Muscarinic M₂ receptors in acetylcholine-isoproterenol functional antagonism in human isolated bronchus

BENJAMIN SARRIA,1 EMMANUEL NALINE,2 YONG ZHANG,3 JULIO CORTIJO,1 MATHIEU MOLIMARD,2 JOELLE MOREAU,2 PATRICE THEROND,2 CHARLES ADVENIER,2 AND ESTEBAN J. MORCILLO1

1Departament de Farmacologia, Facultat de Medicina i Odontologia, Universitat de València, 46010 València, Spain; 2Faculté de Médecine Paris-Ouest, Unité de Formation et de Recherche Biomédicale des Saint Pères, and Centre Hospitalier de Versailles, 78157 Le Chesnay, France; and 3Shanghai Second Medical University, 200025 Shanghai, China

Received 22 March 2002; accepted in final form 12 June 2002

Sarria, Benjamin, Emmanuel Naline, Yong Zhang, Julio Cortijo, Mathieu Molimard, Joelle Moreau, Patrice Therond, Charles Advenier, and Esteban J. Morcillo. Muscarinic M₂ receptors in acetylcholine-isoproterenol functional antagonism in human isolated bronchus. Am J Physiol Lung Cell Mol Physiol 283: L1125–L1132, 2002. First published June 21, 2002; 10.1152/ajplung.00084.2002.—The muscarinic functional antagonism of isoproterenol relaxation and the contribution of muscarinic M₂ receptors were examined in human isolated bronchus. In intact tissues, acetylcholine (ACh) precontraction decreased isoproterenol potency and maximal relaxation (−log EC₅₀ shift = −1.49 ± 0.16 and Eₘₐₓ inhibition for 100 μM ACh = 30%) more than the same levels of histamine contraction. The M₂ receptor-selective antagonist methoctramine (1 μM) reduced this antagonism in ACh-but not histamine-contracted tissues. Similar results were obtained for forskolin-induced relaxation. After selective inactivation of M₃ receptors with 4-diphenylacetoxy-N-(2-chloroethyl)piperadine hydrochloric acid (30 nM), demonstrated by abolishment of contractile and inositol phosphate responses to ACh, muscarinic recontractile responses were obtained in U-46619-precontracted tissues fully relaxed with isoproterenol. Methoctramine antagonized recontraction, with pKᵦ (6.9) higher than in intact tissues (5.4), suggesting participation of M₂ receptors. In M₃-inactivated tissues, methoctramine augmented the isoproterenol relaxant potency in U-46619-contracted bronchus and reversed the ACh-induced inhibition of isoproterenol cAMP accumulation. These results indicate that M₂ receptors cause indirect contraction of human bronchus by reversing sympathetically mediated relaxation and contribute to cholinergic functional antagonism.

HUMAN AIRWAYS ARE DENSELY innervated with parasympathetic nerves, which provide the dominant neural constrictor pathway. Previous studies using in situ hybridization and Northern blot analysis have demonstrated the presence of muscarinic M₂ and M₃ receptors in human airway smooth muscle but no detectable mRNA for M₁, M₄, or M₅ receptors (10). The postjunctional muscarinic receptor subtype mediating contraction of human airways is the M₃ receptor (16, 25). Functional studies have provided evidence of the existence of prejunctional M₂ receptors in human airways that modulate acetylcholine (ACh) release from postganglionic prejunctional cholinergic nerve endings (11, 20). By contrast, the functional role of postjunctional M₂ receptors localized to the human airway smooth muscle is unclear. Functional experiments do not support their direct contribution to the contractile response elicited by muscarinic stimulation in human isolated airways (25). However, a number of studies have implicated these M₂ receptors in the functional antagonism between airway smooth muscle contraction by muscarinic agonists and the relaxation elicited by β-adrenoceptor agonists in canine (7, 13) and guinea pig (24, 30) trachea, but not in bovine trachea (17).

