Arch. Pharm. Res. Vol. 17, No. 6, pp. 443-451, 1994

Selectivity of Oxomemazine for the M_1 Muscarinic Receptors

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(Received August 31, 1994)

The binding characteristics of pirenzepine and oxomemazine to muscarinic receptor were studied to evaluate the selectivity of oxomemazine for the muscarinic receptor subtypes in rat cerebral microsomes. Equilibrium dissociation constant (K_D) of (−)-[^{3}H]quinuclidinyl benzilate([^{3}H]QNB) determined from saturation isotherms was 64 pM. Analysis of the pirenzepine inhibition curve of [^{3}H]QNB binding to cerebral microsome indicated the presence of two receptor subtypes with high (K_i=16 nM, M_1 receptor) and low (K_i=400 nM, M_3 receptor) affinity for pirenzepine. Oxomemazine also identified two receptor subtypes with about 20-fold difference in the affinity for high (K_i=84nM, O_H receptor) and low (K_i=1.65 μM, O_L receptor) affinity sites. The percentage populations of M_1 and M_3 receptors to the total receptors were 61 : 39, and those of O_H and O_L receptors 39 : 61, respectively. Both pirenzepine and oxomemazine increased the K_D value for [^{3}H]QNB without affecting the binding site concentrations and Hill coefficient for the [^{3}H]QNB binding. Oxomemazine had a 10-fold higher affinity at M_1 receptors than at M_3 receptors, and pirenzepine a 8-fold higher affinity at O_H receptors than at O_L receptors. Analysis of the shallow competition binding curves of oxomemazine for M_1 receptors and pirenzepine for O_L receptors yielded that 69% of M_1 receptors were of O_H receptors and the remaining 31% of O_L receptors, and that 29% of O_L receptors were of M_1 receptors and 71% of M_3 receptors. However, M_3 for oxomemazine and O_H for pirenzepine were composed of a uniform population. These results suggest that oxomemazine could be classified as a selective drug for M_1 receptors and also demonstrate that rat cerebral microsomes contain three different subtypes of M_1, M_3 and the other site which is different from M_1, M_2 and M_3 receptors.

Key words: Muscarinic receptor subtype, Oxomemazine, Pirenzepine, Receptor binding, Rat cerebrum

INTRODUCTION

Muscarinic cholinergic receptors mediate a variety of acetylcholine-induced biochemical and physiological responses in the central and peripheral tissues, but the potency of the muscarinic receptor agonists in eliciting these responses varies between tissues. From these functional studies with agonists (Birdsall and Hulme, 1976; Goyal and Rattan, 1978; Barlow *et al.*, 1980), the possible existence of muscarinic receptor subtypes has been hypothesized. Although it was suggested that antagonists might also recognize the heterogeneity of muscarinic receptors (Barlow *et al.*, 1976; Kloog *et al.*, 1979), this was not accepted until the selectivity of pirenzepine for certain tissues was confirmed in functional(Brown *et al.*, 1980) and binding experiments (Hammer *et al.*, 1980; Hammer, 1980).

The discovery of pirenzepine, a selective muscarinic antagonist, have provided substantial evidence for the subclassification of muscarinic receptors into high(M_1) and low(M_2) affinity sites for pirenzepine (Hammer *et al.*, 1980; Hammer and Giachetti, 1982; 1984; Berrie *et al.*, 1983; Watson *et al.*, 1983; Hirschowitz *et al.*, 1984). The M_2 receptors were further subclassified into cardiac M_2 receptors and glandular(ileal) M_3 receptors by their selectivity for 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]-benzodiazepine-6-one(AF-DX 116)(Giachetti *et al.*, 1986; Hammer *et al.*, 1986; Micheletti *et al.*, 1987) and 4-diphenyl-acetoxy-N-methylpiperidinemethiodide (4-DAMP) (Doods *et al.*, 1987; Giraldo *et al.*, 1987; Lazareno and Roberts, 1989), respectively. Thus, the introduction of selective muscarinic antagonists, such as pirenzepine, AF-DX 116 and 4-DAMP, proved the most important pharmacological basis to subclassify

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the muscarinic receptors into M_1, M_2 and M_3.

Oxomemazine, a histamine H_1-receptor blocker which is used clinically for the symptomatic treatment of allergic reaction, may produce antimuscarinic adverse effects because of its pronounced antimuscarinic properties (Ambre *et al.*, 1991). Unlike most traditional antihistamines, it has been demonstrated that oxomemazine exhibited the shallow inhibition curve in [^{3}H]QNB binding to the rat cerebrum (Lee *et al.*, 1990) and showed approximately 10- and 20-fold high affinity for cerebral muscarinic receptors, in comparison to those on the ileum(M_3) and the heart(M_2), respectively (Lee and Kim, 1994). However, these reports have not provided a direct evidence for the M_1 selectivity of oxomemazine in the rat cerebrum which contains a mixed population of M_1 and M_3 receptors (Doods *et al.*, 1987).

