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0031-3998/84/1811-1136$02.00/0
PEDIATRIC RESEARCH

Vol. 18, No. 11, 1984
Printed in U.S.A.

Muscarinic Cholinergic Receptors in Developing Rat Lung

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ABSTRACT. Muscarinic cholinergic receptors were identified and partially characterized in crude membrane fractions of rat lung and trachea from day 17 of gestation to adulthood using (−)-[^{3}H]quinuclidinyl benzilate (QNB). (−)-[^{3}H]QNB binding to rat lung membrane was characteristic of muscarinic cholinergic receptor sites. Binding capacity of muscarinic receptors sites was relatively low in rat lung compared to that in other tissues. The number of (−)-[^{3}H]QNB-binding sites (binding capacity) decreased progressively and significantly from 79 ± 8 fmol·mg^{-1} protein on days 17–18 of gestation to 21 ± 3 fmol·mg^{-1} mean ± SEM on days 21–22 of gestation, $p < 0.01$. Binding capacity did not vary thereafter from birth to adulthood. Affinity of (−)-[^{3}H]QNB binding for lung membranes did not change with age (K_D approximately 70 pM). (−)-[^{3}H]QNB-binding sites were significantly higher in membrane preparations of trachea or tracheal-bronchial tissue than in lung parenchyma from both the adult and newborn rats. (−)-[^{3}H]QNB binding was undetectable in crude membrane preparations of cultured purified type II epithelial cells isolated from the adult rat lung.

Received September 26, 1983; accepted March 33, 1984.
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This work was supported in part by Research Center Development Award HL 10124 from the National Institutes of Health and HL 28623 and HD 11725, and from the Children's Hospital Research Foundation, Cincinnati, OH. An abstract of this work was presented at a meeting of the Society for Pediatric Research, Washington, D. C., May 1983.

Muscarinic cholinergic receptor sites are present in rat lung as early as day 17 of gestation. Since preparations of proximal portions of the lung are relatively enriched in (−)-[^{3}H]QNB binding compared to more peripheral portions of the lung, ontogenic decreases in (−)-[^{3}H]QNB binding may result from the higher contribution of tracheal-bronchial tissue compared to alveolar tissue in the preparations of early fetal lung, rather than to a specific regulation of muscarinic receptor sites. (*Pediatr Res* 18:1136–1140, 1984)

Abbreviations

QNB, quinuclidinyl benzilate
EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid

Cholinergic stimulation of pulmonary tissue alters smooth muscle tone, mucous secretion in the tracheal-bronchial tree, and surfactant secretion (1, 7, 8, 17). These effects are presumably mediated by the release of acetylcholine from muscarinic nerve endings and the subsequent activation of pulmonary muscarinic cholinergic receptors. Neural and humoral regulation of pulmonary function is of vital importance to perinatal respiratory adaptation, and dependent in part upon the presence of neurohormonal receptors on their target cells. Developmental aspects of α- and β-adrenergic receptors have been recently demonstrated in pulmonary tissues, both α_1- and β-adrenergic receptor concentrations increasing dramatically during perinatal development in the rat lung (10, 20–22). Ontogenic aspects of muscarinic influences on lung function are less well defined. In the rat, acetylcholinesterase-positive nerve endings have been identified in paratracheal tissue as early as 13 days gestation and in the lung at birth (9, 14). Their density increased during the perinatal period, primarily along bronchi and major blood vessels. Acetylcholinesterase-positive cells were not present in peripheral alveolar tissue in this species (9, 14). Cholinergic agonists increased surfactant secretion in the perfused, purified rat lung but are not effective in isolated type II epithelial cells, supporting the premise that acetylcholine exerts its action in the type II epithelial cell by indirect mechanisms (3). Muscarinic receptor sites were recently identified in canine and bovine tracheal smooth muscle by direct binding studies using (−)-[^{3}H]QNB (5, 16). Binding capacity was significantly higher in tracheal preparations than in bovine lung parenchyma (5). Ontogenic aspects of muscarinic receptors in pulmonary tissues have not been previously determined. In the present study, we describe muscarinic receptors in rat lung and tracheal-bronchial tissue during perinatal development using (−)-[^{3}H]QNB, a potent muscarinic antagonist. Muscarinic-cholinergic receptors were also determined in membrane preparations of fetal and adult rabbit lung.