The involvement of M₂ receptors in the impairment of the isoproterenol-mediated relaxation of cholinergic tone may be explained by the finding that the activation of these receptors inhibits β-agonist- or forskolin-stimulated cAMP production in airway smooth muscle of different animal species (18, 28). These observations are consistent with findings in other tissues. Thus, although no direct contractile response to M₂ receptor activation can be demonstrated in rat intestinal muscle, guinea pig ileum, rat esophageal muscularis mucosae, or pig urinary bladder, an indirect influence on contraction via inhibition of cAMP-mediated smooth muscle relaxation by β-adrenoceptor agonists, forskolin, 5-hydroxytryptamine, or vasoactive intestinal peptide has been reported (5, 8, 15, 29).

The functional antagonism by muscarinic agonists of the β-adrenoceptor-mediated relaxation has been scarcely studied in human isolated bronchus (23, 26). Therefore, the aim of the present study was to examine
further the functional antagonism between the relaxation produced by the cAMP pathway and the contraction produced by ACh and histamine in human isolated bronchus and the contribution of M2 receptors to this functional antagonism. Relaxation was obtained with isoproterenol, a β-adrenoceptor agonist, and with a direct activator of adenylyl cyclase, forskolin. ACh and histamine were chosen as spasmodgens, because both are relevant mediators in allergic asthma. Methoctramine was chosen as a relatively selective antagonist of M2 receptors (11). To explore better the contribution of M2 receptors, experiments were also performed on tissues after selective inactivation of muscarinic M3 receptors with the alkylating agent 4-diphenylacetoxy-N-(2-chloroethyl)piperidine hydrochloric acid (4-DAMP mustard) (22).

METHODS

Preparation of human tissues for pharmacomechanical experiments. Lung tissue was obtained from patients who were undergoing surgery for lung carcinoma. None of the patients had a history of asthma. The experiments were approved by the local ethics committee. A piece of macroscopically normal tissue at a distance from the malignancy was obtained, and rings (3–4 mm long, 2–4 mm ID) were prepared as previously described (19). Preparations were stored in physiological salt solution (PSS) composed of (mM) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.1 KH2PO4, 25.0 NaHCO3, and 11.7 glucose and equilibrated with 5% CO2 in O2 at 4°C. Experiments were completed within 24 h of initiation of storage (19).

Bronchial rings were suspended for isometric recording of tension changes under an initial load of 2.0 g and equilibrated for 60–90 min with changes in bath PSS every 15–20 min before any pharmacological intervention; at the end of the equilibration period, the resting load was stable at 1–2 g. Under these conditions, responses were optimal and reproducible according to data from this and other laboratories (19, 27).

Experimental protocol in intact tissues. Human bronchi were first contracted with 1 mM ACh and then relaxed with 3 mM theophylline to obtain reference values for maximal contraction. After the samples were washed and re-equilibrated, 0.1–30 nM 4-DAMP mustard or its vehicle was added to the bath for 60 min. The drug was then removed by washing with PSS at 10-min intervals over 30 min, and a cumulative concentration-response curve to ACh was constructed. Only one concentration-response curve to ACh was obtained in each ring. For biochemical studies, the inhibition by 4-DAMP mustard of inositol phosphate formation produced by ACh was determined (see below).

Once the concentration of 4-DAMP mustard that virtually abolished the ACh-induced contraction and inositol phosphate production in human isolated bronchus was established, the protocol to achieve selective M3 receptor inactivation was essentially as previously outlined (9, 29). After 1 mM ACh was added to obtain a reference value for maximal contraction and the tissues were washed, the tissues were incubated with the alkylating agent 4-DAMP mustard (30 nM) for 60 min in the presence of 1 μM methoctramine to protect M2 receptors, with methoctramine added 30 min before the 4-DAMP mustard. The drugs were then removed from the tissues by washing for 60 min with PSS. Then two different protocols were carried out: one to assess the presence of functional antagonism in M3 receptor-inactivated tissues and its reversal by methoctramine and another to detect the presence of recontraction (i.e., relaxation and its antagonism by methoctramine under conditions of stimulated adenylyl cyclase activity (i.e., isoproterenol-induced relaxation of precontracted tissue) (9). In the first protocol, paired tissues were incubated with or without 1 μM methoctramine for 30 min, 100 μM ACh was added, and preparations were contracted with 0.1 μM U-46619 to a plateau level of ~70%; then a concentration-response curve to isoproterenol was constructed. In the second protocol, paired tissues were incubated with or without 1 μM methoctramine and contracted 30 min later with 0.1 μM U-46619; then the stable contractile tone was relaxed to baseline with 10 μM isoproterenol, and 10 min later a concentration-response curve to ACh was constructed.

