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Muscarinic receptor subtypes in the porcine lung during postnatal development

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Abstract

The responsiveness of the pulmonary circulation to acetylcholine changes in the newborn piglet. Therefore muscarinic receptors have been studied in the developing porcine lung from birth to adulthood using ligand binding, Northern blotting and in situ hybridisation. Maximal binding capacity of [*N*-methyl-^{3}H] scopolamine and the affinity of the receptor in lung membranes increased between birth and 16 days ($p < 0.05$). Subtype affinity changed with age, but always $M_3 > M_1 > M_2$. Northern blots of porcine muscarinic receptor subtypes showed m1, m2 and m3 mRNA present in lung membranes. m2 mRNA was present at all ages and decreased with age. m1 mRNA was absent at birth and m3 mRNA was absent at 3 days. Autoradiographic localisation showed ligand binding to the parenchyma and airway smooth muscle and nerves, there was no binding to intrapulmonary vessels. In situ hybridisation localised mRNA of all three subtypes to the smooth muscle cells of both vessels and airways. Changes in the receptor subtypes may explain the pharmacological changes during postnatal adaptation.

Keywords: Lung development; Muscarinic receptor subtype; Pulmonary artery; In situ hybridisation; Ligand binding

1. Introduction

Adaptation of the pulmonary circulation to extra-uterine life involves a decrease in vascular resistance with an increase in flow. Previous studies have shown rapid remodelling of the endothelial and smooth muscle cells during this period (Haworth and Hislop, 1981; Hall and Haworth, 1986). More recent physiological and pharmacological studies have shown maturational changes in response to vasodilators in late fetal life and early infancy in lambs, piglets and other animals (Shaul et al., 1992; Perreault and De Marte, 1993; Kinsella et al., 1994). Acetylcholine stimulates the production of nitric oxide via muscarinic receptors in both systemic and pulmonary arteries (Eglen and Whiting, 1990). This response is thought to be endothelium dependent and in adult man, pulmonary arterial activation is via the M_3 muscarinic receptor subtype (McCormack et al., 1988). Studying porcine pulmonary arteries we found that the relaxant response to acetylcholine was absent at birth and present from three days of age, when it was endothelium dependent (Liu et al., 1992). We have also demonstrated a contractile response to acetylcholine in newborn pulmonary arteries at all concentrations (Tulloh et al., 1997) irrespective of the presence of the endothelium. By three days of age, arteries contracted in response to acetylcholine when endothelium was absent and by six days no contraction was found.

Muscarinic receptors in the lung have been classified into four major subtypes (M_1–M_4) on the basis of their antagonist affinities (Caulfield, 1993). The molecular cloning of five muscarinic receptor genes (m1–m5) has provided the molecular basis of muscarinic receptor subtypes though m5 does not have a functional correlate (Hulme et al., 1990). It has been suggested that muscarinic receptor subtypes M_1 and M_3 are present in the vasculature (Eglen and Whiting, 1990). In man, stimulation of muscarinic M_3 muscle receptors leads to contraction of isolated pulmonary arteries while muscarinic M_1 and M_3 receptor stimulation in the presence of endothelium leads to relaxation (Norel et al., 1996). In rabbits, muscarinic M_3 receptor stimulation leads to an endothelium dependent contraction at resting tone but a relaxation at elevated tone

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(Altiere et al., 1994). Until now no data are available on the constitutive expression of these muscarinic receptor subtypes immediately after birth and because stimulation of muscarinic receptors leads to both relaxation and contraction in the unstable transitional circulation we studied the expression of muscarinic receptor subtypes in the developing porcine lung using receptor ligand binding, Northern blot analysis, and in situ hybridisation.

2. Materials and methods

Lung tissue was obtained from 51 Large White pigs aged less than 5 min (newborn), 3 days, 16 days, and 6 months (adult). Adult pig tissue was obtained from a local abattoir. The piglets were delivered in our laboratory. The young animals were kept in accordance with NIH regulations outlined in the Guide for the Care and Use of Laboratory Animals and were killed in accordance with British Home Office regulations and the approval of the Animal Ethics Committee of the Institute of Child Health. Within 1 h of death peripheral lung tissue which contained no large airway structures was snap frozen in liquid nitrogen and stored at −70°C. In addition, blocks of lung tissue and extrapulmonary arteries, together with aorta, main bronchus and atrium were surrounded by embedding compound (OCT Brights, Cambridge, UK), on cork discs and snap frozen in melting dichlorodifluoro-methane (Arcton) cooled in liquid nitrogen. The blocks were then stored at −70°C. Subsequently, serial 10-μm cryostat sections were cut from each block and thaw mounted on glass slides coated with Vectabond (Vector Laboratories, Peterborough, UK). Slides were stored in sealed boxes with desiccant at −70°C.

Table 1
Inhibition of ^{3}H-NMS binding to pig peripheral lung membrane by muscarinic antagonists

	IC_{50}(nM)	n_H	pK_i
Telenzepine			
Newborn	69 (9.1)[a]	1.04 (0.13)	7.46 (0.09)
3 day	57 (6.2)	0.87 (0.08)	7.67 (0.06)
16 day	50.2 (5.1)	0.99 (0.04)	7.78 (0.04)
Adult	56 (5.0)	1.01 (0.04)	7.82 (0.06)[b]
Methoctramine			
Newborn	171 (29)	0.89 (0.1)	7.08 (0.12)
3 day	204 (20)	0.95 (0.08)	7.26 (0.16)
16 day	174 (26)	0.93 (0.09)	7.29 (0.06)
Adult	592 (81)	1.13 (0.19)	6.8 (0.04)[c]
4-DAMP			
Newborn	19.3 (2.1)	1.01 (0.09)	7.99 (0.04)
3 day	24 (3.1)	0.83 (0.09)	7.98 (0.11)
16 day	25 (3.1)	0.87 (0.08)	8.14 (0.09)
Adult	29 (3.3)	0.98 (0.11)	8.12 (0.07)

[a]Mean and standard error of the mean, $n = 4$ for each age; [b]$P < 0.05$ in comparison with newborn; [c]$P < 0.01$ in comparison with 16 days; n_H = Hill coefficient.

