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Interaction of Antihistaminics with Muscarinic Receptor (III)
—Relationship between binding and functional *in vitro* data—

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Abstract □ The muscarinic antagonist 1-[benzilic 4,4'-^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) bound to a single class of muscarinic receptors with high affinity in rabbit ileal membranes. The K_D and B_{max} values for [^{3}H]QNB calculated from analysis of saturation isotherms were 52.5 pM and 154 fmol/mg, respectively. Chlorpheniramine (CHP), histamine H_1 blocker, increased K_D value for [^{3}H]QNB without affecting the binding site concentrations and Hill coefficient. The K_i value of CHP for inhibition of [^{3}H]QNB binding in ileal membranes was 1.44 μM and the pseudo-Hill coefficient for CHP was close to unit. In the functional assay carbachol, muscarinic agonist, increased the contractile force of ileum with ED_{50} value of 0.11 μM. CHP caused the rightward shift of the dose-response curve to carbachol. The pA_2 value of CHP determined from Schild analysis of carbachol-induced contraction was 5.77 and the slope was unity indicating competitive antagonism with carbachol. The dissociation constant (K_i) of CHP obtained in competitive experiments with [^{3}H]QNB was similar to the K_A value (1.69 μM) of CHP as inhibitor of carbachol-induced contraction in rabbit ileum. This result suggests that the binding of H_1 blocker, CHP, vs [^{3}H]QNB to muscarinic receptors in ileal membranes represents an interaction with a receptor of physiological relevance.

Keywords □ [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB), muscarinic receptor, antihistaminics, chlorpheniramine (CHP), ileum.

Although H_1-antihistaminics, H_1-receptor blockers, have widely used in the treatment of several allergic symptoms[1-3], most of these drugs have other neurotransmitter receptor-blocking action[3-6]. The antimuscarinic properties of H_1-blockers are related to certain adverse effects such as blurred vision, dry mouth, constipation and urinary retention in patients [7,8]. Recently, we reported to certain adverse effects such as blurred vision, dry mouth, constipation and urinary retention in patients[7,8]. Recently, we reported that the affinity of H_1-blockers for muscarinic receptors estimated from the radioligand binding studies using [^{3}H]QNB as a radioligand in cardiac sarcolemma[9] and brain microsomes[10] varies over a wide range. These informations on the muscarinic receptor-blocking potency of H_1-blockers may be helpful in the selection of a H_1-blocker in order to minimize or avoid antimuscarinic side effects.

The radioligand binding techniques are extremely useful in identification of a specific receptor and characterization of its interaction with drugs[11,12]. However, to apply the binding data it should be proved by the relationship between data from binding assays and pharmacological or biochemical studies that a drug binding represents an interaction with a receptor of physiological relevance[13,14].

In this study, the effects of chlorpheniramine, H_1-receptor blocker, on the [^{3}H]QNB binding to muscarinic receptors in homogenates prepared from rabbit ileum were compared to those of this drug on the carbachol-induced contractions of isolated rabbit ileum to investigate whether the binding data correlate with functional *in vitro* data.

EXPERIMENTAL METHODS

Materials

Atropine sulfate, carbamylcholine chloride (car-

bachol) and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Company. (−)-[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, 41.6 Ci/mmol) was obtained from Amersham International. Chlorpheniramine maleate was a gift from Dr. Kwang-Won Ha (National Institute of Safety Research, Korea). All other reagents were of reagent grade.

Tissue preparation

Albino rabbits of either sex weighing 1.5 to 2.2 kg were sacrificed by a blow to the head and the ileum was rapidly removed and dissected in ice-cold 10 mM Tris·Cl (pH 7.4). Tissues were minced with scissors and homogenized in 10 vol. of 10 mM Tris·Cl buffer at 4℃ for 4×15 sec periods with 30 sec cooling between each burst. Homogenates were centrifuged at 3,600×g for 10 min at 4℃ and the pellet was discarded. The supernatant was centrifuged at 45,000×g for 20 min. The resulting pellet was resuspended in an appropriate volume of 10 mM Tris·Cl (pH 7.4), using a hand driven glass-teflon homogenizer to give a final protein concentration of 10 to 15 mg/m*l*. Samples were either used immediately or stored in small aliquots at −70℃ until used in the binding assays. Protein concentrations were determined by the method of Lowry *et al.*[15] using bovine serum albumin as the standard.

