International Journal of Occupational Medicine and Environmental Health, 2004; 17(4): 417—431

VAGUS NERVE PARTICIPATES IN REGULATION OF THE AIRWAYS: INFLAMMATORY RESPONSE AND HYPERREACTIVITY INDUCED BY OCCUPATIONAL ASTHMOGENS

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Abstract. An initial recognition of occupational asthmogens present in dust, fume or aerosol particles is carried out by a specialized subset of immune cells, dendritic cells and macrophages, present in the airway tissues. When activated by asthmogens these cells release proinflammatory molecular signals and not only send them to other cells of the innate immunological system, but also activate sensory pathways that relay information to the central nervous system (CNS). The precise mechanisms by which the peripheral immune system can signal to the CNS the airway injury has been the subject of much debate. Recently, a new pathway of the CNS-mediated regulation of the peripheral immune response has been found. The efferent vagus nerve was proposed as an immune-to-brain pathway and it was suggested that acetycholine, the principal vagal neurotransmitter, may directly modulate the airway immune response to pathogenic invasion or to injury by irritant asthmogens. Sensory innervation of the airways by ascending fibres traveling in the vagus nerve as well as by pain sensory pathways, provides an important input about the status of injurious challenges in the inflammation zone of the airway compartments. These neural inflammation-sensing pathways can function at low thresholds of detection and can activate responses even when inflammatory agents are present in the airway tissues in quantities that are not high enough to reach the brain through the bloodstream. The cholinergic vagus nerves participate not only in the regulation of the airways inflammatory response. The airways function in response to spastic stimuli such as irritants, allergens, and inflammatory mediators is also controlled, in a larger part, by efferent vagal endings present in the airway smooth muscles. Cholinergic mechanisms represent the predominat constrictor neural pathway in human airways. Differences in expression of muscarinic acetylcholine receptors in asthma suggest that cholinergic system may participate in the molecular framework influencing the airway functions in occupational asthma.

Key words:
Airway, Vagus nerve, Muscarinic acetylcholine receptors, Inflammation, Occupational asthmogens

INTRODUCTION

Occupational asthma is a disease in which exposure to chemical agents with antigenic and irritant properties (occupational asthmogens) play an important role. Many high and low molecular weight occupational asthmogens that penetrate through the airways as organic or inorganic dusts, fumes, vapors and aerosols could act as an antigen (high molecular weight) or hapten (low molecular weight) to provide signal to be recognized by specific T cells [1]. Low molecular weight asthmogens and probably the majority of high molecular weight asthmogens also exhibit dose-dependent toxicity and exert direct or indirect irritant

Received: September 3, 2004. Accepted: October 25, 2004.
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effects on the airway cells and may induce inflammatory response [2–4]. A "danger" model, proposed by Matzinger [5] may be a very interesting alternative to study chemically induced occupational asthma. An antigenic signal sent by asthmogen on its own would tend to produce tolerance. In allergic occupational asthma, the presence of antigenic and "danger" (irritant) signals, usually pronounced by irritation of the airway epithelial cells and macrophages, may activate the immune system. Whether an immune or tolerant response occurs depends upon a secondary, irritant signal being delivered to the airway dendritic cells in combination with an antigenic signal [6]. In most cases, antigenic and irritant signals come from the asthmogen, although in an occupational setting, traumatic injury to airway epithelias, and inflammatory response induced by other chemicals, would be the source of an irritant signal. The damage done by irritants helps the development of occupational asthma.

An initial recognition of chemicals present in dust, fume or aerosol particles is carried out by a specialized subset of immune cells present in the airway tissues. The most important of these cells are the airway macrophages and dendritic cells. They express receptors for a wide variety of dust particle-associated chemicals (also asthmogens) and are capable of internalizing such chemicals. When activated by dust, fume or aerosol particle-associated chemicals, dendritic cells or macrophages release proinflammatory molecular signals and not only send them to other cells of the innate immunological system but also activate sensory pathways that relay information to the central nervous system (CNS) [7,8]. The precise mechanisms by which the peripheral immune system can signal the brain has been the subject of much debate [9]. The possibilities include: 1) the direct entry of proinflammatory cytokines into the brain across the blood-brain barrier by a saturable transport mechanism; 2) the interaction of proinflammatory cytokines with circumventricular organs such as the organum vasculosum of the lamina terminalis and area postrema, which lack the blood-brain barrier; and 3) the activation of afferent neurons of the vagus nerve. On the other hand, central proinflammatory cytokines induce activation of both the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis. These sympathetic system and HPA axis are known to be the major mechanisms involved in cross-talk between the brain and immune system [10,11]. Recently, a new pathway of the brain-mediated regualtion of the peripheral immune response has been found [12,13]. The efferent vagus nerve was proposed as an immune-to-brain pathway and it was suggested that acetycholine, the principal vagal neurotransmitter, may directly modulate the airway immune response to pathogenic invasion or to injury by irritant chemicals.

Sensory innervation of the airways by ascending fibres traveling in the vagus nerve and by pain sensory pathways provides an important input about the status of injurious challenges in the inflammation zone of the airway compartments [14]. The neural inflammation-sensing pathways can function at low thresholds of detection and can activate responses even when inflammatory agents are present in the airway tissues in quantities that are not high enough to reach the brain through the bloodstream [15]. Of peripheral nerves that have been investigated so far, only the vagus is recognized as an immunosensory nerve [8]. The cholinergic vagus nerves participate not only in regulation of the airways inflammatory response. The airways function in response to spastic stimuli such as irritants, allergens and inflammatory mediators is also controlled, in a larger part, by efferent vagal endings present in the airway smooth muscles. Cholinergic mechanisms represent the predominat constrictor neural pathway in human airways. The airways hyperresponsiveness is an essential part of the definition of asthma. Differences in the expression of muscarinic receptors (differences in methacholine test) in asthma suggest that cholinergic system may participate in the molecular framework, influencing the airway functions in asthma [16].

