Original Article

M_3 muscarinic receptors mediate acetylcholine-induced pulmonary vasodilation in pulmonary hypertension

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Summary **Information about the muscarinic receptor subtype(s) mediating pulmonary circulatory vasodilator responses to acetylcholine (ACh) is limited. The aim of this study was to pharmacologically characterize the muscarinic receptors associated with ACh-induced pulmonary vasodilation in a pulmonary hypertension model. Vasodilation of rabbit isolated buffer-perfused lungs in which pulmonary hypertension was induced with the thromboxane A_2 analogue U-46619 was evoked by ACh at a just maximally effective concentration (2×10^{-7} M). The effects of cumulative concentrations of three specific muscarinic receptor subtype antagonists [pirenzepine (M_1), methoctramine (M_2), and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP, M_3)] on ACh-induced pulmonary vasodilation were determined. Double vascular occlusion pressure was recorded to locate the muscarinic receptors within the pulmonary vasculature. Based on the 50% inhibitory concentrations (IC_{50}), the rank of order of antagonist potency was 4-DAMP >> pirenzepine > methoctramine. The vascular effects of all three inhibitors were localized to the precapillary segment. These findings suggest that the vasodilator action of ACh on rabbit isolated perfused U-46619 pretreated lungs is mediated by M_3 muscarinic receptors located in the pulmonary arterial bed.**

Keywords: Pulmonary vasodilation, muscarinic receptors, rabbit

1. Introduction

Although acetylcholine (ACh) was the first endothelium-dependent vasorelaxant to be identified (*1*), the complex role of ACh in the regulation of pulmonary vascular tone remains unclear. Under normal resting conditions, ACh induces a vasopressor response of the pulmonary circulatory system, whereas during pulmonary hypertension ACh evokes a vasodilator response (*2-7*). In dogs with oleic acid-induced lung injury, vagotomy markedly increases the pulmonary arterial pressure (Ppa), thereby increasing pulmonary edema (*8*). Moreover, pancuronium bromide, which at clinically used doses is a potent M_2 and M_3 muscarinic receptor antagonist, increases Ppa in this model and this effect is potentiated by hypoxemia (*9*). These findings suggest that cholinergic vasodilation involving M_2 and/or M_3 receptors plays a role in the pulmonary hypertensive state. Limited information is available, however, about the muscarinic receptor subtype(s) mediating ACh-induced vasodilator responses of the pulmonary circulatory system (*10*). Although experiments on isolated pulmonary arteries indicate that ACh-induced vasodilation is mediated by M_3 receptors, the overall function of ACh in a whole lung preparation has not been elucidated (*11*). Furthermore, it is unknown whether the distinct muscarinic receptor subtypes mediating ACh-induced vasodilation are distributed heterogeneously within the pulmonary vasculature.

To pharmacologically characterize the muscarinic receptors associated with ACh-induced pulmonary vasodilation, we evaluated the effects of varying concentrations of specific muscarinic receptor subtype antagonists on ACh-induced vasodilation in a thromboxane A_2 analogue (U-46619)-stimulated pulmonary hypertension model. To localize muscarinic

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receptors within the pulmonary vasculature, double vascular occlusion pressure, a measure of the pulmonary capillary pressure, was recorded (*12-15*).

2. Methods

The protocol for this study was approved by The Tokyo University Laboratory Animal Care Committee. A standard rabbit perfused lung preparation *in vitro* (*2*,*11*,*16*) was modified as described below.

2.1. *Isolated perfused lung preparation*

Fifty-five New Zealand white rabbits of both sexes, weighing 2.5 to 3.5 kg, were anesthetized with sufficient volumes (4-6 mL) of pentobarbital sodium (50 mg/mL) administered through the marginal vein to produce surgical anesthesia. Local anesthesia of the neck was induced by infiltration with 0.5% lidocaine. A tracheostomy was performed and each rabbit was mechanically ventilated with room air at a rate of 30 to 40 breaths/min, a peak airway pressure of 10 to 12 cmH_2O, and a positive end-expiratory pressure of 2.5 cmH_2O. A catheter was inserted in the right jugular vein to administer drugs and to drain the lymph fluid during perfusion. Heparin (1,000 U/kg) was injected into the right jugular vein and additional pentobarbital sodium was injected before starting the pulmonary isolation procedure. After sternotomy, the pulmonary artery (PA) and left atrium (LA) were cannulated *via* the right and left ventricles, respectively, and the lungs were perfused slowly with blood-free Krebs-Henseleit solution [composition (in mM): NaCl 118; KCl 4.74; $CaCl_2 \cdot H_2O$ 2.54; KH_2PO_4 1.19; $NaHCO_3$ 26.2; pH 7.4] containing 3% bovine serum albumin at 37°C and ventilated with 95% air/5% CO_2 at a rate of 5 breaths/min, a tidal volume of 10 mL/kg, and a positive end-expiratory pressure of 2.5 cmH_2O.