Inositol phosphate measurement. Total inositol phosphate accumulation was determined as previously reported (2). Briefly, human bronchi were incubated in PSS containing 50 μCi of myo-[3H]inositol for 4 h at 37°C in 95% O2-5% CO2. 4-DAMP mustard (1, 10, or 30 nM) or its vehicle (control tissues) was added for 60 min, and the preparation was stimulated for 15 min at 37°C with PSS (control) or 1 mM ACh. Inositol phosphates were separated by an HPLC ion-exchange system, and radioactivity was measured in a Flow-
Measurement of cAMP. The content of cAMP in human isolated bronchus was measured as previously outlined (3). Briefly, bronchial rings were subjected to the M3 receptor inactivation process by incubation with 30 nM 4-DAMP as indicated above; then the preparation was exposed for 30 min to 100 μM ACh or its vehicle, and 10 μM isoproterenol or its vehicle was added for 10 min. At the end of experiment, tissues were homogenized and centrifuged, and the amount of cAMP in the soluble fraction was estimated (RPN 225 enzyme immunoassay kit, Amersham Life Sciences) following the instructions of the manufacturer without acetylation.

Drugs and statistical analysis of results. Drugs were obtained from Sigma-Aldrich (St. Louis, MO), except 4-DAMP mustard and methoctramine tetrahydrochloride, which were purchased from Research Biochemical (Natick, MA). Solutions of 4-DAMP mustard were freshly prepared in dimethyl sulfoxide and subsequently diluted in distilled water. Stock solutions of U-46619 were prepared in dimethyl sulfoxide and those of forskolin in ethanol, and these solutions were subsequently diluted in PSS. Isoproterenol solutions contained 22 μM ascorbic acid as an antioxidant. Vehicle controls (drug solvent only) were run in parallel, and no significant vehicle effects were observed. Drug concentrations are expressed as final bath concentrations of the active species.

Contractile and relaxant responses are expressed as a percentage of the reference contractile (1 mM ACh) and relaxant (3 mM theophylline) responses. The EC50 of spasmodgens and relaxants, i.e., that producing half-maximal response (Emax), was calculated by nonlinear regression analysis of log concentration-effect curves (GraphPad, San Diego, CA) and expressed as pD2 (i.e., –log EC50). Antagonist affinity estimates were determined by the following equation: pKB = –log [(antagonist)/CR – 1]), where CR is the concentration ratio determined from EC50 values in the presence and absence of antagonist.

Values are means ± SE; n is the number of preparations examined from different patients. Statistical analysis of the results was performed by analysis of variance followed by Bonferroni’s multiple comparison test or Student’s t-test as appropriate (GraphPad). Differences were considered significant when P < 0.05.

RESULTS

Methoctramine did not influence the level of basal or induced tone in intact tissues. There was no significant effect of 0.3 or 1 μM methoctramine on the basal tone of the preparations (not shown). The level of plateau contraction produced by ACh before the concentration-relaxation curves were performed for isoproterenol or forskolin did not differ between control and methoctramine-treated tissues (not shown). The contractile responses to 0.3, 1, and 10 μM histamine were not significantly different from the corresponding values observed after 1, 10, and 100 μM ACh, and the contraction in response to 10 μM histamine was not reduced by 1 μM methoctramine (not shown). In the experiments where 0.1 μM U-46619 was used to raise the tissue tone, contractions were not affected by pretreatment with 1 μM methoctramine (not shown).

Incubation with 0.3 and 1 μM methoctramine did not significantly displace the concentration-response curve to ACh (not shown), but 10 μM methoctramine produced a small parallel rightward shift from control curves, with a pKB of 5.4 ± 0.3 (n = 4), which is consistent with values reported in this preparation with carbachol as agonist (25).

