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Cholinesterase activity in human pulmonary arteries and veins

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1 Human isolated pulmonary vessels were treated with cholinesterase (ChE) inhibitors to determine the role of these enzymes in regulating vascular muscle tone. In addition, kinetic parameters were determined for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in human pulmonary vessel homogenates.

2 Carbachol (CCh) and acetylcholine (ACh) were equipotent contractile agonists in human pulmonary arteries (pD_2 values, 5.28 ± 0.05 and 5.65 ± 0.16; E_{max}, 0.91 ± 0.26 and 0.98 ± 0.30 g wt. for CCh and ACh, respectively; $n = 7$). In venous preparations, ACh was ineffective and CCh induced small contractions (E_{max}, 0.08 ± 0.04 g wt.; $n = 13$).

3 In human pulmonary arteries following pretreatment with tetraisopropylpyrophosphoramide (iso-OMPA, 100 μM), an increased sensitivity to the contractile agonist ACh was observed (pD_2 values, 5.80 ± 0.13 and 6.37 ± 0.19 for control and treated preparations, respectively; $n = 5$). This pretreatment had no effect on the CCh concentration response curve. In contrast, human pulmonary veins pretreated with iso-OMPA failed to elicit a contractile response to ACh.

4 Neither Iso-OMPA nor neostigmine elicited concentration-dependent contractions in human isolated pulmonary arteries or veins. These results suggest the absence of sufficient spontaneous release of ACh to modulate human pulmonary vessel basal tone.

5 CCh was less potent than ACh in relaxing precontracted human isolated pulmonary arteries (pD_2 value, CCh: 6.55 ± 0.15 and ACh: 7.16 ± 0.13, $n = 4$) and veins (pD_2 value, CCh: 4.95 ± 0.13; $n = 5$ and ACh: 5.56 ± 0.17; $n = 6$). Pretreatment of vessels with either iso-OMPA or neostigmine did not modify ACh relaxant responses in either type of preparation.

6 In human pulmonary veins, the ChE activity was two fold greater than in arteries ($n = 6$). V_{max} for AChE was 1.73 ± 0.24 and 3.36 ± 0.26 miu mg^{-1} protein in arteries and veins, respectively, whereas V_{ss} for BChE was 1.83 ± 0.22 and 4.71 ± 0.17 miu mg^{-1} protein, in these respectively.

7 In human pulmonary arteries, BChE activity may play a role in the smooth muscle contraction but not on the smooth muscle endothelium-dependent relaxation induced by ACh. A role for ChE activity in the control of venous tone is presently difficult to observe, even though this tissue contains a greater amount of enzyme than the artery.

Keywords: Human pulmonary arteries; human pulmonary veins; acetylcholinesterase; butyrylcholinesterase; acetylcholine; carbachol; iso-OMPA; BW284C51; neostigmine

Introduction

Acetylcholine (ACh) produces relaxations in precontracted human isolated pulmonary vessels *via* the activation of endothelial muscarinic receptors, whereas at resting tone, this agonist induced contractions by activation of muscarinic receptors located on the smooth muscle (Greenberg *et al*., 1987; Norel *et al*., 1996). Vasodilatation is mediated by the release of relaxant factors (endothelium-derived relaxing factor, EDRF; Furchgott & Zawadzki, 1980), identified as nitric oxide (NO; Palmer *et al*., 1987) and prostacyclin (PGI_2) (Bunting *et al*., 1976). In canine trachea or human bronchial smooth muscle, ACh may not accumulate at muscarinic receptors, since this mediator is rapidly hydrolysed by cholinesterase (ChE) in the airways (Adler *et al*., 1991; Norel *et al*., 1993). Two different types of ChE (acetylcholinesterase (AChE): EC 3.1.1.7. and butyrylcholinesterase (BChE): EC 3.1.1.8.) have been described based on their sensitivity to different inhibitors and substrate specificity (Massoulié *et al*., 1993). In the anaesthetized dog, anticholinesterases (sarin and ethyl pyrophosphate) provoked an increase of pulmonary vascular resistance which was prevented and abolished by atropine (Daly, 1957). Furthermore, in the feline perfused lung, physostigmine enhanced the decrease of lobar arterial pressure induced by ACh (Nandiwada *et al*., 1983). These results indirectly suggest that inhibition of ChE activity may modulate pulmonary vascular tone.