To reduce the numbers of cells in the recirculating perfusates, the pulmonary circulatory system was initially flushed with 700 mL fresh perfusion solution at a rate of 20 to 40 mL/min. The lungs were then connected to a recirculatory perfusion system in which the perfusate in the venous reservoir was recirculated at a flow rate of 100 mL/min using a peristaltic pump (Masterflex pump) through a water-jacketed heating coil and then through a bubble trap into the pulmonary artery and back to the venous reservoir. The perfusate temperature was maintained at 37°C with a heated water bath and the total volume in the circuit was 200 mL.

The airway (Paw), pulmonary arterial (Ppa), and left atrial (Pla) pressures were monitored continuously and recorded using an 8-channel recorder (Nihon Kohden, Tokyo, Japan). Ppa and Pla were measured *via* ports in the respective cannulas. The vascular pressures were referenced to the mid-level of the LA and the venous reservoir height was adjusted to maintain the Pla at 4 mmHg, thereby maintaining the whole lung under West zone III conditions. The LA was wrapped loosely with gauze and glued to keep its volume constant. In the present study, we used only lungs that *i*) had a homogeneous white appearance with no signs of congestion, edema, or atelectasis and *ii*) maintained a constant pulmonary vascular pressure gradient (Ppa-Pla) of less than 7 mmHg when perfused at 100 mL/min and a peak ventilation pressure within the normal range. Indomethacin (30 μM) was included in the perfusate to reduce the vascular contractile response to ACh (*2*,*16*,*17*).

2.2. *Measurement of the pulmonary capillary pressure (Ppc)*

The Ppc was estimated using the double vascular occlusion method. Briefly, this method is based on the assumption that when the arterial inflow and venous outflow are occluded simultaneously, all vascular pressures are equal to the Ppc, because the majority of the pulmonary vascular compliance is attributable to the microcirculation (*12-15*). To measure the Ppc, the PA and LA cannulas were simultaneously occluded during the apneic period between ventilator breaths and during occlusion. The Ppa and Pla were digitized and sampled at 200 Hz using a Maclab A/D converter. From 2 to 3 s after double vascular occlusion, the difference between the Ppa and Pla was less than 0.5 mmHg, so their average was considered to represent the Ppc. The total vascular resistance (Rt) was determined by calculating the difference between the Ppa and Pla and dividing the result by the flow rate (Q). The precapillary resistance (Ra) was calculated as the difference between Ppa and Ppc divided by Q, and the postcapillary resistance (Rv) was calculated as the difference between Ppc and Pla divided by Q.

2.3. *Effects of ACh on the pulmonary vascular resistance under conditions of U-46619 induced pulmonary hypertension*

The lungs of 9 rabbits were initially perfused with Krebs-Henseleit solution containing 3% bovine serum albumin at 100 mL/min, as described above. To ensure strong stable vascular contraction, the Ppa was pharmacologically increased to 25 mmHg at Q = 50 mL/min providing that the lung preparation remained stable for 15 min (*i.e.*, constant Paw, Ppa, and Pla) after reducing Q. Baseline measurements were recorded. Pulmonary hypertension was induced by infusing the stable thromboxane A_2 analogue U-46619 into the venous reservoir (*6*,*7*,*16*). Initially, U-46619 was infused at 150 ng/min to achieve a Ppa of 25 to 26 mmHg and then the infusion rate was reduced to deliver an hourly dose equal to the initial total dose required

to achieve pulmonary hypertension (*18*). After a 20-min period of stable pulmonary hypertension, the Paw, Ppa, Pla, and Ppc (control values) were measured, after which a bolus of ACh was added to the reservoir every 6 min to produce final cumulative concentrations of 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} M and the vasodilator responses were recorded 5 min after administering each bolus, because the maximal sustained effect was attained within 2 to 3 min.

