0026-895X/95/030485-06$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 47:485–490, 1995.

MOL PHARM

# Regulation of Airway Muscarinic Cholinergic Receptor Subtypes by Chronic Anticholinergic Treatment

PAULA A. WITT-ENDERBY, HENRY I. YAMAMURA, MARILYN HALONEN, JOSEPHINE LAI, JOHN D. PALMER, and JOHN W. BLOOM

*Departments of Pharmacology (P.A.W.-E., H.I.Y., M.H., J.L., J.D.P., J.W.B.) and Medicine (M.H., J.D.P., J.W.B.) and the Respiratory Sciences Center (M.H., J.W.B.) (Westend Laboratories), College of Medicine, University of Arizona Health Sciences Center, Tucson, Arizona 85724*

Received July 21, 1994; Accepted December 19, 1994

**SUMMARY**

Anticholinergic agents are commonly used as bronchodilators for patients with airway obstructive diseases. The effects of chronic anticholinergic therapy on airway function and bronchial responsiveness are not known, but data from clinical studies suggest the possibility of adverse effects. We demonstrated in rabbits that, after atropine treatment for 4 weeks, the efficacy (maximum contraction) of *in vitro* methacholine-induced contraction of mainstem bronchi was increased [control (untreated), 1.0 ± 0.1 g; atropine-treated, 1.6 g ± 0.2 g; $p$ = 0.04]. However, there was no significant change in the potency ($EC_{50}$) of methacholine-induced contraction. Chronic atropine treatment increased the maximum density ($B_{max}$) of muscarinic receptors in the airways, as determined by radioligand binding studies with tritiated quinuclidinyl benzilate. Individual muscarinic receptor subtypes were measured using antibodies selective for the m1–m5 subtypes. Of the subtypes detected in rabbit tracheal smooth muscle (m2, m3, and m4), only the m2 and m3 muscarinic receptor subtypes were significantly up-regulated, compared with control, after chronic atropine treatment. Because cholinergic agent-mediated contraction of smooth muscle has been shown to be mediated by m3 muscarinic receptors, the atropine-induced increase in the methacholine response in airway smooth muscle appears to be the result of the up-regulation of m3 muscarinic cholinergic receptors. Such a mechanism may explain the clinical observations that chronic anticholinergic therapy for asthmatic patients is associated with an increase in bronchial responsiveness and that continuous versus "on demand" anticholinergic bronchodilator therapy may cause an accelerated decline in ventilatory function.

Cholinergic bronchoconstriction is mediated through the parasympathetic division of the autonomic nervous system by contraction of airway smooth muscle via mAChRs (1). Pharmacologic subtypes of mAChRs have been identified in airway smooth muscle (2–5). The existence of pharmacologic subtypes has been confirmed by the identification of five distinct mAChR genes (6, 7). Selective pharmacologic antagonists are available for the m1, m2, and m3 subtypes, but selective antagonists that distinguish the proteins encoded by the m4 and m5 genes have not yet been developed. Recently, antisera specific for the mAChR m1–m5 subtypes have been developed (8–12).

Anticholinergic agents are commonly used as bronchodilators in airway obstructive diseases. Chronic administration of anticholinergic agents has resulted in supersensitivity to cholinergic agonists in salivary gland cells (13), in the central nervous system (14), and in the airways (15). Chronic anticholinergic therapy with an inhaled anticholinergic agent for asthmatic patients produced transient bronchial hyperresponsiveness (15). A long term clinical study using an anticholinergic bronchodilator with patients with asthma and chronic bronchitis demonstrated that continuous versus "on demand" use of this class of bronchodilator resulted in an accelerated decline in ventilatory function (16). The mechanism underlying the adverse effects of chronic anticholinergic drug treatment remains unclear. In this study, we examined the hypothesis that chronic anticholinergic treatment alters the functional airway response to methacholine by specifically up-regulating mAChRs in the airways. As possible alternative mechanisms, we determined whether β-adrenergic relaxation mechanisms had been altered and whether a nonspecific enhancement in contractile activity could be demonstrated.

## Materials and Methods

**Implantation of osmotic mini-pumps.** New Zealand White, specific pathogen-free rabbits of either sex (1.7–2.0 kg) were anesthetized with 40 mg/kg ketamine HCl (Aveco Co., Fort Dodge, IA) and 4 mg/kg xylazine HCl (Lloyd Laboratories, Shenandoah, IA) and implanted with Alza osmotic mini-pumps (model 2ML4; Alza Corp., Palo Alto, CA) filled with either vehicle (control) or atropine sulfate

This work was supported by National Heart, Lung, and Blood Institute Grant HL14136.

