INTRODUCTION

Nicotinic receptor blocking agents are widely used in anesthetic practice as neuromuscular blocking drugs (NMBD). Nevertheless, there is sufficient evidence confirming that the nicotinic receptor blockers would affect also muscarinic receptors on smooth muscle like bronchial, arterial or ileum smooth muscle (1-5). Indeed, the effects of NMBD on bronchial smooth muscle are of particular relevance because it has been frequently observed that some degree not only of laryngospasm but also of bronchoconstriction may accompany induction of the skeletal muscle relaxation in patients undergoing general anesthesia (6). Laryngeal muscles are striated skeletal muscle while those of the airway are smooth muscle. Spasm to laryngeal muscles and bronchial muscle is achieved by distinct and unrelated mechanisms. Laringospasm is relatively easily relieved by an administration of additional NMBDs and, logically, observed bronchoconstriction has been ascribed not to the NMBD effects on muscarinic receptor, but to the capacity of, for example, succinylcholine to liberate histamine. There are however theoretical reasons and some experimental and clinical evidence suggesting that the clinically relevant bronchoconstriction can indeed appear as a result of a selective M2 receptor block (8, 9). The M2 receptors have been documented not only in postganglionic nerve endings of cholinergic nerves (i.e., pre-junctional M2 receptors, promoting inhibition of acetylcholine release particularly when electric field stimulation is being used) but also on the surface of smooth muscle cells, i.e. post-junctional M2 receptors, promoting inhibition of relaxation and thus favoring contractile response, resembling direct M3 postjunctional contractile stimulation (10). Bad experience with rapacuronium - fatal bronchoconstriction (11) - for which it was shown to have greater affinity for M2 than for M3 receptors (12) illustrates well the existence of potential danger that the unexplored new such agents may present.

It is of substantial clinical interest to know whether muscarinic block would relax (postjunctional block) or facilitate contraction (prejunctional block) of the bronchial smooth muscle. This may depend on muscarinic receptor subtypes distribution, the specificity of the blocking agent and concomitant vagal (cholinergic) outflow. Unfortunately, M2/M3 quantitative relations on the smooth muscle and nerve terminals and the importance of each of them for bronchoconstriction have been studied incompletely (12). Recent binding studies have shown that NMBD have high affinities for both M2 and M3 muscarinic receptors (13). However, more exact estimation of the compound resultant effect

SKELETAL MUSCLE RELAXANTS INHIBIT RAT TRACHEAL SMOOTH MUSCLE TONE IN VITRO

Neuromuscular blocking drugs (NMBD) can inhibit not only nicotinic but also muscarinic (M) receptors and thereby affect not only skeletal but also smooth muscle (SM) tone. A selective postjunctional muscarinic inhibition would relax, while prejunctional inhibition of muscarinic M2 receptor might hasten SM contraction thereby increasing the risk of bronchospasm. In rat tracheal rings in vitro we evaluated the effects of cumulative concentrations of some NMBD and M receptor blocking agents for their effects on tracheal smooth muscle (TSM) tone pre-contracted with carbachol (CARB; 5x10^-7M or 10^-6M), pilocarpine (PILO; 5x10^-6M), or by electrical field stimulation. The NMBDs produced relaxation in the preparations precontracted with CARB or PILO. The order of potency after CARB (10^-6M) was (EC50): 4-DAMP (9.8) >atropine (9.2) >methoctramine (6.4) >pancuronium (6.0) >mivacurium (5.8) >cisatracurium (5.6) >gallamine (5.2) >rocuronium (4.8) >succinylcholine (2.9); NMBDs also partially prevented contraction elicited by the electrical field stimulation. We demonstrated that the clinically used NMBD that were examined produced rat TSM relaxation, probably by predominantly blocking postjunctional muscarinic receptors.

Key words: skeletal muscle relaxants, rat, trachea, muscarinic receptor, smooth muscle tone, bronchoconstriction

*The first and the second author contributed equally to this work.
of the NMBD on bronchial smooth muscle tone is needed. Although new NMBDs with faster action and shorter duration of their relaxing effects are permanently developed, comparative studies of their effects on bronchial smooth muscle are missing.

In the present study the main purpose was to examine and compare the bronchial smooth muscle relaxant effects of some most common NMBDs in pre-contracted tracheal smooth muscle (TSM) of rat tracheal ring preparations in vitro. The hypothesis to examine in this study was whether NMBDs could, by simultaneously (and probably by unequally) blocking prejunctional and postjunctional muscarinic receptors, elicit some facilitation of the airway smooth muscle contraction favoring bronchospasm.

