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Characterization of muscarinic receptors that mediate contraction of guinea-pig isolated trachea to choline esters: effect of removing epithelium

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1 The muscarinic receptor subtype that mediates contraction of guinea-pig trachea, in the presence and absence of epithelium, to acetic and carbamic acid choline esters was determined by use of preferential muscarinic receptor antagonists: pirenzepine (M_1 receptor), methoctramine (M_2 receptor) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) (M_3 receptor).

2 Acetylcholine (ACh), methacholine (MeCh), carbachol (CCh), bethanechol (BeCh) and oxotremorine induced concentration-dependent contraction of guinea-pig isolated tracheal strips in the presence and absence of epithelium. Contraction to acetic choline esters (ACh and MeCh) was augmented by removal of the epithelium, whereas contraction to carbamic acid choline esters (CCh and BeCh) and oxotremorine was not influenced by removal of the epithelium.

3 Pirenzepine, methoctramine and 4-DAMP caused parallel rightward displacements of the concentration-contraction curves to the muscarinic agonists. The pA_2 values (determined from Arunlakshana-Schild graphs) for pirenzepine and 4-DAMP in guinea-pig trachea in the presence of epithelium were: ACh as the agonist, 7.6 and 9.0, respectively; CCh as the agonist, 7.6 and 9.1, respectively. The apparent pK_B values for methoctramine with the same system were: ACh as the agonist, 5.6; CCh as the agonist, 5.6. Similar values were obtained with MeCh, BeCh and oxotremorine as the agonists. These values were agonist- and epithelium-independent.

4 It is concluded from the pA_2 and apparent pK_B values obtained for the muscarinic receptor antagonists used in this study that contraction of guinea-pig isolated trachea, with and without epithelium, to both acetic and carbamic acid choline esters is mediated via the muscarinic M_3 receptor subtype. Differential contractile responses of guinea-pig trachea to acetic and carbamic acid choline esters upon the mechanical removal of the epithelium may not be explained by activation of different muscarinic receptor subtypes by these agonists.

Keywords: Trachea; choline esters; epithelium; muscarinic receptors

Introduction

The responsiveness of airway smooth muscle preparations to various stimuli *in vitro*, may be modulated by the presence of a functional epithelium (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; for reviews see Fedan *et al.*, 1988; Goldie *et al.*, 1990; Morrison *et al.*, 1990). Contraction of guinea-pig trachea to acetic choline esters, e.g. acetylcholine (ACh), which is the endogenous cholinergic transmitter, and methacholine (MeCh), but not to the carbamic acid derivatives, e.g. carbachol (CCh) and bethanechol (BeCh), is augmented by the mechanical removal of the epithelium (Goldie *et al.*, 1986; Hay *et al.*, 1986; Holroyde, 1986; Tschirhart *et al.*, 1987; Small *et al.*, 1990). This has suggested that acetic, but not carbamic acid choline esters, may stimulate the release of epithelium-derived relaxing factor (s) (EpDRF (s)) that depress tracheal tone (Goldie *et al.*, 1990). However, both ACh and CCh stimulate the release of a substance (s) from tracheal epithelium that relaxes vascular smooth muscle in the co-axial bioassay preparation (Ilhan & Sahin, 1986; Fernandes & Goldie, 1990). Furthermore, contraction of guinea-pig tracheal strips, denuded of epithelium, to CCh is diminished when this tissue is surrounded by an intact tracheal segment (Guc *et al.*, 1988a). Therefore, the differential contractile responses of guinea-pig trachea, in the presence and absence of epithelium, to acetic and carbamic acid choline esters may be due to a mechanism (s) other than differences in the abilities of these cholinoceptor agonists to stimulate the release of a relaxing substance (s) from the epithelium.

