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CHARACTERISTICS OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN RAT BRAIN

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Abstract. Characteristics of muscarinic acetylcholine (ACh) receptors were studied in the rat central nervous system (CNS) using ^{3}H-quinuclidinyl benzilate (QNB), an antagonist of muscarinic ACh receptors. Scatchard analysis indicated that the rat CNS had a single ^{3}H-QNB binding site with an apparent dissociation constant (Kd) of 5.0×10^{-10} M. Li^+, Zn^{++} and Cu^{++} had strong effects on ^{3}H-QNB binding which indicates that these metal ions might play important roles at muscarinic ACh receptor sites in the brain. Since antidepressants and antischizophrenic drugs displaced the binding of ^{3}H-QNB, the anticholinergic effects of these drugs need to be taken into account when they are applied clinically. The muscarinic ACh receptor was successfully solubilized with lysophosphatidylcholine. By gel chromatography, with a Sepharose 6B column, the solubilized muscarinic ACh receptor molecule eluted at the fraction corresponding to a Stokes' radius of 6.1 nm. With the use of sucrose-density-gradient centrifugation, the molecular weight of the solubilized muscarinic ACh receptor was determined to be about 90,000 daltons. The regional distribution of ^{3}H-QNB binding in rat brain was examined, and the highest level of ^{3}H-QNB binding was found to be in the striatum followed by cerebral cortex and hippocampus, indicating that muscarinic ACh mechanisms affect CNS function mainly through these areas.

Key words: muscarinic acetylcholine receptors, central nervous system, ion effect, solubilization, molecular weight.

The presence of acetylcholine (ACh) in the central nervous system (CNS) had been known for 50 years (1). ACh had been considered a neurotransmitter of the CNS, based on determinations of the distribution of choline acetyltransferase and acetylcholinesterase, changes in the concentration of ACh in the CNS under various conditions, and the release of ACh from the CNS. It is important to investigate the characteristics of the muscarinic ACh receptor in the CNS because most cholinergic synapses in the CNS are thought to be muscarinic rather than nicotinic. Although dramatic advances have been made in recent years in the identification and isolation of the peripheral nicotinic ACh receptor (2-4), few studies have been performed to solubilize and purify the CNS muscarinic ACh receptor (5-8). Since ACh is quickly degraded by biological materials, antimuscarinic drugs such as atropine and quinuclidinyl benzilate (QNB) are used as ligand for radioreceptor assay (RRA). This study was designed to characterize and solubilize

the muscarinic ACh receptor in the rat CNS, using ^{3}H-QNB which specifically binds to muscarinic ACh receptors.

MATERIALS AND METHODS

Materials. ^{3}H-QNB (specific activity 12 Ci/m mol) was purchased from Amersham, U.K.; lysophosphatidylcholine from Sigma Chemical Co., St. Louis, USA; atropine from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Sepharose 6B from Pharmacia, Uppsala, Sweden, and glass fiber filters from Whatman, Ltd., U.K. TRH was given by Tanabe Seiyaku Co., Osaka, Japan. Somatostatin and Met^5-enkephalin were bought from Peptide Institute Inc., Osaka, Japan, and β-endorphin from Peninsula Labs., San Carlos, USA. ACTH was given by Shionogi Pharmaceutical Co., Osaka, Japan. Polyethylene glycol was purchased from Nakarai Chemicals, Tokyo, Japan. All other reagents and chemicals were of reagent grade.

Crude synaptic membranes. Adult male Sprague-Dawley rats (200-250 g) were decapitated, and the brain tissues (except cerebellum) were immediately removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a Brinkman PT-10 homogenizer (dial setting 7, duration 10 sec, repeated once). The homogenate was centrifuged at 900 × G for 10 min, and the resulting supernatant was centrifuged at 11,500 × G for 20 min (9). The pellet (synaptosomal fraction) was homogenized in 10 volumes of Tris-HCl buffer (50 mM, pH 7.6) and centrifuged at 11,500 × G for 20 min. The pellet was resuspended in 10 volumes of the same buffer and stored at -70℃ until assay. This preparation was used as the crude synaptic membrane in most of the experiments.