AIRWAY VAGUS NERVES AND THEIR FUNCTION

Information accumulated in recent years has begun to unveil a previously unsuspected complexity in the innervation of the lungs [17]. The conducting airways and lung parenchyma receive the preponderance of their innervation from the vagus nerves (Fig. 1). Each nerve supplies affer-

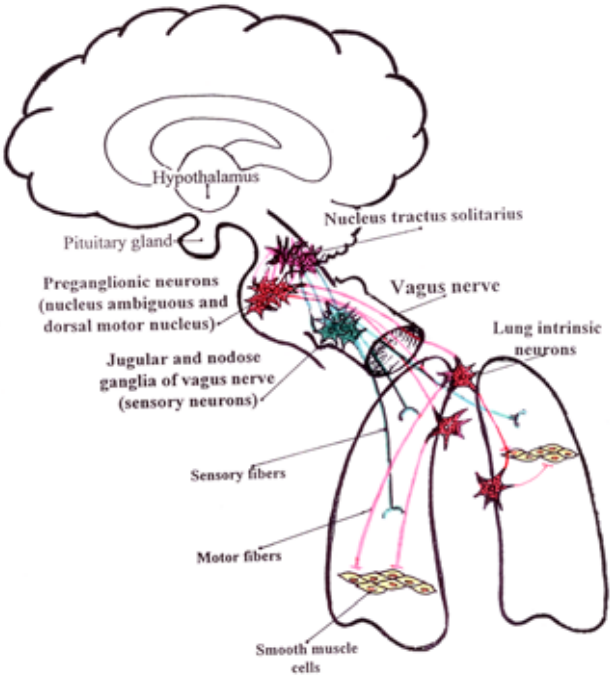

Fig. 1. The motor and sensory innervation of the lung by the vagus nerves. Axons from preganglionic parasympathetic neurons located in nucleus ambiguous and dorsal motor nucleus of the vagus in the medulla travel in the vagus nerve to lung walls, where they are thought to synapse obligatorily with lung intrinsic neurons or ganglia. Axons from lung intrinsic neurons innervate lung smooth muscles. Neuronal bodies for sensory fibers are located in jugular and nodose ganglia of the vagus nerve. Sensory neurons establish synapses with neurons located in nucleus tractus solitarius, a sensory integration area located in the dorsal medula.

ent and efferent fibres to both lungs through multiple rami which exit the nerve trunk directly or with the recurrent laryngeal nerve. Airway motor fibers derive primarily from the nucleus ambiguous and, in smaller numbers, from the dorsal motor nucleus of the vagus. Actually we know that lung-bound motor vagal fibers do not undergo decussation in the brain stem [17]. One single neuron can innervate airways on both sides of the midline. Fontain et al. [18] denoted the unsuspected presence of direct interconnections between the bronchomotor vagal centers. These connections may serve a coordinating function between the two sides of the brain, assuring symmetrical cholinergic outflow. The vagal bronchomotor neurons have a viscerotopic organization by which the cholinergic outflow carried by a given neuron varies, depending on the segmental location of its innervation target in the bronchial tree [18]. The resistance of the peripheral airways is more increased in response to physiological bronchoconstrictive stimuli and more decreased after vagotomy than the resistance of the central airways [19].

The airway preganglionic neurons receive inputs from a complex brain stem cellular network, which integrates and processes afferent information from chemoreceptors, mechanoreceptors and C-fibers, and uses parasympathetic preganglionic nerve fibers carried by the vagus as the main, but not exclusive, efferent pathway [20,21]. Axons from preganglionic parasympathetic neurons are located in nucleus ambiguous and dorsal motor nucleus of the vagus in the medulla travel from the vagus nerve to the airway walls or in their immediate vicinity, where they are thought to synapse obligatorily with airway intrinsic neurons or ganglia. The axons from the airway intrinsic neurons travel a short distance to network with other intrinsic neurons or to innervate local targets such as the airway smooth muscle, vascular smooth muscle, and mucus glands [17].

A substantial proportion of intrinsic neurons lack cholinergic markers and are therefore unlikely to serve as mere way stations for parasympathetic signals [17]. Many contain neuropeptides like substance P, calcitonin gene-related peptide, and vasoactive intestinal peptide in variable patterns of expression, suggesting the existence of differentiated neuronal populations with specific functions [14]. Vagal sensory fibers serve multiple receptor functions and may be myelinated or unmyelinated. Their neuronal bodies are loacted in the jugular and nodose ganglia of the vagus nerve, where they are segregated anatomically by their sensory phenotype. Only a small contingent of fibers originate from thoracic dorsal root ganglia [22]. Sensory neurons (airway and pulmonary) establish synapses with neurons predominantly located in the nucleus of tractus solitarius, a sensory integration area located in the dorsal medulla. There, they synapse with interneurons that relay their inputs to the inspiratory and bronchomotor medullary networks, thereby closing a multineuronal reflex loop, which is initiated by stimulation of airway mechanical or nociceptive receptors and completed by enhancement or inhibition (depending on the stimulus) of cholinergic outflow to the airway smooth muscle, blood vessels and mucous glands [17,23–25].

Noxious stimuli, however, need not travel to the medulla to evoke a defensive response in the airways. C-fibers, a class of unmyelinated sensory neurons, have been known for quite some time to contain proinflammatory neuropeptides, including members of the tachykinin family, primarily substance P and other peptides encoded by the preprotachykinin A gene [26]. These fibers are the main vehicle of a local reflex loop, whereby irritation of sensory terminals elicits neuropeptide release, either locally or via antidromic stimulation, at other points in the distribution territory of the C-fiber. Because C-fiber terminals and tachykinin receptors are both represented in the walls of blood vessels, airway smooth muscle cells, airway epithelium, and airway ganglia, noxious stimuli can cause hyperemia, edema, bronchoconstriction, and increased mucus secretion without ever depolarizing the body of the C-fiber. Moreover, because intrinsic airway neurons receive innervation from local C-fibers and undergo partial depolarization in the presence of substance P, activation of local sensory nerves can also enhance the responses initiated via longer medullary reflexes [27].

