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Rapacuronium Augments Acetylcholine-induced Bronchoconstriction via Positive Allosteric Interactions at the M3 Muscarinic Receptor

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Background: **Neuromuscular blocking agents' detrimental airway effects may occur as a result of interactions with muscarinic receptors, allergic reactions, or histamine release. Rapacuronium, a nondepolarizing muscle relaxant, was withdrawn from clinical use because of its association with fatal bronchospasm. Despite its withdrawal from clinical use, it is imperative that the mechanism by which bronchospasm occurred is understood so that new muscle relaxants introduced to clinical practice do not share these same detrimental airway effects.**

Methods: **Airway smooth muscle force was measured in guinea pig tracheal rings in organ baths exposed to muscle relaxants with or without subthreshold concentrations of acetylcholine. Antagonism of muscarinic, histamine, neurokinin, leukotriene receptors, or blockade of L-type calcium channels or depletion of nonadrenergic, noncholinergic neurotransmitters was performed. Muscle relaxants' potentiation of acetylcholine-stimulated inositol phosphate synthesis and allosteric interactions on the kinetics of atropine-induced [^{3}H]N-methylscopolamine dissociation were measured in cells expressing recombinant human M3 muscarinic receptors.**

Results: **Rapacuronium, within clinically achieved concentrations, contracted tracheal rings in the presence but not in the absence of subthreshold concentrations of acetylcholine. This effect was prevented or reversed only by atropine. The allosteric action of rapacuronium was demonstrated by the slowing of atropine-induced dissociation of [^{3}H]N-methylscopolamine, and positive cooperativity was demonstrated by potentiation of acetylcholine-induced inositol phosphate synthesis.**

Conclusion: **Many muscle relaxants have allosteric properties at muscarinic receptors; however, positive cooperativity at the M3 muscarinic receptor within clinically relevant concentrations is unique to rapacuronium. These findings establish novel parameters that should be considered in the evaluation of airway safety of any newly synthesized neuromuscular blocking agents considered for clinical practice.**

NEUROMUSCULAR blocking agents are widely used in clinical anesthesia to rapidly achieve muscle relaxation for airway intubation and to facilitate some surgical procedures. Succinylcholine continues to be the drug of choice to rapidly achieve optimal intubation conditions, and its short duration of action is advantageous in the event that intubation of the airway is difficult. However, numerous clinical side effects of succinylcholine have stimulated the search for a nondepolarizing muscle relaxant with similar clinical properties. One such muscle relaxant was rapacuronium, which was used clinically for only a short period of time because of the development of severe bronchospasm that accompanied its use in some patients.[1–5] Despite its withdrawal from clinical use, it is imperative to understand the mechanism by which rapacuronium contributed to fatal bronchospasm so that new nondepolarizing muscle relaxants currently in development[6–8] do not share these same airway detrimental effects.

The intended clinical target of neuromuscular blocking agents is the nicotinic receptor on skeletal muscle. However, neuromuscular blocking agents also interact with muscarinic receptors[9–16] because nicotinic and muscarinic receptor share the same endogenous ligand (*i.e.*, acetylcholine). Muscarinic receptors are found on numerous cells within the airway, including postganglionic parasympathetic nerves and the airway smooth muscle, which these nerves innervate. Muscarinic receptors on the parasympathetic nerves are of the M2 subtype and normally function in an inhibitory autofeedback mode to prevent the further release of acetylcholine.[17] Muscarinic receptors on the airway smooth muscle are of the M2 and M3 subtypes, which function to inhibit relaxation and facilitate contraction, respectively.[18] Irritation of the upper trachea (*e.g.*, by the introduction of an endotracheal tube) initiates a neural reflex that results in the release of acetylcholine from parasympathetic nerves. The acetylcholine binds to M2 and M3 muscarinic receptors in airway smooth muscle, resulting in bronchoconstriction. Normally the release of acetylcholine is terminated by activation of presynaptic M2 muscarinic inhibitory autofeedback receptors on the parasympathetic nerves terminals.

We have previously proposed a scenario that could explain the detrimental airway effects of rapacuronium: selective inhibition of the M2 muscarinic receptor during a period of heightened parasympathetic nerve stimulation (*i.e.*, intubation).[10] Therefore, if large intubating doses of rapacuronium were administered, selective blockade of the M2 muscarinic receptor on the nerve would allow for enhanced acetylcholine release to act on relatively unopposed M3 muscarinic receptors on airway

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smooth muscle, resulting in enhanced bronchoconstriction. Indeed, we have shown that rapacuronium displays greater blockade of the M2 muscarinic receptor relative to the M3 muscarinic receptor in both radioligand binding[11] and airway functional assays.[10] However, several other muscle relaxants block M2 muscarinic receptors (*e.g.*, pancuronium, gallamine) within clinically relevant concentrations but have not elicited bronchospasm with the severity and prevalence that has been observed with rapacuronium. This suggests that rapacuronium may have detrimental effects on the airway in addition to M2 muscarinic receptor blockade. One possibility is that rapacuronium, like many other neuromuscular blocking agents (*e.g.*, pancuronium, gallamine, alcuronium), is an allosteric modulator at muscarinic receptors. Positive cooperativity with acetylcholine at the airway smooth muscle M3 muscarinic receptors would promote bronchoconstriction.[10,19–21]

It is well established that muscarinic receptors are susceptible to allosteric modulation by various pharmacologic compounds.[20,21] An allosteric effect is defined by the ability of a substance to bind to a site on the receptor (allosteric site) other than the classic ligand binding site (orthosteric site) and thereby effect the binding of the traditional ligand (*i.e.*, acetylcholine) at the orthosteric site. An allosteric substance can demonstrate positive (facilitating the binding of ligand to the orthosteric site), negative (inhibiting the binding of ligand to the orthosteric site), or neutral (not effecting binding of ligand to orthosteric site but occupying the allosteric site instead of another allosteric substance) cooperativity. Indeed, two nondepolarizing muscle relaxants no longer used clinically, gallamine and alcuronium, are used as classic allosteric modulators for certain muscarinic receptor subtypes.[20,21] Conceptually, allosteric interactions of muscle relaxants with muscarinic receptors in the airways could be of immense clinical importance because these drugs could modulate the effects of acetylcholine in either a beneficial or a detrimental manner.

