[3H]5-Hydroxytryptamine Binding to Reconstituted Fraction with Sulphatides, Phosphatidyserine and Phosphatidylinositol

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Abstract—As a first step toward the examination of the involvement of sulphatides, phosphatidyserine and phosphatidylinositol in 5-HT receptor mechanisms, we performed [3H]5-HT binding experiments on the various reconstituted fractions with these acidic lipids. A binding assay of [3H]5-HT to these fractions was carried out by Sephadex LH20 column chromatography. Among various reconstituted fractions, only the reconstitution system with the three acidic lipids exhibited a saturable [3H]5-HT binding capacity, whereas no binding was seen with [3H]-spiperone. When the binding of [3H]5-HT to this fraction was plotted as a function of the ligand concentration, a multiple binding mode with three classes of binding components (or sites) was observed. Furthermore, the double reciprocal plot indicated that this reconstitution system had three apparent Kd values of 4.7, 15 and 59 nM. The displacement studies with various compounds indicated that only a few 5-HT agonists (5-methoxytryptamine and tryptamine) and neurotransmitters (DA and ACh) inhibited the [3H]5-HT binding to this fraction, but 5-HT antagonists, LSD analogues and neuroleptics had no effect. Moreover, GTP, GDP and Gpp(NH)p clearly inhibited the [3H]5-HT binding in spite of their weak potencies, while GMP did not have any effect.

From a series of our studies on the characteristics of myelin butanol extracts (1-4), i.e., proteolipids of Folch-Lees (5), it had been demonstrated that the myelin butanol extracts have a specific binding capacity for 5-hydroxytryptamine (5-HT) and, moreover, its binding components are mainly sulphatides (CS), phosphatidyserine (PS) and phosphatidylinositol (PI) in nature. Although the primary role of lipids is to provide a molecular "cement" for the construction of biomembranes and other cellular organelles, some dynamic function would also be expected. For example, Loh and Law (6) have suggested that one of the dynamic functions of lipids in receptor mechanisms is to act directly as binding sites themselves and that the most likely candidates for this role are the acidic lipids, as large numbers of neurotransmitters and drug molecules are cationic under a physiological environment. These aspects prompted us to examine the possible involvement of CS, PS and PI in 5-HT receptor mechanisms.

As a first step toward the above examinations, we planned the [3H]5-HT binding experiments for the various reconstituted fractions with CS, PS and PI. We report here that only the reconstitution system with these three acidic lipids regenerate a saturable 5-HT binding capacity, and the present data suggest the involvement of CS, PS and PI in the 5-HT, especially 5-HT1, receptor mechanisms.

Materials and Methods

Chemicals: Sephadex LH20 was obtained from Pharmacia. The following compounds were generously given to us: quipazine (Miles), lysergic acid diethylamide (LSD) analogues (Sandoz), cyproheptadine and methiothepin (Roche), cinanserin (Squibb),

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Preparations of synaptic plasma membranes and acidic lipids: Male Wistar rats (150-200 g) were decapitated, and their brains were removed and dissected on ice by the method of Glowinski and Iversen (7). The tissues were homogenized in 10 vol. of 0.32 M sucrose, and synaptic plasma membranes were prepared from this homogenate by a method reported previously (8) with a minor modification. Briefly, the synaptosomal fraction (0.8-1.2 M sucrose layer) was separated from the crude mitochondrial fraction (P2) by discontinuous sucrose (0.32, 0.8 and 1.2 M) density gradient centrifugation (53,500xg for 2 hr) and then was lysed with water for 30 min at 0°C (10 ml/g tissue). The sample was loaded onto a two-step sucrose gradient (0.8 and 1.2 M). After the same centrifugation, the 0.8 and 1.2 M layers were collected and were designated as the synaptic plasma membrane fraction.

For lipid analysis, membranes were lyophilized, and the lipid extracts from those were prepared by the method of Norton and Poduslo (9), using chloroform-methanol (CM 2:1, v/v) as a solvent. CS, PS and PI were purified from rat brain by silicic acid and DEAE cellulose (acetate form) column chromatographies as reported previously (10). The isolated three acidic lipids were more than 96% pure.

Lipid analysis: Each lipid was determined by the quantitative TLC method as described previously (4). Total lipid galactose, phosphorus and cholesterol were quantified using the method of Svennerholm (11), Chen et al. (12) and Searcy and Bergquist (13), respectively. Only the CS concentration was estimated by the method of Kean (14), since the sensitivity of this method is 30-fold higher than that of the orcinol-method (11). The isolated three acidic lipids were more than 96% pure.

