Effect of Phospholipase A2 on Temperature-Induced High-Affinity $[^3]$H Tryptamine Binding Sites in Rat Brain

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ABSTRACT—To investigate a link between membrane phospholipids and tryptamine binding molecules, we examined the effects of phospholipases A2 and D on the temperature-sensitive high-affinity $[^3]$H tryptamine binding sites in rat brain. When the phospholipase A2-treated membranes were exposed to 1% bovine serum albumin (BSA) before assaying for $[^3]$H tryptamine binding, a complete dose-dependent inhibition curve was observed. At a concentration of 0.03 U, the action of phospholipase A2 resulted in the splitting of phosphatidylserine (PS), choline phosphatides (PC) and ethanolamine phosphatides (PE) by about 32, 34 and 65%, respectively, and reduced $[^3]$H ligand binding by about 32%. On the contrary, in the case of phospholipase D (500 U), PS and PC decreased by about 8% and 33% and PE by about 29% with no significant alteration in the binding capacity. Moreover, Scatchard analysis of the $[^3]$H tryptamine binding showed that phospholipase A2 drastically increased only the $K_D$ value of the high affinity sites, and this was accompanied by a decrement of the $B_{max}$ values of both the high and low affinity binding sites. From these results, it is inferred that certain lipids (PS) may be a modulator for the function of the temperature-induced high-affinity $[^3]$H tryptamine binding molecules.

Recent studies employing radioreceptor-binding assays have found that the $[^3]$H tryptamine binding sites, with a $K_D$ of approx. 3 nM, have the characteristics of specific tryptamine receptors in the brain (1, 2). In previous studies (3–5), we reported the preincubation conditions that induce the enhancement of specific $[^3]$H tryptamine binding in rat brain synaptic membranes. This phenomenon is fairly physiologically temperature-dependent, and it may occur as a result of a structural change in the sites that are transformed from a state of low affinity for the $[^3]$H ligand ($K_D = 32$ nM) to one of higher affinity ($K_D = 0.45$ nM). Moreover, it has been revealed that the specific $[^3]$H tryptamine binding molecule(s) is a thiol protein and that the existence of the thiol group in a hydrophobic environment is essential for the binding of ligands by the high-affinity sites.

There have been many reports investigating the role of membrane lipids in receptor mechanisms by the use of phospholipases or other methods. The authors have recently conducted reconstitution experiments with acidic lipids which indicated that only the reconstituted fractions including PS regenerated a saturable high affinity binding capacity for

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Abbreviations used are: BSA, bovine serum albumin; CS, sulphatides; PC, choline phosphatides; PE, ethanolamine phosphatides; PS, phosphatidylserine.
[\textsuperscript{3}H]tryptamine (6). However, these studies themselves were merely a model experiment in vitro and thus further investigations in the synaptic membranes preparation should be performed. The objective of the present work was to establish the possible involvement of certain acidic lipid(s) in the binding of tryptamine molecules. Through the present studies with phospholipases (A₂ and D), it will be shown that PS may be a modulator for the function of the temperature-induced high-affinity [\textsuperscript{3}H]tryptamine binding sites.

**MATERIALS AND METHODS**

**Preparation of synaptic membranes**

Whole rat brains (male Wistar 150–200 g), except for the cerebellum and olfactory lobes, were homogenized in 10 vol. of 0.32 M sucrose, and synaptic membranes were prepared from this homogenate by a previously reported method (7) with minor modifications. Briefly, the synaptosomal fraction (0.8–1.2 M sucrose layer) was isolated from the crude mitochondrial fraction (P₂) by discontinuous sucrose (0.32, 0.8 and 1.2 M) density gradient centrifugation (53,500 × g for 2 hr) and then was lysed with water for 30 min at 0°C (10 ml/g of original tissue). The sample was loaded onto a two-step sucrose gradient (0.8 and 1.2 M). After similar centrifugation, the 0.8 and 1.2 M layers were collected and designated as the synaptic membrane fraction. Finally, the membranes were suspended in 0.05 M Tris-HCl buffer, [pH 7.4 (i.e., Tris buffer) 10 mg protein/ml] and quick-frozen under liquid N₂ and stored at −75°C until used, generally 1–3 weeks.