To evaluate the selectivity of oxomemazine for M_1 receptors in the rat cerebrum we further investigated the binding characteristics of oxomemazine to M_1 and M_3 receptors for pirenzepine and those of pirenzepine to high and low affinity sites for oxomemazine by a receptor binding technique.

MATERIALS AND METHODS

Materials

(−)-[^{3}H]QNB (43.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Atropine sulfate, pirenzepine dihydrochloride, 2,5-diphenyloxazole(POP), 1,4-bis[2-(5-phenyloxazolyl)]benzene(POPOP) and tris-(hydroxymethyl)aminomethane(Tris) were from Sigma Chemical Co. (St. Louis, MO). Oxomemazine hydrochloride was a generous gift from Dr. K. W. Ha (National Institute of Safety Research, Korea). All other chemicals were of reagent grade.

Preparation of Cerebral Microsome

Male Sprague-Dawley rats (180~220 g, Life Science) were killed by decapitation. Brains were rapidly removed and placed in ice-cold 10 mM Tris·Cl (pH 7.4). Cerebrums were dissected, weighed, minced, and homogenized two times(seperated by 30 sec intervals) on ice with a Ultra Turrax T25 homogenizer (IKA Co.) for 15 sec at a setting of 13,500 rpm in 30 volumes of iced 10mM Tris·Cl buffer (pH 7.4). The homogenates were centrifuged at 1,100×g for 5min to remove unhomogenized particles. The resultant supernatant was further centrifuged at 7,700×g for 20 min to pellet the mitochondrial fraction, and the supernatant from the mitochondrial pellet was then centrifuged again at 55,000×g for 30 min. The final microsomal pellet was resuspended in a small volume of 10 mM Tris·Cl using 5 strokes of a hand driven glass-teflon pestle to give the protein concentrations of 2~3 mg per ml and either used immediately or stored in 0.5 ml aliquots at −70℃ until use in the binding assay. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

[^{3}H]QNB Binding Assays

[^{3}H]QNB binding to cerebral microsomes was carried out by a filtration method. For saturation experiments microsomal protein (50 μg) was incubated in a final volume of 1 ml containing 50 mM Tris·Cl(pH 7.4), 10 mM $MgCl_2$ and increasing concentrations (25~800 pM) of [^{3}H]QNB for 150 min at 37℃. Nonspecific binding was defined by the addition of 10 μM atropine to the incubation medium. Each binding reaction was terminated by the addition of 5 ml of ice-cold assay buffer (50 mM Tris·Cl and 10 mM $MgCl_2$, pH 7.4) and then immediate filtration onto a Whatman GF/B (2.5 cm) glass fiber filter under a vacuum. The filter was rinsed four times with 5 ml of ice-cold buffer, dried for 3 hr at room temperature and placed in plastic scintillation vials with 8 ml of scintillation cocktail (PPO: 6 g, POPOP: 0.225 g, Triton X-100: 500 g, toluene: 1 *l*). Radioactivity was determined at least 12 hr later in a Packard liquid scintillation counter. Specific [^{3}H]QNB binding to the muscarinic receptors was calculated as the difference between [^{3}H]QNB binidngs in the absence(total binding) and the presence(nonspecific binding) of 10 μM atropine. Nonspecific binding was less than 10% of total binding.

For competition experiments, microsomes were incubated in triplicate with 300 pM [^{3}H]QNB in the absence and in the presence of varying concentrations of unlabeled pirenzepine, oxomemazine or atropine. Incubation conditions were similar to those described above.

Data Analysis

Saturation binding data were transformed using the method of Scatchard (1949) and the K_D and B_{max} values were estimated by unweighted linear regression analysis of the transformed data. Competition binding curves were analysed by linear regression analysis of log-logit plots for determination of IC_{50} values and Hill coefficients. The Ki values for unlabeled drugs were calculated from IC_{50} values, using the method of Cheng and Prusoff (1973).

The radioligand binding(B) for the muscarinic receptor subtypes in the presence of a given concentration (I) of pirenzepine or oxomemazine is related to those (B_O) in the absence of these competing drugs by the following equation:

$$B = B_O/(1 + I/IC_{50})$$

where IC_{50} is the pirenzepine or oxomemazine concentration that inhibits 50% of [^{3}H]QNB binding at each subtype. Binding characteristics of oxomemazine for M_3 receptors were evaluated by the analysis of oxomemazine/[^{3}H]QNB competition binding curve in the presence of pirenzepine occupying about 96% of M_1 receptors. Binding characteristics of oxomemazine for M_1 receptors were then calculated by substracting the competition binding curves for M_3 from the total curves (oxomemazine/[^{3}H]QNB competition curve in the absence of pirenzepine). Competition curves were also analysed by a nonlinear least squares curve-fitting program LIGAND (Munson and Rodbard, 1980). The statistical difference between one- and two-site models was assessed with the partial F test implemented in the LIGAND program.