Therefore, in the current study, we used intact guinea pig tracheal rings and recombinantly expressed human M3 muscarinic receptors to determine whether rapacuronium exhibited an allosteric effect with positive cooperativity at M3 muscarinic receptors.

Materials and Methods

Reagents

Clinical formulations of rapacuronium and vecuronium (Organon, West Orange, NJ) were used in these studies. Control vehicle solutions were prepared according to the manufacturer's product insert. Methoctramine, tetrodotoxin, ranitidine, nifedipine, pyrilamine, N-vanillylnonanamide (capsaicin analog), and atropine were purchased from Sigma (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). [^{3}H]N-methylscopolamine (^{3}H-NMS; 81 Ci/mmol) was purchased from PerkinElmer (Boston, MA). [^{3}H]-*myo*-inositol (20 Ci/mmol) was purchased from MP Biomedicals (Irvine, CA). 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%.

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

All studies were approved by the Institutional Animal Care and Use Committee (College of Physicians and Surgeons, Columbia University, New York, New York). Force measurements were performed on closed guinea pig tracheal rings suspended in organ baths as previously described.[10] Briefly, Hartley male guinea pigs (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. Trachea were attached using silk threads to a fixed tissue hook in a 2-ml bath (Radnoti Glass Technology, Inc., Monrovia, CA) and a Grass (Grass Telefactor, West Warwick, RI) force transducer coupled to a computer *via* BioPac hardware and Acqknowledge 7.3.3 software (Biopac Systems, Inc., Goleta, CA) for continuous digital recording of muscle force. Rings were equilibrated at 1 g isotonic force for 1 h with new Krebs-Henseleit buffer[10] (including 10 μM indomethacin) (pH 7.4, 37°C) added every 15 min while gassed with 95% oxygen and 5% carbon dioxide.

Rings were subjected to (1) two cycles of acetylcholine-induced contractions (increasing cumulative concentrations of 0.1 μM–1.0 mM acetylcholine) followed by two washing protocols: washed only three times or washed nine times with Krebs-Henseleit buffer; (2) a single acetylcholine concentration (0.1 μM); or (3) no pretreatment. Cumulatively increasing concentrations of rapacuronium (1 nM–100 μM), vecuronium (10 nM–100 μM), rocuronium (10 nM–200 μM), or pancuronium (100 nM–100 μM) were then added to the organ baths. In the studies where rapacuronium caused an increase in baseline force, antagonists were added at the peak of contraction in an attempt to reverse the increase in baseline tone. The antagonists included atropine (1 μM), pyrilamine (10 μM; H_1 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) and were added to individual baths in an

attempt to antagonize this rapacuronium-induced increase in baseline tone.

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

To determine whether rapacuronium was increasing baseline tone by activation of nonadrenergic, noncholinergic (NANC) nerves, N-vanillylnonanamide (10 μM; capsaicin analog) was added first to activate and deplete NANC contractile tachykinins. After N-vanillylnonanamide–induced force had returned to baseline (60–90 min), rapacuronium was added to determine whether force increased despite depletion of NANC neurotransmitters. To confirm adequate depletion of contractile NANC neurotransmitters, control tissues received a repeated dose of N-vanillylnonanamide.

Inositol Phosphate Assays

The M3 muscarinic receptor classically couples to the stimulation of inositol phosphate synthesis, and this assay was thus used as a measure of M3 receptor function in response to acetylcholine in the absence and presence of rapacuronium. Synthesis of total ^{3}H-inositol phosphates was measured[22] in Chinese hamster ovary (CHO) cells stably expressing the human M3 muscarinic receptor (a kind gift from Tom I. Bonner, Ph.D., Senior Investigator, Laboratory of Genetics, National Institutes of Health, Bethesda, Maryland)[23,24] grown to confluence[9] in 24-well tissue culture plates. Briefly, after overnight loading with ^{3}H-*myo*-inositol (10 μCi/ml, 20 Ci/mmol) in inositol-free and serum-free Dulbecco's modified Eagle's medium, plates were washed three times (37°C, 500 μl Hanks balanced salt solution with 10 mM LiCl). Incubation of cells in a final volume of 300 μl at 37°C for 30 min was performed in the absence and presence of 1 μM acetylcholine. Parallel wells were incubated with 1 μM acetylcholine plus a range of concentrations (0.1–10 μM) of rapacuronium. Reactions were terminated, and total [^{3}H]inositol phosphates recovered by chromatography as described.[22] To confirm that the effects of rapacuronium (and acetylcholine) on inositol phosphate synthesis was *via* the M3 muscarinic receptor, two types of control experiments were performed. The M3 muscarinic receptor expressed in CHO-M3 cells was inactivated by alkylation with 1 μM 4-DAMP mustard for 1 h before exposure to acetylcholine or rapacuronium. In a separate cell line that expresses the human M2 but not the M3 muscarinic receptor (CHO-M2; a kind gift from Tom I. Bonner, Ph.D.).[25] cells were exposed to acetylcholine and rapacuronium as above before the measurement of inositol phosphate synthesis.

Dissociation Kinetic Studies

Allosteric effects are defined by the ability of a substance (*e.g.*, muscle relaxant) to delay the dissociation of a ligand from a receptor.[26–28] We determined whether clinically relevant concentrations of several muscle relaxants (rapacuronium, vecuronium, rocuronium, and gallamine) had allosteric interactions at the M3 muscarinic receptor by slowing the atropine-induced dissociation of ^{3}H-NMS from M3 muscarinic receptors. CHO-M3 membranes (0.2–0.3 mg/ml) were incubated in triplicate in buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) with 0.88 nM ^{3}H-NMS (two times the K_d for these membranes) for 70 min at 32°C. Dissociation of ^{3}H-NMS was induced at time 0 min by the simultaneous addition of 1 μM atropine with or without muscle relaxants. The reaction was terminated at 5 min (3 ml cold wash buffer [50 mM Tris-HCl in 0.9% NaCl, pH 7.4]) and collected on glass fiber filters (GF/B) presoaked in 0.1% polyethylenimine using a cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 3 ml cold wash buffer. Filters were immersed in 5 ml Econo scintillation fluid, stored overnight, and counted (Beckman Liquid Scintillation 5000 TD; Beckman, Fullerton, CA).[16,20,29,30]

Data Analysis

Smooth muscle force was continuously recorded using Acqknowledge 3.7.3 software. Statistical analysis was determined using GraphPad Instat 3.01 software (GraphPad Software, Inc., San Diego, CA) by two-way analysis of variance with Bonferroni multiple comparison post test. Statistical significance was defined by a P value less than 0.05. In all experiments, n represents the number of individual tracheal rings studied or the independent number of experiments for radioligand dissociation studies or inositol phosphate assays.

