Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells

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1 Activation of muscarinic receptor subtypes leads to contraction, an increase in the accumulation of inositol phosphates (IPs) and a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) synthesis in tracheal smooth muscle. The concentrations of carbachol that produced a half-maximal effect (EC_{50}) in inhibition of cyclic AMP generation, stimulation of IPs formation and contraction were 15 nM, 2.0 μ M and 0.17 μ M, respectively.

2 Pirenzepine, a selective M_1 antagonist, displayed a low affinity for antagonizing cyclic AMP inhibition, IPs formation and contraction induced by carbachol (pK_B = 6.8, 7.0, and 7.1, respectively).

3 Methoctramine, a cardioselective M_2 antagonist, blocked cyclic AMP inhibition with a high affinity (pK_B = 7.5), while it antagonized IPs formation and contraction with a low affinity (pK_B = 6.2 and 6.1, respectively).

4 4-Diphenylacetoxy-N-methylpiperidine (4-DAMP), a selective smooth muscle M_3 antagonist, possessed a high affinity in blocking IPs formation (pK_B = 8.8) and contraction (pK_B = 9.2) as well as a low affinity for antagonism of cyclic AMP inhibition (pK_B = 8.1).

5 In conclusion, we have demonstrated that M_2 and M_3 receptor subtypes are coupled to different effector systems in tracheal smooth muscle. An M_1 receptor subtype is not involved in the generation of the second messengers examined. Inhibition of cyclic AMP formation may be coupled to the M_2 receptor subtype. The accumulation of IPs and presumably IP-induced Ca^{2+} release may function as the transducing mechanism for cholinergic contraction of tracheal smooth muscle through the activation of M_3 receptors.

Keywords: Smooth muscle; cyclic AMP; inositol phosphates; muscarinic receptor subtypes; contraction

Introduction

Contractions of the tracheal smooth muscle are mediated in part through muscarinic receptors. One of factors that may contribute to the diversity of physiological responses is the presence of multiple subtypes of the muscarinic receptors. By the use of pirenzepine, these receptors have been subdivided into M_1 and M_2 subtypes (Hammer *et al.*, 1980). Recently, experimental evidence has been obtained for the subclassification of muscarinic receptors into M_1 , M_2 and M_3 , based on studies with selective antagonists (Nathanson, 1987; Doods *et al.*, 1987). Pirenzepine has high affinity for M_1 receptors (Hammer *et al.*, 1980), AF-DX 116 and methoctramine for M_2 receptors (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987; Micheletti *et al.*, 1987; Giraldo *et al.*, 1988) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) as well as HHSiD for M_3 receptors (Barlow *et al.*, 1976; Mutschler & Lambrecht, 1984). Furthermore, genetic studies have indicated that M_1 , M_2 , and M_3 subtypes are encoded by separate m1, m2, and m3 genes (Bonner, 1989; Buckley *et al.*, 1989). In addition, distinct m4 and m5 genes have been detected (Buckley *et al.*, 1989). That different receptor subtypes are coupled to various effector systems has been substantiated by the recent demonstration that the expressed m1, m3, and m5 subtypes are preferentially coupled to hydrolysis of phosphoinositides, whereas the m2 and m4 subtypes are coupled to attenuation of adenylate cyclase (Ashkenazi *et al.*, 1987; Bonner *et al.*, 1988; Shapiro *et al.*, 1988; Peralta *et al.*, 1988; Bonner, 1989). Expression of these receptor genes in cell lines has made it possible to explore the pharmacological properties of each distinct structure, leading to a direct correlation among genes, protein structure and pharmacological properties. Whether a single receptor subtype couples to a single effector system or each muscarinic receptor subtype can interact with multiple effectors remains unclear.

