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Multiple Binding Affinities for Muscarinic Acetylcholine Receptors in Rat Brain

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ABSTRACT

We investigated the binding properties of (^{3}H) QNB and (^{3}H) NMS to mAchR to elucidate the characterstics of mAchR in rat brain by using two different preparations (homogemates & intact brain cell aggregates).

The binding properties of both ligands demonstrated high affinity and saturability in both experiments, however (^{3}H) QNB showed a significantly higher maximal binding capacity than tha ot (^{3}H) NMS.

1. In rat brain homogenates;

Displacement of both lignands with several mAchR antagonists resulted in competition curves in accoradnce with the law of massaction for QNB, atropine & scopolamine in thie preparation, also a similar profile was found for the quaternary ammonium analogs of atropine & scopolamine (methylatropine & methylscopolamine) when (^{3}H) NMS was used to label the receptors in rat brain.

But when these hydrophillic antagonists were used to displace (^{3}H) QNB, they showed interaction with high- and low-affinity binding sites in brain homogenates. Pirenzepine, the nonclassical mAchR antagonist, was able to displace both ligands from binding sites in this preparation.

2. In intact rat brain cell aggregates;

Intact bain cell aggregates were used to elucidate the binding characteristics of (^{3}H) NMS to mAchR in rat. The magnitude of binding of this ligand was related linearly to the amount of cell protein in the binding assay with a high ratio of total to nonspecific binding. mAchR antagonists displaced specific (^{3}H)NMS binding according to the law of mass-action, while it was possible to resolve displacement curves using mAchR agonist into high-& low-affinity component.

3. Our results indicate that more hydrophilic receptor ligand (^{3}H) QNB, displacement experiments in both tissues demonstrated that the lipid solubility of a particulr mAchR ligand might play an important role in determining its profile of binding to the mAchR, and the concentrations of mAchR in rat brain are both on the cell surface (membrane-bound receptor) and in the intracelluar membrane (intermembrane-bound receptor).

4. The results are discussed in terms of the usefulness of dissociated intact rat brain cells in studying mAchR in central nervous system.

Key Words: Binding properties, Brain homogenates, Intact brain cell aggregates, Law of mass-action, Hydrophillic-lipophillic-antagonist, Cell membrane-bound, Intermembrane-bound

Abbreviation: (^{3}H) QNB: (^{3}H) Quinuclidinyl benzilate
(^{3}H)NMS: (^{3}H)N-methylscopolamine
mAchR: muscarinic acetylcholine receptor

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INTRODUCTION

Muscarinic acetylcholine receptors (mAchR) are abundant in the CNS and the density of mAchR in the CNS is regulated by the concentration of muscarinic agonist in the vicinity of the receptor.

In recent years, neurotransmitter receptor binding studies have been very fruitful in demonstrating several of the characteristics of mAchR in the CNS. Especially radiolabeled ligand bindings studies have demonstrated several important features of bain mAchR (Mckinney & richelson, 1984). For example, classical mAchR antagonists bind with high affinity to a single population of the receptors (Hulme *et al.*, 1978), on the other hand, agonists interact with a heterogeneous population of mAchR, consisting of high-and low-affinity binding sites (Birdall *et al.*, 1978), and in some instances, the existance of agonist 'super-high-affinity' sites has been demonstrated (Birdsall *et al.*, 1980).

In general, receptor antagonists bind with high affinity to a homogeneous poupulation of mAchR with exception of pirenzeipne which interacts with high-and low-affinity mAchR, on the other hand, mAchR agonists exhibit binding to multiple afinity states of the receptor (Mckinney & Richelson, 1984; Birdsall & Hulmen, 1983).

Since membrane lipids are believed to be closely associated with mAchR binding sites (Aronstam *et al.*, 1977), we decided to invesigate the characteristics of mAchR in rat brain whether more lipophilic ligand such as (^{3}H)QNB might access to some membrane bound mAchR that are not redily available to more hydrophilic antaonist (^{3}H)NMS, and also we explored the binding characteristics of pairs of tertiary and quaternary mAchR antagonists by studyin thier ability to displace the specific binding of mAchR ligands (^{3}H)QNB & (^{3}H) NMS in rat brain.

Several tissue models have been used to study the binding properties of brain mAchR in vitor. Brain homgenates have been frequently used in some studies from the fact that it is easy to obtain in large quantities. Brain synaptosomes (Aguilar *et al.*, 1982) in a more refined preparation, brain slices (Gilbert *et al.*, 1979) under more physiological condition and intact cell culture in CNS (El-Fakahany & Richelson, 1983) have been utilized to study the mAchR in living system as models (Bir sall *et al.*, 1983).

In the present study, we reported the results from both uses of brain homogenates as a standard model and of disscoiated intact brain cell aggregates as a new model to study the binding properties of mAchR in the brain.