MATERIALS AND METHODS

Membrane preparations of whole lung homogenates were prepared from fetuses and pups obtained from time-dated Sprague-Dawley rats purchased from Charles River, Inc. Rabbit lung (New Zealand albino) was obtained from fetal (day 25) and adult animals and membranes were prepared as described for the rat. Dams (rat) were kept on 12-h light-dark cycles and routinely delivered at 22 days of gestation. Dams were sacrificed by cervical dislocation. The fetuses or pups were weighed and the lungs were carefully dissected from hilar tissues and placed in iced 250 mM sucrose, 10 mM Tris-HCl (pH 7.2), 1 mM EGTA (STE buffer). Tracheal and proximal bronchial tissue (hilar tissue) were obtained from adult and newborn rats. Nontracheal tissue was carefully dissected from trachea and bronchi by direct visualization. Tissue was washed in the same iced buffer and homogenized in 9 volumes of STE by three 5-s bursts at high setting with a Tekmar Tissuemizer (Cincinnati, OH). The homogenate was filtered through four layers of gauze and centrifuged at 3,000 × *g* for 5 min at 4° C. The resulting supernatant was centrifuged at 40,000 × *g* for 30 min. The resulting pellet was resuspended in iced buffer and centrifuged again at 40,000 × *g* for 30 min. This final crude membrane preparation was frozen in dry ice-acetone and stored at −70° C (−)-[^{3}H]QNB binding to these preparations was not altered during storage for up to 6 months. Prenatal and newborn preparations consisted of lungs pooled from entire litters. Postnatal samples consisted of samples of from two to four rats while adult samples were compared from individual animals. Protein concentrations in the membranes were determined by the method of Lowry using bovine serum albumin as standard (13).

Type II cell preparation. Type II epithelial cells were isolated from 200–250-g male Sprague-Dawley rats as described by Brown and Longmore (3). Cells from four rats were plated for 16 hours and adherent cells separated from the culture dishes in STE buffer. Crude membranes were prepared as above and diluted to 1–2 mg/ml in STE and used in the binding assay. These cells are generally 95% viable cells as assessed by trypan blue exclusion and 90% type II cells as assessed by (phosphine 3R staining and electron microscopy of sample preparations). In our preparations, β-adrenergic agents increase the release of [^{3}H] phosphatidylcholine from type II cells in association with increasing cAMP levels. Crude membranes from these cells also contain β-adrenergic receptors defined with (−)-[^{3}H]dihydroalprenolol.

(−)-[^{3}H]QNB-binding assay. (−)-[^{3}H]Quinuclidinyl benzilate (40.2 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Stock solutions were diluted in distilled water and stored at −30° C prior to use. (−)-[^{3}H]QNB binding was determined in an assay similar to that described by Hardin *et al.* (12). Incubations were performed in a 1-ml assay volume containing 10 mM magnesium chloride, 50 mM Tris-HCl (pH 7.2), and 50–100 μg of lung membrane. Tubes were incubated at 37°C for 90 min in a shaking water bath. The reaction was terminated by filtration on Whatman GFC filters. The assay tube was washed with 4 ml of incubation buffer onto the filter and the tube was rinsed twice with 4 ml of the same buffer at 37° C. Filters were dried and placed in 7 ml of scintillation fluid. Nonspecific binding was determined in the presence of 10^{-6} M atropine. All incubations were performed in duplicate or triplicate. Specific binding was determined by subtracting nonspecific binding (in the presence or absence of 10^{-6} M atropine) from total binding. Receptor number and affinity were determined from saturation experiments using increasing concentrations of (−)-[^{3}H]QNB (10–500 pM) and analyzed by the method of Scatchard (19). Preliminary experiments demonstrated that nonspecific binding increased in a linear fashion with increasing (−)-[^{3}H]QNB concentration. Therefore, in some experiments specific binding was calculated by subtracting binding values obtained from a computer-fit regression line generated from "nonspecific" values obtained from two or three ligand concentrations within the range of the assay.

