Cholinergic neurotransmission in human and guinea pig airways Function and dysfunction of muscarinic receptor subtypes

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SUMMARY

The diameter of the airways, and as a result airway resistance, are controlled by the contractile tone of smooth muscle layers within the airway wall. In asthma, there is shortness of breath (bronchial obstruction), wheezing and coughing, as a result of increased contractile tone of the airway smooth muscle. In addition, other factors such as oedema of the airway wall or increased mucus production by airway submucosal glands contribute to the obstruction. In allergic asthma, inhalation of a critical amount of allergen provokes an early allergic response, which in most humans consists of severe shortness of breath during 1 to 3 hours. In guinea pigs, the experimental animal used in the studies described in this thesis, this phase lasts for 2 to 5 hours (**Chapters 7 and 8**). After the early phase, there is an interval of several hours without obstruction, followed at approximately 8 hours after antigen challenge by the late obstructive reaction that lasts for 10 to 15 hours. Both after the early and after the late phase there is increased susceptibility of the airways to non-specific non-allergic, physical, chemical and pharmacological stimuli; this bronchial hyperreactivity (BHR) is one of the major characteristics of asthma. Both the airway obstruction and BHR are partly caused by increased contractility of the smooth muscle tissue.

The most important contractile nervous system in the airways is the parasympathetic or cholinergic (named after its neurotransmitter acetylcholine) system. A further contractile nerve system is the so called excitatory non-adrenergic non-cholinergic (e-NANC) system; it releases contractile neuropeptides called tachykinins like substance P and neurokinin A. Relaxation of airway smooth muscle (and thus dilation of the airways) can be produced by circulating adrenaline or by the neurotransmitter of adrenergic nerves (sympathetic nervous system), noradrenaline. In connection to airway smooth muscle contraction, mediators of the inhibitory NANC system can also be released, leading to decrease of contractile tone (**Chapter 1**). The vagal nerve pathways of the cholinergic system consist of preganglionic nerves, that run from the central nervous system down to the ganglion, and short postganglionic nerves that project at only a few nanometers distance from the smooth muscle cells. Muscarinic receptors have been found to be involved in vagal neurotransmission in ganglia (postjunctional M_1 subtype receptors), as well as in negative feedback of neurotransmitter release (by prejunctional M_2 subtype receptors on postganglionic nerve endings) and in the contraction of smooth muscle (via postganglionic postjunctional M_3 subtype receptors). In **Chapters 3 to 8** of this thesis, the role of these muscarinic receptor subtypes in the airways of guinea pig and man, and the putative alterations in their function under conditions of allergic bronchoconstriction and bronchial hyperreactivity, have been investigated.

In **Chapter 3**, the function of the prejunctional M_2 receptors in isolated guinea pig tracheal ring preparations was examined. To this end, the rings were mounted for isotonic recording, and twitch contractions were elicited by electrical field stimulation (EFS) (30Hz, 0.5ms, 8V over the electrodes, 5s per min). It was found that low concentrations of M_2-selective muscarinic receptor antagonists (gallamine, methoctramine, AF-DX 116, AQ-RA 741) increased the twitch contractions - as a result of blockade of the negative feedback loop - whereas higher concentrations decreased twitch contractions through blockade of postjunctional M_3 receptors. It was observed that the extent of the increase in twitch height induced by these M_2-selective antagonists was related to their selectivity for M_2 over M_3 receptors, and that the concentration dependence of this effect was related to their M_2 receptor affinity. From the additional examination of non- and M_3-selective antagonists (which only produced twitch decrease) it was inferred that the inhibition of the twitch contractions at higher concentrations was strongly correlated to the M_3 receptor affinity of all types of antagonist. The findings in this chapter show that putative dysfunctions of M_2 and M_3 receptors in sensitised and/or challenged animals can be studied using this experimental set-up.

