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CHARACTERIZATION OF CHOLINERGIC MUSCARINIC RECEPTORS IN COW TRACHEAL MUSCLE MEMBRANES

EFFECT OF MATURATION*

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Abstract—The parasympathetic nervous system is important in the control of basal airway muscle tone and caliber. We characterized muscarinic cholinergic receptors in isolated tracheal membranes from cows of three age groups (immature, <2 weeks; transition, 3–5 months; and mature, >5 years) using *l*-[^{3}H]quinuclidinyl benzilate (*l*-[^{3}H]QNB) as the radioligand. There were significant decreases in the densities of *l*-[^{3}H]QNB binding sites with maturation (B_{max}: 2344 ± 169 vs 1381 ± 85 vs 1116 ± 80 fmol/mg protein for tissues from immature, transition and mature cows respectively). No change in the dissociation constant was observed with maturation (K_d: 0.38 ± 0.09 vs 0.55 ± 0.06 vs 0.50 ± 0.07 nM for tissues from immature, transition and mature animals respectively). The association and dissociation rate constants did not vary between tissues from immature and mature animals. The specific activity of the enzyme, acetylcholinesterase, was correlated with the density of *l*-[^{3}H]QNB binding sites present in the tracheal homogenates; that is, with maturation, there were significant decreases in acetylcholinesterase activity [0.28 ± 0.01 vs 0.16 ± 0.02 vs 0.08 ± 0.01 mol·l^{-1}·min^{-1}·(mg protein)$^{-1}$ for tissues from immature, transition and mature animals respectively]. All competition binding studies using muscarinic antagonists exhibited single site binding and did not show any differences in drug affinities between the age groups. In contrast, multiple binding sites were observed with carbachol, methacholine and muscarine, and there were significant decreases in receptor affinities for the muscarinic agonists. No changes in the proportion of high and low affinity sites were found. These results indicate that with maturation there are alterations in the properties of muscarinic receptors in tracheal smooth muscle.

The parasympathetic nervous system is a major contributing factor in the regulation of airway reactivity [1, 2]. Specifically, stimulation of vagally innervated respiratory tissues causes a release of the neurotransmitter, acetylcholine. The released acetylcholine, which interacts with cholinergic muscarinic receptors in the lung, can cause smooth muscle contraction [3, 4], can increase intracellular concentrations of cGMP [5, 6], and can stimulate the secretion of mucus and the development of pulmonary edema [7, 8]. The parasympathetic system has been implicated in several respiratory diseases where there is an alteration of airway caliber, for example asthma [9], chronic obstructive lung disease [10], and intolerance to environmental pollutants such as ozone [1].

Administration of muscarinic antagonists such as atropine to either humans or animals [11, 12] will often attenuate induced bronchoconstriction. In contrast, muscarinic agonists (e.g. carbachol) and acetylcholinesterase inhibitors (e.g. physostigmine) will induce and/or potentiate bronchoconstriction. If humans with hyperreactive airways are treated with hexamethonium, a ganglionic blocking agent which abolishes histamine-induced bronchoconstriction, the airways still remain hyperreactive to muscarinic agonists [13]. These data suggest that airway hyperreactivity to muscarinic agonists may result from (1) alterations in muscarinic receptor densities and drug binding affinities, (2) changes in the release or inactivation of acetylcholine, and/or (3) modification in some post-receptor mechanism.

The use of specific radiolabeled muscarinic antagonists such as *l*-[^{3}H]quinuclidinyl benzilate (*l*-[^{3}H]QNB) has enabled several research groups to characterize muscarinic receptors in pulmonary tissues from rats [14], dogs [15], cows [16], guinea pigs [17] and humans [10]. These studies have focused on a single age group for each species. However, numerous *in vivo* and *in vitro* studies have demonstrated alterations in airway muscle responses to bronchoconstrictors and bronchodilators during ontogenesis [18, 19]. In guinea pigs, there is a 4-fold reduction in the *in vitro* sensitivity (pD_2 values) of tracheal muscle to carbachol as a consequence of maturation [20].

Thus, it is important to determine if the altered responsiveness of airway tissues with maturation may be due to changes in muscarinic receptor densities and/or alterations in drug binding affinities. In the present study, we characterized the ontogenic changes in muscarinic receptors in tracheal smooth muscle.

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METHODS

Materials. Protosol, Atomlight and *l*-[^{3}H]quinuclidinyl benzilate (33.1 Ci/mmole) were purchased from DuPont-New England Nuclear Corp., Boston, MA. Unlabeled *d*-, *l*- and *dl*-QNB were gifts from The Center for Disease Control, Atlanta, GA. All other biochemical and pharmacological agents were obtained from commercial sources: carbamylcholine chloride (carbachol), *dl*-muscarine chloride, oxotremorine sesquifumarate, pilocarpine hydrochloride, *l*-scopolamine hydrobromide, acetyl-β-methyl choline chloride (methacholine), atropine sulfate, acetylthioiodide, 5,5-dithiobis-2-nitrobenzoic acid (Sigma Chemical Co., St. Louis, MO); pyrilamine maleate (R. W. Greeff & Co., Inc., New York, NY).