Statistics

Data were expressed as mean value± standard error (S.E.M.) of the indicated number of experiments and analysed using a two-tailed student's *t* test for determining the statistical significance of the differences of means. The level of significance was accepted at $p< 0.05$.

RESULTS

[^{3}H]QNB Binding to Cerebral Homogenates and Microsomes

In initial experiments, the specific binding of 300 pM [^{3}H]QNB to homogenates and microsomes prepared from rat cerebrum was maximal by 60min at 37℃ without significant decrease up to 150 min and was linear with protein concentrations in the ranges of 25~100 μg (data not shown). All subsequent binding assays were therefore performed for 150 min at 37℃ with 50 μg of protein.

The relative enrichment of muscarinic receptors in the microsomal fractions was assayed using [^{3}H]QNB equilibrium binding technique. As shown in Table I, [^{3}H]QNB binding to homogenates and microsomes in the presence of 300 pM [^{3}H]QNB was appox. 800 and 3000 fmol per mg of protein, respectively, indicating a 3.8-fold enrichment of muscarinic receptors in the microsomes compared to the homogenates. In addition to this high specific binding of [^{3}H]QNB, nonspecific binding to microsomes (50 μg) was less than 5% of total binding. Thus, microsomes prepared from rat cerebrum in this study were considered a proper preparation for muscarinic receptor binding assays.

Table I. [^{3}H]QNB binding to homogenate and microsome prepared from rat cerebrum

	[^{3}H]QNB binding(fmol/mg)
Homogenate	780.3± 37.6
Microsome	2946.6± 171.7
Purification	3.8± 0.24

Homogenates (50 μg) or microsomes (50 μg) were added to the tube containing 50 mM Tris·Cl (pH 7.4), 10 mM $MgCl_2$, and 300 pM [^{3}H]QNB with or without 10 μM atropine in a final volume of 1ml and the tubes were incubated at 37℃ for 150 min. The suspension was then filtered through glass-fiber filter (Whatman GF/B). Specific [^{3}H]QNB binding was calculated by subtracting the nonspecific binding measured in the presence of 10 μM atropine from the total binding measured in the absence of atropine. Values represent the mean± S.E.M. of four separate preparations.

Binding Characteristics of [^{3}H]QNB in the Rat Cerebrum

The [^{3}H]QNB binding was studied by incubating cerebral microsomes with various concentrations of [^{3}H]QNB to determine the dissociation constant (K_D) and the number of binding site (B_{max}) of [^{3}H]QNB for muscarinic receptors. The specific [^{3}H]QNB binding was saturable with respect to the [^{3}H]QNB concentration (Fig. 1). Scatchard analysis of these saturation data gave linear plots (Fig. 1, inset) and the Hill coefficient was close to unity (nH=1.01), which indicated that rat cerebral microsomes contained a single population of high affinity binding site for [^{3}H]QNB. The equilibrium K_D value for [^{3}H]QNB binding determined from nine individual experiments was 63.8± 3.4 pM and the B_{max} was 4282.4± 100.3 fmol/mg of protein.

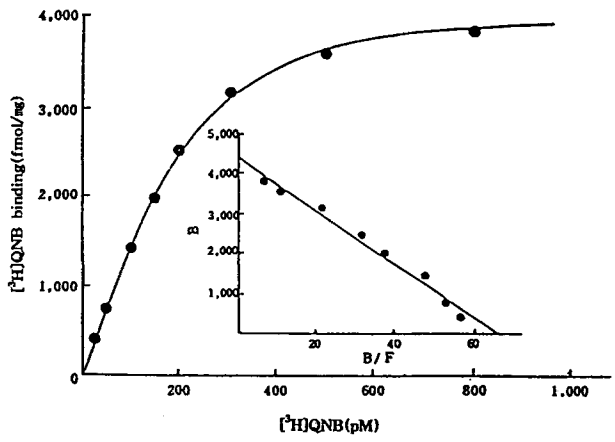

Fig. 1. A typical saturation experiment with [^{3}H]QNB in rat cerebral microsome. Microsomal protein (50 μg) was incubated with various concentrations of [^{3}H]QNB for 150 min at 37℃ in a final volume of 1 ml. The inset shows a Scatchard plot of specific [^{3}H]QNB binding. Bound [^{3}H]QNB(B) was plotted as a function of bound [^{3}H]QNB(B)/free [^{3}H]QNB(F).

