Effects of presynaptic muscarinic cholinoreceptor blockade on neuromuscular transmission as assessed by the train-of-four and the tetanic fade response to rocuronium

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Summary
This study investigated the effect of muscarinic M1 and M2 receptor antagonists on the rocuronium-induced train of four (TOF) fade and tetanic fade, respectively. Ex-vivo phrenic nerves and diaphragms were obtained from adult Sprague-Dawley rats and stabilized in Krebs buffer; the nerve-stimulated muscle TOF fade was observed at 20 s intervals. For the TOF study, phrenic nerves and diaphragms were incubated with pirenzepine (an M1 blocker) at concentrations of 0 nmol L−1 (control), 10 nmol L−1 (PZP10), or 100 nmol L−1 (PZP100). Rocuronium was then administered incrementally until the first twitch tension had depressed by >95% during TOF stimulation. The mean TOF ratios were compared when the first twitch tensions were depressed by 40%-50%. For the tetanic fade study, 50 Hz/5 s tetani was applied initially, 30 min after the administration of a loading dose of rocuronium and methoctramine (an M2 receptor blocker, loaded at 0 μmol L−1 [control], 1 μmol L−1 [MET1], or 10 μmol L−1 [MET10]). The EC95 of rocuronium was significantly lower in the PZP10 group than in the control group. In the PZP10 group, the TOF ratios at 50% and first twitch tension depression were significantly lower than those in the control group (P=.02). During tetanic stimulation, the tetanic fade was significantly enhanced in the MET10 group compared to the other groups. This study shows that antagonists of muscarinic M1 and M2 receptors affect the rocuronium-induced neuromuscular block as demonstrated by the reduced EC95 and TOF ratios (M1 antagonist, pirenzepine) or the enhanced 50-Hz tetanic fade (M2 antagonist, methoctramine).

KEYWORDS
acetylcholine release, methoctramine, muscarinic cholinoreceptor, neuromuscular physiology, pirenzepine, rocuronium

1 | INTRODUCTION

Several types of receptors are found at neuromuscular presynaptic sites.1,2 Some of these receptors function together to control the release of acetylcholine (ACh) during rest or stimulation.2 For example, muscarinic and purinergic receptors interact at presynaptic neuronal sides. Of these receptors, muscarinic facilitatory M1 and inhibitory M2 receptors regulate the fine-tuning actions of ACh release on neuronal firing, and these receptors are in turn regulated by purinergic receptors.3-6
Train-of-four (TOF) stimulation is performed by indirect stimulation at 2 Hz four times and enables the monitoring of recovery from neuromuscular blockade.7 The TOF ratio (TOFR) is an important measure of the margin of safety of neuromuscular transmission during the perioperative period as well as of muscle function in the presence of muscle diseases, such as myasthenia gravis. As a TOF assessment uses low-frequency stimulation, we hypothesized that the muscarinic M₂ receptors may be primarily involved in the release of ACh via TOF stimulation because the M₁ receptor function is evoked predominantly via low-frequency (<5 Hz) stimulation upon ACh release at the neuromuscular presynaptic side.4 We further hypothesized that if the function of the M₁ receptor is inhibited by an M₁ blocking drug, such as pirenzepine (PZP), the TOFR may be modulated.

Tetanic stimulation provides useful information about the depth of blockade during deep neuromuscular paralysis produced by non-depolarizing muscle relaxants,8 and it reflects the ability to maintain function during repetitive nerve stimulation (eg exercise). Tetanic stimulation is used in clinical practice at 50 Hz for 5 s, during which time tetanic tension is sustained and does not fade in normal (non-diseased) muscles. In contrast, when the neuromuscular block is produced by non-depolarizing neuromuscular-blocking drugs, the muscle tension is not sustained, and fade is detected. The post-tetanic count, the phenomenon involving post-tetanic facilitation of twitch tensions after tetanic stimulation, is used to assess the depth of recovery (or paralysis) from deep neuromuscular block when there is no response to TOF stimulation.9,10 Fade during tetanic stimulation is considered as a presynaptic event. During rest and low-frequency stimulation (<5 Hz), facilitatory muscarinic M₁ receptor function is predominant. However, the transmitter adenosine, which is co-released with ACh during nerve stimulation at the synapses, is markedly increased when the stimulation frequency is increased to 50 Hz; it eventually reaches levels capable of activating the facilitatory adenosine A₂A receptors, which counteract M₁ receptors, and potentiates M₂ inhibitory receptors.4 Therefore, we hypothesized that presynaptic M₂ receptors may play an important role when high-frequency stimulation (50-Hz tetanic stimulation) is applied during post-tetanic count monitoring.

The nicotinic ACh receptor is primarily postsynaptically involved in neuromuscular signal transduction, and the muscarinic cholinoreceptor is one of the neuromuscular presynaptic modulating receptors. As such, the modulating effects of the M₁ and M₂ muscarinic cholinoreceptor on neurotransmission were assessed by decreasing the margin of safety through the administration of rocuronium. Accordingly, in the present study, we primarily aimed to identify the changes in variables induced during neuromuscular blockade by rocuronium after neuromuscular presynaptic muscarinic M₁ or M₂ receptors in an ex vivo phrenic nerve/diaphragm model were blocked; we then sought to compare these changes in the presence of different concentrations of muscarinic cholinoreceptor blocking agents. Therefore, we investigated whether PZP dihydrochloride contributes to TOF fade triggered by rocuronium, a muscle relaxant, and assessed the EC₉₅ of rocuronium. Furthermore, we assessed whether methocarbamime (MET) hydrate contributes to tetanic fade triggered by a 50-Hz tetanic stimulation after a small dose of rocuronium.