Tissues. Cow tracheae were obtained from two local abattoirs. Tracheae from milk-fed Holstein calves, 2 weeks old or 3–5 months old, were obtained from J. G. Fortes, North Branford, CT. Tracheae from mature Holstein cows (>than 5 years old) were obtained from Frank DeMartino & Son & Sons, Seymour, CT. The fresh tissues were placed in normal saline (4°) immediately after slaughter of the cows and prior to transportation to the laboratory.

Tissue preparation. Fresh tracheae were placed in Tris buffer (50 mM, pH 7.4) at 4°. The tracheae were stripped free from all extraneous connective tissue. An incision was made down the dorsal medial line in order to expose the trachealis muscle. The cartilage rings were cut, and the rings were everted to reveal the smooth muscle covered with an epithelial layer. This layer was stripped off the muscle with forceps. The trachealis muscle was cut away from the cartilage rings and placed in fresh Tris buffer. Average wet weights of muscle per trachea from immature and mature animals were 1.5–2 and 15–20 g respectively. To obtain sufficient amounts of tissue for the radioligand binding assays, the smooth muscle from immature cows or from cows of an intermediate age were pooled (six to twelve animals per isolation). Each trachea from a mature cow was processed independently.

Tissues were minced with scissors and then homogenized in 5 vol. of buffer at 4° for 2 × 30 sec periods in a Virtis blade homogenizer at full speed. The homogenate was homogenized further in a Tekmar homogenizer (blade 18N) for 4 × 15 sec periods with 1 min cooling between each burst. The homogenate was centrifuged at 755 *g* for 15 min to remove connective tissue and unbroken cells. The supernatant fraction was spun at 29,000 *g* for 15 min. The pellet was resuspended in buffer and spun again at 29,000 *g* for 15 min. The pellet was rewashed and recentrifuged twice. The final pellet was suspended in fresh buffer. Protein concentrations were determined using the method of Lowry *et al.* [21]. Membrane aliquots (concentration 1.0 mg membrane protein/ml) were frozen at −80° until used in the ligand binding assays.

Radioligand binding assays. Saturation binding curves were determined by incubating the 29,000 *g* membrane fraction (approximately 200 μg membrane protein/ml, final tube concentration) in Tris buffer (50 mM, pH 7.4) with increasing concentrations of *l*-[^{3}H]QNB (final tube concentrations, 0.02 to 5 nM) for 20 min at 25°. The total volume of the assay was 250 μl. All samples were assayed in duplicate. Nonspecific binding was determined by incubating the membrane fraction and radioligand with atropine (1 μM, final tube concentration). Specific binding was defined as the total binding minus the nonspecific binding. The reaction was initiated by the addition of protein and terminated by the addition of cold Tris buffer (2.5 ml). The membrane fractions were filtered under vacuum onto Whatman GF/A glass fiber filters that had been presoaked for 20 min in atropine (1 μM). Use of GF/B, GF/C or 934-AH glass fiber filters did not increase the total recovery of bound radioactivity. The tubes were rinsed with Tris buffer (4 ml). Filters were then rapidly washed with aliquots (3 × 10 ml) of Tris buffer and subsequently transferred to glass scintillation vials containing water (100 μl). Protosol (0.6 ml) was added to each vial. Vials were capped and incubated at 60° for 90 min to solubilize protein and release bound radioactivity from the filters. After an overnight incubation at room temperature, scintillation mixture (13 ml) and glacial acetic acid (35 μl) were added to the vials. Samples were counted on a Packard scintillation counter at 45% efficiency.

Kinetic analysis of *l*-[^{3}H]QNB binding to tracheal membranes was done using the method of Rodbard [22] for pseudo first-order rates of reaction. The association rate was determined by incubating membrane fractions for 35 min with *l*-[^{3}H]QNB (1 or 5 nM) for various intervals. For example, a 1-min association time was determined by incubating the membrane fraction for 34 min alone and for 1 min with *l*-[^{3}H]QNB. The time of the reaction did not include the interval between the end of the reaction and vacuum filtration. Parallel samples were incubated with an excess of atropine (10 μM, final tube concentration). After each time interval, reactions were stopped, filtered, and processed as described above. Dissociation rates were determined by incubating the membrane fractions with *l*-[^{3}H]QNB (1 or 5 nM) for 20 min. Then atropine (10 μM, final tube concentration) was added to duplicate tubes at set intervals up to 240 min. At the end of the incubation period, all reactions were stopped, filtered, and processed as described above.

In competition binding experiments, *l*-[^{3}H]QNB (final tube concentrations of 0.3 to 0.5 nM) was incubated with the membrane fraction (200 μg/ml, final tube concentration) in Tris buffer with various concentrations of competing agent. Antagonist concentrations ranged from 10^{-11} M to 10^{-6} M, and agonist concentrations were from 10^{-8} M to 10^{-3} M unless otherwise specified. Total binding was determined in the absence of the drug. Nonspecific binding was determined by incubating the membrane fraction with *l*-[^{3}H]QNB and atropine (1 μM, final tube concentration). Samples were assayed in triplicate and processed as described above.