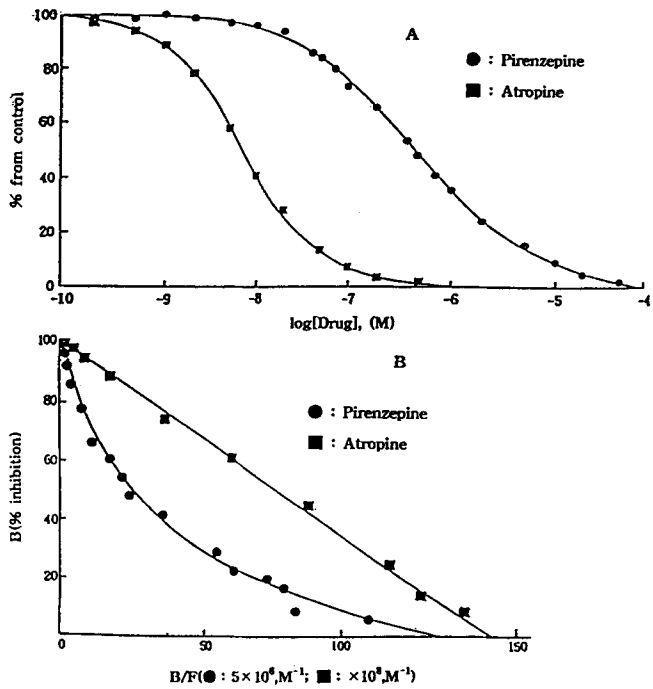

Fig. 2. Inhibition of [^{3}H]QNB binding by atropine and pirenzepine to rat cerebral microsome. The concentration of [^{3}H]QNB was 300 pM. A: Inhibition curve of atropine (■) and pirenzepine (●) with [^{3}H]QNB. B: Hofstee plot of the inhibition data. B represents the percentage inhibition of [^{3}H]QNB binding and F the free pirenzepine or atropine concentration. Each point represents the mean of four separate determinations.

Table II. K_i values of atropine and pirenzepine for the total receptor population (M_1+M_3), M_1 and M_3 receptors in rat brain microsome

	Atropine	Pirenzepine		
		M_1+M_3	M_1	M_3
nH	0.98± 0.02	0.67± 0.01		
K_i (nM)	1.0 ± 0.07	71.2± 19.3	15.6± 4.5	431 ± 37
%	100	100	61.2± 1.8	38.8± 1.8

The competition binding curves shown in Fig. 2 were analysed according to a two-site model by LIGAND as described by Munson and Rodbard (1980). The M_1 and M_3 represent high and low affinity sites for pirenzepine, respectively. Values are the mean± S.E.M. of four experiments.

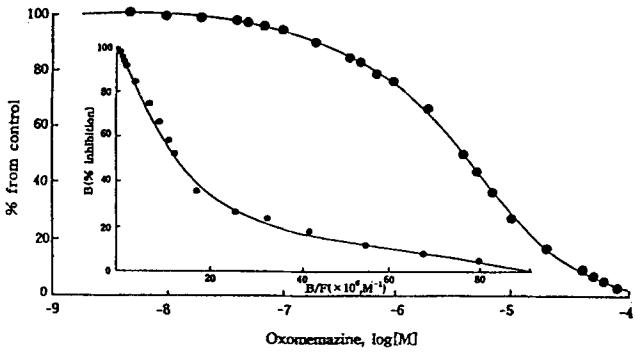

Fig. 3. Inhibition of [^{3}H]QNB binding by oxomemazine to rat cerebral microsome. The concentration of [^{3}H]QNB was 300 pM. Inset: Hofstee plot of the competition binding data. Each point represents the mean of eight separate determinations.

Inhibition of [^{3}H]QNB Binding by Atropine and Pirenzepine

The ability of atropine, a non-selective muscarinic antagonist, and pirenzepine, a selective muscarinic antagonist, to displace the [^{3}H]QNB binding was examined at various drug concentrations. Atropine and pirenzepine inhibited effectively the [^{3}H]QNB binding in a concentration-dependent manner and the complete inhibition was obtained at 1 μM of atropine and 100 μM of pirenzepine (Fig. 2-A). While the inhibition curve of atropine vs [^{3}H]QNB was steep, yielding a Hill coefficient of nearly one (0.98), the inhibition curve of [^{3}H]QNB binding by pirenzepine was shallow and yielded a Hill coefficient of 0.67 (Table II). The Hofstee plot of the pirenzepine inhibition data was also not linear (Fig. 2-B), indicating the presence of multiple muscarinic receptor subclasses with different affinity for pirenzepine in the rat cerebrum.

The atropine and pirenzepine inhibition curves were analysed by using a LIGAND program of Munson and Rodbard (1980). The pirenzepine inhibition data were fitted better to a two-site model than to a one-site model, whereas the atropine inhibition data were fitted to a one-site model. The Ki values of pirenzepine for the high affinity (M_1) and low affinity (M_3) sites, estimated from their IC_{50} values (88.7 nM for M_1 and 2.46 μM for M_3), were 15.6 and 431nM, respectively, with corresponding relative receptor densities of 61.2 and 38.8% (Table II). The Ki value of atropine for a single homogeneous population of binding sites was 1 nM (Table II).