Binding of [3H]5-HT: Various reconstituted fractions of acidic lipids (CS, 17 μg; PS, 108 μg; and PI, 26 μg) were dissolved in 1.0 ml of butanol-water mixture (14% water, v/v). Aliquots of each sample (0.25 ml) were incubated at room temperature for 20 min with [3H]5-HT. After incubation, the mixtures were loaded onto a Sephadex LH20 column (0.6×15 cm), and stepwise elution was carried out with the following solvents: 8.4 ml each of chloroform and CM (10:1), and then 25 ml of CM (9:2). After chromatography, an aliquot of each of the collected fractions (0.4-1.0 ml) was added to a counting vial and evaporated under an air stream. After the evaporation, 10 ml of toluene-ethanol (9:1, v/v) phosphor was poured into the vial, and radioactivity was measured in a liquid scintillation counter. As reported previously (4), in this assay system, a minute amount of the free [3H]5-HT (12-14%) appeared also in the CM (9:2) solvent front, and thus the free or bound [3H]5-HT was easily distinguished.

Results

Before designing the [3H]5-HT binding experiments for the various reconstituted fractions with CS, PS and PI, the lipid composition of rat brain synaptic plasma membranes was determined by a quantitative TLC method (see Table 1). In both cortices (cortex+hippocampus) and stems (midbrain + striatum + hypothalamus + pons-medulla), the contents of all other lipids were nearly equal, except for minor galactolipids, cerebrosides and sulphatides. Thus, we applied the mean value of both samples as the content of each acidic lipid in the reconstitution systems.

[3H]5-HT binding capacities of the various reconstituted fractions with CS, PS and PI were examined by Sephadex LH20 column chromatography (see Fig. 1). The results revealed that only the reconstituted fraction with three acidic lipids exhibited the binding capacity for [3H]5-HT (39.3±2.0% of total input radioactivity, 4 experiments), whereas the others did not show any clear binding peaks. Moreover, displacement studies...
inferred that the reconstituted system of CS, PS and PI was composed of only a saturable component (see Table 2). These observations strongly suggest that some specifically organized complex with three acidic lipids will be necessary to regenerate the 5-HT binding properties. In addition, an interesting find was that this reconstituted fraction had little binding capacity for three doses of [3H]spiperone (10, 100 and 1000 nM).

To investigate the binding mode of the reconstituted fraction with CS, PS and PI, the binding of [3H]5-HT to this fraction has been measured as a function of ligand concentration, 5–35 nM (see Fig. 2). The nature of this binding isotherm was a saturable binding mode and well reflected the results of the displacement experiments. Furthermore, a multiple binding mode with three classes of binding components (or sites) was observed. At the present time, if these multiple binding components (or sites) do not interact with one another, the apparent Kᵦ value can be obtained by a double reciprocal plot. Thus, the multiple binding components of [3H]-5-HT were represented as a double reciprocal plot (see Fig. 2). The results indicated that this reconstituted system had three apparent Kᵦ values of 4.7, 15 and 59 nM. The present observations are in good agreement with those of the previous experiments (4), in cases in which the multiplicity of the reconstituted system with CS, PS and PI has already been found.

### Table 1. Lipid composition of synaptic plasma membranes

| Lipid                              | Cortices | Stems |
|------------------------------------|----------|-------|
| Cholesterol                       | 70.0±5.5 | 76.3± 7.2 |
| Minor galactolipids               | 0.2±0.1  | 1.5± 0.5 |
| Ceramides                         | 1.1±0.1  | 15.6± 1.4 |
| Sulphatides                       | 0.8±0.1  | 7.8± 0.9 |
| Ethanolamine phosphatides         | 94.4±3.4 | 101.9± 6.3 |
| Choline phosphatides              | 120.3±3.3| 128.3±10.1|
| Sphingomyelin                     | 12.3±0.5 | 16.9± 1.3 |
| Phosphatidylserine                | 24.9±1.2 | 29.1± 1.5 |
| Phosphatidylinositol              | 5.9±1.1  | 7.0± 1.0 |

Values are expressed as μg/mg dry weight (mean±S.E.M. of 4 determinations). Cortices: cortex+hippocampus. Stems: midbrain+striatum+hypothalamus+pons-medulla.

**Fig. 1.** Sephadex LH₂₀ chromatograms of the various reconstituted fractions. Samples were incubated with 5 nM of [³H]5-HT. The discontinuous elution system described in the text was used.
By displacement experiments, various compounds were tested for their abilities to inhibit the $[^3]$H$5$-HT binding to the reconstituted fraction with three acidic lipids. To investigate the high affinity binding sites, we chose 10 nM of $[^3]$H$5$-HT as the concentration of radioligand. The results (Table 2) indicated that only a few 5-HT agonists (i.e., 5-methoxytryptamine and tryptamine) inhibited the $[^3]$H$5$-HT binding, but LSD analogues, 5-HT antagonists and neuroleptics had no effect. Among several neurotransmitters, dopamine (DA) and acetylcholine (ACh) showed inhibition, while noradrenaline and $\gamma$-aminobutyric acid (GABA) did not have any effect. Moreover, although they had weak potencies, guanosine triphosphate (GTP), diphosphate (GDP) and guanyl-5'-yl-imidodiphosphate (Gpp(NH)p) clearly decreased the $[^3]$H$5$-HT binding to this reconstituted fraction, while guanosine monophosphate (GMP) did not show any inhibitory effect.