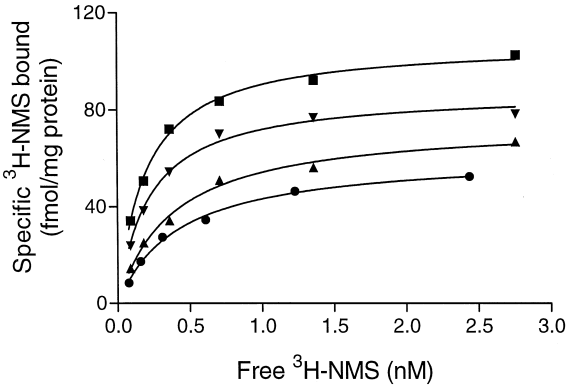

Fig. 1. Saturation isotherms for specific [*N*-methyl-^{3}H]scopolamine binding in pig lung during development. Lung membranes from newborn (●), 3 days (▲), 16 days (▼) and adult (■) were incubated with various concentrations of [*N*-methyl-^{3}H]scopolamine for 2 h at 25°C in the absence (total binding) or presence of 1 μM atropine (non-specific binding). These curves are from a single representative experiment and are the computer generated best fit lines by least-squares non-linear regression analysis.

2.1. Receptor ligand binding studies

2.1.1. Binding to lung membranes

Peripheral lung tissue was homogenised in 10 volumes of ice cold 0.32 M sucrose, 25 mM tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.4) using a Polytron homogeniser (10–15 quick bursts). After centrifugation at $1000 \times g$ for 10 min at 4°C, the pellets were discarded and the supernatants were centrifuged at $40\,000 \times g$ for 20 min at 4°C. The resulting pellets were mashed once and recentrifuged at the same speed. The final pellets were resuspended in incubation buffer (25 mM Tris buffer, pH 7.4 at 25°C) and stored as aliquots at −70°C. Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumen as standard.

For saturation studies the binding reaction was carried out with 0.25 ml of 25 mM Tris buffer (pH 7.4) containing [*N*-methyl-^{3}H]scopolamine (New England Nuclear, Hounslow UK) at increasing concentrations from 0.06–2 nM with appropriate amounts of membrane (approximately 300 μg/ml). Incubations were performed at 25°C for 2 h and terminated by rapid vacuum filtration over 0.2% polyethyleneimine pre-treated GF/C glass fibre filters (Whatman) using a Brandel cell harvester. Binding was counted in a Packard beta counter. Non-specific binding was defined as the binding in the presence of 1 μM atropine. The maximal binding sites (B_{max}) and the equilibrium dissociation constants (K_d) were determined by Scatchard analysis using the LIGAND programme for each case. For competition studies binding of 0.5 nM [*N*-methyl-^{3}H]scopolamine was made in the presence of the relatively selective antagonists, telenzepine (M_1), methoctramine (M_2) and 4-diphenyl acetoxy-*N*-methyl piperidine (4-DAMP) (M_3) at concentrations 0.01 nM to 0.1 mM.

Table 2
Dissociation constants (pK_d) and maximum binding capacity (B_{max}) for porcine peripheral lung membranes

Age	Newborn	3 day	16 day	Adult
n	7	12	5	9
pK_d	9.24 (0.1)[a]	9.49 (0.03)	9.65 (0.05)[b]	9.63 (0.05)[b]
B_{max} (fmol/mg protein)	51.1 (5.8)	69.6 (4.0)	82.5 (8.8)[c]	101.3 (8.6)[d]

n = number of cases; [a]mean and standard error of the mean; [b]$P < 0.01$ in comparison with newborn; [c]$P < 0.05$ in comparison with newborn; [d]$P < 0.01$ in comparison with newborn.

Competition binding data were analysed using the PRISM programme (Graphpad) to provide IC_{50} values and Hill coefficients (n_H) for competing compounds. The K_i values were established after correcting for the presence of radioligand according to the method of Cheng and Prusoff (1973) by PRISM programme. In Table 1 where pK_i values (i.e., $-\log IC_{50}$) are presented, the mean and standard error of the mean were calculated from the individual pK_i values. The mean and standard error for each age group were compared using Analysis of Variance (ANOVA) and Student's *t*-test. When more than one comparison was made, Bonferroni's correction was made. Differences were considered significant when $P < 0.05$.

2.1.2. Binding to lung sections

Sections of lung, bronchus, atrium and extrapulmonary artery were brought to room temperature and pre-incubated in 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl, 5 mM $MgCl_2$, 40 mg/l bacitracin and 1% bovine serum albumin for 15 min at room temperature. Sections were then incubated in 1 nM [^{3}H]quinuclidinyl benzilate made up in Tris–HCl buffer for 90 min and also in the presence of excess antagonists, either atropine (1 μM), telenzepine (100 nM), methoctramine (100 nM) or 4-DAMP (10 nM) to demonstrate the muscarinic receptor subtypes. In addition sections were incubated in 10 nM [^{3}H]pirenzepine (a relatively selective M_1 ligand). Incuba-