Choline esters induce contraction of the guinea-pig trachea and the release of a relaxing substance (s) from the epithelium via the stimulation of muscarinic receptors since these responses are sensitive to atropine (Eglen & Whiting, 1988; Guc *et al.*, 1988b; Fernandes *et al.*, 1989). Muscarinic receptors have been classified, from functional studies, into three major subtypes which are present in the airways: M_1, M_2 and M_3 receptors (Barnes *et al.*, 1988). Both M_1 and M_2 muscarinic receptors may be involved in the epithelial control of smooth muscle tone in response to cholinoceptor agonists in the rabbit trachea (Lev *et al.*, 1990). The aim of the present study was to determine the role of muscarinic receptors in the modulation of smooth muscle reactivity to choline esters by the epithelium in guinea-pig trachea. This study used preferential receptor antagonists to characterize the muscarinic receptor subtypes in this preparation.

Methods

Preparation of tracheal strips

Male, Hartley guinea-pigs (350–500 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and killed by exsanguination. The trachea was excised and placed in cold physiological salt solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium edetate 0.026, and glucose 11.1 Fat and connective tissue were cleaned from the trachea and paired rings (2 cartilage rings wide) were prepared. In one ring of each pair, the epithelium was removed

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by inserting the tips of a watchmaker's forceps into the lumen and rolling the tissue gently over a saline-soaked filter paper. Transverse strips were then prepared from each ring by making a longitudinal cut along the ventral surface of the cartilage. The successful removal of the epithelium was confirmed by histological examination of the luminal surfaces of the tissues at the end of preliminary experiments (e.g. Flavahan *et al.*, 1988). Tracheal strips were suspended between two stainless steel wire hooks, under a resting load of 500 mg (e.g. Tucker *et al.*, 1990), in organ chambers containing 25 ml of physiological salt solution, maintained at 37°C, and gassed with a mixture of 95% O_2:5% CO_2 (pH 7.4). Tissues were connected to a strain gauge (Statham Gould UC2) for recording isometric tension and allowed to equilibrate for 45 min with regular washing before the start of experiments.

Materials

The following drugs were used: acetylcholine chloride; acetyl-β-methyl choline chloride (methacholine); carbamylcholine chloride (carbachol); carbamyl-β-methylcholine chloride (bethanechol); eserine sulphate (physostigmine); indomethacin; oxotremorine sesquifumarate and pirenzepine dihydrochloride (Sigma Chemical Company, St Louis, MO, U.S.A.); hexamethonium bromide (K & K Laboratories, Incorporated, Plainview, NY, U.S.A.); 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and methoctramine 4-hydrochloride (Research Biochemicals Incorporated, Natick, MA, U.S.A.). Most drugs were prepared daily in distilled water, kept on ice and added to organ chambers in volumes not exceeding 250 μl. Indomethacin was prepared with an equimolar concentration of Na_2CO_3. Concentrations of drugs were expressed as the final organ chamber concentrations (M).

Experimental protocol

All experiments were conducted in the presence of physostigmine (0.1 μM; an inhibitor of acetylcholinesterase) and hexamethonium (1 mM; a ganglionic nicotinic receptor antagonist). Tissues, with and without epithelium, were contracted with ACh (100 μM), followed by a washout period of 30 min. Absolute contraction to ACh was not affected significantly by removal of the epithelium (e.g. with epithelium: 483.3 ± 35.3 mg; without epithelium: 440.0 ± 42.5 mg; $n = 6$). Subsequent contraction to agonists was expressed as a percent of the contraction to ACh (100 μM) since this concentration of ACh was equi-effective in the presence or absence of epithelium. Tracheal strips were then incubated with either pirenzepine (0.03–1 μM), methoctramine (3 and 30 μM), or 4-DAMP (1–30 nM) for 30 min. Control and treated tissues were studied in parallel. Cumulative concentration-response curves for ACh, CCh, MeCh (0.01–100 μM), BeCh (0.01 μM–1 mM) and oxotremorine (1 nM–30 μM) were then obtained. One concentration-response curve was obtained from each tissue.

