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Rapacuronium Preferentially Antagonizes the Function of M2 versus *M3 Muscarinic Receptors in Guinea Pig Airway Smooth Muscle*

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Background: **Rapacuronium, a nondepolarizing muscle relaxant that was proposed as a replacement for succinylcholine for rapid intubation, was withdrawn from clinical use as a result of fatal bronchospasm, but the mechanism of this effect is not known. Preferential antagonism of presynaptic M2 muscarinic receptors *versus* postsynpatic M3 muscarinic receptors can facilitate bronchoconstriction. The authors questioned whether rapacuronium preferentially antagonized M2 *versus* M3 muscarinic receptors in intact airway.**

Methods: **Guinea pig tracheal rings were suspended in organ baths and muscle relaxants' antagonism of prejunctional M2 muscarinic autoreceptors was evaluated by augmentation of muscle contraction elicited by electrical field stimulation. Muscle relaxants' antagonism of postjunctional M3 muscarinic receptors was assessed by attenuation of muscle contraction elicited by acetylcholine.**

Results: **Rapacuronium displayed a 50-fold higher affinity for antagonism of the M2 *versus* M3 muscarinic receptor. Moreover, its affinity for the M2 but not the M3 receptor was within concentrations achieved clinically. In addition, rapacuronium caused an increase in baseline tone of airway smooth muscle that was antagonized by atropine but not by previous depletion of nonadrenergic noncholinergic neurotransmitters or by inhibitors of histamine receptors, tachykinin receptors, leukotriene receptors, or calcium channels.**

Conclusion: **These findings are consistent with the hypothesis that rapacuronium may precipitate bronchoconstriction by selective antagonism of the M2 muscarinic receptor on parasympathetic nerves, enhancing acetylcholine release to act upon unopposed M3 muscarinic receptors on airway muscle. An additional mechanism of rapacuronium-induced bronchoconstriction is suggested by increases in baseline muscle tension.**

NEUROMUSCULAR blocking agents are utilized to facilitate tracheal intubation and to maintain muscle relaxation during many surgical procedures. Because of numerous undesirable side effects of succinylcholine the search has continued for a nondepolarizing muscle relaxant than can achieve optimal intubation conditions rapidly with a rapid termination of action in the event of difficulties in managing the airway.

An ultra-short acting nondepolarizing neuromuscular blocking agent, rapacuronium, was introduced clinically to replace succinylcholine for rapid sequence inductions but was withdrawn from clinical use because of frequent incidence of severe bronchospasm and at least five fatalities attributed to irreversible bronchoconstriction.[1–5] Despite its withdrawal from clinical use it is imperative that we understand the mechanism of rapacuronium-induced bronchoconstriction so that newly synthesized neuromuscular blocking agents[6] that might share the same effect will not be introduced clinically.

Bronchospasm during general anesthesia is a potentially life-threatening event. Histamine release is one known risk factor for bronchospasm, and it has been attributed to older neuromuscular blocking agents such as curare and atracurium. An additional mechanism that can facilitate bronchoconstriction during the use of neuromuscular blocking agents and instrumentation of the airway involves neuromuscular blocking agents' interaction with muscarinic receptors in airway nerves and smooth muscle. Instrumentation of the well-innervated upper trachea initiates a reflex that results in the release of acetylcholine from parasympathetic nerves that act on M2 and M3 muscarinic receptors in airway smooth muscle, resulting in bronchoconstriction. Normally the release of acetylcholine is terminated by acetylcholine acting on M2 muscarinic auto-feedback receptors present in the presynaptic terminals of postganglionic parasympathetic nerves.[7,8] However, nondepolarizing muscle relaxants are known to have differential antagonistic affinities for muscarinic receptor subtypes.[9,10] Agents that have a higher affinity for the M2 *versus* the M3 muscarinic receptor can lead to selective inhibition of M2 muscarinic auto-receptors on parasympathetic nerves during periods of parasympathetic nerve stimulation[11] (*e.g.*, intubation) and result in the massive release of acetylcholine to act on unopposed M3 receptors in airway smooth muscle, thereby facilitating bronchospasm.

Therefore the characteristics of a muscle relaxant that would be expected to potentiate vagally induced bronchoconstriction include a higher affinity for the M2 *versus* M3 muscarinic receptors, an affinity for the M2 muscarinic receptor but not the M3 muscarinic receptor within clinically achieved concentration ranges, and use of an intubating dose of this muscle relaxant during a period of heightened parasympathetic tone (*e.g.*, intubation).

