14. J. Carroll and L. M. Armstrong, *Biochem. Soc. Trans.* **4**, 871 (1976).
15. T. Watabe, A. Hiratsuka and K. Ogura, *Carcinogenesis* **8**, 445 (1987).
16. E. Cavalieri, R. Roth and E. Rogan, in *Polynuclear Aromatic Hydrocarbons* (Eds. P. W. Jones and P. Leber), pp. 517–529. Ann Arbor Science, MI (1979).
17. P. G. Wislocki, K. M. Gadek, M. W. Chou, S. K. Yang and A. Y. H. Lu, *Cancer Res.* **40**, 3661 (1980).
18. T. Watabe, Y. Hakamata, A. Hiratsuka and K. Ogura, *Carcinogenesis* **7**, 207 (1986).
19. T. Watabe, T. Ishizuka, M. Isobe and N. Ozawa, *Science* **215**, 403 (1982).
20. T. Watabe, T. Ishizuka, T. Fujieda, A. Hiratsuka and K. Ogura, *Jpn. J. Cancer Res. (Gann)* **76**, 684 (1985).
21. T. Watabe, A. Hiratsuka, K. Ogura and K. Endoh, *Biochem. biophys. Res. Commun.* **131**, 694 (1985).
22. R. D. Sekura, C. J. Marcus, E. S. Lyon and W. B. Jakoby, *Analyt. Biochem.* **95**, 82 (1979).

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[^{3}H]Quinuclidinyl benzilate binding to the human lung muscarinic receptor*

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The parasympathetic (cholinergic) nervous system is an important regulator of airway tone [1] and plays a major role in the diseases of hyperreactive airways. The human lung receives its parasympathetic innervation from the vagi which supply the bronchial tree to the level of the respiratory bronchioles. Impulses from the postganglionic fibers are transmitted by acetylcholine which stimulates muscarinic receptors located on a variety of target cells [1]. Stimulation of muscarinic receptors has been shown to increase total lung cyclic GMP levels [2], increase the immunologic release of mast cell mediators [2], increase mucus release from cultured human airways [3], and contract human airway smooth muscle [4]. Target cell response begins with the number and affinity of the surface receptors for their neurotransmitter. Therefore, it is important to characterize the human lung muscarinic receptor system in order to understand the role of these receptors in peripheral lung responses and to ascertain whether binding parameters differ among patient populations. Using a computer modeling program which allowed us to combine and analyze radioligand binding data from multiple experiments, we determined that human peripheral lung contains a relatively low concentration of single-site, high-affinity muscarinic receptors.

Materials and methods

Reagents. Bovine serum albumin (BSA†), scopolamine, atropine, oxotremorine, methacholine chloride, carbachol, histamine diphosphate and D,L-isoproterenol were purchased from Sigma, St. Louis, MO; sucrose and magnesium chloride were purchased from the Fisher Scientific Co., Fair Lawn, NJ; Tris was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; Dulbecco's medium was purchased from Hazleton, Denver, PA; quinuclidinyl benzilate (QNB) was a gift from Hoffmann-La Roche, Nutley, NJ; and [^{3}H]QNB (39.4 Ci/mmol) was purchased from New England Nuclear, Boston, MA.

Preparation of human lung tissue. Human lung tissue was obtained at the time of resection for lung cancer. Macroscopically normal areas of peripheral lung tissue were dissected free of pleura, large bronchi (>3–5 mm) and large blood vessels, and were washed in Dulbecco's medium. Tissue was frozen at −70° until use.