Particulate fractions. The rat brain was dissected as described by Glowinski and Iversen (10). The brain tissues were homogenized in a glass homogenizer in 120 volumes of ice-cold Tris-HCl buffer. The homogenate was centrifuged at 50,000 × G for 20 min at 4℃, and the pellet was resuspended in the original volume of Tris-HCl buffer and recentrifuged. This pellet was particulate fraction. The pellets obtained from each part of the brain were used as the receptor preparations for the determination of regional distribution.

QNB binding assay with the synaptic membrane. QNB binding studies were carried out according to a slight modification of the method described by Yamamura *et al.* (7). The receptor preparation (0.5 ml, containing 500 μg protein) was incubated at room temperature (25℃) for 90 min with 0.5 ml of Tris-HCl buffer containing ^{3}H-QNB (1 nM). After incubation the contents were passed through a glass fiber filter (Whatman GF/C) positioned over a vacuum. The filter was washed three times under reduced pressure with 3 ml of ice-cold Tris-HCl buffer and was placed in a scintillation vial with 10 ml of scintillation fluor. The radioactivity of the filter was counted with an automatic beta counter. Determinations of binding were perfomed in triplicate, together with triplicate samples containing atropine (1 μM) to determine nonspecific binding. Specific binding was defined as the total binding minus the binding in the presence of 1 μM atropine.

Solubilization of receptor. The crude synaptic membrane of rat brain was suspended in 10 volumes of lysophosphatidylcholine solution (1.0 mg/mg receptor protein), and incubated at 30℃ for 10 min. The suspension was centrifuged at 100,000 × G for 60 min, and the resulting supernatant was used as the solubilized muscarinic ACh receptor preparation.

QNB binding assay with the solubilized receptor. The solubilized receptor (0.3 ml, containing 200 μg protein) was incubated at room temperature (25℃) for 90 min with 0.2 ml of Tris-HCl buffer containing ^{3}H-QNB (1 nM). After incubation, 0.1 ml of ice-cold Tris-HCl buffer containing 0.2 % (W/V) human γ-globulin and 0.6 ml of ice-cold 25 % (W/V) polyethylene glycol

(PEG) (final concentration, 12.5 %) was added. The contents were throughly mixed and placed in ice for 20 min, after which they were passed through a glass fiber filter (Whatman GF/C) positioned over a vacuum. The filter was washed three times with 3 ml of ice-cold 12.5 % PEG and placed in a scintillation vial with 10 ml of scintillation fluor. The radioactivity of the filters was counted with an automatic beta counter. Determinations of binding were performed in triplicate, together with triplicate samples containing atropine (1 μM) to determine nonspecific binding. Specific binding was defined as the total binding minus the binding in the presence of 1 μM atropine.

Gel filtration. Synaptic membrane of rat brain was incubated with ^{3}H-QNB in an excess of atropine or in the absence of atropine for 90 min at room temperature (25℃). After incubation, lysophosphatidylcholine (1.0 mg/mg protein) was added and the mixture was kept at 30℃ for 10 min and then centrifuged at 100,000 × G for 60 min. The supernatant was applied to a column of Sepharose 6B (1 cm × 70 cm) equilibrated and eluted with Tris-HCl buffer containing 0.1 % Triton X-100. One-ml fractions were collected and the radioactivity of each fraction was measured. Seven marker proteins, including thyroglobulin, γ-globulin, bovine serum albumin, hemoglobulin, peroxidase, trypsin and cytochrome C, were dissolved in the same buffer and processed in the same manner.

Sucrose-density-gradient centrifugation. Linear sucrose gradients (4.5 ml) were prepared from 5 % to 20 % sucrose in Tris-HCl buffer. The lysophosphatidylcholine-solubilized receptor (0.5 ml) was applied to the top of the gradient and centrifuged at 2℃ and 100,000 × G for 16 h. After centrifugation the bottom of the tube was puntured, and 21 fractions of 14 drops each were collected. The binding of ^{3}H-QNB to each fraction was determined by the same method as for the solubilized receptor. Several marker proteins including γ-globulin, bovine serum albumin and cytochrome C were dissolved in the same buffer and processed in the same manner.

Measurement of protein concentrations. All protein concentrations of this study were determined by the method of Lowrey *et al.* (11), with bovine serum albumin as the standard.

RESULTS

Time course. At room temperature (25℃), specific binding of ^{3}H-QNB to synaptic membrane attained, within 90 min, a steady state which continued for 3 h (Fig. 1). The time course of specific binding of ^{3}H-QNB to solubilized receptors was similar to specific binding of ^{3}H-QNB to synaptic membrane.