Muscarinic receptors are also known to interact via a G protein regulated process, with multiple effector systems leading to inhibition of adenylate cyclase, increased phosphoinositide breakdown, arachidonate release, and modulation of potassium channels (Nathanson, 1987). In the tracheal smooth muscle, activation of muscarinic receptors leads to phosphoinositide breakdown and contraction of the smooth muscle (Grandordy *et al.*, 1986; Roffel *et al.*, 1990) as well as inhibition of adenylate cyclase (Sankary *et al.*, 1988). Also, M_2 and M_3 receptor subtypes have been characterized in tracheal smooth muscle (Roffel *et al.*, 1988; Yang, 1991). Therefore, the question as to how the receptor subtypes regulate the function of tracheal smooth muscle through the generation of different second messenger systems requires study.

The purpose of the present study was to determine the correlation between muscarinic receptors mediating adenylate cyclase inhibition and receptors mediating the generation of IPs and contraction. The pharmacological investigation performed by use of several muscarinic antagonists and carbachol revealed that these two second messenger responses involved distinct muscarinic receptor subtypes in tracheal smooth muscle. The data suggest that both effectors may play roles in the overall physiological function of tracheal smooth muscle, but phosphoinositide breakdown is the transducing mechanism of muscarinic stimulation for smooth muscle contraction.

Methods

Animals

Mongrel dogs, 20–30 kg, purchased from a local supplier, were used throughout this study. The dogs were fed with standard laboratory chow and tap water *ad libitum* and were housed indoors in the animal facilities under automatically controlled temperature and light cycle. Dogs of either sex were anaesthetized with pentobarbitone (30 mg kg⁻¹, intravenously) and

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the lungs were ventilated mechanically via an orotracheal tube. The trachea was removed from the animal.

Preparation of tracheal smooth muscle

The trachea, about 20 cm long from the larynx to bifurcation, was quickly removed, and transferred into oxygenated (95% O₂ and 5% CO₂; pH 7.2) Krebs Henseleit solution (KHS; Farley & Miles, 1977). The tracheal smooth muscle was maintained in KHS throughout the experiment. The trachea was opened by cutting the cartilage rings opposite the muscle. The muscle was cleaned in two steps. The initial step in each dissection involved removal of the epithelium and submucosal tissue with forceps. The connective tissue on the serosal surface was carefully cleaned under a microscope and cartilage up to the point of insertion of the muscle deep into the cartilage rings, then the trachea was cut into separate individual rings. Each of the tracheal rings was suspended in KHS in a muscle chamber and continuously bubbled with 95% O₂ and 5% CO₂ at 37°C. The tracheal rings were hooked to Gould (BG-25 gm) force displacement transducers with fine silk thread. Isometric muscle tension was recorded with a Gould 3000 recorder (Cleveland, Ohio, U.S.A.). A base line tension of 0.5 g was applied in preliminary experiments and found to be adequate for maximum force generation. The muscles were allowed to stabilize in the muscle chambers for 1 h with two or three changes of the KHS during this period. A minimum of six muscles from different animals was used to generate the dose-response curves.

Isolation of tracheal smooth muscle cells

For studying the effects of activation of muscarinic receptors on the phosphoinositide breakdown and adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation, enzymatically isolated tracheal smooth muscle cells (TSMCs) were used in this study. The smooth muscle was dissected out, minced and transferred to the dissociation medium containing 0.2% collagenase I, 0.01% DNase I, and 0.01% elastase IV as well as antibiotics in physiological solution. The physiological solution used contained (mM): NaCl 137, KCl 5, CaCl₂ 1.1, NaHCO₃ 20, NaH₂PO₄ 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were then gently agitated at 37°C in a rotator for 1 h. The released cells were collected and the remaining tissue was digested in the same fresh enzyme solution for an additional 1 h at 37°C. All the released cells were collected and washed with KHS. The cell number was counted and diluted with KHS to 10⁶ cells ml⁻¹.

In order to characterize the isolated TSMCs and to exclude the possibility of epithelial cells and fibroblast contamination, the TSMCs were identified by an indirect immunofluorescence method (Gown *et al.*, 1985). Over 95% of the cells isolated in this study were smooth muscle cells.