**Pretreatment of membranes with phospholipases**

After rapid thawing of the frozen membranes, samples were pretreated with phospholipases A₂ and D in Tris buffer, containing 2 mM CaCl₂ for 55 min at 37°C (2 mg protein/ml). Preincubation was terminated by adding EGTA at a final concentration of 5 mM and a brief immersion in an ice bath, followed immediately by centrifugation (27,000 × g for 25 min). All the treated membranes were washed once by centrifugation (27,000 × g for 25 min) with Tris buffer. The enzyme-treated membranes and the control membranes were resuspended in the above-mentioned buffer to a final suspension of 1 mg protein/ml, containing 1% (w/v) fatty acid-poor BSA, unless otherwise stated. The mixtures were incubated for 30 min at 0°C and then centrifuged at 27,000 × g for 40 min. The pellets were resuspended in Tris buffer and washed once by the same centrifugation method. All the resultant pellets were resuspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.1% ascorbic acid and 10 μM pargyline (i.e., standard assay medium) and were supplied for the binding assay of [\textsuperscript{3}H]tryptamine. Before use, to eliminate any proteolytic activities that may be present, phospholipase A₂ was heated at 100°C for 10 min as reported previously (8).

**\textsuperscript{3}H]Tryptamine binding assay**

The radioreceptor-binding assay was carried out essentially as described by Cascio and Kellar (1). A 0.5-ml aliquot of membrane suspension (approx. 500 μg protein) was added to tubes containing [\textsuperscript{3}H]tryptamine and standard assay medium. Half of the tubes also contained 10 μM of unlabelled tryptamine to measure nonspecific binding. The tubes (1.0 ml) were incubated at 0°C for 60 min and then rapidly filtered under vacuum through Whatman GF/B filters with five rinses of 5 ml ice-cold Tris buffer. The filters were counted by a liquid scintillation counter in 10 ml of toluene/Triton X-100 (3:1) emulsion phosphor after extraction for 3 hr with shaking. The specific binding of [\textsuperscript{3}H]tryptamine was defined as the total minus the nonspecific binding. Every determination of binding was performed in triplicate, and the bound radioactivities were identified as [\textsuperscript{3}H]tryptamine itself by a quantitative TLC method reported previously (3). The membranous protein concentration was determined by the SDS-Lowry method of Lees and Paxman (9). The
Scatchard plot data were computer-assessed by nonlinear regression analysis as reported previously (10). F-test analysis (P < 0.01) was used to determine whether a one-site or two-site model was more appropriate.

**Lipid analysis**

For lipid analysis, the phospholipase-treated and control membranes were lyophilized, and lipids were extracted according to the method of Norton et al. (11, 12), using chloroform-methanol (2:1, v/v) as the solvent. Individual phospholipids were determined by the quantitative TLC method using a two dimensional solvent system with chloroform-methanol-ammonium hydroxide (14:6:1) followed by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1) as described previously (13). Total lipid phosphorus was assayed by the method of Chen et al. (14). To calculate the molar concentration of each lipid, the following average molecular weights were used: PE, 732; PC, 804 and PS, 820.

**Materials**

[^3]H]Tryptamine (28.6-40.0 Ci/mmol) and Triton X-100 were obtained from New England Nuclear. Phospholipases A<sub>2</sub> (EC 3.1.1.4, from Naja naja, 535 U/mg solid) and D (EC 3.1.4.4, from peanut, 155 U/mg solid), fatty acid-poor BSA (less than 0.005%) and other chemicals were from Sigma Chemical Co. Thin-layer of Kieselgel 60 was from E. Merck.

**RESULTS**

The interaction of[^3]H]tryptamine with rat brain synaptic membranes was substantially altered by pretreatment of the membranes with phospholipase A<sub>2</sub>, as shown in Fig. 1. When the phospholipase A<sub>2</sub>-treated membranes were exposed to defatted BSA before assaying for tryptamine binding, the complete dose-dependent inhibition curve was established, and 50% inhibition was obtained at an enzyme concentration of approx. 0.1 U/mg protein. Furthermore, there was a relative correspondence between the decrease of[^3]H]-ligand binding capacity and the degree of hydrolysis of total membrane phospholipids. On the other hand, in the enzyme-treated membranes without BSA washing, significant inhibition was also achieved at the enzyme concentration of 0.03 U/mg protein, but this loss of[^3]H]tryptamine binding peculiarly did not increase by the further addition of enzyme.