Acetylcholinesterase assay. Acetylcholinesterase activity was measured in homogenates of tracheal muscle colorimetrically by the method of Ellman *et al.* [23] as modified by Benke *et al.* [24]. Acetylthiocholine iodide was used as the substrate. Units

of activity were defined as the rate of substrate degradation ($mol \cdot l^{-1} \cdot min^{-1}$) per mg protein at 25°. An aliquot of tracheal muscle homogenate (0.7 to 1.1 mg protein/0.1 ml) was incubated with acetylthiocholine iodide (0.4 mM) and 5,5-dithiobis-2-nitrobenzoic acid (0.1 mM) in sodium phosphate buffer (0.1 M, pH 8.0). The total assay volume was 5 ml. The absorbance at 412 nm was read immediately after the addition of the protein and again after a 30-min incubation at 25° (Bausch & Lomb, Spectronic 88 with microflothru cell). Nonspecific absorbances of substrate blanks, boiled enzyme blanks, and the initial nonspecific absorbance due to the 5,5-dithiobis-2-nitrobenzoic acid were subtracted from the final absorbance reading for calculation of the rate of substrate hydrolysis. Pseudocholinesterase activity present in the homogenates was measured by incubating the reaction mixture with tetraisopropyl pyrophosphoramide (100 μmol/l).

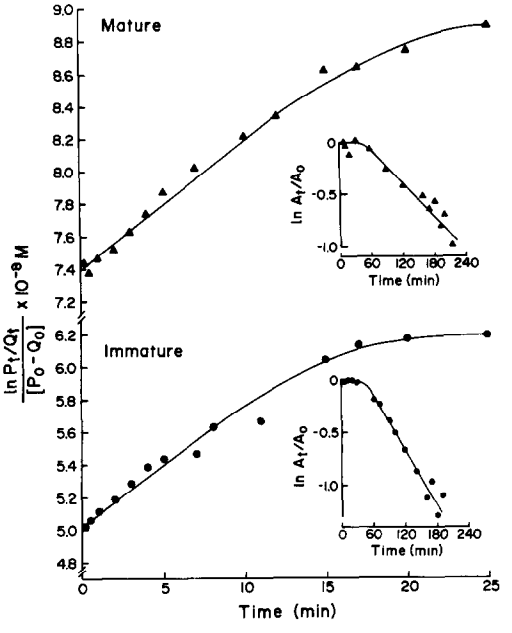

Fig. 1. Kinetics of *l*-[^{3}H]QNB binding to tracheal muscle membranes. Association kinetics for tracheal muscle membranes from mature (upper panel; ▲) and immature (lower panel; ●) cows. Each panel is a typical experiment and shows a pseudo first-order plot. Association rates were determined by adding *l*-[^{3}H]QNB (5 nM) in the presence or absence of atropine (1 μM) to tracheal membranes (200 μg membrane protein/ml) in Tris buffer (50 mM, pH 7.4) at 25°. P_t = concentration of unbound *l*-[^{3}H]QNB at time t, Q_t = concentration of free receptor at time t, P_o = total concentration of *l*-[^{3}H]QNB added (5 nM), and Q_o = total concentration of receptors as determined from the saturation binding isotherms. Total receptor concentrations for tissues from mature and immature tracheae were 1.76×10^{-10} M and 5.54×10^{-10} M respectively. Similar experiments were done at *l*-[^{3}H]QNB concentration (P_o) of 1 nM. The reaction proceeded linearly with time for 20 min (see Methods). Dissociation kinetics (insets) for tracheal muscle membranes from mature (upper panel, ▲) and immature (lower panel, ●) cows are also shown. Tracheal membranes (200 μg protein/ml) were preincubated for 20 min with *l*-[^{3}H]QNB (1 or 5 nM). Atropine (1 μM) was added to duplicate tubes at set intervals such that the ligand was always incubated with the membrane for a total of 240 min, but contact with the atropine varied from 1 to 240 min. Nonspecific binding was defined as the binding in the presence of atropine after a 240-min contact with both ligand and atropine. A_t = specific binding at time t, and A_0 = total binding in the absence of atropine corrected for nonspecific binding. Values are the means of one to three determinations.

Data analysis. Data from both saturation binding curves and competition experiments were analyzed using the weighted nonlinear least squares computer curves fitting program, SCTFIT, developed by Hancock *et al.* [25]. Values are mean ± SE. Data were analyzed using either Student's *t*-test for unpaired values or by analysis of variance. Multiple sites of the data from competition binding experiments were analyzed for "goodness of fit" using a partial F-test [25] where:

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

SS_1 and SS_2 are the sum of squares of residuals for the one and two sit fits, respectively, and dF_1 and dF_2 are the corresponding degrees of freedom.

RESULTS

The characterization of muscarinic cholinergic receptors in cow tracheal smooth muscle membranes was investigated using the radiolabeled antagonist *l*-[^{3}H]QNB. The binding of *l*-[^{3}H]QNB to tracheal membranes from tissues of both immature and mature cows was saturable at 25°. Maximum binding was achieved within 20 min (Fig. 1). Association rate constants for membranes from immature and mature cows were determined at both 1 and 5 nM *l*-[^{3}H]QNB. Association rate constants were neither concentration nor age dependent; the rates were: $5.88 \pm 0.62 \times 10^6\ M^{-1}\ min^{-1}$ vs $9.29 \pm 2.83 \times 10^6\ M^{-1}\ min^{-1}$ for membranes from immature and mature cows, respectively. The binding of *l*-[^{3}H]QNB was stable with time but, in the presence of atropine (10 μM), the ligand dissociated from membranes with a half-time of between 120 and 140 min (Fig. 1, insets). Dissociation rate constants (N = 3) for membranes from immature and mature cows were $0.0093 \pm 0.0002\ min^{-1}$ and $0.0095 \pm 0.0001\ min^{-1}$ respectively. Estimates of the dissociation constant using the association and dissociation rates gave K_d values of 1.58 ± 0.24 nM for membranes from immature cows and 1.01 ± 0.32 nM for membranes from mature cows. These K_d values are in good agreement with the values obtained from saturation binding experiments with *l*-[^{3}H]QNB (Table 1).