IRRITANT CHEMICALS MAY INDUCE NEUROGENIC INFLAMMATORY RESPONSE

The airway tissues damaged by irritant chemicals (also occupational asthmogens) unleash up to several types of go-signals. One of them appears in response to intracellular substances released by damaged cells present in the inflammatory zone. Neurons and other cells (e.g., macrophages. eosinophils) release bioactive compoumds, cytokines, bioactive peptides, enzymes and prostaglandins. These compounds may activate the airway mast cells that release histamine, eikosanoids, pre-formed tumor necrosis factor-α (TNF-α), newly synthesized cytokines, tryptase and other proteases and chemokines that attract inflammatory leukocytes [27,28]. There is a close interaction between the airway nerves and chemicaly induced inflammation. Many mediators that are released in the inflammatory zone may modulate sensory and cholinergic nerves in the airways through the activation of receptors on nerve terminals [29]. However, sensory nerves in turn may also amplify inflammation in the airways through the release of peptide neurotransmitters. This neurogenic inflammation has been documented in the upper and lower respiratory tract in several species [30]. The idea that sensory nerves may amplify and spread the inflammatory response has attracted considerable attention as it may contribute to the inflammation in the airway disease such as asthma.

The presence of substance P in sensory fibers is the cornerstone of our current understanding of neurogenic inflammation. Substance P is a member of the family of peptides sharing the carboxy-terminal sequence Phe – X – Gly – Leu – Met – amide, collectively termed “tachykinins” for their ability to produce fast smooth muscle contraction. Substance P can be synthesized from three alternative spliced forms (α, β, γ) of the pretachykinin-A (PPT-A) gene. The β and γ splice variants also contain the coding sequence for neurokinin A. Neurokinin B is formed from a separate gene [31]. Both pre-protachykinin A mRNAs and mature peptides have been detected in the nodose, jugular and dorsal root ganglia, where they can undergo up-regulation by stimuli generated during airway inflammation. In the past few years, however, pre-protachykinin A gene products have also been identified in non-sensory cells in the airways and lungs [32]. Lung ganglia, for instance, contain substance P immunoreactivity in a number of species, including humans. It is also possible that inflammatory cells use preprotachykinin A gene-encoded neurokinins as a paracrine or autocrine signaling mechanism to propagate inflammation beyond the limited topographic spread of C-fibers and intrinsic neurons [33].

The presence or absence of tachykinins and other neuropeptides appears to be an important element in the differentiation of the airway cells, not only from a functional point of view, but also anatomically. At least in the trachea, the majority of neuronal somata that contain substance P and vasoactive intestinal peptide are located in the superficial muscular plexus, where choline acetyltransferase (ChAT)-immunoreactive neurons are rare. Conversely, ChAT immunoreactive neurons are abundant in the longitudinal nerve plexus, where few peptidergic neurons are found [17]. These findings have important implications. Neurons can serve as a networked tachykinin

reservoir available via direct stimulation by cytokines or though neural inputs from other neurons, or via the local sensory reflex, by sensory fibers. In addition, the absence of a characteristic cholinergic phenotype from a large proportion of the airway neurons suggests that these neurons do not function as a way station for the cholinergic outflow of the airway medullary premotor neurons. A notion that these neurons receive inputs from the CNS or from other neurons is in itself questionable. Evidence also exists that non-neuronal cell lines can express pre-protachykinin A gene products. Macrophages, lymphocytes, and eosinophils have been reported to contain pre-protachykinin A mRNAs substance P immunoreactivity [26]. Because many of these cells also display neurokinin-1 receptors (NK-1Rs) in their membranes, their ability to produce substance P offers an autocrine alternative to the neuronal mechanism of amplification of inflammation by tachykinins.

Experiments utilizing residual oil fly ash [34] have demonstrated that afferent neural fibers play a crucial role in mediating a variety of inflammatory mechanisms following airborne pollutant exposure. Additional studies have indicated that these nerves are sensitive to many air polluttants such as O_3 [35], NO_2 [36], SO_2 [37] and cigarette smoke [35]. It seems reasonable to speculate that exposure to airborne chemicals present in organic and/or inorganic dust may result in neurogenic inflammation through bronchopulmonary C-fiber afferents. Wong et al. [27] hypothesized that inhaled chemicals present in dust, fume, smoke, and areosol forms induce bronchopulmonary neurogenic inflammation that is mediated by tachykinins released from sensory C-fiber endings, which act via NK-1R. According to Lu-Yauan and Widdicombe [35], bronchopulmonary C-fiber endings and rapidly adapting pulmonary receptors (RARs) are primarily responsible for eliciting the defense reflexes in protecting the lungs against inhaled irritants. In animals, inhalation of cigarette smoke into the lungs elicits pulmonary chemoreflexes that are mediated through the stimulation of pulmonary C-fibers. When the C-fiber conduction is selectively blocked in the vagus nerve, the same smoke inhalation triggered only augmented breath, a reflex effect of activating RARs, in the same animals. An electrophysiologic study shows that inhaled smoke exerts a direct stimulatory effect on both C-fiber endings and RARs. The excitability of C-fiber endings and RARs, and thus their reflex actions are enhanced by airway mucosal inflammation, like in the airway hyperresponsiveness induced by acute exposure to ozone. Although the mechanism underlying the inflammation-induced hypersensitivity of C-fiber endings is not fully understood, a possible involvement of local release of certain inflammatory mediators, such as histamine and prostaglandin E_2 (PGE_2), should be considered. It is likely that changes in the membrane properties mediated by the activation of certain specific receptor proteins located on the membrane of these nerve terminals are involved, since the sensitizing effects of PGE_2 can be also demonstrated in cultured pulmonary C neurons [38].

