SUMMARY

The airway diseases asthma, chronic bronchitis and emphysema all share as a main characteristic shortness of breath, which is the result of airway obstruction. The parasympathetic (cholinergic) nervous system, being the major bronchoconstrictor neural pathway in (human) airways, has therefore been implied in both the pathogenesis and the therapy of these disease states. The effects of the cholinergic nervous system on target cells in the airways (smooth muscle, exocrine glands, blood vessel walls) are elicited via release of acetylcholine, which binds to muscarinic type receptors on the target cells. The present thesis deals with investigations concerning binding properties, transductional properties as well as functional properties of these muscarinic receptors in airway smooth muscle (in comparison with cardiac and brain tissue), in view of the notion emerged during the past decade that muscarinic receptors can be distinguished into discrete subtypes.

In the first part of the thesis (Chapters 2-5), the discovery of the presence of a majority of M2 (cardiac type) as well as a minority of M3 (smooth muscle/glandular type) muscarinic binding sites in bovine tracheal smooth muscle membranes is described. Initially it was found that the binding properties of pirenzepine towards muscarinic receptors in bovine cardiac and tracheal membranes agreed very well with affinities reported in functional experiments using mainly guinea pig tissues (low affinity in all cases), but that this was not the case for AF-DX 116. In cardiac membranes, the AF-DX 116 binding affinity was relatively high (K_d = 96 nM) in agreement with reported functional affinities (M1 receptor subtype). However, in tracheal membranes two binding sites could be distinguished, of which the majority (74%) had high (M3, heart-like) affinity and the minority low (M1, gland-like) affinity for AF-DX 116. Since these membranes had been prepared from bovine tracheal smooth muscle with intact epithelium and submucosa, it was suggested that the minority of low affinity muscarinic binding sites represented glandular muscarinic receptors, the majority of high affinity binding sites presumably representing smooth muscle muscarinic receptors (Chapter 2). The binding properties of AF-DX 116 in bovine tracheal membranes were especially important because they predicted discrepancies between binding and functional properties for muscarinic receptors in smooth muscle, contraction being mediated by the M3 subtype. This was further investigated in Chapter 3. It was established that functional affinities of muscarinic antagonists (AF-DX 116 and six others) in bovine tracheal smooth muscle correlated extremely well with those reported for guinea pig smooth muscle preparations, suggesting discrepancies between binding and functional properties of muscarinic antagonists in bovine tracheal smooth muscle rather than species differences. These, occasionally marked, discrepancies between binding and function indeed appeared to be present with all compounds tested. However, in contrast to the non-selective (atropine, pirenzepine) and smooth muscle selective antagonists (4-DAMP methobromide, hexahydro-
siladifenidol), which gave monophasic displacement curves with $M_2$-like affinities, the cardioselective antagonists AF-DX 116, gallamine and methoctramine still distinguished a majority of $M_2$-like and a minority of $M_3$-like binding sites, although the membranes had been prepared using bovine tracheal smooth muscle fully denuded of epithelium and submucosa (i.e. no exocrine glands present). These results suggested that in smooth muscle membranes only a small proportion of the muscarinic binding sites is involved in contraction, but the rather clear quantitative differences between $pA_2$ values for contraction and $pK_i$ values for binding to the minority of $M_3$-like binding sites as well as the presence of the majority of $M_2$ type binding sites precluded a definite conclusion.

