Rhinology, 34, 136-139, 1996

The roles of muscarinic receptor subtypes in modulation of nasal ciliary action*

Bin Yang[1], Thomas V. McCaffrey[2]

[1] Department of Physiology and Biophysics, Mayo Clinic, Rochester, Minnesota, USA
[2] Department of Otorhinolaryngology, Mayo Clinic, Rochester, Minnesota, USA

SUMMARY

Muscarinic receptors are believed to play an important role in modulation of ciliary action in respiratory system. We studied the in vitro effect of methacholine, a ß-methyl ester of acetylcholine, on the ciliary beat frequency (CBF). Adenoid explants were cultured in Minimum Essential Medium Eagle (MEM). CBF was determined using microphotometry. Methacholine (10^{-6} M) increased CBF a maximum of 10.34±0.42% ($p<0.001$). The non-selective muscarinic antagonist atropine (10^{-6} M) significantly inhibited the ciliostimulatory effects of methacholine ($p<0.001$). To characterize the muscarinic receptor subtypes in nasal mucosa, the selective M_1-, M_2- and M_3-muscarinic antagonists pirenzepine dihydrochloride (PZ), gallamine triethiodide (gallamine), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) were used prior to addition of methacholine. PZ and 4-DAMP, at concentrations of 10^{-6} to 10^{-8} M, significantly inhibited the ciliostimulatory effects of methacholine ($p<0.0001$). There was no significant inhibition of methacholine-induced ciliostimulation by gallamine ($p>0.3$). Our study showed that ciliostimulation by methacholine in human upper airway mucosa involves M_1- and M_3-muscarinic receptor subtypes, but not the M_2-receptor subtype. The identification of the muscarinic receptor subtypes and intracellular signalling mechanisms involved in CBF modulation will permit the selection appropriate of pharmacological agents for treating the cholinergic symptoms of rhinitis.

Key words: M_1- and M_3 receptors, M_2 receptors, methacholine, CBF, nasal cilia

INTRODUCTION

The parasympathetic nervous system is believed to play an essential role in the airway defense system. Muscarinic receptors are involved in modulation of ciliary action in respiratory system (Hybbinette and Mercke, 1982). Methacholine, a ß-methyl ester of acetylcholine, accelerates the mucociliary wave frequency, and this effect can be blocked by the muscarinic-receptor antagonist atropine.

Muscarinic acetylcholine receptors are cell surface receptors, which participate in a wide variety of physiological actions. Several classes of muscarinic receptors have been described. These are members of a superfamily with at least five receptor subtypes (M_1-M_5; Humle et al., 1990). These subtypes differ in their tissue distribution, agonist- and antagonist-binding affinities, structures, and functions. M_1, M_3 and M_5 subtypes couple to inositol polyphosphate generation which activates phospholipase C (PLC), while the M_2 and M_4 subtypes inhibit the generation of cAMP (Humle et al., 1990; Lambert, 1993). M_1- and M_3-muscarinic receptor subtypes have been shown to regulate mucus glycoprotein secretion from human nasal mucosa (Mullol et al., 1992).

The aims of the present study were to demonstrate the *in vitro* effects of methacholine and the non-selective muscarinic antagonist atropine on the ciliary beat frequency (CBF) of ciliated human adenoid explants and to further study the selective muscarinic antagonists to identify the receptor subtypes affecting the CBF in human upper airway mucosa. Determining the actions of the muscarinic receptor subtypes in nasal mucosa may contribute to the specificity of anti-cholinergic therapy for allergy and inflammation of the airway in such clinical conditions as allergic rhinitis, bronchial asthma, and chronic bronchitis.

* Received for publication October 6, 1995; accepted February 27, 1996

MATERIAL AND METHODS

Tissue preparation

Ciliary activity was studied *in vitro* in human adenoid explants. Human adenoid tissue was obtained in the operating room from patients undergoing surgery for nasal obstruction. The tissue was immersed in Medium 199 (Sigma, St. Louis, MO, USA) and transported to the laboratory. Specimens were cut into 3×3×2 mm pieces and washed three times in clean Minimum Essential Medium Eagle (MEM; Sigma, St. Louis, MO, USA) to remove blood and debris. Specimens were screened for viable ciliated edges. Those pieces with ciliated edges were placed onto sterile 35-mm plastic culture dishes, which had been coated with type I collagen (Sigma, St. Louis, MO, USA) diluted 1:10 in MEM and sterilized overnight under ultraviolet light. The ciliated specimens were covered with 1.0 ml of culture medium MEM and placed in a 95%-humidified 5%-CO_2 incubator at 35 °C until used for experiments. The culture medium in each dish was replaced every two days. At the time of experiment, specimens were examined under the phase-contrast microscope and screened for bacterial or fungal contaminants. Only explants free of any bacterial or fungal contamination were used. Specimens were used for experiments only once every 24 h to allow the tissue to return to equilibrium. A minimum of five trials were done to establish the average change in CBF for each concentration of the various experiments. Reversibility of the receptor blockade by the muscarinic antagonists was evaluated 24 h after removal of the antagonist from the culture medium by stimulation with methacholine.