**ABBREVIATIONS:** mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; DHA, dihydroalprenolol hydrochloride.



MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

(Sigma Chemical Co., St. Louis, MO). The pumps were designed to deliver a volume of 2.34 μl/hr at a concentration that was adjusted to the weight of the rabbit, to yield a drug concentration delivery of 4 mg/kg/day. The pumps were implanted subcutaneously in the midline of the back at the level of the shoulder. Blood was drawn from the central ear artery of the rabbits 2 weeks after the beginning of the infusion and plasma samples were analyzed for atropine concentrations by high performance liquid chromatography and gas chromatography/mass spectroscopy (National Medical Services, Willow Grove, PA). The pumps were removed on day 27 of the infusion and the rabbits were sacrificed 24 hr later by overdose with 390 mg of sodium pentobarbital (Vet Labs, Lenexa, KS). The heart and lungs were removed *en bloc* from the rabbit and immediately submerged in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 20.3 mM $NaH_2PO_4$, 3.2 mM HCl, 10 mM D-glucose, pH 7.4) aerated with 95% $O_2$/5% $CO_2$. The trachea, mainstem bronchi, peripheral lung, and heart were dissected, cleaned of connective tissue, and either used immediately (functional studies) or frozen at −70° until use (used within 1 month for radioligand binding and antibody studies). Time course studies were performed in an identical manner except that infusion pumps were removed on day 7 or day 14.

**Functional tissue bath analysis.** Two rings (2 mm in height) were cut from the distal portion of the trachea (nearest the carina) and two rings (1 mm in height) were cut from the mainstem bronchi. The rings were suspended between two stainless steel wires in a 10-ml, water-jacketed, organ bath containing Krebs buffer bubbled with 95% $O_2$/5% $CO_2$ and maintained at 37°. The rings were stretched with an isometric tension of 1.0 g until a steady base-line was reached (approximately 1 hr). For methacholine and KCl dose-response studies, increasing concentrations of methacholine ($10^{-9}$ to $10^{-3}$ M final concentration) or KCl (1–500 mM final concentration) were cumulatively added to the baths and the amount of contraction (in grams of force) was determined. The response to each dose was expressed as a percentage of the maximum contraction of each ring. The log $EC_{50}$ values for each tissue pair from each rabbit were averaged together to yield an $n$ value of 1/rabbit. The group $EC_{50}$ values are expressed as the geometric mean with the range of the standard errors; the efficacy values are expressed as the arithmetic mean ± standard error of the grams of force generated. All data were analyzed by analysis of variance with the App-Stat statistical package (StatSoft, Tulsa, OK), and significance was defined as $p < 0.05$.

For isoproterenol dose-response studies, the rings were initially contracted with increasing concentrations of methacholine ($10^{-9}$ to $10^{-3}$ M final concentration). When the maximum contraction for each ring was achieved, the tissue was washed five times and allowed to stabilize for 15 min, at which time half-maximal contraction and stabilization were achieved. Increasing doses of isoproterenol ($10^{-9}$ to $10^{-3}$ M final concentration; Sigma) were dissolved in Krebs buffer with 0.5% bismetabisulfite and cumulatively added to the baths. The amount of relaxation was determined as a reduction in grams of force. Dose-response curves were derived as described above.

**[$^3$H]QNB and [$^3$H]DHA saturation isotherms.** Radioligand binding assays were performed using L-[*benzilic*-4,4′-$^3$H(N)]QNB at 44.9 Ci/mmol and *l*-[*ring,propyl*-$^3$H(N)]DHA at 107.0 Ci/mmol (NEN Research Products, Boston, MA). Control and atropine-treated tissues (heart, peripheral lung, and tracheal and mainstem bronchial smooth muscle) were analyzed in parallel. Tracheal smooth muscle was excised from the trachea by cutting out the posterior muscle band. Because only a limited amount of tissue (approximately 150 mg) could be obtained from the tracheal and mainstem bronchial muscle, tissues from two rabbits were pooled and treated as an $n$ value of 1. Tissues were homogenized in ice-cold modified Krebs phosphate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 20.3 mM $NaH_2PO_4$, 10 mM D-glucose, pH 7.4) to yield a 5% (w/v) solution, which was then homogenized with two 15-sec pulses separated by a 30-sec pause, using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 8. The homogenates were centrifuged at 1200 × $g$ for 10 min at 4° and the pellet was discarded. The supernatant was centrifuged at 40,000 × $g$ for 15 min and the resulting pellet was resuspended in a volume of ice-cold modified Krebs phosphate buffer to yield a 5% (w/v) solution. Protein concentration was determined by colorimetric assay using bovine serum albumin as the standard (Pierce, Rockford, IL).