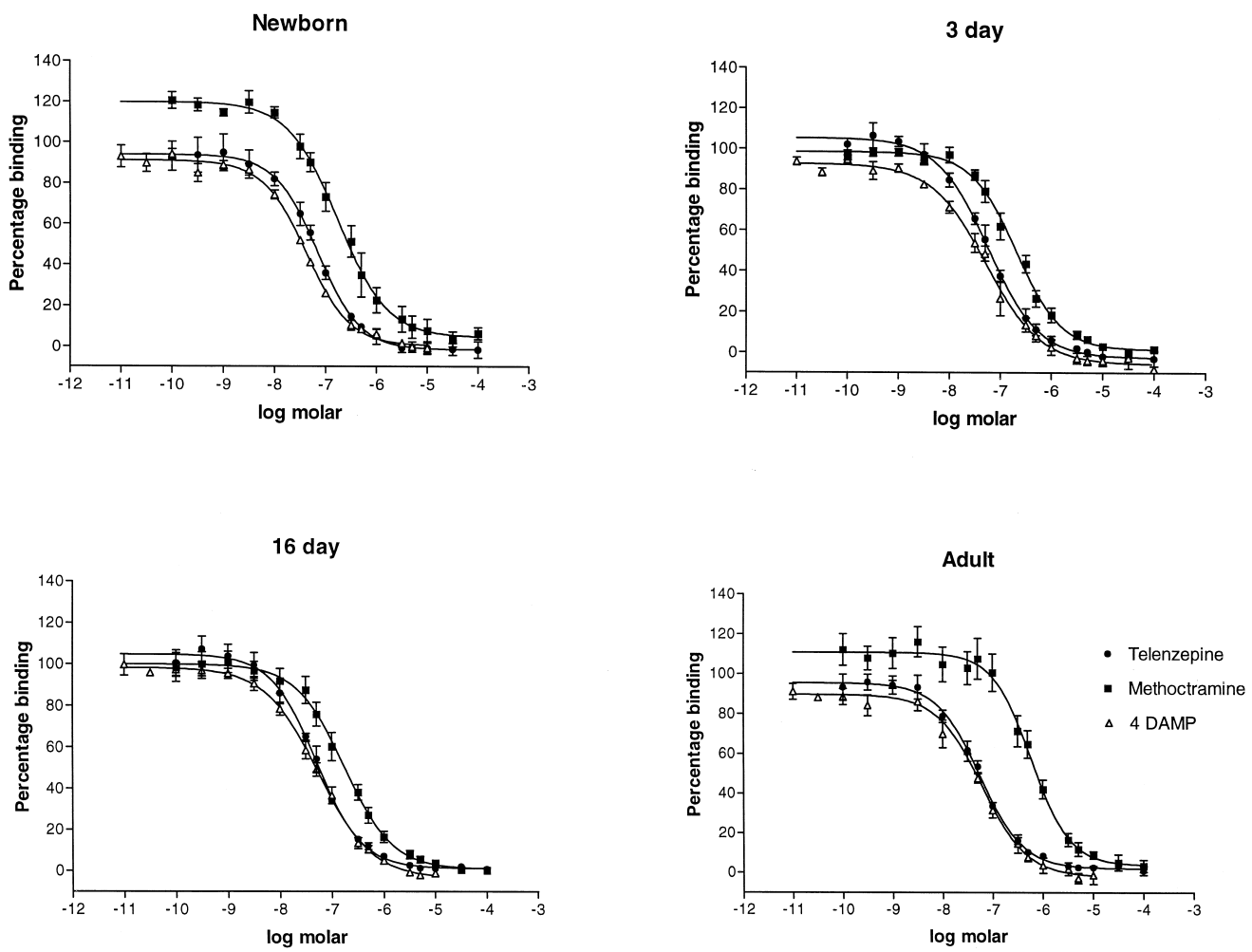

Fig. 2. Inhibition of [*N*-methyl-^{3}H]scopolamine (0.5 nM) binding by muscarinic M_1-selective antagonist, telenzepine; muscarinic M_2 selective antagonist, methoctramine; and muscarinic M_3-selective antagonist, 4-DAMP in peripheral lung from newborn, 3-day-old, 16-day-old and adult pigs. Values are mean and standard error of the mean for four animals at each age and each antagonist performed in duplicate on separate membrane preparations.

tion was terminated by washing in buffer at 4°C (two changes of 5 min each), the sections rinsed in distilled water at 4°C for 2 min and then dried rapidly in cold air. Sections were exposed to Hyperfilm ^{3}H (Amersham, UK) for up to 4 months at 4°C and then developed in Kodak D19 developer for 5 min at 18°C and fixed in Amfix.

2.2. Probe labelling

2.2.1. RNA probes

Porcine muscarinic receptor cDNAs (m1, m2 and m3) were generously provided by Dr T Kubo, Kyoto, Japan. A *Pst*I/*Sac*I fragment (241 bp) of m1 cDNA, a *Sma*I/*Sac*I fragment (327 bp) of m2 cDNA and a *Pst*I/*Hin*dIII fragment (241 bp) of m3 cDNA, corresponding to the coding region, were subcloned into pGEM-3Z vector, respectively. The plasmid DNAs linearized with *Eco*RI or *Hin*dIII were used as templates to generate antisense and sense riboprobes by in vitro transcription in the presence of SP6 or T7 RNA polymerase (Promega; Southampton, UK) and [^{35}S]UTP (1000 Ci/mmol; Amersham, UK). The specifications of the reaction were according to Promega recommendations.

2.2.2. DNA probes

A *Sac*I fragment (797 bp) of m1 cDNA, a *Bam*HI/*Sma*I fragment (1048 bp) of m2 cDNA, a *Xba*I/*Pst*I fragment (883 bp) of m3 cDNA and a *Pst*I fragment (1200 bp) of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) corresponding to the coding region were cut out. The cDNA fragments were labelled with [^{32}P]dCTP by the random primer labelling kit (Amersham, UK).