In previous radioligand binding assays using cell lines that express M2 or M3 muscarinic receptors, rapacuronium was found to have a higher affinity for M2 *versus*

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M3 muscarinic receptors.[9] However, these studies were not performed in intact functional airway smooth muscle but in cell lines overexpressing muscarinic receptor subtypes. The current studies were designed to address the hypothesis that rapacuronium has detrimental effects mediated by muscarinic receptors in intact functional airways. Therefore, in the current study we questioned whether in intact guinea pig airway tissue rapacuronium potentiated electrical field stimulated nerve-induced contractions, had a higher functional antagonism for the M2 *versus* M3 muscarinic receptor, and itself increased smooth muscle tension by a mechanism independent of preferential M2 muscarinic receptor antagonism.

Materials and Methods

Reagents

Clinical formulations of rapacuronium, rocuronium, vecuronium (Organon, West Orange, NJ); cisatracurium (GlaxoSmithKline, Research Triangle Park, NC) and mivacurium (Abbott Laboratories, North Chicago, IL) were used in these studies. Control vehicle solutions were prepared according to manufacturer product inserts. Methoctramine, pancuronium, gallamine, tetrodotoxin, diphenhydramine, ranitidine, nifedipine, pyrilamine, N-vanillylnonanamide (capsaicin analog), and atropine were purchased from Sigma Chemical (St. Louis, MO). The peptide MEN10376 (NK_2 antagonist), sendide (NK_1 antagonist), BAY-u9773 (nonselective leukotriene antagonist), and MK571 (D4 leukotriene antagonist) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All drugs were dissolved in distilled water, except for nifedipine, which was dissolved in dimethyl sulfoxide such that the final concentration of dimethyl sulfoxide in the baths was 0.01%.

Electrical Field Stimulation-induced Contractions of Guinea Pig Trachea to Characterize Muscarinic M2 Receptor Antagonism

All animal protocols were approved by the Columbia University Animal Care and Use Committee. Hartley male guinea pigs weighing approximately 400 g were anesthetized with 50 mg intraperitoneal pentobarbital, and the tracheas were removed promptly and dissected into closed rings comprised of two cartilaginous rings from which mucosa, connective tissue, and epithelium were removed. Silk threads were tied to the rings such that the threads were at each end of the posterior aspect of the ring (lacking in cartilage), approximately 180° from one another. One thread was attached to a fixed point at the bottom of the baths and the opposing thread was attached to a Grass force transducer (Grass-Telefactor, West Warwick, RI) coupled to a computer *via* BioPac hardware and Acknowledge 7.3.3 software (Biopac Systems, Inc., Goleta, CA) for continuous digital recording of muscle tension. The rings were suspended in 4 ml organ baths in Krebs-Henseleit buffer solution (composition in mM: NaCl 118, KCl 5.6, $CaCl_2$ 0.5, $MgSO_4$ 0.2, $NaHCO_3$ 25, NaH_2PO_4 1.3, D-glucose 5.6) with 10 μM indomethacin (dimethyl sulfoxide vehicle final concentration of 0.01%) added and gassed with 95% oxygen and 5% carbon dioxide at a pH 7.4 and a temperature of 37°C. The rings were equilibrated at 1g of isotonic tension for 1 h with new Krebs-Henseleit buffer added every 15 min. After equilibration, the preparations were precontracted twice with a full dose response of acetylcholine (0.1um to 1 mM). After multiple washes (6–9 times) of Krebs-Henseleit buffer solution to stabilize the baseline, electrical field stimulation (EFS) was delivered by constant voltage *via* two platinum electrodes on opposite sides of the preparation about 0.8 cm apart by a Grass RPS 107 stimulator (Grass-Telefactor) with signal enhancement through a Med-Lab Stimu-Splitter II (Grass-Telefactor) at 24V, pulse duration 0.5 ms, frequency 32 Hz, train duration of 5 s every 80 s.[12–14] These stimulation parameters were chosen on the basis of the literature and preliminary results to ensure stable twitch contractions and sensitivity to modulation. After a minimum period of 60 min and when the EFS induced contraction height had become constant, cumulative doses of muscle relaxants were added to the baths to determine the 50% inhibitory concentration (IC_{50}) at which tension of contractions was accentuated by antagonism of the presynaptic M2 muscarinic autoreceptors.[12,15] The effect of muscle relaxants on the EFS-induced contraction was expressed as a percentage of the stable EFS-induced contraction before muscle relaxants were added. To confirm that contractions elicited by the EFS parameters were attributable to neural release of acetylcholine acting on muscarinic receptors, preliminary studies were performed with methoctramine, a well known M2 muscarinic receptor antagonist, tetrodotoxin (1 μM), a sodium channel blocking agent, and atropine (1 μM) a nonselective antimuscarinic agent.