MATERIALS AND METHOD

In rat brain homogenates studies

Adult male and female Sprague-Dawley rats were sacrificed by decapitation and their brains rapidly removed, washed and weighed. Whole brains without the cerebellum were homogenized at 4℃ by Polytron (Brinkman, setting 7, 30 sec) in a buffer of the following composition (millimolar): NaCl 110; KCl, 3.5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; glucose, 25; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20. The pH was adjusted to 7.4 and sucrose was added to adjust osomolarity to 340 mosmol. Brain homogenates were centrifuged for 10 min at 1,000×g at 4℃ and the supernatant was the centrifuged for 30 min at 30,000×g at 4℃. The final pellet was suspended in buffer to give a final concentration of 2%(w/v) and was used fresh for the binding experiments. The assay was performed using a modification of the method of Yamaura and Snyder (1974). Membranes (0.07–0.1 mg of protein per assay tube) were incubated with 0.2 nM (^{3}H)QNB (33.2 or 56 Ci/mmol from New England Nuclear, Boston, MA or Amersham, Arligton Heights, IL) or (^{3}H)NMS (84.8 Ci/mmol, New Enqland nuclear). Incubations were carried in a final volume of 1 ml of buffer in triplicate in the absence and in the presence of increasing concentrations of the unlabeled mucarinic receptor antagonists. Nospecific binding was measured in the presence 2 μM atropine and was subtacted from all values to yield specfic binding. For saturation studies, membranes were incubated in triplicate with increasing concentration (0.01～1.0 nM) of the radioactively labeled ligands in the absence (total binding) or in the presence (nonspecific binding) of 2 μM atropine. In all assay, incubations were carried out for 90 min at 15℃ where equilibrium was attained. The binding reaction was terminated by filtration under vacuum through GF/B glass fiber filters (Whatman Inc., Clifton, NJ) using a Cell Harvester (Brandel, Gatithersburg, MD). Filters were washed three times with 5 ml of

ice-cold isotonic saline.

Each filter was placed into a scintillation vial and then 4.0 ml of a toluene-based scintilation fluid was added. Radioactivity was determined at least 6 hr later in a Beckman LS-6800 liquid scintillation counter with automatic correction for the counting efficiency of each sample, which averaged about 50%. Protein was determined according to Lowry *et al.*, (1951) using bovine serum albumin as standard.

In intact rat brain cell aggregates studies

Adult male or female Sprague-Dawley rats were decapitated, and brains were immediately dissected on ice to remove the cerebellum. Tissue was dissociated at 4℃ using a modification of the sieving technique of Honegger and Richelson(1976). Brains were minced into a paste using a razor blade, then placed in a nylon mesh bag (210 μm pore diameter, Nitex 210, Tetko, Elmsford, NY), and submerged in a modified Puck's D_1 solution (medium I) of the following composition-(mM): NaCl, 138; KCl, 5.4; Na_2HPO_4, 0.17; KH_2PO_4, 0.22; glucose, 5.5; and sucrose, 58.4 (pH 7.35, 340 mOsm). Tissue was dissociated by gently stroking the bag from the ouside with a glass rod. The resulting suspension was filtered by gravity flow through a tighter nylon mesh bag (130 μm pore diameter, Nitex 130), and the resulting tissue was washed twice by centrifugation (400 g for 3 min at 4℃) in a physiological buffer solution (medium II) of the following composition (mM): NaCl, 110; KCl, 5.3; $CaCl_2$, 1.8; $MgSO_4$, 1; glucose, 25; sucrose, 70; and HEPES (4-2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 20 (pH 7.4, 340 mOsm). Viability test performed by the trypan blue exclusion method usually yielded viability values greater than 90%.

For displacement studies, intact brain cell aggregates (0.07 to 0.1 mg protein/assay tube) were incubated with 0.2 nM (^{3}H)N-methyl scopolamine ((^{3}H)NMS). Incubations were carried out in final volume of 1 ml of medium II in triplicate in the absence and in the presence of increasing concentration of the unlabeled muscarinic recetor agonists of antagonists. Nospecific binding was measured in the presence of 2 μM atropine and was subtacted from all values to yield specific binding. For saturation studies, cell aggregates were incubated in triplicate with increasing concentrations (0.01 to 1.0 nM) of the radiocatively labeled ligand in the absence (total binding) or in the presence (nonspecific binding) of 2 μM atropine. In all assays, incubations were carried out for 90 min at 15℃ where equilibrium was attained. This temperature was chosen to increase the stability of the preparation and to minimize desensitization in the presence of high agonist concentratios. The following binding experiment was the same to those mentioned above.

Data analysis

Displacement curves were analyzed by computerized iterative nonlinear least-squares regression using the LIGAND program (Munson & Rodbar, 1980) adapted for an Apple II computer. The statistical difference between one-site and two-site models was analyzed by comparing the residual variance between the predicted and actual data points. and the F statistic was computed according to the following equation:

$$F = ((SS_1 - SS_2) / (dF_1 - dF_2)) / (SS_2 / dF_2)$$

where SS_1 and SS_2 are the sum of squares of residuals for the one-and two-site fits, respectively, and dF_1 and dF_2 are the corresponding degrees of freedom. Saturation isotherms were analyzed by the method of Scatchard (1949) using linear lines-squares regression analysis.

