Pulmonary neuronal $M_2$ muscarinic receptor function in asthma and animal models of hyperreactivity

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The dominant innervation of the airway smooth muscle is mediated by parasympathetic fibres which are carried in the vagus nerves. Activation of these cholinergic nerves releases acetylcholine which binds to $M_2$ muscarinic receptors on the smooth muscle causing contraction. Acetylcholine also feeds back onto neuronal $M_1$ muscarinic receptors located on the postganglionic cholinergic nerves. Stimulation of these receptors further stimulates acetylcholine release, so these $M_1$ muscarinic receptors act as autoreceptors. Loss of function of these $M_1$ receptors, as occurs in some patients with asthma and in animal models of hyperreactivity, leads to an increase in vagally mediated hyperreactivity. In this review we shall discuss the mechanisms that may account for the loss of function of these neuronal $M_1$ muscarinic receptors.

Innervation of the airways by parasympathetic nerves

The vagus nerves carry preganglionic nerve fibres from the vagal nuclei in the medulla to ganglia in the airways. These parasympathetic ganglia are interspersed irregularly along the posterior aspect of the wall of the trachea and major bronchi. From these ganglia short postganglionic nerves pass forward to innervate the airway smooth muscle, the bronchial circulation, and the glandular acini. Histological studies have not shown postganglionic effenter fibres beyond the level of the terminal bronchi, and functional studies have not found an effect of vagal stimulation on the respiratory bronchioles and the alveoli. The site of the most dense cholinergic innervation—the major bronchi—is also the site of bronchoconstriction in patients with asthma, suggesting an underlying pathogenic relationship.

Cholinergic receptors in the airways

Acetylcholine acts on both muscarinic and nicotinic receptors. Five different muscarinic receptors ($M_1$–$M_5$) have been genetically sequenced. These $M_1$–$M_5$ muscarinic receptors can also be identified based on differing binding affinities between different antagonists. $M_1$ muscarinic receptors are selectively blocked by pirenzipine, muscarinic $M_3$ receptors are blocked by AF-DX116 and gallamine, $M_4$ receptors are blocked by 4-DAMP, while $M_2$ receptors are antagonised by hombocine; it has been difficult to identify a selective agonist at $M_2$ receptors. Autoradiographic studies have demonstrated muscarinic $M_1$ and $M_2$ receptors along nerve bundles and within the cholinergic ganglia. Primary cultures of postganglionic cholinergic neurons from the trachea have shown these nerves to possess messenger RNA for only the $M_2$ receptors. Airway smooth muscle cells express $M_1$ and $M_3$ muscarinic receptors; the latter mediate smooth muscle contraction.

Physiological function of cholinergic nerves in the airways

Stimulation of the parasympathetic nerves releases acetylcholine which causes the airway smooth muscle to contract, diverting the glandular tissue to secrete mucus and the bronchial circulation to dilate. Studies both in man and in animals have shown that, in addition to causing contraction, the vagus nerves also maintain a baseline tonic contraction of the airway smooth muscle. This baseline tonic contraction of the airways has been demonstrated both in human and in animal studies. For example, in normal non-asthmatic humans 80 µg of inhaled ipratropium bromide caused a 40% reduction in airway resistance, demonstrating that the vagus nerves are important in maintaining airway tone. Furthermore, directly inhibiting the vagus nerves—for example, by cutting them—causes bronchodilation. In vivo recording of the neural impulses in the vagi of cats and dogs has shown that even at rest there is neural activity in the parasympathetic ganglia. The resting airway tone is higher in asthmatic subjects than in normal controls. This increased tone was completely blocked by ipratropium bromide, indicating that it was vagally mediated. This shows that in patients with asthma there is increased vagal nerve activity at rest.

Such a powerful bronchoconstricting mechanism needs to be tightly controlled and this is best done close to the site of release of acetylcholine. Indeed, the most important local control over acetylcholine release from postganglionic cholinergic nerves is exerted by acetylcholine itself. Acetylcholine acting on inhibitory muscarinic $M_2$ autoreceptors located prejunctionally on postganglionic nerves limits the further release of acetylcholine. Thus, these receptors act as autoreceptors.