Muscarinic receptor blockers

MEM was used as the substrate medium in the preparation of all experimental solutions. It was supplemented with 160 U/ml penicillin and 0.16 mg/ml streptomycin (Sigma, St. Louis, MO, USA). Experimental solutions were made using 1% albumin (Sigma, St. Louis, MO, USA) in MEM and adding methacholine, atropine (all from Sigma, St. Louis, MO, USA), pirenzepine dihydrochloride (selective M_1-muscarinic antagonist), gallamine triethiodide (selective M_2-muscarinic antagonist) or 4-diphenylacetoxy-N-methylpiperidine methiodide (selective M_3-muscarinic antagonist). Methacholine was used in concentrations of 10^{-6} and 10^{-8} M; atropine, pirenzepine dihydrochloride (PZ), gallamine triethiodide (gallamine), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) at 10^{-6} and 10^{-8} M. Stock solutions were made and frozen until the day of the experiment. MEM was supplemented with antibiotics as above and used as culture medium.

Analysis of ciliary beat frequency

The specimens were placed on the heated stage of an inverted phase-contrast microscope (Nikon 8328) and allowed to equilibrate for 15 min. The stage was heated to 33 °C to approximate *in vivo* temperatures. The cilia were viewed at ×400 and oriented to interrupt the passage of light through a 0.2-mm slit in the diaphragm of the attached photometer (Nikon P-1). The fluctuating light produced by the ciliary beats was processed into an electrical analogue signal. The signal was amplified, filtered through a 5-Hz high-pass filter and a 30-Hz low-pass filter and digitized at 200 Hz. Using ASYST software (Keithley), a fast Fourier transform was performed on the signal to determine modal CBF in Hz. For comparison, CBF was expressed as percentage change from control values. This was done to account for differences in baseline CBF between preparations.

Effects of methacholine

After equilibration, baseline CBF of a specimen was determined by averaging 20 successive measurements obtained from the same ciliated site. The medium covering the specimen was removed and replaced with 1.0 ml of MEM containing methacholine. The same site on the specimen was relocated, and the first CBF measurement was made within 2 min. Fifty measurements were averaged to determine the effect of methacholine alone at concentrations of 10^{-10} M and 10^{-8} M.

Effects of non-selective muscarinic antagonist

Baseline CBF was determined by averaging 20 successive measurements. The MEM covering the specimen was removed and replaced with 1.0 ml of MEM containing atropine, a non-selective muscarinic antagonist. Thirty CBF measurements were performed over 15 min to determine the effect of atropine alone on baseline CBF. After 15 min, the atropine was removed and methacholine was added. Thirty subsequent measurements were obtained at the same site on each specimen to determine the response to methacholine after muscarinic-receptor blockade by atropine.

Effects of selective muscarinic-receptor antagonists

Experiments investigating the role of selective M_1-, M_2- and M_3-muscarinic receptor antagonists were performed after determining baseline CBF. The MEM covering the specimen was removed and replaced with 1.0 ml of MEM containing either pirenzepine dihydrochloride (PZ), a selective M_1-muscarinic antagonist, gallamine triethiodide (gallamine), a selective M_2-muscarinic antagonist, or 4-DAMP, a selective M_3-muscarinic antagonist. Thirty CBF measurements were made over 15 min to determine the effect of the antagonists on baseline CBF. After 15 min, the antagonist was removed and methacholine was added. Thirty subsequent measurements were obtained for the given site on each specimen to determine the effect of receptor blockade on the activity of methacholine.

Statistics

CBF values were expressed as means±S.E.M. (standard error of the mean) and the response as percentage change from the baseline CBF. Statistical differences between values were determined by a paired Student's t-test. Significance was accepted when $p<0.05$.

RESULTS

Effects of methacholine

Methacholine alone (10^{-8} M and 10^{-6} M) in MEM produced increases in CBF of 6.46±0.27% and 10.34±0.42%, respectively. These increases were statistically significant by Student's paired t-test with $p<0.001$.