Acetylcholine-induced Contractions of Guinea Pig Trachea to Characterize Muscarinic M3 Receptor Antagonism

Guinea pig trachea rings were prepared as above. After two cycles of acetylcholine-induced contractions (cumulative concentrations of 0.1 μM to 1 mM) the rings were repetitively washed (6–9 buffer changes) and the rings were repetitively contracted with a single dose of acetylcholine approximately equal to the EC_{50} of acetylcholine (typically $\sim$1 μM) until the contraction height was consistent. Increasing doses of muscle relaxants were added, allowing a 35-min incubation period between doses to allow for each concentration to reach apparent equilibrium before the muscle was contracted with an EC_{50} acetylcholine dose. Increasing muscle relaxant

doses were used to determine IC_{50} concentrations at which tension of contractions was attenuated by muscarinic M3 receptor antagonism.

In additional preliminary experiments with muscle relaxants (rapacuronium, vecuronium, and pancuronium), a second method was used to determine the IC_{50} at which these muscle relaxants blocked the M3 muscarinic receptor. After two cycles of acetylcholine-induced contractions with cumulative concentrations of acetylcholine and washing, EFS was initiated until the resultant contraction became consistent. Increasing concentrations of rapacuronium or vecuronium were added, allowing a 35 min incubation period between doses to achieve equilibrium, after which time a burst of five EFS-induced contractions were elicited. The concentration of rapacuronium or vecuronium that antagonized the EFS-induced contractions[12,15] was compared with the concentration that antagonized acetylcholine-induced contractions.

To address the possibility that postjunctional M2 muscarinic receptor antagonism contributed to the calculated IC_{50} of rapacuronium acting at postjunctional M3 muscarinic receptors, rapacuronium-induced decrease of acetylcholine-mediated contraction was repeated in the presence of the selective M2 antagonist methoctramine. The methoctramine concentration that effectively antagonized the M2 muscarinic receptor in this preparation was first defined by measuring augmentation of EFS-induced contractions. Furthermore, it was determined whether the selected concentration of methoctramine (0.5 μM) inhibited acetylcholine-induced contractions. A second experimental strategy was also employed to address the potential contribution of antagonism of postjunctional M2 muscarinic receptors and relaxation of acetylcholine-induced contractions. Methoctramine (1 nM–100 μM) or 4-DAMP (0.1 nM–1 μM) were added in half-log increasing increments 15 min before the addition of an EC_{50} concentration of acetylcholine. The resulting decrease in acetylcholine-induced muscle tension was recorded and IC_{50} values were calculated.

Characterization of the Effect of Rapacuronium on Baseline Resting Tone of Tracheal Rings

Guinea pig tracheal rings were prepared as above. Rings were either not precontracted or were subjected to two cycles of acetylcholine-induced contractions (increasing cumulative concentrations of acetylcholine 0.1 μM–1.0 mM) followed by two washing protocols: washed only three times or washed 6–9 times with Krebs-Henseleit buffer. Cumulative increasing concentrations of rapacuronium (1 nM–100 μM) were then added to the organ baths. Once the rapacuronium-induced increase in baseline tension was achieved, antagonists were added in an attempt to reverse the increase in baseline tone. Atropine (1 μM) (muscarinic antagonist), pyrilamine (10 μM) (H_1 histamine receptor antagonist), diphenhydramine (1 μM) (H_2 histamine receptor antagonist), nifedipine (10 μM) (calcium channel blocker), sendide (30 μM) (NK_1 receptor antagonist), MEN10376 (30 μM) (NK_2 receptor antagonist), MK571 (10 μM) (leukotriene D_4 antagonist), or BAY-u9773 (10 μM) (nonselective leukotriene antagonist) were added to individual baths in an attempt to antagonize this rapacuronium-induced increase in baseline tone.

In a separate series of experiments these antagonists were added after the acetylcholine-induced contractions and washing but 30 min before the addition of cumulatively increasing concentrations of rapacuronium.

In an attempt to determine if rapacuronium was increasing baseline tone by activation of noncholinergic, nonadrenergic (NANC) nerves, N-vanillylnonanamide (10 μM) (capsaicin analog) was added first to activate and deplete NANC contractile tachykinins.[14] After N-vanillylnonanamide-induced tension had returned to baseline (60–90 min) rapacuronium was added to determine if tension still increased after depletion of NANC neurotransmitters. To confirm adequate depletion of contractile NANC neurotransmitters control tissues received a repeated dose of N-vanillylnonanamide that did not result in a repeated contraction.

Data Analysis

Smooth muscle tension was measured in Acqknowledge 3.7.3 software (Biopac Systems Inc., Goleta, CA) and muscle relaxant IC_{50}s for potentiating EFS effects at M2 muscarinic receptors or inhibiting acetylcholine-induced contractions at M3 muscarinic receptors were calculated by nonlinear regression analysis using Prism 3.0 software (GraphPad Software, Inc., San Diego, CA). All values are given as mean ± SD. In all experiments n represents the number of individual tracheal rings studied.