Plasma membrane preparation. To determine the optimal lung plasma membrane preparation for the radioligand binding assays, the specific binding of [^{3}H]QNB to five different plasma membrane preparations was compared (Fig. 1). Specific binding was calculated as the difference in binding when 4 nM [^{3}H]QNB was incubated at 37° for 60 min in the absence or presence of 1 μM unlabeled QNB. The lung was minced, placed in ice-cold 10 mM Tris (pH 7.4) containing 0.25 M sucrose and 0.5% BSA (10 ml/g tissue), homogenized, gauze filtered, and centrifuged slowly. The resultant supernatant fraction was tested directly for specific binding or was centrifuged further at either 27,000 *g* for 20 min or 40,000 *g* for 45 min. The pellets and supernatant fractions were then resuspended in the buffer used for the radioligand binding assays (50 mM Tris with 25 mM $MgCl_2$, pH 7.2), and analyzed for specific binding. As shown in Fig. 1, the lowest specific [^{3}H]QNB binding occurred in the supernatant fractions (<7 fmol/mg protein), whereas the highest specific [^{3}H]QNB binding occurred in pellet 3 (27.8 fmol/mg protein). The specific binding measured in pellet 3 was 88% of the total binding. Therefore, all subsequent radioligand binding studies used the plasma membrane isolation procedure outlined for pellet 3 (Fig. 1). The protein content [5] of this plasma membrane preparation was adjusted to 1–3 mg/ml with Tris $MgCl_2$ buffer.

Radioligand binding assays. Radioligand binding assays were done as described previously [6]. Aliquots (100 μl) of the plasma membrane preparations suspended in the Tris $MgCl_2$ buffer were used in the binding assays in a final volume of 160 μl. The assay mixtures were incubated at 37° for 60 min with either increasing concentrations of the radioligand, [^{3}H]QNB, or a fixed concentration of radioligand and different concentrations of various agonists and antagonists. Incubations were terminated by adding 4 ml of ice-cold incubation buffer followed by rapid vacuum filtration through Whatman GF/C Glass Microfibre Filters. The filters were washed immediately thereafter with 20 ml of ice-cold buffer, dried, and then assayed in a liquid scintillation system using a Beckman LS-7000 counter. All samples were run in duplicate to triplicate, and replicates differed from each other by less than 10%.

Modeling and statistical evaluation of receptor binding. The binding data were analyzed using a weighted, nonlinear, least-squares curve fitting as provided in the computer program LIGAND [7]. The binding curves were first re-expressed in terms of bound [^{3}H]QNB concentration versus total concentration added, considering both the labeled and unlabeled ligand. Contrary to common custom for binding studies, nonspecific binding (*N*) was not measured for each individual concentration by use of a 100-fold excess unlabeled ligand concentration. Rather, *N* was modeled directly as an extra, very low-affinity, nonsaturable class of

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† Abbreviations include: BSA, bovine serum albumin; K_d, dissociation constant; *N*, ratio of nonspecifically bound to free ligand; QNB, quinuclidinyl benzilate; *R*, binding capacities; and *RMS*, root-mean square.

