BINDING OF H³-IMIPRAMINE, H³-DIMETACRINE AND S³⁵-CHLORPROMAZINE TO SYNAPTOSONES

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Abstract—Binding of H³-imipramine, H³-dimetacrine and S³⁵-chlorpromazine to synaptosomes of rat cerebral cortex was studied using a centrifugation method, and kinetic analysis of the experimental data. Three psychotropic drugs were shown to be rapidly bound to synaptosomes at 2°C, representing a typical binding mode with two classes of binding components, i.e., saturable and non-saturable binding. A double reciprocal plot of the saturable binding component of these drugs revealed that H³-dimetacrine and S³⁵-chlorpromazine represented a single binding mode, whereas H³-imipramine showed a multiple one. When the synaptosomes were treated by freezing and thawing 15 times, a high affinity binding component of H³-imipramine was not observed, while the other two drugs showed a single binding mode as well as those of the undisrupted synaptosomes. To investigate the specificity of this multiple binding mode, comparative binding studies of H³-imipramine were carried out using myelin fragments of rat cerebral cortex. In the myelin fragments preparation, two typical classes of binding mode as shown in the synaptosomes were also recognized. However, a double reciprocal plot of the saturable binding component showed only a straight line, i.e., single binding mode. These findings suggest that imipramine has multiple binding sites to synaptosomes and a high affinity binding component is affected by freezing and thawing procedure.

Combined electron microscopic and biochemical studies of subcellular fractions (1–3) have demonstrated the isolation of pinched-off nerve endings or synaptosomes and sub-synaptosomal organelles (synaptic vesicles, synaptic membranes, junctional complexes, etc.) from the central nervous tissue. On the nerve transmission, Whittaker (4) and De Robertis (5) pointed out the physiological significances of these subcellular organelles. Their findings suggested that in order to study the mode of action of drugs and toxins on the central nervous system, the interaction between elements of the synapse and those agents should be investigated in detail.

In previous experiments (6–8), we found that imipramine, dimetacrine and chlorpromazine were highly concentrated in the synaptic areas. Furthermore, the results obtained by 5-stepwise sucrose density gradient centrifugation (8) suggested that these psychotropic drugs were concentrated in the membrane components of synaptosomes, but that binding of these three compounds to synaptosomes differed. Based on these observations, we planned binding experiments of psychotropic drugs to synaptosomes.

The present paper deals with the kinetic analysis of H³-imipramine, H³-dimetacrine and S³⁵-chlorpromazine binding to synaptosomes. In addition, H³-imipramine binding to myelin fragments is reported to confirm the specific binding mode of this drug to
MATERIALS AND METHODS

Radioactive drugs

Labeled imipramine (12.6 μCi/mg) was prepared according to the method of Kawazoe and Ohnishi (9) using tritium water as the labeled precursor. Preparation of H3-dimetacrine (22.0 μCi/mg) was as reported previously (6). S35-Chlorpromazine (12.0 μCi/mg) was purchased from Radiochemical Center, Amersham, England.

Preparation of synaptosomes and myelin fragments

Male Wistar rats (200-250 g) were decapitated and the cerebral cortex was separated in a cold room (4°C). The pooled cerebral cortex was homogenized in 0.32 M sucrose at a final concentration of 10%. F-A (myelin fragments-rich fraction) and F-B (synaptosomes-rich fraction) were separated from the homogenate by the method of Whittaker et al. (2), and the morphological examination of both fractions was performed by electron microscopy (Hitachi HU-12A, 100 kV).

Binding of radioactive drugs

To study the binding of psychotropic drugs to synaptosomes and myelin fragments, the following techniques were used. The separated F-A and F-B fraction from rat cerebral cortex was diluted with equal volumes of water and centrifuged at 100,000 × g for 30 min. The resultant pellet was resuspended in 0.05 M Tris-maleate buffer containing 0.16 M NaCl (pH 7.1) and aliquots of suspension (0.6 ml, about 3.3 mg protein) were incubated with the various concentrations of radioactive drugs in centrifuge tubes of RP55-rotor of the Hitachi 65P ultracentrifuge (total vol. of 2 ml). Incubations were carried out for 30 min at 2°C, and the tubes were centrifuged at 60,000 × g for 30 min. The surface of resultant pellets and a wall of tubes was carefully rinsed twice with cold Tris-maleate buffer, and then re-suspended in water. An aliquot of each of the suspensions (0.2-0.5 ml) was added to counting vials containing 10 ml of tT-21 emulsion phosphor (10), and radioactivity was counted in a Aloka LSC 651 liquid scintillation counter. Recorded counts were corrected to 100% efficiency using an channel ratio method. Counting efficiency of H3 ans S35 radioactivity was approx. 30 and 83%, respectively.