The simultaneous occurrence of $M_2$ and $M_3$ type muscarinic binding sites in bovine tracheal smooth muscle could also be demonstrated using a kinetic approach, i.e. radioligand dissociation measurements (Chapter 4). It appeared that $[^3H]$dextetimide dissociation from muscarinic receptors in this tissue was biphasic, with a fast phase (half-life < 1 min) followed after 4-5 min by a slow phase (half-life ~ 40 min). The fast phase was shown to represent the cardiac type receptors in bovine tracheal smooth muscle because (i) a similar dissociation half-life was found for $[^3H]$dextetimide in bovine cardiac left ventricular membranes, (ii) the relative proportion of muscarinic binding sites in bovine tracheal smooth muscle exhibiting this fast dissociation behaviour agreed with the proportion of cardiac ($M_2$) muscarinic sites as detected in equilibrium binding studies, and (iii) a virtually identical allosteric (negative cooperative) modulation of radioligand dissociation from $M_2$ muscarinic receptors in bovine cardiac and tracheal smooth muscle membranes by AF-DX 116, gallamine and methoctramine was observed. The slow phase was concluded to represent the smooth muscle/glandular type receptors in this tissue based on its relative proportion (30%) and comparison to $[^3H]N$-methylscopolamine dissociation behaviour in cardiac and glandular membranes (as reported by others). It was concluded that the $M_2$ type muscarinic binding sites in bovine tracheal smooth muscle membranes resemble $M_2$ muscarinic receptors in cardiac membranes not only regarding equilibrium binding affinities but also regarding radioligand dissociation and allosteric modulation. Moreover, $M_3$ type binding sites in bovine tracheal smooth muscle appeared much less sensitive to allosteric modulation than the cardiac receptor subtype. The studies concerning the $M_2$ and $M_3$ muscarinic binding sites in (bovine tracheal) smooth muscle were completed with Chapter 5. Using computer fitting and simulation procedures, it was shown that the presumed non-selective muscarinic radioligand applied in our experiments, $[^3H]$dextetimide, actually possesses a small (about 6-fold) selectivity for $M_3$ over $M_2$ receptors. The assumption of non-selectivity of this radioligand (as in Chapters 2 and 3) was shown to result in erroneous estimates of binding parameters for cardioselective muscarinic antagonists, and the small radioligand selectivity actually present was shown to lead to an inability to detect both $M_2$ and $M_3$ receptor populations using smooth muscle selective muscarinic antagonists with selectivities less than 30-fold. Using fore-
knowledge acquired with the cardioselective compounds, however, the monophasic displacement curves obtained with the smooth muscle selective antagonists could be resolved into high (M2) and low (M3) affinity populations. As a consequence of the findings in Chapter 5, all results obtained in Chapters 2-4 could be accommodated into the model that smooth muscle membranes contain a majority of M2 (cardiac) and a minority of M3 (smooth muscle/glandular) type binding sites, the latter representing the receptors involved in smooth muscle contraction.

The second part of the thesis (Chapters 6-8) presents investigations on the recognition by classical quaternary muscarinic antagonists (like ipratropium, N-methylscopolamine and oxyphenonium bromide) of two discrete binding sites, as initially observed in calf and rat brain tissue. It was found that this binding heterogeneity, which we have called Q1/Q2, occurs in bovine striatal membranes in a manner similar to bovine total brain membranes, indicating that the high (Q1) and low (Q2) affinity binding populations do not originate from different brain areas (Chapter 6). It was also found that the Q1/Q2 binding heterogeneity is independent of the pirenzipine binding heterogeneity (M1/M2). In search of the peripheral tissue distribution of the Q1/Q2 binding heterogeneity it was established that quaternary muscarinic antagonists also distinguish the two classes of binding sites in bovine tracheal smooth muscle membranes but not in bovine cardiac (atrial and ventricular) membranes. The findings from Chapter 6 were extended by investigating the molecular basis of the Q1/Q2 binding heterogeneity (Chapter 7) and the possible functional consequences of this binding behaviour in bovine tracheal smooth muscle contraction (Chapter 8). Using agents that are known to modulate (muscarinic) receptor binding (Na+ and Mg²⁺, GppNHp, dithiothreitol, N-ethylmaleimide), no indications were obtained that the Q1 and Q2 binding site populations are molecularly different muscarinic receptor subtypes since they were not differentially modulated by these agents. Using membrane modulating agents (sodium laurylsulfate, digitonine, polyethylene glycol 6000, cholesteryl hemisuccinate), no evidence was obtained either that the low affinity (Q2) binding sites represent muscarinic receptors located in a different membrane environment less accessible to (charged) quaternary compounds, since these agents did not bring about changes in the appearance of the heterogeneous binding behaviour. However, a qualitative correlation could be observed between the octanol:buffer partition coefficients (as a measure of lipid solubility) and the appearance of Q1/Q2 binding heterogeneity for six quaternary muscarinic antagonists and therefore the low affinity binding sites may after all be (normal) muscarinic receptors located in a hydrophobic membrane domain (Chapter 7).