pA_2 values (estimates of the equilibrium dissociation constants) for pirenzepine and 4-DAMP were determined from graphs of log concentration-ratios minus 1 (CR-1) versus log concentration of antagonist (Arunlakshana & Schild, 1959). The concentration-ratio is defined as the concentration of agonist required to induce a 50% maximal contraction (EC_{50}) in the presence of the antagonist, divided by the agonist EC_{50} value in the absence of antagonist. The slopes and intercepts on the abscissae of the Arunlakshana-Schild graphs were determined by linear regression (Statview II, Berkeley, CA, U.S.A.). Since only 2 concentrations of methoctramine were used, pA_2 values for this antagonist could not be determined from Arunlakshana-Schild graphs. Apparent pK_B values (equivalent to the negative logarithm of the dissociation constant) for methoctramine at each concentration were calculated by single point analysis using the formulae:

$$K_B = \frac{\text{Concentration of methoctramine}}{\text{Concentration ratio-1}}$$

$$pK_B = -\log K_B$$

(Furchgott, 1972)

Analysis of data

Results are expressed as mean ± s.e.mean. All n values refer to the number of animals. For single comparisons within or between groups, Student's paired or unpaired t test was used. For multiple comparisons between groups, one-way analysis of variance was used and where significance was indicated, Scheffe's test (Scheffe, 1959) was applied. Statistical significance was accepted at P values of less than 0.05.

Results

ACh, MeCh, CCh (0.01–100 μM), BeCh (0.01 μM–1 mM) and oxotremorine (1 nM–30 μM) induced concentration-dependent contraction of guinea-pig tracheal strips. Contraction induced by ACh (Figure 1a) and MeCh was augmented by removal of the epithelium whereas contraction to CCh (Figure 1b), BeCh and oxotremorine was not influenced by the presence or absence of the epithelium (Table 1). The maximal responses to each agonist, in the presence and absence of epithelium, were not significantly different (Table 1).

Indomethacin (5 μM) caused a small, leftward and parallel displacement of the concentration-response curves for ACh and CCh both in the presence and absence of epithelium. There was no significant change in the maximal responses induced by these agonists. Indomethacin did not abolish the augmentation of contraction to ACh upon denudation of the epithelium (Table 1).

Pirenzepine (0.03–1 μM), methoctramine (3 and 30 μM), and 4-DAMP (1–30 nM) caused parallel rightward displacements of the concentration-response curves (at the linear portion of the curves) to ACh, MeCh, CCh, BeCh and oxotremorine with no significant changes in the maximal contraction of the tissues, both in the presence and absence of epithelium. The effects of 4-DAMP on contraction induced by ACh or CCh, in the presence and absence of epithelium, are shown in Figures 2 and 3, respectively. 4-DAMP caused concentration-dependent parallel rightward displacements of the concentration-response curves for ACh and CCh. The concentration-ratio shifts (CR) induced by 4-DAMP (1 nM, 10 nM and 30 nM) were, respectively: ACh as the agonist, 2, 8 and 20 with epithelium and 2, 14 and 22 without epithelium; CCh as the agonist, 2, 10 and 19 with epithelium and 2, 7 and 16 without epithelium.

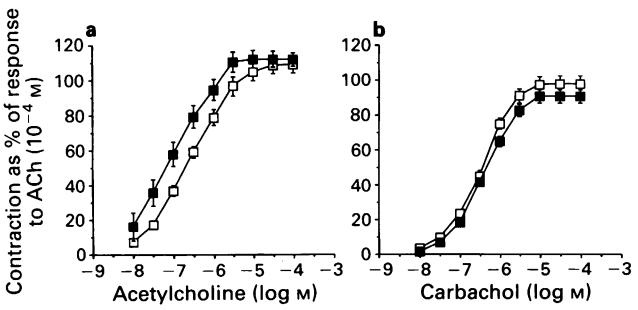

Figure 1 Concentration-response curves for acetylcholine (ACh, a) and carbachol (b) in guinea-pig trachea in the presence (□) and absence (■) of epithelium. Results are shown as mean values (with s.e.mean indicated by vertical bars) and are expressed as a percentage of the contraction to ACh (100 μM). $n = 6$.