RESULTS

In rat brain homogenates studies

When specific binding was analysed using Scatchard plots, both (^{3}H)QNB and (^{3}H)NMS

Table 1. Maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) of (^{3}H) QNB in rat brain homogenates

Ligand	n	B_{max} (fmol/mg protein)	K_d (nM)
(^{3}H) QNB	7	507.1 ± 59[a]	0.19 ± 0.01
(^{3}H) NMS	7	331.3 ± 22	0.12 ± 0.01
(^{3}H) NMS/ (^{3}H) QNB		65.3%	

Values are represented as the mean ± S.E. for the number (n) of independent experiments.

a : Significantly higher than the value of (^{3}H)NMS, $p > 0.0125$

showed high-affinity saturable binding to a single set of sites in rat brain homogenates, with equilibrium dissociation constants (K_d, mean±S.E.M.) of 0.19±0.01 nM (n=7) and 0.12±0.01 nM (n=7), respectively. The high K_d values for both ligands obtained in our experiments might be due to the low incubation temperature (Aronstam *et al.*, 1977). Howeve,r under our experimental conditions, (^{3}H)QNB labeled a higher concentration of mAchR (507.1±59 fmol/mg protein) than that labeled by (^{3}H)NMS (331.3±22 fmol/mg protein), suggesting that (^{3}H)NMS interacts with high affinity only with a subpopulation of (^{3}H)QNB binding sites (Table 1).

Since the observed diffference in the density of muscarinc receptors labeled by (^{3}H) QNB and (^{3}H) NMS could well be a consequence of compartmentalization of binding sites during homogenization due to the formation of a popultion of outside in/inside out vesicles, we performed identical saturation experiments in two hypotonic media commonly used for muscarinic receptor binding studies.

In addition, unlabeled QNB and NMS (0.01 -100 nM) were used to study their ability to displace the specific binding of 0.2 nM (^{3}H)QNB or (^{3}H)NMS in rat brain homgoenates. Under experimental conditions simlar to those mentioned above, NMS demonstrated paradoxical properties that were ot shared by QNB. Both unlabeld NMS and QNB displaced specific (^{3}H) NMS binding accoring to the law of mass action with a Hill coefficient close to unity. On the other hand, although the dispacement curve of (^{3}H) QNB binding by QNB was steep, NMS displacement curve of this binding was ratehr shallow, suggesting the possible involvement of more than one binding site for NMS (data not shown). Analysis of displacement curves of both ligands by QNB resulted in a single affinity state.

On the other hand, although NMS displaced (^{3}H)NMS from a single population of receptor sites, displacement of (^{3}H)QNB binding by NMS displayed binding to a high-affinity receptor population (K_d=0.22 nM, 81.7% of sites) in addition to another low-affinity population (K_d=68.7 nM, 18.3% of sites) (Table 2 & 3).

Several muscarinc receptor antagonists were tested fro their ability to displace. (^{3}H)QNB (0.2 nM) or (^{3}H)NMS (0.2 nM) from their specific binding sites in brain homogenates. These antagonists included QNB, atropine and scopolamine, in addition to the quaternary amine analogs of the latter two antagonists (methylsocpolamine & methylatropine) in order to investigate wheter tertiary and quaternary analogs of the same antagonist would interact differently with mAchR. Under our experimental conditions, QNB, atropine and scopolamine displaced specific binding of (^{3}H)QNB according to the law of mass-action with steep slopes, resulting in Hill coefficients not significantly different from unity although methylatropine and methylscopolamine binding deviate slightly from a simple bimolecular reaction

Table 2. Displacement of specific (^{3}H) QNB and (^{3}H) NMS binding by unlabeled QNB and NMS in rat brain homogenates

Displacer	n	Parameter	Ligand	
			(^{3}H)QNB	(^{3}H)NMS
QNB	8	K_H	0.34 ± 0.04 nM	0.46 ± 0.04 nM
		K_L	–	–
		$\%R_H$	100%	100%
		$\%R_L$	–	–
NMS	8	K_H	0.22 ± 0.03 nM	0.26 ± 0.04 nM
		K_L	68.7 ± 7.6 nM	–
		$\%R_H$	81.7 ± 3.34 %	100%
		$\%R_L$	18.3 ± 3.34 %	–
		P	< 0.01	

Values are represented as the mean ± S.E. for the number (n) of independent experiments.
K_H & K_L represent the equilibrium dissociation constants of the high-and low-affinity sites respectively.
$\%R_H$ & $\%R_L$ are their respective relative densities

Table 3. Hill coefficients of muscarinic receptor antagonists using (^{3}H) QNB or (^{3}H) NMS as ligands in rat brain homogenates