[^{3}H]QNB binding assays

Binding of [^{3}H]QNB was determined by a filtration assay. Homogenates (500 μg of protein) were incubated with [^{3}H]QNB in the incubation medium (Tris·Cl, 50 mM and $MgCl_2$, 10 mM; pH 7.4) at 37℃ in a final volume of 5 m*l*. The saturation binding curve was determined by incubating the homogenate with increasing concentrations of [^{3}H]QNB (10 to 500 pM) for 150 min. In competition binding experiments, homogenates were incubated with 100 pM of [^{3}H]QNB in the incubation medium with various concentrations of chlorpheniramine as a competing agents. Nonspecific binding was measured in the presence of 10^{-5} M atropine. Specific binding was defined as the total binding minus the nonspecific binding. Binding reactions were terminated by filtering the incubation media under vacuum on Whatman GF/B glass fiber filter. The tubes were rinsed twice with 5 m*l* of ice-cold buffer (Tris·Cl, 50 mM and $MgCl_2$, 10 mM, pH 7.4). Filters were then rapidly washed twice the 5 m*l* of buffer and subsequently transferred to scientillation vials. After the filters were dried for at least 2 hrs, 8 m*l* of scintillation cocktail (PPO; 6g, POPOP; 0.225 g, Triton X-100; 500 g, Toluene; 1 *l*) were added and the radioactivity trapped on the filters was counted by a Packard scientillation counter with an efficiency of 45 percent. All measurements were made in duplicate at least three independent experiments.

Functional studies

Segments of the rabbit ileum (about 2 cm long) were removed and suspended in a 30 m*l* organ bath containing Tyrode's solution (mM: NaCl 137, KCl 2.86, $CaCl_2$ 1.84, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.0, and pH 7.4) at 37℃ and continuously gassed with 95% O_2 and 5% CO_2 mixture. Contractions were recorded at 1 g of tension with a Grass FT 03 force transducer. The ileal strips were allowed to stabilize for 1 hr and exposed to carbachol (10^{-7} M) with an interval of 20 min, until a reproducible contractile response was observed. A variation of less than 10% between three subsequent carbachol-induced contractions was accepted as reproducible. In each experiment two preparations were used in parallel. Two control dose-response curves to carbachol were obtained on each by use of cumulative dosing and then exposed to chlorpheniramine for 20 min. Next two dose-response curves were then obtained in the presence of chlorpheniramine. Contractile response to a given concentration of carbachol was expressed as a percentage of the maximum response to carbachol and plotted against the concentration of carbachol to determine the EC_{50} value.

Data analysis

The K_D value and concentration of binding sites (B_{max}) were determined by Scatchard analysis[16] of the saturation data. The Hill coefficient (nH) was determined using equation:

$$\log[Y/(1-Y)] = nH \cdot \log[F] - \log[K_D]$$

where Y is the B/B_{max} and F is concentration of free (unbound) [^{3}H]QNB. The Ki value for chlorpheniramine was calculated according to the method of Cheng and Prusoff[17] from the IC_{50} value, the concentration of chlorpheniramine needed to inhibit 50% of the [^{3}H]QNB specific binding.

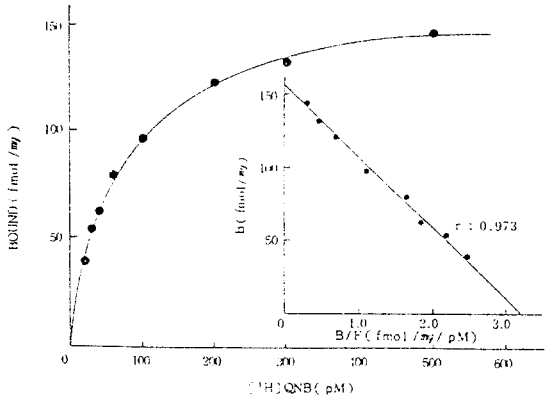

Fig. 1. Saturation isotherms of [³H]QNB binding in rabbit ileal membranes.
Protein (0.5 mg) was incubated with various concentrations of [³H]QNB for 150 min at 37℃ in a final volume of 5 ml. Other assay conditions were as described under "Methods". The inset shows a Scatchard plot of specific [³H]QNB binding. Bound [³H]QNB(B) was plotted as a function of bound [³H]QNB(B)/free [³H]QNB (F). Each ponit represents the mean of five separate determinations.

