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Role of the muscarinic M_2 receptor in human nasal mucosa*

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SUMMARY

Introduction: *Neurally mediated secretory hyperresponsiveness is a feature of allergic rhinitis (AR). Muscarinic M_2 receptors are inhibitory autoreceptors which limit acetylcholine release at postganglionic parasympathetic nerve terminals, dysfunction of which has been implicated as a cause of bronchial hyperresponsiveness in asthma. The purpose of this study was to investigate the presence and function of M_2 receptors in the human nose.*

Methods: *In the first part of the study, nasal biopsies from subjects with AR (n=12) and normal controls (n=10) were examined for the presence of M_2 receptor mRNA using polymerase chain reaction (PCR). In the second part, subjects with symptomatic AR (n=17), with established contralateral secretory reflexes to bradykinin, underwent unilateral bradykinin challenge after premedication with pilocarpine, a M_2 receptor agonist. The effect of pilocarpine on contralateral secretory reflexes was studied.*

Results: *M_2 receptor mRNA was detected in 12 subjects (54%). Pilocarpine premedication led to a significant reduction of the contralateral secretory reflex to bradykinin.*

Conclusion: *M_2 receptors are present in the human nasal mucosa, though not universally. The function of M_2 receptors in subjects with AR can be demonstrated using pilocarpine, however, more work is required to define their importance and potential role in nasal hyperresponsiveness*

Key-words: muscarinic, acetylcholine, receptors, allergic rhinitis, nose

INTRODUCTION

When subjects with active allergic rhinitis (AR) undergo nasal challenge with bradykinin, hyperosmolar saline, or other non-allergenic irritants, they typically show an enhanced symptomatic and secretory response compared to that seen in normal subjects [1-4]. This tendency, termed nasal hyperresponsiveness, is clearly manifested after unilateral nasal bradykinin challenge by a contralateral secretory reflex, which is absent in normal subjects [1,2]. This reflex is blocked by atropine or ipratropium bromide premedication, demonstrating that the efferent limb is mediated by cholinergic nerves [2,5].

Muscarinic M_2 receptors are inhibitory autoreceptors that are present prejunctionally on postganglionic parasympathetic nerves. Activation of these receptors by acetylcholine serves to inhibit further acetylcholine release [6,7]. In subjects with atopic asthma, M_2 receptor dysfunction has been suggested as a cause of bronchial hyperresponsiveness [8]. This is believed to arise as a result of allosteric inhibition of M_2 receptors by eosinophil major basic protein (MBP), leading to loss of auto-inhibition of acetylcholine release, and enhancement of vagally mediated bronchoconstriction [9-11].

The function of M_2 receptors has clearly been demonstrated in the lower airways [12], however, their presence in the nasal airways is controversial [13-15]. The purpose of the present study was to investigate whether functional M_2 receptors are present in the nose in human subjects with AR. In order to do this, we first established the presence of M_2 receptor mRNA in nasal biopsies using polymerase chain reaction (PCR). We then determined whether nasal M_2 receptors are functional by investigating the effect of pilocarpine, an M_2 receptor agonist, on contralateral bradykinin-induced secretory reflexes.

MATERIALS & METHODS

Subjects

Subjects were recruited by volunteer advertisement. Subjects were not offered remuneration for participation. Twenty-two (14 female) participated in the first part of the study, to determine whether M_2 receptor mRNA is present in the nose. Ten were normal controls; 8 had perennial allergic rhinitis (PAR), defined as nasal symptoms for $>$ 9 months of the year, and positive skin prick tests to at least one perennial allergen

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(house dust-mite), but not to seasonal allergens (HAL Allergenen Laboratorium BV, Haarlem, The Netherlands), and 4 had seasonal allergic rhinitis (SAR), defined as strictly seasonal symptoms, and positive skin prick tests to seasonal (grass pollen), but not perennial allergens. The 4 subjects with SAR were studied outside the pollen season.