The functional presence of ganglionic M_1 receptors that had been suggested to be involved in facilitatory neurotransmission in human lungs had not been satisfactorily demonstrated in the guinea pig at the start of our experiments (**Chapter 4**); in fact such receptors were postulated in sympathetic and parasympathetic ganglia, but also denied in the latter. In our experiments, we used isolated preparations of the main bronchus which were carefully dissected with the supplying vagus nerve left intact. Twitch contractions were alternately elicited by (1) EFS, which releases acetylcholine from the vagus nerve endings without the intervention of the ganglia, and (2) electrical stimulation of the vagus nerve (VS) which includes neurotransmission through the ganglia. The inhibitory effect of the M_1 receptor selective muscarinic antagonist pirenzepine on VS- and EFS-induced twitch contractions was established under various conditions. Using identical stimulation conditions for VS and EFS (30Hz, 0.5ms, 8V over the electrodes, 5s per min), VS-induced contractions were smaller than EFS-induced contractions, probably due to ganglionic filtering, and pirenzepine was 2.3-fold selective in antagonising VS-induced contractions, which small selectivity does not readily indicate the involvement of M_1 receptors in vagal bronchoconstriction. This was confirmed by experiments in which stimulation frequency for EFS was decreased to obtain twitch contractions that matched those elicited by VS; under these conditions pirenzepine showed no preferential inhibition of VS-induced twitches at all. However, high affinity inhibition of VS-induced twitches was achieved when stimulation was prolonged (for both VS and EFS) to reach plateau contractions, in the presence of ß-adrenoceptor and M_2 receptor blockade to exclude any feedback regulation, and when the major component of ganglionic transmission, i.e. nicotinic receptors, were partially blocked using hexamethonium. It thus appears that muscarinic M_1 receptors are indeed

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operational in parasympathetic ganglia of the guinea pig bronchus, as in human lungs, and are involved in the amplification of postganglionic impulses. Future studies are indicated to establish whether alterations in the involvement of these M_1 receptors are of importance in bronchial hyperreactivity, be it in response to allergic reactions or not.

In **Chapter 5**, experiments were aimed at measuring directly the concentrations of endogenous (i.e. not radiolabeled) acetylcholine that are released from vagus nerve endings in isolated guinea pig trachea, in order to obtain *direct* information concerning the function of inhibitory M_2 autoreceptors on these nerve endings; the measurement of endogenous acetylcholine may have relevant advantages, since there is evidence to indicate that radiolabeling does not affect all pools of acetylcholine equally and differences in modulation of radiolabeled and endogenous acetylcholine release have been reported. To achieve this, tracheas were electrically stimulated in a small volume of buffer, and samples were immediately analysed using high performance liquid chromatography with electrochemical detection (HPLC/ECD). It was found that under relatively high stimulation intensity (30Hz, 0.5ms, 8V, for 5 min in the presence of neostigmine), resulting in high levels of acetylcholine release, the muscarinic receptor antagonist atropine more than doubled this release, whereas the muscarinic receptor agonist methacholine did not show any effect. These results suggested that under these conditions synaptic concentrations of acetylcholine are that high that prejunctional M_2 receptors are maximally activated; consequently, they can be blocked but not further activated. Under mild stimulation conditions (16Hz, 0.1ms, 8V, for 5 min in the absence of neostigmine), resulting in 20-fold lower acetylcholine levels, methacholine indeed inhibited this release; this was similarly true for the partial muscarinic agonist pilocarpine, which under high stimulation behaved as an antagonist (i.e. like atropine). These results showed that the negative feedback control of acetylcholine release through prejunctional M_2 autoreceptors is directly dependent on the synaptic levels of neurotransmitter, and thus on nerve activity.