The Hill coefficient (nH) of chlorophenir-amine was determined from the corresponding Hill plot using equation:

$$\log[I/(100-I)]=nH\cdot\log[D]-\log[IC_{50}]$$

where I is the percentage inhibition of [³H]QNB binding and D is the concentration of chlorphenir-amine.

The pA_2 value (-log K_A) of chlorpheniramine on the ileum was estimated as described by Arunlak-shana and Schild[18]. Dose ratios (ratio of the ED_{50} values of carbachol in the presence and absence of chlorpheniramine) were calculated for each chlor-pheniramine concentration. K_A value was obtained from the relationship, K_A=[chlorpheniramine]/(dose ratio −1). The logarithm of (dose ratio −1) was plotted against the negative logarithm of the molar concentration of chlorpheniramine and the pA_2 value was then determined from X-intercept of the linear regression line. Data were expressed as means± standard error of the mean and analysed by student's *t* test at a 5% significance level.

Table I. The binding parameters of [³H]QNB to rabbit ileum

	K_D(pM)	B_{max}(fmol/mg)	nH
Control	2.48± 5.63	153.88± 18.47	1.02± 0.05
Chlorpheniramine (5 μM)	217.23± 4.90*	170.11± 12.03	1.05± 0.03

K_D and B_{max} were calculated from Scatchard analysis. Hill coefficient (nH) was calculated from Hill plot. Values are the mean± S.E.M of five independent experiments.
*Significantly different from corresponding values of control ($p<0.01$).

RESULTS

Binding of [³H]QNB to homogenates of the rabbit ileum

The specific binding of 100 pM [³H]QNB to homogenates reached equilibrium by 90 min at 37℃ without significant decrease up to 180 min and was linear with tissue concentrations in the ranges 0.05-1.0 mg of protein (data no shown). All subsequent binding assays were therefore performed for 150 min at 37℃ with 0.5 mg of protein.

The saturability of specific [³H]QNB binding in ileal homogenates was examined as a function of the added [³H]QNB concentration (Fig. 1). The specific [³H]QNB binding was saturated with increasing concentration of [³H]QNB, showing a rectangular hyperbola. When these saturation data were replotted as a straight line according to the method of Scatchard (Fig. 1, inset), correlation coefficient for this straight that [³H]QNB bound to a single population of sites. The apparent K_D value and B_{max} for [³H]QNB binding were about 53 pM and 154 fmol per mg of protein, respectively (Table I). Hill plot of the data in Fig. 1 was also linear with a Hill coefficient of 1.02, again indicating single class of high affinity [³H]QNB binding sites in ileal homogenates.

Inhibition of specific [³H]QNB binding by chlorphenir-amine

The effects of chlorpheniramine on [³H]QNB binding to rabbit ileal homogenates were examined. As shown in Fig. 2, the inhibition of [³H]QNB binding by chlorpheniramine in the presence of 100

Table II. Inhibition by chlorpheniramine in [^{3}H]QNB binding and in carbachol-induced contraction to rabbit ileum

K_i (μM)[a]	nH[b]	pA_2[c]	K_A (μM)[d]	Schild's slope
1.44± 0.15	0.953± 0.05	5.77± 0.04	1.69± 0.18	0.934± 0.06

[a]Inhibition constant determined from inhibition of [^{3}H]QNB binding in the presence of various chlorpheniramine concentrations.
[b]Hill coefficient (nH) for chlorpheniramine calculated from Hill plot of [^{3}H]QNB/chlorpheniramine competition binding.
[c]$pA_2 = -\log K_A$
[d]K_A is the inhibition constant determined from Schild plot of carbachol-induced smooth muscle contractions in the presence of chlorpheniramine.