Saturation curve and Scatchard plot. Fig. 2 and Fig. 3 show the saturation curve and Scatchard plot of ^{3}H-QNB binding to rat brain synaptic membrane. Scatchard analysis suggested that the rat brain had a single distinct binding site with an apparent dissociation constant (Kd) of 5.0×10^{-10} M. When brain particulate fraction, synaptic membrane or solubilized receptor was used as the sourse of the muscarinic ACh receptor, there were no significant differences in the dissociation constants. This finding suggested that there was no difference in the nature of ^{3}H-QNB binding according to binding site.

Effects of various cations on specific ^{3}H-QNB binding. Fig. 4 shows the effects of various ions in the incubation medium. Lithium chloride (LiCl), sodium chloride (NaCl) or potassium chloride (KCl) was added to the reaction mixture at concen-

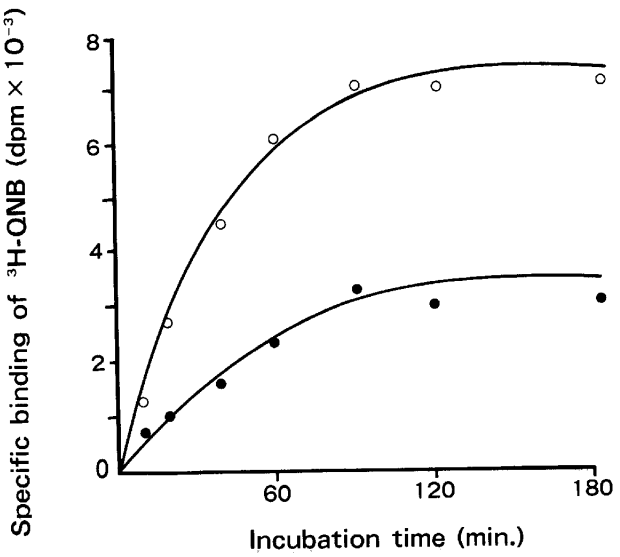

Fig. 1. Effect of time on the binding of ^{3}H-QNB to the synaptic membrane and solubilized receptor. One nM of ^{3}H-QNB was incubated with 500 μg synaptic membrane (○) or 200 μg solubilized receptor (●). Values are the means of three determinations.

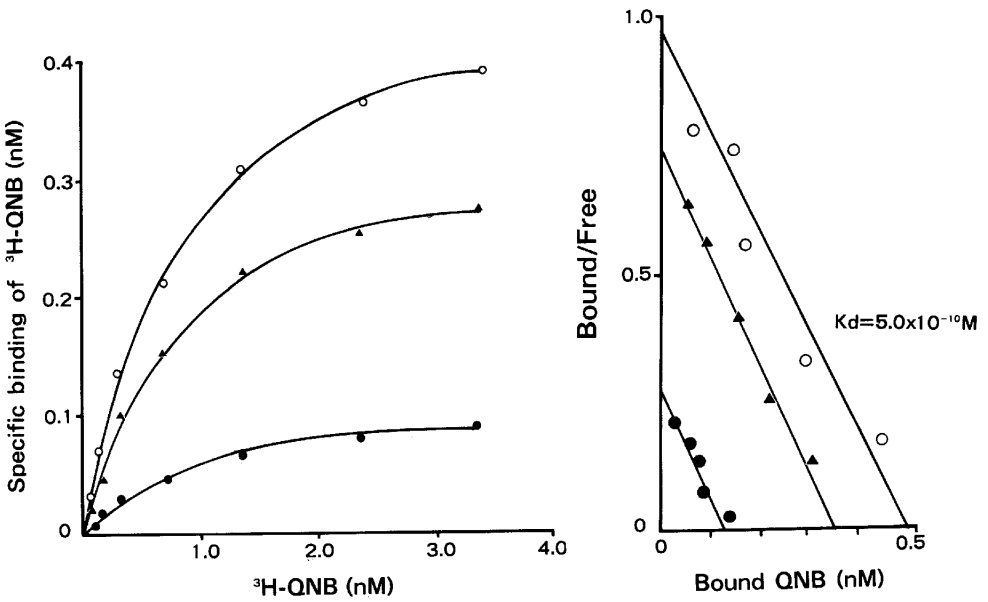

Fig. 2. Saturation curve using crude synaptic membrane (○), solubilized receptor (●) or particulate fraction (▲). Values represent the means of three determinations.