Effects of atropine

After treatment with 10^{-6} M atropine, the ciliary response to methacholine alone at concentrations of 10^{-8} M and 10^{-6} M was -2.95±0.64% and 2.06±0.85%, respectively. The inhibition of methacholine-induced ciliostimulation by atropine was statistically significant ($p<0.001$, by unpaired t-test) at both concentrations of atropine. Methacholine ciliostimulation returned within 24 h following removal of atropine from the culture medium.

Effects of selective M_1-muscarinic antagonist

After treatment with 10^{-6} M PZ, the ciliary response to methacholine at concentrations of 10^{-8} M and 10^{-6} M was 1.72±1.08% and -1.21±0.84%, respectively. After treatment with 10^{-8} M PZ, the ciliary response to methacholine at concentrations of 10^{-8} M and 10^{-6} M was -0.60±0.33% and 4.58±0.40%, respectively (Figure 1). The inhibition of methacholine-induced ciliostimulation by PZ was statistically significant ($p<0.0001$, by unpaired t-test) at both concentrations of PZ. Methacholine ciliostimulation returned within 24 h following removal of PZ from the culture medium.

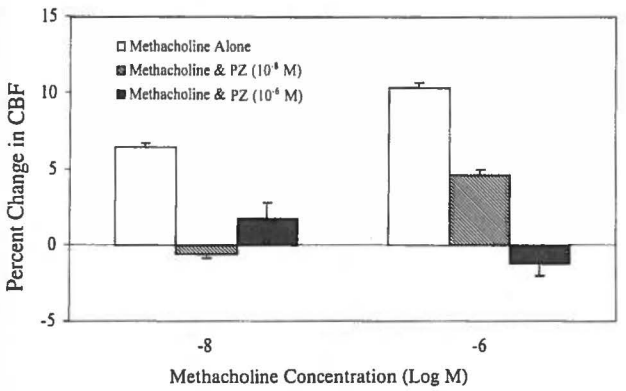

Figure 1. The increases in CBF with methacholine alone in MEM (open bars) at 10-8 M and 10-6 M were significant ($p<0.001$). The ciliostimulation seen with methacholine alone at 10-8 M and 10-6 M was significantly inhibited by 10-6 M PZ (slashed bars; $p<0.0001$) and 10-8 M PZ (cross-hatched bars; $p<0.0001$).

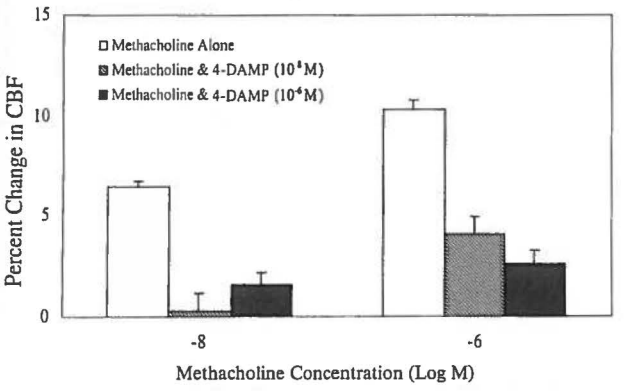

Figure 3. The increases in CBF with methacholine alone in MEM (open bars) at 10-8 M and 10-6 M were significant ($p<0.001$). The ciliostimulation seen with methacholine alone at 10-8 M and 10-6 M was significantly inhibited by 10-6 M 4-DAMP (slashed bars; $p<0.0001$) and 10-8 M 4-DAMP (cross-hatched bars; $p< 0.0001$).

Effects of selective M_2-muscarinic antagonist

After treatment with 10^{-6} M gallamine, the ciliary response to methacholine at concentrations of 10^{-8} M and 10^{-6} M was 6.51±0.54% and 10.62±0.84%, respectively (Figure 2). There was no significant inhibition of methacholine-induced ciliostimulation by gallamine ($p>0.3$).