Protein determination

Protein was determined by the method of Lowry et al. (11), using crystalline bovine serum albumin as a standard.

Kinetic analysis

When the binding pattern appeared, as shown in Fig. 1 (curve A), it was reasonable to assume that this type of binding curve was a composite constituted with the saturated and non-saturated groups. In this case, a commonly used procedure (12) can be adopted to resolve the adsorption isotherm into its two presumed components. The observed linear slope in the high concentration range is used for the slope of a straight line which goes through the origin (curve B). This is taken to represent the non-saturating component. The differ-
ence between curves A and B represents the binding to the presumed group of high affinity
sites which saturate and is represented by curve C. This curve approximates a rectangular
hyperbola.

If only one type of binding component is present in curve C, the amount of drug bound
(a) is given by:

$$a = x \cdot \frac{1}{\frac{1}{Ka[S]} + 1}$$

where $x$; the amount of binding component, $Ka$; the association constant of the binding
reaction and $[S]$; the concentration of free drug, and a double reciprocal plot of curve C
represents a straight line.

The above described kinetic analysis was used in this experiment.

RESULTS

Binding of three psychotropic drugs as a function of concentration

H$^3$-Imipramine, H$^3$-dimetacrine and S$^{35}$-chlorpromazine were incubated with the
isolated synaptosomes for various periods of time, i.e., 30, 60 and 120 min at 2°C. Binding
of these drugs to the particulate fraction was the same value each time.

The binding of three psychotropic drugs to synaptosomes has been measured as a
function of drug concentration. Figure 2 shows the saturation curves of these drugs. The
nature of these binding curves suggests that the binding may be resolved into a saturable
(curve C) and a non-saturable (curve B) binding components as shown in Fig. 1.

Double reciprocal plot of a saturable binding component

To investigate the saturable binding component of these psychotropic drugs kinetically,
the binding curves shown in Fig. 2 were resolved into a saturable and non-saturable binding component by the method described in Materials and Methods. As shown in Fig. 3, the saturable binding components of these three drugs were represented as a double reciprocal plot. Double reciprocal plots of H<sub>3</sub>-dimetacrine and S<sub>35</sub>-chlorpromazine bindings showed a straight line. On the other hand, a double reciprocal plot of H<sub>3</sub>-imipramine showed a multiple binding mode.

*Psychotropic drugs binding to the disrupted synaptosomes*

In a previous experiment (8), we found that when the synaptosomes-rich fraction was disrupted by freezing and thawing 15 times, H<sub>3</sub>-imipramine showed a 74.1% of release from the synaptosomes, while releases with H<sub>3</sub>-dimetacrine and S<sub>35</sub>-chlorpromazine were 3.9 and 11.3%, respectively. From these results it was suggested that binding of these three compounds to synaptosomes differed and only one part of the binding components of imipramine present in synaptosomes would be affected by freezing and thawing procedures.

In the present study, we also used disrupted synaptosomes to observe the multiple binding mode of H<sub>3</sub>-imipramine in detail. These synaptosomes were incubated with various concentrations of three radioactive drugs as for the undisrupted synaptosomes. The
saturation curves were quite similar to those obtained previously (Fig. 2) and resolved into a saturable and non-saturable binding component in the same way, by the method described in Materials and Methods. As shown in Fig. 4, the saturable binding components of three drugs were plotted as a double reciprocal. A saturable binding component of each of these drugs is shown as a double reciprocal plot. IP; $H^3$-imipramine, DT; $H^3$-dimetacrine and CPZ; $S^{35}$-chlorpromazine.

**Fig. 4.** Psychotropic drugs binding to the disrupted synaptosomes. The isolated synaptosomes were treated by freezing and thawing 15 times. Binding curve of $H^3$-imipramine, $H^3$-dimetacrine and $S^{35}$-chlorpromazine to the disrupted synaptosomes was resolved into a saturable and non-saturable binding component. A saturable binding component of each of these drugs is shown as a double reciprocal plot. IP; $H^3$-imipramine, DT; $H^3$-dimetacrine and CPZ; $S^{35}$-chlorpromazine.

**Fig. 5.** Double reciprocal plot of $H^3$-imipramine binding to myelin fragments. $H^3$-Imipramine binding curve to myelin fragments was resolved into a saturable and non-saturable binding, and a saturable binding component is plotted as a double reciprocal.

$H^3$-Imipramine binding to myelin fragments

To determine whether or not the multiple binding mode of $H^3$-imipramine is specifically observed in synaptosomes, myelin fragments-rich fraction was selected as the binding tissue fraction because of the higher purity of this fraction than the synaptosomes-rich fraction and a constituent of the central nervous system.