2.4. *Effects of muscarinic receptor antagonists on ACh-induced pulmonary vasodilation*

Twenty-eight rabbits were randomly divided into 4 subgroups of 7 and their isolated lungs were perfused under baseline conditions and then with U-46619 to produce stable pulmonary hypertension, as described above. When the Ppa reading had been stable for 15 min, baseline Ppc, Ppa, Pla, and Paw values under conditions of U-46619 induced pulmonary hypertension were recorded and then a bolus of ACh was injected into the venous reservoir to produce a final concentration of 2×10^{-7} M. After a 20-min equilibration period, to confirm the stable decrease in Ppa induced by ACh, the Ppc, Ppa, Pla, and Paw were recorded, after which normal saline or the muscarinic receptor antagonist dissolved in normal saline was administered cumulatively at 6-min intervals, as described below, and Ppa, Pla, Ppc, and Paw were measured 5 min after each drug addition.

The magnitude of the ACh-elicited vasodilator responses of the preparations varied. Therefore, each Ra value is expressed as a ratio (B2/B1). Here, B1 is the decrease in Ra (Ra before saline or antagonist administration − Ra after administration of ACh). B2 is defined as increase of Ra after the administration of saline or antagonist.

2.5. *Four subgroups (n = 6) received the following treatment regimens*

Group 1 (saline alone, vehicle control) – Normal saline (3 mL) was injected into the venous reservoir 20, 26, 32, and 38 min after ACh administration and Ppa, Pla, Ppc, and Paw were recorded to assess the changes in pulmonary vascular resistance (PVR) during the course of the experiment.

Group 2 (selective M_1-receptor antagonist) – The M_1-receptor antagonist pirenzepine (2×10^{-8}, 10^{-7}, 5×10^{-7}, and 10^{-6} M) was administered cumulatively to assess the effects of M_1-receptor blockade on pulmonary vascular tone.

Group 3 (selective M_2-receptor antagonist) – The selective M_2-receptor antagonist methoctramine (10^{-7}, 5×10^{-7}, 2.5×10^{-6}, and 4×10^{-6} M) was administered cumulatively to assess the effect of M_2-receptor blockade on vascular tone after pulmonary vasodilation with ACh.

Group 4 (selective M_3-receptor antagonist) ─ The selective M_3-receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; 2×10^{-10}, 10^{-9}, 5×10^{-9}, and 10^{-8} M) was administered cumulatively to evaluate the effect of M_3 receptor blockade on the PVR after inducing pulmonary vasodilation with ACh.

2.6. *Drugs*

The drugs used in this study were all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.7. *Statistical analysis*

The values are expressed as mean ± S.D. The statistical analysis was performed using one-way analysis of variance with repeated measures. For within-group comparisons, Student's *t* tests were used with Bonferroni's correction for multiple comparisons, as appropriate. Differences between means at $p < 0.05$ were considered significant.

3. Results

3.1. *Effects of ACh on the resistance of the pulmonary vasculature constricted with U-46619*

After recording the baseline pulmonary vascular pressures, rabbit isolated buffer-perfused lungs ($n = 9$) were treated with U-46619, followed by cumulative administration of ACh at 6-min intervals. U-46619 increased pulmonary vascular resistance (Rt) from 146.57 ± 12.76 to 421.71 ± 8.71 mmHg/L/min, with proportional increases in the right atrium (Ra) and right ventricle (Rv) (Figure 1). Under these conditions of elevated vascular tone, administration of ACh resulted in concentration-dependent decreases in Rt and Ra. The maximal vasodilatory effect was obtained with 10^{-7} M ACh and higher concentrations evoked no

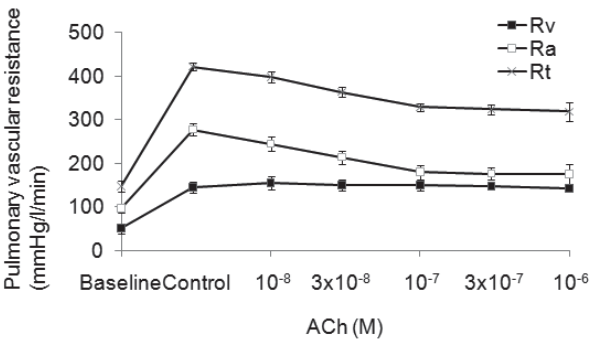

Figure 1. Effects of ACh (10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} M) on the pulmonary vascular resistance in rabbit isolated lungs pretreated with U-46619. * and # $p < 0.05$ *versus* baseline and control values, respectively. Values are mean ± S.D.