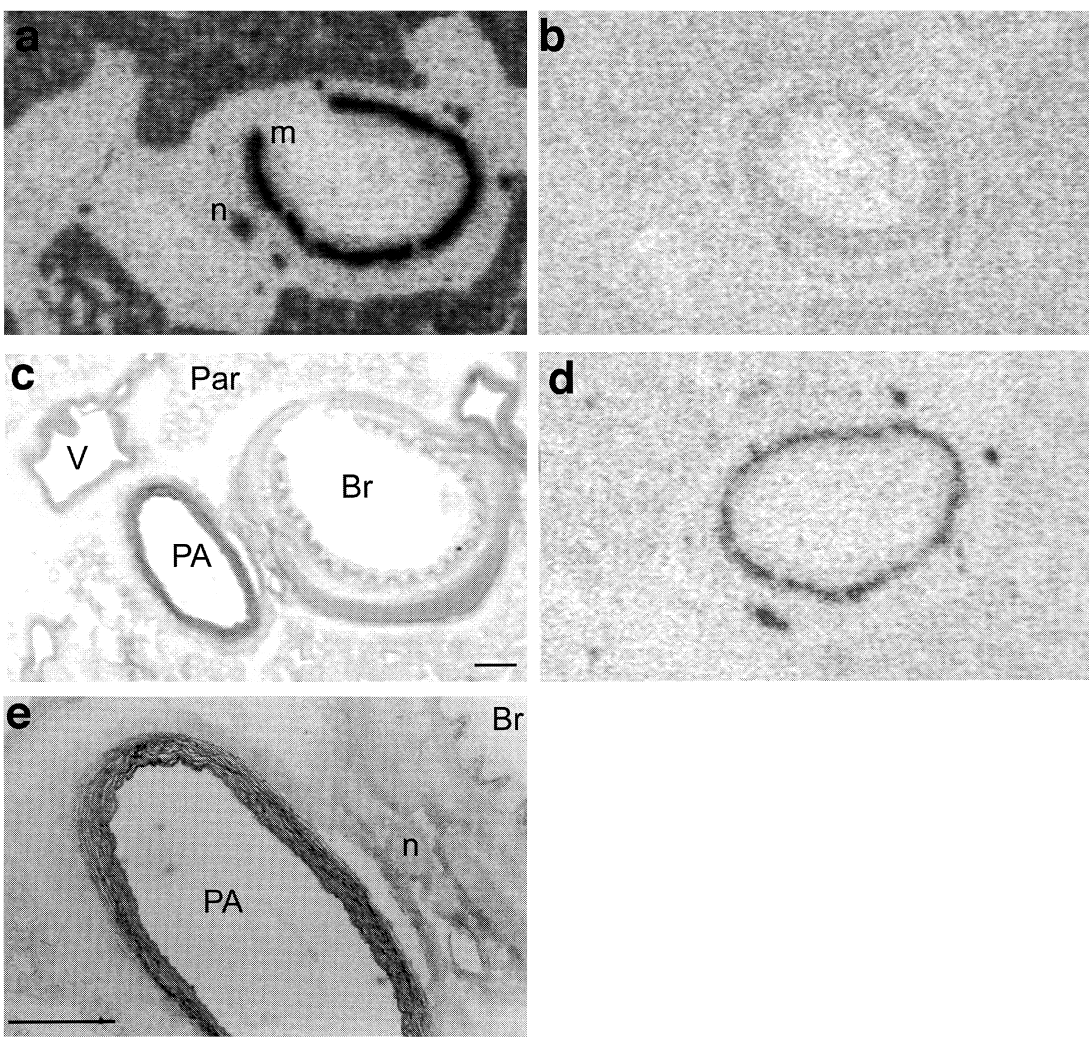

Fig. 3. Images of autoradiographs of newborn lung sections after incubation with [^{3}H]quinuclidinyl benzilate or [^{3}H]pirenzepine and exposure to X-ray film for 10 weeks. (a) [^{3}H]quinuclidinyl benzilate with Tris buffer. Positive binding is seen on parenchyma, bronchial smooth muscle and nerves (compare with (e)). (b) [^{3}H]quinuclidinyl benzilate in the presence of atropine (1 μM) binding is displaced. (c) Lung section stained with elastic van Gieson at the same magnification to show lung structure. (d) [^{3}H]pirenzepine with Tris buffer. Positive binding is seen over bronchial smooth muscle and nerves. (e) Photomicrograph of the artery and airway seen in (a) and (c) with a nerve bundle between. There is no specific binding over the pulmonary artery or vein with [^{3}H]quinuclidinyl benzilate or [^{3}H]pirenzepine. Bar = 500 μm. PA = pulmonary artery, Br = bronchus, V = vein, Par = parenchyma, n = nerve, m = muscle.

2.3. Northern blot analysis

Total RNA was isolated from homogenised frozen peripheral lung tissues by guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was obtained by using PolyAT tract mRNA Isolation System (Promega). Samples of poly(A)$^+$ RNA were size fractionated on a 1% denaturing gel and blotted onto Hybond-N membranes (Amersham) by capillary action. Blots were prehybridised for 4 h at 42°C in hybridization buffer containing 5 × standard saline citrate (SSC; 1 × SSC—0.15 M NaCl, 0.015 M trisodium citrate), 50 mM Na_2HPO_4, 0.1% sodium dodecyl sulphate (SDS), 250 μg/ml sonicated denatured salmon sperm DNA and 50% formamide. Labelled cDNA probes ($(1–2) \times 10^6$ cpm/ml) were then added. Hybridization was carried out at 42°C overnight. Blots were washed thoroughly to a high stringency of 0.1 × SSC/0.1% SDS at 55°C, before apposition to Kodak OMAT-XS film for 3–7 days. After exposure, blots were stripped before subsequent rehybridization. Autoradiographs were scanned with a densitometer where appropriate (Quantity One Software, PDI, NY, USA). The ratio of the receptor subtype to GAPDH was calculated and the mean established for each age group. These were compared using Student's *t*-test.

2.4. In situ hybridization

After rinsing in phosphate buffered saline (PBS) frozen tissue sections were fixed in 4% paraformaldehyde for 60 min and permeabilized in 0.3% Triton X-100 in phosphate buffered saline for 15 min. 10 μg/ml proteinase K in 10 mM Tris–HCl at pH 8.0 in 5 mM EDTA was used for deproteinization for 7.5 min. After refixing in 4% paraformaldehyde/PBS for 5 min, acetylation was made in 0.1 M triethanolamine/0.25% acetic anhydride for 10 min, followed by dehydration through ethanol. After this pretreatment the sections were hybridised in buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 10 mM NaH_2 PO_4 (pH 8.0), 10% dextran sulphate, 1 × Denhardt's solution and 500 μg/ml yeast RNA with m1, m2 and m3 sense and antisense RNA probes (1×10^6 cpm/section) in a humid chamber at 50°C overnight. Some sections were pre-treated with RNase A solution (20 μg/ml) at 37°C for 1 h prior to hybridisation to use as negative controls.

After hybridisation the unhybridised single stranded RNA probes were removed with 20 μg/ml RNase A solution in 4 × standard sodium citrate (SSC) for 30 min and then sections were washed in decreasing concentrations of SSC at 37–50°C. Sections were dehydrated through ethanol containing 0.3 M ammonium acetate and air-dried. Dry sections were dipped in liquid emulsion (Ilford K-5) at 40°C, and exposed for 10 days in light-tight boxes at 4°C. Sections were then developed in Kodak D19 developer for 5 min at 20°C and fixed in Amfix.