Methoctramine reduced the functional antagonism of the relaxation to isoproterenol and forskolin produced by ACh in intact tissues. When the level of contraction was increased with ACh, a rightward and downward shift of the concentration-response curves to isoproterenol was observed. This indicates a functional antagonism between the isoproterenol relaxation and the ACh contraction. The displacement of the concentration-response curves of isoproterenol produced by ACh was partially reversed in the presence of methoctramine (Fig. 1).
A rightward and downward shift of the concentration-relaxation curves to forskolin was also noted in preparations contracted with ACh. As observed for isoproterenol, this displacement was partially reversed in the presence of methoctramine (Fig. 2).

Hexamethonium and tetrodotoxin did not influence the ACh-isoproterenol functional antagonism or the reduction of this antagonism by methoctramine in intact tissues. Pretreatment with 10 μM hexamethonium or 1 μM tetrodotoxin, in the absence or presence of 1 μM methoctramine, did not depress the contraction in response to 10 μM ACh (not shown). Hexamethonium and tetrodotoxin did not modify the functional antagonism of the isoproterenol relaxation exerted by ACh: shifts of isoproterenol pD2 values of −0.61 ± 0.09 and −0.46 ± 0.03 were obtained in the presence of hexamethonium and tetrodotoxin, respectively, which did not differ from the corresponding control values of −0.58 ± 0.07 and −0.48 ± 0.03 (n = 5 per group). The facilitatory effect of 1 μM methoctramine on the isoproterenol relaxation of ACh-contracted tissues was not altered by hexamethonium and tetrodotoxin (not shown).

Methoctramine failed to increase the reduced relaxation to isoproterenol and forskolin in histamine-contracted intact tissues. When the level of tone was increased with histamine, the concentration-response curves to isoproterenol were shifted rightward, but the maximal relaxation to isoproterenol and its potency were scarcely affected compared with similar levels of contraction elicited by ACh (cf. Figs. 1 and 3). Similar results were obtained with forskolin (cf. Figs. 2 and 3). Methoctramine (1 μM) did not increase the relaxations elicited by isoproterenol and forskolin in preparations contracted with 10 μM histamine (Fig. 3).

Contraction and inositol phosphate production in response to ACh were virtually abolished in M3 receptor-inactivated tissues. 4-DAMP mustard produced a concentration-dependent inhibition of the contractions evoked by ACh, with a progressive reduction of maximal responses without significant changes in potency (Fig. 4). 4-DAMP mustard (30 nM) produced a virtually complete inhibition of the ACh contraction without affecting the contraction elicited by U-46619: Emax and pD2 values were 89 ± 6% and 7.40 ± 0.06, respectively, in the absence and 83 ± 8% and 7.37 ± 0.07, respectively, in the presence of 30 nM 4-DAMP mustard (n = 3 for each group).

Incubation with 4-DAMP mustard also produced a concentration-related decrease of inositol phosphate production by 1 mM ACh that reaches near complete inhibition for 30 nM 4-DAMP (Fig. 4). Consequently, experiments performed to study the effects of methoctramine and the role of muscarinic M3 receptors in the interaction between isoproterenol and ACh were conducted after incubation of bronchi with 30 nM 4-DAMP mustard.

Methoctramine reduced the antagonism produced by ACh in the relaxation and cAMP elevation elicited by isoproterenol in M3 receptor-inactivated tissues. Because 100 μM ACh produced no contraction in M3 receptor-inactivated tissues (see above), tone was increased with 0.1 μM U-46619 in the presence of 100 μM ACh to a level of contraction (71 ± 6% of 1 mM ACh, n = 5) similar to that produced by 100 μM ACh in intact tissues. In this situation, a rightward displacement of the concentration-response curves to isoproterenol was observed (Fig. 5). These results indicate the persistence of a functional antagonism produced by ACh against the relaxant effects of isoproterenol in M3 receptor-inactivated tissues. This antagonism was partially reversed by methoctramine (Fig. 5).