Since few studies have been performed to examine the ChE activity in the pulmonary vascular bed, this investigation was undertaken to explore the involvement of ChE in the regulation of human isolated pulmonary vessel tone and to determine the kinetic parameters of AChE and BChE in human pulmonary vessels.

Methods

Isolated preparations

Human lung tissues were obtained from patients (23 male and 3 female) who had undergone surgery for lung carcinoma. The mean age was 60 ± 02 years. Vascular tissues were dissected from those macroscopically normal regions of the diseased lung. Arteries and veins (3–6 mm internal diameter) were removed, dissected free of adjoining connective tissue and lung parenchyma, placed in Tyrode solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5 and maintained at 4°C. All tissues were used within 1 to 12 h of surgery.

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Physiological studies

Arteries and veins were cut as rings (3 to 5 mm in length). In some preparations the endothelium was mechanically removed by inserting both smooth-edged arms of a dissecting forceps into the lumen of the vessel and gently rolling the moistened preparation between the surface of a forefinger and the forceps for 10 s without undue stretch. Histological confirmation of the presence or the absence of the endothelium was made with the silver nitrate staining procedure. The rings were then set up in 10 ml organ baths containing Tyrode solution, gassed with 5% CO_2 in O_2, at 37°C and pH 7.4. An optimal load (1.5 g), which ensured maximal responses to contractile agonists used, was applied to each ring. Changes in force were recorded by isometric force displacement transducers (Narco F-60) and physiographs (Linseis). Subsequently, preparations were allowed to equilibrate for 90 min with bath fluid changes taking place every 10 min.

Contraction studies In preparations without endothelium, after a precontraction with carbachol (CCh, 10 μM), the rings were washed with fresh Tyrode solution and allowed to return passively to their resting tone. Two different protocols were then followed. In the first protocol, increasing concentrations of each ChE inhibitor (tetraisopropylpyrophosphoramide (iso-OMPA) or neostigmine) were added to the bath fluid in a cumulative fashion. In the second protocol, tissues were incubated for 30 min with either Tyrode solution or Tyrode solution containing a ChE inhibitor (iso-OMPA, 100 μM or neostigmine, 10 μM). Subsequently, the preparations were stimulated by serial additions of ACh or CCh in a cumulative fashion. The same procedure was performed, without a ChE inhibitor in experiments with atropine (30 min incubation, 1 μM), except that a second agonist curve was performed after a washout period.

Relaxation studies In preparations with an intact endothelium, a 30 min incubation with Tyrode solution or Tyrode solution containing a ChE inhibitor (iso-OMPA, 100 μM or neostigmine, 10 μM) was performed. The rings were then precontracted with noradrenaline (NA, 10 μM). When the response reached a plateau, increasing concentrations of ACh or CCh were applied in a cumulative fashion.

Biochemical studies

Human pulmonary arteries and veins were longitudinally cut, rinsed in Tyrode solution to eliminate any trace of blood and minced. The preparations were then submitted to an osmotic shock and centrifuged (10 min, 5000 *g* at 4°C). Supernatant was discarded and pellet was resuspended in cold phosphate buffer (0.1 M, pH 7.4) at 250 mg ml^{-1}. Tissues were homogenised at 4°C by a polytron (400 W) at speed setting 7 (7 times 10 s). The homogenates were rapidly filtered through gauze and stored at −80°C.