Fig. 3. Scatchard plot of saturation experiments using crude synaptic membrane (○), solubilized receptor (●) or particulate fraction (▲). Each value represents the mean of three determinations.

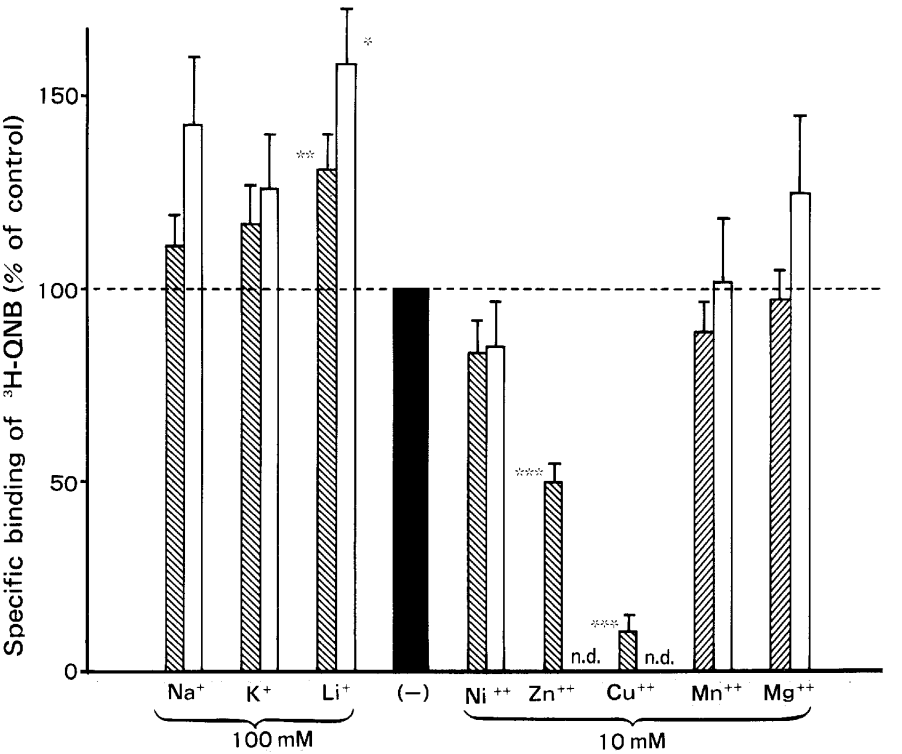

Fig. 4. Effects of various ions on specific ^{3}H-QNB binding. The crude synaptic membrane (▨) or the solubilized receptor (□) was incubated with 1 nM of ^{3}H-QNB with various ions. Values are expressed as percent of the control. Each bar represents mean ± SEM. n.d. = not detectable. $^*P < 0.05$ $^{**}P < 0.02$ $^{***}P < 0.01$.

trations of 25 mM-100 mM. Magnesium chloride ($MgCl_2$), nickel chloride ($NiCl_2$), cupric chloride ($CuCl_2$) or zinc chloride ($ZnCl_2$) was added to the reaction mixture at concentrations of 2.5 mM-10 mM. LiCl increased the specific binding of ^{3}H-QNB, while the other monovalent chloride salts had little effect on the specific binding of ^{3}H-QNB. $ZnCl_2$ and $CuCl_2$ significantly decreased the specific binding of ^{3}H-QNB. Specific binding of ^{3}H-QNB to solubilized receptors were similar to specific binding to synaptic membrane except when $ZnCl_2$ and $CuCl_2$ were present. $ZnCl_2$ and $CuCl_2$ completely inhibited the specific binding of ^{3}H-QNB to solubilized receptors, although low but significant binding was detected using synaptic membrane receptors. Since the medium already contained chloride ion, these results appear to be due to the effect of the cation. Scatchard plot revealed that changes in ^{3}H-QNB binding were caused by changes in receptor number, not in affinity (Fig. 5).