In M3 receptor-inactivated tissues, 10 μM isoproterenol elevated cAMP levels from basal values of 8.7 ± 0.9 to 33.9 ± 4.8 pmol/mg protein (P < 0.05). ACh (100 μM) did not change the resting levels of cAMP (6.2 ± 1.3 pmol/mg protein) but reduced the isoproterenol-stimulated cAMP accumulation (17.3 ± 1.8 pmol/mg protein)
protein, \( P < 0.05 \). Methoctramine (1 \( \mu \)M) did not affect basal cAMP levels (7.7 ± 0.8 pmol/mg protein) but fully reversed the inhibitory effect produced by ACh (29.6 ± 3.6 pmol/mg protein, \( n = 4 \) for each group).

ACh-induced recontraction in \( M_3 \) receptor-inactivated tissues is antagonized by methoctramine. In tissues subjected to selective \( M_3 \) receptor alkylation, precontracted with 0.1 \( \mu \)M U-46619 (71 ± 5\%), and fully relaxed with 10 \( \mu \)M isoproterenol, ACh produced a concentration-dependent recontraction with \( E_{\text{max}} \) of 55 ± 3\% and \( pD_2 \) of 4.23 ± 0.23 (\( n = 4 \)). These values were significantly lower than those obtained in paired intact tissues (\( E_{\text{max}} \) = 98 ± 2\% and \( pD_2 \) = 5.05 ± 0.08). The maximal recontraction was close to the level of contraction produced by U-46619, thus indicating near-complete reversal of relaxations to isoproterenol.

The curves for recontraction were antagonized in a surmountable fashion by 1 \( \mu \)M methoctramine, with a \( pK_B \) of 6.92 ± 0.41 (\( n = 4 \)). This value is significantly higher than that found in intact tissues (see above), and it would be consistent with activation of \( M_2 \) receptors.

**DISCUSSION**

Increased functional antagonism of cholinergic tone against isoproterenol and forskolin relaxation of human isolated bronchus. This study was carried out in human isolated bronchus to assess the functional antagonism between spasmogens, such as ACh and histamine, and relaxants, such as isoproterenol (a \( \beta \)-adrenoceptor agonist) and forskolin (an adenylyl cyclase activator), and the role that \( M_2 \) receptors may play in this antagonism. We found that a gradual increase of tone promoted by ACh from low to half- or near-maximal levels resulted in a pronounced, ACh concentration-dependent decrease in the ability of isoproterenol and forskolin to relax the airway smooth muscle. However, when tone was increased with histamine to levels similar to those obtained with ACh, the ability of isoproterenol or forskolin to relax the airway smooth muscle was decreased to a lesser extent.

These results differ from those of Van Amsterdam et al. (23), which show equivalent decreases of maximal relaxation and shifts of potency for isoproterenol and forskolin in human bronchus maximally contracted with methacholine or histamine. By contrast, and consistent with this study, Watson et al. (26) found increased func-

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**Fig. 3.** Functional antagonism of isoproterenol- and forskolin-induced relaxations of human isolated bronchus by histamine (HA). Isoproterenol and forskolin inhibited basal tone and tone increased by addition of 0.3–10 \( \mu \)M histamine. Methoctramine (1 \( \mu \)M) was without effect on isoproterenol- and forskolin-induced relaxations. Insets: shift of \( pD_2 \) values for isoproterenol and forskolin as relaxants of histamine contraction compared with values in preparations with basal tone. \( pD_2 \) values for isoproterenol and forskolin in preparations with basal tone were 7.79 ± 0.08 and 7.36 ± 0.07, respectively. Values are means ± SE (\( n = 4–5 \) for each group). All shifts were significant (\( * P < 0.05 \)), but the shift in methoctramine-treated tissues failed to reach significance vs. its corresponding control.