Ligand-binding saturation analyses were performed as described previously (17). The final ligand concentration ranges used in the saturation experiments were 10–1000 pM [$^3$H]QNB and 100-5000 pM [$^3$H]DHA. The maximum binding assays were carried out in 0.5-ml final volumes. The final concentrations used in the maximum binding experiments were 1 nM and 5 nM for [$^3$H]QNB and [$^3$H]DHA, respectively. Six-point [$^3$H]QNB and [$^3$H]DHA saturation curves were constructed. Nonspecific binding for [$^3$H]QNB was measured in the presence of 1 μM atropine sulfate (Sigma) and that for [$^3$H]DHA was determined in the presence of 10 μM (*S*)-(−)-propranolol (Sigma). The incubations were carried out in modified Krebs phosphate buffer, pH 7.4, at 37° for 1 hr. The rapid filtration assay, as described (18), was used to separate bound from unbound ligand. Filtration was performed with Whatman GF/B glass fiber filters, using a Brandel cell harvester apparatus (Gaithersburg, MD). The filters were presoaked in distilled water for the [$^3$H]QNB binding assays and in distilled water containing 0.5% polyethylenimine (Sigma) for the [$^3$H]DHA binding assays. The radioactivity of the samples was determined by liquid scintillation counting (Beckman LS 5000CE counter). The saturation data were analyzed by the GRAFIT program (Erithacus Software), using a one-site fit model. Group comparisons were analyzed by one-way analysis of variance using the App-Stat statistical package, and significance was defined as $p < 0.05$.

**Immunoprecipitation analysis of tracheal smooth muscle.** The immunoprecipitation assays were carried out using antibodies specific for the m1–m5 mAChRs (a gift from Dr. Barry Wolfe, Georgetown University). The m1, m2, m4, and m5 mAChR-specific antibodies were directed against the third intracellular loops of the receptors (8, 9, 11). The m3 receptor-specific antibody was directed against a synthetic peptide encoding an 18-amino acid sequence in the carboxyl terminus (amino acids 561–578) of the receptor (10).

In initial experiments characterizing tracheal smooth muscle for mAChR subtype identity, the tracheae were obtained from PelFreez (Rogers, AR). For the experiments comparing control versus atropine-treated tracheal smooth muscle, control tissues were always assayed in parallel with atropine-treated tissues, with eight tracheal smooth muscle strips combined for each experiment ($n$ = 1).

**Solubilization of mAChRs.** Tissues were homogenized as described above for saturation studies, except that they were resuspended in TE buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA). Maximum [$^3$H]QNB binding and protein analyses were performed with an aliquot of the homogenate. All binding conditions were identical to those described above. The remaining homogenate was then solubilized in TEDC buffer (TE buffer plus 0.4% digitonin and 0.04% cholic acid) for 2 hr at 4° and centrifuged at 12,000 × $g$ for 10 min. Maximum [$^3$H]QNB binding was determined in the solubilized fractions the following day, with the preparations having been kept at 4°. Bound and free ligand were separated by chromatography using 3-ml Sephadex G-25 columns (Pharmacia LKB Biotechnology, Piscataway, NJ) that had been pre-equilibrated with 3 column volumes of 0.1% TEDC buffer (TE buffer plus 0.1% digitonin and 0.01% cholic acid). Solubilization efficiency was determined by comparing the femtomoles of receptor in the soluble fraction with those in the membrane fraction. Receptor solubilization efficiency under these conditions was 40–60%.

**Immunoprecipitation of m1–m5 mAChRs.** Immunoprecipitation analysis was performed as described previously (8–11), with some modifications. Immediately after the chromatography, 250-μl portions of the total and nonspecifically [$^3$H]QNB-labeled solubilized fractions were mixed with each antibody (m1–m5) to yield a 0.5 mg/ml final concentration and were incubated for 2 hr at 4°. Goat anti-rabbit IgG (PelFreez) was diluted 1/2 in TE buffer, 70 μl were

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

added to each tube containing the primary antibody, and tubes were incubated for 16–24 hr at 4°. The resulting precipitate was recovered by centrifugation at 2000 × *g* for 10 min at 4°, and the supernatant was discarded (by aspiration). The pellet was resuspended in 300 μl of 0.1% TEDC buffer and centrifuged at 10,000 × *g* for 10 min. The pellet was washed, resuspended in 100 μl of TE buffer plus 1% sodium dodecyl sulfate, and incubated at 37° for 30 min, and the radioactivity was quantified. Control antiserum was used to define nonspecific immunoprecipitation. Groups were compared by Student's unpaired *t* test, with significance defined as $p < 0.05$.

## Results

**Effect of chronic atropine exposure on mAChRs and β-adrenergic receptors.** Twenty-seven rabbits (18 control and nine atropine treated) were implanted with osmotic pumps and 25 rabbits survived to complete the study. The mean plasma level of atropine in the atropine-treated animals 2 weeks after the beginning of the infusion was 22 ± 9 ng/ml.

On day 28 (1 day after removal of the osmotic pumps), airway smooth muscle contraction was studied *in vitro*. Maximum methacholine-induced contraction for the atropine-treated mainstem bronchial rings was increased by 60% over control ($p = 0.04$). A similar trend was observed in tracheal tissues (31% increase in efficacy over control, $p = 0.1$) (Table 1). Potency of the methacholine-induced contractions was not significantly different between the groups for either the mainstem bronchus or trachea.