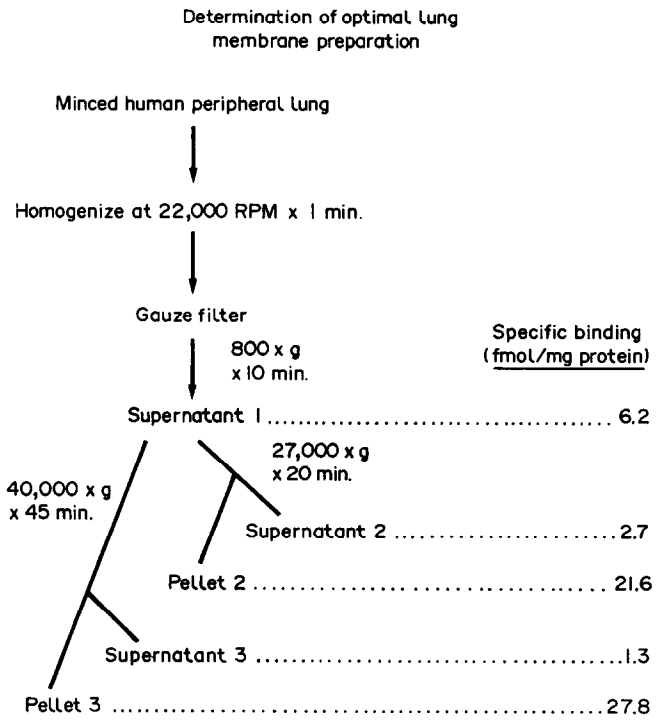

Fig. 1. Specific binding of [^{3}H]QNB to five different human lung plasma membrane preparations. [^{3}H]QNB (4 nM) and the human peripheral lung membrane preparations were incubated in the presence or absence of 1 μM unlabeled QNB for 60 min at 37° and specific binding was compared.

receptors, and expressed as the ratio of nonspecifically bound to free ligand [7]. Equilibrium binding models with one and two classes of specific binding sites were fitted to the data, and the best-fitting model was chosen on the basis of the "extra sum-of-squares" F-test criterion [7]. Finally, values for the dissociation constants (K_d), binding capacities (R), and N for the chosen model were estimated, along with their standard errors. The predicted curve was then plotted superimposed on the data, and the goodness-of-fit was evaluated using a "number-of-runs" tests on the residuals and by evaluation of the root-mean-square (*RMS*) residual error.

Results and discussion

Initial experiments were done to determine when [^{3}H]QNB binding to human peripheral lung tissue reached equilibrium. Four nanomolar [^{3}H]QNB was incubated for 0–120 min at 37° in the presence and absence of 1 μM unlabeled QNB. The binding of [^{3}H]QNB to lung plasma membranes occurred rapidly, reached equilibrium by 60 min, and remained stable for an additional 60 min. Therefore, all subsequent radioligand binding assays employed a 60-min incubation.

Experiments were then done to determine if incubation temperature influenced the amount of specific [^{3}H]QNB binding to human lung plasma membranes. Lung membranes were incubated with 0.1 nM [^{3}H]QNB in the presence and absence of 1 μM unlabeled QNB at 23° and 37° for 60 min. The specific binding of [^{3}H]QNB to lung membranes was 2.55 ± 0.53 fmol/mg protein at 23°, and 4.21 ± 0.47 fmol/mg protein at 37° ($P < 0.05$). Therefore, all subsequent radioligand binding assays were performed at 37°. It should be noted, however, that the lower specific binding observed at 23° may be due to non-equilibrium binding conditions.

To analyze the binding of QNB to human lung membranes over a wide range of QNB concentrations, it is necessary to combine data from many different experiments. Unlike traditional Scatchard analyses [8], the LIGAND program enables the investigator to pool and fit data from multiple experiments by providing normative correction factors that allow for proportional fluctuations in saturable and nonsaturable binding with constant K_d values [7]. Two types of experiments were performed. In the first type, various concentrations of labeled QNB (10^{-11} to 10^{-8} M) were incubated with lung membranes at 37° for 60 min. In the second type, a fixed concentration of labeled QNB (4 nM) plus various concentrations of unlabeled QNB (10^{-9} to 10^{-3} M) were incubated with lung membranes at 37° for 60 min. The combined data generated from seven different equilibrium binding experiments (60 points) on a single subject's lung tissue using QNB concentrations of 10^{-11} to 10^{-3} M were most compatible with a single high-affinity binding site with a K_d of 0.04 nM and a receptor concentration of 24 fmol/mg protein. The average scatter of a point around the fitted curve (Fig. 2) corresponded to only a ±19% error in the value of [Bound/Total] for any given value of [Total]. The calculated K_d and R values had errors of only 17 and 8% respectively. The overall value for N was very well determined (±7%), as were the correction factors for experiments 2 through 7 (6–33%). The model involving a single class of high-affinity binding sites was significantly better than models involving multiple classes of binding sites. When the "extra sum of squares" principle was employed, the use of only a single class of binding sites decreased the *RMS* value with a corresponding F value that was highly significant ($P < 0.001$).