Competition experiments were determined in the presence of 100–200 pM (−)-[^{3}H]QNB and increasing concentrations of the cholinergic agents. Affinity and slope of the inhibition curves were obtained using the program Direct Fit (11), Significance of differences in binding capacity at various ages was determined by a one-way analysis of variance. Atropine, oxotremorine, methacholine, *d*-tubocurarine, acetylcholine, carbamylcholine, and guanosine triphosphate were obtained from Sigma Chemical Co., St. Louis, MO. Gpp(NH)p was purchased from ICN, Irvin, CA.

RESULTS

(−)-[^{3}H]QNB binding to particulate fractions of neonatal and adult rat lung membranes was time dependent and reversible. Nonspecific binding was less than 20% of total binding near saturation. Specific binding increased in relation to time (Fig. 1), reaching maximal binding within 90 min of incubation at lower

ligand concentrations. Incubation time of 120 min and longer resulted in decreased binding. Specific binding increased in direct proportion to protein (40–200 μg) (Fig. 2). Binding increased nonlinearly in relation to increasing concentrations of (−)-[^{3}H]QNB (Fig. 3*a*). Scatchard analyses of these saturation curves were entirely linear in both fetal and adult samples, suggesting the presence of a single class of binding sites (Fig. 3*b*). Affinity of (−)-[^{3}H]QNB was approximately 70 pM in both fetal and rat lung membranes and did not change during development. In contrast, binding capacity decreased in late gestation, from approximately 79 ± 8.3 fmol·mg^{-1} protein on days 17–18 of gestation reaching adult levels of binding by 21–22 days of gestation, 21 ± 3 fmol·mg^{-1} protein. Binding capacity did not change thereafter (determined on postnatal days 2, 6, 14, and adulthood) (Table 1).

Specificity of the receptor site was assessed by competition experiments with (−)-[^{3}H]QNB and cholinergic agents (Table 2). Cholinergic antagonists inhibited specific (−)-[^{3}H]QNB binding with characteristics of muscarinic cholinergic receptors, atropine being approximately 10,000 times more potent than *d*-tubocurarine. The slopes of inhibition curves for cholinergic antagonists

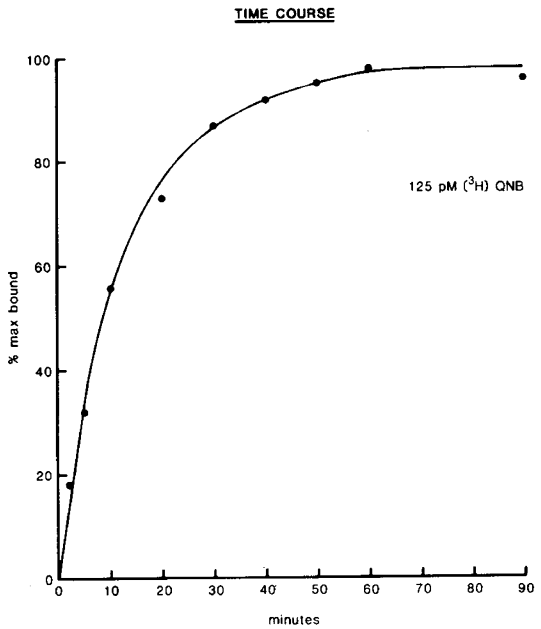

Fig. 1. Time course of specific (−)-[^{3}H]QNB binding to 19-day fetal rat lung membranes. Lung membrane (100 μg) was incubated in the presence of 125 pM (−)-[^{3}H]QNB in the presence or absence of 10^{-6} atropine as described in "Materials and Methods" for 1–90 min. Specific binding was determined in duplicate assays at each time point.