To investigate the possible involvement of certain phospholipid(s) in specific[^3]H]-tryptamine binding molecules, the enzyme-treated membranes were then extracted with chloroform-methanol, and the major phospholipids, i.e., PC, PE and PS (and also sphingomyelin and phosphatidylinositol), were separated by thin layer chromatography and analyzed as described elsewhere. Figure 2 shows that there was a striking correlation

![Fig. 1. Concentration-dependent inhibition by phospholipase A<sub>2</sub> of[^3]H]tryptamine binding and content of total lipid phosphorus. Rat brain synaptic membranes were pretreated with increasing concentrations of phospholipase A<sub>2</sub>, either in the presence (○) or in the absence (●) of 1% bovine serum albumin, and then the specific binding capacities (with 3 nM[^3]H]tryptamine) and total lipid phosphorus were determined as described in the text. The data (mean of 3-4 experiments, the results of which varied by less than 6%) are expressed as a percent of the control values. The absolute values of each control are shown in Table 1.](image-url)
between PS as well as PC hydrolysis and the loss of $[^3H]$tryptamine binding. Namely, the hydrolysis ratio of PS or PC and the loss of $[^3H]$ligand binding in percent were well-superimposable, while a similar correlation could not be made with PE. In addition, the content of sphingomyelin and phosphatidylinositol did not change by the treatment of phospholipase A$_2$ (data not shown).

A composite of the data for $[^3H]$ligand binding, total phospholipid hydrolysis, and hydrolysis of the major phospholipids for an appropriate concentration of phospholipase A$_2$ or D is presented in Table 1. The action of phospholipase A$_2$ (0.03 unit/mg protein) resulted in the splitting of PS, PC and PE by about 32, 34 and 65%, respectively and reduced $[^3H]$tryptamine binding by about 32%. In the case of phospholipase D (500 unit/mg protein), PS and PC decreased by about 8% and 33% and PE by about 29% with no reduction of $[^3H]$tryptamine binding. Moreover, an approx. 8% decrement of PS content was seen in all the enzyme-treated membranes, without significant alteration in their binding capacities.

To clarify the mode of action of phospholipase A$_2$ on the high-affinity tryptamine binding sites, we performed a Scatchard analysis of $[^3H]$tryptamine binding to the membrane prep-

![Graph](image1.png)

**Fig. 2.** Correlations between the loss of $[^3H]$tryptamine binding capacity and degree of hydrolysis of phosphatidylserine (C), choline phosphatides (●) and ethanolamine phosphatides (△). Rat brain synaptic membranes were pretreated with several concentrations of phospholipase A$_2$ and then were further incubated with 1% bovine serum albumin. Lipid analysis of the control membranes and the treated membranes was carried out as described in the text. The data (mean of 3–4 experiments, which varied by less than 5%) are expressed as a percent of the control values. The absolute values of each control are shown in Table 1. The dashed line indicates the $[^3H]$tryptamine bound (data from Fig. 1).

**Table 1.** Effects of phospholipases A$_2$ and D pretreatment on $[^3H]$tryptamine binding capacity and phospholipid content in synaptic membranes

<table>
<thead>
<tr>
<th>Phospholipase (U/mg protein)</th>
<th>$[^3H]$Tryptamine bound (dpm/mg protein)</th>
<th>Lipid phosphorus (μg/mg dry weight)</th>
<th>PS</th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15000 ± 800</td>
<td>11.8 ± 0.2</td>
<td>27.0 ± 1.0</td>
<td>124.3 ± 3.0</td>
<td>98.2 ± 3.3</td>
</tr>
<tr>
<td>A$_2$ 0.01</td>
<td>15150 ± 435</td>
<td>9.9 ± 0.3**</td>
<td>25.2 ± 0.5</td>
<td>104.3 ± 0.5**</td>
<td>57.5 ± 3.1**</td>
</tr>
<tr>
<td>A$_2$ 0.03</td>
<td>10200 ± 225*</td>
<td>8.4 ± 0.2**</td>
<td>18.3 ± 0.8**</td>
<td>82.1 ± 2.7**</td>
<td>34.6 ± 1.3**</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>15270 ± 480</td>
<td>10.3 ± 0.2**</td>
<td>24.8 ± 1.0</td>
<td>107.1 ± 1.9**</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>15450 ± 935</td>
<td>9.2 ± 0.1**</td>
<td>24.9 ± 0.6</td>
<td>83.3 ± 0.6**</td>
</tr>
</tbody>
</table>