Effects of various drugs on specific ^{3}H-QNB binding. After establishing the optimal conditions for ^{3}H-QNB binding, the assay was employed for examining the interaction of neurotransmitters, neuropeptides and drugs which act on the CNS with

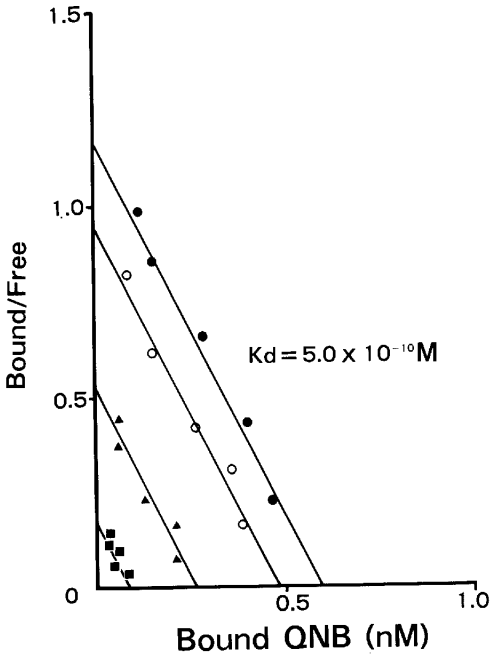

Fig. 5. Scatchard plot of saturation experiments in the presence of 100mM Li^+ (●), 10mM Zn^{++} (▲) or 10mM Cu^{++} (■). Control (○) was in the absence of these metal ions.

muscarinic ACh receptors. Table 1 shows the concentrations of endgenous ligands or drugs required to inhibit specific binding by 50 % (IC_{50}). Atropine and scopolamine, muscarinic antagonists, were the most potent inhibitors of ^{3}H-QNB binding of those drugs tested. These 2 agents inhibited specific ^{3}H-QNB binding by 50 % at 1.2-2 nM. By contrast, nicotine and hexamethonium, which are nicotinic agents, did not alter ^{3}H-QNB binding at concentrations up to 1 mM. Neurotransmitters such as catecholamines, serotonin and GABA did not alter specific ^{3}H-QNB binding at a 1 mM concentration. Many other drugs which act on the CNS had little or no effect on specific ^{3}H-QNB binding, but antidepressants and antischizophrenic drugs inhibited specific ^{3}H-QNB binding in a dose dependent fashion. Amitriptyline and thioridazine had the highest affinity to ^{3}H-QNB binding sites in the brain except for anticholinergic drugs.

Fig. 6 shows the log-probit plots for chlorpromazine, thioridazine and atropine. The slopes of the log-probit plots for atropine and the other drugs are parallel, which indicates that the drugs interacte with muscarinic ACh receptors in the same manner as standard atropine. Other drugs which had an effect on ^{3}H-QNB binding also had similar to that of atropine (data not shown).

Molecular weight of the muscarinic ACh receptor. Synaptic membrane of rat brain was incubated with ^{3}H-QNB in an excess of atropine or in the absence of atropine. After incubation, lysophosphatidylcholine was added and the mixture was kept

TABLE 1. INHIBITION OF ^{3}H-QNB BINDING BY NEUROTRANSMITTERS, NEUROPEPTIDES AND DRUGS ACTING ON THE CNS

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Atropine	0.0012	Serotonin	*
Scopolamine	0.002	Amitriptyline	0.008
Trihexyphenidyl	0.002	Trimipramine maleate	0.103
Protenamine	0.013	Imipramine	0.12
Metixene	0.003	Maprotiline	2.4
Biperiden	0.003	Propranolol	*
Acetylcholine[a]	4.8	Bufetolol	*
Oxotremorine	3	Indenolol	*
Pilocarpine	8	Oxpranolol	*
Carbamylcholine	10	Alprenolol	*
Nicotine	*	Pindolol	*
Hexamethonium	*	Clonidine	106
Dopamine	*	Regitine	92
Haloperidol	82	Dihydroergotoxine	*
Chlorpromazine	2	Pyritinol	*
Levomepromazine maleate	3.2	Citicholine	*
Thioridazine	0.05	TRH[b]	*
Sulpiride	306	ACTH[b]	*
GABA	*	β-endorphin[b]	*
Hopantenate calcium	*	Met^5-enkephalin[b]	*
Picrotoxine	*	Somatostatin[b]	*
Diazepam	*		