Seventeen volunteers (9 female) participated in the second part of the study, to investigate the function of nasal M_2 receptors. Twelve had PAR, and 5 had SAR. These 5 subjects with SAR were studied during the pollen season, while they were symptomatic. All 12 subjects with PAR were allergic to house dust-mite. Four of these were additionally allergic to grass pollen. All 5 subjects were SAR were allergic to both house-dust mite and grass pollen.

None of the subjects had asthma, the diagnosis of which was made on clinical grounds (no history of wheezing or use of inhalers) All participants including normal controls underwent skin-prick testing. Subjects were excluded if they had suffered from a respiratory infection or used oral, nasal, or inhaled corticosteroids within the previous month; used astemizole within the previous 3 months; or used short-acting antihistamines or nasal decongestants within the previous 2 days. All participants gave written informed consent, and the study was approved by the Hospital Research Ethics Committee.

Nasal mucosal biopsy

Nasal biopsies were taken from the anterior edge of the inferior turbinate under topical anaesthesia using a Blakesley 45° upwards tru-cutting forceps[16]. For topical anaesthesia, copheylcaine (lignocaine [4%]/phenylephrine spray, Paedpharm, Perth, WA, Australia) was sprayed onto a piece of unravelled cotton wool which was placed inside the nose for 10 minutes. Nasal biopsies were immediately snap frozen in liquid nitrogen, and stored at -80°C.

Determination of M_2 receptor mRNA

Messenger RNA was isolated from nasal biopsies using the RNeasy Mini Kit and QIAshredder spin column (Qiagen, Valencia, CA, USA). Tissue was first physically disrupted using a Pellet pestle and a pellet pestle motor (Kontes, Vineland, NJ, USA), and then homogenized by centrifugation at 14,000 rpm in a QIAshredder spin column for two minutes also containing 350 µl of RNeasy RLT lysis buffer. The homogenized tissue lysate was then added to 350 µl of 70% ethanol and transferred into an RNeasy spin column, and centrifuged at 10,000 rpm for 15 seconds. This was then washed with RNeasy RW1 and RPE wash buffers. RNA was eluted by adding 45 µl of RNAse-free water directly onto the RNeasy silica-gel membrane, and centrifuging at 10,000 rpm for 1 minute. Isolated RNA was stored at -80°C. The quality and quantity of the isolated RNA was checked by subsequent PCR for β-actin.

Complementary DNA synthesis from the isolated mRNA was then performed using Roche 1st strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Indianapolis, IN, USA). A master mix was prepared containing 2 µl of 10x reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), 4 µl of $MgCl_2$ solution (25 mM), 2 µl of deoxynucleotide mix (containing 10 mM of each of dATP, dCTP, dTTP, and dGTP), 2 µl of oligo-$p(dT)_{15}$ primer (0.02 A_{260} units/µl), 1 µl of RNAse inhibitor (50 units/µl), and 0.8 µl of AMV reverse transcriptase. 11.8 µl of this master mix was added to 8.2 µl of sample RNA. The resulting mixtures were incubated at 25°C for 10 minutes, and then at 42°C for 60 minutes, followed by cooling to 4°C, in a PTC200 DNA Engine (MJ Research, Waltham, MA, USA). Subsequent storage was at -20°C.

PCR using cDNA was performed in the Roche LightCycler apparatus (Roche Diagnostics GmbH, Mannheim, Germany), using Qiagen QuantiTect SYBR Green PCR Kits (Qiagen GmbH, Hilden, Germany). Primers were obtained from MWG Biotech (Cedex, France). The primer sequences used were 5′ – TCC TGT GGC ATC CAC GAA ACT – 3′ (β-actin forward); 5′ - GAA GCA TTT GCG GTG GAC GAT - 3′ (β-actin reverse); 5′ - ATA GTG CCC GAC TAC ATC GC - 3′ (vChAT forward); 5′ - TCT TCG CTC TCC GTA GGG TA - 3′ (vChAT reverse); 5′ - GTG GTC AGC AAT GCC TCA GTT AT - 3′ (M_2 receptor forward); 5′ - TCC CCA TCC TCC ACA GTT CTC - 3′ (M_2 receptor reverse). A master mix was prepared containing 10 µl of QuantiTect SYBR Green PCR master mix, 7 µl of RNAse-free water, and 0.5 µl of forward and reverse primers (20 pM), per reaction. 18 µl of this master mix was added to 2 µl of cDNA inside the LightCycler capillaries. Negative controls were used in all experiments. vChAT (vesicular choline acetyltransferase) is a specific marker of cholinergic nerves and was used to confirm that the biopsies contained cholinergic neural tissue [19].