The binding of $H^3$-imipramine to myelin fragments was plotted as a function of the drug concentration. The obtained binding curve was similar to that of the synaptosomes
and thus resolved into a saturable and non-saturable binding component. As shown in Fig. 5, a double reciprocal plot of the saturable binding component represented only a straight line and the multiple binding mode which had appeared in the synaptosomes was not observed.

DISCUSSION

Investigation of the binding of pharmacological agents to particulate fractions from various tissues is well established as a means of examining the mechanism of action of those agents. In the case of psychotropic drugs, Bickel (13) reported the binding of chlorpromazine and imipramine to blood components, i.e., red cells, albumin and lipoproteins. Furthermore, several investigators (14-16) demonstrated the binding of chlorpromazine and imipramine to liver microsomes and mitochondria. However, to our knowledge, the binding of psychotropic drugs to synaptosomes has not been documented in detail.

In the present experiment, we used 0.05 M Tris-maleate buffer containing 0.16 M NaCl (pH 7.1) as the incubation medium according to the method of Weinstein et al. (17). We consider this media would preserve the biological function of the isolated synaptosomes, but at present we have no information on the Na⁺-effect on the binding of H³-imipramine, H³-dimetacrine and S³5-chlorpromazine to synaptosomes. Before determining the amount of drug bound, the resultant pellet was carefully rinsed with the incubation medium, but this process did not remove the radioactivity present in extraparticulate fluid. However, for the binding of H³-imipramine to the crude synaptosomes preparation, it has been demonstrated that the value of the correction factor (F) based on inulin space measurement (i.e., the ratio of the extraparticulate fluid volume over the total volume of the suspension) was 0.98 and the corrected binding curve revealed a relatively insignificant shift in the shape of the adsorption isotherm (17). The type of binding is difficult to define because of the interaction of various systems (e.g., adsorption, uptake, diffusion, etc.) but, at least, the binding of these three compounds to synaptosomes does not involve an active transport process. In fact, it has been reported that the binding of H³-imipramine to synaptosomes did not require any metabolically derived energy source (18).

All of the particulate fractions used here were examined under electron microscopy. The isolated F-A and F-B fraction was the myelin fragments-rich and synaptosomes-rich fraction, respectively and the F-A fraction showed a higher purity than the F-B fraction. In addition, morphological examination of the disrupted F-B fraction revealed that this fraction contained the damaged synaptosomes, synaptic ghost membranes and the released synaptic vesicles. Full detailed morphological information on these fractions was reported previously (6, 8).

When the binding of three radioactive drugs to synaptosomes was plotted as a function of the drug concentration, a typical binding mode with two classes of binding components (i.e., saturable and non-saturable binding) was observed with all three drugs. For the H³-imipramine binding to the synaptosomal fraction, Weinstein et al. (17) and Hunt et al. (18) have reported the same type of saturation curve.
On the double reciprocal plot of a saturable binding component of each of the drugs, H3-dimetacrine and S35-chlorpromazine showed a single binding mode, whereas H3-imipramine represented a multiple one. In the comparative binding studies of H3-imipramine to myelin fragments, the multiple binding mode seen in synaptosomes was not observed. From these observations it is plausible that a multiple binding mode is evident only on the binding of H3-imipramine to synaptosomes. Furthermore, when the synaptosomes were treated by freezing and thawing 15 times, the high affinity binding component which should be represented in the lowest concentration range of this drug did not appear (see Fig. 4). This result suggests that the high affinity binding component of imipramine is specifically destroyed by freezing and thawing, and thus this component may be located on the outer membrane of synaptosomes.

Marchbanks (19) also reported that synaptosomes contained three binding components (high, medium and low affinity) for 5-HT and the high affinity binding component was destroyed by freezing. Moreover, in the binding of 5-HT to synaptosomes, Fiszer and De Robertis (20) demonstrated that the high affinity binding of 5-HT was inhibited by desmethylimipramine. In a preliminary series, we also found that imipramine inhibited the high affinity binding of 5-HT to synaptosomes more so than did dimetacrine and chlorpromazine (unpublished observations). Additionally, several investigators (21-24) have reported that the clinical activity of imipramine-like tricyclic anti-depressants is correlated with their inhibitory action on the re-uptake of 5-HT into synaptosomes.

Finally, the results obtained in this study indicate that the difference of the binding mode of psychotropic drugs to synaptosomes may implicate the clinical property of those agents.