*: No effect at 1 mM *a*: Physostigmine (10 μM) was added to the incubation medium to prevent enzymatic (cholinesterase) hydrolysis. *b*: Bacitracin (25 μg/ml) was added to the incubation medium to prevent enzymatic degeneration. Abbreviations: GABA= γ-aminobutyric acid, TRH= thyrotropin releasing hormone, ACTH= adrenocorticotropic hormone.
Each IC_{50} determination used five concentrations of drugs in triplicate in the competing standard RRA for ONB. Results were converted to percentage of maximal specific binding and plotted on log-probit paper to estimate the concentration of 50% binding.

at 30℃ for 10 min, and then centrifuged at 100,000 × G for 60 min. Approximately 20 % of the QNB receptor complex of synaptic membrane were recovered in the supernatant. The supernatant was subjected to gel filtration on a Sepharose 6B column. Two distinct peaks of radioactivity were noted after gel filtration in the absence of atropine, whereas most of the radioactivity was eluted in the same fraction coincident with free ^{3}H-QNB in the presence of excess atropine (Fig. 7). These findings indicated that the first peak was the muscarinic ACh receptor complex. Seven marker proteins were applied on the same column, and by comparing with their elution volumes, the Stokes' radius of the muscarinic ACh receptor was estimated to be 6.1 nm.

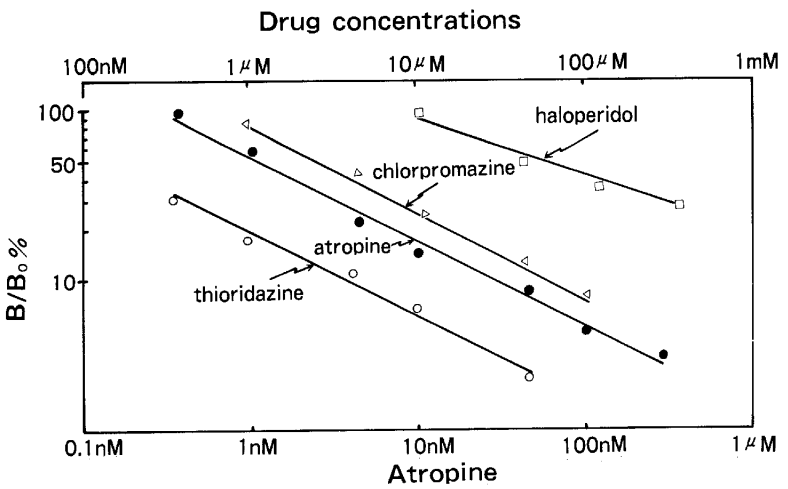

Fig. 6. Effects of thioridazine, chlorpromazine, haloperidol and atropine on ^{3}H-QNB binding. Crude synaptic membrane (500 μg protein) was incubated with 1nM of ^{3}H-QNB and five concentrations of each drug. Percent inhibition of ^{3}H-QNB specific binding was determined and plotted as a function of drug concentration on log-probit paper. Points are the mean values of three determinations.