Results

Organ Bath Studies

Figure 1A illustrates a representative force recording of a tracheal ring exposed to two cycles of cumulatively increasing acetylcholine concentrations followed by three washings and then exposed to 1–100 μM rapacuronium, resulting in increased force that returned to baseline after extensive washing (nine times). After extensive washing (*i.e.*, removing residual acetylcholine), this tracheal ring was unresponsive to another rapacuronium dose response. Figure 1B illustrates the failure of rapacuronium (1–100 μM) to induce contraction in the absence of a precontraction with acetylcholine; however, the contractile effect of rapacuronium subsequently occurs after the same ring is subjected to two cycles of cumulatively increasing acetylcholine concentrations followed by three washes. To further confirm that subthreshold acetylcholine concentrations were necessary for the effect of rapacuronium, we added a low concentration of acetylcholine (0.1 μM) to the baths.

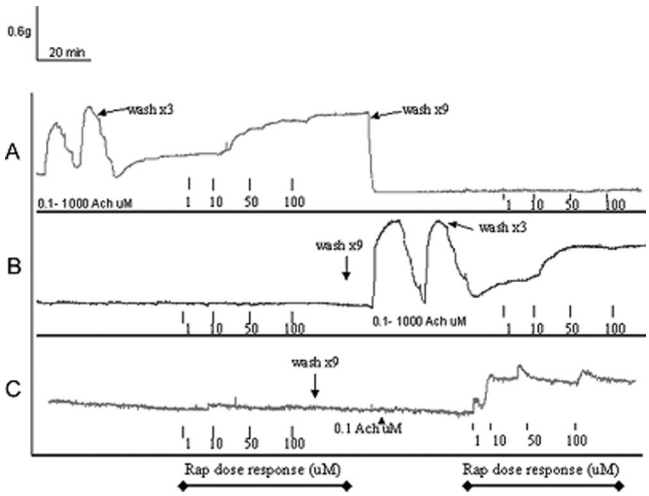

Fig. 1. Representative force recordings of guinea pig tracheal ring preparations suspended in organ baths illustrating allosteric effects of rapacuronium (Rap). In *A*, rings were first precontracted twice with an increasing concentrations of acetylcholine, washed three times, and then exposed to 1–100 μM rapacuronium, resulting in an increase in force; after thorough washing (nine times), a repeat addition of 1–100 μM rapacuronium was without effect. (*B*) In the absence of acetylcholine precontraction, 1–100 μM rapacuronium was without effect; after thorough washing (nine times), an acetylcholine precontraction, and three washes, rapacuronium now increased force. (*C*) In the absence of acetylcholine precontraction, 1–100 μM rapacuronium was without effect; after thorough washing (nine times), a single 0.1-μM acetylcholine dose was added, and rapacuronium then resulted in increased force. These results illustrate that rapacuronium only increased baseline force in the presence of subthreshold concentrations of acetylcholine, consistent with a positive allosteric cooperativity with acetylcholine at the M3 muscarinic receptor. Illustrations are typical of at least five independent experiments for each condition.

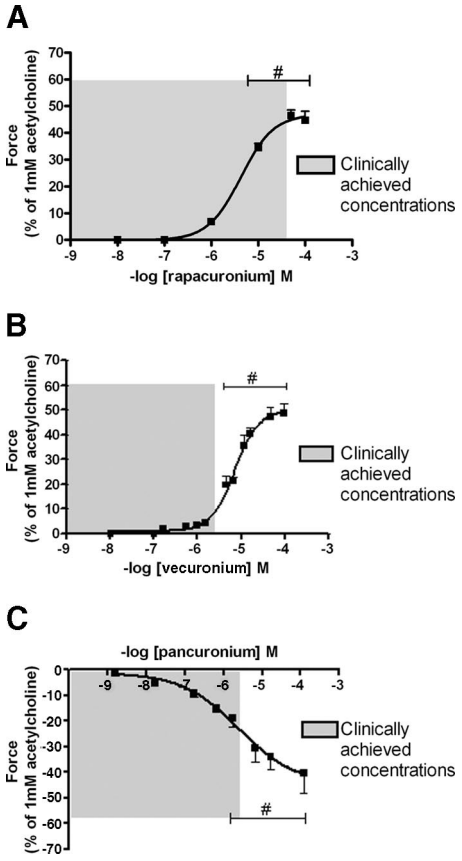

Fig. 2. (*A*) Rapacuronium (10–100 μM) significantly increased baseline force (expressed as a percent of the maximum contraction achieved with 1 mM acetylcholine) when subthreshold acetylcholine was present in the organ baths after acetylcholine precontraction and limited washing (three times). (*B*) Vecuronium (5–100 μM) significantly increased baseline force when subthreshold acetylcholine was present, but this only occurred at concentrations of vecuronium above those achieved clinically. (*C*) Pancuronium (2—100 μM), within clinically achieved concentrations, significantly decreased baseline force when subthreshold acetylcholine was present. # $P < 0.001$ compared with baseline; n = 4 or 5.