Inhibition of [^{3}H]QNB Binding by Oxomemazine

The studies on the inhibition of [^{3}H]QNB binding by oxomemazine were conducted to evaluate whether this drug could discriminate M_1 and M_3 muscarinic receptors which coexist in our cerebral microsomes. Oxomemazine inhibited the [^{3}H]QNB binding to muscarinic receptors in a concentration-dependent manner, showing 100% inhibition at a concentration of 100 μM (Fig. 3). The resulting inhibition curve was shallow with a Hill coefficient of 0.81 (Table III) which was significantly less than unity. The Hofstee plot of the inhibition data was not linear (Fig. 3, inset). Furthermore, nonlinear least-square regression analysis of these inhibition data was significantly better fitted by

Table III. K_i values of oxomemazine for the total receptor population (O_H+O_L), O_H and O_L receptors in rat cerebral microsome

	O_H+O_L	O_H	O_L
nH	0.81± 0.01		
K_i (μM)	0.49± 0.03	0.08± 0.01	1.65± 0.28
%	100	38.6 ± 2.03	61.4 ± 2.03

The competition binding curve shown in Fig. 3 was analysed according to a two-site model. The O_H and O_L represent high and low affinity sites for oxomemazine, respectively. Values are the mean± S.E.M. of eight experiments.

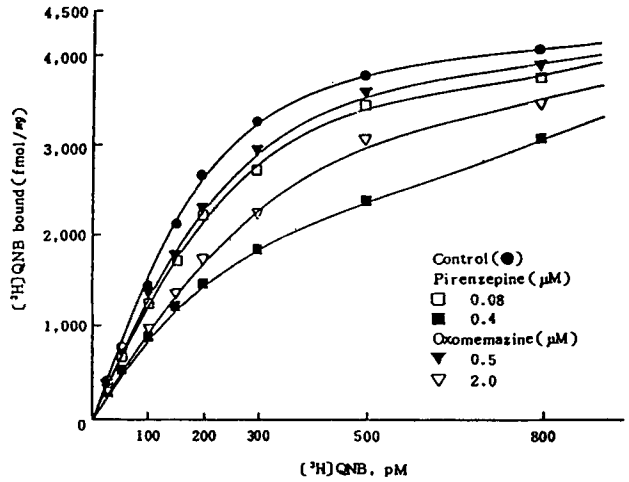

Fig. 4. Effect of pirenzepine and oxomemazine on the saturation isotherms of [^{3}H]QNB binding to rat cerebral microsome. Cerebral microsomes (0.05 mg protein) were incubated with increasing concentration of [^{3}H]QNB in the absence of unlabeled drug (●) or presence of pirenzepine (□: 0.08 μM, ■: 0.4 μM) or oxomemazine (▼: 0.5 μM, ▽: 2 μM). Nonspecific binding was defined in the presence of 10 μM atropine. The data shown are the result obtained from three independent experiments.

Table IV. Effect of pirenzepine and oxomemazine on the binding parameters of [^{3}H]QNB to rat cerebral microsome

	K_D (pM)	B_{max} (fmol/mg protein)	nH
Control	60.3± 4.7	4486.3± 52.7	1.00± 0.003
Pirenzepine (nM)			
80	99.2± 10.5*	4384.9± 316.3	1.03± 0.03
400	175 ± 10.0*	4128.3± 123.5	1.06± 0.04
Oxomemazine (μM)			
0.5	91.8± 6.5*	4597.1± 299.5	1.00± 0.01
2.0	194.0± 8.6*	4412.3± 258.8	1.06± 0.04

Legends are the same as described in Fig. 4.
Values are the mean± S.E.M. of three independent experiments.
*: Significantly different from corresponding value of control ($P<0.01$).

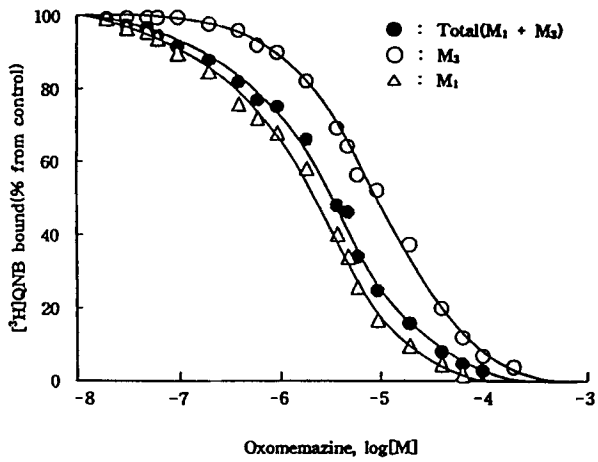

Fig. 5. Oxomemazine/[^{3}H]QNB competition binding to the total population (M_1+M_3, ●), M_3 (○) and M_1 (△) receptors. Competition binding for M_3 was performed in the presence of 2 μM pirenzepine. The competition binding data for M_1 were calculated by subtracting the curve for M_3 from the total curves. Each point represents the mean of four independent experiments.

a two-site model, supporting the presence of both high (O_H) and low (O_L) affinity sites for oxomemazine. The O_H and O_L sites comprised 38.6 and 61.4% of the total muscarinic receptor population, respectively, with corresponding K_i values of 0.08 and 1.65 μM (Table III). Thus, oxomemazine recognized the different muscarinic receptor subtypes in the rat cerebrum.