Accumulation of inositol phosphates

The effects of muscarinic agents on the hydrolysis of phosphoinositide were assessed by monitoring the accumulation of ³H-labelled inositol phosphates as described by Berridge *et al.* (1983). Isolated TSMCs were incubated with 10 µCi ml⁻¹ of myo-[2-³H]-inositol at 37°C for 2 h. TSMCs were washed 3 times with and incubated in Krebs Henseleit buffer (pH 7.4) containing (mM): NaCl 117, KCl 4.7, MgSO₄ 1.1, KH₂PO₄ 1.2, NaHCO₃ 20, CaCl₂ 2.4, glucose 1, HEPES 20 and LiCl 10, at 37°C for 10 min. After 10 min, carbachol was added at the indicated concentration and incubation was continued for a further 30 min. When an antagonist was used, it was added 10 min before the addition of the agonist. Reactions were terminated by adding 5% perchloric acid followed by sonication and centrifugation at 3000 g for 15 min.

The perchloric acid-soluble supernatants were extracted 4 times with ether, neutralized with KOH and applied to a column of the anion exchange resin (AG1-X8, formate form,

100–200 mesh). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate/5 mM sodium tetraborate to eliminate free [³H]-myo-inositol and glycerophosphoinositol, respectively. A total IPs fraction was then eluted with 10 ml of 1 M ammonium formate/0.1 M formic acid. The amount of [³H]-inositol phosphates were determined by scintillation counting of 0.5 ml samples.

Cyclic AMP assay

TSMCs were incubated with 3-isobutyl-1-methylxanthine (IBMX) at a final concentration of 0.5 mM at 37°C for 10 min and then exposed to 1 µM isoprenaline alone or combined with the specific muscarinic agonist carbachol used at the indicated concentrations and continuously incubated at 37°C for 10 min. Muscarinic antagonists, when used, were added at the indicated concentrations 10 min before the addition of carbachol and/or isoprenaline. Reactions were stopped by boiling the cells for 3 min and followed by centrifugation at 10,000 g for 20 min at 4°C. Cyclic AMP was assessed by Amersham cyclic AMP assay kit. Cyclic AMP levels were expressed as pmol/10⁶ cells. Inhibition of cyclic AMP accumulation mediated by carbachol was evaluated as the difference between cyclic AMP due to isoprenaline and cyclic AMP obtained in the simultaneous presence of isoprenaline and carbachol. Alternatively, inhibition was expressed as the percentage of the inhibitory response to a maximally effective concentration of carbachol.

Data analysis

The EC₅₀ values of carbachol for inhibition of cyclic AMP formation, IPs accumulation and contraction were estimated by Graph Pad programme (Graph Pad, San Diego, California, U.S.A.), sharing the same estimate of the maximum response between the control data and the data measured in the presence of muscarinic antagonists. When several concentrations of antagonists were used, the dissociation constants (*K_B*) of muscarinic antagonists were estimated by measurement of their abilities to antagonize carbachol-mediated inhibition of cyclic AMP formation, stimulation of phosphoinositide hydrolysis and contraction of the tracheal smooth muscle. The *K_B* values were calculated as described by Furchgott (1972).

All the data are expressed as the means ± standard error (s.e.) of at least three experiments with statistical comparisons based on a two-tailed Student's *t* test. The level of significance was chosen at the *P* < 0.05 level. The Graph Pad programme was used to generate graphical data and in nonlinear regression analyses.

Drugs

Myo-[2-³H]-inositol (18 Ci mmol⁻¹) and [³H]-cyclic AMP assay kit were obtained from Amersham (Buckinghamshire). 4-DAMP and methocitramine were purchased from RBI (Boston, MA, U.S.A.). Pirenzepine was a gift from Dr K. Noll at Dr Karl Thomae, GmbH. AG1-X8 was ordered from Bio-Rad (Richmond, CA, U.S.A.). Other enzymes and chemicals were purchased from Sigma (St Louis, MO, U.S.A.).