Displacer	Ligand	
	(^{3}H) QNB	(^{3}H)NMS
QNB	0.94 ± 0.05 (8)	1.00 ± 0.01 (8)
Atropine	1.04 ± 0.02 (6)	0.82 ± 0.08 (6)
Scopolamine	0.92 ± 0.03 (9)	0.90 ± 0.06 (7)
Methylscopolamine	0.56 ± 0.05[a] (6)	0.74 ± 0.03[a] (6)
Methylatropine	0.61 ± 0.05[a] (6)	0.73 ± 0.07[a] (6)
Pirenzepine	0.59 ± 0.02[a] (6)	0.61 ± 0.04[a] (5)

Numbers in parentheses, number of independent experiments.
a : Significantly less than unity, $p < 0.01$

Table 4. Binding parameters of pirenzepine determined by using (^{3}H) QNB or (^{3}H) NMS as ligands in rat homogenates

Parameter	Ligand	
	(^{3}H) QNB	(^{3}H) NMS
K_H	$7.33 \pm 0.35 \times 10^{-8}$ M	$6.91 \pm 1.91 \times 10^{-8}$ M
K_L	$8.76 \pm 2.18 \times 10^{-6}$ M	$8.06 \pm 1.48 \times 10^{-6}$ M
K_L/K_H	119.5	116.6
$\%R_H$	49.6 ± 4.09 %	36.0 ± 1.89 %
$\%R_L$	50.4 ± 4.09 %	64.0 ± 1.89 %
n	(6)	(5)
p	< 0.01	< 0.01

Values are represented as the mean ± S.E.
Numbers in parentheses, number of experiments. P values were obtained by evaluating the improvement of fitting the data by using a two-site as compared to a one site model.

(Table 3).

Pirenzepine was also used to displace specific binding of both (^{3}H)QNB and (^{3}H)NMS. In both cases, displacement curves were very shallow exhibiting Hill coefficients that were significantly lower than unity. Displacement curves were better fitted according to a two-site model as compared to a one-site model ($P<0.01$). For pirenzepine/(^{3}H)QNB experiements, the curves were resolved into a high-affinity site ($K_d = 73.3$ nM, 49.6% of total sites) and a lowaffinity site ($K_d = 8.76$ μM, 50.4% of sites). When (^{3}H)NMS was used to lalbel the receptors, the values of the quilibrium dissociation constants were 69.1 nM (36% of total sites) and 8.06 μ M (64 % of total sites) for the high-and law affinity receptor populations, respectively. In addition, the ratios of the equilibrium dissocation constants for the low-and high-affinity binding sites were similar when either (^{3}H)QNB or (^{3}H)NMS were used as ligands (119.5: 1 and 116.6: 1, respectively) (Table 4).

In intact rat brain cell aggregates studies

When different concentrations of rat brain cell aggregates were incubated with 1 nM (^{3}H)NMS in the presence or in the absence of 2 μM atropine, specific (^{3}H)NMS binding to muscarinic acetylcholine receptors was linearly related to the amount of cell protein included in the binding assay, up to 400 μg of protein. All subsequent binding experiments were conducted usin 100 μg protein per assay tube. Increasing the concentraition of (^{3}H)NMS in the range of 0.01 to 2 nM resulted in increased total binding, while speific binding of (^{3}H)NMS to muscarinic acetylcholine receptors demonstrated saturability at higher ligand concentrations, and nonspecific binding measured in the presence of 2 μM atropine increased linearly with the lignand concentration (Fig. 1A). It is noteworthy that the level of nonspecific binding was reasonably low compared to total binding. Analysis of the averaged saturation isotherms of specific (^{3}H)NMS binding shown in Fig. 1A, using Scatchard plots and linear lestsquares regression (Fig. 1B), demonstrated a maximal binding capacity (B_{max}) of 451 ± 44 fmoles/mgprotein with K_d of 0.175 nM, on the other hand (^{3}H)QNB Binding shown in Table 5 demonstrated a maximal binding capacity (B_{max}) of 638 ± 26 fmoles/mg protein with K_d of 0.199 ± 0.04 nM.

Scatchard plots were linear with an average correlation coefficient of 0.992 ± 0.001, suggesting that (^{3}H)NMS binds to a single homogenous population of muscarinic acetylcholine receptors in rat brain cell aggregates, with no evidence for cooperative interactions, as indicated by a Hill coefficient of unity (Fig. 1B).

Displacement of the specific bidning of 0.2 nM (^{3}H)NMS in intact brain cell agregates by muscarinic receptor antagonists resulted in steep displacement curves (Table 6 & Fig. 2).