The binding of *l*-[^{3}H]QNB to tracheal membranes from both immature and mature cows reached half-maximal saturation at 0.5 nM *l*-[^{3}H]QNB at 25° (Fig. 2). Nonspecific binding (i.e. binding in the presence of 1 μM atropine) was linear with increasing *l*-[^{3}H]QNB concentrations and equal to less than 20% of the total binding at saturating *l*-[^{3}H]QNB concentrations. There was a significant difference in the maximum number of receptors per mg protein (B_{max}) between tracheal membranes from immature and mature cows (Fig. 1, Table 1). Tracheal membranes

Table 1. Kinetic parameters of muscarinic receptors from cow tracheal membranes

	K_d (nM)	B_{max} (fmol/mg protein)	Acetylcholinesterase activity [mol·l^{-1}·min^{-1}·(mg protein)$^{-1}$]
Immature	0.38 ± 0.09 (10)	2344 ± 169* (10)	0.28 ± 0.01* (6)
Transition	0.55 ± 0.06 (3)	1381 ± 85† (3)	0.16 ± 0.02‡ (2)
Mature	0.50 ± 0.07 (8)	1116 ± 80 (8)	0.08 ± 0.01 (11)

Values are mean ± SE for (N) experiments where the numbers of tracheae used in the preparation of the muscle membranes from immature, transition, and mature cows were >90, >20 and 8 respectively. K_d = dissociation constant for *l*-[^{3}H]QNB and B_{max} = maximum density of *l*-[^{3}H]QNB binding sites in muscle membranes. Values are derived from data analyzed using the computer curve fitting program, SCTFIT.

* Significantly greater than corresponding values from muscle membranes from transition ($P < 0.01$) and mature ($P < 0.001$) animals.

† Significantly greater than corresponding value for tracheal muscle membranes from mature animals ($P < 0.05$).

‡ Significantly greater than corresponding value for tracheal muscle membranes from mature animals ($P < 0.01$).

from a third "transitional" age group were also studied (Fig. 3, Table 1). This tissue was obtained from the same abattoir as the tissues from immature cows but the tracheae were obtained from animals that were 3–5 months old. The inner–outer diameters of the tracheae were 1.4/1.7 cm for the 2-week-old calves and 1.9/2.9 cm for the 3- to 5-month-old calves. In contrast, the inner–outer diameters of the mature tracheae were 4.4/5.8 cm. There were no differences between the dissociation constants (K_d) of the membrane binding sites in the preparations from the cows in the different age groups.

The kinetic parameters of *l*-[^{3}H]QNB binding were not affected by the buffer system used in the assay. For example, results similar to those described above, were obtained when tracheal membranes were incubated with *l*-[^{3}H]QNB in Tyrode's solution (pH 7.4). Receptor densities at saturating concentrations of *l*-[^{3}H]QNB were 2550 and 1392 fmol/mg protein and dissociation constants were 0.54 and 0.35 nM for tracheal membranes from immature and mature cows respectively.

An alternative way to distinguish between the different age groups was to determine the acetylcholinesterase activities in the tracheal homogenates. There was a 1.75-fold decrease in true acetylcholinesterase activity; that is, the total acetylcholinesterase activity minus pseudocholinesterase activity, between tracheal membranes from immature and transitional cows ($P < 0.01$) and a further 2-fold decrease in activity between tissues from transitional and mature cows ($P < 0.001$) (Table 1).

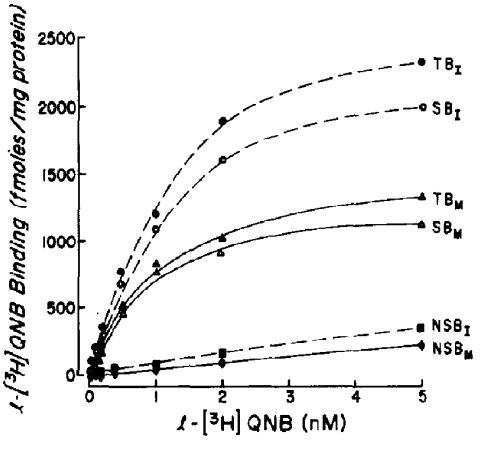

Fig. 2. Saturation binding curves of *l*-[^{3}H]QNB binding in tracheal muscle membranes. Total binding (TB) was determined for tracheal muscle membranes from immature (I, ●) and mature (M, ▲) animals by incubating the membranes (approximately 200 μg/ml) with increasing concentrations of *l*-[^{3}H]QNB for 20 min at 25°. Nonspecific binding (NSB; I, ■; M, ◆) was measured by incubating membranes with atropine (1 μM). Specific binding (SB; I, ○; M, △) was equivalent to total binding minus nonspecific binding. Samples were run in duplicate. Concentrations (abscissa) are final tube concentrations.