Results

Effects of muscarinic antagonists on muscarinic receptor-mediated inhibition of cyclic AMP generation

The data obtained in Figure 1, show the high affinity interaction of carbachol with the inhibitory pathway of adenylate cyclase system in TSMCs. Carbachol inhibited isoprenaline-stimulated cyclic AMP formation in a dose-dependent manner with maximal inhibition at 1 µM carbachol. The mean values ± s.e. of cyclic AMP levels from carbachol inhibition

Figure 1 Competitive antagonism of carbachol-mediated inhibition of isoprenaline-stimulated cyclic AMP formation by (a) atropine, (b) pirenzepine, (c) methoctramine, and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) in tracheal smooth muscle cells. Each point represents the mean of three experiments determined in triplicate; s.e. shown by vertical bars. Basal levels of cyclic AMP, in the absence of isoprenaline, were 110 ± 20 and 95 ± 15 pmol/ 10^6 cells in the absence and the presence of carbachol, respectively. The amount of cyclic AMP inhibited by carbachol was evaluated as the amount of cyclic AMP stimulated by isoprenaline ($1 \mu\text{M}$) alone (190 ± 30 pmol/ 10^6 cells) minus the amount of cyclic AMP generated by the simultaneous presence of isoprenaline and carbachol ($1 \mu\text{M}$). Results are expressed as the percentage of response to a maximally effective concentration of carbachol (maximal inhibition = 95 ± 20 pmol/ 10^6 cells). The dose-response curves were constructed in the presence of various concentrations of carbachol in the absence ($\circ$) and in the presence of various antagonists: (a) atropine, ($\blacktriangle$) 10 nM and ($\square$) 100 nM; (b) pirenzepine, ($\blacktriangle$) 1 μM and ($\square$) 10 μM ; (c) methoctramine, ($\blacktriangle$) 1 μM and ($\square$) 10 μM ; (d) 4-DAMP, ($\blacktriangle$) 0.1 μM and ($\square$) 1 μM .

experiments in the absence of antagonists were as follows: maximal level produced by $1 \mu\text{M}$ isoprenaline was 190 ± 30 pmol/ 10^6 cells; minimal level remaining after the maximal inhibition by $100 \mu\text{M}$ carbachol was 95 ± 15 pmol/ 10^6 cells; and basal level, 110 ± 20 pmol/ 10^6 cells. EC_{50} for the inhibitory effect of carbachol on the accumulation of cyclic

Table 1 The dissociation constants (K_B) for muscarinic antagonists to antagonize the carbachol-mediated inhibition of adenylate cyclase activity

Antagonist	EC_{50} (μM)	Dose-ratio	pK_B	n
Atropine				
(10 nM)	0.39 ± 0.11	26	9.4 ± 0.2	3
(100 nM)	2.0 ± 0.7	130	9.1 ± 0.1	3
Pirenzepine				
(1 μM)	0.11 ± 0.03	7	6.8 ± 0.2	3
(10 μM)	0.63 ± 0.14	43	6.6 ± 0.3	3
Methoctramine				
(1 μM)	0.44 ± 0.13	30	7.5 ± 0.2	3
(10 μM)	56 ± 1.4	380	7.6 ± 0.2	3
4-DAMP				
(0.1 μM)	0.22 ± 0.09	15	8.1 ± 0.1	3
(1 μM)	2.2 ± 0.7	150	8.2 ± 0.2	3

Values [mean $\pm$ s.e. for number (n) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 1. EC_{50} for the inhibitory effect of carbachol on the isoprenaline-stimulated cyclic AMP formation was 15 ± 3 nM.

4-DAMP = 4-diphenylacetoxy-*N*-methylpiperidine.