This concentration alone had no effect on muscle force but allowed for the dose-dependent potentiation of muscle force of rapacuronium (fig. 1C). Although we did not measure the concentration of acetylcholine in the organ baths, both methods resulted in approximately the same percent increase in baseline force when rapacuronium was added. Rapacuronium (10–100 μM) significantly increased baseline muscle force in the presence (but not in the absence) of subthreshold acetylcholine ($P < 0.001$; n = 5; fig. 2A). Similarly, vecuronium (5–100 μM) significantly increased baseline force in the presence of subthreshold acetylcholine but only at vecuronium concentrations above those achieved clinically ($P < 0.001$; n = 5; fig. 2B). Conversely, pancuronium (2–100 μM) significantly decreased baseline force within clinically achieved concentrations in the presence of subthreshold acetylcholine concentrations ($P < 0.001$; n = 4; fig. 2C). High concentrations of rocuronium (50–100 μM) also increased baseline force in the presence of subthreshold concentrations of acetylcholine, but this contraction was qualitatively different from the other relaxants studied in that it spontaneously and rapidly returned to baseline (data not shown). Taken together, these results suggest that rapacuronium, vecuronium, and rocuronium can increase baseline force under conditions where subthreshold concentrations of acetylcholine are present and are consistent with a positive allosteric effect at M3 muscarinic receptors. Most significantly, however, rapacuronium was the only muscle relaxant studied to increase force within clinically relevant concentrations.

In an attempt to confirm that this rapacuronium-induced increase in resting force in the presence of low acetylcholine concentrations involved muscarinic receptors and did not involve other classic contractile agonists in airway smooth muscle, a series of inhibitors was used either before the addition of rapacuronium to the baths or after the rapacuronium-induced force had reached a peak. Figures 3 and 4 illustrate representative force recordings of tracheal rings prepared as above with two acetylcholine dose responses followed by three washes, pretreatment with an inhibitor, and then the addition of rapacuronium. Only atropine prevented (fig. 3A) or reversed (fig. 3C) the rapacuronium-induced increase in

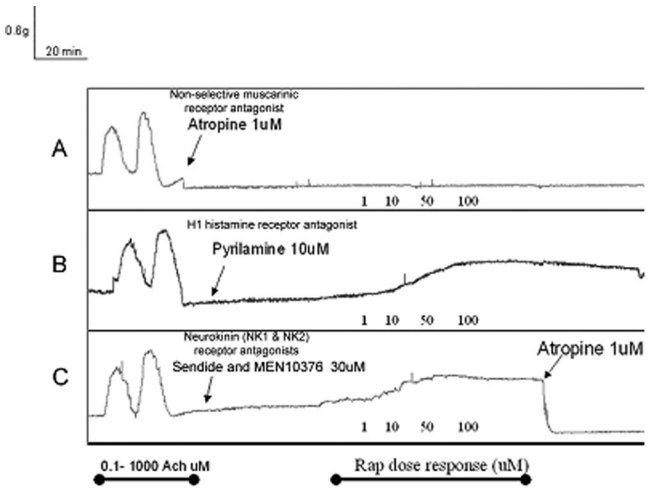

Fig. 3. Representative force recordings of guinea pig tracheal ring preparations suspended in organ baths illustrating the effect of various antagonists on the contractile effects of rapacuronium (Rap). After precontraction with acetylcholine (ACh) and three washes, antagonists were added before 1–100 μM rapacuronium. (*A*) Atropine (1 μM) completely inhibited rapacuronium-induced contraction. (*B*) Pyrilamine (10 μM) or (*C*) sendide and MEN10376 (each 30 μM) were without effect on rapacuronium-induced contractions. Conversely, atropine added at the peak of a sustained contraction completely inhibited the rapacuronium-induced contraction. Illustrations are typical of at least three independent experiments for each condition.

baseline force. Pretreatment of tracheal rings with pyrilamine (H1 histamine receptor antagonist; fig. 3B), sendide plus MEN10376 (neurokinin receptor 1 and 2 antagonists; fig. 3C), MK-571 (leukotriene LTD4 antagonist; fig. 4A), BAY-u9773 (nonselective leukotriene antagonist; fig. 4B), or nifedipine (L-type calcium channel

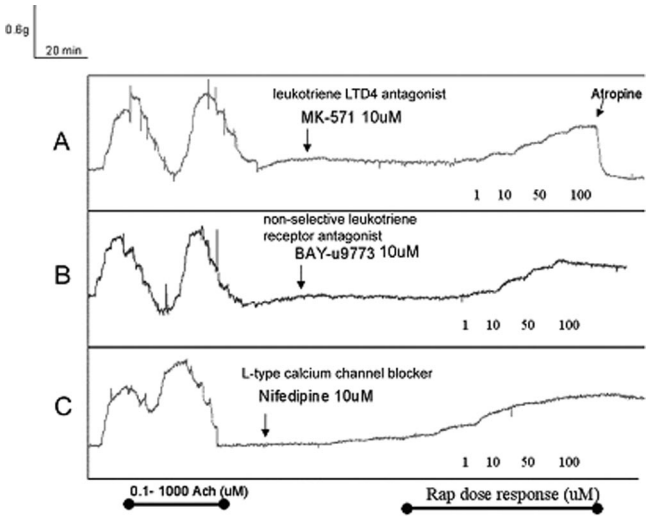

Fig. 4. Representative force recordings of guinea pig tracheal ring preparations suspended in organ baths illustrating the effect of various antagonists on the contractile effects of rapacuronium (Rap). After precontraction with acetylcholine (ACh) and three washes, antagonists were added before 1–100 μM rapacuronium. (*A*) MK-571 (10 μM), (*B*) Bay-u9773 (10 μM), or (*C*) nifedipine (10 μM) were without effect on rapacuronium-induced contractions. Illustrations are typical of at least three independent experiments for each condition.