To determine whether the increase in efficacy of methacholine in the atropine-treated tissues was a mAChR-dependent event, the tissues were contracted with KCl, a receptor-independent contractile stimulus. The maximum contraction in response to KCl in the atropine-treated tissues was not significantly different from that in control tissues (control, 0.9 ± 0.1 g; atropine-treated, 1.0 ± 0.2 g). Similarly, the potency of the response to KCl was not significantly different between the groups [control $EC_{50}$, 19 mM (range, 17–20 mM); atropine-treated $EC_{50}$, 14 mM (range, 14–16 mM)].

Radioligand saturation binding assays in peripheral lung and heart and maximum binding assays in all tissues were performed with [$^3$H]QNB. There was a significant increase in mAChR density ($B_{max}$) in the mainstem bronchi (44% increase over control, $p = 0.0006$) and trachea (62% increase over control, $p = 0.01$) of atropine-treated animals, compared with control. The mAChR densities in peripheral lung and heart were also increased with chronic atropine treatment (59% over control, $p = 0.02$, and 94% over control, $p = 0.03$, respectively). In tissues with sufficient material for saturation analyses (peripheral lung and heart), no change in affinity of [$^3$H]QNB was observed (Table 2).

To determine whether chronic atropine treatment also had an effect on β-adrenergic receptors, we measured β-adrenergic receptor function, affinity, and number. Chronic atropine exposure did not significantly alter either the potency or the efficacy of isoproterenol-induced relaxation responses in the mainstem bronchi or trachea (Table 3). Similarly, there was no significant change in either the affinity ($K_d$) or the β-adrenergic receptor density ($B_{max}$), as determined by radioligand binding saturation isotherms and maximum binding assays using the radioligand [$^3$H]DHA (Table 4).

**Effect of chronic atropine exposure on the mAChR subtypes.** To determine the distribution and extent of up-regulation for each mAChR subtype (m1–m5) upon chronic atropine exposure, the individual subtypes were measured by immunoprecipitation analyses of tracheal smooth muscle, using antibodies selective for the m1–m5 mAChR subtypes. Tracheal smooth muscle contained predominantly the m2 mAChR subtype, with a minority of the m3 mAChR subtype (Fig. 1). In addition, we found that rabbit tracheal smooth muscle also contained a small percentage of the m4 mAChR, but no significant amounts of either the m1 or the m5 mAChR subtype were detected. Only the m2 and m3 mAChR subtypes were significantly up-regulated in tracheal smooth muscle with chronic atropine exposure, compared with control (Fig. 1). The actual amounts of atropine-induced up-regulation of the m2 and m3 mAChRs appeared to be similar (an increase of 35 fmol/mg of protein for m2 mAChR and 24 fmol/mg of protein for m3 mAChR), although when calculated as percentage increases the m3 receptor increase was greater (66%, versus 27% for m2).

TABLE 1

**Effect of chronic atropine treatment on methacholine-induced contraction in rabbit mainstem bronchi and tracheae *in vitro***

| | $EC_{50}$[a] | Maximum contraction[b] |
|---|---|---|
| | μM | g |
| Mainstem bronchial smooth muscle | | |
| Control ($n = 17$) | 1.4 (0.9–2.2) | 1.0 ± 0.1 |
| Atropine-treated ($n = 8$) | 0.6 (0.4–0.9) | 1.6 ± 0.2[c] |
| Tracheal smooth muscle | | |
| Control ($n = 17$) | 1.5 (0.9–2.7) | 1.6 ± 0.2 |
| Atropine-treated ($n = 8$) | 0.6 (0.3–1.0) | 2.1 ± 0.4 |

[a] All $EC_{50}$ values represent the geometric means, with the range of the standard errors in parentheses.
[b] The maximum contraction values are shown in grams of tension and represent the arithmetic means ± standard errors.
[c] $p < 0.05$, as determined by one-way analysis of variance.

TABLE 2

**Effect of chronic atropine treatment on mAChR affinity and density**

Radioligand binding studies were performed as described in Materials and Methods, using [$^3$H]QNB. Saturation experiments are the sum of four to six individual experiments performed in duplicate. The total binding experiments are the sum of four individual experiments performed in quadruplicate. All data were analyzed by one-way analysis of variance.

| | $K_d$[a] | $B_{max}$[b] |
|---|---|---|
| | pM | fmol/mg of protein |
| Mainstem bronchial smooth muscle | | |
| Control | ND[c] | 267 ± 10 |
| Atropine-treated | ND[c] | 385 ± 14[d] |
| Tracheal smooth muscle | | |
| Control | ND[c] | 641 ± 39 |
| Atropine-treated | ND[c] | 1040 ± 125[e] |
| Peripheral lung | | |
| Control | 28 (20–38) | 129 ± 37 |
| Atropine-treated | 26 (20–33) | 205 ± 28[e] |
| Heart | | |
| Control | 19 (17–22) | 131 ± 21 |
| Atropine-treated | 29 (25–33) | 254 ± 28[e] |

[a] $K_d$ values represent the geometric means, with the range of the standard errors in parentheses.
[b] $B_{max}$ values represent the arithmetic means ± standard errors.
[c] ND, not determined (due to insufficient amounts of tissue).
[d] $p < 0.01$.
[e] $p < 0.05$.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