3. Results

3.1. Receptor ligand binding studies

3.1.1. Peripheral lung membranes

The binding of [*N*-methyl-^{3}H]scopolamine to membranes showed that the binding of this non-selective antagonist was specific and saturable (Fig. 1). Non-specific binding represented about 5% of total binding. Scatchard analysis showed a linear plot for all age groups. The pK_d increased with age and the newborn value was significantly lower than that for the 16 day and adult tissue

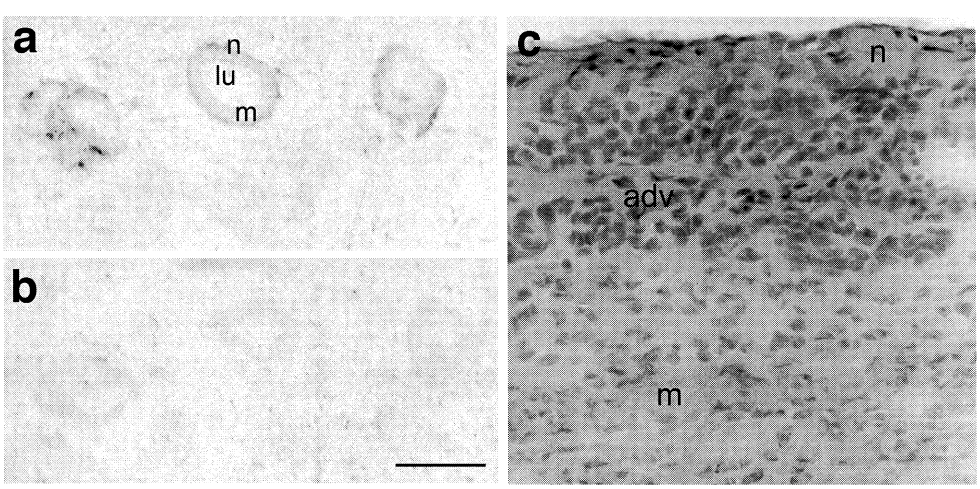

Fig. 4. Images of autoradiographs of extrapulmonary arteries from 3-day-old piglets after [^{3}H]quinuclidinyl benzilate incubation and exposure to X-ray film for 4 months. (a) [^{3}H]quinuclidinyl benzilate with Tris buffer. There is some binding to the outside of the media and in nerves. (b) [^{3}H]quinuclidinyl benzilate with atropine (1 μM) all binding is displaced. (c) Photomicrograph of the outside of the arterial wall stained with haematoxylin and eosin showing nerves in the adventitia. Bar = 8 mm, for (a) and (b), bar = 40 μm for (c). n = Nerves, lu = lumen, m = media, adv = adventitia.

($P < 0.01$) (Table 2). The increase in affinity was associated with a significant increase in maximal binding capacity (B_{max}) between newborn and 16 days. The competitive binding studies showed that with the relatively selective muscarinic receptor M_1, M_2 and M_3 antagonists there was a steep inhibitory curve at all ages which could be described by a single binding site model and a Hill coefficient close to unity (Table 1). At all ages, the pK_i values showed that the greatest affinity was for 4-DAMP followed by telenzepine and then methoctramine (Fig. 2). The pK_i value for telenzepine at birth was significantly lower than in the adult, suggesting lower affinity at birth (Table 1). The pK_i values for methoctramine were higher in the young cases than the adult and there was a significant decrease in the pK_i between 16 days and adulthood (Table 1). The pK_i values for 4-DAMP remained similar at all ages.

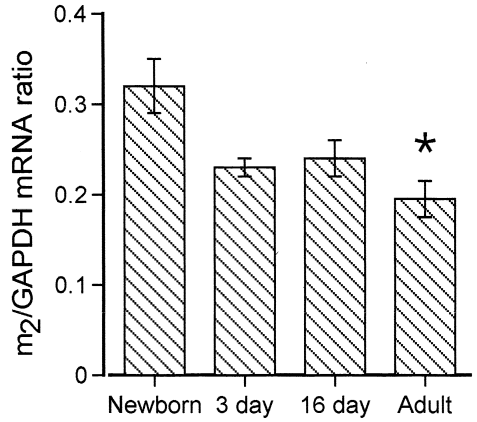

Fig. 6. The ratio of density of m2 mRNA/GAPDH (mean and standard error) on Northern blots for newborn ($n = 7$), 3 day ($n = 7$), 16 day ($n = 6$) and adult ($n = 6$). The adult has significantly less m2 mRNA than the newborn ($P < 0.05$).

3.1.2. Location of binding

Lung sections incubated in [^{3}H]quinuclidinyl benzilate, and exposed to X-ray film for 10 weeks showed binding to the peripheral parenchymal region and dense binding over bronchial smooth muscle and individual nerve bundles (Fig. 3a and c). All binding was displaced by incubating in the presence of atropine (Fig. 3b). No binding was demonstrated over the large intrapulmonary arteries or veins. By exposing the X-ray films for four months binding was demonstrated on the extrapulmonary arteries (Fig. 4), where a low density of binding was present over the outer half of the media. There was no evidence of binding over the endothelium. There were discreet regions around the outside of the media with a high density of binding (Fig. 4a). These regions corresponded with nerves in the adventitia (Fig. 4c). Co-incubation with atropine reduced the density of binding over the media and displaced binding over the nerves (Fig. 4b). Incubation in the presence of the antagonists telenzepine, methoctramine or 4-DAMP did not alter the binding pattern to the extrapulmonary arteries. However, on lung sections there was a reduction in binding to the parenchyma and bronchial smooth muscle in the presence of methoctramine suggesting the presence of muscarinic M_2 receptors.

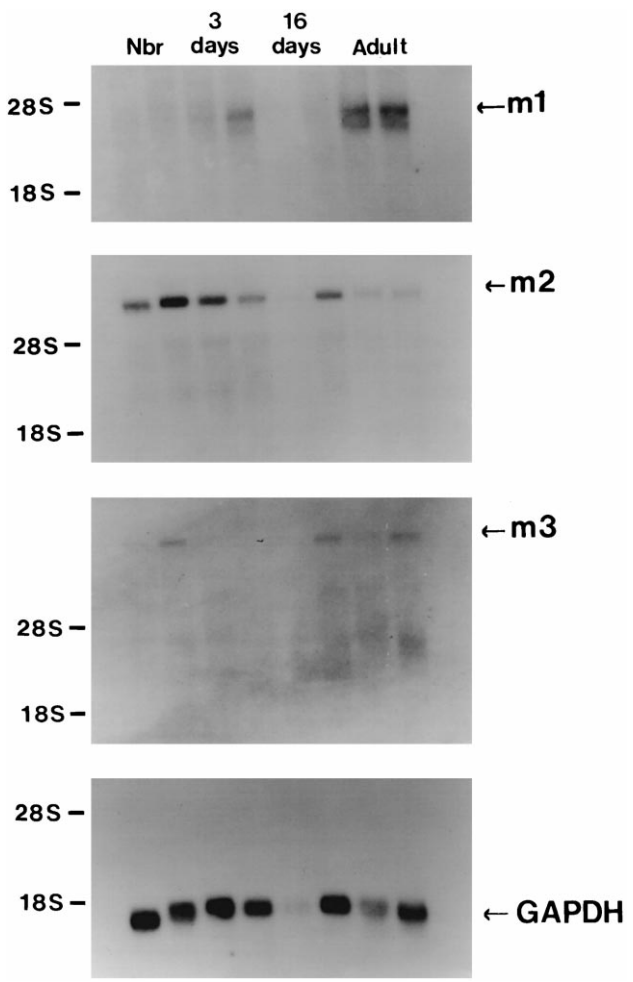

Fig. 5. Representative Northern blot showing in the adult m1, m2 and m3 bands. In the newborn and 16 day m1 mRNA is absent and in the 3-day-old m3 mRNA is absent.