A large proportion of axons from the airway intrinsic neurons, which contain proinflammatory neuropeptides, also express proteinase-activated receptor 2 (PAR2) [39]. Certain proteinases released from the airway cells injured by irritant chemicals signal molecules that they are cleaving and activating PARs. Proteinases cleave PARs within the extracellular N-terminal domains to expose tethered ligands that bind to and activate the cleaved receptors. Activated PARs stimulate the release of tachykinins from nerve fibers of afferent neurons. It is interesting that mast cells containing proteinase-tryptase which activate PARs, are in close proximity to afferent fibers containing tachykinins. Such properties has also trypsin present in the airway epithelial cells as trypsinogen. Trypsin and tryptase stimulate the release of tachykinins from the peripheral endings of afferent neurons by a local, Ca^{2+}-dependent mechanism. Thus, tryptase released from degranulated mast cells and trypsin released from injured epithelial cells and PARs may play a central role in neurogenic inflammation that is induced by irritant chemicals [40].

Neurogenic inflammation and peptides released from sensory nerves might be important as an amplifying mechanism in asthmatic inflammation. However, in humans this idea has little direct, supportive evidence. While we possess convincing data that neuropeptides released from sensory nerves contribute to airway inflammation in rodents and some other species [22,27], there is relatively

little information that neurogenic inflammation is important in human asthma, and also in occupational asthma [14,41–43]. Sensory neuropeptides are not prominent in human airways, and initial studies showing an apparent increase in substance P-immunoreactive nerves in asthmatic airways have not been confirmed. However, substance P and neurokinin A are released in asthmatic airways and may have some effect, particularly if their metabolism is impaired through defective expression or function of the neutral endopeptidase, which is a key enzyme in the degradation of tachykinins in airways, or if there is an increased expression or sensitivity of tachykinin receptors [40]. Subsequent to its release from afferent nerve endings, substance P increases substantial responses, such as an increase in microvascular permeability, promotion of plasma extravasation, and priming of other inflammatory mediators. These effects are mostly modulated by neutral endopeptidase through degradative cleavage of substance P. Many of the asthmogenic agents that exacerbate asthma appear to reduce the activity of neutral endopeptidase at the airway surface, leading to exaggerated responses to tachykinins (and other peptides) and thus to the increased airway inflammation [14].

So far the results of clinical studies with potent tachykinin antagonists have shown little effect in challenge studies. However, it is possible that sensory neuropeptides may only be involved in more severe asthma or in its exacerbation. There is little doubt that afferent nerves are sensitised in asthma, resulting in symptoms of cough and chest tightness, but this does not necessarily result in neurogenic inflammation [14].

NEURAL INHIBITION OF THE LUNG INFLAMMATION

The nervous system not only participates in the induction of lung inflammation by chemical agents, but also reflexively monitors the inflammatory response. Recent insights have identified a neural pathway mediated by the vagus nerve, termed the "cholinergic anti-inflammatory pathway", that may act together with immunological regulatory mechanisms [13,44]. But first, the immune system must alert the CNS to the presence of an inflammation. It is not completely clear how the vagus nerve "detects" the presence of low doses of inflammatory agents. It was suggested [9] that inflammatory products, e.g., TNF-α and IL-1, released in the inflammatory zone, may activate afferent signals that are relayed to synapse in the nucleus tractus solitarius. Subsequent activation of the vagus nerve efferent activity and increased efferent signals in the vagus nerve innervate the airway tissues and suppress proinflammatory cytokines release through immune cells present in the inflammatory zone. Such "inflammatory reflex" may be described as rapid, discrete, and localized to injured lung tissue. Probably, molecular dovetail between the cholinergic nervous system and the innate immune response system is a macrophage nicotinic acetylcholine receptor (N AChR) [45]. Interaction between the macrophage N AChR and acetylcholine, released from vagus nerve endings, can specifically inhibit macrophage activation and decreases the synthesis of proinflammatory cytokines, TNF-α, IL-1α and IL-18 but not anti-inflammatory cytokines such as IL-10 [13].

It may be possible to activate neural anti-inflammatory mechanisms using small molecules, e.g., a tetravalent guanylhydrazone (CNI-1493), that initiate signals in proximal components of the inflammatory reflex in the CNS. CNI-1493 was originally described as an inhibitor of macrophage activation and TNF-α release in macrophage cell cultures [46]. Recent evidence has shown that the TNF-α-supressing activities of CNI-1493 *in vivo* are dependent on the cholinergic anti-inflammatory pathway and this low molecular weight chemical functions as a stimulator of the vagus nerve. Intracerebral application of small doses of CNI-1493 significantly inhibited peripheral TNF-α synthesis, and intact vagus nerves were required to prevent increases in serum TNF-α [47]. The mechanism by which CNI-1693 activates the vagus nerve is unknown, but increased vagus nerve firing has been observed after either intracerebral or intravenous administartion of CNI-1493. Direct delivery of CNI-1493 into the cerebral ventricles was significantly (> 100 000-fold) more potent than the effective intravenous dose. It may be hypothesized that CNI-1493 might inhibit systemic TNF-α through activa-

tion of efferent neural signals. It is interesting that electrical stimulation of the intact vagus nerve in experimental animals receiving implantable vagus nerve stimulators significantly attenuated TNF-α serum levels [48]. Hypnosis, meditation, and acupuncture can substantially increase the vagus nerve output and may be used to reduce inflammatory responses [13].

The cholinergic anti-inflammatory pathway can also induce systemic humoral anti-inflammatory responses because, vagus nerve efferent activity can be relayed to the medullary thereticular formation, locus cereleus and hypothalamus, leading to increased release of ACTH from anterior pituitary, and stimulated release of adrenal glucocorticoids and epinephrine [13]. Glucocorticoids are the main effector end-point of this neuroendocrine system (hypothalamic-pituitary-adrenal axis) and, through the glucocorticoid receptor, have multiple effects on immune cells [11]. The diffusible anti-inflammatory network, which includes adrenal glucocorticoids and epinephrine, is rather slow, distributed, non-integrated, dependent on concentration gradients, and important in the late inflammation stage [10,49].