**Fig. 4.** Incubation of human isolated bronchus with 4-diphenylacetox-N-(2-chloroethyl)piperadine hydrochloric acid (4-DAMP mustard) depresses bronchial contraction and inositol phosphate production in response to ACh. A: concentration-response curves to ACh in human bronchial rings incubated in the absence and presence of 0.1–30 nM 4-DAMP mustard. Results are expressed as percentage of initial 1 mM ACh contraction. B: increase in [\( ^{3} \)H]inositol phosphate (IP) induced by 1 mM ACh in the absence (control) and presence of 4-DAMP mustard in segments of human bronchi. Results are expressed as percentage of control responses produced by 1 mM ACh. Values are means ± SE (\( n = 3–6 \) for each group). *\( P < 0.05 \) vs. control.
Fig. 5. Isoproterenol-induced relaxations of human bronchus in M₃ receptor-inactivated tissues with tone increased by 0.1 μM U-46619 in the absence (basal) or presence of 100 μM ACh. A: functional antagonism of relaxation to isoproterenol imposed by ACh. B: relaxation to isoproterenol obtained in the presence of 1 μM methoctramine. Functional antagonism was significantly reduced by methoctramine. Inset: shifts of pD₂ values of isoproterenol in the absence and presence of methoctramine. pD₂ values for isoproterenol in preparations not exposed to ACh were 7.78 ± 0.05 and 7.72 ± 0.08 in the absence and presence of methoctramine, respectively. Values are means ± SE (n = 5 for each group). *P < 0.05 vs. control preparations not treated with ACh; †P < 0.05 compared with corresponding values in the absence of methoctramine.

Muscarinic functional antagonism of isoproterenol relaxation of human isolated bronchus for carbachol vs. histamine. Although a definite explanation for these different results is not apparent, methodological differences as well as the variable degree of inflammation in samples from different individuals may have contributed.

Methoctramine enhanced relaxation by isoproterenol and forskolin in ACh- but not histamine-contracted human bronchus. Whether M₂ receptors contribute to the functional antagonism between ACh- and isoprotere-

Isoproterenol and forskolin relaxations of ACh-contracted human bronchus were facilitated in the presence of methoctramine, both in terms of pD₂ values and maximal effects. This finding suggests that M₂ receptors play a role in the above-mentioned functional antagonism that is in keeping with results obtained in canine, rabbit, and guinea pig trachea with the use of methoctramine and other selective M₂ receptor antagonists (7, 13, 24, 30). Consistent with these results is our finding that the relaxation by isoproterenol or forskolin of histamine-induced tone, which does not involve M₂ receptors, was not facilitated in the presence of methoctramine.

Hexamethonium and tetrodotoxin did not influence the effect of methoctramine on functional antagonism between ACh and isoproterenol. Human bronchus contains intramural parasympathetic ganglia, and exogenously administered ACh could activate nicotinic receptors located on ganglionic neurons. On the other hand, micromolar concentrations of methoctramine show a significant antagonist activity at nicotinic receptors (11). We found that 10 μM hexamethonium did not depress the contraction produced by 10 μM ACh in human isolated bronchus, nor did it influence the facilitation produced by 1 μM methoctramine of the isoproterenol relaxation in preparations contracted with ACh. Similar results were obtained by inhibiting nerve conduction with the sodium channel blocker tetrodotoxin (1 μM). These results indicate that, consistent with other studies (1), neural mechanisms do not contribute to the contraction to exogenously added ACh and that interaction with nicotinic receptors is not likely involved in the action of methoctramine on the functional antagonism between ACh and isoproterenol in human isolated bronchus.

Methoctramine augmented isoproterenol-induced relaxation and antagonized cholinergic recontraction in M₃ receptor-inactivated tissues. Because the augmentation by an M₂ receptor antagonist of the relaxant potencies of isoproterenol and forskolin in ACh-contracted tissues provides somewhat ambiguous evidence for an involvement of the M₂ receptor in this functional antagonism (5), we performed additional studies in M₃ receptor-inactivated tissues. Selective alkylation of M₃ receptors after incubation of tissues with 4-DAMP mustard, in the presence of methoctramine to protect M₂ receptors, has successfully unmasked a functional role for M₂ receptors in a number of previous studies (5, 6, 9, 14, 15, 21, 29).