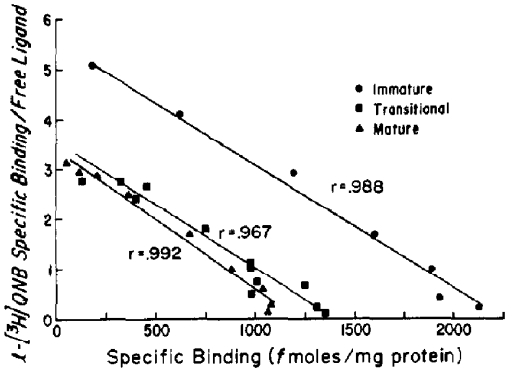

Fig. 3. Scatchard analysis of *l*-[^{3}H]QNB binding in tracheal smooth muscle membranes. Data are representative of experiments from immature (N = 10), transition (N = 8) and mature (N = 8) membrane preparations. The total numbers of tracheae used in the membrane preparations for determination of the maximum binding capacity (B_{max}) and dissociation constant (K_d) for immature, transition and mature cows were: >90, >20 and 8 respectively. Data were analyzed using linear regression following a SCTFIT analysis which indicated that a one-site fit was optimal. The data derived for these analyses are shown in Table 1.

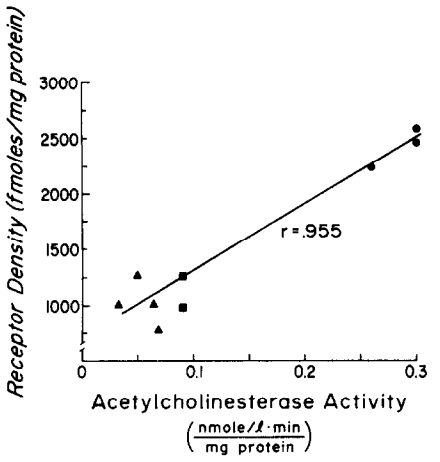

Fig. 4. Acetylcholinesterase activity in tracheal muscle homogenates. Acetylcholinesterase activity was determined in muscle homogenates from immature (●), transition (■) and mature (▲) animals as described in Methods. Pseudocholinesterase activities were subtracted from total acetylcholinesterase activities. Receptor densities were obtained from saturation binding experiments on tracheal muscle membranes as determined for a one site fit with the weighted nonlinear least squares curve fitting program, SCTFIT (e.g. Fig. 2). The graph shows the relationship between the specific activity of acetylcholinesterase (abscissa) and receptor density (ordinate). Note that the data points represent values from pooled tissues (see Table 1).

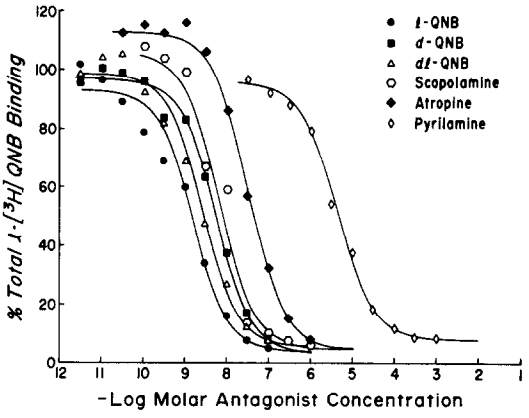

Fig. 5. Antagonist inhibition of total *l*-[^{3}H]QNB binding in tracheal muscle membranes from immature animals. Data are representative of three experiments for each antagonist. Plots of the data for antagonist inhibition of *l*-[^{3}H]QNB binding in membranes from animals of different age groups were not different. The curves were computer generated by the curve fitting program SCTFIT, utilizing the data points (N = 10) which are also shown for each antagonist.

Pseudocholinesterase activity did not vary between the different age groups. Activity of pseudocholinesterase ranged from 0.037 ± 0.005 to $0.035 \pm .005$ mol·l^{-1}·min^{-1}·(mg protein)$^{-1}$ in tissues from immature and mature cows respectively. The numbers of *l*-[^{3}H]QNB muscarinic receptors present in tracheal membrane fractions were correlated ($r = 0.955$) with the activities of acetylcholinesterase in tracheal homogenates from animals of the same age (Fig. 4).

To analyze the interactions of muscarinic receptors in cow tracheal membranes to muscarinic agonists and antagonists, competition experiments were done using *l*-[^{3}H]QNB as the radioligand. All antagonist competition curves yielded steep dissociation plots indicative of single site binding. As seen in Fig. 5, the order of potency for the specific antagonists was *l*-QNB ≥ *d*-QNB ≥ *dl*-QNB > scopolamine > atropine > pyrilamine maleate. No stereospecificity was observed between the isomers of QNB. The reason for the lack of stereospecificity for QNB in tracheal smooth muscle is not known. Other compounds such as ipratropium bromide and its stereoisomer, SCH1178, and *d*- and *l*-thiazinamium chloride exhibited stereoselectivity in tracheal tissues from immature and mature animals (unpublished observations). There were no significant differences in the affinity constants for each drug in tissues from animals of different ages (Table 2).