Rat brain synaptic membranes were pretreated with phospholipases A$_2$ and D at several concentrations. After pretreatment, the specific $[^3H]$tryptamine (3 nM) binding capacities and the amounts of PS, PC and PE present in the control membranes and the treated membranes were measured as described in the text. All the membrane preparations were further incubated with 1% BSA. The values are the mean ± S.E.M. of 4 experiments. Significantly different from the control value at *P < 0.05 and **P < 0.01.
arations pretreated with this enzyme. As can be seen in Fig. 3, the Scatchard plot of $[^3H]$tryptamine binding in the control membranes conformed to a curved line, and nonlinear regression analysis demonstrated the presence of high and low affinity sites, as shown with the dashed lines. The phospholipase A$_2$-pretreated membranes also yielded curvilinear Scatchard plots. However, two types of inhibitory modes were observed. One is a mixed type of inhibition, and this type occurred in the pretreated membranes with further BSA washing (termed "with BSA"). The other was seen in the case of preparations without BSA washing (termed "without BSA"), which showed an apparently noncompetitive type of inhibition. The Scatchard plot data were computer-assessed by nonlinear regression analysis, and the results obtained are summarized in Table 2. Interesting differences in the effect of BSA treatment on the binding parameters were observed, as can be seen in Table 2. Namely, the "without BSA" treatment decreased the $B_{max}$ values of both the high and low affinity sites (no significance) and had no effect on the $K_D$ values. On the other hand, the "with BSA" treatment drastically increased the $K_D$ value of only the high affinity sites in correspondence with the applied doses, although this treatment decreased the $B_{max}$ values of both sites. In addition, we could not find any statistically significant difference (at $P < 0.05$) in the decrement of the $B_{max}$ values between the high and low affinity sites.

![Fig. 3. Scatchard plots of $[^3H]$tryptamine binding: Effect of phospholipase A$_2$ pretreatment. Membrane preparations were pretreated with 0.1 U/mg protein of phospholipase A$_2$, either in the presence (●) or in the absence (○) of 1% bovine serum albumin. After pretreatment, the subsequent $[^3H]$tryptamine (1–25 nM) binding capacities of the control membranes (□) and the treated membranes were estimated as described in the text. The results shown are those of a typical experiment, performed 4 times. The computer-generated curves were obtained by nonlinear regression analysis, and the two dashed lines were generated from the curvilinear plot of the control using a two-site model.](image)

### Table 2. Scatchard analysis of $[^3H]$tryptamine binding to synaptic membranes pretreated with phospholipase A$_2$.

<table>
<thead>
<tr>
<th>Phospholipase A$_2$ (U/mg protein)</th>
<th>High-affinity sites</th>
<th>Low-affinity sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (nM)</td>
<td>$B_{max}$ (pmoles/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>0.45 ± 0.01</td>
<td>107.1 ± 12.7 (100)</td>
</tr>
<tr>
<td>0.03</td>
<td>0.68 ± 0.04**</td>
<td>88.2 ± 3.4 (82)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.94 ± 0.07**</td>
<td>75.0 ± 4.4 (70)</td>
</tr>
<tr>
<td>0.1-without BSA</td>
<td>0.46 ± 0.01</td>
<td>83.5 ± 1.9 (78)</td>
</tr>
</tbody>
</table>

Rat brain synaptic membranes were pretreated with phospholipase A$_2$. After pretreatment, the control membranes and the treated membranes were incubated with various concentrations of $[^3H]$tryptamine, and Scatchard plots were performed as shown in Fig. 3. All the membrane preparations were further incubated with 1% BSA, unless otherwise stated. The plot data were computer-assessed by nonlinear regression analysis as described in the text. Values are expressed as the mean ± S.E.M. of 4 experiments. Significantly different from the control value at *$P < 0.05$ and **$P < 0.01$. 
DISCUSSION