AMP stimulated by isoprenaline was 15 ± 3 nM. The abilities of muscarinic antagonists, atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (1 and 10 μM) and 4-DAMP (0.1 and 1 μM) to antagonize carbachol-mediated inhibition of cyclic AMP accumulation were investigated in TSMCs (Figure 1). In general, these antagonists caused parallel rightward shifts in the concentration-effect curves of carbachol. The dissociation constants (K_B) of each antagonist were calculated from dose-ratios by the method of Furchgott (1972). Two concentrations of each antagonist were used in these experiments; consequently, two K_B values were calculated for each antagonist. Table 1 lists the K_B values of the muscarinic antagonists. There was no significant difference between these two values ($P > 0.05$). The rank order of potency for preventing the inhibition of cyclic AMP accumulation was atropine $>$ 4-DAMP $>$ methoctramine $>$ pirenzepine. The pK_B values of pirenzepine, methoctramine and 4-DAMP for antagonizing cyclic AMP inhibition were 6.7, 7.5 and 8.2 (Table 1), respectively, which corresponded to the high affinity for methoctramine and low affinity for pirenzepine and 4-DAMP in this response (see review Hulme *et al.*, 1990).

Effects of muscarinic antagonists on muscarinic receptor-mediated stimulation of inositol phosphates accumulation

Carbachol caused a concentration-dependent accumulation of IPs in TSMCs, with a maximal effect being a 2.5 fold increase over the basal levels (8000 d.p.m./ 10^6 cells per 0.5 ml). The EC_{50} value for the carbachol-stimulated IPs accumulation was approximately $2.0 \pm 0.8 \mu\text{M}$ (Figure 2). The concentration-effect relationship of carbachol was shifted to the right in a nearly parallel fashion, without a reduction in maximal response, upon the addition of atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (10 and 100 μM), and 4-DAMP (0.1 and 1 μM) (see Figure 2). The dissociation constants (K_B) were calculated from dose-ratios by the method developed by Furchgott (1972). There was no significant difference between these two K_B values for each antagonist (Table 2). The order of potency of the antagonists in antagonizing the carbachol-stimulated IPs accumulation was atropine $>$ 4-DAMP $>$ pirenzepine $>$ methoctramine. The pK_B values of pirenzepine, methoctramine and 4-DAMP for antagonizing the carbachol-mediated IPs accumulation were 7.0, 6.2, and 8.8 (Table 2), respectively, which were in good agreement with the low affinity for pirenzepine and methoctramine and high affinity for 4-DAMP in this response (see review Hulme *et al.*, 1990).

Figure 2 Competitive antagonism of carbachol-mediated stimulation of inositol phosphates accumulation in tracheal smooth muscle cells by (a) atropine, (b) pirenzepine, (c) methoctramine, and (d) 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP). Each point represents the mean of three experiments determined in triplicate; s.e. shown by vertical bars. Total [^3H]-inositol phosphates (IPs) were measured as d.p.m./ 10^6 cells. Results for the measurement of IPs production are expressed as the percentage of the IPs accumulation (19300 ± 1200 d.p.m./ 10^6 cells) stimulated by a maximal concentration of carbachol in the absence of antagonists. Carbachol-stimulated IPs accumulation was measured in the absence (○) and presence of atropine (▲, 10 nM and □, 100 nM), pirenzepine (▲, 1 μM and □, 10 μM), methoctramine (▲, 10 μM and □, 100 μM), and 4-DAMP (▲, 0.1 μM and △, 1 μM).

Effects of muscarinic antagonists on muscarinic receptor-mediated contractile responses

Carbachol caused a dose-dependent contraction of dog tracheal smooth muscle and the geometric mean EC_{50} for carbachol was $0.17 \pm 0.10 \mu\text{M}$ (Figure 3). When the muscarinic antagonists, atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (10 and 100 μM), and 4-DAMP (10 and 100 nM), were examined for their ability to antagonize the carbachol-mediated contractile response, the concentration-effect relationship of carbachol was shifted to the right in a parallel fashion, without a change in the maximal response

Table 2 The dissociation constants (K_B) for muscarinic antagonists antagonizing the carbachol-mediated accumulation of inositol phosphates

Antagonist	EC_{50} (μM)	Dose-ratio	pK_B	n
Atropine (10 nM)	70 ± 15	34	9.5 ± 0.3	3
(100 nM)	600 ± 160	290	9.5 ± 0.3	3
Pirenzepine (1 μM)	23 ± 9	11	7.0 ± 0.2	3
(10 μM)	210 ± 80	100	7.0 ± 0.2	3
Methoctramine (10 μM)	38 ± 13	18	6.2 ± 0.2	3
(100 μM)	275 ± 110	130	6.1 ± 0.3	3
4-DAMP (0.1 μM)	150 ± 70	72	8.9 ± 0.2	3
(1 μM)	990 ± 170	480	8.7 ± 0.3	3