To measure the binding affinity of different drugs relative to the affinity of [^{3}H]QNB for the muscarinic receptor, K_d values were calculated from competition binding studies.

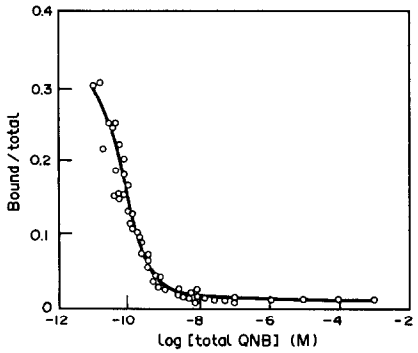

Fig. 2. Equilibrium binding of QNB to human peripheral lung. Various concentrations of labeled and unlabeled QNB were incubated with lung membranes for 60 min at 37°. The computer-generated curve plots the amount of [^{3}H]QNB bound over the total QNB added versus the log of the total QNB concentration (considering both the labeled and unlabeled ligand). The data from seven experiments were combined and analyzed using the LIGAND program. The best fit curve has been superimposed over the data points. The calculated values for the binding parameters are: $K_d = 0.04 \pm 0.01$ nM; $R = 24 \pm 2$ fmol/mg protein; $N = 1.4 \pm 0.1\%$; and *RMS* residual error = 19%.

These experiments involved the displacement of 4 nM [^{3}H]QNB from the lung muscarinic receptor site by unlabeled drugs added in concentrations from 10^{-9} to 10^{-3} M. This relatively high radiolabeled concentration (50-fold > K_d value) was used for these and the time course experiments because it enabled us to get higher counts with easily interpretable results using smaller lung tissue samples. The data were analyzed using the computer program LIGAND and are summarized in Table 1. The K_d values for the cholinergic antagonists were less than those for the cholinergic agonists. The rank order of potency for displacement of [^{3}H]QNB from the muscarinic receptor was QNB > scopolamine = atropine > oxotremorine > methacholine > carbachol. As expected, histamine and isoproterenol, which are not muscarinic agents, did not interact with the [^{3}H]QNB identified receptor. The affinity of unlabeld QNB was the same as that of [^{3}H]QNB, indicating that the addition of tritium to the molecule did not change its interaction with the receptor.

Table 1. Dissociation constants of various agents for human lung muscarinic receptors

Agent	K_d (nM)
Cholinergic antagonists	
[^{3}H]QNB	0.04 ± 0.01
QNB	0.06 ± 0.06
Scopolamine	1.22 ± 0.39
Atropine	1.63 ± 0.48
Cholinergic agonists	
Oxotremorine	370 ± 70
Methacholine	870 ± 380
Carbachol	2653 ± 1114
Other	
Histamine	No effect
Isoproterenol	No effect

Various concentrations of drugs were incubated with 4 nM [^{3}H]QNB, and the binding data were analyzed with the LIGAND program. Values represent the mean ± SE; number of experiments varied from three to eight.

We did not ascertain which lung cells possess the identified receptors. Since our data were most compatible with a single affinity binding site, it is likely that the muscarinic receptors on the various lung cells have similar affinities for muscarinic ligands. Autoradiographic studies on mammalian lung indicate that the density of these receptors is greatest in large airways, tracheal smooth muscle and exocrine glands, less in bronchiolar smooth muscle, airway epithelium and vascular smooth muscle, and least in alveoli [9, 10].

Using computer analyses of equilibrium binding experiments, we determined the binding parameters for [^{3}H]QNB to lung tissue from twenty-five different subjects. Twenty-two subjects were between the ages of 50 and 70, twenty-three were cigarette smokers, and all but three had lung cancer. The K_d values ranged from 0.01 to 0.16 nM with the geometric mean = 0.06 nM. The receptor concentrations ranged from 7 to 72 fmol/mg protein with the geometric mean = 24 fmol/mg protein. The *RMS* residual errors for the experiments ranged from 6 to 53%, indicating good fits for each data set. When binding experiments were repeated, estimated binding parameter values between experiments usually agreed within 30% of each other. We did not find a significant difference in binding parameters obtained for the subjects based on age, sex or pack years of smoking.