Table 1 Responses of guinea-pig trachea to muscarinic agonists

Agonist	$-\log$ M EC_{50} with epithelium	$-\log$ M EC_{50} without epithelium	Maximal response with epithelium	Maximal response without epithelium
ACh	6.61 ± 0.05	7.04 ± 0.06*	109.2 ± 4.7	110 ± 6.2
ACh + indomethacin	6.88 ± 0.05	7.17 ± 0.05*	105.8 ± 3.3	120.5 ± 10.6
MeCh	6.49 ± 0.04	6.75 ± 0.05*	98.3 ± 5.2	96.3 ± 6.3
CCh	6.38 ± 0.05	6.41 ± 0.07	97.0 ± 7.1	94.0 ± 4.5
CCh + indomethacin	6.55 ± 0.09	6.76 ± 0.06	129.8 ± 12.6	118.3 ± 8.8
BeCh	5.05 ± 0.15	5.09 ± 0.12	106.3 ± 6.1	113.9 ± 7.5
Oxotremorine	6.85 ± 0.06	6.91 ± 0.03	102.5 ± 7.3	97.9 ± 7.1

Results are expressed as mean ± s.e.mean. $n = 5$–6. ACh: acetylcholine; MeCh: methacholine; CCh: carbachol; BeCh: bethanechol. *Values are significantly different from tissues with epithelium.
Maximal response is expressed as percentage of the contraction to ACh (100 μM).

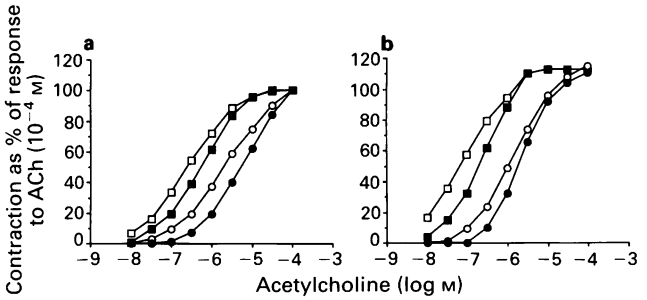

Figure 2 Effect of 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, 1–30 nM) on contraction to acetylcholine (ACh) in guinea-pig trachea in the presence (a) and absence (b) of epithelium. Responses of control tissues (□) and effects of 4-DAMP 1 nM (■), 10 nM (○), and 30 nM (●) are shown. Results are shown as mean values and are expressed as a percentage of the contraction to ACh (100 μM). Error bars (s.e.mean) are omitted for clarity but did not exceed 10%. $n = 5$.

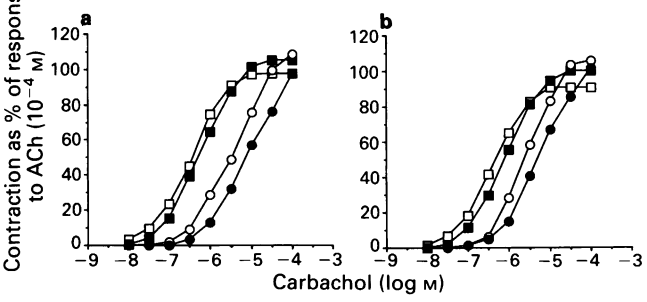

Figure 3 Effect of 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, 1–30 nM) on contraction to carbachol in guinea-pig trachea in the presence (a) and absence (b) of epithelium. Responses of control tissues (□) and effects of 4-DAMP 1 nM (■), 10 nM (○), and 30 nM (●) are shown. Results are shown as mean values and are expressed as a percentage of the contraction to ACh (100 μM). Error bars (s.e.mean) are omitted for clarity but did not exceed 10%. $n = 5$.