TABLE 3

**Effect of chronic atropine treatment on isoproterenol-induced relaxation of rabbit mainstem bronchi and tracheae *in vitro***

There are no significant differences in either the $EC_{50}$ or maximum relaxation values in tissues treated with atropine, compared with control tissues. All data were analyzed by Student's unpaired *t* test, where significance is defined as $p < 0.05$.

| | $EC_{50}$[a] | Maximum relaxation[b] |
|---|---|---|
| | *μM* | *g* |
| Mainstem bronchial smooth muscle | | |
| Control ($n$ = 9) | 0.46 (0.34–0.62) | 0.7 ± 0.1 |
| Atropine-treated ($n$ = 6) | 0.18 (0.06–0.51) | 0.6 ± 0.08 |
| Tracheal smooth muscle | | |
| Control ($n$ = 9) | 0.31 (0.20–0.46) | 1.0 ± 0.08 |
| Atropine-treated ($n$ = 6) | 0.31 (0.16–0.59) | 1.1 ± 0.1 |

[a] All $EC_{50}$ values represent the geometric means, with the range of the standard errors in parentheses.
[b] The maximum relaxation values represent the arithmetic means ± standard errors.

**Time course of the effect of chronic atropine exposure on mAChRs.** The time course of the effect of atropine treatment was investigated at 7 and 14 days of exposure. Twelve rabbits (three control and three atropine-treated rabbits at each time point) were studied. In contrast to the 4-week atropine exposure period, there was no effect of chronic atropine treatment on the efficacy of methacholine-induced contraction in mainstem bronchi either at 7 days (maximum contraction: atropine-treated, 1.1 ± 0.4 g; control, 0.8 ± 0.2 g) or at 14 days (maximum contraction: atropine-treated, 1.2 ± 0.3 g; control, 1.1 ± 0.3 g) of exposure. In addition, radioligand binding saturation isotherms with [$^3$H]QNB were performed in lung homogenates at both the 7-day and 14-day time points. In contrast to the 28-day atropine exposure, there was no increase in the mAChR density after 7 days ($B_{max}$: atropine-treated, 172.7 ± 48.9 fmol/mg of protein; control, 142 ± 12.7 fmol/mg of protein) or 14 days of atropine treatment ($B_{max}$: atropine-treated, 102.7 ± 12.9 fmol/mg of protein; control, 143.5 ± 23.3 fmol/mg of protein).

## Discussion

Chronic anticholinergic treatment has been shown to be associated with an increase in bronchial responsiveness and worsening of asthma (15, 16). The results obtained in this study suggest that chronic treatment with the anticholinergic agent atropine can induce an increase in airway smooth muscle contractility in response to methacholine *in vitro* and that this increased contractility is associated with an up-regulation of the m2 and m3 mAChR subtypes in airway smooth muscle.

We used a dose of atropine of 4 mg/kg/day, which had previously been shown to produce 100% inhibition of vagally induced bronchoconstriction in rabbits (19). This dose produced plasma atropine levels in rabbits that were comparable to plasma levels in humans after an intravenous dose of 1 mg (20). Atropine treatment produced an increase in the efficacy of methacholine-induced contraction of isolated rabbit airways *in vitro*, when assessed after 4 weeks of treatment. Time course studies showed that the alterations associated with atropine treatment for 4 weeks were not evident at or before 14 days of treatment. The osmotic pumps were removed 24 hr before muscle contraction and receptor studies, to allow atropine clearance from the tissues. Evidence that atropine was cleared from the tissues was provided by the lack of a significant difference in mAChR affinity for the radiolabeled antagonist (−)-[$^3$H]QNB between control and treated tissues. Also, in studies by other investigators, 24 hr were sufficient for atropine clearance from tissues (21).

TABLE 4

**Effect of chronic atropine treatment on β-adrenergic receptor affinity and density**

Radioligand binding studies were performed as described in Materials and Methods, using [$^3$H]DHA. There are no significant differences in either the β-adrenergic receptor affinity or density in atropine-treated tissues, compared with control, as determined by Student's unpaired *t* test.

| | $K_d$[a] | $B_{max}$[b] |
|---|---|---|
| | *nM* | *fmol/mg of protein* |
| Tracheal smooth muscle | | |
| Control ($n$ = 4) | ND[c] | 254 ± 41 |
| Atropine-treated ($n$ = 4) | ND[c] | 350 ± 34 |
| Peripheral lung | | |
| Control ($n$ = 9) | 0.87 (0.78–0.98) | 781 ± 50 |
| Atropine-treated ($n$ = 5) | 0.91 (0.89–0.93) | 799 ± 48 |
| Heart | | |
| Control ($n$ = 5) | 1.3 (1.1–1.5) | 173 ± 22 |
| Atropine-treated ($n$ = 5) | 1.6 (1.4–1.9) | 276 ± 52 |

[a] All $K_d$ values represent the geometric means, with the range of the standard errors in parentheses.
[b] The $B_{max}$ values represent the arithmetic means ± standard errors.
[c] ND, not determined (due to insufficient amounts of tissue).