Effects of selective M_3-muscarinic antagonist

After treatment with 10^{-6} M 4-DAMP, the ciliary response to methacholine at concentrations of 10^{-8} M and 10^{-6} M was 1.54±0.60% and 2.60±0.64%, respectively. After treatment with 10^{-8} M 4-DAMP, the ciliary response to methacholine at concentrations of 10^{-8} M and 10^{-6} M was 0.85±0.32% and 4.10±0.84%, respectively (Figure 3). The inhibition of methacholine-induced ciliostimulation by 4-DAMP was statistically significant ($p<0.0001$) at both concentrations of 4-DAMP. Methacholine ciliostimulation returned within 24 h following removal of 4-DAMP from the culture medium. The inhibition of methacholine-induced ciliostimulation by pirenzepine (PZ),

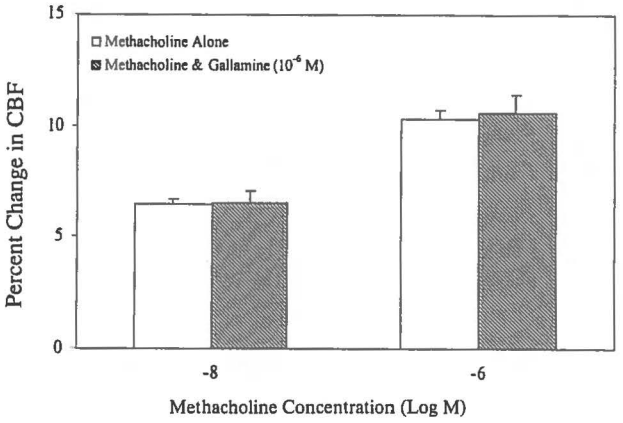

Figure 2. The increases in CBF with methacholine alone in MEM (open bars) at 10-8 M and 10-6 M were significant ($p<0.001$). There was no significant inhibition of methacholine-induced ciliostimulation by gallamine (slashed bars; $p>0.3$).

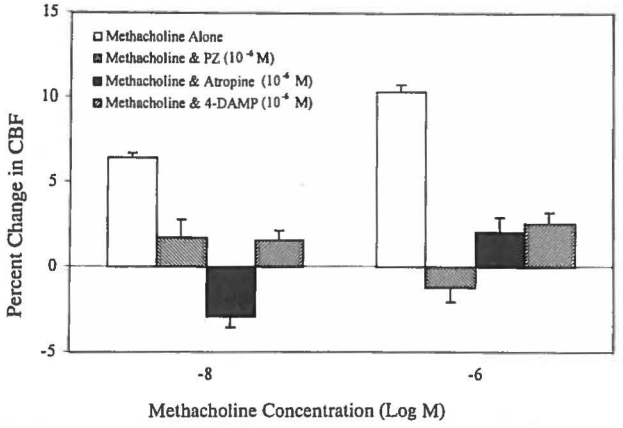

Figure 4. Methacholine alone in MEM (open bars) increased CBF in a dose-dependent manner. Increases were significant at 10-8 M and 10-6 M ($p<0.001$). The ciliostimulation seen with methacholine alone at 10-8 M and 10-6 M was significantly inhibited by 10-6 M PZ (left-slashed bars; $p<0.0001$), 10-6 M atropine (cross-hatched bars; $p<0.001$), and 10-6 M 4-DAMP (right-slashed bars; $p<0.0001$).

atropine and 4-DAMP were of similar magnitude with essentially complete inhibition of the effect of methacholine in all cases (Figure 4).

DISCUSSION

Effects of atropine

The non-selective muscarinic-receptor antagonist atropine significantly blocked methacholine-induced ciliostimulation. There was no significant increase in CBF in response to methacholine after non-specific muscarinic-receptor blockade by atropine, indicating that one or more of the muscarinic-receptor subtypes is responsible for the observed ciliostimulatory effects of methacholine.

Effects of M_1- and M_3-muscarinic receptors

It has been shown that at least five muscarinic-receptor subtypes are present in animal and human tissues (Baraniuk et al., 1990). Excitatory M_1 receptors have been demonstrated functionally and electrophysiologically in autonomic ganglia, including parasympathetic ganglia of the human airways (Bloom et al., 1988). Binding studies in guinea pig and human lung membranes have demonstrated the presence of M_3 receptors (Mak and Barnes, 1989). M_1- and M_3-muscarinic receptor subtypes have been identified in human nasal and bronchial tissues, with the M_3 subtype being most predominant (Mullol et al., 1992). M_3 receptors have been localized in submucosal glands in human airways, which appear to have a mixed population of M_1 and M_3 receptors in a proportion of 1:2 (Mak and Barnes, 1990). In this study, the selective M_1-muscarinic receptor antagonist pirenzepine dihydrochloride (PZ) and the selective M_3 muscarinic receptor antagonist 4-DAMP significantly blocked methacholine-induced ciliostimulation, indicating that M_1- and M_3-muscarinic receptors are involved in the transduction of the muscarinic acetylcholine-receptor signal responsible for ciliostimulation.