The pA_2 values for pirenzepine and 4-DAMP (Table 2) and the apparent pK_B values for methoctramine (Table 3) did not differ significantly with the muscarinic agonist chosen for study, nor by removal of the epithelium. The values of log (CR − 1) plotted on the Arunlakshana-Schild graphs for 4-DAMP versus ACh or CCh, in the presence and absence of epithelium, were not significantly different (Figure 4). Furthermore, the slopes of the Arunlakshana-Schild plots for pirenzepine and 4-DAMP versus each agonist did not differ significantly from unity (Table 2).

Table 2 Summary of pA_2 values for muscarinic antagonists in guinea-pig trachea

Agonist	Antagonist: Pirenzepine	Antagonist: 4-DAMP
Acetylcholine with epithelium	7.6 ± 0.09 (0.84)	9.0 ± 0.06 (0.80)
Acetylcholine without epithelium	7.5 ± 0.08 (0.83)	9.0 ± 0.05 (0.90)
Methacholine with epithelium	7.4 ± 0.07 (0.84)	8.8 ± 0.05 (0.98)
Methacholine without epithelium	7.5 ± 0.06 (0.91)	9.0 ± 0.08 (0.89)
Carbachol with epithelium	7.6 ± 0.05 (0.91)	9.1 ± 0.06 (0.89)
Carbachol without epithelium	7.6 ± 0.10 (0.80)	9.1 ± 0.07 (0.79)
Bethanechol with epithelium	7.4 ± 0.06 (0.77)	8.9 ± 0.05 (0.90)
Bethanechol without epithelium	7.6 ± 0.09 (1.18)	8.8 ± 0.07 (0.88)
Oxotremorine with epithelium	7.7 ± 0.07 (0.97)	8.9 ± 0.04 (0.88)
Oxotremorine without epithelium	7.8 ± 0.04 (0.75)	9.0 ± 0.03 (0.80)

Results are shown as mean ± s.e.mean. $n = 4$–6. 4-DAMP: 4-diphenylacetoxy-N-methylpiperidine.
Values in parentheses are the slopes of the Arunlakshana-Schild graphs, and are not significantly different from unity.

Discussion

This study used preferential muscarinic receptor antagonists to characterize the muscarinic receptor subtypes that mediate contraction of guinea-pig tracheal strips to acetic and carbamic acid choline esters in the presence and absence of epithelium.

In guinea-pig trachea, muscarinic M_3 receptors are located on the smooth muscle cells (Mak & Barnes, 1990). However, muscarinic receptors may also be present on epithelial cells since atropine prevents the release of a relaxing substance (s) from the epithelium in response to choline esters in the co-axial bioassay assembly (Ilhan & Sahin, 1986; Guc *et al.*, 1988b; Fernandes *et al.*, 1989).

Contraction of tracheal strips to the cholinergic transmitter, ACh and its methyl derivative, MeCh, was augmented by removal of the epithelium. However, contraction to the carbamic acid choline esters CCh and BeCh, was not altered by the presence of the epithelium. This confirms previous findings and demonstrates that contraction of guinea-pig trachea to acetic, but not carbamic acid choline esters is

Table 3 Summary of apparent pK_B values for methoctramine in guinea-pig trachea

Agonist	*pK_B methoctramine 3 μM*	*pK_B methoctramine 30 μM*
ACh	5.5 ± 0.12	5.7 ± 0.10
with epithelium		
without epithelium	5.2 ± 0.11	5.2 ± 0.12
MeCh	4.9 ± 0.11	5.3 ± 0.07
with epithelium		
without epithelium	5.0 ± 0.14	5.5 ± 0.05
CCh	5.7 ± 0.08	5.6 ± 0.06
with epithelium		
without epithelium	5.6 ± 0.04	5.4 ± 0.06
BeCh	5.8 ± 0.05	5.5 ± 0.06
with epithelium		
without epithelium	5.6 ± 0.04	5.4 ± 0.06
Oxotremorine	5.7 ± 0.03	5.7 ± 0.04
with epithelium		
without epithelium	5.5 ± 0.06	5.6 ± 0.03

ACh: acetylcholine; MeCh: methacholine; CCh: carbachol; BeCh: bethanechol.
Results are shown as − log M and are expressed as mean ± s.e.mean. $n = 4-6$.