We considered several possible mechanisms as potential explanations for the altered response after chronic anticholinergic treatment. The data support homologous up-regulation of the mAChRs by atropine as the mechanism for the altered response. Heterologous down-regulation of the β-adrenergic receptors was ruled out because the functional response (isoproterenol-induced relaxation of the airway *in vitro*) was not altered and neither the affinity ($K_d$) nor the density ($B_{max}$) of the β-adrenergic receptors was decreased in response to anticholinergic treatment. To examine the possibility that an alteration independent of cholinergic receptors had occurred, we investigated the contractile response of the atropine-treated tissue to the receptor-independent stimulus KCl and found that this stimulus produced a contractile response that was not significantly different from control. Thus, we conclude that the functional increase in response to methacholine was the result of some change in the mAChRs. Direct evidence for homologous up-regulation of the mAChRs was provided by our studies demonstrating that chronic exposure to atropine was associated with an increase in mAChR density in the airway.

Studies by others have demonstrated that treatment with anticholinergic agents can lead to up-regulation of mAChRs in brain and heart (22–25). This up-regulation of mAChRs by anticholinergic agents has been shown to be associated with an increase in mAChR function. In rat brain, chronic atropine exposure resulted in an increase in mAChR number and an increase in sensitivity of an atropine-induced behavioral response in rats (24). Similarly, Majocha and Baldessarini (25) demonstrated that chronic anticholinergic agent exposure resulted in an increase in cholinergic agent-induced behavioral responses that was paralleled by an increase in brain mAChRs. In contrast to our findings, however, chronic anticholinergic agent treatment did not increase mAChRs in guinea pig airways (26). The lack of effect in the guinea pig

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

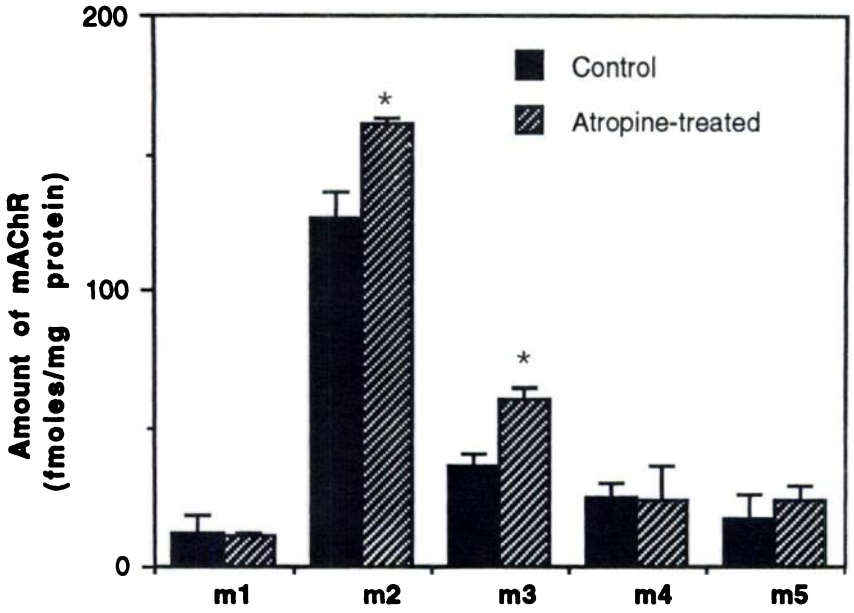


**Fig. 1.** Effect of chronic atropine treatment on mAChR subtypes in rabbit tracheal smooth muscle. Chronic treatment with atropine increased the mAChR density of the m2 and m3 subtypes, compared with control values. The percentage of each subtype was initially calculated as the percentage of the total counts immunoprecipitated. The amount (fmol/mg of protein) of each mAChR subtype was calculated by multiplying the percentage of the mAChR subtype by the total amount of mAChRs, as determined by radioligand binding analysis. Each *bar* represents the arithmetic mean ± standard error from three individual experiments, using eight individual rabbit tracheae/experiment. Control and atropine-treated tissues were assayed in parallel. All data were analyzed by Student's unpaired *t* test, and significance (*) is defined as $p < 0.05$.

studies is most likely due to the shorter duration of anticholinergic agent treatment in those studies.