In **Chapter 6**, the presence and function of prejunctional inhibitory muscarinic autoreceptors in human airway preparations (derived from human lung tissue obtained from thoracotomies and transplant donors) was investigated. Isolated smooth muscle strips from trachea and main bronchus and ring preparations from various generations of bronchi and bronchioli (3.5-0.7 mm I.D.) were subjected to EFS, and using the method described in Chapter 3, it was studied whether M_2-selective antagonists (gallamine, AQ-RA 741) would potentiate twitch contractions as previously observed in guinea pig trachea. Remarkably, such increase in twitch contractions was observed in all preparations from central airways (trachea, main bronchus), and in half of the terminal bronchi (1-2 mm I.D.); no prejunctional M_2 receptor function was found in smaller bronchi (<1 mm I.D.). In addition, the direct release of acetylcholine from bronchial preparations (1.5-3.5 mm I.D.) was significantly increased by the addition of atropine in all tests performed. In conclusion, prejunctional inhibitory M_2 autoreceptors

are functionally present in human airways, but it remains to be established why there is (individual) variability in peripheral airways.

In **Chapter 7** it was investigated to what extent prejunctional M_2 receptor function in guinea pig trachea is changed after the event of an allergic asthmatic reaction. In these experiments we made use of an animal model of allergic asthma developed in our laboratory, in which lung function is being measured on-line in conscious, unrestrained guinea pigs that have been sensitised to and subsequently challenged by inhalation with the allergen ovalbumin; lung function is monitored as pleural pressure using a small fluid-filled balloon-cannula implanted by microsurgery. As in human asthmatics, these animals develop early and late phase obstructive reactions in response to antigen; in between (5-8 hours after antigen challenge) lung function is normal. Immediately after the early and after the late reaction (i.e. 6 and 24 hours after challenge, respectively), bronchial responsiveness to histamine was measured. Prejunctional muscarinic M_2 receptor function was investigated using the method described in chapter 3, in isolated tracheal preparations obtained from animals that had been terminated just after the early or late response. It was observed that after the early allergic reaction, when BHR was at its highest level, M_2 receptors on cholinergic nerve terminals were largely dysfunctional, because the potentiation of the twitch responses by all M_2-selective antagonists (gallamine, methoctramine, AQ-RA 741, AF-DX 116) was significantly and markedly decreased compared to controls. Moreover, the degree of dysfunction was correlated to the magnitude of the early response. After the late reaction, dysfunction was already restored to a large extent, but BHR was still present, although smaller than after the early response. These results strongly suggest that BHR to histamine after the early allergic response is partly due to the fact that prejunctional inhibitory M_2 autoreceptors are severely dysfunctional, since it has been shown that histamine activates sensory nerve endings in the airways leading to vagal reflex activity and, consequently, to acetylcholine release from vagal nerve endings. Dysfunctional M_2 receptors would lead to increased acetylcholine release and thus to increased bronchoconstriction. Recently, it was indeed shown by Santing that inhaled ipratropium bromide strongly decreased BHR to histamine in our guinea pigs. Finally, it should be mentioned that postsynaptic M_2 receptors in the hearts of the animals were not affected by the allergic responses, indicating that inhalation of antigen only induces these effects locally.

In **Chapter 8**, M_2 receptor function after the allergic response was investigated again, but now *in vivo*, in conscious, unrestrained animals, in order to establish the extent and time course of the putative dysfunction in the intact respiratory tract, and under physiological conditions. Guinea pigs were subjected to microsurgery to implant an intrapleural balloon-cannula (as in Chapter 7) as well as a bipolar stimulation electrode around the right vagus nerve; in contrast to other studies using anaesthetized animals in which the vagus nerve was not sectioned, native nervous activity was preserved. In this way, electrical stimulation of the nerve with stepwise-increasing