pM [^{3}H]QNB occurred in a dose-dependent manner with IC_{50} value of about 5 μM. Hofstee plot of the inhibition data was linear (Fig. 2, inset) and Hill coefficient for the inhibition of [^{3}H]QNB binding was close to one (Table II), which suggest that chlorpheniramine was bound to homogeneous population of sites. In order to assess the nature of interaction of chlorpheniramine with [^{3}H]QNB binding sites in rabbit ileum, its effects on the saturation isotherms of [^{3}H]QNB were investigated in ileal homogenates. Table I shows that chlorpheniramine (5 μM) increased the equilibrium dissociation constant (K_D value) for [^{3}H]QNB binding to about four-fold, with no change in the concentration of binding sites (B_{max}) and Hill coefficient (nH). From the competitive inhibition of [^{3}H]QNB binding by chlorpheniramine for the same set of muscarinic receptors, the IC_{50} value was normalized using equation of Cheng and Prusoff. The Ki value for chlorpheniramine calculated from its IC_{50} value was 1.44 μM (Table I).

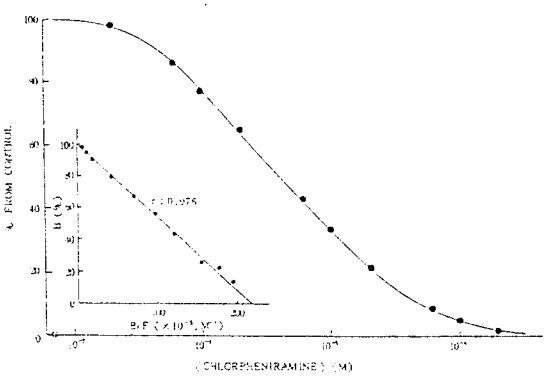

Fig. 2. Inhibition of [^{3}H]QNB binding to muscarinic receptors in rabbit ileum by chlorpheniramine (5 μM).
The concentration of [^{3}H]QNB was 100 pM. Assay conditions were as described under "Methods". Binding is expressed as percentage of maximal. Inset; Hofstee plot of the competition binding data. B represents the percentage inhibition of [^{3}H]QNB binding and F the free chlorpheniramine concentration. Each point represents the mean of three separate determinations.

Inhibition of the carbachol-induced contraction by chlorpheniramine

Dose-response curves to carbachol with the ED_{50} value of 0.11 μM in the isolated rabbit ileum are shown in Fig. 3. Chlorpheniramine inhibited the contractile response induced by carbachol and caused a parallel shift to the right of the dose-response curve to carbachol without any decrease in the maximal response obtainable to carbachol (Fig. 3). The Schild plot of the antagonism between carbachol and chlorpheniramine are illustrated in Fig. 4. The Schild plot was linear and the slope of the regression line was not significantly different from unity indicating competitive antagonism for a uniform population of muscarinic receptors. This linear regression line gave the pA_2 value of 5.77, corresponding to the inhibition constant (K_A) of 1.69 μM for chlorpheniramine (Table II).

DISCUSSION

The present data obtained in the rabbit ileum demonstrate that chlorpheniramine is a competitive antagonist of specific [^{3}H]QNB binding and carbachol-induced contraction, and that its binding potency for muscarinic receptors agrees well with its functional anticholinergic potency.

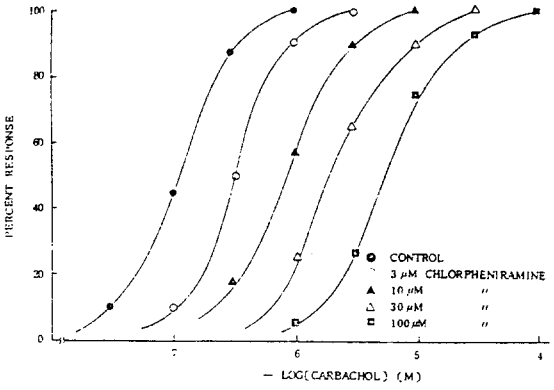

Fig. 3. Dose-response curve of carbachol in the presence of chlorpheniramine to the isolated rabbit ileum. Responses are expressed as a percentage of the maximum contraction elicited by carbachol. Each point is the mean response of three different preparations.

