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_3 muscarinic receptors on the smooth muscle causing contraction.[1] Acetylcholine also feeds back onto neuronal M_2 muscarinic receptors located on the postganglionic cholinergic nerves. Stimulation of these receptors further limits acetylcholine release, so these M_2 muscarinic receptors act as autoreceptors.[2 3] Loss of function of these M_2 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_2 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.[4] These parasympathetic ganglia are interspersed irregularly along the posterior aspect of the wall of the trachea and major bronchi.[5] From these ganglia short postganglionic nerves pass forward to innervate the airway smooth muscle, the bronchial circulation, and the glandular acini.[6–9] Histological studies have not shown postganglionic efferent fibres beyond the level of the terminal bronchi,[4] and functional studies have not found an effect of vagal stimulation on the respiratory bronchioles and the alveoli.[10 11] 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_2 receptors are blocked by AF-DX116 and gallamine, M_3 receptors are blocked by 4-DAMP, while M_4 receptors are antagonised by hombocine; it has been difficult to identify a selective agonist at M_5 receptors. Autoradiographic studies have demonstrated muscarinic M_1 and M_2 receptors along nerve bundles and within the cholinergic ganglia.[12 13] Primary cultures of postganglionic cholinergic neurons from the trachea have shown these nerves to possess messenger RNA for only the M_2 receptors.[14] Airway smooth muscle cells express M_2 and M_3 muscarinic receptors; the latter mediate smooth muscle contraction.[15 16]

Physiological function of cholinergic nerves in the airways

Stimulation of the parasympathetic nerves releases acetylcholine which causes the airway smooth muscle to contract,[15–19] the glandular tissue to secrete mucus,[20 21] and the bronchial circulation to dilate.[22 23] 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.[17 24–26] 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.[27] Furthermore, directly inhibiting the vagus nerves—for example, by cutting them—causes bronchodilation.[28] 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.[22 29 30] 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.[26] 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 (fig 1). Thus, these receptors act as autoreceptors.[2 3 31]

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 (fig 2A).[32] Conversely, the muscarinic agonist pilocarpine stimulates neuronal M_2 muscarinic receptors and so decreases vagally induced bronchoconstriction. Pilocarpine 100 μg/kg reduces vagally

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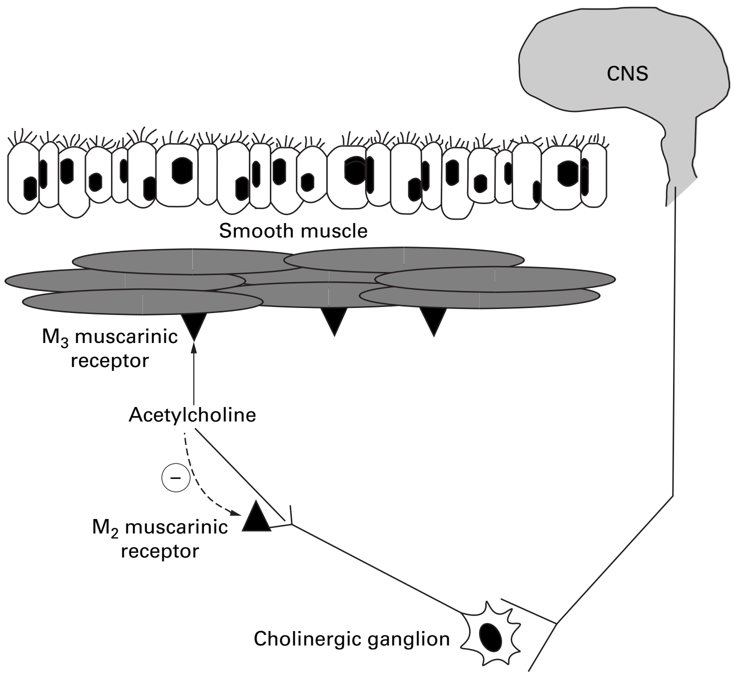

Figure 1 The pulmonary neuronal M_2 muscarinic receptor. Acetylcholine released from pulmonary vagal nerves stimulates muscarinic M_3 receptors on airway smooth muscle causing smooth muscle contraction; at the same time acetylcholine stimulates M_2 muscarinic receptors located on the postganglionic nerves. Stimulation of these neuronal M_2 muscarinic receptors limits further acetylcholine release.