All PCR reactions commenced with initial preincubation (95°C for 15 minutes), followed by amplification (45 cycles for β-actin and vChAT, 65 cycles for M_2 receptor; this was necessary to sufficiently amplify the small amount of RNA present), melting (from 65°C to 95°C, at 0.1°C/second), and cooling (to 40°C). The amplification step consisted of 3 segments: denaturation (94°C for 15 seconds); annealing (55°C for 20 seconds); and elongation (72°C for 16 seconds for M_2 receptor, and for 11 seconds for β-actin and vChAT). In our laboratory we have found these settings to provide optimum amplification. Fluorescence was measured at the end of the elongation phase during amplification using single acquisition mode, and continuously during melting using continuous acquisition mode. Analysis of specificity of PCR products was performed by melting curve analysis and agarose gel electrophoresis. Melting curve analysis was performed using the LightCycler Data Analysis (LCDA) program of the LightCycler software, which calculated the melting peaks of the amplified products. The melting peaks for β-actin, M_2 receptor, and vChAT cDNA were 86°C, 83°C, and 91°C, respectively. Agarose gel was stained with 4 µl of ethidium bromide (2 mg/ml) (Gibco BRL, Grand Island, NY, USA). 10 µl of loading dye (prepared by adding 7.5 g of Ficoll, 0.125 g of bromophenol blue, and 0.125

g of xylene cyanole FF to 50 ml of distilled water) was added to 20 µl of PCR product, and placed into the wells (10 µl per well). A 100 bp DNA ladder (New England Biolabs, Hertfordshire, UK) was used in one well. The ethidium bromide- bound PCR products were visualized with a Syngene ultraviolet light source and camera attached to a G6-300 Gateway computer, using GeneSnap image acquisition systems software for Windows 98 (version 2.60.0.14, 1998, Synoptics, Cambridge, UK). The size of the cDNA products were 314 bp (base pairs) for β-actin, 218 bp for vChAT, and 211 bp for M_2 receptor.

Nasal bradykinin challenge experiments

Bradykinin was obtained from Bachem (Basle, Switzerland) and prepared for nasal challenges as described in previous publications[17]. Hartman's solution (Baxter, Pharmaceuticals, Norfolk, UK; pH 6-6.2, osmolarity 278 mOsm/l) was used as diluent. Nasal challenges were performed using paper disks punched from Shandon filter cards (Shandon, Pittsburgh, PA, USA)[18], impregnated with 10 µl of challenge substance (diluent, bradykinin 50 µg, or bradykinin 100 µg). Nasal challenges were performed by placement of a challenge disk onto the left side of the anterior nasal septum, beyond the mucocutaneous junction. This was removed after 60 seconds.

Secretions were collected from both sides of the nose using filter paper collection disks. These collection disks were capable of absorbing up to 50 µl of water. These were kept in numbered collection tubes and weighed before use. Collection disks were placed on both sides of the anterior nasal septum (left side first). After 30 seconds, the collection disks were removed and replaced into their original collection tubes and re-weighed.

Prior to performing nasal challenges, anterior rhinoscopy was performed, and any crusts or dried secretions removed. Baseline secretions were then collected. Nasal challenge with diluent was then performed. Thirty seconds after removal of the challenge disk, secretions were again collected, this collection representing secretions obtained for the first two minutes after the challenge. Three minutes later, secretions were again collected, this time representing secretions obtained at 5 minutes. Following this, subjects were allowed to blow their noses. At ten minute intervals after diluent challenge, nasal challenge with bradykinin 50 µg and bradykinin 100 µg was performed. For each challenge, secretion weights obtained at 2 minutes and 5 minutes were added.