Results

It is well known that antagonism of presynaptic M2 muscarinic receptors results in a potentiation of EFS-induced contractions. This is the result of blockade of the M2 muscarinic receptor that normally functions in a negative auto-feedback mode after stimulation of parasympathetic nerves and release of acetylcholine. Figure 1 is a representative tension recording of guinea pig airway smooth muscle after two cycles of preexposure to cumulative increases in acetylcholine followed by the initiation of EFS. Gallamine (a known M2 antagonist) and rapacuronium caused an increase in the magnitude of the EFS-induced contraction (at approximately 1 μM) whereas mivacurium required higher concentrations to accentuate the EFS-induced contraction (fig. 1). IC_{50}s for all the relaxants tested are presented in table 1 with a rank order of potency for this M2 muscarinic receptor

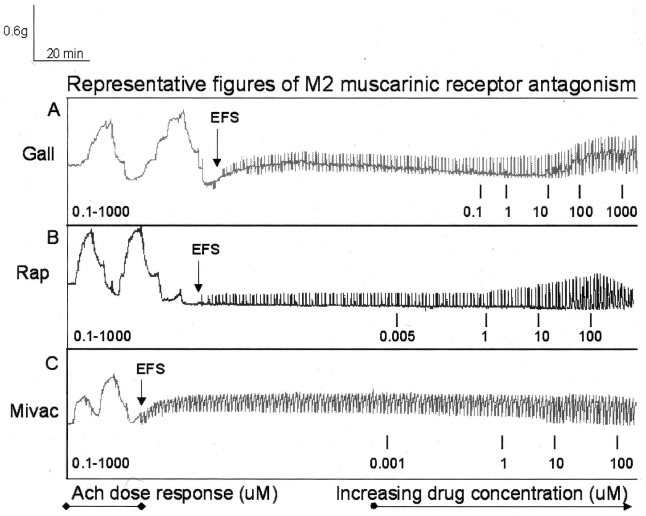

Fig. 1. Representative tension measurements of guinea pig tracheal rings suspended in organ baths illustrating muscle relaxant M2 muscarinic receptor antagonism resulting in an increase contraction elicited by electrical field stimulation (EFS). After two cycles of precontractions induced by acetylcholine (0.1–1000 μM) and washing, EFS was initiated. (*A*) Gallamine (Gall), a muscle relaxant with known M2 muscarinic receptor effects, increased EFS-induced contraction at concentrations 10 μM and 1000 μM. (*B*) Rapacuronium (Rap) increased EFS-induced contractions at concentrations between 1 μM and 100 μM. (*C*) Mivacurium (Mivac) exhibited minimal effects on EFS-induced contractions. Shown are representative tracings for 6–8 experiments performed for each muscle relaxant.

antagonism of pancuronium, rapacuronium, vecuronium, cisatracurium, mivacurium, and rocuronium. The IC_{50}s for the M2 muscarinic receptor were well within the concentrations achieved clinically for rapacuronium and pancuronium but greater than the clinical concentrations typically achieved with vecuronium or rocuronium (fig. 2).

Methoctramine, as expected, potentiated the EFS-induced contraction as a result of its presynaptic M2 muscarinic receptor antagonism, with a peak effect at 0.5 μM. Tetrodotoxin (1 μM) completely abolished the EFS-induced contractions, confirming that the contractions were neural in nature and not attributable to direct muscle stimulation. Atropine (1 μM) also abolished both EFS-induced and acetylcholine-induced contractions confirming the role of muscarinic receptors in these contractions. Studies performed with the vehicles in which the muscle relaxants are supplied showed no effect on the EFS-induced contractions.

Table 1. Comparative functional activities of muscle relaxants for antagonism of M2 or M3 muscarinic receptors

Muscle relaxants	IC_{50} at M2 with EFS (μM)	IC_{50} at M3 with Ach (μM)	Reported plasma concentration (μM)
Rapacuronium	2.4 ± 1.9	125.3 ± 11	7.39–59.1[17;37]
Vecuronium	5.6 ± 3.1	279.0 ± 90	0.63–3.14[38]
Cisatracurium	7.8 ± 2.9	>240	0.08–1.13[39]
Rocuronium	27.8 ± 16.7	572.5 ± 146	4.7 (peak serum)[40]
Mivacurium	25.6 ± 14.9	>150	1.4 (peak serum)[41]
Pancuronium	0.6 ± 0.6	6.7 ± 5.5	2.2–4[40]
Gallamine	5.7 ± 1.5	>1000	81 (peak serum)[42]

Ach = acetylcholine; EFS = electrical field stimulation; M2 = M2 muscarinic receptors; M3 = M3 muscarinic receptors.