MUSCARINIC ACETYLCHOLINE RECEPTORS AND AIRWAY HYPERRESPONSIVENESS IN ASTHMA

Cholinergic vagus nerves participate not only in regulation of lung anti-inflammatory response. The lung function in response to bronchospastic stimuli such as irritants, allergens and inflammatory mediators is also controlled, in a larger part, by efferent vagal endings present in the lung smooth muscles. Cholinergic mechanisms represent the predominat bronchoconstrictor neural pathway in human airways. Two muscarinic acetylcholine receptor (M AChR) subtypes (M_2 and M_3) are involved in the vagal control of lung bronchomotor responses. Dysfunction of these receptors probably contributes to the development of lung hyperresponsiveness and to bronchomotor responses associated with asthma [50]. Stimulation of the vagus nerve releases acetylcholine onto post-junctional M_3 AChRs, located in lung smooth muscle cells, causing their contraction and bronchoconstriction. At the same

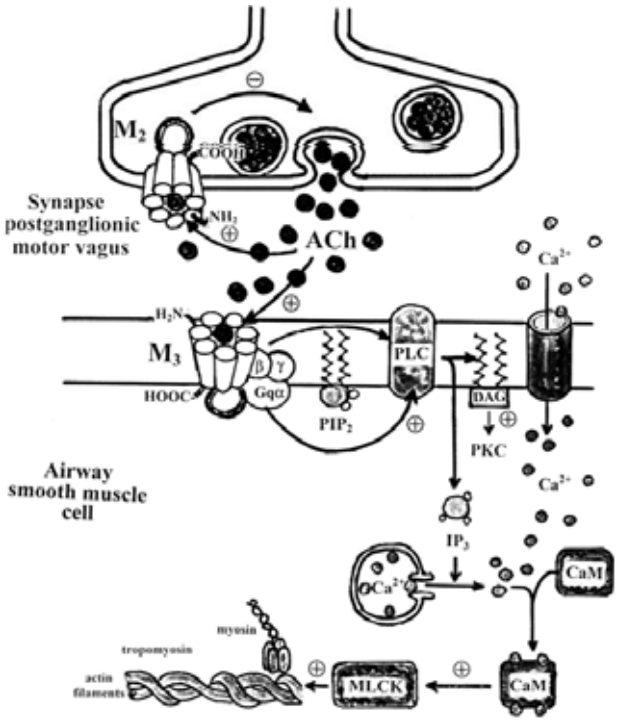

Fig. 2. M_3 muscarinic acetylcholine receptor (mAChR)-mediated signaling in the airway smooth muscle. Acetylcholine (ACh) released from synapse postganglionic motor vagus nerve binds M_3 mAChRs on the airway smooth muscles and initiates a conformational change in the M_3 receptors that promotes their association with and activation of heterotrimeric G proteins Gq. The activated subunits of Gq in turn activate membrane-bound phospholipase C (PLC) that hydrolyzes phosphoinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 promotes Ca^{2+} release from specialized intracellular compartments. Flux from voltage-dependent Ca^{2+} channels also modulates intracellular Ca^{2+} levels. DAG promotes the activation of protein kinase C (PKC) that phosphorylates numerous cellular enzymes. In the airway smooth muscle, PKC-mediated phosphorylation of actin-binding proteins such as calponin facilitates cross-bridge cycling. Increased Ca2+ induces the formation of Ca^{2+}/calmodulin (CaM) complexes capable of activating myosin light chain kinase (MLCK). The subsequent phosphorylation of myosin light chain allows actin activation of myosin ATPase, cross-bridge cycling and generation of force. At the same time, ACh released from synapse feeds back onto prejunctional inhibitory M_2 AChRs on the vagal nerve endings, inhibiting further release of ACh and limiting the airway smooth muscle constriction. Dysfunction of the neuronal M_2 AChRs increases synaptic ACh concentration and potentiates vagally-induced airway smooth muscle constriction.

time, acetylcholine feeds back onto prejunctional inhibitory M_2 AChRs on the vagal nerve endings, inhibiting further release of acetylcholine and limiting bronchoconstriction (Fig. 2). Dysfunction of the neuronal M_2 AChRs increases synaptic acetylcholine concentration and potentiates vagally-induced bronchoconstriction [51,52]. M_2 mAChRs have been shown to be also expressed on airway smooth

muscle cells, but probably do not play a significant role in muscarinic bronchoconstrictor responses *in vivo* [53]. According to Ehlert [54], activation of M_2 receptors modulates contraction by preventing relaxation or potentiating M_3 receptor-mediated contractions.

M_3 AChRs play an important role in the airway function by mediating the effects of acetylcholine on multiple airway cell types. They promote not only an increased airway smooth muscle tension, but in addition these receptors are implicated in the regulation of mucous secretion in submucosal glands and in chemotactic mediator release in alveoloar macrophages. Thus, multiple cellular functions that influence resistance to airflow are under control of M_3 AChRs [55]. Although M_3 AChRs have the capacity to activate multiple signaling pathways in various cell systems, activation of phospholipase C (PLC) via the intermediary heterodimeric G protein Gq is the predominat pathway through which M_3 AChRs regulate important cell functions such as airway smooth muscle contraction. PLC activation induces protein kinase C (PKC) activation and inositol 1,4,5-triphosphate (IP_3) generation, which serve to increase intracellular Ca^{2+}, sensitize and activate the cell's contractile machinery. The increased $[Ca^{2+}]_i$ (intracellular Ca^{2+} level) induces the formation of Ca^{2+}/calmodulin complexes capable of activating myosin light chain kinase. The subsequent phosphorylation of myosin light chain allows actin activation of myosin ATPase, cross-bridge cycling and the generation of force for muscle contraction [56,57].