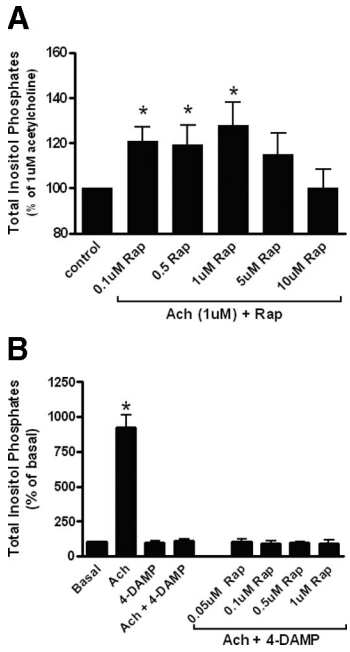

Fig. 5. (*A*) Rapacuronium (Rap) (0.1–1 μM) accentuates acetylcholine (1 μM; ACh)–induced increases in inositol phosphate synthesis *via* human M3 muscarinic receptors stably expressed in Chinese hamster ovary-M3 cells. * $P < 0.05$ compared with acetylcholine alone (control); n = 6. (*B*) One-hour pretreatment with 1 μM 4-DAMP mustard (4-DAMP) to inactivate M3 muscarinic receptors eliminated 1 μM acetylcholine or acetylcholine plus effects of rapacuronium (0.05–1 μM) on inositol phosphate synthesis. * $P < 0.05$ compared with basal inositol phosphate synthesis (control); n = 3.

blocker; fig. 4C) had no effect on the subsequent muscle contraction induced by rapacuronium. 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). The functional studies in tracheal rings suggesting a positive allosteric effect of rapacuronium at muscarinic receptors led us to perform biochemical (inositol phosphate) and radioligand (dissociation rates) assays *in vitro* using human recombinant M3 muscarinic receptors expressed in CHO cells.

Inositol Phosphate Assays

Rapacuronium (0.1–1 μM) accentuated the acetylcholine-induced increase in inositol phosphate synthesis in CHO cells expressing the human M3 muscarinic receptor consistent with a positive allosteric effect ($P < 0.05$ compared with acetylcholine alone; n = 6; fig. 5A). Rapacuronium alone had no effect on inositol phosphate synthesis. Higher concentrations of rapacuronium (5–10 μM) decreased inositol phosphate synthesis likely due to either an apparent classic orthosteric antagonist effect or steric hindrance of the orthosteric site by the allosteric binding of rapacuronium on the M3 muscarinic receptor (fig. 5A). Inactivation of the M3 muscarinic receptor by alkylation (1 h of 1 μM 4-DAMP mustard) blocked the ability of acetylcholine or acetylcholine plus

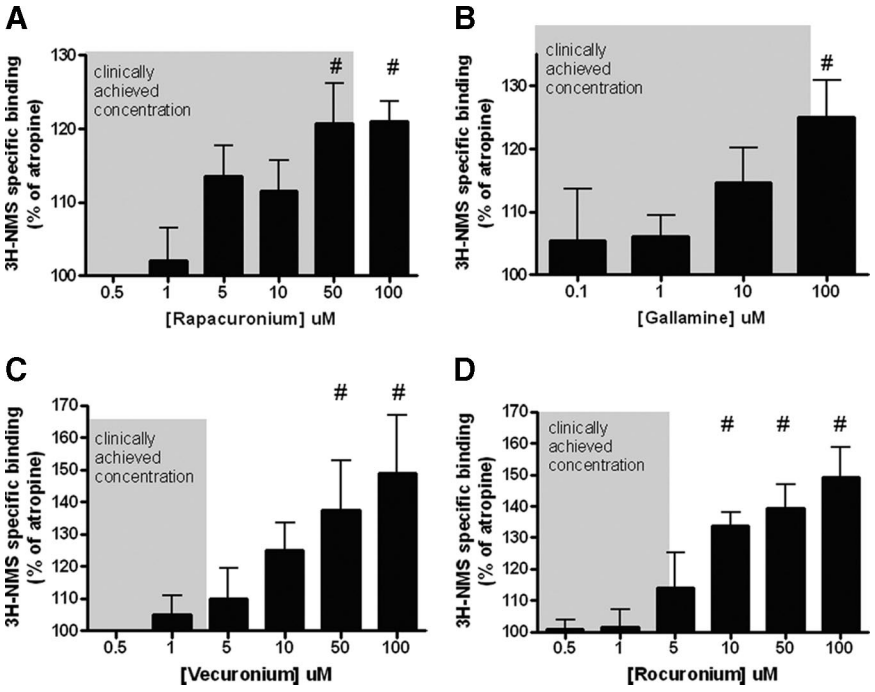

Fig. 6. Inhibition of atropine (1 μM)–induced [^{3}H]N-methylscopolamine (^{3}H-NMS) dissociation from the human M3 muscarinic receptors in membranes prepared from Chinese hamster ovary-M3 cells by (*A*) rapacuronium, (*B*) gallamine, (*C*) vecuronium, and (*D*) rocuronium. Rapacuronium (50–100 μM), gallamine (100 μM), vecuronium (50–100 μM), and rocuronium (10–100 μM) each significantly inhibited atropine-induced ^{3}H-NMS dissociation, demonstrating allosteric interactions with the M3 muscarinic receptor. Rapacuronium was the only relaxant studied that had allosteric interactions within clinically achieved concentrations. # $P < 0.01$ compared with atropine alone; n = 6.

rapacuronium to increase inositol phosphate synthesis (fig. 5B). To further confirm that rapacuronium's potentiation of acetylcholine-induced inositol phosphate synthesis was mediated *via* the effect of both acetylcholine and rapacuronium at the M3 muscarinic receptor, these assays were repeated in a CHO cell line devoid of M3 muscarinic receptors (but expressing human M2 muscarinic receptors, which do not couple to inositol phosphate synthesis). Neither rapacuronium nor acetylcholine increased inositol phosphate synthesis in this human M2 muscarinic receptor–expressing cell line (0.5 μM rapacuronium, 93.8 ± 5.3% of control; 1 μM acetylcholine, 99.2 ± 5.1% of control).

Radioligand Binding Dissociation Kinetic Assays

Further evidence for the allosteric effects of muscle relaxants at M3 muscarinic receptors was provided by dissociation kinetic radioligand binding studies. Rapacuronium (50–100 μM) significantly inhibited atropine induced ^{3}H-NMS dissociation from the CHO-M3 muscarinic receptors ($P < 0.01$; n = 6; fig. 6A) within clinically relevant concentrations. Gallamine, a muscle relaxant with known allosteric interactions with muscarinic receptors (positive control), significantly inhibited ^{3}H-NMS dissociation at 100 μM ($P < 0.001$; n = 6; fig. 6B). Similarly, vecuronium (50–100 μM) ($P < 0.001$; n = 6; fig. 6C) and rocuronium ($P < 0.05$; n = 5; fig. 6D) significantly inhibited atropine-induced ^{3}H-NMS dissociation; however, the concentrations required are above those concentrations achieved with routine clinical use of these relaxants (peak serum concentrations: vecuronium, 3.2 μM; rocuronium, 4.7 μM).