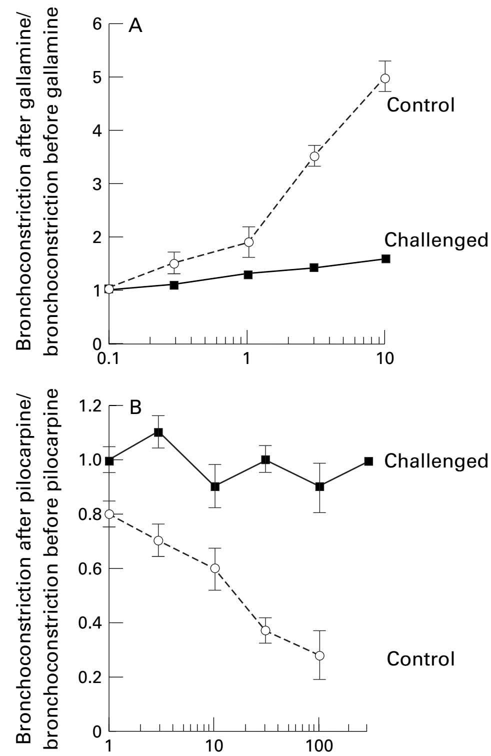

Figure 2 The neuronal M_2 muscarinic receptor does not function in antigen sensitised guinea pigs after challenge. Results are expressed as a ratio of the response to vagal stimulation after the antagonist gallamine (A) or the selective agonist pilocarpine (B) to the response before the compound. In control animals gallamine potentiates vagally induced bronchoconstriction and pilocarpine inhibits vagally induced bronchoconstriction. In contrast, in antigen sensitised animals after challenge gallamine does not potentiate and pilocarpine does not inhibit vagally induced bronchoconstriction, indicating loss of M_2 receptor function. Adapted from Fryer and Wills-Karp.[38]

mediated bronchoconstriction by about 75% (fig 2B). The presence of an M_2 autoreceptor has also been confirmed by measuring changes in induced acetylcholine release using high performance liquid chromatography in the presence of selective M_2 receptor antagonists.[33] Although first described in the airways of guinea pigs, M_2 receptors have been described in the airways of all species studied, including humans.[33–35]

Loss of function of neuronal muscarinic M_2 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.[36 37]

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 M_2 muscarinic receptor is impaired. These alternative explanations were tested in an animal model of hyperreactivity where the function of the neuronal M_2 muscarinic receptor and the smooth muscle response to acetylcholine were compared between control and antigen sensitised guinea pigs.[38] The bronchoconstriction induced by acetylcholine was the same in vagotomised control and antigen challenged animals, indicating that the muscarinic M_3 receptor on airway smooth muscle was functioning normally.[38] 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 M_2 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.[39–42] Increased concentrations of acetylcholine have been reported in the airways of other antigen challenged animals including mice,[43] dogs,[44] and guinea pigs,[43] providing indirect supportive evidence that there is loss of function of neuronal M_2 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.[45 46] 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 M_2 receptor, the role of eosinophils in the loss of function of M_2 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 M_2 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 M_2 muscarinic receptors.[49] Thus, eosinophils are responsible for loss of pulmonary neuronal M_2 muscarinic receptor function in the airways of antigen challenged guinea pigs.

A potential mechanism for this eosinophil dependent loss of M_2 receptor function in antigen challenged animals was suggested by the finding that some eosinophil products are antagonists at M_2 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 M_2 muscarinic receptors such as gallamine and protamine[52] are positively charged. The cationic nature of these antagonists is important in binding to M_2 muscarinic receptors, possibly because the receptor is heavily sialated giving it a net negative charge. In receptor binding studies on M_2 and M_3 receptors MBP and, to a lesser extent, EPO displaced the agonist [3H]*N*-methylscopolamine ([3H]NMS) from guinea pig and human M_2 but not M_3 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 M_2 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 M_2 receptors was allosteric rather than competitive. Eosinophil MBP may have a physiologically relevant role in the loss of M_2 receptor function since the dissociation constant for MBP at M_2 receptors is 1.4×10^{-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 M_2 receptors and so does not appear to be physiologically relevant as an antagonist at M_2 receptors.