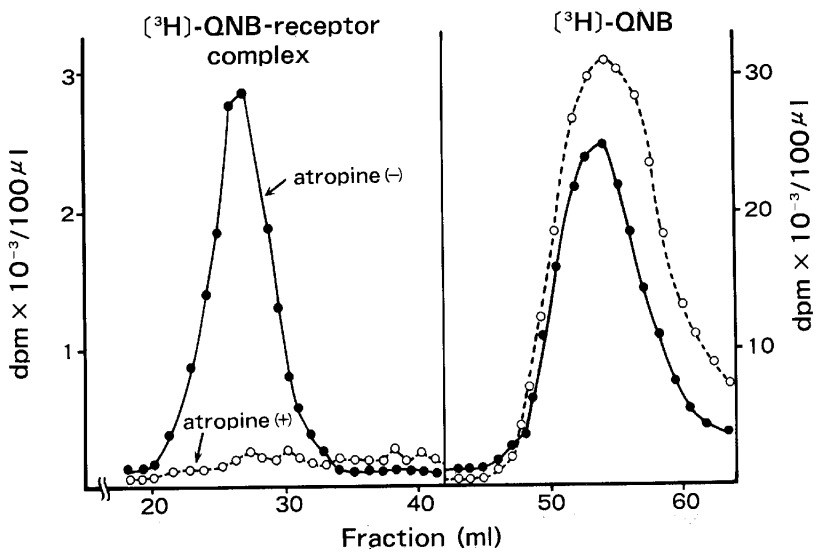

Fig. 7. Distribution of solubilized muscarinic ACh receptor complex after gel filtration through a Sepharose 6B column. Synaptic membrane of rat brain was incubated in a glass flask with 1nM ^{3}H-QNB in the presence (○---○) or absence (●—●) of 1 μM atropine. After incubation for 90min at room temperature (25℃), lysophosphatidylcholine (1.0mg/mg protein) was added to the mixture, the mixture was incubated for 10min at 30C, and then centrifuged at 100,000 × G for 60min at 2℃. The supernatant was applied to the column as described in the text. One-ml fractions were collected and radioactivity of each fraction was measured with an automatic beta counter. The first peak corresponds to the ^{3}H-QNB receptor complex and the second peak to the free ^{3}H-QNB.

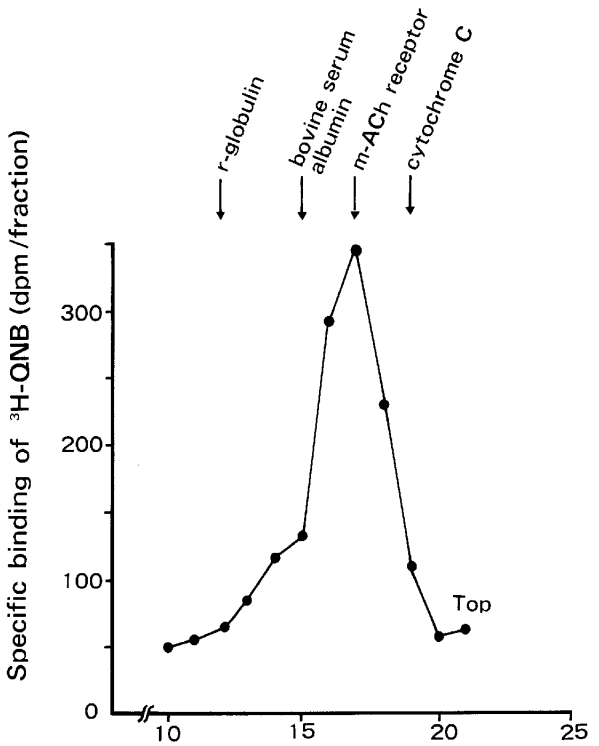

Fig. 8. Sucrose-density-gradient centrifugation. Lysophosphatidylcholine-solubilized receptor was subjected to sucrose-density-gradient centrifugation as described in the text. Binding to ^{3}H-QNB in the each fraction was determined. Three marker proteins were processed in the same manner and the positions of the protein peaks are indicated by arrows.

TABLE 2. REGIONAL DISTRIBUTION OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN THE RAT CENTRAL NERVOUS SYSTEM

Region	Specific binding of QNB (p moles/mg protein)	N
striatum	1.61±0.25	(5)
cerebral cortex	1.25±0.21	(5)
hippocampus	1.21±0.24	(4)
olfactory bulb	0.88±0.09	(5)
midbrain+thalamus	0.62±0.07	(5)
hypothalamus	0.41±0.08	(4)
pons+medulla oblongata	0.31±0.05	(5)
cerebellum	0.15±0.03	(5)

Data are presented as mean±SD for the indicated number of experiments.

After sucrose-density-gradient centrifugation of the solubilized receptor, the binding of ^{3}H-QNB to each fraction was determined by the same method as for

solubilized receptor. The sedimentation pattern is shown in Fig. 8. A single peak of specific ^{3}H-QNB binding was noted. Three marker proteins were applied on the same sucrose-density-gradient system, and as compared with the peaks of these proteins, the molecular weight of solubilized muscarinic ACh receptor was about 90,000 daltons.

Regional distribution of specific ^{3}H-QNB binding in rat brain. Since regional distribution of specific ^{3}H-QNB binding should parallel that of muscarinic synapses, specific ^{3}H-QNB binding in eight discrete regions of rat brain was measured. Muscarinic ACh receptor varied dramatically indistribution, with more than a ten-fold variation between some regions (Table 2). The highest level of specific ^{3}H-QNB binding was in the striatum, and high levels of specific ^{3}H-QNB binding were in the cerebral cortex and hippocampus. On the other hand, the lowest level of specific ^{3}H-QNB binding was in the cerebellum, which was only 9 % of the striatum binding.