Initially, two methods were compared for measuring the affinity of rapacuronium, pancuronium, or vecuronium for the M3 muscarinic receptor.[12,15] Representative tracings of these two methods, attenuation of the height of the contraction elicited by EFS or attenuation of the height of the contraction elicited by a single dose (approximately the EC_{50}) of acetylcholine, are presented in figure 3. Pancuronium (*top panel,* fig. 3) and rapacuronium (*middle panel,* fig. 3) initially augmented the height of the contraction elicited by EFS (evidence of their M2 muscarinic receptor antagonism). However, at higher concentrations both pancuronium and rapacuronium attenuated the height of the EFS-induced contraction as a result of postjunctional M3 muscarinic receptor antagonism. Increasing concentrations of rapacuronium (*bottom panel,* fig. 3) attenuated the height of the acetylcholine-induced contractions. The IC_{50} for rapacuronium at the M3 muscarinic receptor was 125 ± 7.8 μM and 152 ± 10.7 μM measured by inhibiting acetylcholine-induced or EFS-induced contractions, respectively, whereas the IC_{50} for vecuronium at the M3 muscarinic receptor was 279 ± 51.9 μM and 214 ± 6.3 μM measured by inhibiting acetylcholine-induced or EFS-induced contractions, respectively. As these methods yielded similar results, one method (attenuation of acetylcholine-induced contraction) was used for subsequent studies.

To address the possibility that antagonism of postjunctional M2 muscarinic receptors may have contributed to the calculated IC_{50} for rapacuronium's inhibition of acetylcholine-induced contraction, studies were repeated in the presence of 0.5 μM methoctramine (a concentration shown in preliminary studies to augment EFS-induced contraction indicative of M2 antagonism). The IC_{50} for rapacuronium's inhibition of acetylcholine-induced contraction was not different ($P = 0.18$) in the absence or presence of this concentration of methoctramine (125 ± 7.8 μM [n = 4] and 98.0 ± 26.3 μM [n = 3], respectively). In addition, we compared the ability of classic and selective M2 (methoctramine) and M3 (4-DAMP) muscarinic receptor antagonists to inhibit acetylcholine induced contractions in this preparation. 4-DAMP demonstrated dose-dependent inhibition of acetylcholine-induced contractions with an IC_{50} of 4 nM and complete inhibition at 50 nM, but methoctramine did not demonstrate dose-dependent inhibition and even at very high and nonselective concentrations (1 μM) demonstrated only a 25% inhibition of acetylcholine-induced contractions.

The potencies of muscle relaxants for attenuating the acetylcholine-induced contractions are presented in table 1 and figure 4 with a rank order of potency of pancuronium, rapacuronium, vecuronium, and rocuro-

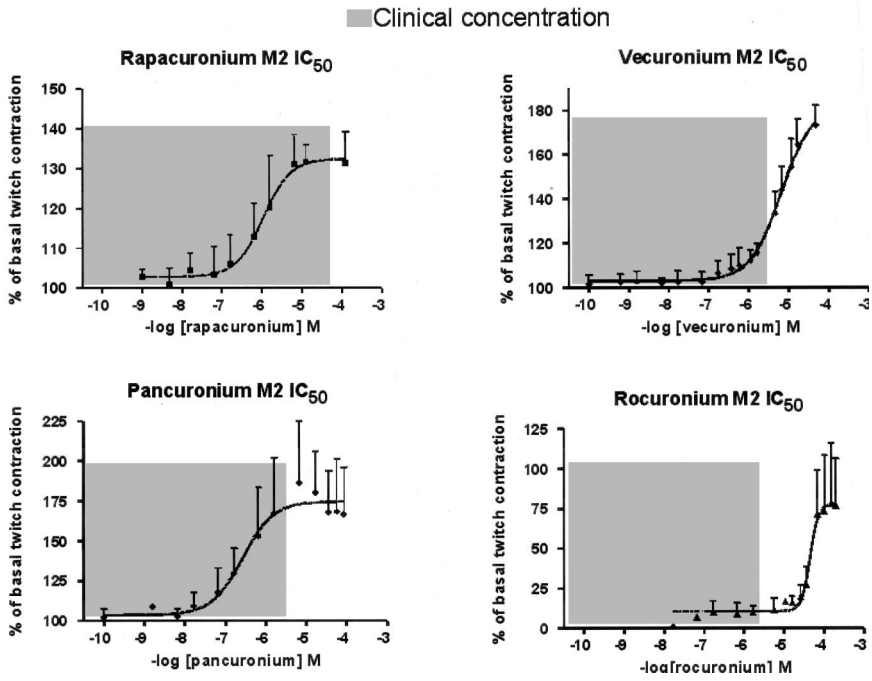

Fig. 2. Nonlinear regression analysis results of muscle relaxants abilities to augment EFS-induced contraction *via* M2 muscarinic receptor antagonism. *Shaded areas* of each graph indicate clinical plasma concentrations reported. Rapacuronium and pancuronium's 50% inhibitory concentrations are well within clinical achieved concentrations. Data represents means ± SD from 6–8 experiments.

nium. The potencies of cisatracurium, mivacurium, and gallamine for the M3 muscarinic receptor were so low that sufficient concentrations could not be reached to attenuate acetylcholine-induced contractions (table 1). Although pancuronium, rapacuronium, vecuronium, and rocuronium have measurable potencies, only pancuronium had an M3 muscarinic receptor potency within clinically relevant concentrations (fig. 4).