ChE activities were determined according to Ellman's colorimetric technique (Ellman *et al*., 1961). This technique was adapted for use on microplates. In each well of a microplate homogenates were first incubated (30 min, at room temperature with gentle shaking) in phosphate buffer (0.1 M, pH 8.0) containing a ChE inhibitor at a fixed concentration. To inhibit selectively either AChE or BChE activities, BW284C51 (1 μM) or iso-OMPA (10 μM) were respectively used (Austin & Berry, 1953). Homogenates were then incubated 10 min in the presence of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, 1.85 mM). Acetylthiocholine iodide (ACTI), at different concentrations (AChE activity: ACTI, 0.1 – 1 mM; BChE activity: ACTI, 0.25 – 10 mM), was added to wells and plates were immediately shaken for 5 s. Subsequently, absorbance was measured at 414 nm every 2 min for 40 min at room temperature with a Titertek Multiscan plus II. The same procedure was followed with only one concentration of ACTI (1 mM) after an incubation with neostigmine (10 μM) or iso-OMPA (100 μM). In the microplates, non-enzymatic hydrolysis of ACTI (control) was determined at each concentration in the absence of homogenate.

For each homogenate, the protein concentration was measured with the method of Lowry (Lowry *et al*., 1951). The standard curves were performed with bovine serum albumin.

Data analysis

Physiological studies Contractile responses are expressed in grams weight (g wt.) or as % of the CCh (10 μM) precontraction. The maximal responses (E_{max}) produced by cholinergic agonists and the half-maximum effective concentration values (EC_{50}) were interpolated from the different concentration-effect curves. The pD_2 values were calculated as the negative log of EC_{50}.

Biochemical studies The kinetic parameters (K_m, K_{ss}, V_m and V_{ss}) were therefore estimated from S/V against S plots of substrate-velocity data (correlation coefficients: $r^2 = 0.988 \pm 0.004$). K_m and K_{ss} were expressed in μM, V_m and V_{ss} in miu mg^{-1} protein. At low ACTI concentrations (<0.25 mM), detection of BChE activity in the vascular homogenates varied considerably. A higher range of substrate concentrations was therefore used. According to Brown and co-workers (1981), this higher range of substrate concentration allowed a determination of K_{ss} values (second substrage dissociation constant) and V_{ss} values (maximal velocity after second substrate fixation). For a substrate concentration range (0.01 – 100 mM), BChE enzymatic kinetics were of a biphasic form reflecting substrate activation (Brown *et al*., 1981). When, the inhibition of ChE activity (ACTI, 1 mM) was determined, for each vascular homogenate derived from a lung sample, the resultant activity was expressed as % of the control value determined without inhibitor. Results are expressed as means ± s.e.mean and were derived from different lung samples (*n*). Statistical analyses were performed by use of Student's *t* test or Student's paired *t* test with a confidence level of 95%.

Drugs

Acetylcholine chloride, carbamylcholine chloride (carbachol), noradrenaline, tetraisopropylpyrophosphoramide (iso-OMPA), 1,5-bis (4-allyldimethylammoniumphenyl) pentane-3-one dibromide (BW284C51), atropine, acetylthiocholine iodide (ACTI), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Neostigmine methylsulphate (Prostigmine) was obtained from Roche (92521 Neuilly sur Seine, France).

All agonists and neostigmine were dissolved in Tyrode solution, each subsequent dilution was made in Tyrode solution. BW284C51, ACTI and bovine serum albumin were dissolved in distilled water. Iso-OMPA (0.1 M) was dissolved in 100% ethanol and subsequent dilutions were made in Tyrode solution for the physiological studies or in water for the biochemical studies. DTNB was dissolved in phosphate buffer (0.1 M, pH 7.0) with $NaHCO_3$ (0.15%).