MATERIALS AND METHODS

Experiments (an approval to perform this study was obtained from the Animal Protection Commission, LALLF, State Ministry of Economics, Rostock, Germany) were performed on tracheas taken from Lewis rats (Lew. 1A, Department for Laboratory Animal Science, Greifswald University) weighting 250-420 g. Care was taken that animal treatment and experimental procedure would be in accordance with the local Instructions for Animal Care of Greifswald University. All animals were housed in individual cages and received water and food ad libitum. For the experiments they were stunned, the abdomen was opened, abdominal aorta and inferior vena cava cut and animals quickly exsanguinated. The tracheas were then excised, immersed in Krebs-Henseleit solution (in mM: 113 NaCl, 4.8 KCl, 1.3 MgCl₂ x 6H₂O, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 5.7 glucose) and cleaned from surrounding tissue. The tracheal ring preparations (each consisting of 2 rings) were suspended between two stainless hooks. The lower hook served as a fixed point and the upper hook was attached to an isometric force transducer (Entran, ELJ-S045C-35G IT1-25, purchased from EMKA Technologies, Paris, France), the latter being attached to micromanipulator that permitted displacement of the upper hook along a strict vertical axis. The signal from the transducer was amplified (STA 2808, EMKA Technologies) and displayed on paper recorder (Rikadenki multipen recorder, R-50 series, Hugo Sachs Elektronik, March-Hugstetten, Germany). The Krebs solution of the double-jacket organ bath (at 37°C, pH 7.4, bubbled with 95% O₂/5% CO₂) was exchanged every 20 min. After a period of stabilization of the preparation (45 min) the tracheal muscle was stretched to its optimal length, which was established, in preliminary experiments, to correspond to a counter - weight of 1.5 g (14).

For the electrical field stimulation (EFS) rectangular platinum electrodes, 1 cm² large, were used; they were connected to a stimulator Grass S48K equipped with a Stimulus Isolation Unit (both from Grass Instrument Division, Astrp-med. Inc., West Warwick, UK) coupled to the stimulator stimulus booster (STC4808, EMKA Technologie).

Procedure

1. Effects of NMBD and muscarinic antagonists on pharmacologically induced tracheal smooth muscle tone

The effects of skeletal muscle relaxants on basic tension or agonist elicited contraction were examined. In each preparation we used only one agonist and one antagonist. The preparations were precontracted either with carbachol (CARB 10⁻⁶M) or pilocarpine (Pило 5x10⁻⁶M, M₁ receptor specific agonist) - the concentrations corresponded to their EC₅₀. One weaker concentration of CARB (5 x 10⁻⁷ M) was also used in an attempt to eventually distinguish a selective block of an antagonist. When the tension reached a plateau, preparations under the resting tension, or precontracted preparations, were tested with cumulative concentrations of NMBD, pancuronium (PANC), cisatracurium (CIS), rocuronium (ROC), mivacurium (MIV), or succinylcholine (SCC). Both clinically used substances as well as pure NMBD were tested. Additionally, we examined the effects of muscarinic blockers atropine (ATRO, non-specific), gallamine (GALL, selective M₂ blocker), or methoctramine (METHO, selective M₁ blocker), or 4-DAMP (selective M₁ blocker).

2. Effects of NMBD on electrically induced tracheal smooth muscle tone

The effects of cumulative applications of increasing concentrations of the NMBD pure substance on the contractions elicited by the electrical field stimulation (EFS) were examined. About 6 minutes were allowed to achieve maximal effects after adding new concentration of each NMBD agent and then, DC square pulses, of 0.5 ms duration, 25 Hz, for 25 sec were delivered.

3. Effects of stimulation with low dose pilocarpine on muscle relaxation

To test for possible facilitation of contraction - a prejunctional M₂ receptor effect, the preparations were preincubated for 30 minutes with a low concentration of PILO (10⁻⁹M), and then one contraction was elicited with CARB (10⁻⁶M). In another set of preparations, to test for inhibition of relaxation mediated by postjunctional M₁ receptor, after the precontraction elicited with carbachol (10⁻⁶) reached a plateau, the preparations were preincubated for 10 minutes with PILO (10⁻⁹) and then cumulative concentrations of PANC were added to the bath solution. The effects were compared to preparations that were not preincubated with pilocarpine.