An unexpected finding was that rapacuronium caused an increase in baseline resting tension in the absence of EFS only if the tracheal rings had been subjected to contraction with acetylcholine followed by a limited amount of buffer changes. This effect of rapacuronium was eliminated if the baths were washed more extensively after the initial acetylcholine-induced contractions (6–9 washings instead of the usual three washings). In an attempt to elucidate the mechanism of this rapacuronium-induced increase in tone a series of inhibitors were used either before rapacuronium or after the rapacuronium-induced tension had reached a peak. Only atropine could antagonize or prevent the rapacuronium-induced increase in baseline tension. Inhibition of histamine, tachykinin or leukotriene receptors or calcium channels was without effect (data not shown). Furthermore, preactivation and depletion of NANC neurotransmitters by a capsaicin analog did not prevent the subsequent increase in baseline tone elicited by rapacuronium (data not shown).

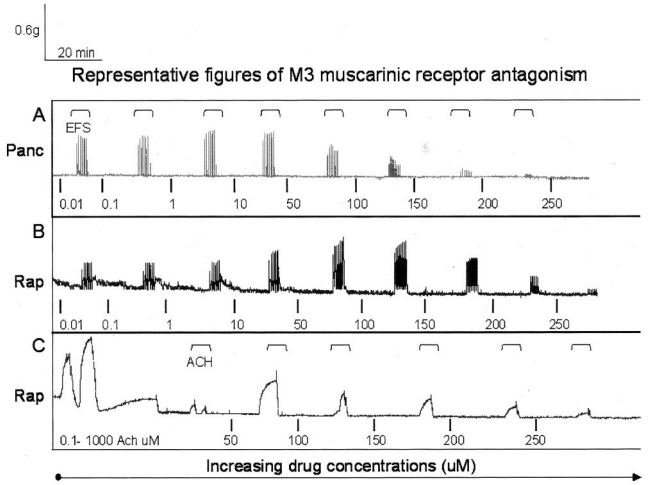

Fig. 3. Representative tension recordings of guinea pig tracheal ring preparations suspended in organ baths illustrating two methods for measuring the effect of M3 muscarinic receptor antagonism with increasing concentrations of pancuronium (Panc) or rapacuronium (Rap). The twitch response of electrical field stimulation (EFS) is attenuated by increasing concentrations of (*A*) pancuronium (Panc) or (*B*) rapacuronium (Rap). (*C*) The contraction height of a fixed acetylcholine concentration is attenuated by increasing concentrations of rapacuronium. Three to four experiments were performed for each muscle relaxant.

Discussion

The primary finding of this study is that although rapacuronium was found to be an antagonist at both M2 and M3 muscarinic receptors in intact guinea pig airway tissue, rapacuronium had an approximately 50-fold greater ability to potentiate muscle contraction through its M2 muscarinic receptor antagonism than its ability to inhibit contractions through its antagonism of the M3 muscarinic receptor. Moreover, the IC_{50} of rapacuronium for the M2 but not the M3 muscarinic receptor was well within a clinically relevant concentration range.[16–19]