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frequencies induced increasing levels of bronchoconstriction in conscious animals, monitored as increase in respiration amplitude. The function of prejunctional M₂ autoreceptors was studied using the M₂-selective antagonist gallamine which, when inhaled in relatively low concentrations, increased nerve stimulation-induced bronchoconstriction at each frequency, as a result of the blockade of these inhibitory prejunctional receptors; with higher concentrations of gallamine, the left-shift of the frequency-response curve was reveiled and the maximum obstruction was depressed as a result of blockade of postjunctional (smooth muscle) M₃ receptors. When these experiments were repeated (in the same animal) after the early allergic response, it was observed that gallamine no longer potentiated nerve-induced bronchoconstriction, but still blocked constriction in the highest dose. After the late reaction, gallamine effects were rather similar to control, i.e. before antigen challenge. As in the previous chapter, BHR to histamine was measured and found to be rather high after the early and smaller (but still significant) after the late response. These results fully confirmed those obtained in isolated tracheal preparations *in vitro*, in showing that guinea pig airways develop a severe dysfunction of prejunctional M₂ receptors already during the early allergic response after antigen challenge. The resulting vagal hyperactivity may strongly contribute to the bronchial hyperreactivity to inhaled substances stimulating sensory nerve endings.

The mechanism underlying the almost complete M₂ receptor dysfunction after an allergic response has been proposed to be related to the eosinophilic granulocyte, being an inflammatory cell that infiltrates the airways already during the early response. It has been shown that these cells produce and release a cytotoxic protein called major basic protein, which appears to block muscarinic M₂ (but not M₃) receptors in a reversible (allosteric) fashion. In agreement with this, postjunctional smooth muscle M₃ receptors were found to be unaffected by the allergic responses, in the *in vitro* as well as in the *in vivo* studies.

Finally, in **Chapter 9** it was investigated to what extent cholinergic and e-NANC neurotransmission in guinea pig main bronchus are under the inhibitory control of prejunctional ß-adrenoceptors; this has been postulated by others but the subtype identity of these ß-adrenoceptors remained controversial. EFS was performed for 20s (30Hz, 0.5ms, 8V over the electrodes) to produce both a rapid cholinergic twitch contraction and a slow, 15-min e-NANC wave-like contraction, which results from the release of the neuropeptide transmitter neurokinin A. The effects of ß₂- and ß₃-selective adrenoceptor agonists (fenoterol and salbutamol, and BRL 37344, respectively) were studied both on the height of the cholinergic twitch and on the magnitude of the e-NANC contraction, in the absence and as well as in the presence of the ß₂-selective receptor antagonist ICI 118,551; these inhibitory effects were compared to those on contractions induced by exogenous methacholine and neurokinin A, respectively. It was found that e-NANC contractions were inhibited by lower concentrations of the ß-adrenergic agonists compared to neurokinin A-induced contractions, and that this

response only involved β_2 receptors; such preferential inhibition of EFS-induced contractions was not observed for the cholinergic pathway. It was concluded that the release of e-NANC neurotransmitter(s) in guinea pig main bronchus is under the inhibitory control of prejunctional β_2-adrenoceptors, but that there is no such control on cholinergic nerve endings.

In conclusion, the studies described in this thesis have demonstrated the functional presence of M_1 receptors in parasympathetic ganglia of isolated guinea pig airways, and of postganglionic, prejunctional M_2 autoreceptors in guinea pig and human airways, both by indirect (contraction) and direct (endogenous acetylcholine release) measurement. Most importantly, it was shown both *in vitro* and *in vivo* that the prejunctional M_2 receptors become dysfunctional after the early allergic response in the guinea pig isolated trachea and in the respiratory tract of the intact (freely moving) animal, but recover during the late phase, which indicates that M_2 receptor dysfunction may contribute to bronchial hyperresponsiveness to sensory stimuli after the early but not after the late response following a single allergen challenge. It remains to be established whether dysfunction (i.e. increased reactivity) of ganglionic M_1 receptors contributes to BHR as well. Similarly, further investigations are necessary to establish the precise relationship between M_2 receptor dysfunction in human asthmatics (which has been observed by studying SO_2-induced bronchoconstriction) and the observations made in this thesis.

The new methods developed in this study may be of great value for future investigations on prejunctional control mechanisms of acetylcholine release in isolated human and guinea pig airways and the regulation of vagally induced bronchoconstriction in conscious, unrestrained guinea pigs, both under physiological and pathophysiological conditions. Especially investigations on the direct and indirect role of the e-NANC and i-NANC systems are challenging.