In contrast, muscarinic agonists bind to multiple binding sites—a high and a low affinity site. In cow tracheal membranes, there was a decrease in the sensitivity of the tissue to agonists with maturation

Table 2. Affinity constants of muscarinic antagonists for cow tracheal membranes

Antagonist	Negative logarithm of affinity constant		
	Immature	Transition	Mature
l-QNB	9.28 ± 0.26 (3)	9.40 ± 0.44 (2)	9.08 ± 0.11 (3)
d-QNB	9.23 ± 0.57 (3)	9.28 ± 0.23 (2)	8.70 ± 0.11 (2)
dl-QNB	9.18 ± 0.12 (4)	9.26 ± 0.21 (2)	9.46 ± 0.36 (4)
Scopolamine	8.77 ± 0.10 (3)	8.55 ± 0.12 (3)	8.73 ± 0.07 (3)
Atropine	8.28 ± 0.39 (3)	9.20 ± 0.27 (3)	8.40 ± 0.30 (4)
Pyrilamine maleate	5.55 ± 0.05 (3)	5.63 ± 0.05 (3)	5.84 ± 0.14 (3)

Values are mean ± SE of the negative logarithms of the affinity constants which were determined for data from competition experiments (e.g. Fig. 5) using a multiple site analysis with the weighted nonlinear least squares computer curve fitting program, SCTFIT. The number of determinations is shown in parentheses.

Table 3. Affinity constants of agonists for cow tracheal membranes*

Agonist	Age	N	pK_H†	pK_L†
Carbachol	I	4	6.81 ± 0.10‡	5.14 ± 0.08‡
	T	4	6.45 ± 0.09§	4.72 ± 0.11§
	M	6	6.16 ± 0.19	4.51 ± 0.15
Methacholine	I	5	6.63 ± 0.13	4.84 ± 0.05‡
	T	3	6.83 ± 0.29	5.02 ± 0.18‡
	M	5	6.22 ± 0.27	4.33 ± 0.16
Muscarine	I	5	6.25 ± 0.13	4.74 ± 0.03‡
	T	3	6.04 ± 0.35	4.31 ± 0.16§
	M	4	5.90 ± 0.32	4.20 ± 0.22
Oxotremorine	I	3	7.34 ± 0.20	6.08 ± 0.12
	T	4	7.69 ± 0.18	6.24 ± 0.06
	M·	3	7.48 ± 0.05	6.23 ± 0.21
Pilocarpine	I	2		5.76 ± 0.21
		1	6.39	5.39
	T	2		5.98 ± 0.05‡
		1	6.00	5.15
	M	2		5.86 ± 0.16
		2	6.42 ± 0.02	5.27 ± 0.03

* Data from competition experiments (e.g. Fig. 6) were analyzed for multiple binding sites using the weighted nonlinear least squares curve fitting computer program, SCTFIT. The best fits were for two site binding from which a high (K_H) and a low (K_L) affinity site were calculated. Values are mean ± SE for (N) determinations for tracheal muscle membranes from immature (I), transition (T), and mature (M) animals.

† $pK_H = -\log K_H$ (M); $pK_L = -\log K_L$ (M).

‡ Significantly greater than corresponding value from mature animals ($P < 0.05$).

§ Significantly less than corresponding value from immature animals ($P < 0.05$).

(Table 3). Specifically, the affinity constants for the low affinity receptor site (pK_L) for carbachol, methacholine and muscarine were significantly less ($P < 0.05$) in mature tissues as compared with the affinity constants in immature tissues (Table 3). Furthermore, the low affinity constants in the transitional age group for muscarine and carbachol were also less than the corresponding pK_L value from immature tissues. The low affinity constants for muscarine and carbachol in the transitional age group were not different from pK_L values from mature tissues. The affinity of the receptor for the agonist high affinity binding site did not vary between the age groups except for the agonist, carbachol. For this agonist, there was a 4.5-fold decrease in the pK_H with maturation (Table 3). No differences in the affinity constants of partial agonists, such as oxotremorine or pilocarpine, were observed in tissues isolated from tracheal membranes. Pilocarpine bound competitively to two binding sites in tissues from animals of each age group, but in several experiments it was impossible to distinguish between the high and low affinity binding sites.

There were no significant changes in the relative proportion of high and low affinity sites between age groups with a given agonist (Table 4). Furthermore, there were no differences in the proportions of high and low affinity sites between the agonists with the exception of pilocarpine. With pilocarpine, a partial agonist, 70–80% of the receptors were high affinity binding sites (Table 4). Kent *et al.* [26] demonstrated that the ratio of the affinity constants (K_L/K_H) correlates with the intrinsic activity of the agonists. In tracheal muscle, the order of efficacy for agonists was: methacholine > carbachol > muscarine > oxotremorine > pilocarpine. Although there were increases in these ratios with maturation, they were not significantly different.

In summary, in cow tracheal membranes, there was a decrease in both the density of the muscarinic receptors and in the affinity of the muscarinic receptors for full agonists with maturation.