Study design

In the first part of the study, to investigate whether M_2 receptor mRNA is present in nasal tissue, nasal biopsy was performed on both normal and allergic volunteers. RNA extraction, cDNA synthesis, and PCR were performed, and the presence of β-actin and vChAT cDNA (a marker of cholinergic nerves[19]) established by melting curve analysis. PCR runs for M2 receptor were then performed. The presence of M_2 receptor product was checked using both melting curve analysis and agarose gel electrophoresis.

The second part of the study was performed in order to investigate whether M_2 receptors have any functional role in the nose. For this part of the study, subjects with PAR or in-season SAR, with established contralateral secretory reflexes to bradykinin, were recruited. Asymptomatic subjects were not recruited as they did not, in general, demonstrate this reflex.

At their first visit, subjects underwent control bradykinin challenge according to the protocol described. Subjects then returned for a second challenge, at least 48 hours, but not more than seven days, later. On this occasion, 200 µg pilocarpine (Minims, Clauvin Pharmaceuticals, London, UK) diluted in sterile water [15] was administered to the contralateral nasal cavity using a metered dose spray bottle. This was performed after diluent challenge (after the collection of secretions at 5 minutes), before bradykinin 50 µg challenge. Nasal secretory responses were expressed as the increase in secretion weights induced by bradykinin (performed after administration of pilocarpine) over that induced by diluent (which had performed before the administration of pilocarpine). The contralateral secretory responses after pilocarpine pre-medication were then compared to the corresponding responses obtained on the control study day (Figure 1).

Statistical analysis

Statistical analysis was performed using WinStat for Microsoft Excel (version 2001.1). In order to test whether data were distributed normally, a Chi-squared test for discrete variables was used. Non-normal data was analyzed using non-parametric tests. A Friedman's test was used to test whether bradykinin induced significant increases in secretion weights, with a Wilcoxon ranked-pairs test performed to test for differences between individual doses. In order to test whether nasal secretory responses were altered by pilocarpine premedication, the increases in bradykinin-induced secretion weights over those

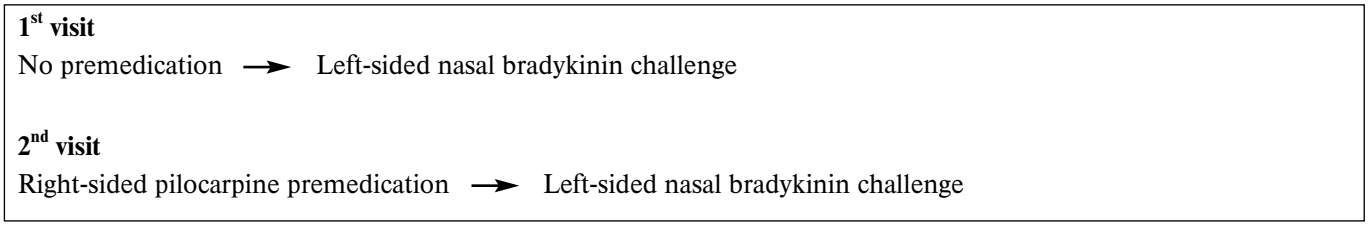

Figure 1. Study design.

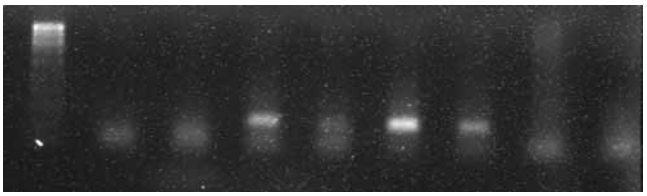

Figure 2. Agarose gel electrophoresis for M_2 receptor mRNA. From left, 100 bp DNA ladder, normal subject (negative), PAR subject (negative), normal subject (positive), normal subject (positive), normal subject (positive), normal subject (positive), normal subject (negative), normal subject (negative).