The inhibition constants of 0.42 ± 0.08, 1.27 ± 0.12 and 0.25 ± 0.01 nM for atropine, scopolamine

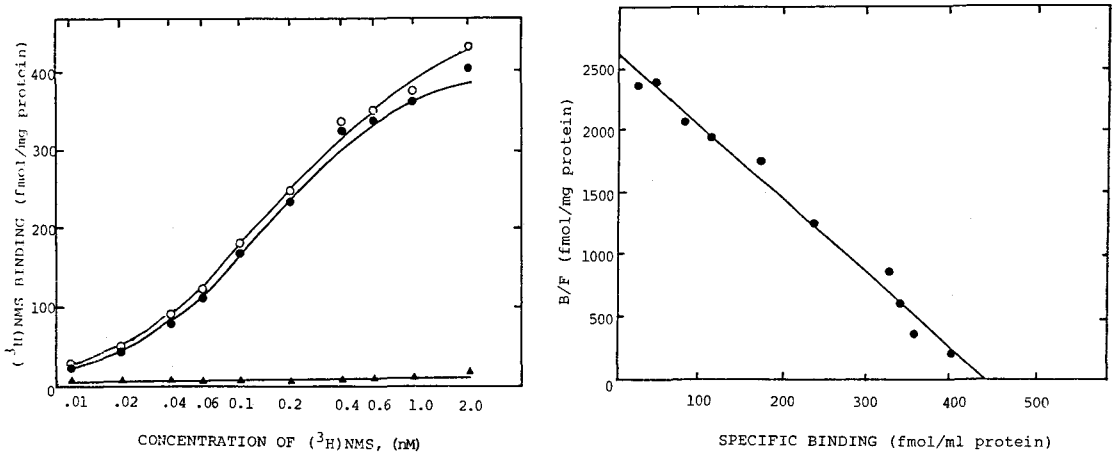

Fig. 1. Saturation isoterm of (^{3}H) NMS binding in rat brain cell aggregates. (A) Rat brain cells (100 μg protein) were incubated in triplicate with increasing concentrations of (^{3}H) NMS in the absence (○, total binding) and the presence (▲, nonspecific binding) of 2 μM atropines. Specific binding(●) was obtained by subtracting non-specific binding from total binding. The data presented are the average of fourteen independent experiments. (B) Scatchard plot of (^{3}H) NMS specific binding presented in (A) (r = 0.992).

Table 5. Maximum binding capacity (B_{max}), Equilibrium idssociation constatn (K_d) and Hill coefficient (n_H) of (^{3}H) QNB & (^{3}H) NMS in intact brain cell aggregates

Ligand	n	B_{max} (fmol/mg protein)	K_d (nM)	n_H
(^{3}H) QNB	8	638 ± 26	0.199 ± 0.04	0.998 ± 0.02
(^{3}H) NMS	8	451 ± 44	0.175 ± 0.03	0.992 ± 0.01
(^{3}H) NMS/ (^{3}H) QNB		70.7%		

Values are represented as the mean ± S.E. for the number (n) of independent experiments.

Table 6. Binding parameters of (^{3}H) NMS in intact rat brain cell aggregates

Displacer	n	Parameter				
		K_H (M)	K_L (M)	$\%R_H$	$\%R_L$	n_H
Antagonists						
Atropine	3	$0.42 \pm 0.08 \times 10^{-9}$		100		0.96
Scopolamine	5	$1.27 \pm 0.12 \times 10^{-9}$		100		0.94
QNB	5	$0.25 \pm 0.01 \times 10^{-9}$		100		0.81
Agonists						
Carbamylcholine	3	$4.3 \pm 1.8 \times 10^{-6}$	$8.9 \pm 1.3 \times 10^{-5}$	18.5	81.5	0.64
Oxotremorine	4	8.98×10^{-8}	1.8×10^{-6}	18.9	81.1	0.65
Pilocarpine	5	1.6×10^{-6}	1.81×10^{-5}	34.5	65.5	0.78

Values are represented as the mean ± S.E. for the number (n) of independent experiments.
A better fit resulted using a two-site model compared to a one-site model, $P < 0.01$.
K_H & K_L represent the equilibrium dissociation constants of the high-and low-affinity sites respectively.
$\%R_H$ & $\%R_L$ are their respective relative densities.
n_H represents the Hill coefficient.