Results

Physiological studies

The data presented in Figure 1 show the effect of ACh and CCh in isolated human pulmonary vessels at resting tone. While ACh produced concentration-dependent contractions in human pulmonary arteries, veins were not responsive to this agonist. In arteries, ACh and CCh were equipotent contractile agonists (pD_2 values, 5.65 ± 0.16 and 5.28 ± 0.05 for ACh and CCh, respectively). CCh also induced a small significant contractile response in some venous preparations (5/13), the combined data are shown in Figure 1. This response was in-

hibited by atropine (1 μM; $n=2$, data not shown). Addition of iso-OMPA (100 μM) potentiated the ACh-induced contractile response in arteries (pD_2 values, 5.80 ± 0.13 and 6.37 ± 0.19 control and pretreated preparations, respectively; $P<0.05$, Figure 2a). In contrast, pretreatment of arteries with iso-OMPA did not modify CCh curves (Figure 2b) and addition of neostigmine (10 μM) was without effect on ACh-induced contractions on pulmonary arteries ($n=4$, data not shown). Neither iso-OMPA nor neostigmine provoked contraction in human pulmonary arterial preparations at resting tone ($n=2$, data not shown). The ChE inhibitors (iso-OMPA, 100 μM; $n=5$ and neostigmine, 10 μM; $n=4$) did not modify either the lack of contractile response to ACh or basal tone in pulmonary veins (data not shown).

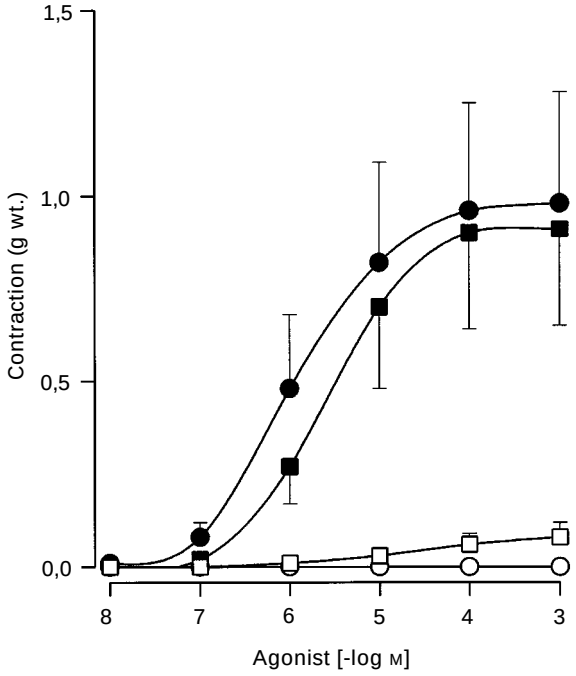

Figure 1 Cumulative concentration-response curves induced by (●, ○) acetylcholine (ACh) or (■, □) carbachol (CCh) in human pulmonary arteries (●, ■) and veins (○, □). Responses are expressed in (g wt.). Values are means from 7 to 13 different lung samples for arteries and veins, respectively; vertical lines show s.e.mean.

ACh-induced relaxations were performed on precontracted (NA, 10 μM) human pulmonary vessels (precontraction, 1.80 ± 0.48 and 1.73 ± 0.46 g wt. for arteries and veins derived from 4–6 lung samples, respectively). Arteries were significantly more responsive to ACh than veins (Table 1). CCh was less potent than ACh in relaxing vessels (Table 1). In addition, ChE inhibitors: iso-OMPA (100 μM, Table 2) and neostigmine (10 μM; $n=4-5$, data not shown) did not modify the ACh relaxations in arteries or veins.

Biochemical studies

The effect of the inhibitors on ChE activity in vascular homogenates, when a single concentration of ACTI (1 mM) was used as the substrate, are shown in Table 3. While, neostigmine (10 μM) inhibited almost completely ChE activity, BW284C51 (1 μM) and iso-OMPA (10 or 100 μM) inhibited approximately half the activity in both types of vessel.

The kinetic parameters for both ChE were obtained with different ranges of ACTI concentrations (see Methods). K_m values for AChE were 97 ± 17 and 106 ± 19 μM in arteries and veins, respectively, whereas K_{ss} values for BChE were 362 ± 72 and 491 ± 87 μM, respectively. V_{max} and V_{ss} values for AChE and BChE, respectively, are shown in Figure 3. For each enzyme, a two fold greater activity was observed in pulmonary veins when compared with pulmonary arteries.