mAChR subtype diversity exists within tracheal smooth muscle. Radioligand binding analyses have demonstrated that tracheal smooth muscle contains predominantly the m2 mAChR subtype, with a minority of the m3 mAChR subtype (2–4). In agreement with the binding data, using antibodies selective for the m1–m5 mAChR subtypes we demonstrated that rabbit tracheal smooth muscle contained predominantly the m2 mAChR subtype and a minority of the m3 mAChR subtype. Evidence suggests that it is the minority receptor (m3 mAChR subtype), linked to the phosphoinositide transduction pathway (27), that is responsible for cholinergic agent-induced contraction of the airway smooth muscle (2, 5). Because cholinergic agent-mediated contraction of airway smooth muscle is mediated by the m3 mAChR, the atropine-induced increase in the methacholine-induced response appears to be the result of up-regulation of the m3 mAChR. The role of the m2 mAChR is less clear. The m2 mAChR is preferentially linked to inhibition of adenylyl cyclase activity and may play a role in modulating relaxation responses to β-adrenergic receptor agonists in airway smooth muscle (28, 29). Up-regulation of the m2 mAChR by chronic atropine administration in our studies does not appear to have produced an effect on the relaxation response in airway smooth muscle. Our data show no effect of atropine treatment on isoproterenol-induced relaxation of airway smooth muscle precontracted with methacholine. In addition to the m2 and m3 mAChR subtypes, the immunoprecipitation studies showed a small proportion of the receptors to be of the m4 mAChR subtype. The m4 mAChR has been identified previously as the predominant subtype in rabbit peripheral lung (11, 12, 30). The function of the m4 mAChR, if any, in airway smooth muscle is unknown.

Chronic exposure to atropine up-regulated only the m2 and m3 mAChR subtypes and not the m4 mAChR subtype in tracheal smooth muscle. Data from several studies in brain tissue or neurally derived cell lines demonstrate that significant up-regulation of mAChRs after chronic exposure to antagonist requires the presence of a functional synapse (31, 32). This finding suggests that the mAChRs that are up-regulated after chronic blockade are under continuous "tone" from endogenously released acetylcholine. Thus, although the function of the m2 mAChR in airway smooth muscle is unclear, the up-regulation of the m2 mAChR after chronic atropine exposure suggests that it is vagally innervated and under at least some tonic stimulation.

In conclusion, chronic exposure to the anticholinergic agent atropine produced an up-regulation of the m2 and m3 mAChR subtypes that was associated with enhanced efficacy of airway smooth muscle contraction in response to cholinergic agonist. These data provide a plausible explanation for the clinical observations, with asthmatic patients, of increased methacholine-induced responsiveness and worsening of lung function with chronic anticholinergic treatment.

**Acknowledgments**

The authors thank Linda Nunan, Carla Lohman, and MaryAnn Raymond for technical assistance.

**References**

1. Richardson, J. B. Nerve supply to the lungs. *Am. Rev. Respir. Dis.* **119:** 785–802 (1979).
2. Mahesh, V. K., L. M. Nunan, M. Halonen, H. I. Yamamura, J. D. Palmer, and J. W. Bloom. A minority of muscarinic receptors mediate rabbit tracheal smooth muscle contraction. *Am. J. Respir. Cell. Mol. Biol.* **6:**279–286 (1992).
3. Fryer, A. D., and E. E. El-Fakahany. Identification of three muscarinic receptor subtypes in rat lung using binding studies with selective antagonists. *Life Sci.* **47:**611–618 (1990).
4. Lucchesi, P. A., C. R. Scheid, F. D. Romano, M. E. Kargacin, D. Mullikin-Kilpatrick, H. Yamaguchi, and T. W. Honeyman. Ligand binding and G protein coupling of muscarinic receptors in airway smooth muscle. *Am. J. Physiol.* **258:**C730–C738 (1990).
5. Roffel, A. F., C. R. S. Elzinga, R. G. M. Van Amsterdam, R. A. De Zeeuw, and J. Zaagsma. Muscarinic M2 receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur. J. Pharmacol.* **153:** 73–82 (1988).
6. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science (Washington D. C.)* **237:**527–532 (1987).
7. Bonner, T. I., A. C. Young, M. R. Brann, and N. J. Buckley. Cloning and expression of the rat and human m5 muscarinic receptor genes. *Neuron* **1:**403–410 (1988).
8. Wall, S. J., R. P. Yasuda, F. Hory, S. Flagg, B. M. Martin, E. I. Ginns, and B. B. Wolfe. Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. *Mol. Pharmacol.* **39:**643–649 (1991).