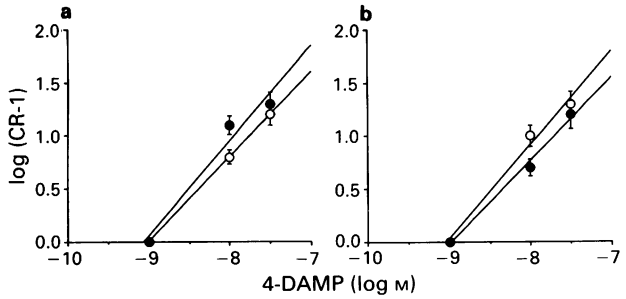

Figure 4 Arunlakshana-Schild graphs for 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) versus acetylcholine (a) or carbachol, (b) in guinea-pig trachea in the presence (○) and absence (●) of epithelium. Results are shown as mean with s.e.mean indicated by vertical bars. $n = 5$.

modulated by the presence of a functional epithelium (Goldie *et al.*, 1986; Hay *et al.*, 1986; Holroyde 1986; Tschirhart *et al.*, 1987; Small *et al.*, 1990).

Pirenzepine, methoctramine and 4-DAMP caused concentration-dependent parallel rightward displacements of the concentration-contraction curves for each cholinoceptor agonist that was studied, suggesting competitive antagonism at the muscarinic receptors. This is emphasized by the facts that: (a) the slopes of the Arunlakshana-Schild graphs for pirenzepine and 4-DAMP were not significantly different from unity (Arunlakshana & Schild, 1959); and (b) the apparent pK_B values for methoctramine were not significantly different from each other at the two concentrations used (Kenakin, 1984). The pA_2 values obtained for pirenzepine and 4-DAMP, and the apparent pK_B values calculated for methoctramine are consistent with muscarinic M_3 receptors mediating contraction of guinea-pig trachea to choline esters, in the presence and absence of epithelium (Eglen & Whiting, 1988). The apparent affinities of the receptor antagonists for muscarinic receptors were not influenced by the cholinoceptor agonist chosen for study. Furthermore, these affinity values were not altered significantly by removal of the epithelium. It appears from these results, therefore, that contraction of guinea-pig trachea in the presence and absence of epithelium in response to acetic and carbamic acid choline esters and oxotremorine is mediated via muscarinic M_3 receptors. This is consistent with other findings (Eglen *et al.*, 1991).

The augmentation of contraction of airway smooth muscle preparations to various agonists upon removal of the epithelium has suggested that it is stimulated release of a non-prostanoid EpDRF (s) that depresses muscle tone (Barnes *et al.*, 1985; Flavahan *et al.*, 1985). The evaluation of antagonist potency in the present experiments was conducted in the absence of indomethacin and therefore, the involvement of epithelium-derived relaxant prostanoids in the modulation of smooth muscle tone cannot be excluded. The effect of removing the epithelium on contraction to ACh may be mimicked by indomethacin in intact guinea-pig tracheal strips (Hay *et al.*, 1986). Also, indomethacin can impair the augmentation of contraction to acetic choline esters in this preparation (Eglen *et al.*, 1991). In contrast, several studies have demonstrated that augmentation of contraction of guinea-pig trachea to ACh and MCh upon removal of the epithelium is not impaired by indomethacin (e.g. Holroyde, 1986; Murlas, 1986). Furthermore, in the present study, inhibition of cyclooxygenase by indomethacin caused a small parallel leftward displacement of the concentration-response curves to both ACh and CCh in the presence and absence of epithelium. However, the augmentation of contraction to ACh upon denudation of the epithelium was not inhibited by indomethacin. These results suggest that the putative EpDRF(s) may not be a prostanoid. The release of EpDRF(s) from the same tissue induced by muscarinic receptor agonists in the co-axial bioassay system is insensitive to indomethacin (Guc *et al.*, 1988b; Fernandes *et al.*, 1989; Eglen *et al.*, 1991). Although the release of prostanoid and non-prostanoid epithelium-derived relaxing substances was not measured in this study, the results obtained are consistent with the proposal that the release of EpDRF(s) induced by both acetic and carbamic acid choline esters from the epithelium of guinea-pig trachea is mediated via muscarinic M_3 receptors (Eglen *et al.*, 1991). However, it must be recognised that the relaxing substance (s) that is detected in the co-axial bioassay assembly, may be distinct from the putative EpDRF(s) that may modulate airway smooth muscle tone (Goldie *et al.*, 1990). Failure to observe a modulatory effect of the epithelium on contraction to carbamic acid choline esters in tracheal strips suspended in conventional organ chambers does not imply that CCh cannot stimulate the release of EpDRF(s). CCh may induce the release of EpDRF(s) that is detected by tracheal strips denuded of epithelium in the co-axial bioassay preparation (Guc *et al.*, 1988a).