Using somewhat different techniques, Raaijmakers and co-workers [11] studied the binding of [^{3}H]QNB to human lung tissue from eight normals and four subjects with chronic obstructive lung disease. These investigators found that the two groups of subjects had similar K_d values (0.09 and 0.12 nM respectively), but different receptor concentrations (83 and 26 fmol/mg protein respectively). Of the twenty-five subjects in our study, six were found to have no evidence of chronic obstructive lung disease by chart review (no chronic lung medications, no history of respiratory difficulties, and pulmonary function tests > 80% of predicted). When we compared this group of six normal subjects to the other nineteen subjects in our study, the two groups were not found to have significant differences in either the K_d (0.06 ± 0.01 and 0.07 ± 0.01 nM respectively) or receptor concentration values (21 ± 5 and 29 ± 4 fmol/mg protein respectively). Thus, unlike Raaijmakers and co-workers, we did not find the normal subjects in our study to have a greater lung muscarinic receptor density. Since receptor concentrations are dependent upon the degree of membrane purification [12], differences in methodology between these two studies make direct comparisons of calculated binding parameters somewhat difficult. However, our data suggest that caution should be used when comparing estimated binding parameters between study groups. Large numbers of subjects are required for meaningful conclusions since there appears to be a range (>1 log) for K_d and receptor concentration values for muscarinic receptors on human lung.

In summary, we have determined that human peripheral lung tissue contains a single high-affinity [^{3}H]QNB binding site of moderately low concentration. The calculated [^{3}H]QNB binding parameters to twenty-five different lungs were found to differ by up to 1 log, but these differences did not appear dependent upon the clinical or demographic characteristics of the subjects studied. The methodologies employed herein will allow the accurate determination of receptor density and the affinity of the receptors for various drugs and hormones using small amounts (<1 g) of tissue samples. Thus, direct and meaningful comparisons of these parameters between large patient groups and in response to experimental manipulations can be done. These future studies should provide much information on the effect of many important clinical parameters such as age, smoking

history, allergic state, and drug exposure on the role of the muscarinic-receptor system in peripheral lung responses in health and disease.

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REFERENCES

1. J. B. Richardson, *Am. Rev. resp. Dis.* **119**, 785 (1979).
2. M. Kaliner, *J. Allergy clin. Immun.* **60**, 204 (1977).
3. J. H. Shelhamer, Z. Marom and M. Kaliner, *J. clin. Invest.* **66**, 1400 (1980).
4. B. P. Avner and J. W. Jenne, *J. Allergy clin. Immun.* **68**, 51 (1981).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
6. T. B. Casale, D. Rodbard and M. Kaliner, *Biochem. Pharmac.* **34**, 3285 (1985).
7. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
8. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
9. P. J. Barnes, J. A. Nadel, J. M. Roberts and C. B. Basbaum, *Eur. J. Pharmac.* **86**, 103 (1983).
10. P. J. Barnes, C. B. Basbaum and J. Nadel, *Am. Rev. resp. Dis.* **127**, 758 (1983).
11. J. A. M. Raaijmakers, G. K. Terpstra, A. J. van Rozen, A. Witter and J. Kreukniet, *Clin. Sci.* **66**, 585 (1983).
12. T. B. Casale, M. Friedman, N. Parada, J. Plekes and M. Kaliner, *J. membr. Biol.* **79**, 33 (1984).