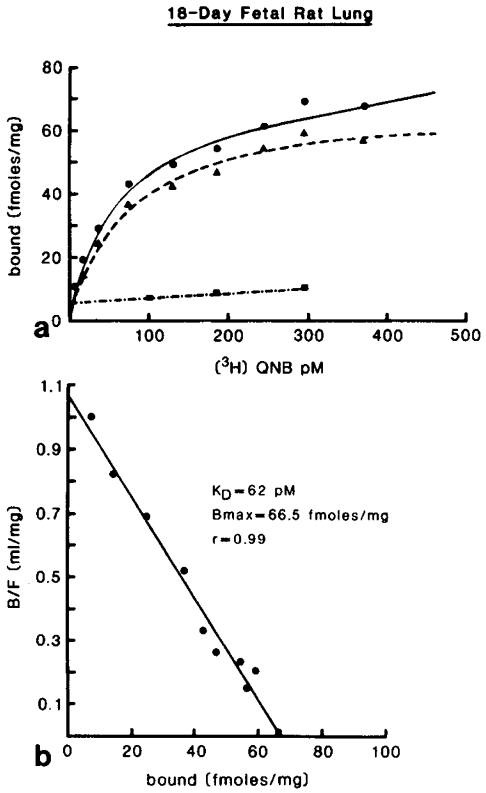

Fig. 3. Saturation experiment and Scatchard plot of (−)-[^{3}H]QNB binding to lung membranes from day 18 of gestation. Specific and nonspecific (−)-[^{3}H]QNB binding was determined using approximately 100 μg membrane protein and 10–500 pM (−)-[^{3}H]QNB as described in "Materials and Methods." Scatchard plot of the saturation data in *a*.

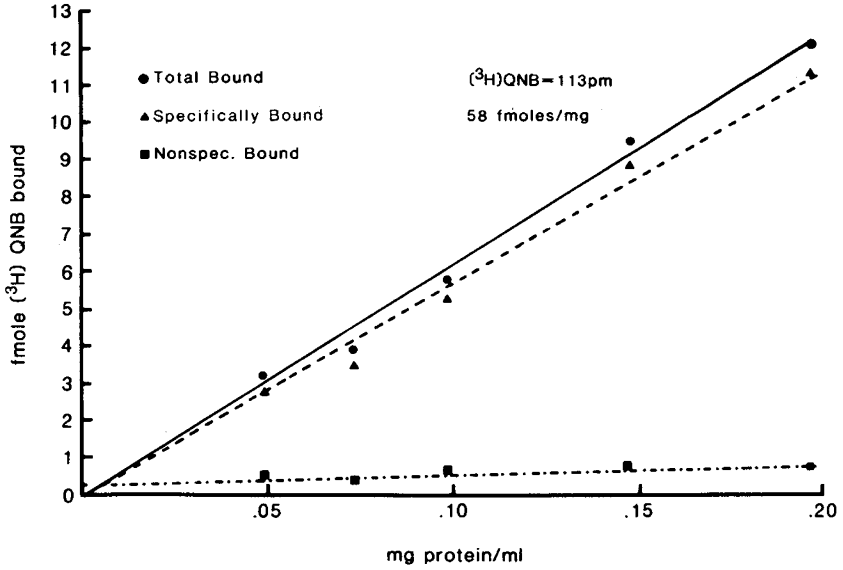

Fig. 2. Relationship between (−)-[^{3}H]QNB binding and membrane protein. Membrane (0–200 μg) from a 19-day gestation fetal rat lung was incubated with (−)-[^{3}H]QNB in the presence and absence of 10^{-6} M atropine for 90 minutes at 37° C as described in "Materials and Methods." Binding capacity in this preparation was 58 fmol·mg^{-1} protein.