DISCUSSION

Cholinergic muscarinic receptors in pulmonary tissues regulate both the basal tone and airway caliber and may induce airway contraction in disease states such as asthma or during natural processes such as aging. The present study examined the binding properties of muscarinic receptors in isolated cow tracheal membranes as a function of animal age. We report that significant decreases in the total number of *l*-[^{3}H]QNB binding sites occurred with maturation. The use of *l*-[^{3}H]QNB as a radiolabeled antagonist to characterize muscarinic receptors has been well established in, for example, brain [27], heart [28, 29], ileum [30] and other peripheral smooth muscle systems [31]. Muscarinic receptors in pulmonary tissues have been investigated, either as isolated tracheal muscle membranes [15, 16] or more commonly as parenchymal homogenates from several species including guinea pigs [17], rats [14] and humans [10]. However, since the responsiveness of isolated guinea pig tracheal tissues to carbachol is reduced significantly with maturation [20], it was critical to determine if the alteration in potency could be due in part, to changes in the total number of

Table 4. Percentages of high and low affinity binding sites of agonists in cow tracheal membranes*

Agonist	Age	N	$\%K_H$†	$\%K_L$†	Ratio‡ K_L/K_H
Carbachol	I	4	54 ± 6	46 ± 6	49 ± 10
	T	4	61 ± 7	39 ± 7	53 ± 4
	M	6	56 ± 5	44 ± 5	73 ± 18
Methacholine	I	5	57 ± 2	43 ± 2	68 ± 17
	T	3	55 ± 8	45 ± 8	70 ± 16
	M	5	48 ± 8	52 ± 8	103 ± 41
Muscarine	I	5	51 ± 5	49 ± 5	42 ± 18
	T	3	72 ± 4§	28 ± 4	66 ± 32
	M	4	48 ± 5	52 ± 5	60 ± 24
Oxotremorine	I	3	66 ± 7	34 ± 7	30 ± 8
	T	4	58 ± 6	42 ± 6	36 ± 13
	M	3	49 ± 7	51 ± 7	26 ± 15
Pilocarpine	I	1	72	28	11
	T	1	74	26	7
	M	2	81 ± 2‖	19 ± 2	14 ± 2

* Data from competition experiments (e.g. Fig. 6) were analyzed for multiple binding sites using the weighted nonlinear least squares computer curve fitting program, SCTFIT. Values obtained from a two site analysis yielded the best fit. Values are mean ± SE for (N) determinations.

† $\%K_H$ is the percent of high affinity binding sites, whereas $\%K_L$ is the percent of low affinity site binding in the tracheal membranes from immature (I), transitional (T), and mature (M) animals.

‡ Ratio K_L/K_H = low affinity constant (K_L)/high affinity constant (K_H).

§ Significantly greater than $\%K_H$ for membranes from either immature or mature animals ($P < 0.05$).

‖ Significantly greater than $\%K_H$ for other agonists in membranes from immature, transitional or mature animals ($P < 0.05$).

cholinergic receptors and/or to alterations in agonist binding affinities.

The binding of *l*-[^{3}H]QNB in these preparations achieved saturation within 20 min but dissociated at a slow dissociation rate. Our results for the dissociation constants in isolated tracheal membranes from immature and mature cows (Table 1) varied little from the range of values (0.27 to 0.62 nM) observed in pulmonary tissues from other species. In contrast, the total number of muscarinic receptors varied significantly, not only between species but also between regions within the lung. Homogenates of parenchymal tissues from humans [10], guinea pigs [17], or rats [14] have significantly lower densities of *l*-[^{3}H]QNB binding sites than we and other investigators have found in isolated cow [16] or dog [15] trachealis membranes. The higher levels of *l*-[^{3}H]QNB binding in trachealis membranes support autoradiographic data presented by Barnes *et al.* [32] in ferret lungs, where *l*-[^{3}H]QNB binding is predominantly localized in tracheal muscle and intrapulmonary cartilagenous airways. Species variation also exists in the densities of *l*-[^{3}H]QNB binding sites in trachealis membranes, since we report that cow tracheal membranes have three to six times more specific binding than dog tracheal membranes [15]. These differences in *l*-[^{3}H]QNB binding between species in tracheal membranes and the differences in the regional distribution of *l*-[^{3}H]QNB binding may follow the pattern of cholinergic nerve distribution within the lung [33].

Muscarinic agonists administered to humans and animals [11, 12] are believed to interact with membrane bound receptors. The physiologically active agonist at muscarinic receptors is acetylcholine. Regulation of acetylcholine concentrations at the cell membrane is achieved via cholinesterases which rapidly inactivate the neurotransmitter. We report that the specific activity of acetylcholinesterase in tracheal homogenates correlated directly with the density of *l*-[^{3}H]QNB binding sites in tracheal muscle membranes (Fig. 4). Simon *et al.* [34] observed similar findings in the brain, where *l*-[^{3}H]QNB binding to muscarinic receptors was highest in regions of the brain where the activities of acetylcholinesterase and choline acetyltransferase were the highest. A close correlation between acetylcholinesterase activity and density of muscarinic receptors has also been observed in cerebral cells [35] and chicken muscle cells [36] during development. The increase in both the density of receptors and enzyme activity coincided with synaptogenesis [36]. It is not known if the converse is true; namely, that a decrease in nerve input to the tissue results in both a decrease in the density of muscarinic receptors and a decrease in acetylcholinesterase activity.