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

9. Li, M., R. P. Yasuda, S. J. Wall, A. Wellstein, and B. B. Wolfe. Distribution of m2 muscarinic receptors in rat brain using antisera selective for m2 receptors. *Mol. Pharmacol.* **40:**28–35 (1991).
10. Wall, S. J., R. P. Yasuda, M. Li, and B. B. Wolfe. Development of an antiserum against m3 muscarinic receptors: distribution of m3 receptors in rat tissues and clonal cell lines. *Mol. Pharmacol.* **40:**783–789 (1991).
11. Yasuda, R. P., W. Ciesla, L. R. Flores, S. J. Wall, M. Li, S. A. Satkus, J. S. Weisstein, B. V. Spagnola, and B. B. Wolfe. Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: distribution of m4 and m5 receptors in rat brain. *Mol. Pharmacol.* **43:**149–157 (1993).
12. Dorje, F., A. I. Levey, and M. R. Brann. Immunological detection of muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. *Mol. Pharmacol.* **40:**459–462 (1991).
13. Emmelin, N., and A. Muren. Paralytic secretion in cats after treatment with atropine. *Acta Physiol. Scand.* **22:**277–280 (1951).
14. Friedman, M. J., and J. H. Jaffe. Changes in CNS sensitivity to cholinergic (muscarinic) agonists following withdrawal of chronically administered scopolamine. *Pharmacologist* **8:**199 (1966).
15. Newcomb, R., D. P. Tashkin, K. K. Hui, and M. E. Conolly. Rebound hyperresponsiveness to muscarinic stimulation after chronic therapy with an inhaled muscarinic antagonist. *Am. Rev. Respir. Dis.* **132:**12–15 (1985).
16. van Schayck, C. P., E. Dompeling, C. L. A. van Herwaarden, H. Folgering, A. L. M. Verbeek, H. J. M. van der Hoogen, and C. van Weel. Bronchodilator treatment in moderate asthma or chronic bronchitis: continuous or on demand? A randomised controlled study. *Br. Med. J.* **303:**1426–1431 (1991).
17. Bloom, J. W., M. Halonen, L. Lawrence, E. Rould, N. A. Seaver, and H. I. Yamamura. Characterization of high affinity [$^3$H]pirenzepine and (−)-[$^3$H]quinuclidinyl benzilate binding to muscarinic cholinergic receptors in rabbit peripheral lung. *J. Pharmacol. Exp. Ther.* **240:**51–58 (1987).
18. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. USA* **71:**1725–1729 (1974).
19. Bloom, J. W., H. I. Yamamura, C. Baumgartener, and M. Halonen. A muscarinic receptor with high affinity for pirenzepine mediates vagally induced bronchoconstriction. *Eur. J. Pharmacol.* **133:**21–27 (1987).
20. Berghem, L., U. Bergman, B. Schildt, and B. Sorbo. Plasma atropine concentrations determined by radioimmunoassay after single-dose i.v. and i.m. administration. *Br. J. Anaesth.* **52:**597–601 (1980).
21. Lee, W., and B. B. Wolfe. Regulation of muscarinic receptor subtypes and their responsiveness in rat brain following chronic atropine administration. *Mol. Pharmacol.* **36:**749–757 (1989).
22. Wall, S. J., R. P. Yasuda, M. Li, W. Ciesla, and B. B Wolfe. Differental regulation of subtypes m1–m5 of muscarinic receptors in forebrain by chronic atropine administration. *J. Pharmacol. Exp. Ther.* **262:**584–588 (1992).
23. Chevalier, B., P. Mansier, E. Teiger, F. Callens-El Amrani, and B. Swynghedauw. Alterations in β-adrenergic and muscarinic receptors in aged rat heart: effects of chronic administration of propranolol and atropine. *Mech. Ageing Dev.* **60:**215–224 (1991).
24. Takeyasu, K., S. Uchida, Y. Noguchi, N. Fujita, K. Saito, F. Hata, and H. Yoshida. Changes in brain muscarinic acetylcholine receptors and behavioral responses to atropine and apomorphine in chronic atropine-treated rats. *Life Sci.* **25:**585–592 (1979).
25. Majocha, R., and R. J. Baldessarini. Tolerance to an anticholinergic agent is paralleled by increased binding to muscarinic receptors in rat brain and increased behavioral response to a centrally active cholinomimetic. *Life Sci.* **35:**2247–2255 (1984).
26. Suzuki, R., K. Takagi, and T. Satake. Changes in muscarinic acetylcholine receptors in guinea-pig lung: effects of aging, inhalation of an allergen, administration of drugs, and vagotomy. *Lung* **163:**173–182 (1985).
27. Grandordy, B. M., N. Frossard, K. J. Rhoden, and P. J. Barnes. Tachykinin-induced phosphoinositide breakdown in airway smooth muscle and epithelium: relationship to contraction. *Mol. Pharmacol.* **33:**515–519 (1988).
28. Jones, C. A., J. M. Madison, M. Tom-Moy, and J. K. Brown. Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. *Am. J. Physiol.* **253:**C97–C104 (1987).
29. Fernandes, L. B., A. D. Fryer, and C. A. Hirshman. M2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.* **262:**119–126 (1992).
30. Lazareno, S., N. J. Buckley, and F. Roberts. Characterization of muscarinic M4 binding sites in rabbit lung, chicken heart, and NG108–15 cells. *Mol. Pharmacol.* **38:**805–815 (1990).
31. Siman, R. G., and W. L. Klein. Cholinergic activity regulates muscarinic receptors in central nervous system cultures. *Proc. Natl. Acad. Sci. USA* **76:**4141–4145 (1979).
32. Vige, X., and M. Briley. Muscarinic receptor plasticity in rats lesioned in the nucleus basalis of Meynert. *Neuropharmacology* **28:**727–732 (1989).


**Send reprint requests to:** John W. Bloom, Respiratory Sciences Center, Arizona Health Sciences Center, 1501 N. Campbell Ave., Tucson, AZ 85724.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012