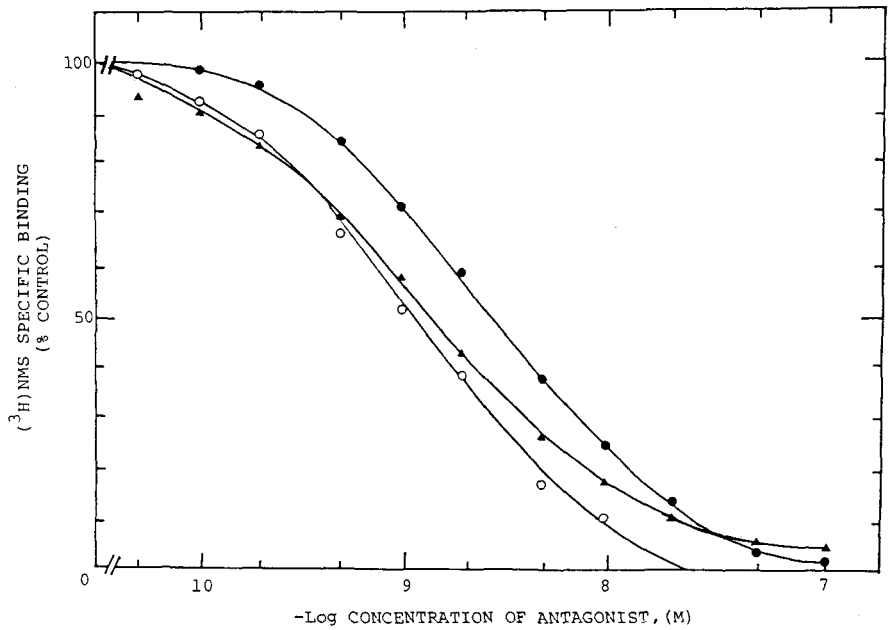

Fig. 2. Displacement of the specific binding of (^{3}H) NMS by muscarinic receptor antagonists in adult intact rat brain cells. Cells were incubated in triplicate with 0.2 nM (^{3}H) NMS in the presence of increasing concentrations of atropine (○), scopolamine (●) or quinuclidinyl benizilate (▲). Data are presented as a percentage of specific binding obtained in the absence of displacers (with a mean of 18 fmoles/assay tube), by averaging the results of three to five independent experiments.

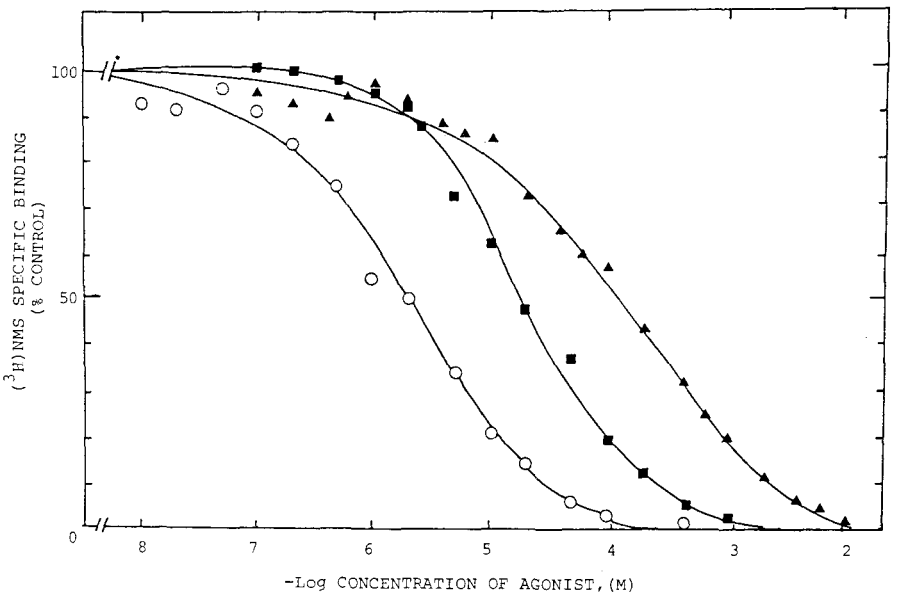

Fig. 3. Displacement of specific (^{3}H) NMS binding by muscarinic receptor agonists in intact adult rat brain cell aggregates. Cells were incubated in triplicate with 0.2 nM (^{3}H) NMS in the presence of increasing concentrations of carbamylcholine (▲), oxotremorine (○), or pilocarpine (■). Data are shown as a percentage of control binding in the absence of displacers, which averaged 14 fmole/assay tube, and presented as the mean of three to five independent experiments.

and QNB were abtained respectively (Table 6 & Fig. 2).

On the other hand, muscarinic receptor agonists displaced specific (^{3}H)NMS bindings in intact brain cell aggregates with very shallow inhibition curves extending over three log units (Table 6 & Fig. 3). Hence, Hill coefficients for carbamylcholine, oxotremorine and pilocarpine were 0.64, 0.65 and 0.78 nM respectively (Table 6). Nonlinear least-squares regression analysis of displacemtn of (^{3}H)NMS specific binding in brain cells by agonists resolved the displacement curves into luigh-and low-affinity components. The equilibrium dissociation constants of the high-affinity sites for carbamylcholine, oxotremorine and pilocarpine were 4.3 μM, 89.8 μM and 1.6 μM, respectively, while the respective values for the low affinity sites were 89 μM, 1.8 μM and 18.1 μM (Table 6). In addition, the proportion of the density of high-affnity sites to total receptor concentration was 18.5, 18.9 and 34.5% for carbamylcholine, oxotremorine and pilocarpine respectively (Table 6).

DISCUSSION

Receptor binding studies for mAchR in brain have been advanced by the accumulation of significant amount of valuable information regarding the properties of receptor itself. It has been generally proposed that mAchR antagonists bind to a homogenous populaion of receptors according to the law of mass-action, on the other hand, mAchR agonists can discriminate between multiple affinity sites which resulted in heterogeniety state (Birdsall *et al.*, 1978). However, it has been suggested recenlty that binding characteristics of mAchR antagonist might not be as simple as once thought to be, for instance, it has been reported that gallamine or pancronium interacts with cholinergic receptor though hinding to a site which is allosterically coupled to the receptor (Dunlap & Brown, 1983), and similar results have been observed with quinidine (Waelbracek *et al.*, 1984), phencyclidine (El-Fakahany *et al.*, 1984) and 4-aminopyridine (Lai *et al.*, 1985).