Airway inflammation induced by biological and chemical irritant agents increases the level of bronchomotor tone (smooth muscle contraction) in asthma, and M AChRs are a potential target that mediates bronchomotor activity in asthma by regulating both baseline responsiveness and inflammatory-mediated contraction of parasympathetic neuromuscular activity in the lung [58]. Bronchial hyperresponsiveness is an essential part of the definition of asthma [16]. Differences in expression of muscarinic receptors (differences in methacholine test) in asthma suggests that cholinergic system may participate in the molecular framework, influencing the lung functions in asthma. Subjects who do not respond to direct bronchoconstriction to methacholine or histamine, and currently have symptoms of coughing or weezing, are highly unlikely to have asthma [59]. In methacholine test, the cholinergic receptor agonist methacholine (Fig. 3) induces airway obstruction in asthmatics with a quantifiable stimulus known to be tolerated by healthy individuals. Methacholine or histamine directly induces bronchial smooth-muscle constriction, whereas exercise, inhalation, or cold dry air, or inhalation of either nebulized distilled water or hypertonic saline (osmotic challenges) appear to provide more indirect mechanisms such as mast-cell mediator release and stimulation of sensory C-fibers in the airways. The direct challenges provide a more sensitive parameter than the indirect stimuli. However, the indirect challenges generally provide higher specificity [60].

The degree of airway hyperresponsiveness correlates with the severity of asthma [61,62]. Furthmore, an understanding of the mechanisms of airway responsiveness is essential to the elucidation of the pathogenesis of asthma. Exposure to environmental stimuli such as allergens, infection with certain viruses or pollutants, e.g., ozone and other factors associated with occupational asthma, is temporally associ-

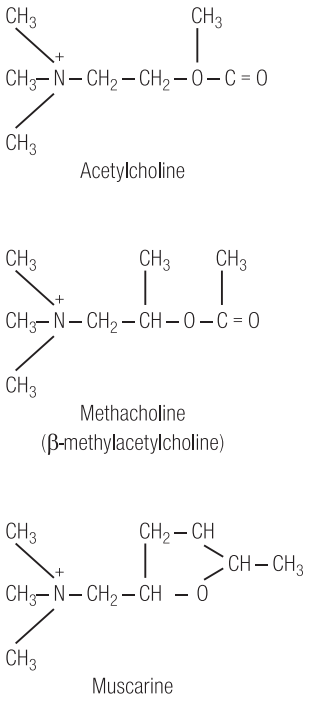

Fig. 3. Chemical structures of acetylcholine, methacholine, and muscarine.

ated with increased airway responsiveness [63]. It has been suggested that a certain degree of mAChRs (M_2 and/or M_3) dysfunction may be present in asthmatics and may be responsible for this airway responsiveness. Evidence for this comes from studies using pilocarpine, an M_2 receptor agonist, which exerts an inhibitory effect on SO_2-mediated bronchoconstriction in healthy individuals, fails to do so in asthmatics [64]. Dysfunctionality of muscarinic receptors has been shown to occur in the presence of inflammatory proteins normally present in asthmatic airways such as major basic protein and eosinophil peroxidase [65,66].

The above mechanism might account for the effects of inflammation on muscarinic receptor functioning, but does not explain the inter-individual variation in responsiveness, which has been reported in the clinical response to anticholinergic therapy [67]. Fenech et al. [68] were unable to identify any polymorphic variation within the M_3 AChR gene coding region or flanking regions. Within the M_2 AChR gene, they identified two degenerate single base substitutions in asthmatics and a common 3'UTR (untranslated region) polymorphism (T→A) was found at base pair (bp) 1696, but this did not alter transcription factor recognition sites. According to these authors, the coding regions for the human muscarinic M_2 and M_3 AChR genes are both highly conserved. They suggest that polymorphic variation within these coding sequences is unlikely to account for inter-individual variability in response to methacholine. Probably this variability is due to an increased density or to more efficient coupling transduction of airway M_2 and/or M_3 AChRs in asthamtics [50].

The cloning of the M_3 AChR gene is significant in that it provides a reductionist tool for determining whether any agent relevant to obstructive airway disease pathogenesis or its management has the capacity to influence M_3 AChR expression in the airway. Some human and experimental studies have suggested that changes in AChRs expression, their density in the airway smooth muscle cells can be dynamically up- or down-regulated by widely-administered therapies, and that such changes in expression may affect the airway contractile state. For example, up-regulation of M_3 AChRs is the mechanism underlying increased bronchial hyperresponsiveness observed in patients with asthma treated chronically with ipratropium bromide [55,69]. Chronic treatment of dogs with glucocorticoids decreases M_2 and M_3 AChRs in airway smooth muscle cells [70]. A convincing series of studies suggests that exaggerated cholinergic discharge of acetylcholine, caused by a viral- or inflammation-driven inhibition of autoinhibitory M_2 AChRs expressed on postganglionic cholinergic nerves, contributes to increased airway resistance in animal models [66]. Prolonged exposure of postganglionic cholinergic nerves M_2 AChRs to acetylcholine, after the vagus nerve stimulation, may lead to attenuation of the receptor responses towards acetylcholine and increase its effect on the M_3 AChRs of the airway smooth muscle.

An important regulatory pathway of muscarinic AChRs is the internalization of receptors into the cell interior [71]. It is interesting that the M_2 AChR internalization leads to receptor down-regulation, while M_3 AChRs are internalized into clathrin-coated vesicles and recycle back to the plasma membrane. Internalization of M_2 AChRs requires dynamin, but proceeds in an apparent β-arrestin-, c-Src- and clathrin-independent manner. M_2 AChRs internalization is required for receptor down-regulation, probably by targeting at the receptors for degradation in the lysosomes. The internalization pathway of M_3 AChRs is dependent on the concerted action of β-arrestin, c-Scr and the GTPase dynamin, which "catalyses" the budding of clathrin-coated vesicles from the plasma membrane. Such coated M_3 AChRs are not targeting at lysosomes [72].