Incubation of human isolated bronchus with the alkylating agent 4-DAMP mustard inhibited the ACh-induced contraction and inositol phosphate production in a concentration-related manner, with responses virtually abolished for 30 nM 4-DAMP. Therefore, we demonstrate that alkylation conditions can be achieved in human bronchus that selectively and extensively deplete the muscarinic M₂ receptor population, thus facilitating exploration of a functional role for M₂ receptors.

After M₃ receptor inactivation, the relaxant potency of isoproterenol in U-46619-contracted human bronchus was reduced in the presence of 100 μM ACh, indicating the persistence of a cholinergic functional antagonism in these experimental conditions. ACh was also able to reduce the increase of cAMP elicited by isoproterenol in M₃ receptor-inactivated tissues. The isoproterenol relaxation and the cAMP accumulation were augmented in the presence of methoctramine, suggesting the participation of M₂ receptors in this functional antagonism. These results confirm and extend previous findings in cultured human airway...
smooth muscle cells where carbachol was shown to inhibit the isoproterenol-induced augmentation of cAMP, and methoctramine reversed this effect by blockade of M₂ receptors (28).

Furthermore, in U-46619-contracted tissues that were exposed to isoproterenol to provide full relaxation via elevation of cAMP, ACh induced concentration-dependent reconstructions (i.e., indirect contraction or reversal of relaxation). The maximal reconstruction to ACh, while reversing the contracture to U-46619, remained less than that mediated by direct M₃ receptor activation. However, the magnitude of the response was markedly larger than that in the alkylated tissues. This provides some evidence that M₂, rather than M₃, receptors mediate the response. Further evidence to implicate M₂ receptors was obtained from studies with muscarinic receptors, M₂ receptors appear to be involved in the postjunctional M₂ receptors may gain in the human airway smooth muscle. Thus activation of cAMP, and methoctramine reversed this effect by blockade of M₂ receptors (28).

The possibility that M₂ receptor activation inhibits M₂ receptors mediate the response. Further evidence to implicate M₂ receptors was obtained from studies with methoctramine, because this drug antagonized the reconstructions, with pKᵢ values (6.9) that are higher than those obtained in intact tissues (5.4) and more consistent with activation of muscarinic M₂ receptors than with activation of M₃ receptors. These findings would be consistent with results obtained in guinea pig ileum (15, 21) and in rat (9) and pig (29) urinary bladder, in which an M₂ receptor-mediated contraction to muscarinic stimulation has been demonstrated after M₃ receptor inactivation and elevation of cAMP levels.

In conclusion, these results indicate that, in addition to mechanisms derived from the activation of M₃ receptors, M₂ receptors appear to be involved in the functional antagonism of relaxant responses to isoproterenol produced by cholinergic stimulation. This effect may be related to the inhibition of adenyl cyclase, because methoctramine was able also to reduce the functional antagonism elicited by ACh against forskolin-induced relaxation and reversed the muscarinic inhibition of isoproterenol cAMP accumulation in M₃ receptor-inactivated tissues. These findings may have relevance in the pathophysiology and pharmacology of the human airway smooth muscle. Thus activation of postjunctional M₂ receptors may gain influence under conditions of high sympathoinhibitory tone acting in concert with M₃ receptors to reinforce bronchoconstriction. The possibility that M₂ receptor activation inhibits cAMP accumulation promoted by some inhibitory neurotransmitters widens the potential modulation of this receptor over bronchial tone. Also, nonselective muscarinic antagonists, by blocking the postjunctional M₂ receptor-mediated inhibition of relaxation, might be more efficient for treating bronchospasm than M₃ receptor-selective antagonists, yet the relative clinical importance of this mechanism over the blockade of autoinhibitory M₂ receptors remains to be determined.

The authors are indebted to the teams of the Services of Thoracic Surgery and Pathology of the Hospital La Fe and Hospital Clínico of the University of Valencia for making the human lung tissue available to us. The authors also thank P. Santamaria and S. Martí for expert technical assistance.

The present work was supported in part by Comisión de Investigación Científica y Técnica Grants SAF1999-0111 and SAF2000-0144 (Spain) and funds from Generalitat Valenciana.

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