The role of lipids in modulating hormone and neurotransmitter receptor mechanisms is now well-recognized. Loh and Law (15) have suggested that one of the dynamic functions of lipids in receptor mechanisms is to regulate directly the active sites and that the most likely candidates for this role are the acidic lipids, as large numbers of neurotransmitters and drug molecules are cationic in the physiological environment. In fact, several studies have demonstrated direct interactions between acidic lipids and various receptors (8, 16–19). Besides as already described elsewhere, from reconstitution experiments with acidic lipids, it was inferred that PS and CS may be involved in the regulation of tryptamine binding activities (6). Furthermore, it has been found that Azure A, which has the greatest affinity for acidic lipids, especially for CS, affects both temperature-dependent and independent [3H]-tryptamine binding to the same degree (4). All these considerations prompted us to assume the possible involvement of PS in temperature-induced high-affinity [3H]tryptamine binding.

The pretreatment condition of phospholipases A2 and D (i.e., 55 min incubation at 37°C) also corresponds to the preincubation conditions of the membranes that induce the augmentation of specific [3H]tryptamine binding (3). As shown in Fig. 1, further treatment with BSA resulted in a synergistic inhibitory effect on the [3H]tryptamine binding capacity of the phospholipase A2-treated membranes. On the contrary, several investigations (20, 21) have shown that the phospholipase A2 inhibition of [3H]ligand binding is reversed almost completely by BSA washing. Although we have no explanation for this discrepancy until the active binding molecules in soluble form can be studied, Scatchard analysis of [3H]tryptamine binding to the enzyme-treated membranes with or without BSA washing (Table 2) lead to one speculation that the inhibition of [3H]tryptamine binding by phospholipase A2 is due to the change of the lipid environment, which induces the deformation of its binding molecules, originating from the loss of specific lipid moieties, whereas the products of phospholipolysis partially prevent this conformational change.

When rat brain synaptic membranes were treated with phospholipase A2, there was a striking correlation between PS or PC hydrolysis and inhibition of [3H]tryptamine binding (Figs. 1 and 2). Since phospholipases A2 and D possess substrate specificity for the phospholipid molecule, they may provide more precise information about the role of endogenous phospholipids on the regulation of [3H]tryptamine binding. As presented in Table 1, 0.03 U of phospholipase A2 gave cleavage rates of 32 and 34% for PS and PC, respectively, and reduced [3H]tryptamine binding by about 32%, while phospholipase D (at 500 U) showed cleavage rates of 8 and 33% for PS and PC with no reduction of [3H]ligand binding. Moreover, an approx. 8% decrement of PS was estimated in all enzyme-treated membranes, without significant alteration in their binding capacities. These observations strongly suggest a direct correspondence between PS hydrolysis and reduction in specific [3H]-tryptamine binding capacity. Several reports also have shown that PS directly interacts with the activities of glutamate (17), benzodiazepine (18) and dopamine D2 (19) receptors. In addition, it is well-documented that the substrate preference of phospholipases A2 and D is PS > PC > PE and PC > PE > PS, respectively (22). However, our results showed that the substrate preference of phospholipases A2 and D is PE > PC > PE and PC > PE > PS, respectively (Table 1). At present, we have no explanation for the discrepancy with that of phospholipase A2.

To clarify the mode of action of phospholipase A2 on the temperature-induced high-affinity [3H]tryptamine binding sites, Scatchard analysis was performed (Fig. 3 and Table 2). The data obtained clearly revealed that phospholipase A2 drastically increased only the K_D value of the high-affinity sites, and this was accompanied by a decrement of
the $B_{\text{max}}$ values of both the high and low affinity binding sites. In other words, the high-affinity binding sites are more lipophilic in nature than the low affinity sites. This concept is further supported by other recent findings of our group (5). From these studies, we have predicted that the presence of a thiol group in a hydrophobic environment such as the intramembrane site is essential for the binding of ligands by the high-affinity sites. Taken together, these data strongly suggest that PS may be a modulator for the function of the temperature-induced high-affinity $[^3\text{H}]$tryptamine binding molecules.

REFERENCES