The role of altered M_2 and M_3 muscarinic AChRs expression in the asthma pathogenesis is as yet not established. Studies to date have tended to discount any role of M_3 AChRs dysfunction *per se* in the development of hyperreactive airway disease [73,74]. Some of studies suggest that exaggerated cholinergic discharge of acetylcholine on lung efferent vagal endings may be the result of decreased M_2 AChRs density of postganglionic cholinergic nerves [54]. Now, we do not know if irritant chemicals may activate M_2 AChRs internalization and degradation. It is suggested [75] that changes in neuronal M_2 AChR expression are mediated by cytokines produced at the sites of inflammation induced by chemical irritants or biological pathogens. These cytokines may then circulate to the air-

way nerves, where they decrease expression of M_2 AChRs and cause airway hyperreactivity. According to these authors double-stranded RNA, a product of viral replication, promotes the expression of interferons (IFNs). IFN-γ decreases the M_2 AChR gene expression in cultured airway parasympathetic neurons.

β-ADRENERGIC RESPONSES AND AIRWAY SMOOTH MUSCLE HYPERRESPONSIVENESS

The reduced sensitivity of β_2-adrenergic-receptor-induced excitation-contraction uncoupling in the airway of asthmatic subjects is one of the hypotheses that could explain the increased sensitivity of airway smooth muscle to acetylcholine (or other agonists such as metacholine) [63]. It is important to recognize that stimulation of β_2-adrenergic receptors causes hyperpolarization of airway smooth muscle and inhibits its tension. β_2-adrenergic receptors elicit changes in the airway smooth muscle are coupled to intracellular guanine nucleotide-binding regulatory proteins, designated as G_S-proteins (Fig. 4). G_S-proteins are responsible for relaying the activity of various effector systems, e.g., adenylate cyclase [76]. One hypothesis for the hypersensitive reaction of airway smooth muscle to contractile agents (acetylcholine) could be the reduced sensitivity of β_2-adrenergic receptor-induced excitation-contraction uncoupling in the airway of asthmatic subjects [77]. This could result from abnormal G_S-protein regulation of adenylate cyclase. Protein G_S is a substrate of protein kinase C (PKC) phosphorylation, which is likely to elicit a profound inhibitory effect on subsequent GTP-stimulated adenyl-cyclase activity. It has been hypothetized [63,77] that in asthmatic airways, the expression of Gs may be decreased because of its down-regulation, and thus less Gs be available to couple to β_2-receptors. It is also clear that there is a significant cross-communication between second-messenger pathways, involving airway relaxation through the adenylate cyclase path activated by β_2-adrenoreceptors and inositol phospholipid path activated by M_3 AchRs, controlled by the vagus nerve. As mentioned earlier, M_3 AChRs have the capacity to activate phospholipase C via protein Gq. PLC activation induces IP_3 and

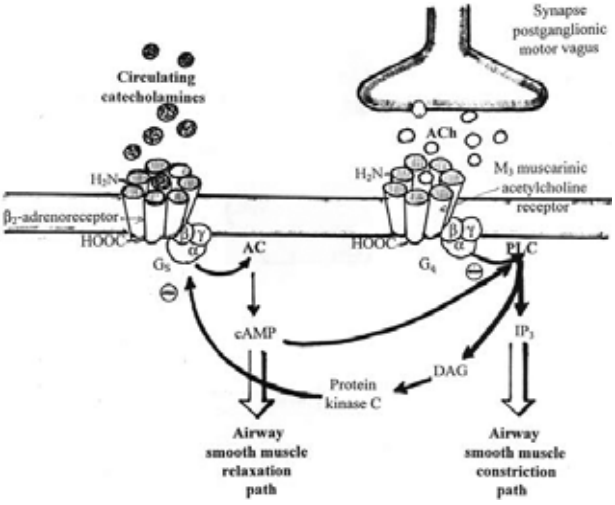

Fig. 4. Schematic representation of molecular cross-communication between adenylate cyclase (AC) and phospholipase C (PLC) signal cascades in the airway smooth muscles. Hyperactivation of phospholipase C leads to a decreased expression of G_S, and thus to its lower availability to couple to β_2-adrenergic receptors. In asthma, such an event may lead to reduced β_2-adrenergic receptor sensitivity. ACh – acetylcholine; cAMP – cyclic adenosine-monophosphate; IP3 – inositol 1,4,5-triphosphate; DAG – 1,2-diacylglycerol.

diacylglycerol (DAG) generation and PKC activation. In asthma, such an event may lead to reduced β_2-adrenergic-receptor sensitivity in airway smooth muscle.

Genetic polymorphisms of β_2-adrenergic receptors are thought to act as disease modifiers in asthma and may be responsible for potentiating bronchoconstricton caused by acetylcholine. There are at least four different polymorphic forms of β_2-adrenergic receptors: Arg16→Gly, Gln27→Glu, Val34→Met, and Thr164→Ile. The two most common polymorphisms are at positions 16 and 27. The Ile164 polymorphism is less common, and the Met34 variant is rare (<1%) [78,79]. According to some authors these polymorphic forms, resulting form site-directed mutagenesis, may account for some of the clinical heterogeneity among patients with the airway hyperresponsiveness [80]. The ferquency of these polymorphisms did not differ between the asthmatic and normal groups, so at least genetic variability of β_2-adrenergic receptors did not appear to play a major causative role [81]. Probably β_2-adrenergic polymorphisms may only modify asthmatic phenotype and the response to β-agonist therapy [76].