As binding studies have shown that heparin displaces MBP from M_2 muscarinic receptors, and because eosinophil MBP may be responsible for the loss of function of the neuronal M_2 muscarinic receptor, the effect of heparin on M_2 receptor function was tested in antigen challenged animals. In these studies it was shown that the administration of heparin acutely restored M_2 receptor function in antigen challenged guinea pigs and rats[40] (Belmonte *et al*, unpublished). These findings suggest that M_2 receptors become dysfunctional after antigen challenge and that positively charged proteins such as MBP are acting as endogenous antagonists at M_2 receptors.

In order to establish a role for eosinophil MBP in the loss of M_2 receptor function, in vivo studies were performed with a specific neutralising antibody to eosinophil MBP.[56] In these studies antigen sensitised guinea pigs were pretreated with an antibody to MBP before challenge and the function of the M_2 receptor as well as vagally mediated hyperreactivity were tested 24 hours after challenge. In sensitised animals studied after antigen challenge there was loss of function of M_2 receptors and an increase in vagally mediated bronchoconstriction. In contrast, in antigen sensitised animals pretreated with the antibody to MBP the function of M_2 receptors was preserved and hyperreactivity inhibited (fig 3). Thus, it is likely that eosinophil products, in particular MBP, are responsible for the loss of M_2 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 M_2 muscarinic receptors.[42] Thus, there is good evidence to indicate that eosinophil MBP is involved in the loss of function of neuronal M_2 muscarinic receptors in antigen challenged animals.

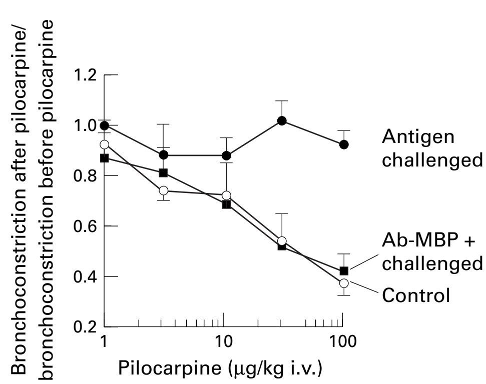

Figure 3 An antibody to eosinophil MBP protects neuronal M_2 muscarinic receptor function in antigen challenged guinea pigs. Results are expressed as a ratio of the response to vagal stimulation after pilocarpine to the response before pilocarpine. In control animals (open circles) pilocarpine inhibited vagally induced bronchoconstriction but in antigen challenged guinea pigs (closed circles) it did not. Pretreatment with an antibody to MBP before antigen challenge protected the response to pilocarpine. Adapted from Evans et al.[56]

Loss of function of neuronal M_2 muscarinic receptors after exposure to a respiratory viral infection

Infection with a respiratory virus causes an increase in vagally mediated hyperreactivity.[57 58] Studies in guinea pigs and in rats infected with parainfluenza virus indicate that there is loss of function of the neuronal M_2 muscarinic receptor.[59 60] The central binding site of the M_2 muscarinic receptor is composed of negatively charged sialated glycoproteins.[61] Respiratory viruses, in particular parainfluenza virus, contain the enzyme neuraminidase which cleaves sialic acid residues.[62 63] In receptor binding studies it has been shown that the affinity of M_2 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.[64] These data suggest that the viral enzyme neuraminidase may be involved in the loss of function of neuronal M_2 muscarinic receptors. Inflammatory cells also appear to play a part in the loss of function of the neuronal M_2 receptor.[65] Since heparin does not restore the function of the M_2 receptor in non-sensitised virally infected animals, the inflammatory cell causing this impaired neuronal M_2 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.[66] In antigen sensitised guinea pigs viral infections cause pulmonary eosinophilia and heparin restores the function of M_2 receptors, which suggests that the M_2 receptor dysfunction may also be eosinophil mediated (Fryer *et al*, unpublished).

Loss of function of neuronal M_2 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 M_2 muscarinic receptor after exposure to ozone that is long lasting.[67 68] The mechanisms responsible for the loss of function of the neuronal M_2 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.[69] 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 M_2 receptor function (Fryer *et al*, unpublished observation).