Substances

The following substances were used: carbachol (carbamylcholine chloride), pilocarpine (pilocarpine hydrochloride), (all from Sigma Chemical, Saint Louis, Missouri, USA); muscarinic blockers: gallamine, methoctramine (methoctramine tetrachloride), 4-DAMP (4-Diphenylacetoxy-N-methylpyperidine methide) (all from Sigma Chemical, Saint Louis, Missouri, USA), atropine (B. Braun, Melsungen AG, Germany); depolarizing NMBD: succinylcholine (Curasan AG, Kleinostheim, Germany); pure or commercially available steroidal nondepolarizing NMBD: pancuronium bromide (ORG NA97 or Pancuronium) and rocuronium bromide (ORG 9429 and Esmeron) were purchased or, when pure substances, they were kindly supplied by Organon, Akzo-Nobel, N.V. Organon, BH Oss, Netherlands; benzylisoquinolinium nondepolarizing NMBD: cisatracurium (Nimbex) and pure substance (Cisatracurium besylate) and mivacurium chloride (Mivacron), i.e. pure substance 950U77 (mivacurium chloride related) were purchased from or, if pure substances, they were kindly supplied by GlaxoSmithKlein, Durham, UK. All solutions were prepared using distilled water, with exception of gallamine, which was prepared as 0.1 M stock solutions in DMSO (dimethyl sulphoxide, from Sigma, Sigma-Aldrich Corp., St Luis, Missouri, USA) and further dilutions were made with distilled water.

Data analysis

The data are expressed in percent of maximal response and in absolute values and given as means±SD. Half-maximal concentration (EC₅₀) values were calculated by means of non-linear regression (Hill-Langmuir equation) and the results are given as means of -log EC₅₀ values obtained. Statistical assessment was conducted by use of analysis of variance and the
Student's t-test for paired or unpaired data and obtained P values were adjusted for multiple comparisons, when appropriate. To test its applicability, the False Discovery Rate procedure (FDR, (15, 16)) was also applied to some multiple comparisons. The value of P<0.05 was regarded as being statistically significant.

RESULTS

Effects of NMBD and muscarinic antagonists on tracheal smooth muscle tone

Both, NMBD and mucarinc antagonists, relaxed the preparations pre-contracted with CARB (5x10⁻⁷M and 10⁻⁶M) or PILO (5x10⁻⁴M) (Figs. 1 and 2, and Table 1 and 2). Relaxing potency of each NMBD and muscarinic antagonist, which were examined in this study, was similar either in CARB and in PILO precontracted preparations (Table 1 and 2). PANC, out of all skeletal muscle relaxants tested, was the most effective both in CARB and in PILO precontractions. The effects of the NMBD pure substances were similar to the corresponding galenic preparations. (legend for Table 1). NMBD did not affect basic tension of the preparations.

Precontractions with low versus higher concentrations of CARB

In the preparations precontracted with CARB 5x10⁻⁷M, as compared to those precontracted with CARB 10⁻⁶M, dose-response
curves of ROC and PANC were displaced to the left, while CIS and MIV dose-response curves did not differ (Fig. 1 and Table 1).

Precontractions with EC50 concentrations of CARB and PILO

The dose-response curves of the preparations precontracted with concentrations of PILO (5 x 10^-6) were more on the left, as compared to the preparations precontracted with CARB (10^-6), indicating increased sensitivity of these preparations to the relaxing agent. This was most pronounced in ROC, METHO and SCC preparations (Table 1 and 2).

Effects of NMBD on electrically induced tracheal smooth muscle tone

Electrical field stimulation produced twitch contractions that increased only slightly (although p<0.05) after the administration of lower concentrations of NMBD indicating minimal, if at all, M2 receptor block (Table 3a). Higher concentrations of the agents produced partial or complete relaxation of the preparations (Table 3b). Mivacurium had little effect in the concentration used and its EC50 could not be calculated.

**Fig. 2.** Muscarinic antagonists’ and neuromuscular blocking drugs (NMBDs) dose-response curves following precontraction elicited with (a) CARB 10^-6 M or (b) PILO 5x10^-6 M which was taken as 100% (Tmax). Values are presented as means±/s.e. mean.