Table 1. *(−)-[³H]QNB-binding capacity and affinity in crude lung and tracheal-bronchial membranes**

	n	(−)-[³H]QNB-binding capacity (fmol·mg⁻¹ protein)	K_D (pM)
Fetal, days 17–18	4	79 ± 8	71 ± 16
Fetal, day 19	3	49 ± 10	62 ± 19
Fetal, days 21–22	5	21 ± 3	74 ± 19
Adult	4	20 ± 3	70 ± 14
Newborn tracheal-bronchial (hilar) membranes	1	90	28
Adult tracheal-bronchial	5	68 ± 17	75 ± 16

* Binding capacity and affinity constant (K_D) were determined in membrane preparation of lung or tracheal-bronchial tissue by saturation experiments as described in "Materials and Methods." Tracheal-bronchial tissue was dissected from other hilar tissue in adult samples under direct visualization. Tracheal-bronchial tissues from an entire litter of newborn pups were pooled and included other hilar tissue which could not be clearly dissected from the major bronchi. Values are mean ± SEM of multiple preparations (n). Statistical differences were determined by one-way analysis of variance. Age-related differences were significant at $p < 0.001$. Binding to adult lung membranes were significantly less than to adult tracheal-bronchial membranes, $p = 0.007$.

Table 2. *Competition experiments with cholinergic agents and (−)-[³H]QNB with adult rat lung membranes**

Cholinergic agent	K_I(M)	Slope
Atropine	$4.8 \pm 1.7 \times 10^{-9}$	0.86 ± 0.1
Oxotremorine	$2.2 \pm 0.20 \times 10^{-7}$	0.53 ± 0.06
Oxotremorine (Gpp(NH)p)	$2.5 \pm 0.69 \times 10^{-7}$	0.46 ± 0.05
Methacholine	$3.1 \pm 1.0 \times 10^{-6}$	0.54 ± 0.004
Methacholine (GTP)	$4.7 \pm 1.5 \times 10^{-6}$	0.54 ± 0.08
Tubocurarine	$3.8 \pm 1.0 \times 10^{-5}$	0.84 ± 0.1
Acetylcholine ($n = 1$)	3.2×10^{-6}	0.52
Carbamylcholine ($n = 1$)	2.1×10^{-6}	0.48

* Increasing concentrations of cholinergic agents (10^{-11}–10^{-3} M) were added to incubations with rat lung membrane and 100 pM (−)-[³H]QNB as described in "Materials and Methods." The K_I and slope of the inhibition curves were determined using the program Direct Fit. Values are mean ± SD of three experiments with each agent except where indicated $n = 1$. Gpp(NH)p and GTP were added at 10 μM where indicated.

were approximately 1.0 in the presence or absence of 10 μM GTP (Fig. 4). Cholinergic agonists also inhibited (−)-[³H]QNB binding in the order of potency oxotremorine > methacholine = acetylcholine = methacholine = carbamylcholine (Table 2). Slopes of inhibition of (−)-[³H]QNB binding observed with the agonists were considerably less than 1, ranging from 0.48–0.53, Fig. 4). Addition of 10 μM GTP or 10 μM Gpp(NH)p did not affect either the affinity or slope of competition of (−)-[³H]QNB binding by either oxotremorine or methacholine (Table 2).

While the specific activity of lung (−)-[³H]QNB-binding sites decreased significantly in late gestation, it was unclear whether this related to specific regulation of muscarinic receptors in the target tissue or to changes in the growth and distribution of tracheal-bronchial as compared to alveolar tissues. The specific activity of (−)-[³H]QNB binding to tracheal-bronchial tissue was therefore assessed in both neonatal and adult samples. (−)-[³H]QNB bound to tracheal membranes with characteristics identical to those observed in lung preparation. Specific activity was higher in adult tracheal-bronchial membranes, approximately 70 fmol·mg⁻¹, than in adult rat lung membrane, 20 fmol·mg⁻¹. Likewise, (−)-[³H]QNB-binding activity was higher in tracheal-bronchial tissues than in whole membranes from the newborn rat lung. Dissection of adequate tracheal-bronchial tissue from prenatal samples was not feasible due to small tissue size. Thus, proximal