Neuronal M_2 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,[70 71] or the investigators have not adequately inhibited vagal nerve function.[72–74] One particular problem with the currently available anticholinergic agents is their non-selective nature; they antagonise both M_3 and M_2 receptors. Antagonism of the M_2 receptor by these agents will potentiate vagally induced bronchoconstriction and thus counteract the effect of inhibiting the M_3 receptor on the smooth muscle.[75]

The presence of neuronal M_2 muscarinic receptors has also been described in humans. In these in vivo studies vagally induced bronchoconstriction is induced indirectly via a

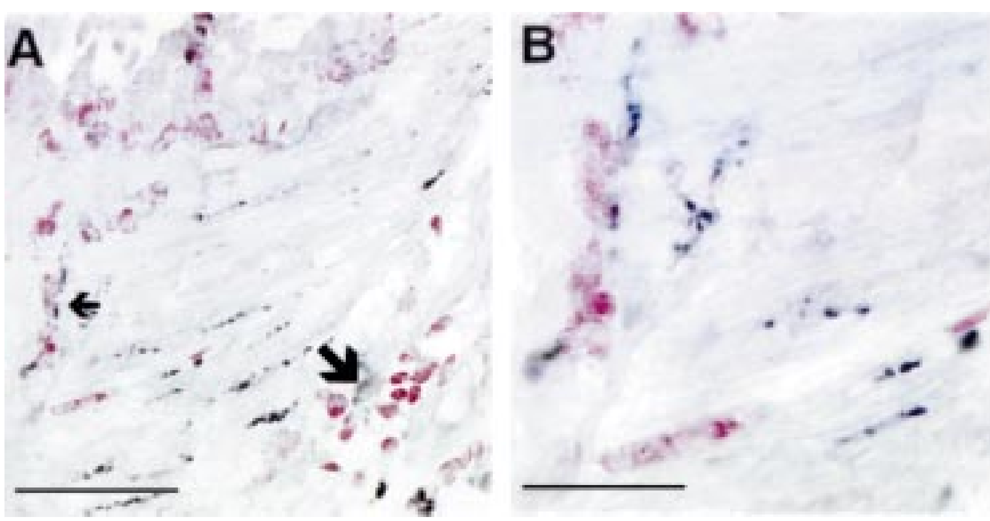

Figure 4 Eosinophils are found in association with nerve fibres in the airways of antigen challenged guinea pigs. The photomicrograph is from a paraffin embedded section of antigen challenged guinea pig bronchus. Eosinophils (detected with chromotrope 2R) are seen in close proximity to airway nerve fibres 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.[56]

vagal reflex with an agent such as sulphur dioxide or histamine.[35 76] In the presence of a normal M_2 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 M_2 receptor.[33]

Most studies on the function of the neuronal M_2 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 which could be used for in vitro studies is rarely resected from patients with asthma. However, there is evidence that there is loss of function of neuronal M_2 receptors in some patients with asthma.[35 76 77] In these in vivo studies the subjects had stable allergic asthma and in all but one of these studies inhaled pilocarpine had no effect on vagally induced bronchoconstriction, indicating impaired function of these receptors. The reasons for the differences in the results of these studies may reflect the techniques used to induce the vagal reflex bronchoconstriction or, alternatively, the severity of asthma. The function of the neuronal M_2 muscarinic receptor has been recently tested in people with very mild asthma with a history of wheeze during a viral infection. In this preliminary study there was normal function of the M_2 receptor at baseline and a transient loss of function of the M_2 receptor during a viral respiratory infection (Costello *et al*, unpublished observation).

The underlying mechanism of loss of function of M_2 receptors has been investigated. One recent study has shown that inhaled heparin prevented late phase hyperreactivity to allergen in sensitive asthmatic subjects. The late phase response is characterised by an inflammatory cell influx, in particular of eosinophils. It is to tempting to speculate that this effect of heparin was due to an effect on M_2 receptor function analogous to that seen in antigen challenged guinea pigs, although further studies will be required to test this hypothesis.[78]

Summary

In the lungs neuronal M_2 muscarinic receptors limit acetylcholine release from postganglionic cholinergic nerves. These inhibitory M_2 receptors are dysfunctional in antigen challenged guinea pigs and in humans with asthma which leads to an increase in vagally mediated hyperreactivity. In vitro, eosinophil products act as allosteric antagonists at neuronal M_2 muscarinic receptors. In vivo, displacing or neutralising MBP preserves neuronal M_2 muscarinic receptor function and prevents hyperreactivity. Thus, there is good evidence from animal studies that after antigen challenge pulmonary M_2 muscarinic receptors become dysfunctional because MBP inhibits their function. Loss of function of pulmonary neuronal M_2 muscarinic receptors has also been reported in patients with asthma, although the clinical significance of this dysfunction and the mechanisms underlying it are not yet established.

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