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Inhibition by H_1-antihistamines of the uptake of noradrenaline and 5-HT into rat brain synaptosomes

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There are a number of reports that a range of structurally diverse H_1-receptor antagonists can interfere with the reuptake of the two biogenic amine neurotransmitters, noradrenaline and 5-hydroxytryptamine (5-HT), into presynaptic nerve endings in both the peripheral and central nervous system [1–4]. Isaac and Goth [1] first showed that diphenhyramine and chlorpheniramine potentiated the cardiovascular responses of noradrenaline by reducing the neuronal uptake of this neurotransmitter. Similar *in vitro* [2–6] and *in vivo* [2] studies have shown that mepyramine, diphenhyramine and chlorpheniramine can inhibit the neuronal uptake of noradrenaline and 5-HT in the mammalian central nervous system. Few studies, however, have attempted to quantify the effectiveness of H_1-receptor antagonists as inhibitors of the different neurotransmitter reuptake processes. In those studies that have been made, there is an indication that mepyramine [4, 5] and diphenhyramine [4, 6] can discriminate between the high affinity transport systems for noradrenaline and 5-HT. The present study was undertaken to quantify and compare the specificity of seven representative H_1-receptor antagonists as inhibitors of noradrenaline and 5-HT uptake.

Materials and methods

Measurement of uptake. A crude synaptosomal preparation of rat (Wistar, males, 250 g) cerebral cortex was prepared as described by Kellar *et al.* [7]. Aliquots (100 μl) of the synaptosomal suspension were added to incubation tubes containing H_1-receptor-antagonist in 660 μl of Krebs–Tris–Ringer medium (mM concentrations: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; Tris base, 50.4; glucose, 0.07; pargyline, 0.05, pH 7.4), gassed with O_2/CO_2 (95:5) and incubated at 37° for 10 min. [^{3}H]noradrenaline or [^{3}H]5-HT was then added in 40 μl of Krebs medium, to give a final concentration of 10 nM [^{3}H]noradrenaline or 2 nM [^{3}H]5-HT, and the incubation continued for a further 5 min. Incubations were terminated by addition of 4 ml of ice-cold 0.9% saline and filtered immediately through Whatman GF/B glass fibre filters under vacuum. The tubes were rinsed and the filters washed twice with 4 ml 0.9% saline and tritium was determined by liquid scintillation counting. Triplicate measurements were made at each incubation condition. The extent of the non-specific transport of [^{3}H]noradrenaline and [^{3}H]5-HT into rat brain synaptosomes was determined to be the uptake measured in the presence of 10 μM imipramine. Inhibitor constants (K_Is) for blockade of monoamine uptake were calculated from the concentration of drug (IC_{50}) required for 50% inhibition of the imipramine-sensitive transport of noradrenaline or 5-HT using the relationship $K_I = IC_{50}/(A/K_t + 1)$ where A is the concentration of [^{3}H]monoamine and K_t is its transport constant (i.e. the concentration at one half maximal imipramine-sensitive transport). K_t values were calculated from curves of inhibition of [^{3}H]monoamine uptake by the corresponding nonradioactive monoamine (added simultaneously with the radioactive ligand) according to the expression $K_t = IC_{50} - A$.

Materials. [^{3}H]5-HT creatinine sulphate (20 Ci/mmol) and [^{3}H]noradrenaline hydrochloride (13.5 and 15 Ci/mmol) were purchased from Amersham International. Mepyramine maleate, 5-HT creatinine sulphate, noradrenaline hydrochloride and methapyrilene hydrochloride were obtained from Sigma. Gifts of (+)-chlorpheniramine maleate (Schering), imipramine hydrochloride (Courtin & Warner), diphenhydramine (Parke Davis), promethazine hydrochloride (May & Baker), triprolidine hydrochloride and chlorcyclizine hydrochloride (Wellcome) are gratefully acknowledged.

Results and discussion

The transport constants (K_t) for [^{3}H]noradrenaline and [^{3}H]5-HT (0.17 and 0.09 μM respectively, Table 1) in the present study agreed well with those reported previously [6]. The antidepressant drug imipramine inhibited the uptake of both monoamines in a dose related fashion yielding similar inhibitor constants (K_I) of 0.04 and 0.11 μM for