**Effects of stimulation with low dose of pilocarpine on muscle relaxation**

This was done to test for the effects of selective stimulation of the pre-junctional M2 muscarinic receptors and inhibition of relaxation that could be mediated by pre-junctional M2 receptor. Preincubation with low concentrations of PILO (10^-6 M) either before or after carbachol precontraction reached a plateau, did not influence PANC induced relaxation, as compared to the controls (PANC, EC50; 30 min, 10 min preincubation, and control: 6.21±0.15; 6.06±0.44; 6.03±0.041; mean±SD; differences not significant). This indicated that low concentration of PILO was not producing visible prejunctional inhibition.

**DISCUSSION**

The present study aimed to depict existence of potential facilitation of the smooth muscle constriction produced by various contractile stimuli, by concomitant application of some most commonly used NMBDs. This should have revealed the potential risk from bronchoconstriction - if one existed, by some most
Table 1. EC_{50} of skeletal muscle relaxants in carbachol or pilocarpine pre-contracted rat tracheal preparations.

<table>
<thead>
<tr>
<th>CASB 5x10^{-7}M (n)</th>
<th>CASB 10^{-6}M (n)</th>
<th>Pilocarpine 5x10^{-5}M (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rocuronium</td>
<td>5.83 +/-1.26 (7)</td>
<td>4.84 +/-0.64 (7)</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>5.57 +/-1.02 (6)</td>
<td>5.59 +/-0.49 (5)</td>
</tr>
<tr>
<td>Mivacurium</td>
<td>5.41 +/-0.35 (5)</td>
<td>5.77 +/-0.72 (5)</td>
</tr>
<tr>
<td>Pancuronium</td>
<td>6.72 +/-0.56 (6)</td>
<td>6.03 +/-0.41 (6)</td>
</tr>
</tbody>
</table>

Values are presented as means +/-SD of log EC_{50} values obtained in tracheal rings precontracted with CASB 5x10^{-7}M, CASB 10^{-6} M and PILO 5x10^{-5} M, and relaxed with NMBD. Obtained by non-linear regression (Mean +/-SD). * indicating significant differences for comparison between NMBD within given column, (ANOVA) with Student's t-test for paired and unpaired data (corrected for multiple comparisons); n: number of preparations. Some reported peak serum concentrations (M): Rocuronium 2.8x10^{-5} (human; cited by Appadu et al. (26)), Cisatracurium 1.13x10^{-6} (human; cited in: Jooste et al. (10)), Mivacurium 1.4x10^{-6} (human; Basta et al. (27)), Pancuronium 4x10^{-6} (human; as cited by Appadu et al. (26)).

For comparison, the results obtained with pure NMBD in tracheal rings precontracted with CASB 10^{-6} M were as follows (EC_{50} +/-SD): Rocuronium 4.72 +/-0.15 (n=5), Cisatracurium 5.27 +/-0.31 (n=5), Mivacurium 4.23 +/-0.69 (n=5), Pancuronium 6.13 +/-0.35 (n=6).

Table 2. EC_{50} of some neuromuscular blocking drugs (NMBD) agents and muscarinic antagonists in carbachol or pilocarpine pre-contracted rat tracheal preparations.

<table>
<thead>
<tr>
<th>CASB 10^{-6}M (n)</th>
<th>Pilocarpine 5x10^{-5}M (n)</th>
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<tbody>
<tr>
<td>Succinylcholine</td>
<td>2.9 +/-0.63 (9)</td>
</tr>
<tr>
<td>Gallamine</td>
<td>5.18 +/-0.24 (7)</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>6.45 +/-0.45 (6)</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.22 +/-0.22 (11)</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>9.8 +/-0.64 (6)</td>
</tr>
</tbody>
</table>

Values are presented as means +/-SD of log EC_{50} values obtained in tracheal rings precontracted with carbachol 10^{-6} M and pilocarpine 5x10^{-5} M, and relaxed with NMBDs or other muscarinic antagonists. * indicates significant differences for comparison. n: number of preparations. (Full dose-response curve for SCC could not be produced and calculated EC_{50} value is less reliable).

Effects of NMBD and muscarinic antagonists on tracheal smooth muscle tone

When comparing CASB and PILO preconstricted preparations (Table 1 and 2), the relaxing effects of each particular NMBD and muscarinic antagonist in these preparations, were similar. Since the PILO is a M_{2} agonist, and CASB a non-selective muscarinic agonist, this indicates high importance of M_{3} postjunctional receptors for tracheal smooth muscle contraction (17-20). The exceptions were PILO precontracted preparations which were more sensitive to ROC METHO and SCC (Table 1 and 2). This could indicate slightly more of M_{3} receptor inhibition by these agents.