Developmental alterations in the densities of cholinergic muscarinic receptors have also been observed in brain [37], heart [29] and skeletal muscle [38]. In most systems, the density of the muscarinic receptors decreases with age, and there is no alteration in the affinity. The decrease in the number of muscarinic receptors by itself could explain altered responsiveness (i.e. contractile and relaxant properties) in tracheal tissues with maturation. However, in the case of muscarinic receptors, less than 5% of the

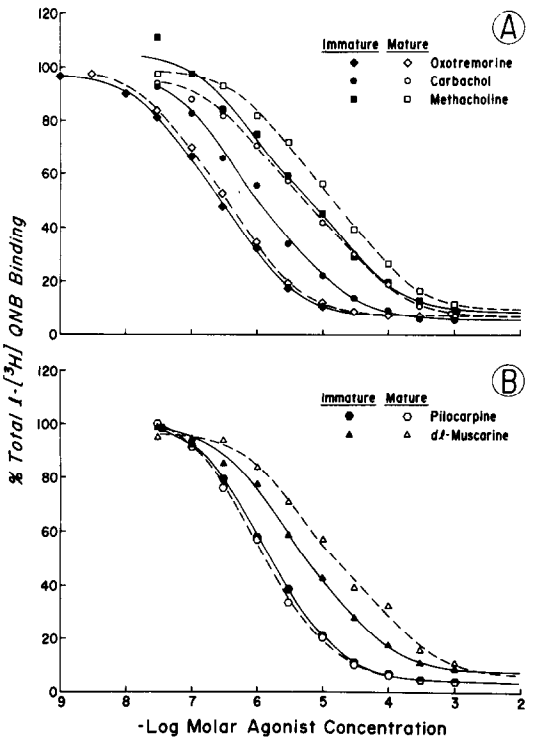

Fig. 6. Agonist inhibition of *l*-[^{3}H]QNB binding in tracheal muscle membranes from immature (closed symbols) and mature (open symbols) animals. Data are representative of three to six experiments for each agonist. Curves were computer generated with the curve fitting program SCTFIT, utilizing the data points (N = 10) which are also shown for each agonist. Note that there were significant differences in the position of the inhibition curves with maturation when full agonists were used. A complete analysis of these results is shown in Table 3.

receptors is needed to produce a response in guinea pig airways [39]. Preliminary *in vitro* organ bath experiments with cow tracheal smooth muscle suggests that with maturation there are significant decreases in both the pD_2 values for carbacol and the magnitude of the tension produced in the tissue after carbachol-induced contraction. In this study, we observed that there was both a decrease in the density of muscarinic receptors with maturation and an alteration in the affinity of the receptor for muscarinic agonists (Fig. 6, Table 3). For full agonists, such as carbachol, muscarine or methylcholine, there were significant decreases in the affinity of the drugs for the low affinity binding site. In agonist-induced desensitization of tissues, there is a decrease in the physiological responsiveness of the tissue to the agonist [40]. Our results initially suggested that a similiar agonist-induced down-regulation of muscarinic receptors was occurring in tracheal tissues with maturation. However, in several systems in which agonists such as acetylcholine or carbachol have induced alterations in the characteristics of the muscarinic receptors, the changes have been limited to either decreases in the densities of muscarinic receptors [41, 42] and/or decreases in the affinity of the low affinity binding site with a concomitant decrease in the proportion of low affinity binding receptor sites [43, 44]. Furthermore, the effect of agonist-induced desensitization or down-regulation was readily reversible. In our system, there were no changes in the proportions of high and low affinity sites (Table 4). The changes with maturation in tracheal smooth muscle were linked to alterations in the K_i values of the agonists for the muscarinic receptors. Similar age-related decreases in agonist affinities have been reported for heart tissues [29]. Alterations in agonist affinities during differentiation have also been linked to the development of synapses [45]. Recently, Freund *et al.* [46] suggested that changes in the membrane microenvironment (i.e. alteration in membrane fluidity) with aging can affect binding to muscarinic receptors. The likelihood of similar phenomena occurring in lung tissue is not known.

The binding characteristics of pilocarpine and oxotremorine in cow tracheal membranes were different from those observed for carbachol, methacholine or muscarine. There was no decrease in binding affinities with maturation. Furthermore, pilocarpine exhibited inconsistent binding to multiple muscarinic binding sites (Table 3). The reason for these anomalous results is not fully understood. One possible explanation is that pilocarpine has a very low K_L/K_H ratio. Birdsall *et al.* [47, 48] and, more recently, Brown and Brown [49] have suggested that low K_L/K_H ratios are indicative of low efficacy of the drug. Agonists with low efficacy are poor at promoting or stabilizing conformational changes in receptor structure [50] as seen with single site binding of pilocarpine in tracheal smooth muscle.

In summary, our experiments demonstrate in tracheal tissues from cows a significant decrease with maturation in both the density of muscarinic receptor sites and in the activity of the enzyme acetylcholinesterase. There were also changes in the affinities of agonists for muscarinic binding sites. These changes in membrane receptor properties may play a role in the alterations of airway caliber observed in a diseased state such as asthma and thus contribute to remission of asthma in children with age.

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