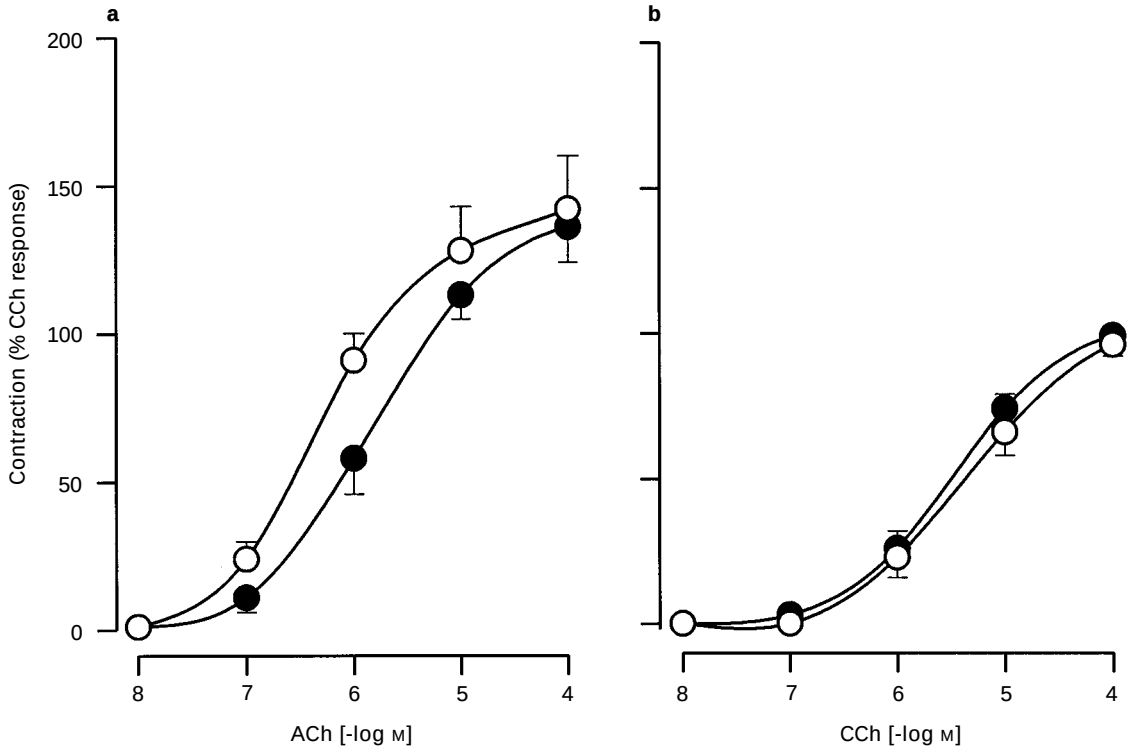

Figure 2 Effect of iso-OMPA (○,100 μM) on contraction induced by (a) ACh or (b) CCh in human pulmonary arteries; (●) control responses. Responses are expressed as % of precontraction induced by CCh (10 μM; 0.95 ± 0.21 g wt.). Values are means from 5 different lung samples; vertical lines show s.e.mean.

Table 1 Relaxations induced by ACh and CCh in human pulmonary arteries and veins precontracted with noradrenaline (10 μM)

	ACh			*CCh*		
	E_{max}	pD_2 *values*	n	E_{max}	pD_2 *values*	n
Artery	1.21±0.16	7.16±0.13	4	1.14±0.34	6.55±0.15*	4
Vein	0.60±0.15	5.56±0.17	6	0.74±0.12	4.95± >0.13*	5

E_{max} are expressed in (g wt.). Values are means±s.e.mean derived from different lung samples (*n*). *Indicates data significantly different ($P<0.05$) from corresponding values obtained with ACh stimulation (arteries, Student's paired *t*-test; veins, Student's test).