The efficiency of almost all NMBD in CASB preconstricted tracheas in these experiments corresponded roughly to the binding results reported by Hou et al. (13). In our experiment we found that the preparations were the most sensitive to PANC, and less sensitive to MIV, GAL and SCC in the given order, which might but does not necessarily indicate lesser importance of M_{3} over M_{1} receptors in the postsynaptic membrane for muscle relaxation with NMBD in TSM of the rats. In addition, the findings of Chiba et al. (21) in rat bronchus are also in accordance with our conclusion. Out of all NMBD tested, PANC was the strongest as relaxant of the preparations precontracted with EFS. Presynaptic M_{2} block could initiate bronchospasm (by facilitating acetylcholine liberation) and PAN, as at the same time a potent M_{3} antagonist, could inhibit bronchoconstriction, despite of the M_{3} presynaptic muscarinic receptor blockade (11), as already suggested (13).

Precontractions with low versus higher concentrations of CASB

CASB seems to be more potent as a M_{3} agonist in the rat trachea (22), at least when used in lower concentrations. We presumed that precontraction with lower concentration of CASB.
would engage a subtype of muscarinic receptors for which it has more specificity. Block of these receptors (by NMBD) should have produced more important displacement of the corresponding dose-response curves of the agents that are more specific for these particular receptors. In the preparations precontracted with CARB 5x10^-7M, as compared to those precontracted with CARB 10^-6M, we found that only dose-response curves of ROC and PANC moved to the left, while CIS and MIV dose-response curves were not affected (Fig. 1). The fact that ROC and PANC appeared to be more powerful blocking agents in preparations precontracted with weak concentrations of CARB, could indicate that they acted predominantly on M1 muscarinic receptors. This assumption was further tested in the experiments using electrical field stimulation. Whether MIV and CIS carry a risk of insufficient M1 block and eventually provide lesser protection from bronchoconstriction, is to be further investigated. This is also in accordance with the suggestions of Jooste et al. concerning CIS (12).

Effects of NMBD on electrically induced tracheal smooth muscle tone

Lower concentrations of the all NMBD examined produced some increase of EFS elicited contraction, indicating some possible prejunctional M1 receptor block. Higher concentrations of these NMBD relaxed tracheal smooth muscle. The order of potency of pure substances, following EFS did not differ much from their potency when examined following CARB precontraction. Surprisingly the EC50 values were different and these agents appeared to have much weaker effects on EFS elicited contraction as compared to carbachol elicited contraction. This may be a result of compound effects on M2 prejunctional and M1 postjunctional receptors and acetylcholine liberation, together with the effects of other neuromediators (substance P, vasoactive intestinal polypeptide - VIP, calcitonine gene-related peptide - CGRP, neurokinin A and B, or other non-adrenergic non-cholinergic agents that could be liberated by the electrical stimulation (23). The finding that mivacurium has very low potency in inhibiting EFS elicited contractions of rat tracheal smooth muscle is interesting and merit further examination.

Effects of stimulation with low dose of pilocarpine on muscle relaxation

We wanted to examine the importance of M1 muscarinic receptor stimulation on the prejunctional membrane that would engage negative feedback and cause a decrease of acetylcholine secretion and thus promote better relaxation with NMBD. Our result indicates that in this model PILO was not producing visible effects on prejunctional receptors. The relative high concentration of carbachol probably produced strong contractile stimulation post-junctionally, and a small diminution of endogenous acetylcholine may have passed unobserved if existed.

Lack of selectivity of the muscarinic blocking agents makes the results of studies as this study, relatively difficult to interpret. It could be concluded, however, that the compound effect of pancuronium, rocuronium, mivacurium, and cisatracurium in rat airway is a relaxation, probably a resultant effects of stimulation of both, yet opposing, postjunctional M1 and M2 receptors. Although there are both M1 and M2 receptor subtypes in the human airways (10, 24), conclusions in respect to humans would not be warranted. In addition, the density of muscarinic receptors may vary among airway generations (10, 25) and an extrapolation from these results to the entire airways would also not be warranted. The skeletal muscle relaxants should be further examined in human airway preparations, as some of NMBD may appear more advantageous for the patients who present a risk of bronchoconstriction.

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