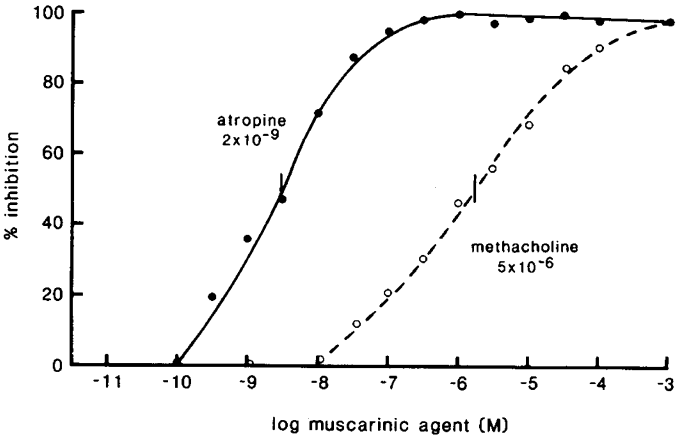

Fig. 4. Inhibition of (−)-[³H]QNB binding by atropine and methacholine. Membrane (100 μg) was incubated for 60 min at 37° C in the presence of 100 pM (−)-[³H]QNB and increasing concentrations of atropine or methacholine. K_D is indicated for each agent. The slope of the inhibition curve was 1.03 for atropine and 0.56 for methacholine. Neither GTP nor Gpp(NH)p (10 μM) altered the slope.

airway tissues appear to contain significantly more muscarinic cholinergic receptor sites than parenchymal-alveolar tissue in rat lung. To further address specific cellular localization of (−)-[³H]QNB-binding sites in alveolar tissues, (−)-[³H]QNB binding was assessed in membrane preparations of purified type II epithelial cells after isolation and tissue culture using the method of Brown and Longmore, as previously described (3, 23). Specific (−)-[³H]QNB binding could not be detected in membrane preparations of type II epithelial cells in multiple experiments. In contrast, membranes from these cells contained β-adrenergic receptors (demonstrated with (−)-[³H]dihydroalprenolol), increased intracellular cAMP concentrations and secreted phosphatidylcholine in response to β-adrenergic stimulation with terbutaline.

In order to test for possible species differences in muscarinic receptor activity, (−)-[³H]QNB binding was assessed in fetal and adult rabbit lung by saturation experiments in membrane prepared as described in "Materials and Methods." (−)-[³H]QNB-binding capacity was considerably higher in rabbit than in rat lung (148 ± 20 fmol·mg⁻¹ protein; K_D = 135 ± 13 pM; mean ± SEM; $n = 5$) and did not differ from that in fetal rabbit lung on day 25 of a 32-day gestation, (122 ± 12 fmol·mg⁻¹ protein; K_D = 167 ± 19 pM; $n = 4$ separate preparations).

DISCUSSION

The present study demonstrates muscarinic-cholinergic receptors in membrane preparations of rat lung and tracheal-bronchial tissue during perinatal development. Both rat lung and tracheal-bronchial tissues contained muscarinic-cholinergic receptor sites characteristic of those in other tissues, including canine tracheal smooth muscle (5, 16). In the rat, binding capacity for (−)-[³H]QNB decreased in late gestation. Specific activity of (−)-[³H]QNB binding in rabbit lung membranes was considerably higher than in those from rat and age-related differences were not detected between preparations of fetal (day 25 of gestation) and adult animals. Specific activity of (−)-[³H]QNB binding was higher in tracheal-bronchial tissue than in more distal portions of the adult and newborn rat lung parenchyma. Crude membranes from purified rat type II epithelial cells did not contain detectable (−)-[³H]QNB-binding sites.