In human airways, there is greater parasympathetic

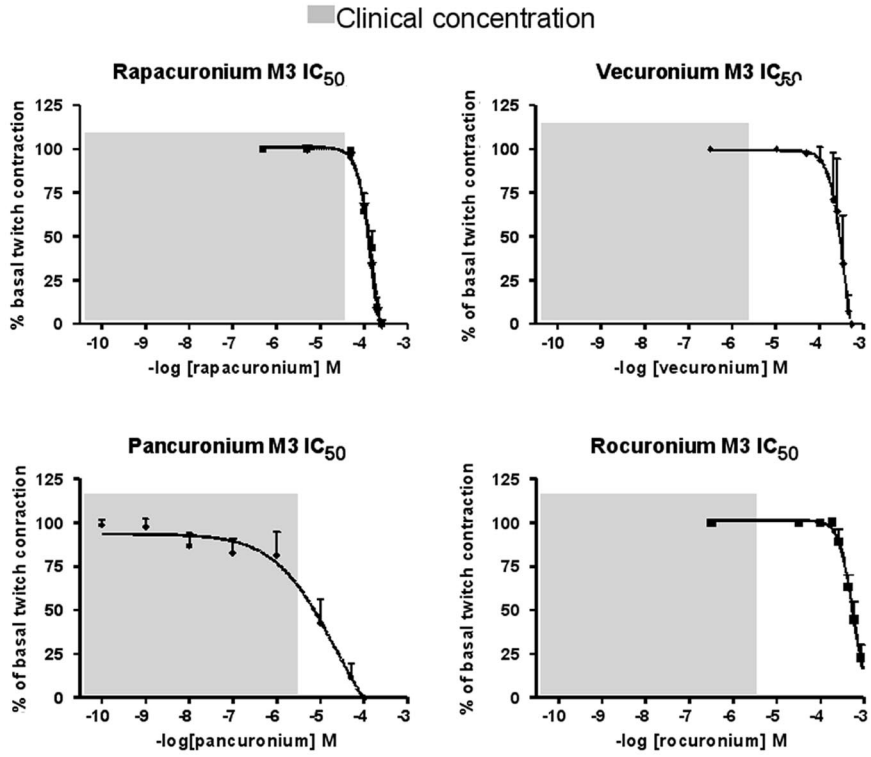

Fig. 4. Nonlinear regression analysis results of muscle relaxants abilities to attenuate acetylcholine-induced contractions *via* M3 muscarinic receptor antagonism. Shaded areas of each graph indicate clinical plasma concentrations reported. Only pancuronium's 50% inhibitory concentration was within clinical achieved concentrations. Data represents means ± SD from 3–4 experiments.

than sympathetic innervation in central airways. The presence of a foreign body such as an endotracheal tube or other irritating substance in these central airways activates irritant receptors that are found just beneath the epithelial lining of the airways, which leads to a heightened parasympathetic tone that in turn[20] results in rapid reflex changes in airway diameter. The afferent and efferent connections of these receptors that form the reflex arch travel in the vagus nerve. Acetylcholine administered exogenously or released from parasympathetic postganglionic nerves induces airway constriction by activating muscarinic receptors on airway smooth muscle.[21,21] Airway smooth muscle expresses both M2 and M3 muscarinic receptors. The M3 muscarinic receptors initiate contraction whereas M2 muscarinic receptors inhibit relaxation. Therefore, blockade of M3 muscarinic receptors by any drug in airway smooth muscle would be extremely beneficial, as it would inhibit both vagally induced and exogenously administered acetylcholine-induced airway constriction. In addition, M2 muscarinic receptors are expressed on presynaptic postganglionic nerves, where they function as negative feedback autoreceptors. After activation of parasympathetic nerve activity they function to attenuate further release of acetylcholine. Therefore, any drug with a preferential affinity to antagonize the M2 *versus* M3 muscarinic receptors at clinically relevant concentrations would interrupt this protective feedback mechanism, allowing for the uncontrolled excessive release of acetylcholine to act on unopposed M3 muscarinic receptors facilitating bronchoconstriction. Studies in animal models show that pancuronium and gallamine at clinically relevant concentrations can potentiate bronchospasm in the setting of vagally induced acetylcholine release.[22,23] Antagonism of presynaptic M2 muscarinic receptors was thought to be the mechanism of action.

Therefore, the criteria for a drug that could potentiate vagally induced bronchospasm are a drug with greater antagonism of the M2 *versus* M3 muscarinic receptor, M2 but not M3 antagonism at clinically relevant concentrations, and use of this drug during a period of intense parasympathetic stimulation (*e.g.*, intubation).

In the current study we measured the ability of muscle relaxants to either enhance (*via* prejunctional M2 muscarinic receptor antagonism) or antagonize (*via* postjunctional M3 muscarinic receptor antagonism) tension in closed guinea pig trachea rings suspended in organ baths. EFS is used to mimic vagal nerve activity in the airways. This method in intact tissue has been used extensively[24,25] to define M2 and M3 muscarinic antagonism in airway smooth muscle. M2 receptor antagonism leads to a potentiation of EFS-induced muscle contraction by inhibiting the negative feedback mechanism (fig. 1), whereas M3 receptor antagonism inhibits EFS induced muscle contraction by preventing muscle contraction (fig. 2). To further define and confirm the muscle relaxants M3 muscarinic antagonism we contracted the trachea rings with an EC_{50} dose of acetylcholine and then inhibited this contraction with increasing concentrations of muscle relaxants to antagonize acetylcholine's effect at the M3 muscarinic receptor on the muscle (fig. 2). These two methods (blockade of EFS-induced or

acetylcholine-induced contraction) yielded similar muscle relaxant IC_{50} values for antagonism of the postjunctional M3 muscarinic receptor.