It seems that circulating catecholamines would exert a primary effect in regulating bronchomotor tone, since human

airway smooth muscle does not directly contain adrenergic nerves [63]. The airways of asthmatic patients fail to relax normally to isoproterenol, which suggests a possible defect in β-receptor function in the airway smooth muscle [82]. It is well-reconized that β-adrenergic blocking agents are contraindicated in patients with asthma and accentuate the immediate response to an allergen as well as to mediators that act directly on airway smooth muscles such as histamine, methacholine, and serotonin [62]. However, β-adrenergic blockade has a much greater effect on the sensitivity to an antigen than on the increased sensitivity to histamine, methacholine, and serotonin. This may well be because of the fact that β_2-adrenergic receptors are located in a wide variety of target tissues of asthma, not just in the airway smooth muscle, where direct acting agents (histamine or methacholine) could exert their primary effect. Pretreatment of asthmatic subjects with propranolol potentiates bronchoconstriction caused by histamine, methacholine, and ACh. However, in normal subjects, the bronchomotor response to methacholine or histamine is not increased by pretreatment with propranolol [83–85]. In propranolol-induced bronchoconstriction, it is believed that unopposed parasympathetic (the lung vagus) tone may be involved, since atropine prevents and partially reverses this effect in patients with mild asthma [86].

CAN ORGANOPHOSPHATE PESTICIDE EXPOSURE INDUCE VAGALLY-MEDIATED AIRWAY HYPERREACTIVITY?

Over the past 20 years there has been a significant increase in the incidence of asthma in industrialized countries, particularly in children in urban settings. At the same time, the use of insecticides, particularly organophosphate insecticides, has increased significantly not only in agricultural, but also in residential and urban settings. A number of clinical and epidemiological studies have linked exposure to organophosphates with airway hyperreactivity and other symptoms of asthma. In humans, exposure to organophosphate insecticides and other pesticides has been associated with a variety of respiratory symptoms, including decreased forced expiratory volume in 1 minute, wheeze, cough, and shortness of breath [87]. Many of the organophosphate insecticides have been restricted or banned due to their developmental neurotoxicity in animals. However, many of these compounds, including chlorpyrifos, are still used commercially in both agricultural settings and urban environments. These pesticide usage patterns correlate positively with reports of high incidence of asthma morbidity in agricultural workers and in residents of the inner cities [88–90].

Organophosphates are known to alter cholinergic function in the brain. A generally accepted mechanism of organophosphate neurotoxicity following acute exposure to high doses is the inhibition of acetylcholinesterase (AChE). It has been suggested that the same mechanism underlies the effects of organophosphate insecticides on bronchoconstriction [88]. Observations that not only organophosphate insecticides, but also other structurally unrelated AChE-inibiting insceticides, e.g., carbaryl, enhance airway hyperreactivity in rats and humans support this hypothesis [91,92]. However, there is evidence that in the brain, low-level doses of organophosphate pesticides that do not inhibit AchE, may alter cholinergic neurotransmission via direct effects on M AChRs and N AChRs function [93].

We would like to remind here once again that in the lung, cholinergic nerves in the vagus mediate airway tone and reactivity. These nerves release acetylcholine onto the lung smooth muscle and M_3 AChRs are causing contraction of these muscles, which results in bronchoconstriction. Vagally-induced bronchoconstriction is limited by autoinhibitory prejunctional M_2 AChRs present in parasympathetic nerves. Earlier studies on animal models of asthma and in patients with asthma have shown that neuronal M_2 AChRs are dysfunctional, and are present in less amount on prejunctional synapse. Decrease in M_2 AChRs leads to an increased release of acetylcholine from vagal nerve endings resulting in potentiation of vagally-mediated bronchoconstriction, which contributes to airway hyperreactivity [55].

Fryer et al. [91] show that neuronal M_2 AChR function is inhibited by both high and low doses of chlorpyrifos, which is consistent with other findings that organophosphate insecticides act on muscarinic receptors in the brain. This effect of chlorpyrifos on vagally-induced bronchoconstric-

tion is dependent on the dosing regimen. Vagally-induced bronchoconstriction was significantly greater in animals treated with the high dose of chlorpyrifos relative to animals treated with the low dose. A similar dependency was observed for the effects of chlorpyrifos on M_2 AChRs function as determined by pilocarpine dose-response curves. In contrast, none of chlorpyrifos doses changed the response to intravenous methacholine, demonstrating that the function of M_3 AChRs in airway smooth muscle was not altered in animals, in which M_2 AChRs mediating ACh release were not present. Selective loss of neuronal M_2 receptor function in the lungs is also associated with other models of airway hyperreactivity, including antigen challenge, viral infection, exposure to ozone, suggesting that the decreased M_2 AChRs function in airway nerves is a generalized mechanism underlying airway hyperreactivity [54,55].

Mechanisms by which the same organophosphate insecticide compounds alter M_2 AChRs function in neurons include down-regulation of the expression of these receptors, modulation of ligand binding to receptor, and alteration of signal transduction pathway downstream of M_2 AChRs activation. *In vitro* studies of cardiac M_2 AChRs have demonstrated that acute exposure to oxon metabolite of chlorpyrifos alters ligand binding via diethylphosphorylation of the receptor itself [94]. Whether these mechanisms underlie the effects of organophosphates on neuronal M_2 AChRs function in the lung has yet to be determined.

Data presented by Fryer et al. [91] indicate that organophosphate insecticides potentiate vagally-induced bronchoconstriction via disruption of the cholinergic control of airway responsiveness. A significant finding from these studies is that chlorpyrifos altered neuronal M_2 AChRs function in the lung at concentrations that did not inhibit AChE. Although the threshold concentration for this effect was not determined in this study, it has been shown that ligand binding to muscarinic receptors in the brain as well as signaling pathways downstream of muscarinic receptor binding can be disrupted by very low (nanomolar to picomolar) concentrations of organophosphate insecticides. These data suggest that exposure not only to occupational, but also to environmental levels of these compounds may entail biological consequences.

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