further decreases. In contrast, ACh did not alter Rv at any concentration tested, suggesting that ACh primarily induced precapillary vasodilation (*i.e.*, reduced Ra) without affecting the postcapillary vessels. ACh did not completely reverse U-46619-induced vasoconstriction and Rt and Ra after ACh administration were consequently higher than their baseline values ($p < 0.05$).

3.2. *Effects of muscarinic antagonists on ACh-induced pulmonary vasodilation*

Four injections of 3 mL saline at 6-min intervals had no effect on ACh-induced pulmonary vasodilation, which remained constant without changes in Rt, Ra, or Rv throughout the experiment. Therefore, a single bolus of ACh induced a persistent decrease in the PVR, which remained constant throughout the experiment with each antagonist.

The selective M_1- (pirenzepine), M_2- (methoctramine), and M_3- (4-DAMP) receptor antagonists all inhibited ACh-induced vasodilation in a concentration-dependent manner (Figures 2-4). Their inhibitory potencies, however, indicated by their different mean IC_{50} (the concentration of antagonist at which the control response was inhibited by 50%) values,

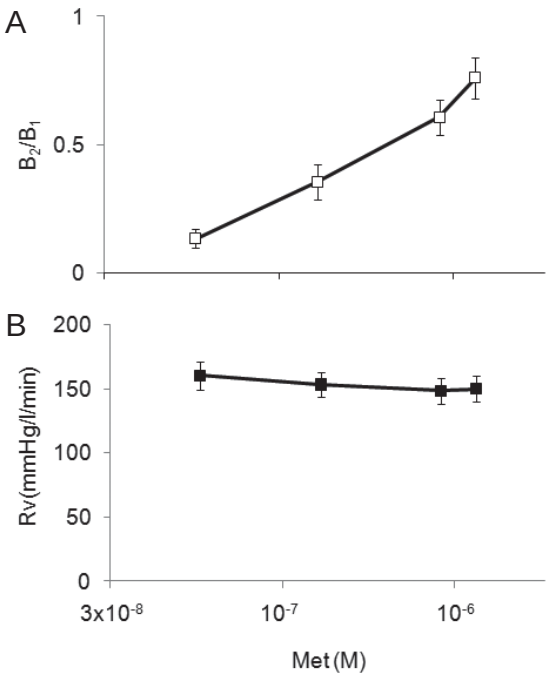

Figure 3. Effects of methoctramine (Met) on ACh-induced pulmonary vasodilation in the precapillary (A) and postcapillary (B) segments (*n* = 6). The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

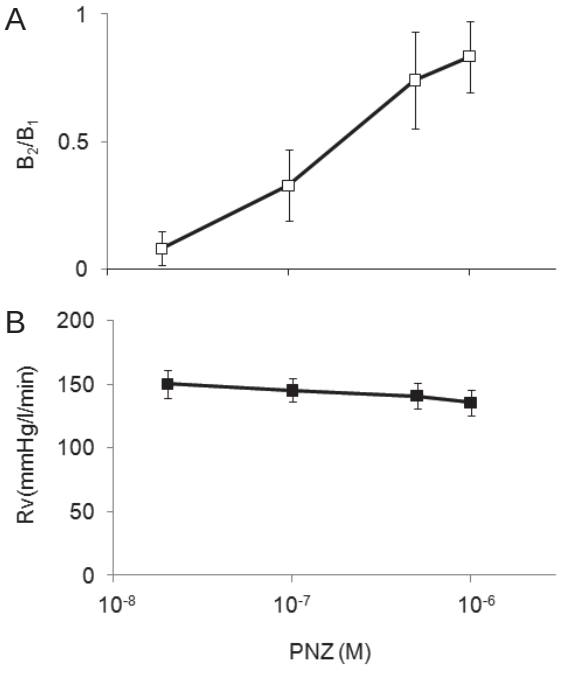

Figure 2. Effects of cumulative concentrations of pirenzepine (PNZ) on ACh-induced vasodilation of the precapillary (A) and postcapillary (B) segments of the rabbit perfused lung preparation (*n* = 6). The inhibitory effect of pirenzepine on the ACh-induced reduction in precapillary resistance is shown as an increase in the B2/B1 ratio [response after (B2) divided by that before (B1) exposure to pirenzepine]. The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