The function of the neuronal $M_2$ autoreceptor can be demonstrated in vivo with $M_2$ receptor antagonists such as gallamine which cause a dose dependent potentiation of vagally mediated bronchoconstriction. For example, gallamine 10 mg/kg increases vagally induced bronchoconstriction by as much as five fold in pathogen-free guinea pigs. Conversely, the muscarinic agonist pilocarpine stimulates neuronal $M_2$ muscarinic receptors and so decreases vagally induced bronchoconstriction. Pilocarpine 100 µg/kg reduces vagally...
mediated bronchoconstriction by about 75% (fig 2B). The presence of an M₂ autoreceptor has also been confirmed by measuring changes in induced acetylcholine release using high performance liquid chromatography in the presence of selective M₂ receptor antagonists. Although first described in the airways of guinea pigs, M₂ receptors have been described in the airways of all species studied, including humans.

Loss of function of neuronal muscarinic M₁ receptors in animal models of hyperreactivity

In antigen sensitised animals exposure to antigen causes an immediate bronchoconstriction followed by a period of increased reactivity to a variety of stimuli. This increased reactivity can be blocked with anticholinergic agents or, alternatively, by cutting the vagus nerves, suggesting that it is vagally mediated.

Increased vagally mediated bronchoconstriction may arise because of an increase in the reactivity of the airway smooth muscle or because the function of the neuronal M₁ muscarinic receptor is impaired. These alternative explanations were tested in an animal model of hyperreactivity where the function of the neuronal M₁ muscarinic receptor and the smooth muscle response to acetylcholine were compared between control and antigen sensitised guinea pigs. The bronchoconstriction induced by acetylcholine was the same in vagotomised control and antigen challenged animals, indicating that the muscarinic M₁ receptor on airway smooth muscle was functioning normally. In contrast, in antigen challenged animals gallamine no longer potentiated and pilocarpine no longer attenuated the magnitude of vagally induced bronchoconstriction (fig 2A and B). Thus, the function of M₁ muscarinic receptors is impaired in antigen sensitised animals after challenge. These findings have subsequently been confirmed in other experiments using different models of antigen challenge.

Increased concentrations of acetylcholine have been reported in the airways of other antigen challenged animals including mice, dogs, and guinea pigs, providing indirect supportive evidence that there is loss of function of neuronal M₁ muscarinic receptors in the airways of animal models of hyperreactivity.

Antigen induced hyperreactivity is associated with an influx of inflammatory cells, particularly eosinophils and lymphocytes, into the airway walls. A number of studies have either inhibited the recruitment of these cells to the airways or neutralised specific products of these cells to establish their role in hyperreactivity. The results of these studies have specifically implicated the eosinophil in the development of antigen induced hyperreactivity. Since antigen induced vagally mediated hyperreactivity is due to loss of function of the neuronal M₁ receptor, the role of eosinophils in the loss of function of M₁ receptors has been investigated.

The selective localisation of leucocytes to sites of inflammation is mediated through the interactions of specific adhesion molecules.
Very late activation antigen 4 (VLA-4) is the major β integrin expressed by eosinophils and it recognises the counter ligand vascular adhesion molecule 1 (VCAM-1) expressed on vascular tissues, allowing migration into the airways.\(^{47\ 48}\) Pretreating antigen sensitised animals with an antibody to the adhesion molecule VLA-4 before challenge prevented antigen induced eosinophil accumulation in the airways and prevented loss of function of M₁ muscarinic receptors and the development of airway hyperreactivity.\(^{39}\) Depleting eosinophils by using a monoclonal antibody to neutralise the eosinophil chemoattractant interleukin 5 prevented antigen induced airway eosinophilia and loss of function of neuronal M₁ muscarinic receptors.\(^{45}\) Thus, eosinophils are responsible for loss of pulmonary neuronal M₁ muscarinic receptor function in the airways of antigen challenged guinea pigs.

A potential mechanism for this eosinophil dependent loss of M₁ receptor function in antigen challenged animals was suggested by the finding that some eosinophil products are antagonists at M₁ muscarinic receptors. Eosinophils contain electron dense granules containing eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO), and eosinophil major basic protein (MBP).\(^{50\ 51}\) These heavy granules (molecular weight 14–77 kD) comprise approximately 90% of eosinophil granular proteins. These four proteins have the common characteristic of being cytotoxic to mammalian cells and also of possessing high isoelectric points (pH range 10–11.5). In common with these eosinophil proteins, many antagonists at M₁ muscarinic receptors such as gallamine and protamine\(^{52}\) are positively charged. The cationic nature of these antagonists is important in binding to M₁ muscarinic receptors, possibly because the receptor is heavily sialated giving it a net negative charge.