In the rat, characteristics of (−)-[³H]QNB binding in these tissues were similar to those previously reported in tracheal smooth muscle, heart, and brain. However, binding capacity was relatively low in lung membrane compared to other tissues (5, 6, 12, 24). Affinity of (−)-[³H]QNB for muscarinic-cholinergic receptors in both fetal and adult lung was estimated from saturation experiments and was similar to that previously described (5, 16,

18, 24). Binding appears to occur to a single class of binding sites and competition experiments with muscarinic-cholinergic agents demonstrated an order of potency characteristic of muscarinic, rather than nicotinic, receptor sites. The slope of inhibition of (−)-[^{3}H]QNB binding by antagonists was approximately 1, consistent with interaction of a single class of receptor sites. In contrast, the slopes of competition experiments with cholinergic agonists were significantly less than 1 and were unaltered by the addition of GTP or its nonhydrolyzable analogue Gpp(NH)p. Guanine nucleotide regulation of muscarinic receptor affinity has been previously demonstrated in both heart and brain (18, 24). Guanine nucleotide effects on bovine tracheal muscarinic receptors appear complex, altering both (−)-[^{3}H]QNB binding (antagonist) and agonist affinity; effects of guanine nucleotides were small and potentiated by Mg^{2+} (6). We are unable to identify changes in either affinity or slope of the inhibition of binding by agonists or antagonists in the presence of guanine nucleotide. This might be related to excessive GTP hydrolysis during the assay; however, agonist affinity was not significantly altered by Gpp(NH)p, its nonhydrolyzable analogue.

Demonstration of increased muscarinic-cholinergic receptor sites in tracheal-bronchial tissue, as compared to lung parenchymal tissue, correlates well with the demonstration of acetylcholinesterase staining in histochemical studies of rat lung and trachea during development (9, 14, 15). In those studies, acetylcholinesterase-positive nerves were identified as early as day 18 of gestation and were located primarily along tracheal and bronchial smooth muscle in the rat (9). Neuroepithelial bodies were also acetylcholinesterase positive in the rat lung (14). Recent studies with bovine lung and trachea support this distribution, (−)-[^{3}H]QNB binding being much greater in bovine tracheal as compared to parenchymal membrane preparations (5). Recent autoradiographic studies of muscarinic receptors in ferret lung demonstrated (−)-[^{3}H]QNB binding primarily in trachea, cartilagenous airways, and submucosal glands and lacking in alveolar structures (2). The apparent lack of muscarinic-cholinergic receptor sites in type II epithelial cells presently described supports previous studies documenting the absence of surfactant secretion after cholinergic stimulation in isolated type II cells (3). The observation that pilocarpine-induced surfactant secretion in the perfused rat lung was blocked by treatment with propanolol supports the hypothesis that cholinergic stimulation of surfactant release is mediated by activation of the β-adrenergic system rather than by direct cholinergic stimulation of the type II epithelial cells (3).

The ontogenic decrease in muscarinic cholinergic receptor sites in developing rat lung was not observed in rabbit lung (day 25 of gestation and adult) and contrasts sharply with the increases in α_1- and β-adrenergic receptor sites which occur in both rat and rabbit lung during perinatal development. β-Adrenergic receptor sites increase during the perinatal period in the rat and rabbit lung and these increases are apparently mediated by both corticosteroids (prenatally) and thyroxine (postnally) in the developing lung (4, 10, 20–22). There is evidence that developmental changes in acetylcholinesterase-positive pulmonary cells are also hormonally regulated, being enhanced by thyroxine *in vitro* (15). While it is possible that the ontogenic decreases in cholinergic receptors in developing rat lung occur on individual pulmonary cells, our data are compatible with the concept that muscarinic receptors are located primarily in the tracheal-bronchial tree rather than in the parenchymal (alveolar) components of the lung. Growth of the trachea and bronchi of the rat lung proceed much more rapidly than alveolar components during fetal life, the latter proliferating during the neonatal and postnatal periods. Thus, in fetal preparations from 17–18 days of gestation, the greater contribution of bronchial or other hilar tissues to lung membrane preparations may account for their increased (−)-[^{3}H]QNB-binding capacity compared to samples from older animals.

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