Pirenzepine (PZ) binds to M_1 receptors with high affinity, but can also bind to M_3 receptors with low affinity (Baraniuk et al., 1990). 4-DAMP has its greatest specificity for the M_3-receptor subtype. Our results showed that PZ, atropine, and 4-DAMP inhibited the ciliostimulation induced by methacholine about equally (Figure 4). Since these blockers are apparently inhibiting different receptor subtypes this finding requires explanation. If it is assumed that M_1 and M_3 receptors are present in a 1:2 ratio as previously shown in human airway submucosal glands, it would he expected that 4-DAMP would be more effective than PZ at blocking the effects of methacholine. However, since PZ does have some non-specific binding to M_3, the combination of M_1 and partial M_3 blockade could make its effect comparable to 4-DAMP. It is also possible that cooperative interaction between the M_1 and M_3 receptors results in complete inhibition even it only one receptor subtype is blocked.

Effects of M_2-muscarinic receptors

There was no significant inhibition of methacholine-induced ciliostimulation by the selective M_2-muscarinic receptor antagonist gallamine triethiodide (gallamine), demonstrating that M_2-muscarinic receptors are not involved in stimulation of respiratory cilia by methacholine. This could be anticipated since M_2-muscarinic receptors are located prejunctionally on post-ganglionic parasympathetic nerves and have a powerful inhibition on acetylcholine release (Barnes, 1992). The M_2-muscarinic receptor subtype was found to be absent from human nasal mucosa when a M_2-muscarinic antagonist was used in autoradiography and kinetic ligand-binding studies (Mak and Barnes, 1990). Although M_2 receptors are not present in the respiratory epithelium, the blockade of M_2 receptors in prejunctional post-ganglionic parasympathetic nerves by M_2 antagonists could increase acetylcholine release. Highly selective M_3- or mixed M_1/M_3-muscarinic antagonists would not increase acetylcholine release from cholinergic nerves, and therefore they may be preferable to the non-selective anti-cholinergic drugs when used for anti-cholinergic therapy.

CONCLUSION

Ciliostimulation by methacholine in human upper airway mucosa involves M_1- and M_3-muscarinic receptor subtypes, but not the M_2-receptor subtype. The relative proportion of M_1- and M_3 receptors on ciliated epithelium cannot be determined using the techniques of this study since the receptor-specific blockers may have the cross-reactivity with the receptor subtypes. Specific binding studies will be necessary to clarify the relative numbers of M_1- and M_3 receptors on ciliated upper airway epithelium.

REFERENCES

1. Baraniuk JN, Lundgern JD, Okayama M, Mullol MM, Shelhamer JH, Kaliner MA (1990) Vasoactive intestinal peptide in human nasal mucosa. J Clin Invest 86: 825-831.
2. Barnes PJ (1992) Modulation of neurotransmission in airways. Physiol Rev 72: 699-729.
3. Bloom JW, Baumgartener-Folkerts C, Palmer JD, Yamamura HI, Halonen M (1988) A muscarinic receptor subtype modulates vaguely stimulated bronchial contraction. J Appl Physiol 65: 2144-2150.
4. Humle EC, Birdsall NJM, Muckley NJ (1990) Muscarinic receptor subtypes. Ann Rev Pharmacol Toxicol 30: 633-673.
5. Hybbinette JC, Mercke U (1982) Effects of the parasympathomimetic drug methacholine and its antagonist atropine on mucociliary activity. Acta Otolaryngol (Stock) 93: 465-473.
6. Lambert DG (1993) Signal transduction: G protein and second messengers. Br J Anaes 72: 86-95.
7. Mak JCW, Barnes PJ (1989) Muscarinic receptor subtypes in human and guinea pig lung. Eur J Pharmacol 164: 223-230.
8. Mak JCW, Barnes PJ (1990) Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. Am Rev Respir Dis 141: 1559-1568.
9. Mullol J, Baraniuk JN, Logun C, Merida M, Hausfeld J, Shelhamer JH, Kaliner MA (1992) M_1 and M_3 muscarinic antagonists inhibit human nasal glandular secretion in vitro. J Appl Physiol 73: 2069-2073.
10. Yousufzai SY, Akhtar RA, Abdel-Latif AA (1986) Effects of substance P on inositol triphosphate accumulation, on contractile responses and on arachidonic acid release and prostaglandin biosynthesis in rabbit iris sphincter muscle. Exp Eye Res 43: 215-226.

Thomas V. McCaffrey, MD
Dept. of Otorhinolaryngology
Mayo Clinic
200 First Street SW
Rochester, MN 55905
U.S.A.