Values [mean $\pm$ s.e. for number (n) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 2. EC_{50} of carbachol-stimulated inositol phosphates accumulation was $2.0 \pm 0.8 \mu\text{M}$. 4-DAMP = 4-diphenylacetoxy-*N*-methylpiperidine.

(Figure 3). The dissociation constants (K_B) of muscarinic antagonists were calculated from dose-ratios by the method of Furchgott (1972). Table 3 lists the K_B values for muscarinic antagonists used in these experiments. Two K_B values were calculated for each antagonist, since two concentrations of each antagonist were used in this study. There was no significant difference between these two K_B values ($P > 0.05$). The rank order of potency of muscarinic antagonists in antagonizing the carbachol-mediated contraction was atropine $>$ 4-DAMP $>$ pirenzepine $>$ methoctramine. As in the IPs accumulation, the pK_B values of pirenzepine, methoctramine and 4-DAMP were 7.1, 6.1 and 9.3 (Table 3), respectively, which were consistent with the low affinity for pirenzepine and methoctramine and high affinity for 4-DAMP (see review (Hulme *et al.*, 1990).

Discussion

The activation of muscarinic receptor subtypes coupled to the generation of different second messengers was investigated in tracheal smooth muscle. It was found that (1) activation of muscarinic receptors in tracheal smooth muscle leads to accumulation of IPs, inhibition of cyclic AMP formation, and generation of the contractile response; (2) an M_1 subtype is not involved in the observed muscarinic-mediated second messenger responses; (3) inhibition of adenylate cyclase seems to be coupled to M_2 subtype distinct from the M_3 subtype coupled

Table 3 The dissociation constants (K_B) for muscarinic antagonists antagonizing the carbachol-mediated contraction

Antagonist	EC_{50} (μM)	Dose-ratio	pK_B	n
Atropine (10 nM)	5.8 ± 1.4	35	9.5 ± 0.2	8
(100 nM)	110 ± 50	650	9.8 ± 0.3	8
Pirenzepine (1 μM)	2.1 ± 0.8	13	7.1 ± 0.2	10
(10 μM)	21 ± 5	130	7.1 ± 0.1	10
Methoctramine (10 μM)	1.7 ± 0.5	10	6.0 ± 0.2	8
(100 μM)	24 ± 8	150	6.2 ± 0.2	8
4-DAMP (0.1 μM)	30 ± 0.9	19	9.2 ± 0.2	12
(1 μM)	36 ± 7	220	9.3 ± 0.1	12

Values [mean $\pm$ s.e. for number (n) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 3. EC_{50} of carbachol-mediated contraction of tracheal smooth muscle was $0.17 \pm 0.10 \mu\text{M}$. 4-DAMP = 4-diphenylacetoxy-*N*-methylpiperidine.

Figure 3 Competitive antagonism of carbachol-mediated contraction in tracheal smooth muscle by (a) atropine, (b) pirenzepine, (c) methoctramine, and (d) 4-diphenylacetoxy-N-methylpiperidine (4-DAMP). Each point represents the mean $\pm$ s.e. of at least eight experiments; s.e. shown by vertical bars. Curves of the contraction induced by carbachol (5 min exposure) were generated and expressed as the percentage of the response to a maximal effect of carbachol. Carbachol-mediated smooth muscle contraction was measured in the absence ($\circ$) and presence of atropine ($\blacktriangle$, 10 nM and $\square$, 100 nM), pirenzepine ($\blacktriangle$, 1 μ M and $\square$, 10 μ M), methoctramine ($\blacktriangle$, 10 μ M and $\square$, 100 μ M), and 4-DAMP ($\blacktriangle$, 0.1 μ M and $\square$, 1 μ M).

to phospholipase C activation; and (4) the contractile response was triggered mainly by the M_3 subtype via the IP₃ pathway.