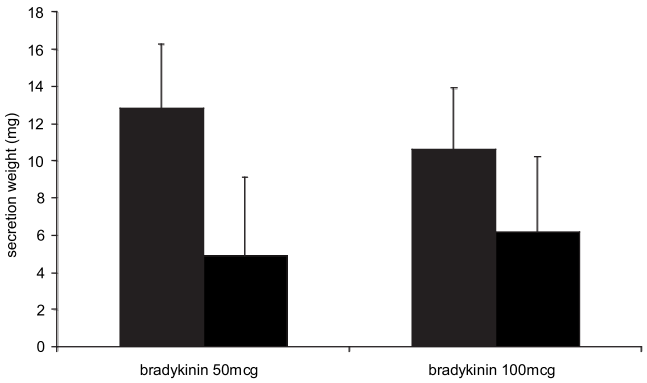

Figure 3. Increases in bradykinin-induced secretion weights over those induced by diluent on the ipsilateral side of the nose, on the control study day (black) and on the pilocarpine study day (grey).

induced by diluent, in response to each of the two doses of bradykinin was compared to the corresponding increase obtained in the same subject on the control study day, using a Wilcoxon ranked-pairs test.

RESULTS

Presence of M_2 receptor mRNA

β-actin (to confirm the presence of RNA) and vChAT (to confirm the presence of cholinergic neural tissue) mRNA was detected by PCR in the nasal biopsies of all subjects. M_2 receptor mRNA was detected in the nasal biopsies of twelve subjects (54%). Six of these were normal controls (60%), three had PAR (37.5%), and 3 had SAR (75%). In all cases, the specificity of the PCR products was tested by both melting curve analysis and agarose gel electrophoresis (Figure 2).

Effect of pilocarpine on bradykinin-induced nasal secretions:

Seventeen atopic subjects with established contralateral secretory reflexes to bradykinin underwent nasal bradykinin challenge protocol on two occasions: once on a control study day, and, 2-7 days later, after pilocarpine premedication. Comparisons of bradykinin-induced secretory weights were done with respect to the effect of diluent in each case.On the control study day, ipsilateral secretion weights went from 13.9 ± 3.3 mg after diluent, to 26.7 ± 4.2 mg after bradykinin 50 μg, to 24.5 ± 4.0 mg after bradykinin 100 μg; (Friedman's test $p = 0.002$). On the pilocarpine study day, ipsilateral secretion weights were 14.9 ± 3.5 mg after diluent; 19.8 ± 3.5 mg after bradykinin 50 μg; and 21.1 ± 3.4 mg after bradykinin 100 μg ($p = 0.02$). When the increases in bradykinin-induced secretion weights over those induced by diluent were compared between the control and pilocarpine study days, the differences were not significant (Figure 3).

On the contralateral side, on the control study day, secretion weights went from 13.6 ± 2.6 mg after diluent, to 20.3 ± 2.5 mg after bradykinin 50 μg, to 21.8 ± 3.8 mg after bradykinin 100 μg (Friedman's test $p < 0.001$). On the pilocarpine study day, contralateral secretion weights in response to bradykinin went from 20.6 ± 4.3 mg after diluent, to 22.3 ± 3.1 mg after bradykinin 50 μg, to 20.0 ± 3.5 mg after bradykinin 100 μg ($p = 0.05$). There were no significant differences in induced secretion weights between control and bradykinin 50 μg challenge, between control and bradykinin 100 μg challenge, or between the two bradykinin challenges (50 μg and 100 μg).

The increases in bradykinin-induced secretion weights over diluent after challenge with bradykinin 100 μg were significantly less on the pilocarpine study day than on the control study day (8.2 ± 2.3 mg on the control study day, versus a decrease of 0.6 ± 2.7 mg on the pilocarpine day, $p = 0.003$). The differences after challenge with bradykinin 50 μg were not significant (6.7 ± 1.9 mg on the control study day versus 1.7 ± 2.4 mg on the pilocarpine day, $p = 0.09$) (Figure 4).