Discussion

These data, along with previously published data,[10] demonstrate that rapacuronium has at least two detrimental effects on airway muscarinic receptors leading to bronchospasm. Rapacuronium exhibits a positive allosteric effect at M3 muscarinic receptors, which potentiated the contractile effect of acetylcholine at airway muscarinic receptors. This detrimental airway effect is in addition to and is additive to the previously described preferential antagonism of presynaptic M2 muscarinic receptors described for rapacuronium.[10]

Muscarinic receptors on airway parasympathetic nerves are of the M2 subtype and normally function in an inhibitory autofeedback mode to prevent the further release of acetylcholine.[17] Muscarinic receptors on the airway smooth muscle are of the M2 and M3 subtypes, which function to inhibit relaxation and facilitate contraction, respectively.[18] Irritation of the well-innervated upper trachea (*e.g.*, by the introduction of an endotracheal tube) initiates a neural reflex that results in the release of acetylcholine from parasympathetic nerves that acts on M2 and M3 muscarinic receptors in airway smooth muscle, resulting in bronchoconstriction. Normally, the release of acetylcholine is terminated by activation of the inhibitory autofeedback receptors on the parasympathetic nerves. The introduction of a large intubating dose of a muscle relaxant with the properties of rapacuronium during this period of parasympathetic nerve activation creates a scenario where negative feedback inhibition of acetylcholine release is blocked (antagonism of neural M2 muscarinic receptor) coincident with augmentation of the contractile effect of acetylcholine at the mus-

cle M3 muscarinic receptor (positive allosteric effect at M3 muscarinic receptor). This dual detrimental effect of rapacuronium at airway muscarinic receptors within clinically relevant concentrations is unique among the muscle relaxants we have studied.[10,11]

Increased baseline force of tracheal rings seen when rapacuronium was added in the presence of subthreshold acetylcholine (*i.e.*, after an acetylcholine dose response with minimal washing or after a single small dose of acetylcholine) suggests that rapacuronium increases airway tone by a positive allosteric effect at the M3 muscarinic receptor. Two clinical studies are also consistent with this mechanism. Increased peak airway inflating pressures[31] or a decrease in maximal expiratory flow[32] occurred with the administration of rapacuronium during steady state anesthesia, a period not associated with heightened activity of airway parasympathetic nerves.

The increase in muscle force by rapacuronium in the presence of subthreshold acetylcholine in the organ baths was inhibited by atropine but was unaffected by antagonists of histamine, neurokinin, or leukotriene receptors or by inhibition of calcium channels or by previous depletion of NANC neurotransmitters. Taken together, these findings suggest that the mechanism of the effect of rapacuronium on baseline force required acetylcholine and involved muscarinic receptors. These findings again are consistent with a positive allosteric effect of rapacuronium on M3 muscarinic receptors that allows acetylcholine to have an increased effect as an agonist at its orthosteric site.

Neuromuscular blocking agents are known to have the potential for both orthosteric[9,11] and allosteric interactions with muscarinic receptors.[15,16,33,34] Gallamine and alcuronium are the most extensively characterized muscle relaxants in terms of muscarinic allosteric interactions, and gallamine serves as the prototypical muscarinic allosteric ligand.[35] Similarly, alcuronium has been shown to enhance binding of muscarinic *antagonists*,[36] but neither gallamine nor alcuronium has been shown to enhance the binding of *agonists* at the muscarinic receptor.[37,38] In our previous studies, gallamine displayed antagonist affinities for the M2 *versus* M3 muscarinic receptors that were similar to the antagonist affinity profile seen with rapacuronium (*i.e.*, M2 $>$ M3),[11] but clinical bronchospasm was not a widely reported problem with gallamine. One interpretation of these findings that may explain the different clinical experiences with these two drugs is that positive allosteric effects at the M3 muscarinic receptor (by rapacuronium) has more deleterious effects in the airway than antagonism of prejunctional M2 muscarinic receptors (by gallamine or rapacuronium). Alternatively, the combination of prejunctional M2 antagonism and postjunctional M3 positive allosterism may synergize to account for the detrimental airway effects seen with rapacuronium. Indeed, rapacuronium is the only muscle relaxant studied that within clinically relevant concentrations displays both M2 muscarinic receptor antagonism[10] and M3 muscarinic receptor positive allosteric effects. Although M1 muscarinic receptors have been described in the parasympathetic ganglia of some species, they were not relevant to the current study, which was performed without activation of the cholinergic nerves in the tracheal rings (*i.e.*, without electrical field stimulation).

Interestingly vecuronium, another commonly used muscle relaxant, had a similar increase in baseline force in the organ baths (fig. 2B), but this effect occurred at much greater concentrations than those achieved clinically. Likewise, rocuronium led to an increase in baseline force; however, it was not sustained, and it only occurred at concentrations of rocuronium above those achieved clinically. Conversely, pancuronium potently decreased the baseline force, demonstrating either classic orthosteric antagonism or strong negative cooperativity at the airway smooth muscle M3 muscarinic receptor.