In addition to its location on the prejunctional parasympathetic nerves, M2 muscarinic receptors are also expressed alongside M3 muscarinic receptors on the postjunctional smooth muscle. Although some studies in central airways suggest that the muscle M2 muscarinic receptor is less important for cholinergic contractions,[26] other studies in peripheral airways suggest a more important role for M2 muscarinic receptors in cholinergic contraction.[27] This distinction is important in our studies because we calculated an IC_{50} for rapacuronium's interaction with M3 muscarinic receptors. If both M2 and M3 postjunctional receptors contribute to reversal of acetylcholine-induced contractions, than our calculated IC_{50} would be a contribution from both receptor subtypes. However, our studies revealed a similar IC_{50} for rapacuronium's reversal of acetylcholine-induced contraction in the presence or absence of previous inhibition of M2 muscarinic receptors. This suggests that our calculated IC_{50} for rapacuronium truly represents an IC_{50} for the M3 muscarinic receptor. Moreover, we further demonstrated that a selective M3 muscarinic antagonist, 4-DAMP, dose-dependently inhibited acetylcholine-induced contractions with an IC_{50} of 4 nM and complete inhibition at 50 nM, whereas methoctramine did not demonstrate dose-dependent inhibition and even at high and nonselective concentations (1 μM) only inhibited acetylcholine-induced contractions by 25%. Taken together, these studies demonstrate that in the central airways of guinea pigs, M3 muscarinic receptors are responsible for acetylcholine-induced contractions and that our calculated muscle relaxant IC_{50} values represent M3 muscarinic receptor antagonism.

An intriguing and unexpected finding of these studies was that the baseline tone in the tracheal rings, in experiments with or without EFS, increased above baseline when rapacuronium was present in concentrations 10 μM and above only if there was residual acetylcholine present in the organ baths (*i.e.*, after an acetylcholine dose response had been performed and partially washed out). The mechanism of rapacuronium-induced increase in the presence of residual acetylcholine and its blockade by atropine is not clear. It is known that several classic neuromuscular blocking agents can have allosteric interactions with muscarinic receptor subtypes that modulate the interaction of acetylcholine with its orthosteric binding site.[28–30] A speculation that could explain rapacuronium-induced contraction with residual concentrations of acetylcholine would be an allosteric interaction of rapacuronium with the M3 muscarinic receptor that enhances acetylcholine's interaction with its orthosteric site (*i.e.*, positive cooperativity). Clinical observations are consistent with this speculation. Two recent studies demonstrated an increase in peak airway inflating pressures[31] or a decrease in maximal expiratory flow[32] with the administration of rapacuronium during steady state anesthesia in intubated patients. This suggests that during steady state general anesthesia rapacuronium is capable of increasing bronchial tone either by facilitating further acetylcholine release from postganglionic parasympathetic nerves or by acting at an allosteric site on airway smooth muscle, augmenting endogenous acetylcholine effects. Alternatively, rapacuronium may increase airway tone by other mechanisms such as histamine release. However, neither of these two studies reported other signs of histamine release and both studies proposed selective M2 muscarinic receptor antagonism as a mechanism to account for their findings.[31,32]

A further alternative mechanism to account for rapacuronium-induced bronchospasm is the release of histamine from mast cells lining the vessels into which the drug is given. Histamine release is described after the administration of several nondepolarizing muscle relaxants including curare, atracurium, and mivacurium.[33–35] However, in a study of 47 adult patients receiving rapacuronium during elective general anesthesia, seven developed bronchospasm without increases in plasma histamine concentrations.[36] Our inability in the current study to attenuate the rapacuronium-induced increase in baseline tone with histamine receptor antagonists is consistent with these findings. Therefore, it does not appear that histamine release can account for rapacuronium-induced bronchospasm. Although β_2-adrenoceptor antagonism is another potential mechanism to precipitate bronchospasm, there is also no current evidence that rapacuronium or any other clinically used muscle relaxants behave as β_2-adrenoceptor antagonists.

Despite rapacuronium's removal from clinical practice, it is important to understand the mechanism by which rapacuronium apparently contributed to fatal bronchospasm. All muscle relaxants developed to date have affinity for muscarinic receptors and it is likely that the next generation of muscle relaxants that attempt to replace succinylcholine will also have affinities for muscarinic receptors. Therefore, if the higher affinity of rapacuronium for the M2 *versus* the M3 muscarinic receptor accounts for induced bronchospasm, it seems prudent that all newly designed muscle relaxants should be evaluated for their potential to selectively inhibit M2 muscarinic receptors at clinically used concentrations. In summary, the bronchospasm caused by rapacuronium is likely in part attributable to its M2 > M3 muscarinic receptor affinity; however, additional mechanisms, such as allosteric interactions with muscarinic receptors, may contribute to rapacuronium's detrimental airway effects.

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