DISCUSSION

In the present study, we have shown that M_2 receptors exist in human nasal tissue, however, the amount of M_2 receptor present is small, and, in many subjects, it is not possible to detect it. Nevertheless, there appears to be a sufficient amount of M_2 receptor present to cause a measurable effect for pilocarpine on contralateral secretory reflexes in subjects with symptomatic allergic rhinitis. Thus, it is interesting to speculate whether M_2 receptors may be involved in nasal hyperresponsiveness in allergic rhinitis.

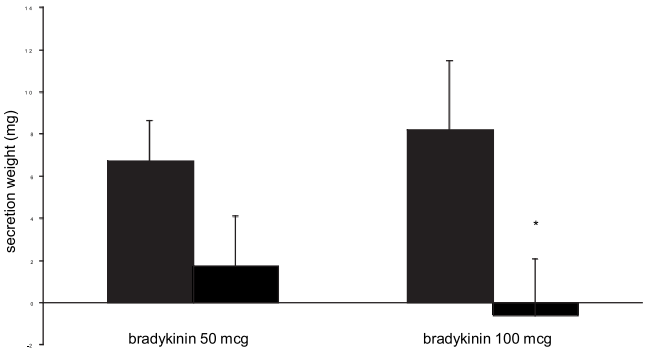

Figure 4. Increases in bradykinin-induced secretion weights over those induced by diluent on the contralateral side of the nose, on the control study day (black) and on the pilocarpine study day (grey).

M_2 receptor function has been more extensively studied in the lower airways [8-12]. In normal subjects, inhalation of sulphur dioxide causes vagally mediated reflex bronchoconstriction. This reflex is blocked by premedication with pilocarpine, an M_2 receptor agonist. The effect of pilocarpine is believed to be due to M_2 receptor activation at the prejunctional nerve terminal, which inhibits further acetylcholine release. Interestingly, in patients with atopic asthma, pilocarpine does not block sulphur dioxide –induced reflex bronchoconstriction, suggesting the presence of M_2 receptor dysfunction in these patients [8]. M_2 receptor dysfunction in atopic asthma is believed to arise as a result of eosinophil localization to airway nerves, with subsequent release of products such as MBP [11], which is an allosteric inhibitor of M_2 receptors [10]. In sensitized guinea-pigs, pre-treatment with antibody to MBP prevents allergen-induced loss of M_2 receptor function and vagal hyperresponsiveness to electrical stimulation [9].

Eosinophil recruitment to nasal tissues with release of MBP occurs during the late phase of the allergic response in AR. It is thus tempting to speculate whether M_2 receptor dysfunction is also involved in nasal hyperresponsiveness in AR. However, to date, there has been little firm evidence supporting the existence of M_2 receptors in the nose. Autoradiographic studies failed to find evidence of M_2 receptors in the nose [14], while immunohistochemical studies have reported sparse presence of nasal M_2 receptors [13]. Evidence is also lacking from functional studies. Whereas in the lower airway, the function of M_2 receptors has been demonstrated by the effects of gallamine, an M_2 receptor antagonist, and pilocarpine, an M_2 receptor agonist, on vagally mediated bronchoconstriction [12], neither of these agents was found to have any significant effect on the contralateral secretory response to nasal challenge with histamine in normal subjects [15].

In the present study, we investigated whether M_2 receptor mRNA is present in the human nose by PCR using the LightCycler, a highly sensitive technique which can detect very small amounts of target cDNA. Our data demonstrate that nasal M_2 receptors are present, albeit in small amounts. We were not able to definitively establish whether this represented neural or glandular M_2 receptor mRNA, however, all biopsies examined contained vChAT mRNA, a specific marker of cholinergic nerves [19]. M_2 receptor mRNA was not detected in the nasal biopsy material of all subjects; it is possible that the explanation for this is that M_2 receptors are not universally present in human subjects. It is interesting to speculate whether the late phase of the allergic response leads to any diminution of M_2 receptor expression in subjects with more active allergic rhinitis; this might explain why M_2 receptor mRNA was only detected in a minority of patients with PAR. This hypothesis could be investigated by a larger study, preferably with biopsies performed before and after allergen challenge.