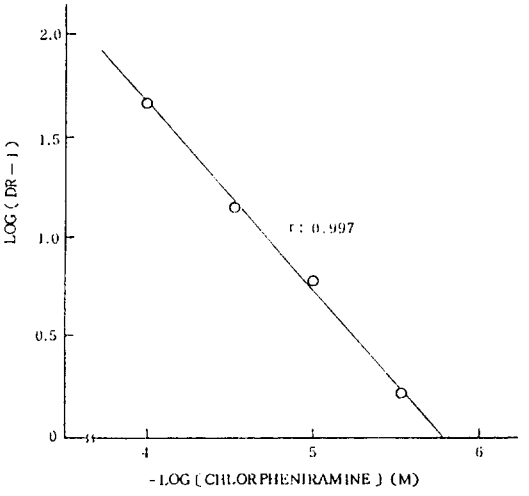

Fig. 4. Schild plot of the antagonism between carbachol and chlorpheniramine to rabbit ileum. Dose ratio ($DR = ED'_{50}/ED_{50}$) was calculated from the dose-response curve to carbachol in the absence and presence of chlorpheniramine. The slope of regression line is not significantly different from unity, indicating competitive antagonism. The intercept on the abscissa equals the pA_2 value. Each point is the mean of three different preparations.

[^{3}H]QNB is a well characterized ligand for muscarinic receptors from a variety of tissues[19-21]. The present results also indicate that [^{3}H]QNB binds to a single population of specific and saturable high affinity muscarinic receptors in rabbit ileum. The [^{3}H]QNB binding characteristics of these receptors are similar to those of the muscarinic receptors in other tissues[20,21]. Therefore, the [^{3}H]QNB binding assay was utilized to investigate how the histamine H_1-blockers interact with muscarinic receptors.

In the present study, the histamine H_1-blocker, chlorpheniramine, fully displaced [^{3}H]QNB binding to the ileal membranes with a Ki value of 1.44 μM. The chlorpheniramine inhibition curve was compatible with interaction at one binding site, as indicated by the linear Hofstee plot and the Hill coefficient which was close to unity. These inhibition data were in agreement with our previous studies in dog heart[9] and rat brain[10]. In addition, a decrease in the apparent affinity of [^{3}H]QNB without influencing the binding site concentration by chlorpheniramine supports further the view that chlorpheniramine interacts in a competitive fashoin with [^{3}H]QNB binding sites in rabbits ileum.

Muscarinic receptors are generally classified into three subtypes, denoted as M_1, M_2 and M_3, by the affinity for selective antagonists. M_1 receptors with a high affinity for pirenzepine are located mainly in neural tissue including cerebral cortex, whereas M_2 receptors with a low affinity for pirenzepine exist in nerves, glands, heart and smooth muscle[22,23]. These peripheral M_2 receptors have been further subdivided into the cardiac M_2 receptors and the ileal M_3 receptors according to their affinity towards AF-DX 116 (11-[[2-[(diethylamino)methyl]1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b] [1,4]-benzodiazepine-6-one)[24], himbacine[25] and 4-DAMP (4-diphenyl-acetoxy-N-methylpiperidine)[26]. Hence, the selectivity of a certain drug for these subtypes can be determined by its affinity for brain, heart and ileum. In the present study, the Ki value for chlorpheniramine in rabbit ileum was similar to previously reported Ki values for chlorpheniramine in brain and heart. It can therfore be concluded that chlorpheniramine is a nonselective antagonist for the muscarinic receptor subtypes.

Although the [^{3}H]QNB binding study gave detailed information about the interaction of chlorpheniramine with the [^{3}H]QNB binding sites, to apply the binding data for characterization of its interaction with muscarinic receptor, it must be proved that chlorpheniramine binds to a functionally

relevant receptor. In our functional studies in rabbit ileum, chlorpheniramine inhibited the carbachol-induces contraction in a competitive manner. The affinity (K_A) estimated from the functional studies was almost identical to the binding affinity (Ki) of chlorpheniramine for muscarinic receptors. These results demonstrate that chlorpheniramine binding occurs at the physiological relevant muscarinic receptor.

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