The discrepancy in the dose-response relationships for the effect of carbachol on the metabolism of cyclic AMP and phosphoinositide in tracheal smooth muscle is consistent with those reported by others using different tissue and cell preparations (Brown & Brown, 1984). Our findings may reflect the presence of muscarinic receptor subtypes (M_2 and M_3) in the intact TSMCs, each coupled selectively to a single effector system. The presence of spare receptors is one of the explanations which may be used to rationalize the low and high affinity agonist interactions (Harden *et al.*, 1986). The differences in

the properties of the two second messenger responses could then lie at the level of receptor-effector coupling: the partial receptor occupancy would trigger a maximal inhibition of adenylate cyclase whereas a total receptor occupancy should be required for the generation of IP₃s.

As an alternative approach to define receptor subtypes, the determination of antagonist affinities can provide more accurate information than the use of agonist relative potencies (Birdsall *et al.*, 1978). From the experiments, the effects of discriminating antagonists have been analysed at the level of functional and biochemical responses. Atropine was non-selective in antagonizing the different muscarinic responses, displaying K_i values in the nanomolar range corresponded to pK_B values. Furthermore, pirenzepine could not differentially antagonize the different muscarinic effects. The pK_B values of pirenzepine for inhibiting the generation of IP₃s, tension and for reversing cyclic AMP attenuation (7.0, 7.1, and 6.7, respectively) were of low affinity (Hammer *et al.*, 1980; Hulme *et al.*, 1990). This suggests that the M_1 receptor subtype does not contribute to these two biochemical responses. This was confirmed by the competitive inhibition of [3 H]-N-methylscopolamine binding which excluded the presence of M_1 subtype in the TSMCs (Roffel *et al.*, 1988; Yang, 1991).

The results obtained with methoctramine and 4-DAMP, differentiated between the receptors mediating the IP₃s and cyclic AMP responses. The muscarinic receptors possessing high affinity for methoctramine (pK_B = 7.6) and low affinity for 4-DAMP (pK_B = 8.1) on carbachol-mediated attenuation of the cyclic AMP response can be considered as the M_2 subtype (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987; Micheletti *et al.*, 1987). The receptor with low affinity for methoctramine (pK_B = 6.1) and a high affinity for 4-DAMP (pK_B = 8.8) can be classified as the M_3 subtype which is involved in carbachol-mediated generation of IP₃s and consequently contraction (Roffel *et al.*, 1990; Hulme *et al.*, 1990).

The pK_B values for inhibiting the carbachol-mediated contraction are similar to those for antagonizing carbachol-induced accumulation of IP₃s (Tables 2 and 3). This is in agreement with reports by Baron *et al.* (1984), which showed that muscarinic contraction is associated with the generation of inositol trisphosphate (Berridge *et al.*, 1984; Roffel *et al.*, 1990) which mobilizes Ca^{2+} from intracellular stores in the smooth muscle cells (Hashimoto *et al.*, 1985). Contraction of tracheal smooth muscle by muscarinic agonists is not dependent on extracellular Ca^{2+} (Farley & Miles, 1977). Therefore, the demonstration that muscarinic agonists stimulate the accumulation of IP₃s in tracheal smooth muscle concurs with the assumption that phosphoinositide breakdown is the transducing mechanism for muscarinic contraction through the release of Ca^{2+} from its internal stores. The contribution of the M_2 subtype, via the cyclic AMP system, to the modulation of the contractile or another response in smooth muscle remains an unsolved problem.

In addition, our data can be considered as indirect evidence for the presence of both M_2 and M_3 receptor subtypes in tracheal smooth muscle which is supported by radioligand binding studies in this tissue (Roffel *et al.*, 1988; Yang, 1991). Furthermore, our results are in agreement with those of a recent paper published whilst this article was in review by Candell *et al.* (1990) on rat longitudinal smooth muscle of ileum, which comes to a similar conclusion.

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