Table 2 Effect of iso-OMPA (100 μM) on relaxation produced by ACh in human pulmonary arteries precontracted with noradrenaline (10 μM)

	Control		*Iso-OMPA*	
	E_{max}	pD_2 *values*	E_{max}	pD_2 *values*
Artery	1.03±0.09	7.25±0.12	0.70±0.32	7.35±0.20
Vein	0.48±0.07	5.88±0.20	0.78±0.32	6.08±0.04

E_{max} are expressed in (g wt.). Values are means±s.e.mean derived from 3 different lung samples.

Table 3 Inhibition of ChE activity in vascular homogenates

	ChE activity (% control)	
Inhibitors	*Artery*	*Vein*
BW284C51 (1 μM)	62±4	47±5
Iso-OMPA (10 μM)	62±6	46±6
Iso-OMPA (100 μM)	59±5	47±6
Neostigmine (10 μM)	16±3	6±3

The inhibitions of ChE activities were determined in arterial and venous homogenates in the presence of a single concentration of substrate (ACTI, 1mM) according to Ellman's technique. For each lung sample, the resultant activity (with inhibitor) is expressed as % of the control value (without inhibitor). The control values were 2.32± 0.22 miu/mg^{-1} protein and 6.10±0.17 miu/mg^{-1} protein for arteries and veins, respectively. Values are means± s.e.mean derived from 6 different lung samples. All data are significantly different ($P<0.05$) from control values (Student's paired *t*-test).

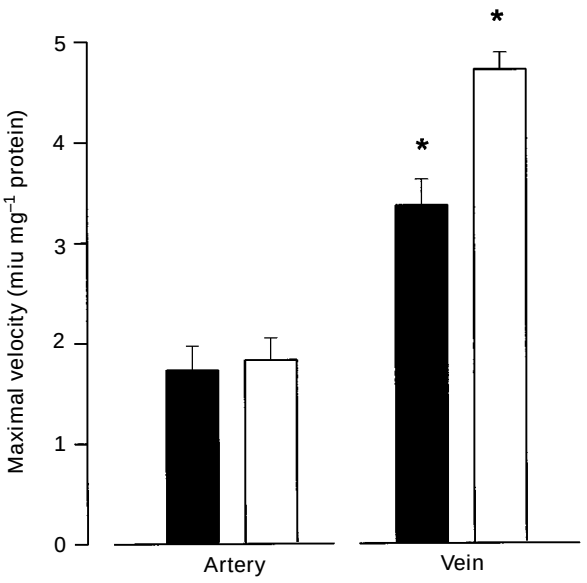

Figure 3 Maximal velocity of AChE (solid columns) and BChE (open columns) in human pulmonary arteries and veins. Values are means±s.e.mean from 6 different lung samples. *Indicates data significantly different ($P<0.05$) from corresponding values in arteries (Student's paired *t* test).

Discussion

ACh induced both contractions and endothelium-dependent relaxations in human isolated pulmonary arteries. However, iso-OMPA modified only the contractile response in these vessels. ChE activities were detected in pulmonary arteries and veins. Even though a greater enzyme activity was observed in veins, no physiological role for these enzymes in either the contraction or relaxation was observed.