**Discussion**

To our knowledge, the CS concentration in synaptic plasma membranes is obscure. Norton (15) has reported that galactolipids are preferentially distributed in the myelin membranes. In general, the cross contamination of each of the subcellular organelles is unavoidable using the density gradient centrifugation method. In fact, the electron microscopic examination of the synaptic plasma membrane fraction used here could not absolutely exclude the presence of the myelin fragments in this fraction as reported previously (8). Nevertheless, we used the mean value of both membrane preparations (i.e., cortices and stems) as the content of each acidic lipid in the reconstitution systems. In addition, the incubation mixture (0.25 ml) contained the concentration of each lipid corresponding to that of 1 mg dry weight of the synaptic plasma membranes.

As shown in Fig. 1 and Table 2, the
reconstitution experiments clearly indicated that only the reconstituted fraction with CS, PS and PI possessed a saturable binding capacity, whereas the others did not show any binding potencies. These results strongly suggest that some specifically organized complex with these three acidic lipids is necessary for regenerating the 5-HT binding properties. Recently, several investigations have implied that acidic lipids are involved in the binding of a biological active substance to its receptor(s). For example, Loh et al. (16) have reported that CS appears to fulfill most of the structural requirements of a hypothetical opiate receptor. Ebadi and Chweh (17) have found that some components of GABA recognition sites may also contain sulpholipid(s) in addition to phospholipid(s). Moreover, it has been suggested that PS may regulate the activity of synaptic L-glutamate
receptors (18). On the other hand, a double reciprocal plot (see Fig. 2) indicated that the reconstituted fraction with CS, PS and PI had three apparent \( K_i \) values of 4.7, 15 and 59 nM. For the 5-HT receptors, it has been found that their affinity constants \( (K_i) \) for \([3H]5-HT\) are 1.6-8 nM (19-21). These observations infer that a certain lipid complex of CS, PS and PI may play a role in the 5-HT receptor mechanisms as a recognition site(s).

The radioreceptor-binding techniques have permitted the differentiation of multiple neurotransmitter receptors. For example, Peroutka et al. (22, 23) have demonstrated the existence of 5-HT\(_1\) and 5-HT\(_2\) receptors in the rat central nervous system. The binding to 5-HT\(_1\) receptors, which can be labelled by \([3H]5-HT\) or \([3H]LSD\), is regulated by guanine nucleotides and may be associated with adenylate cyclase. In addition, classical 5-HT agonists display greater affinity for the former ones. On the other hand, 5-HT\(_2\) receptors have been labelled by \([3H]\)-spiperone, \([3H]\)-LSD, \([3H]\)-mianserin and \([3H]\)-ketanserin and are not affected by guanine nucleotides, but are sensitive to 5-HT antagonists and neuroleptics. Therefore, the latter ones appear to mediate serotonergic behavioral syndromes. To investigate whether or not this reconstituted fraction of three acidic lipids interact with any of the multiple 5-HT receptors, displacement studies with various compounds were performed. As presented in Table 2, the results revealed that only a few 5-HT agonists (5-methoxytryptamine and tryptamine) inhibited the \([3H]5-HT\) binding, while 5-HT antagonists, LSD analogues and neuroleptics had no effect. Moreover, they have weak potencies. GTP, GDP and Gpp(NH)p clearly decreased the \([3H]5-HT\) binding, but GMP had no effect. For the inhibitory effect of guanine nucleotides on the \([3H]5-HT\) binding to its receptors, Peroutka et al. (22) have demonstrated the rank order potency of Gpp(NH)p > GTP > GDP. In contrast, the present results indicated that Gpp(NH)p had a lesser effect than GTP or GDP. It has been well documented that guanine nucleotide regulatory protein is indispensable to the appearance of the decrease of \([3H]\)-agonist binding to its receptors by GTP (24). Thus, for the observation of this GTP effect on the reconstituted system of CS, PS and PI, we can only refer to one report (25) which has suggested that GTP and glucagon bind to a common site involved both in the activation of adenylate cyclase and in the dissociation of glucagon from its receptor and that acidic phospholipids are required for the concerted effects of glucagon and GTP at this site. In addition, an interesting find was that this reconstituted fraction did not exhibit any binding capacity for \([3H]\)-spiperone. Although these correspondences are only partial, these findings suggest that CS, PS and PI may be implicated in the 5-HT\(_1\) receptor mechanisms, rather than 5-HT\(_2\) receptors. At the present time, we have no explanation for the significant decrease of \([3H]5-HT\) binding by DA and also ACh. However, Lindberg et al. (26) have pointed out the structural similarity between DA and 5-HT receptor agonists.

Finally, the present studies themselves are merely a model experiment in vitro for examining the possible involvement of acidic lipids in 5-HT receptor mechanisms. Therefore, the results described here require further investigations in synaptic plasma membranes with respect to the reliability of the inference that CS, PS and PI may be involved in the 5-HT receptor mechanisms. We will offer data which more definitely verifies the above possibility in the following report (27). Nevertheless, the present work will provide an experimental approach for characterizing lipophilic receptor molecules.

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