Effects of Pirenzepine and Oxomemazine on the [^{3}H]QNB Binding Parameters

In order to further assess the nature of interaction of pirenzepine and oxomemazine with [^{3}H]QNB binding sites, effects of pirenzepine and oxomemazine on the saturation isotherms of [^{3}H]QNB binding were investigated (Fig. 4). In the presence of pirenzepine of 80 and 400 nM there was a stepwise increase in the equilibrium dissociation constant of [^{3}H]QNB to 100 pM and 175 pM, respectively, with no changes in receptor density and Hill coefficient for [^{3}H]QNB binding (Table IV). Similar results were obtained with oxomemazine of 0.5 and 2.0 μM: oxomemazine caused an increase in the K_D value to 1.5- and 3-fold without a significant change in the number of binding sites (Table IV), suggesting that the binding of oxomemazine and [^{3}H]QNB to muscarinic receptors is of a competitive nature.

Dissociation Constant of Oxomemazine for M_1 and M_3 Receptors

The binding characteristics of oxomemazine for M_1 and M_3 receptors were investigated to assess whether this drug exhibits the same heterogeneity in rat cereb-

Table V. Apparent K_i values of oxomemazine for the total receptor population (M_1+M_3), M_1 and M_3 receptors

		M_1+M_3	M_3	M_1
nH		0.82± 0.01	0.99± 0.02	0.83± 0.02
K_i (μM)		0.46± 0.04	2.07± 0.18	0.22± 0.04
Subclassification by affinity of oxomemazine				
High	%	38.5 ± 2.7		69.3 ± 2.9
(O_H)	K_i (μM)	0.08± 0.01		0.11± 0.01
Low	%	61.5± 2.7		30.7 ± 2.9
(O_L)	K_i (μM)	1.46± 0.24		1.83± 0.20

The competition binding curves shown in Fig. 5 for M_1+M_3, M_1, and M_3 were analysed according to a two-site model. Values are mean± S.E.M. of four separate determinations.

Table VI. Apparent K_i values of pirenzepine to the total receptor population (O_H+O_L), O_H and O_L receptors

		O_H+O_L	O_L	O_H
nH		0.69± 0.02	0.59± 0.02	1.04± 0.12
K_i (nM)		49.1 ± 7.0	160.3 ± 20.6	19.1 ± 3.7
Subclassification by affinity of pirenzepine				
High	%	57.9± 4.3	29.1± 3.9	
(M_1)	K_i (nM)	13.7± 2.4	12.1± 3.1	
Low	%	42.1± 4.3	70.9± 3.9	
(M_2)	K_i (nM)	380 ± 30	440 ± 40	

The competition binding curves shown in Fig. 6 for O_H+O_L, O_H, and O_L were analysed accoding to a two-site model. Values are mean± S.E.M. of three separate determinations.

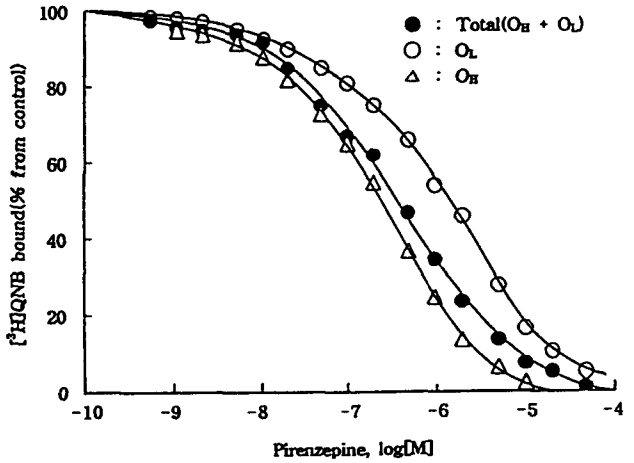

Fig. 6. Pirenzepine/[^{3}H]QNB competition binding to the total population (O_H+O_L, ●), the O_L sites (○) and the O_H sites (△). Competition binding for the O_L sites was performed in the presence of 8 μM oxomemazine. The competition binding data for O_H sites were calculated by subtracting the curve for O_L sites from the total curves. Each point represents the mean of three independent experiments.

rum as that of pirenzepine. As described in Materials and Methods, it could be calculated that 2 μM pirenzepine inhibits 96% of [^{3}H]QNB binding to M_1 receptors and that 90% of the [^{3}H]QNB binding sites are of M_3 receptors when the binding assay was carried out in the presence of 2 μM pirenzepine. Therefore, the competition binding experiments of oxomemazine with [^{3}H]QNB were performed in the presence of 2 μM pirenzepine to evaluate the binding characteristics of oxomemazine for M_3 receptors.