DISCUSSION

Recently the presence of muscarinic ACh receptors within the brain had been demonstrated by biochemical methods, but few studies have been performed to solubilize and identify the muscarinic ACh receptors in mammalian CNS (5, 6). This paper investigated the biochemical character of CNS muscarinic ACh receptors by the use of ^{3}H-QNB which specifically binds to muscarinic ACh receptors, and the binding of which is inhibited by muscarinic drugs in correlation with their pharmacologic effects (7.8), unlike nicotinic agents which have no affinity to the receptors.

Scatchard analysis suggested that the rat brain had a single distinct binding site with an apparent dissociation constant of 5.0×10^{-10}M. When solubilized receptor, synaptic membrane or particulate fraction was used, there was no significant difference between the dissociation constants. This correlation indicated that the ^{3}H-QNB binding sites solubilized by lysophosphatidylcholine were the muscarinic ACh receptor sites of the synaptic membrane.

Monovalent cations such as Na^+ and K^+ had a little effect on ^{3}H-QNB binding. Li^+ is widely used in the treatment of affective disorders. Since Li^+ increased ^{3}H-QNB binding, it is possible that at least a part of the therapeutic efficacy of Li^+ is due to its modulation of ACh neurotransmission in the CNS. The presence of Zn^{++} and Cu^{++} in the CNS is well known. As Zn^{++} and Cu^{++} decreased the binding of ^{3}H-QNB, perhaps these two divalent cations also play a role as a cholinergic transmission modulater in the CNS, but the physiological significance of this was unclear. Zn^{++} and Cu^{++} completely inhibited the specific binding of ^{3}H-QNB to solubilized receptors, although low but significant specific binding was detected using synaptic membrane receptors. It has been shown that divalent cations bind to receptor molecules (12, 13). These findings suggest that an intact membrane structure might be required for muscarinic ACh receptor binding ac-

tivity in the presence of Zn^{++} or Cu^{++}. Mg^{++} is an essential nontransition metal which is present in the mammalian body in large amounts. It, however, produced no change in ^{3}H-QNB binding.

Many drugs which act on the CNS had no effect on ^{3}H-QNB binding except for anticholinergic drugs, antidepressants and antischizophrenic drugs (Table 1). As for antidepressants and antischizophrenic drugs, there seems to be a positive correlation between their anti-muscarinic effects as determined by their ability to inhibit ^{3}H-QNB binding and the ability of the drugs to produce anticholinergic side effects *in vivo* (14). antidepressants with a very low IC_{50}, such as amitriptyline, often produce anticholinergic side effects in clinical use. Antischizophrenic drugs with a very low IC_{50}, such as thiolidazine, seldom produce extrapyramidal side effects in clinical use (15), which agrees with the fact that antischizophrenic drugs with high anticholinergic potencies can decrease extrapyramidal symptoms (15, 16).

Recently, receptors for polypeptide hormones and neurotransmitters have been solubilized (5, 17-19). The muscarinic ACh receptor in the rat CNS was successfully solubilized with lysophosphatidylcholine. By gel chromatography the Stokes' radius of the solubilized muscarinic ACh receptor molecule was estimated to be 6.1 nm, and by sucrose-density-gradient centrifugation, the molecular weight to be about 90,000 daltons, or one quarter that of nicotinic ACh receptor in rat brain (20).

The distribution of biochemical markers of cholinergic synapses, including ACh (21, 22); choline acetyltransferase (21, 23-25); choline, that is, its high affinity uptake (26, 27), and acetylcholinesterase (28-32) have been extensively investigated. In this study, distribution of ^{3}H-QNB binding in eight discrete regions of the rat brain was determined to clarify the site of ACh action. The result was similar to previous reports of the monkey brain (33), dog brain (34), guinea pig brain (35) and rat brain (36). Moreover the distribution of ^{3}H-QNB binding correlated with the distribution of ACh, choline acetyltransferase, high affinity choline uptake and acetylcholinesterase.

The radioreceptor assay for muscarinic ACh receptors is useful not only for identification of potential physiological sites of action but also for understanding the mechanism of ACh action.

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