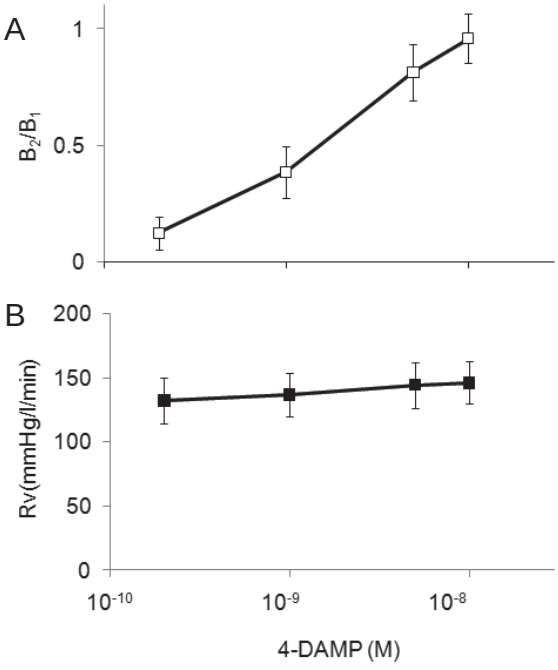

Figure 4. Effects of 4-DAMP on ACh-induced pulmonary vasodilation in the precapillary (A) and postcapillary (B) segments (*n* = 6). The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

differed. Based on their IC_{50} values, the rank order of antagonist potency was 4-DAMP (1.58×10^{-9} M) >> pirenzepine (2.51×10^{-7} M) > methoctramine (1.25×10^{-6} M). Therefore, 4-DAMP was approximately 160 and 1,000 times more potent as an antagonist of ACh-induced pulmonary vasodilation than pirenzepine and methoctramine, respectively. It is noteworthy that the effects of all three antagonists were exerted exclusively on the precapillary segment, as none of them altered the Rv at the concentrations tested.

4. Discussion

The main finding of this study is that M_3-subtype muscarinic receptors mediated the vasodilator action of ACh on the pulmonary vascular bed of the rabbit whole lung preparation with induced pulmonary hypertension. Furthermore, ACh dilated the pulmonary arterial bed exclusively, which we attribute to the preferential distribution of M_3 receptors in the pulmonary arterial, rather than venous, bed. Functional integrity of the vascular tone may be necessary to activate the mechanisms governing regional blood flow and vascular resistance in the pulmonary circulatory system. Identification of pulmonary vascular muscarinic receptors is important toward understanding the process whereby ACh contributes to the regulation of pulmonary circulatory tone.

Muscarinic receptors are classified pharmacologically into five main subtypes: M_1, M_2, M_3, M_4, and M_5 (*19-22*). Differences between antagonist affinities are useful for differentiating receptor subtypes (*23*) and therefore the full concentration-response curves for a multiple set of selective muscarinic antagonists should be obtained, although this may not be easy to achieve in a perfused lung model. In the present study, three specific muscarinic subtype-receptor antagonists were used: pirenzepine, methoctramine, and 4-DAMP. Previous binding studies demonstrated that pirenzepine has high affinity for M_1 receptors and low affinity for M_2, M_3, M_4, and M_5 receptors (*10*,*22*,*24-26*). Methoctramine, a polymethylene tetraamine, is a more specific M_2-receptor antagonist and 4-DAMP has high affinity for M_3 receptors with a relatively low affinity for M_1 receptors (*10*,*22*,*24*,*25*). In the present study, pharmacodynamic characterization of muscarinic receptor subtypes was achieved by determining the effects of these three selective antagonists on the response to a just maximally effective concentration of ACh, determined from a concentration-response curve. Based on their IC_{50} values, the rank of order of antagonist potency was 4-DAMP (1.58×10^{-9} M) >> pirenzepine (2.51×10^{-7} M) > methoctramine (1.25×10^{-6} M). As M_3 receptors have a high affinity for 4-DAMP and a low affinity for pirenzepine (*22*), our findings show that ACh decreases the PVR in rabbit isolated lungs by activating M_3 receptors. ACh evokes a biphasic response in the rabbit pulmonary vascular bed (*2*,*3*,*5-7*) and selective abolition of ACh-induced contractile responses by inhibiting cyclooxygenase enables examination of the relaxant responses without functional interference from the opposing contractile response (*2*,*27*). Therefore, in this study, we used indomethacin to abolish the contractile response to ACh (*11*,*16*,*17*,*28*).