Such heterogeneity of receptor binding sites for antagonist is perhaps best exemplified by the nonclassical mAchR antagonist piren zepine (Hammer *et al.*, 1980; Lee & El-Fakahany, 1985).

Although homogenates prepartion has provided a vast amount of information, it suffers from several potential drawbacks, resulting mainly from disrupting the cell membrane integrity.

In these studeis, it has been shown that a significant differences between teritary and quaternary mAchR antagonists for binding to the receptor sites in rat brain homogenates demonstrated both in saturation and displacment studies in homoqentates preparation. So, we designed the next experiment by using intact rat brain cell aggreagate to eleucidate these two antagonists binding properties.

In brain homogenates studies, it was demonstrate significant differences in the interaction of lipophilic and hydrophilic ligands with rat brain muscarinic receptors, as evidenced by the ability of methylscopolamine and methylatropine to interact with high affinity only with a subpopulation of (^{3}H)QNB binding sites. These data indicate that one should be cautions when (^{3}H)QNB and (^{3}H)NMS are used interchangeably to label muscarinic receptors in the brain.

(^{3}H)NMS labeled only a fraction of the receptor population available to (^{3}H)QNB in brain homogenates studeis suggesting that (^{3}H)NMS might label only a subpopulation of (^{3}H)QNB binding sites with high affinity (Table 1). One possibility to be considered is that the difference in the binding site density for (^{3}H)QNB and (^{3}H) NMS obtained in the present experiment might be due to the use of an iso-osmotic buffer both for tissue homogenization and for the binding assays.

Although the difference in maximum receptor binding of (^{3}H)QNB and (^{3}H)NMS found in the present study is not compatible with the assumption that they identify one and the same binding site, similar finding using these ligands and others have been reported in the literature. For example, (^{3}H) propylbenzilycholine mustard and (^{3}H)QNB label different molecular size species of muscarinic receptors solubilized from rat forebrain homogenates (Berrie *et al.*, 1984), (^{3}H)QNB has been found to label double the receptor number obtained using (^{3}H)NMS, which is similar to our present findings. In addition, the quarternary ammonium analog of (^{3}H)QNB, (^{3}H)QNB-methiodie, interacts only with half of the receptor population that binds (^{3}H)QNB in dog ventricle homogenates (Gibson *et al.*, 1984) and in intact chick heart cells (Goldstein and Brown, 1985).

To make sure of the results from rat brain homogenates studies, we explored (^{3}H)NMS binding properties for mAchR by displacement experiments with cholinergic antagonist or cholinergic

agonist in intact rat brain cell aggregates.

The binding studies for mAchR presented in this work demonstrated the usefulness of dissociated adult rat brain cell aggregates in charcterizing the binding properties of these receptors in an intact cell system. Binding of the potent and speific muscarinic receptor ligand, (^{3}H)NMS, in these cells was proportional to the protein concentration used in the binding assay. In addition, specific binding of this ligand was saturable with a very favorable ratio of total and nonspecific binding values. Scatchard plots of saturation isotherms of specific (^{3}H)NMS binding are linear, suggesting the involvement of a single homogenous population of muscarinic acetylcholine receptors (Fig. 1). In addition (^{3}H)NMS binding in rat brain cells demonstrated high affinity (Table 5). The muscarinic receptor antagonists atropine, scopolamine and QNB displaced specific (^{3}H) NMS binding with high potency and with Hill coefficients close to unity, indication their interaction with a monogenous population of receptors, and the absence of cooperative interaction (Table 6 & Fig. 2), these findings are similar to those obtained in rat brain homogenate (McKinney & Richelson, 1984; Hulme et al., 1978; Yamamura & Snyder, 1974).

On the other hand, the muscarinic receptor agonists carbamylcholine, oxotremorine and pilocarpine displaced specific (^{3}H)NMS binding in these cells with Hill coefficients that were significantly less than unity (Table 6 & Fig. 3), this binding profile of muscarnic receptor agonistis a common finding in different preparation used to study muscarinic receptor binding, and is probably due to their interation with multiple receptor sites (Birdsall *et al.*, 1978; El-Fakahany & Richelson, 1980; Mckinney *et al.*, 1984; Burgerneister *et al.*, 1978).

Another important finding reported here is that displacement curves of (^{3}H)NMS binding by muscarinic receptor antagonists show a different profile from that observed in (^{3}H)QNB binding experiments. The more lipophilic antagonists (QNB, atopine and scopolamine) displace both (^{3}H)NMS and (^{3}H)QNB binding according to the law of mass-action, suggesting that they bind with high affinity to a single population of binding site (Table 3). Alternatively, they might be interacting with two binding sites with an equally high affinity. On the other hand, whereas hydrophilc antagonists displace (^{3}H)NMS binding from a single site, they demonstrate binding to two receptor populations in displacing (^{3}H)QNB binding (Table 2).