In order to ascertain if there were muscarinic M_1 receptors present, sections of lung and extrapulmonary arteries were incubated with [^{3}H]pirenzepine, a relatively selective M_1 ligand. Autoradiographs showed no binding over the media of the pulmonary arteries and there was little binding over the parenchyma (Fig. 3d). There was specific binding on the smooth muscle of large airways but the density was less than that with the [^{3}H]quinuclidinyl benzilate suggesting that only some of the muscarinic receptors were of the M_1 subtype. There was dense binding over the large nerve bundles (Fig. 3d and e).

3.2. Northern blot analysis

A representative of the Northern blot analysis of porcine lung peripheral tissue is shown in Fig. 5. The

Table 3
Distribution of mRNA for receptor subtypes by age from Northern blots (number of cases)

Age	Newborn	3 day	16 day	Adult
m1	1/7	7/7	1/6	5/6
m2	7/7	7/7	6/6	6/6
m3	6/7	0/7	6/6	6/6

poly(A)$^+$RNA hybridised to the m1 probe giving a 3.2-kb band, the m2 probe with a 6.1-kb band and the m3 probe with a 9-kb band. The density of the m1 and m3 mRNA bands was generally lower than that of the m2 mRNA. The amount of m2 mRNA in the newborn was greater than the adult ($P < 0.01$) (Fig. 6). It was not possible to quantify the amounts of m1 and m3 mRNA because of the low density of the bands but both seemed greater in the adult than earlier. The m2 mRNA was present in all cases at all four ages studied (Table 3). The m1 mRNA was absent in six of the seven cases at birth but was present in all seven cases at 3 days of age. At 16 days of age it was only present in one of six cases but was present in five of six adults. The m3 mRNA was present in six of the seven cases at birth, absent in all cases at 3 days and present in

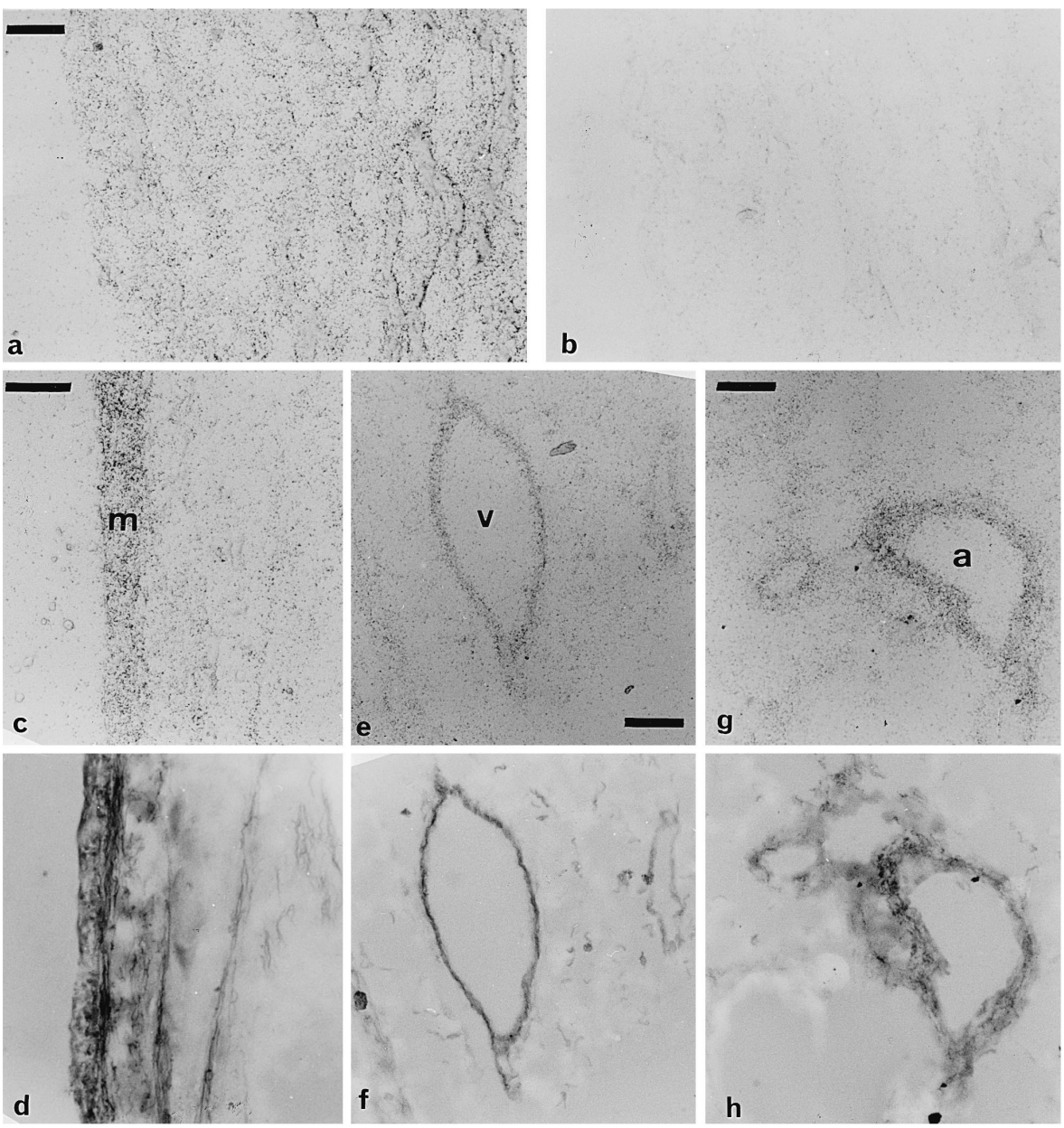

Fig. 7. Photomicrographs of tissue sections after in situ hybridization (a) Extrapulmonary artery of adult pig with m1 probe (antisense) silver grains over muscle cells of media (Bar = 50 μm). (b) Adjacent section with sense m1 probe. (c) Muscular pulmonary artery of adult pig with m1 antisense probe (m = media, Bar = 50 μm). (d) The same section stained with elastic van Gieson stain. (e) Pulmonary vein of adult (v) and (g) pulmonary artery of adult (a) with m2 antisense probe (Bar = 100 μm). (f and h) The same vessels stained with elastic van Giesen stain.

all at 16 days and in the adult. The one case in which m3 was absent at birth was the case in which m1 was present.