We then proceeded to examine whether nasal M_2 receptors are functional. To this end, we recruited subjects with active AR, who had established contralateral secretory reflexes, unlike deTineo, who investigated normal subjects [15]. We employed a nasal bradykinin challenge protocol using filter paper disks. Previous authors have shown that unilateral nasal bradykinin challenge in subjects with PAR leads to a parasympathetic contralateral secretory reflex [2]. In previous work, we subjected normal control subjects to the exact same bradykinin challenge protocol used in the present study. Normal subjects do not show a contralateral secretory reflex; furthermore, bradykinin-induced secretions are significantly less than in PAR subjects (for example, the secretory response to bradykinin 50 μg is 4.6 ± 0.5 μg on the ipsilateral side, and 3.0 ± 0.7 μg on the contralateral side) [20]. We also showed that subjects with SAR who are out-of-season and asymptomatic do not show a contralateral secretory response to bradykinin. However, 24 hours after allergen challenge, a contralateral secretory response is induced [17]. This suggests that a contralateral secretory response to bradykinin is present during the late phase of the allergic response. Thus, the subjects of the second part of the present study were characterized by ongoing allergic inflammation and an active late-phase allergic reaction.

Our finding of an attenuation of the contralateral secretory increase in response to bradykinin 100 μg compared to diluent on the pilocarpine study day suggests that as well as being present in the nose, M_2 receptors are also functional.

The finding of functional M_2 receptors in subjects with symptomatic AR would appear to be at odds with the hypothesis that M_2 receptor dysfunction may have a role in neural hyperresponsiveness in allergic rhinitis. However, it should be noted that we were unable to investigate the effect of pilocarpine in normal subjects, as these subjects do not demonstrate a contralateral secretory response to bradykinin [1,2]. Therefore, no conclusions can be drawn from our work regarding whether M_2 receptor function in allergic rhinitis is different to that in normal subjects. In addition, most subjects undergoing bradykinin challenge in the present study had relatively mild rhinitis that did not require regular treatment with nasal steroids; thus the presence of functional M_2 receptors in our study group does not rule out the possibility of M_2 receptor dysfunction in subjects with more severe rhinitis. However, it is interesting to note that among subjects with out-of-season SAR, a significantly higher proportion of nasal biopsies contained M_2 receptor mRNA compared to subjects with PAR. Clearly, more work is required to better clarify the role and functionality of M_2 receptors in rhinitic patients.

There are some shortcomings in the design of the present study. Most importantly, subjects in the second part of the study were not randomized into control and pilocarpine groups. Instead, all subjects underwent the control bradykinin challenge on their first visit. However, only subjects with established contralateral secretory reflexes were suitable for

the study, and these subjects were identified at the time of the control bradykinin challenge at the first visit.

While we believe the present study to be the first to demonstrate the presence and function of M_2 receptor mRNA in human nasal mucosa, the findings are still preliminary and will require further investigation in future studies with larger numbers of subjects. Nevertheless, recognition of the presence of nasal M_2 receptors may steer treatment options. Currently, ipratropium bromide, a non-selective muscarinic antagonist, is used to treat rhinorrhoea in patients with allergic rhinitis. Tiotropium bromide is a new anti-cholinergic agent which has long-lasting affinity for M_1 and M_3 receptors, and more short-acting affinity for M_2 receptors [21]. Therefore, it blocks the effects of acetylcholine on end-organs, but has less effect on auto-inhibition of acetylcholine release. This compound has been used to treat patients with chronic obstructive pulmonary disease with much success [22-24]. Such a medication may prove to be of significant benefit in patients with allergic rhinitis and functional nasal M_2 receptors.

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