The differential contractile responses of guinea-pig trachea to acetic and carbamic acid choline esters upon removal of the epithelium may be explained by differences in the abilities of these choline esters to diffuse to the muscarinic receptor sites in the tracheal model used in this and other studies. It is clear that the epithelium functions as a barrier to the diffusion of various bronchoactive agents (Boucher *et al.*, 1978; 1981; Nadel *et al.*, 1985; Weibel, 1985; Holroyde, 1986; Undem *et al.*, 1988). Indeed, contraction of perfused tracheal segments to CCh was augmented by removal of the epithelium when this agonist was applied exclusively to the luminal surface (Small *et al.*, 1990). The present experiments were performed under apparent equilibrium conditions since the slopes of the Arunlakshana-Schild graphs were not significantly different from unity, and similar pA_2 and apparent pK_B values were calculated for the receptor antagonists in the presence and absence of epithelium (Arunlakshana & Schild, 1959). This suggests that the two classes of choline ester had equal access to the muscarinic receptor population in the guinea-pig tracheal strip preparation (Kenakin, 1984). Alternatively, if the epithelium functioned as a non-saturable barrier over the full range of agonist concentrations, then parallel rightward shifts in the agonist concentration-effect curves would be obtained in response to the muscarinic antagonists. Thus, these antagonists would exhibit competitive antagonism and the Arunlakshana-Schild plots

would yield slopes of unity. However, if the barrier capability of the epithelium became saturated at higher concentrations of agonists, then the equilibrium state of the agonist, antagonist and receptor population would be changed and the slope of the Arunlakshana-Schild plot would be less than unity (Kenakin, 1984). Therefore, the present results are consistent with the epithelium behaving as a non-saturable diffusion barrier. The relative contributions of the epithelium as a diffusion barrier and source of EpDRF(s) to the regulation of tracheal smooth muscle tone, cannot be determined from the results of this study.

Differences in the susceptibilities of cholinoceptor agonists to hydrolysis by cholinesterase may also explain the differential contractile responses to acetic and carbamic acid choline esters in guinea-pig trachea. However, cholinesterase activity does not account for the augmented contractions to cholinoceptor agonists upon removal of the epithelium in this tissue (Small *et al.*, 1990). Furthermore, the present experiments were conducted in the presence of physostigmine (0.1 μM), an inhibitor of cholinesterase, and augmentation of contraction to acetic choline esters was still observed.

The present study demonstrates that contraction of guinea-pig trachea, in the presence and absence of epithelium, to both acetic and carbamic acid choline esters is mediated by muscarinic M_3 receptors. This suggests that the differential contractile responses of guinea-pig trachea to acetic and carbamic acid choline esters upon removal of the epithelium, may not be explained by activation of different muscarinic receptor subtypes.

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