We also demonstrate that although conventional hydrophilic muscarnic receptor antagonists share this bidning characteristic of pirenzepine, their binding profile is still different from that of the latter, as only pirenzepine can distinguish between various recoptor populations regardless of the nature of the ligand used in the assay (Table 3). In addition, there is evidence in the literature that typical hydrophilic muscarinc antagonists do not possess (possess) the distinct pharmacoloigcl selectivity of pirenzepine. This sugget that the multiple affinity states recongnized by hydrophilic muscarinic receptor antagonists might not be identical to M_1 and M_2 receptors identified by pirenzeipine. In conclusion, the lipid solubility of a particular muscarinic receptor antagonist should be taken into consideration before complex binding data are interpreted in terms of multiple muscarinic receptor (M_1 and M_2) subtypes.

We report that the dissociated adult rat brain cell aggregates may provide a very useful and physiologically-relevant model to study muscarine acetylcholine receptor binding in vitro. This technique offers an easy method to obtain intact differentiated brain cells with minimal diffusion barriers. In addition to the usefulness of these cells in receptor binding experiments, they also provide a tool to study muscarinic receptor-mediated biochemical responses that require intact cells, e.g. cyclic GMP formation and phosphatidylinositol turnover.

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＝국문초록＝

흰쥐 뇌내(腦內)의 무수카린성 콜린 수용체의 이질성(異質性)

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중추신경계 특히 뇌내(腦內)의 무수카린성 콜린 수용체(mAchR)에 대한 수용체 특성의 연구의 하나로, 물리·화학적 성상에 다른 두 종류의 콜린길항제를 사용하여 서로 다른 두 형태의 조직에서 약물의 작용양상 및 다른 약물과의 상호작용을 검초하였다.

실험동물로는 흰쥐를 일정기간 규정사료를 사육하였고, 사용한 Radioactive ligands는 (^{3}H)QNB와 (^{3}H)NMS였으며 그외에 다른 수종의 길항제 또는 효능제와의 치환작용을 brain homogenates와 intact brain cell aggregates에서 관찰하여 다음과 같은 결과를 얻었다.

1. (^{3}H)QNB와 (^{3}H)NMS는 모두 질량작용의 법칙에 비례하여 수용체와의 결합에서 높은 친화력과 포화를 보였으며 또한 높은 결합 능력을 나타내었다. 더욱이 homogenates 제제와 intact cell aggregates제제에서의 결과 사이에는 유사한 점이 많았다.
2. Homogenates제제를 사용한 실험에서, 제 3 급아민콜린길항제인 QNB, atropine과 scopolamine 또는 제 4 급 암모늄콜린 길항제인 methylatropine과 methylscopolamine을 사용하여 위의 두 radioactive ligands와의 치환작용를 검토하였다. (^{3}H)NMS 실험군에서는 제 3 급아민 및 제 4 급 암모늄길항제 모두가 구조의 구별없이 질량작용의 법칙에 따라 치환되었으나 (^{3}H)QNB 실험군에서는 제 4 급 암모늄콜린 길항제들을 단일성(unity)이 아닌 높고 낮은 두 종류의 친화도를 가진 결합부위의 양상을 나타내었다. 또 비특이성 콜린길항제인 pirenzepine을 사용한 실험군에서는 두 ligands을 모두 치환시켰고 서로 다른 결합부위가 있음을 보였다.
3. Intact cell aggregates 제제를 사용한 실험에서, (^{3}H)NMS와 (^{3}H)QNB 모두 homogenats 제제에서와 같은 양상의 반응을 보였다. 또 (^{3}H)NMS를 radioligand로 하여 수종의 콜린길항제와 수종의 콜린 효능제를 사용하여 약물 상호작용으로 수용체의 성질을 검토하였다. 그 결과 콜린 길항제들은 질량작용의 법칙에 따라 치환되었으나 콜린 효능제 투여군에서는 높고 낮은 두 종류의 다른 친화력의 결합부위를 나타내었다.
4. 위의 실험의 결과로,
   (a) 친유성콜린 길항제인 (^{3}H)QNB는 친수성 콜린길항제인 (^{3}H)NMS보다 훨씬 높은 결합능력을 보였으며 이것으로 수용체 특히 mAchR의 존재 장소 또는 mAchR의 형상의 일부는 세포막 표면 뿐 아니라 세포막내의 어떤 부위와도 관계가 되는 것으로 간주되는데 이것이 (^{3}H)QNB가 (^{3}H)NMS보다 높은 최대 결합능력(B_{max})을 나타낸 이유이다.
   (b) 두 종류의 다른 제제에서 우리는 같은 양상의 결과를 관찰하였기에 결점이 많은 homogenates 제제보다는 intact cell aggregates 제제를 수용체 연구에 대한 새로운 실험모형(experiment model)으로 사용할 수 있는 가능성을 제시하고자 한다