3.3. In situ hybridisation

At all ages studied (newborn, 3, 16 day and adult) the distribution of muscarinic receptor subtype mRNA on lung sections (Figs. 7 and 8) showed m1, m2 and m3 expression on the media of pulmonary arteries and veins and on bronchial smooth muscle. There was no expression on the lung parenchyma. Labelling was greater with antisense than with sense probes (Fig. 7a,b and Fig. 8a,b). Within the same tissue section there was greater labelling in the large than the small pulmonary arteries. The greatest labelling was seen over the extrapulmonary arteries. The receptor mRNA was localised across the entire width of the media. It could not be distinguished on the endothelium or on the adventitia of the pulmonary arteries (Fig. 8c). It was not possible to quantify change in distribution with age.

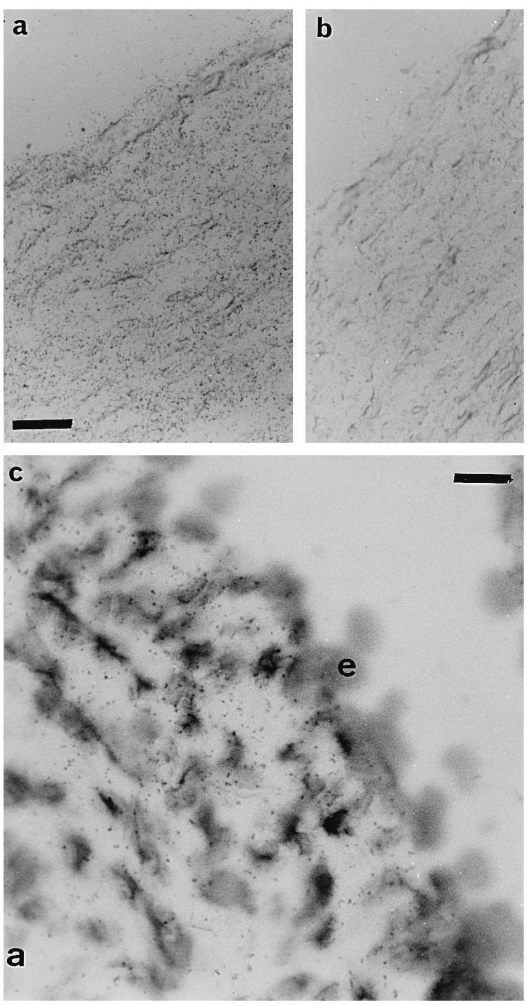

Fig. 8. Photomicrographs of tissue sections after in situ hybridization (a) Main pulmonary artery of 16-day-old piglet with m3 antisense probe (Bar = 50 μm) (b) m3 sense probe on adjacent section (c) Intrapulmonary artery of 16-day-old piglet with m3 antisense probe. Nuclei stained with haematoxylin. Silver grains are located over medial muscle cells and not endothelial cells (e) or adventitia (a) (Bar = 14 μm).

4. Discussion

In this study, using a variety of methods, we sought to find out if there were changes in the number or subtype of the muscarinic receptors after birth which might help explain the instability of the transitional pulmonary circulation during the first 2 weeks of life. Ligand binding to membranes of lung parenchyma using the nonselective muscarinic antagonist [*N*-methyl-^{3}H]scopolamine showed that the number of binding sites increased between birth and adulthood. The affinity for [*N*-methyl-^{3}H]scopolamine also increased gradually with age. Competition studies suggested that this was due to an increase in affinity of muscarinic M_1 receptors together with a decrease in affinity of muscarinic M_2 receptors. Northern blots on the lung periphery demonstrated a change in receptor subtype mRNA (m1 and m3) between birth and 3 days of age. A decrease in m2 mRNA in the adult was associated with a decrease in the affinity for the muscarinic M_2 receptor. These results all suggest changes in the muscarinic receptor subtypes in the lung parenchyma during the period of adaptation to extrauterine life.

In the present study, we found that the density and the affinity of the receptors in the peripheral lung tissue taken from adult pigs was similar to that shown previously by Haddad et al. (1994). These investigators had also demonstrated muscarinic M_1 and M_2 receptors in the adult lung periphery but because of the limited selectivity of the available antagonists were not able to determine whether or not muscarinic M_3 receptors were present. Our competition studies at all ages showed a single binding population for each antagonist with a rank order of 4-DAMP > telenzepine > methoctramine. This suggests that there are likely to be M_3 receptors present because the 4-DAMP is a relatively selective antagonist for muscarinic M_3 receptors. However, the high affinity for 4-DAMP does not necessarily suggest a high number of binding sites because of its high potency and we found a relatively small amount of m3 mRNA in peripheral lung membranes. Gies et al. (1989) found a high affinity for pirenzepine, a relatively selective M_1 antagonist in the human lung, in the presence of a smaller population of muscarinic M_3 receptors. They could not exclude the presence of the muscarinic M_2 receptors in man but we were able to demonstrate these in the pig. In the lung periphery we have found an increase in B_{max} for total muscarinic receptors with age, both during and after the first 2 weeks of life. In a study on piglet tracheal smooth muscle (Haxhiu-Poskurica et al., 1993), an increase in B_{max} occurred later, between three weeks and adulthood. The B_{max} of muscarinic receptors also increases with age in the mouse brain (Falkeborn et al., 1983) and

chick myocardium (Hosey et al., 1985). In our present study, muscarinic receptor affinity increased with age from birth while in porcine tracheal muscle it was reported that the K_d did not change during the first 3 weeks of life, although there was a weaker response to methacholine at 2–4 days than at 1–3 weeks (Haxhiu-Poskurica et al., 1993). It is reported that fetal rat heart membranes (Borda et al., 1997) and newborn rabbit gastric smooth muscle (Tomomasa et al., 1988) had a lower K_d than respective adult membranes.