In bovine tracheal smooth muscle contraction experiments, some quaternary muscarinic antagonists showed anomalous behaviour as well. When antagonizing methacholine-induced contraction, ipratropium, N-methylscopolamine, oxyphenonium, and N-methyldeprpine bromide gave Schild plots with slopes significantly greater than unity (up to 2.0), indicating uncompetitive interaction. By contrast, the tertiary analogues
atropine and scopolamine as well as two quaternary compounds, 4-
DAMP methobromide and thiazinamium, behaved as classical antagonists
with Schöld slopes of unity, as did ipratropium and N-methyldepropine in
guinea pig trachea. The high Schöld plot slopes in bovine tracheal smooth
muscle were found not to be due to inadequate equilibration of the
antagonists or to the presence of an atropinesterase in the tissue, but
positive cooperativity of the quaternary muscarinic antagonists was shown
to be the underlying mechanism instead (Chapter 8). It should be men-
tioned that apparently no relationship exists between heterogeneous binding
behaviour of classical quaternary muscarinic antagonists and positive
cooperativity in contraction studies, since N-methyldepropine gave the
highest Schöld plot slopes (Chapter 8) but did not exhibit Q1/Q2 binding
heterogeneity (Chapter 7).

In the third and last part of the thesis (Chapters 9-11), experiments
are described concerning the transduction mechanism(s) coupled to
muscarinic receptors in bovine tracheal smooth muscle as well as the
characterization of the muscarinic receptor subtype mediating contraction
of human airway smooth muscle. In Chapter 9, the relationship between
bovine tracheal smooth muscle contraction and phosphoinositide metabolism
was studied using muscarinic agonists. It was established that with all
three agonists used, methacholine (non-selective), McN-A-343 (M₁-
selective), oxotremorine (M₂-selective), 50% contraction was elicited by
concentrations which only gave 3.5% of the maximal attainable inositol
phosphates accumulation, indicating (i) a considerable reserve of inositol
phosphates production for the full contractile agonists methacholine and
oxotremorine, and (ii) a direct relationship between phosphoinositide
metabolism and smooth muscle contraction. Such a direct relationship
could also be concluded from Chapter 10, where the muscarinic receptor
subtype mediating phosphoinositide metabolism in bovine tracheal smooth
muscle was pharmacologically identified using the competitive antagonists
pirenzepine (M₁-selective), AF-DX 116 (M₂-selective), and 4-DAMP
methobromide (M₃-selective). The M₃ character found in these experiments
coincided with the M₃ muscarinic receptor subtype mediating smooth
muscle contraction and thus provided additional evidence for the involve-
ment of phosphoinositide metabolism in the pharmacomechanical coupling
between muscarinic receptor stimulation and contraction of (bovine
tracheal) smooth muscle. Hence, the function of the major population of
cardiac type (M₂) muscarinic receptors and the transduction mechanism
involved remain to be elucidated.

As a "Grande Finale", the thesis closes with the characterization of the
muscarinic receptor subtype involved in contraction of human airway
smooth muscle (Chapter 11). It was found, using a Schöld analysis with five
selective muscarinic antagonists, that contraction of human peripheral
(small bronchi) and central (trachea) airway smooth muscle is mediated by
the M₃ muscarinic receptor subtype, just as is the case with bovine and
guinea pig tracheal smooth muscle. These animal tissues therefore provide
excellent models for the development of M₃ subtype-selective muscarinic
antagonists to be used as receptor) blocking probes.

In conclusion, the thesis has significantly added to the information over the past years
important questions regarding smooth muscle and the pharmacology of smooth muscle, especially
the relationship between muscarinic receptors and smooth muscle function. Subsequently, studies in additional regions with smooth muscle receptors were undertaken.

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antagonists to be used as bronchodilators free of M₂-(cardiac or auto-
receptor) blocking properties.

In conclusion, the studies described in this thesis have contributed
significantly to the increasing knowledge on muscarinic receptor subtypes
over the past years, especially concerning (airway) smooth muscle.
Important questions remaining include the function of the M₂ receptors in
smooth muscle and the extrapolation of selected findings to human (airway)
smooth muscle, especially regarding the presence of M₂ receptors and the
relationship between phosphoinositide metabolism and contraction.
Subsequently, studies on the possible disturbance of (pre- and postjunc-
tional) muscarinic receptor mechanisms in obstructive airway disease are at
hand.