Studies of the muscarinic receptors that mediate ACh-induced pulmonary vasodilation have provided conflicting results. Several factors confound the available data, including interspecies differences, differences in the specific vascular bed under consideration, and the use of different experimental models. Our finding that the M_3 receptor is responsible for ACh-induced pulmonary vasodilation in a rabbit isolated perfused lung preparation extends the findings of studies on rabbit and feline lungs (*6*,*7*), which showed that the vasoconstrictor responses to ACh are blocked by pirenzepine, but neither pirenzepine nor galamine inhibits the vasodilator responses, suggesting that a third subtype of muscarinic receptor is responsible for the inhibitory effects.

Our results are also consistent with the findings of studies of isolated pulmonary arteries of several species: the selective M_3-receptor antagonist 4-DAMP inhibits ACh-induced relaxation of rat isolated pulmonary arterial strips at lower concentrations than does pirenzepine and methoctramine (*17*). A study on rabbit isolated pulmonary arteries also showed that 4-DAMP is a more potent inhibitor of the vasodilator response to ACh than pirenzepine or AF-DX116, a selective M_2-receptor antagonist (*10*). Our findings, however, conflict with the report that ACh-induced vasodilation in rat isolated perfused lungs preconstricted with U-46619 is mediated by M_1 receptors (*29*). Although the discrepant results may be partially due to the different species and experimental preparations used, only single high concentrations (μM) of the selective M_1 agonist and antagonist were used in that study, and therefore the possibility that muscarinic receptors other than M_1 were involved cannot be excluded.

In the present study, the double occlusion technique was used to partition the total pulmonary vascular resistance into segmental resistances (*12-14*). Consistent with previous observations, U-46619 significantly increased the PVR (represented by Rt) and both Ra and Rv (*18*). Under conditions of increased vascular tone, ACh reduced Ppa, Rt, and Ra, but had no effect on Rv at any concentration tested. These results demonstrate that ACh decreases the PVR of the pulmonary circulatory system of the rabbit solely by dilating the precapillary segment of the pulmonary vascular bed, which suggests an arterial predominance of vasodilatory muscarinic receptors. The selective M_3 antagonist 4-DAMP completely reversed ACh-induced vasodilation and the other antagonists,

which have much lower affinities for the M_3 receptor, inhibited ACh-induced vasodilation when much higher concentrations were used. The vascular effects of all three inhibitors were localized exclusively to the precapillary segment. These results strongly suggest that M_3 receptors are localized in the pulmonary arterial segment, although the exact anatomic distribution of the vasodilator muscarinic receptors in the lung remains to be determined.

We previously demonstrated that the pulmonary intravascular pressure in dogs with induced lung edema increases to a greater extent in dogs that are vagotomized than in those with normal innervation (*8*). Furthermore, the extravascular lung water content resulting from lung injury is greater in vagotomized than in normally innervated dogs. These findings suggest that vagal innervation plays an important role in controlling pulmonary vascular pressure under conditions of lung injury. Further, pancuronium, which at clinical doses is a potent M_2 and M_3 receptor antagonist, increases PAP and pulmonary vascular resistance, but does not affect systemic blood pressure and reduces the systemic vascular resistance in dogs with oleic acid-induced lung injury (*9*,*30*,*31*). These results suggest that the responses of the pulmonary and systemic vasculature to muscarinic inhibitors may be totally different under conditions of pulmonary hypertension and normotension and raise the possibility that cholinergic innervation and endogenous ACh play crucial roles in the physiology of the pulmonary vasculature.

In conclusion, these findings demonstrated that the vasodilator action of ACh in the isolated perfused rabbit lung pretreated with U-46619 is mediated mainly by M_3 muscarinic receptors. ACh dilates the pulmonary arterial bed, but not the venous bed, which is likely due to the preferential distribution of M_3 receptors in the pulmonary arterial bed.

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