These functional studies in the organ baths suggesting allosteric interactions led us to perform biochemical (*i.e.*, inositol phosphate) and radioligand (*i.e.*, dissociation kinetic) studies to further characterize the interaction of rapacuronium on the allosteric site on recombinantly overexpressed human M3 muscarinic receptors in CHO cells. Low concentrations of rapacuronium alone had no effect on inositol phosphate synthesis but enhanced acetylcholine-induced increases in inositol phosphate synthesis *via* the M3 muscarinic receptor, whereas higher concentrations of rapacuronium decreased acetylcholine-induced inositol phosphate synthesis. This biphasic effect of rapacuronium on acetylcholine-induced inositol phosphate synthesis mimicked the effects on force in intact airway smooth muscle, suggesting that at low concentrations (but still within clinically achieved ranges), rapacuronium has a positive allosteric effect at M3 muscarinic receptors, whereas at high concentrations (above those achieved clinically), rapacuronium can antagonize M3 muscarinic receptors *via* blockade of the orthosteric site. The selectivity of this effect for the M3 muscarinic receptor was further confirmed by inactivation of the M3 muscarinic receptor by alkylation with 4-DAMP mustard, which resulted in complete attenuation of the increase in inositol phosphate synthesis seen with acetylcholine with or without rapacuronium. Acetylcholine or rapacuronium also had no effect on inositol phosphate synthesis in another cell line (CHO-M2) that expresses the human M2 muscarinic receptor.

A radioligand dissociation kinetic assay evaluates whether an agent has an allosteric interaction with a receptor. Allosteric agents inhibit the rate of dissociation of a radioligand induced by an orthosteric competitive ligand (*e.g.*, atropine or acetylcholine for the M3 muscarinic receptor). Rapacuronium at clinically significant

doses inhibited atropine-induced dissociation of ^{3}H-NMS, confirming that rapacuronium interacts with the M3 muscarinic receptor in an allosteric manner. Vecuronium and rocuronium also exhibited allosteric interactions with the M3 muscarinic receptor; however, this only occurred at concentrations of vecuronium or rocuronium above those achieved with clinical use.

Another common mechanism associated with the potential of muscle relaxants for bronchoconstriction is histamine release. Histamine release is described after administration of several nondepolarizing muscle relaxants, including curare, atracurium, and mivacurium.[39–42] However, this seems to be an unlikely mechanism for rapacuronium because two studies of increased airway tone during the maintenance phase of general anesthesia attributed their results to selective M2 muscarinic receptor antagonism,[31,32] and in a study of seven adult patients who had development of bronchospasm, histamine concentrations did not increase while the patients were receiving rapacuronium.[43] Our current study is consistent with this in that pyrilamine pretreatment had no effect on rapacuronium-induced increases in muscle force, ruling out release of endogenous histamine from cells within the tracheal rings.

The search for a muscle relaxant with rapid onset and offset has led to the clinical introduction of relaxants with low potency, requiring larger doses to achieve a targeted clinical effect. In the current study, rapacuronium and vecuronium both demonstrated similar increases in baseline force at similar concentrations. However, because rapacuronium is a much less potent agent, its clinically recommended intubation dose was 10–20 times greater (1.5–2.5 mg/kg) than the clinically recommended dose of vecuronium (0.1 mg/kg). Therefore, this allosteric effect at M3 muscarinic receptors is not unique to rapacuronium, because many muscle relaxants act allosterically with muscarinic receptors. The detrimental consequences of rapacuronium at airway muscarinic receptors are most likely due to the requirement of large clinical doses and the achievement of clinical concentrations that allow for positive cooperativity at the airway smooth muscle M3 muscarinic receptor and antagonism of the M2 muscarinic receptor.

In summary, rapacuronium has dual detrimental and unique effects at airway muscarinic receptors. Not only does rapacuronium block M2 muscarinic receptors, potentiating vagal induced acetylcholine release,[10,11] but rapacuronium also binds to an allosteric site on the M3 muscarinic receptor, resulting in an enhancement of acetylcholine activation of this receptor. Both of these mechanisms lead to increased smooth muscle tone and would be additive in their ability to provoke bronchospasm. Despite the removal of rapacuronium 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 are developed to attempt to replace succinylcholine will also have affinities for muscarinic receptors. We have defined two muscarinic receptor–mediated effects of rapacuronium that likely contribute to its potential to induce bronchospasm. It is therefore prudent that all newly designed muscle relaxants should be evaluated for these properties at concentrations likely to be achieved clinically.

References

1. Goudsouzian NG: Rapacuronium and bronchospasm. ANESTHESIOLOGY 2001; 94:727–8
2. Adams C: Anesthesia drug is being pulled from market after five deaths. The Wall Street Journal March 30, 2001; B3
3. Kron SS: Severe bronchospasm and desaturation in a child associated with rapacuronium. ANESTHESIOLOGY 2001; 94:923–4
4. Naguib M: How serious is the bronchospasm induced by rapacuronium? ANESTHESIOLOGY 2001; 94:924–5
5. Meakin GH, Pronske EH, Lerman J, Orr R, Joffe D, Savaree AM, Lynn AM: Bronchospasm after rapacuronium in infants and children. ANESTHESIOLOGY 2001; 94:926–7
6. Heerdt PM, Kang R, The' A, Hashim M, Mook RJ Jr, Savarese JJ: Cardiopulmonary effects of the novel neuromuscular blocking drug GW280430A (AV430A) in dogs. ANESTHESIOLOGY 2004; 100:846–51
7. Savarese JJ, Belmont MR, Hashim MA, Mook RA Jr, Boros EE, Samano V, Patel SS, Feldman PL, Schultz JA, McNulty M, Spitzer T, Cohn DL, Morgan P, Wastila WB: Preclinical pharmacology of GW280430A (AV430A) in the rhesus monkey and in the cat: A comparison with mivacurium. ANESTHESIOLOGY 2004; 100:835–45
8. Belmont MR, Lien CA, Tjan J, Bradley E, Stein B, Patel SS, Savarese JJ: Clinical pharmacology of GW280430A in humans. ANESTHESIOLOGY 2004; 100: 768–73
9. Hou VY, Hirshman CA, Emala CW: Neuromuscular relaxants as antagonists for M2 and M3 muscarinic receptors. ANESTHESIOLOGY 1998; 88:744–50
10. Jooste E, Zhang Y, Emala CW: Rapacuronium preferentially antagonizes the function of M2 versus M3 muscarinic receptors in guinea pig airway smooth muscle. ANESTHESIOLOGY 2005; 102:117–24
11. Jooste E, Klafter F, Hirshman CA, Emala CW: A mechanism for rapacuronium-induced bronchospasm: M2 muscarinic receptor antagonism. ANESTHESIOLOGY 2003; 98:906–11
12. Fryer AD, Maclagan J: Pancuronium and gallamine are antagonists for pre- and post-junctional muscarinic receptors in the guinea pig lung. Naunyn Schmiedebergs Arch Pharmacol 1987; 335:367–71
13. Okanlami OA, Fryer AD, Hirshman CA: Interaction of nondepolarizing muscle relaxants with m_2 and m_3 muscarinic receptors in guinea pig lung and heart. ANESTHESIOLOGY 1996; 84:155–61
14. Jakubik J, Bacakova L, el-Fakahany EE, Tucek S: Subtype selectivity of the positive allosteric action of alcuronium at cloned M1-M5 muscarinic acetylcholine receptors. J Pharmacol Exp Ther 1995; 274:1077–83
15. Trankle C, Kostenis E, Mohr K: Muscarinic allosteric modulation: M2/M3 subtype selectivity of gallamine is independent of G-protein coupling specificity. Naunyn Schmiedebergs Arch Pharmacol 2001; 364:172–8
16. Waelbroeck M: Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors. Mol Pharmacol 1994; 46: 685–92
17. Fryer AD, Maclagan J: Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. Br J Pharmacol 1984; 83:973–8
18. Emala CW, Aryana A, Levine MA, Yasuda RP, Satkus SA, Wolfe BB, Hirshman CA: Basenji-greyhound dog: Increased m2 muscarinic receptor expression in trachealis muscle. Am J Physiol 1995; 268:L935–40
19. Gilani AH, Shaheen F, Christopoulos A, Mitchelson F: Interaction of ebeinone, an alkaloid from Fritillaria imperialis, at two muscarinic acetylcholine receptor subtypes. Life Sci 1997; 60:535–44
20. Lazareno S, Popham A, Birdsall NJ: Analogs of WIN 62,577 define a second allosteric site on muscarinic receptors. Mol Pharmacol 2002; 62:1492–505
21. Ellis J, Seidenberg M: Interactions of alcuronium, TMB-8, and other allosteric ligands with muscarinic acetylcholine receptors: Studies with chimeric receptors. Mol Pharmacol 2000; 58:1451–60
22. Hotta K, Emala CW, Hirshman CA: TNF-alpha upregulates Gialpha and Gqalpha protein expression and function in human airway smooth muscle cells. Am J Physiol 1999; 276:L405–11
23. Buckley NJ, Bonner TI, Buckley CM, Brann MR: Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol 1989; 35:469–76