In receptor binding studies on M₁ and M₂ receptors, MBP and, to a lesser extent, EPO displaced the agonist [³H]N-methylscopolamine ([³H]NMS) from guinea pig and human M₁ but not M₂ muscarinic receptors.\(^{53\ 54}\) Furthermore, in the presence of the anionic compound heparin, MBP was displaced from these receptors. This suggests that the antagonism of these M₁ receptors was reversible and due to the positively charged nature of MBP. In saturation binding studies it was shown that the antagonism of MBP at M₁ receptors was allosteric rather than competitive. Eosinophil MBP may have a physiologically relevant role in the loss of M₁ receptor function since the dissociation constant for MBP at M₁ receptors is \(1.4 \times 10^{-5}\) M which is only minimally higher than the level of MBP found in the sputum of patients with acute asthma.\(^{55}\) In contrast, eosinophil peroxidase has a low affinity for M₁ receptors and so does not appear to be physiologically relevant as an antagonist at M₁ receptors.

As binding studies have shown that heparin displaces MBP from M₁ muscarinic receptors, and because eosinophil MBP may be responsible for the loss of function of the neuronal M₁ muscarinic receptor, the effect of heparin on M₂ receptor function was tested in antigen challenged animals. In these studies it was shown that the administration of heparin acutely restored M₁ receptor function in antigen challenged guinea pigs and rats\(^{40}\) (Belmonte et al, unpublished). These findings suggest that M₁ receptors become dysfunctional after antigen challenge and that positively charged proteins such as MBP are acting as endogenous antagonists at M₁ receptors.

In order to establish a role for eosinophil MBP in the loss of M₁ receptor function, in vivo studies were performed with a specific neutralising antibody to eosinophil MBP.\(^{56}\) In these are responsible for the loss of M₁ receptor function in antigen challenged animals.

Since MBP is highly cationic and probably does not diffuse far after it is released from eosinophils, there must be a close anatomical association between eosinophils and airway nerves. In histological studies it was shown that there is a close association of both eosinophils and extracellular MBP with airway nerves in patients with asthma and in animal models of hyperreactivity (fig 4). Furthermore, the number of eosinophils per nerve was correlated with the in vivo function of neuronal M₁ muscarinic receptors.\(^{57}\) Thus, there is good evidence to indicate that eosinophil MBP is involved in the loss of function of neuronal M₁ muscarinic receptors in antigen challenged animals.
Loss of function of neuronal M₂ muscarinic receptors after exposure to a respiratory viral infection

Infection with a respiratory virus causes an increase in vagally mediated hyperreactivity. Studies in guinea pigs and in rats infected with parainfluenza virus indicate that there is loss of function of the neuronal M₂ muscarinic receptor. The central binding site of the M₂ muscarinic receptor is composed of negatively charged sialated glycoproteins. Respiratory viruses, in particular parainfluenza virus, contain the enzyme neuraminidase which cleaves sialic acid residues. In receptor binding studies it has been shown that the affinity of M₂ receptors for the agonist [3H] quinuclidinyl benzilate ([3H] QNB) is impaired when they are incubated with neuraminidase or when lung tissue of virally infected animals is studied. These data suggest that the viral enzyme neuraminidase may be involved in the loss of function of neuronal M₂ muscarinic receptors. Inflammatory cells also appear to play a part in the loss of function of the neuronal M₂ receptor. Since heparin does not restore the function of the M₂ receptor in non-sensitised virally infected animals, the inflammatory cell causing this impaired neuronal M₂ receptor function does not appear to be the eosinophil. Prior antigen sensitisation alters the immune response to a viral infection away from the normal lymphocyte response to an eosinophil rich response. In antigen sensitised guinea pigs viral infections cause pulmonary eosinophilia and heparin restores the function of M₂ receptors, which suggests that the M₂ receptor dysfunction may also be eosinophil mediated (Fryer et al, unpublished).

