibition of MAO-B in these platelets by the elevated PS could offer an explanation for the controversial observations of lower platelet-MAO activity associated with this mental disorder. In this regard we have found, in studies now in progress in our laboratory, that platelets from a population of paranoid schizophrenics with lower basal MAO levels showed lower sensitivity to inhibition by added PS liposomes.

In view of the striking effects of PS on other important membrane enzymes involved in neurotransmission, it is intriguing to spectulate that this phospholipid could play some overall role in the regulation of these processes.

The catalytic mechanism for MAO has been well established as having the kinetic characteristics of a ping-pong, bi-reactant system [39]. The Lineweaver–Burk plot shown in Fig. 6 is consistent with the action of a noncompetitive inhibitor on an enzyme of this type [40]. As the inhibitor in this case is most likely to be an integral component of the membrane to which the enzyme is bound, the inhibition would be expected to be reversible only on membrane disruption, as was seen with the octyl glucoside solubilization. The decreased effectiveness of solubilization in reversing the inhibition at higher PS concentrations may reflect a greater component of PS in the MAO boundary lipid layer, at least a

* R. M. Cawthon, J. E. Pintar, F. P. Haseltine, M. S. Buchsbaum, D. L. Murphy, and X. O. Breakefield, *Society for Neuroscience: Tenth Annual Meeting*, A69, p. 4 (1980).

portion of which would be expected to remain associated with the enzyme on transfer to a detergent micelle.

The similar effects of PS on PEA oxidation and binding of the reversible inhibitor, harmaline, as well as of the irreversible inhibitor, clorgyline, for membrane bound rat liver MAO suggest that the inhibition mechanism involves more than just decreased substrate binding. If only initial binding of substrate and structurally related inhibitors to the MAO-B active site were affected, then at least an attenuation of this effect would be expected on prolonged incubation with the covalently binding suicide inhibitor, as the irreversible phase of inhibition proceeds toward completion. Contrary to this prediction, the apparent fractional decrease in MAO-B calculated from increased clorgyline sensitivity (MAO-A specific inhibitor) correlated quantitatively with effects on PEA activity.

To explore further which subsequent steps in catalysis and inhibitor binding are affected by PS, we are currently undertaking more detailed studies of alterations in kinetic and thermodynamic parameters involved in the inhibition.

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REFERENCES

1. M. D. Houslay and K. F. Tipton, *Biochem. J.* **135**, 173 (1973).
2. H. L. White and A. T. Glassman, *J. Neurochem.* **29**, 987 (1977).
3. S.. P. Baker and B. A. Hemsworth, *Eur. J. Biochem.* **92**, 165 (1975).
4. B. Eksted and L. Oreland, *Biochem. Pharmac.* **25**, 119 (1976).
5. S. Eiduson and T. D. Buckman, in *Monoamine Oxidase: Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), p. 213. Academic Press, New York (1979).
6. P. H. Yu, in *Monoamine Oxidase: Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), p. 233. Academic Press, New York (1979).
7. R. H. Huang and R. Faulkner, *J. biol. Chem.* **256**, 9211 (1981).
8. A. Schurr, B. T. Ho and J. C. Schoolar, *J. Pharm. Pharmac.* **33**, 165 (1981).
9. M. D. Houslay, *Biochem. Pharmac.* **29**, 3211 (1980).
10. R. E. Pagano and H. Takeichi, *J. Cell Biol.* **74**, 531 (1977).
11. M. D. Houslay, G. B. Warren, N. J. M. Birdsall and J. C. Metcalfe, *Fedn Eur. Biochem. Soc. Lett.* **51**, 146 (1975).
12. H. K. Kimelberg and E. G. Mayhew, *CRC Crit. Rev. Toxic.* **6**, 25 (1978).
13. T. D. Buckman, S. Eiduson, M. S. Sutphin and R. Chang, *J. biol. Chem.* **258**, 8670 (1983).
14. T. Lloyd and S. Kaufman, *Biochem. biophys. Res. Commun.* **59**, 1262 (1974).
15. J. Tanaka, *J. Neurochem.* **16**, 1301 (1969).
16. G. S. Levey and D. C. Lehotoy, in *The Enzymes of Biological Membranes* (Ed. A. Martanosi), p. 259. Plenum Press, New York (1976).
17. Y. C. Awashti, T. F. Chuang, T. W. Keenan and F. L. Crane, *Biochim. biophys. Acta* **226**, 42 (1971).
18. F. Dabbeni-Sali, R. Furlan and A. Pitotti, *Biochim. biophys. Acta* **347**, 77 (1974).
19. G. H. Dodd, *Eur. J. Biochem.* **33**, 418 (1973).
20. E. B. Kearney, J. I. Salach, W. H. Walker, R. L. Seng, W. Kenney, E. Zeszotek and T. P. Singer, *Eur. J. Biochem.* **24**, 321 (1971).
21. T. P. Singer, *Meth. biochemic. Analysis* **22**, 123 (1974).
22. C. Baron and T. E. Thompson, *Biochim. biophys. Acta* **382**, 276 (1975).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. R. J. Wurtman and J. Axelrod, *Biochem. Pharmac.* **12**, 1439 (1963).
25. O. Suzuki, M. Hattori, M. Oya, Y. Katsumata and T. Matsumoto, *Life Sci.* **25**, 1943 (1979).
26. W. Stoffel and H-G. Schiefer, *Hoppe Sylers' Z. Physiol. Chem.* **349**, 1017 (1968).
27. D. F. Parsons, G. R. Williams, W. Thompson, D. Wilson and B. Chance, in *Round Table Discussion on Mitochondrial Structure and Compartmentation* (Eds. E. Quagliariello, S. Papa, E. C. Slater and J. M. Tager), p. 29. Adriatica Editrice, Bari (1967).
28. L. Oreland and T. Oliverona, *Archs Biochem. Biophys.* **142**, 710 (1971).
29. G. Poste and D. Papahadjopoulos, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1603 (1976).
30. D. B. Zilversmit and M. E. Hughes, in *Methods in Membrane Biology* (Ed. E. D. Korn), Vol. 7, p. 211. Plenum Press, New York (1976).
31. M. L. Karnovsky, *Physiol. Rev.* **42**, 143 (1962).
32. K. Jacobson and D. Papahadjopoulos, *Biochemistry* **14**, 152 (1975).
33. J. K. Raison and E. J. McMurchie, *Biochim. biophys. Acta* **363**, 135 (1974).
34. B. A. Callingham and D. Parkinson, in *Monoamine Oxidase: Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), p. 81. Academic Press, New York (1979).
35. R. M. Denney, R. R. Fritz, N. T. Patel and C. W. Abell, *Science* **215**, 1400 (1982).
36. R. M. Cawthon and X. O. Breakefield, *Nature, Lond.* **281**, 692 (1979).
37. G. K. Brown, J. F. Powell and I. W. Saig, *Biochem. Pharmac.* **29**, 2595 (1980).
38. N. Sengupta, S. Datta and D. Sengupta, *Biochem. Med.* **25**, 267 (1981).
39. K. F. Tipton, *Eur. J. Biochem.* **5**, 316 (1968).
40. W. W. Cleland, in *The Enzymes* (Ed. P. D. Boyer), Vol. II. p. 1. Academic Press, New York (1970).