A number of investigations based on both binding assay studies (Oettling *et al*., 1985) and smooth muscle contraction (Latifpour *et al*., 1989) have shown that CCh is a less potent agonist than ACh both for binding and activation of muscarinic receptors. Furthermore, the differences between ACh and CCh contractions may be less apparent when rank-order potency experiments are performed in tissues with ChE activity. In human pulmonary artery, CCh was as potent as ACh at evoking contractile response. This result suggests that ChE activity may modify the contraction resulting from muscarinic receptor activation of smooth muscle in human pulmonary arteries. The potentiation by iso-OMPA on arterial ACh-contractile response corroborates with this hypothesis (Figure 2a). Biochemical results on arterial homogenates showed that this inhibitor even at 100 μM did not inhibit more than 50% of ChE activity which was principally BChE (Table 3). Under these conditions, the effect of iso-OMPA on ACh-induced contractions in artery (Figure 2a) may be attributed to BChE activity. Neostigmine which was shown to inhibit completely ChE activity in arterial homogenates, failed to modify ACh-induced contractions in arteries. This observation may be partially explained by the low lipid solubility of neostigmine when compared with iso-OMPA (Silver, 1974). In marked contrast to the arterial preparations, ACh failed to elicit a contraction of the pulmonary vein. Significantly, CCh produced only a small contraction (Bourdillat *et al*., 1987; this study). It is noteworthy that the ChE inhibitors examined failed either to elicit a contraction (enhance the activity of endogenous ACh) or to uncover contractions of exogenous ACh. These observations indicate that venous ChE activity, while higher than the corresponding activity in artery, cannot account for the failure of ACh to produce a contractile response. The most likely explanation, therefore, is that a limited number of muscarinic receptors, on human venous smooth muscle, is associated with the contractile events. The relaxations produced in human pulmonary vessels confirmed that CCh was less potent than ACh. In addition, the ACh-induced relaxations were not modified by cholinesterase inhibitors. In human pulmonary artery the ACh contractions are in part regulated by cholinesterase activity, whereas the ACh relaxations are not. Since the ACh relaxations are endothelium-dependent (Furchgott & Zawadzki, 1980), these results indirectly suggest that the cho-

linesterase activity is probably not associated with the endothelium but rather with the vascular smooth muscle.

Similar physiological results were obtained by Altiere and co-workers (1994), ChE activity modified smooth muscle contraction but not the relaxation induced by ACh in the rabbit pulmonary arteries. Among the different types of canine venous preparations, only in tissues where the contractile response to ACh could be modified by neostigmine, was cholinesterase activity detected in smooth muscle cells by histochemical methods (Furuta *et al*., 1987). In rabbit and human pulmonary vessels, histochemical studies have demonstrated a cholinergic innervation in the adventitial-medial transitional zone (Cech, 1973; Amenta *et al*., 1983). These latter studies also demonstrated that nerve fibres penetrate into the media of pulmonary artery and no cholinesterase staining was detected in association with the endothelium. These histochemical results corroborated with the finding that no ChE activity was involved in the relaxant response of human pulmonary vessels in the present study. However, a study on cultured endothelial cell from microvessels of human foetal cortex has revealed the presence of both AChE and BChE activity (Kasa *et al*., 1991).

The ChE inhibitors, neostigmine and iso-OMPA, had no effect on the basal tone of either pulmonary arteries or veins. These results indirectly suggest that in these vessels there is little or not spontaneous release of ACh. These data are in contrast with results obtained in human isolated bronchial preparations where ChE inhibitors produced a marked contraction by accumulation of endogenous ACh (Norel *et al*., 1993).

Biochemical studies in rabbit intrapulmonary artery showed that ChE activity is due to AChE (Altiere *et al*., 1994). ChE activities were also measured in brain microcapillary suspensions. In foetal human cortex microcapillaries, the AChE activity was about fifteen fold greater than BChE activity (Kasa *et al*., 1991). In rat isolated forebrain capillaries, the AChE activity is about four fold greater than BChE activity (Shimon *et al*., 1989). In contrast, in human pulmonary arterial and venous homogenates the AChE and BChE activities were similar. In different studies the range of BChE activities (1.3–4.71 miu mg^{-1} protein) was generally smaller than that of AChE activity (1.73–28.87 miu mg^{-1} protein). There was a greater variability of AChE activity in comparison with BChE activity in various vascular preparations. The reason for these differences remains to be explored. The amount of ChE activity measured in human pulmonary arterial homogenates was smaller than that in rabbit pulmonary arterial homogenates, suggesting that total ChE activity may vary between species.

Together the data derived from physiological experiments in human pulmonary arteries (present study) and data obtained in human bronchus (Norel *et al*., 1993) suggest that, in human lung, BChE activity may play a role in modifying contractile activities of smooth muscle. In human pulmonary veins, the involvement of ChE activity in the control of smooth muscle tone is difficult to observe even though this tissue was shown to contain a considerable amount of cholinesterase enzymes.

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