As shown in Fig. 5, the competition binding curve of oxomemazine with [^{3}H]QNB for M_3 receptors was steep with a Hill coefficient of 0.99 but that for M_1 receptors, which was obtained by subtracting the binding curve for M_3 receptors from the total curve, was shallow with a Hill coefficient significantly less than unity. Computer-assisted analysis of these curves demonstrated that M_3 receptors were composed of a uniform population of O_L receptors with a K_i value of 2.07 μM, whereas M_1 receptors comprised 69% of O_H receptors with a K_i value of 0.11 μM and 31% of O_L receptors (K_i=1.83 μM)(Table V).

Dissociation Constant of Pirenzepine for O_H and O_L Receptors

To evaluate the binding characteristics of pirenzepine for O_H and O_L receptors, the competition binding assays of pirenzepine/[^{3}H]QNB were carried out in the presence of 8 μM oxomemazine. When this concentration of oxomemazine is included in the [^{3}H]QNB binding medium, 92% of [^{3}H]QNB binds to O_L receptors. Therefore, the competition binding experiments performed under this condition allow a good evaluation of the binding natures of pirenzepine for O_L receptors. As shown in Fig. 6, the pirenzepine/[^{3}H]QNB competition curve was shallow for O_L receptors but was steep for O_H receptors. Hill coefficient estimated by the Hill plot of competition curve for O_L and O_H receptors was 0.59, which is far less than unity, and 1.04, respectively. Analysis of these curves revealed that whereas the competition binding data for O_H receptors obeyed the law of mass action with a K_i value of 19.1 nM (Table VI), those data for O_L receptors were fitted better to a two-site than a one-site model, indicating that O_L receptors were composed of high (29.1%) and low (70.9%) affinity receptors for pirenzepine with a K_i value of 12.1 and 440 nM, respectively (Table VI).

DISCUSSION

The present results obtained from studies of the nature of interaction of pirenzepine and oxomemazine with muscarinic receptors demonstrate that oxomemazine like pirenzepine is selective for M_1 receptors and the rat cerebral microsomes prepared in this study co-

ntain three different muscarinic receptor subtypes of M_1, M_3 and the other site.

The specific binding of [^{3}H]QNB to rat cerebral microsomes was saturable, of high affinity and inhibited by muscarinic antagonists such as atropine and pirenzepine, satisfying common criteria applied to receptor identification. The linear Scatchard plot of the [^{3}H]QNB saturation binding data and the corresponding Hill coefficient close to unity indicated strongly the presence of a uniform population of [^{3}H]QNB binding sites in rat cerebral microsomes. Therefore, we used [^{3}H]QNB for labeling all of muscarinic receptors in rat cerebrum.

Muscarinic receptors have been subclassified into neuronal M_1, cardiac M_2 and glandular M_3 receptors on the basis of the discriminative capability of pirenzepine, AF-DX 116 and 4-DAMP at these respective subtypes(Delmendo *et al.*, 1989; Doods *et al.*, 1987; Lazareno and Roberts, 1989). M_1 receptors have high affinity for pirenzepine and 4-DAMP, and intermediate affinity for AF-DX 116, M_2 receptors show high affinity for AF-DX 116, low affinity for pirenzepine and 4-DAMP, and M_3 receptors have high affinity for 4-DAMP, intermediate affinity for pirenzepine and low affinity for AF-DX 116. The inhibition data of [^{3}H]QNB binding by nonselective antagonist atropine and selective antagonist pirenzepine were analyzed to determine the muscarinic receptor subtypes found in rat cerebral microsomes. The Hill coefficient for inhibition of [^{3}H]QNB binding with atropine was close to unity, exhibiting a typical binding pattern of nonselective muscarinic antagonist to muscarinic receptors. However, the pirenzepine/[^{3}H]QNB competition curve was most consistent with two receptor populations with different affinities for pirenzepine. About 60% and 40% of total muscarinic receptors were of the high(M_1) and low affinity sites for pirenzepine with the K_i values of 16 and 430nM, respectively. The K_i value of pirenzepine for M_1 receptor is in good agreement with the published affinity values for pirenzepine at these M_1 sites(Luthin and Wolfe, 1984; El-Fakahany *et al.*, 1986; Michel and Whiting, 1988; Delmendo *et al.*, 1989, Dauphin and Hamel, 1992). As described already, pirenzepine discriminated another population of sites in our cerebral preparations, which were of lower affinity. The affinity of pirenzepine at these low affinity sites was related better to its intermediate affinity at glandular and ileal M_3 than to its low affinity at cardiac M_2 (Michel and Whiting, 1988; Delmendo *et al.*, 1989; Lazareno and Roberts, 1989; Lee and Kim, 1994), indicating that the low affinity sites for pirenzepine in rat cerebral microsomes are of M_3 receptors. Thus, pirenzepine recognized M_1 and M_3 receptors in cerebral microsomes with a 28-fold difference in the affinities for the two sites. These results are further supported by a report, which described that the rat cerebral cortex contains a mixed population of M_1 and M_3 binding sites (Doods *et al.*, 1987).