We sought to locate the muscarinic receptors on the pulmonary vasculature. We demonstrated binding of the non-selective antagonist [^{3}H]quinuclidinyl benzilate to the bronchial smooth muscle in the intrapulmonary airways and the parenchymal area but not to the large intrapulmonary arteries or veins. These findings are similar to those in the adult human lung (Mak and Barnes, 1990) where binding sites were located on the airways but not the pulmonary or bronchial arteries despite the fact that pulmonary and systemic arteries dilate in response to acetylcholine via stimulation of the parasympathetic nerves. In the present study on porcine extrapulmonary arteries ligand binding to [^{3}H]quinuclidinyl benzilate was demonstrated to the outer edge of the media and to nerves in the adventitia, findings similar to those on sections of main pulmonary arteries and aorta from rabbits and cats (Stephenson et al., 1988). The in situ hybridisation studies localised the mRNA of all three muscarinic subtypes to the media of extra and intrapulmonary blood vessels, with a small amount in the airway walls and very little over the alveoli. This suggests that a large number of the muscarinic receptors demonstrated in the peripheral lung membranes may be located on the pulmonary blood vessels. Like other investigators working on different vascular beds (Stephenson et al., 1988; Amenta et al., 1992), we were not able to demonstrate muscarinic receptors on the pulmonary endothelium although acetylcholine stimulation leads to endothelium dependent relaxation. Aronstam et al. (1992), using membranes of cultured bovine pulmonary artery endothelial cells, suggested that muscarinic M_3 but not M_1 receptors were present, but Brunner and Kukovetz (1991) used freshly isolated bovine aortic endothelial cells and found only M_1 receptors. In such cells m1, m2 and m3 mRNA has been found (Tracey and Peach, 1992), with m1 and m3 mRNA in vascular smooth muscle cells. Our in situ studies showed the presence of all three mRNA subtypes (m1, m2, m3) located in the walls of arteries and veins over the smooth muscle cells. The failure to locate endothelial receptors in tissue sections may be because there are relatively few muscarinic receptors expressed at any one time and in situ hybridisation may be too insensitive a technique.

Our autoradiographic studies suggested by indirect competition that muscarinic M_2 receptors were present in the lung parenchyma, and the peripheral lung tissue expressed predominantly m2 mRNA. This mRNA decreased with age and was associated with a lower affinity for methoctramine in the adult parenchyma. Guinea pig lung tissue also shows a decrease in the percentage of the muscarinic M_2 receptor subtype with age (i.e., young vs. old) from 73% of the total to 37%, with a corresponding increase in muscarinic M_1 and M_3 receptors (Wills-Karp, 1993). In the present study, the total number of muscarinic receptors increased with age, though the amount of m2 mRNA decreased, suggesting that there may be an increase in muscarinic M_1 and M_3 receptors. However, the level of m1 and m3 mRNA in the porcine lung tissue was too low to quantify. Although M_2 receptors are generally associated with nerves (Langer, 1974), they have also been described in small cerebral arteries in man and cat, comprising 35% of the total muscarinic receptors and here they are considered to be vasoconstrictor (Dauphin and Hamel, 1992). Our in situ hybridisation studies located m2 mRNA on the smooth muscle cells of pulmonary arteries and veins. The M_2 receptor has been shown to be the major subtype in adult porcine membranes of bronchial smooth muscle (Haddad et al., 1994) and in the present study we have located both M_1 and M_2 muscarinic receptors on the bronchial smooth muscle.

Most of the information on the function of the muscarinic receptors refers to the adult lung. There is little information on the developing lung and therefore the functional implications of the present receptor study are speculative. There is evidence that the muscarinic M_2 receptor is likely to inhibit cAMP accumulation and therefore oppose the relaxation caused by β-adrenoreceptor agonist stimulation (Eglen et al., 1994). Our finding of a decrease in m2 mRNA with age may therefore be related to the observed increase in bronchial responsiveness to β-adrenoreceptor agonists with age (Tepper, 1987). Muscarinic M_1 and M_3 receptors have been located on trachael smooth muscle in young and adult pigs and were associated with bronchoconstriction to methacholine (Haxhiu-Poskurica et al., 1993). Muscarinic M_3 receptors are also located in airway epithelium, more in neonatal than adult pigs, where they facilitate release of nitric oxide (Jakupaj et al., 1997).

For the vasculature, muscarinic M_1 and M_3 receptors play an important part in the regulation of tone and response to stimulation depends upon baseline tone, vascular bed and species (Obi et al., 1994). In rabbits, pulmonary arteries have an endothelium dependent contraction in response to acetylcholine at resting tone but relax at elevated tone both via the activation of the muscarinic M_3 subtype (Altiere et al., 1994). Isolated human pulmonary arteries without endothelium contracted in response to muscarinic M_3 receptor stimulation, but both muscarinic M_1 and M_3 receptors were involved in endothelium dependent relaxation (Norel et al., 1996). Our present studies demonstrated changes in muscarinic mRNA (m1 not present at birth but present by 3 days) and the binding affinity for muscarinic M_1 receptors increased with age. The relax-

ation to acetylcholine increases after birth in the ovine lung (Abman et al., 1990) and our previous studies on isolated porcine intrapulmonary arteries have shown absence of dilatation to acetylcholine at birth, the response appearing by 3 days of age (Liu et al., 1992; Tulloh et al., 1997). When stimulated relaxation first appeared at 3 days we found that both the muscarinic M_1 and M_3 receptor were probably responsible since both pirenzepine and 4-DAMP prevented dilatation (Levy et al., 1993). In animals of 10 days and older, the muscarinic M_3 receptor alone was responsible, possibly reflecting the higher expression of m3 mRNA in the 16 day and adult animals observed in the present study. Acetylcholine also leads to vasoconstriction via muscarinic receptors on the smooth muscle cells in the adult human lung (Casale and Ecklund, 1988; Norel et al., 1996). In newborn piglets, contraction in response to acetylcholine has been described (Tulloh et al., 1997) both in the presence or absence of endothelium.

In conclusion therefore, changes in the number, affinity and subtype of the muscarinic receptors after birth may be related to the postnatal fall in pulmonary vascular resistance and contribute to the instability of the transitional circulation.

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