24. Bonner TI, Young AC, Brann MR, Buckley NJ: Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron 1988; 1:403–10

25. Bonner TI, Buckley NJ, Young AC, Brann MR: Identification of a family of muscarinic acetylcholine receptor genes. Science 1987; 237:527–32

26. Lee NH, el Fakahany EE: Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. J Pharmacol Exp Ther 1991; 256:468–79

27. Lee NH, el Fakahany EE: Allosteric antagonists of the muscarinic acetylcholine receptor. Biochem Pharmacol 1991; 42:199–205

28. Michel AD, Delmendo RE, Lopez M, Whiting RL: On the interaction of gallamine with muscarinic receptor subtypes. Eur J Pharmacol 1990; 182:335–45

29. Tanito Y, Miwa T, Endou M, Hirose Y, Gamoh M, Nakaya H, Okumura F: Interaction of edrophonium with muscarinic acetylcholine M2 and M3 receptors. ANESTHESIOLOGY 2001; 94:804–14

30. Jacoby DB, Gleich GJ, Fryer AD: Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M_2 receptor. J Clin Invest 1993; 91:1314–8

31. Tobias JD, Johnson JO, Sprague K, Johnson G: Effects of rapacuronium on respiratory function during general anesthesia: A comparison with cis-atracurium. ANESTHESIOLOGY 2001; 95:908–12

32. Fine GF, Motoyama EK, Brandom BW, Fertal KM, Mutich R, Davis PJ: The effect on lung mechanics in anesthetized children with rapacuronium: A comparative study with mivacurium. Anesth Analg 2002; 95:56–61

33. Stockton JM, Birdsall NJ, Burgen AS, Hulme EC: Modification of the binding properties of muscarinic receptors by gallamine. Mol Pharmacol 1983; 23:551–7

34. Ellis J, Huyler J, Brann MR: Allosteric regulation of cloned m1-m5 muscarinic receptor subtypes. Biochem Pharmacol 1991; 42:1927–32

35. Lazareno S, Gharagozloo P, Kuonen D, Popham A, Birdsall NJ: Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: Radioligand binding studies. Mol Pharmacol 1998; 53:573–89

36. Krejci A, Tucek S: Changes of cooperativity between N-methylscopolamine and allosteric modulators alcuronium and gallamine induced by mutations of external loops of muscarinic M(3) receptors. Mol Pharmacol 2001; 60:761–7

37. Lazareno S, Birdsall NJ: Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. Mol Pharmacol 1995; 48:362–78

38. Jakubik J, Bacakova L, el Fakahany EE, Tucek S: Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. Mol Pharmacol 1997; 52:172–9

39. Naguib M, Samarkandi AH, Bakhamees HS, Magboul MA, el Bakry AK: Histamine-release haemodynamic changes produced by rocuronium, vecuronium, mivacurium, atracurium and tubocurarine. Br J Anaesth 1995; 75:588–92

40. Takeda J, Ueda E, Takahashi J, Fukushima K: Plasma N-methylhistamine concentration as an indicator of histamine release by intravenous d-tubocurarine in humans: Preliminary study in five patients by radioimmunoassay kits. Anesth Analg 1995; 80:1015–7

41. Koppert W, Blunk JA, Petersen LJ, Skov P, Rentsch K, Schmelz M: Different patterns of mast cell activation by muscle relaxants in human skin. ANESTHESIOLOGY 2001; 95:659–67

42. Habre W, Babik B, Chalier M, Petak F: Role of endogenous histamine in altered lung mechanics in rabbits. ANESTHESIOLOGY 2002; 96:409–15

43. Levy JH, Pitts M, Thanopoulos A, Szlam F, Bastian R, Kim J: The effects of rapacuronium on histamine release and hemodynamics in adult patients undergoing general anesthesia. Anesth Analg 1999; 89:290–5