Oxomemazine also inhibited [^{3}H]QNB binding to cerebral microsomes in a similar manner to pirenzepine. The Hill coefficient (0.81) deviated from the law of mass action and the inhibition data exhibited a significantly better fit to a two-site model, revealing that 40% of total receptors are of high affinity (O_H) sites (K_i=84 nM) and the remaining 60% of low affinity (O_L) sites (K_i=1650 nM). If receptor selectivity is defined as K_D at one receptor subtype$\leq$0.1 K_D at the other receptor subtypes, this 20-fold difference between the K_i values for the O_H and O_L sites indicates that oxomemazine is selective for either M_1 or M_3 receptors. The inverse relationship in the relative densities of M_1 and M_3 receptors (60 : 40) compared with the O_H and O_L sites (40 : 60) may reflect that the O_H sites correspond to M_3 receptors. However, in our previous study (Lee and Kim, 1994) we demonstrated that oxomemazine bound to ventricular (M_2) and ileal muscarinic (M_3) receptors with a low affinity, suggesting that oxomemazine is selective for M_1 receptors.

To evaluate the underlying mechanism of the differences in proportions of M_1 and O_H receptors to the total receptors, oxomemazine binding properties to M_1 and M_3 receptors were investigated in the same cerebral microsomes. The binding profiles of oxomemazine for M_3 receptors were approximated by oxomemazine vs [^{3}H]QNB competition binding studies in the presence of 2 μM pirenzepine. Under these condition, 90% of [^{3}H]QNB binding sites are of M_3 receptors. In this study the oxomemazine vs [^{3}H]QNB competition binding curve for M_3 receptor was compatible with an interaction at one binding site with the K_i value of about 2000 nM. In contrast, the oxomemazine competition curve for M_1 receptors did not obey the law of mass action, as indicated by a Hill coefficient (0.83) significantly differing from unity. Analysis of this curve revealed that 70% of M_1 receptors were of high affinity (O_H) sites ($K_i \simeq$ 110 nM) and the remain 30% of low affinity(O_L) sites ($K_i \simeq$ 1800 nM). These results suggest that M_3 receptors are only composed of a uniform population of the low affinity (O_L) sites for oxomemazine, but M_1 receptors comprise the high(O_H) and low (O_L) affinity sites for this drug. The presence of O_L sites in M_1 receptors was further evidenced by pirenzepine vs [^{3}H]QNB competition binding studies performed in the presence of 8 μM oxomemazine. The pirenzepine competition curve for the O_L sites was best explained by a two-site model with 30% and 70% of high(M_1) and low(M_3) affinity sites, respectively, with corresponding K_i values of 12 and 440 nM. These K_i values of pirenzepine for M_1 and M_3 receptors were consistent with the respective Ki values estimated by

analysis of the pirenzepine vs [^{3}H]QNB competition data for the total receptors performed in the absence of 8 μM oxomemazine. However, the pirenzepine competition curve for the O_H sites indicated that the O_H sites were composed of a single population of the high affinity (M_1) sites for pirenzepine. Thus, our competition binding studies under M_1 or O_H suppressive conditions suggest that the rat cerebral microsomes prepared in this study would contain three populations of muscarinic receptor subtypes, a high affinity (M_1) sites for both pirenzepine and oxomemazine, a low affinity (M_3) sites for both drugs and an another sites exhibiting high affinity for pirenzepine and low affinity for oxomemazine with respective relative proportions of 40 : 40 : 20.

Because M_2 receptors were not detected in our preparations, it would appear that another muscarinic receptor subtype, which is still not characterized well, would be present. Molecular cloning of muscarinic receptors in mammalian and human tissues has demonstrated the presence of five different gene products (m_1~m_5) (Bonner *et al.*, 1987; 1988; Peralta *et al.*, 1987). Three main pharmacological subtypes of M_1, M_2 and M_3 most likely correspond to m_1, m_2 and m_3 gene products, respectively (Bonner, 1989). In addition to these three subtypes, the pharmacological profiles of the five cloned muscarinic receptor subtypes expressed in Chinese hamster ovary cells were reported (Buckley *et al.*, 1989; Dörje *et al.*, 1991; Wess *et al.*, 1991; Bolden *et al.*, 1991). These five subtypes could be divided into two groups of the high (M_1, M_4) and low (M_2, M_3, M_5) affinity subtypes by their affinity for pirenzepine (Dörje *et al.*, 1991; Bolden *et al.*, 1992). Based on its high affinity for pirenzepine and the expression of m_4 gene in rat brain (Buckley *et al.*, 1988), a small proportion (20%) of M_4 receptors seems to be present in our rat cerebral microsomes. To further characterize this subtype, however, the introduction of new selective antagonists for the M_4 and M_5 subtypes of muscarinic receptors is necessary.

ACKNOWLEDGMENT

This work was supported by the research grant (1993) from the Ministry of Health and Social Affairs, Korea. The authors thank Joo-Yeun Kim for artistic work and Jae-Seok Lee for preparation of manuscript.

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