REFERENCES

1) GRAY, E.G. AND WHITTAKER, V.P.: The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat., Lond. 96, 79-87 (1962)
2) WHITTAKER, V.P., MICHAELSON, I.A. AND KIRKLAND, R.J.A.: The separation of synaptic vesicles from nerve-ending particles ('synaptosomes'). Biochem. J. 90, 293-303 (1964)
3) DE ROBERTIS, E., PELLEGRINO DE Iraldi, A., RODRIGUEZ DE LORES ARNAIZ, G. AND SALGANICOFF, L.: Cholinergic and non-cholinergic nerve endings in rat brain-I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. J. Neurochem. 9, 23-35 (1962)
4) WHITTAKER, V.P.: The application of subcellular fractionation techniques to the study of brain function. Prog. Biophys. mol. Biol. 15, 39-96 (1965)
5) DE ROBERTIS, E.: Ultrastructure and cytochemistry of the synaptic region. Science 156, 907-914 (1967)
6) ISHITANI, R., SATOH, T., SUGA, T. AND KITAGAWA, H.: Studies on the ultrastructural distribution of H3-dimetacrine in rat cerebral cortex. Japan. J. Pharmacol. 22, 313-323 (1972)
7) ISHITANI, R. AND IWAMOTO, T.: Distribution of H3-dimetacrine in rat cerebral cortex by electron microscopic autoradiography. Experientia 32, 1306-1308 (1976)
8) ISHITANI, R. AND IWAMOTO, T.: Studies on the subsynaptosomal distribution of psychotropic drugs in rat cerebral cortex. Japana. J. Pharmacol. 27, 755-762 (1977)
9) KAWAZOE, Y. AND OHNISHI, M.: Studies on hydrogen exchange. V. Electrophilic deuteration of quinoline and its 1-oxide Chem. Pharm. Bull. 15, 826-832 (1967)
10) Paterson, M.S. and Greene, R.C.: Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Analyt. Chem.* 37, 854-857 (1965)

11) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275 (1951)

12) Christensen, H.N.: Kinetic approach to transport. *Biological Transport*, p. 50-51. Edited by Benjamin, W.A., New York (1962)

13) Bickel, M.H.: Binding of chlorpromazine and imipramine to red cells, albumin, lipoproteins and other blood components. *J. Pharm. Pharmacol.* 27, 733-738 (1975)

14) Teller, D.N., Denber, H.C.B. and Kopac, M.J.: Binding of chlorpromazine and thioridazine in vitro-I. Results of centrifugation methods with tissue and mitochondria from rat liver and human leukocytes. *Biochem. Pharmacol.* 16, 1397-1410 (1967)

15) Bickel, M.H. and Steele, J.W.: Binding of basic and acidic drugs to rat tissue subcellular fractions. *Chem. Biol. Interactions* 8, 151-162 (1974)

16) Kappus, H. and Remmer, H.: Irreversible protein binding of [14C]imipramine with rat and human liver microsomes. *Biochem. Pharmacol.* 24, 1079-1084 (1975)

17) Weinstein, H., Varon, S. and Roberts, E.: Effects of imipramine on the Na⁺-dependent exchange and retention of γ-aminobutyric acid by mouse brain subcellular particles. *Biochem. Pharmacol.* 20, 103-117 (1971)

18) Hunt, P.F., Kannengiesser, M.H. and Raynaud, J.P.: The nature of [3H]imipramine binding to synaptosomes. *Biochem. Pharmacol.* 24, 681-685 (1975)

19) Marchbanks, R.M.: Serotonin binding to nerve ending particles and other preparations from rat brain. *J. Neurochem.* 13, 1481-1493 (1966)

20) Fiszer, S. and De Robertis, E.: Subcellular distribution and chemical nature of the receptor for 5-hydroxytryptamine in the central nervous system. *J. Neurochem.* 16, 1201-1209 (1969)

21) Carlsson, A., Corrodi, H., Fuxe, K. and Hökfelt, T.: Effect of antidepressant drugs on the depletion of intraneuronal brain 5-hydroxytryptamine stores caused by 4-methyl-α-ethyl-metaamphetamine. *Lancet.* J. Pharmacol. 5, 357-366 (1969)

22) Carlsson, A.: Structural specificity for inhibition of [3H]-5-hydroxytryptamine uptake by cerebral slices. *J. Pharm. Pharmacol.* 22, 729-732 (1970)

23) Lidbrink, P., Jonsson, G. and Fuxe, K.: The effect of imipramine-like drugs and antihistamine drugs on uptake mechanisms in the central noradrenaline and 5-hydroxytryptamine neurons. *Neuropharmacology* 10, 521-536 (1971)

24) Kannengiesser, M.H., Hunt, P.F. and Raynaud, J.P.: An in vitro model for the study of psychotropic drugs and as a criterion of antidepressant activity. *Biochem. Pharmacol.* 22, 73-84 (1973)