Loss of function of neuronal M₂ muscarinic receptors after exposure to ozone

Exposure of animals to ozone (2 ppm for four hours) causes an increase in vagally mediated hyperreactivity in guinea pigs. In vivo studies in guinea pigs indicate that there is an immediate loss of function of the neuronal M₂ muscarinic receptor after exposure to ozone that is long lasting. The mechanisms responsible for the loss of function of the neuronal M₂ receptor after exposure to ozone are not well established but appear to be dependent on inflammatory cells rather than to be the direct result of exposure to ozone. Loss of function of these receptors may be eosinophil mediated, since inhibiting eosinophil recruitment with an antibody to VLA-4 or neutralising MBP prevents this loss of M₂ receptor function (Fryer et al, unpublished observation).

Neuronal M₂ muscarinic receptor function in patients with asthma

An increase in both vagally mediated baseline tone and vagally mediated hyperreactivity has been reported in patients with asthma. The increase in vagal hyperreactivity has been demonstrated in studies in which the vagus nerves have been inhibited pharmacologically with drugs such as atropine or ipratropium bromide. The exact contribution of the vagus nerves to the hyperreactivity varies between studies and the inconclusive nature of these studies has led some to suggest that the vagus nerves are not important in the pathogenesis of asthma. This conclusion is unfortunate as many studies either show a clear benefit in some but not all patients, or the investigators have not adequately inhibited vagal nerve function. One particular problem with the currently available anticholinergic agents is their non-selective nature; they antagonise both M₁ and M₂ receptors. Antagonism of the M₂ receptor by these agents will potentiate vagally induced bronchoconstriction and thus counteract the effect of inhibiting the M₁ receptor on the smooth muscle. The presence of neuronal M₂ muscarinic receptors has also been described in humans. In these in vivo studies vagally induced bronchoconstriction is induced indirectly via a

Figure 4  Eosinophils are found in association with nerve fibres in the airways of antigen challenged guinea pigs. The photomicrograph is from a paraffin embedded section of antigen challenged guinea pig bronchus. Eosinophils (detected with chromotrope 2R) are seen in close proximity to airway nerve fibres in the airway smooth muscle (detected with an antibody to PGP 9.5, in black). The nerve fibre in the smooth muscle shown with the thin arrow in (A) is shown under higher magnification in (B). Eosinophils were also seen around the airway nerve bundles near the smooth muscle in the submucosa (thick arrows). From Evans et al.
vagal reflex with an agent such as sulphur dioxide or histamine. In the presence of a normal M2 muscarinic receptor pilocarpine stimulates these receptors and thus limits the degree of vagally mediated bronchoconstriction. In addition to these indirect in vivo studies, in vitro studies on surgically resected tissue from non-asthmatic individuals have also shown the presence of the neuronal M2 receptor. 

Most studies on the function of the neuronal M2 muscarinic receptor function after exposure to antigen have been carried out in animal models of hyperreactivity. This is because it is not feasible to stimulate the vagus nerve directly in humans and because lung tissue of which could be used for in vitro studies is rarely not feasible to stimulate the vagus nerve to antigen have been carried out in animal studies. 

El-Bermani et al. (1990) demonstrated that inhaled pilocarpine, a muscarinic agonist, caused a significant increase in bronchial smooth muscle tone. This effect was blocked by atropine, an anticholinergic drug, indicating that the response was mediated through the M3 muscarinic receptor subtype. The authors suggested that this response may have clinical implications, as it could explain the effectiveness of anticholinergic treatments in asthma. 

In a subsequent study by El-Bermani et al. (1991), it was found that the M2 receptor subtype was also involved in the hyperreactive state of the airways in allergic asthma. The authors demonstrated that the M2 receptor was downregulated in the airways of allergic asthmatic patients, which was associated with an increase in M3 receptor expression. These changes were reversed by treatment with the M2 receptor agonist carbachol, indicating a potential therapeutic role for this receptor subtype. 

Other studies have shown that the M2 receptor is also involved in the regulation of airway tone in non-asthmatic individuals. For example, a study by El-Bermani et al. (1992) demonstrated that the administration of a selective M2 receptor agonist, tiotidine, caused a decrease in bronchial smooth muscle tone in healthy volunteers. This effect was blocked by atropine, indicating that the response was mediated through the M2 receptor subtype. 

In conclusion, the M2 muscarinic receptor plays a significant role in the regulation of airway tone in both asthmatic and non-asthmatic individuals. Further studies are needed to fully delineate the role of this receptor in the pathophysiology of airway hyperreactivity and to determine the potential therapeutic implications of M2 receptor agonists and antagonists.
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