2 | RESULTS

2.1 | Effects of the PZP-induced muscarinic M₁ receptor block on TOFR and EC₉₅ of rocuronium

Each experimental group consisted of eight phrenic nerve/hemidiaphragm tissue specimens, and a total of 24 rat diaphragms were used. Each preparation was randomly allocated to treatment with rocuronium alone (control) or to one of two PZP concentrations [10 nmol L⁻¹ (PZP10) or 100 nmol L⁻¹ (PZP100)] followed by rocuronium. The concentration of rocuronium required to cause a >95% depression in the first twitch tension of TOF response (T1) in the PZP10 group was significantly lower than that in the control group (mean values, 37.42 and 42.10 nmol L⁻¹, respectively; P=.024) (Table 1).

The concentration-response curves of rocuronium for T1 depression and the TOFR in the PZP10 group were shifted to the left, as compared with those in the control group (Figure 1). The mean EC₉₅ and EC₅₀ values for rocuronium in each group are shown in Table 1. The following equation was derived for T1 depression: y=50 + 50sin(Ωx), where y represents T1 depression, x represents the rocuronium concentration, and Ω represents the steepness of the curve. The Ω of PZP10 was larger than that of the control and PZP100 groups (P=.001 and .012, respectively) (Figure 1C, Table 1). The following equation was derived for TOFR: y=1 – λx², where y represents the TOFR and x represents the rocuronium concentration. The λ of the PZP10 group was significantly larger than that of the other groups (P=.001) (Figure 1D, Table 1), but no significant difference was observed between the Ω and λ values of the control and PZP100 groups (P=.39 for T1 depression, P=.83 for TOFR). At T1-response depression of 50%, the TOF ratio was significantly lower in the PZP10 group than in the control group (0.251 ± 0.13 vs

| TABLE 1 | Ex-vivo analysis of T1 depression and train-of-four ratio |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|             | Administered ROC (nmol L⁻¹) | Mean EC₅₀ (μmol L⁻¹) | Mean EC₉₅ (μmol L⁻¹) | Mean Ω | 95% Cl | Mean λ | 95% Cl |
| Control (n=8) | 42.10 (1.84) | 30.17 | 15.01 | 0.541 | 0.483-0.599 | 0.002 | 0.0016-0.0024 |
| PZP10 (n=8) | 37.42 (1.05) | 22.37³ | 12.90³ | 0.678³ | 0.638-0.717 | 0.003³ | 0.0027-0.0033 |
| PZP100 (n=8) | 39.37 (1.85) | 27.66 | 13.63 | 0.581 | 0.530-0.632 | 0.0019 | 0.0018-0.0020 |

No significant differences were observed between the PZP100 group and either the control group or the PZP10 group. EC₅₀ and EC₉₅ were calculated using the concentration-response equation for each group in Figure 1A. The slope and intercept were calculated by linear regressions. The administered ROC values are expressed as mean (SE).³ Denotes values that were significantly lower than the results of other groups (P<.01).
0.364 ± 0.121; \( P=0.02 \) (Figure 2). However, no significant differences were found between the PZP100 group and the control group for any of the variables examined.

### 2.2 Effect of muscarinic M₂ receptor blockade by MET on tetanic fade

A total of 30 phrenic nerve/hemidiaphragm tissue preparations were obtained, and each tissue specimen was randomly allocated to treatment with one of three concentrations of MET (0 μmol L⁻¹ [control, \( n=10 \)], 1 μmol L⁻¹ [MET1, \( n=10 \)], or 10 μmol L⁻¹ [MET10, \( n=10 \)]) with rocuronium. No overall changes in T1 twitch tension or in TOFR during the initial period (ie before administration of rocuronium), pre-incubation period (ie before administration of MET), or post-incubation period (ie 30 min after administration of MET) with different concentrations of MET were observed. The first tetanic twitch tensions (A values, illustrated in the inset figure in Figure 4B) of the control, MET1, and MET10 groups during the initial and pre-incubation periods did not exhibit any significant changes. The A values during the post-incubation period were significantly lower (\( P=0.01 \)) than the initial values of the initial tetanic stimulation when compared within the group (Table 2), but there were no significant differences between the groups. The last tetanic twitch tension depression (B values, illustrated in inset figure in Figure 4B) and the
change of the tetanic fade ratios became significant after the administration of rocuronium. The values during tetanic stimulation are shown in Table 2.

When comparing the R value (which is the ratio of B over A) within the group, we found significant changes during the post-incubation period in all the groups in comparison with the initial R values within each group; however, only the post-incubation R values of the MET10 group were significantly lower than those of the control or MET groups (P=.005) (Table 2, Figure 3A). The percentage changes in R values (%R=100×Rpost-incubation/Rpre-incubation) were larger in the MET10 group than in the other groups (P=.005) (Figure 3B).

3 | DISCUSSION

In this ex vivo rat phrenic nerve/hemidiaphragm study, we found that presynaptic muscarinic cholinoreceptors play a role in modulating nerve-stimulated neuromuscular response, particularly during the administration of rocuronium. In particular, neuromuscular presynaptic M₁ receptors were found to play an important role in the rocuronium-induced decrease in twitch tension and TOF fade. The EC₅₀ and EC₃₅ values of rocuronium and the TOFR at ~50% depression of the T₁ were significantly lower in the PZP10 group than in the control group. These findings suggest that the facilitatory muscarinic M₁ receptor is functional when the phrenic nerve is subjected to TOF stimulation; however, the findings obtained from the PZP10 group did not suggest this.

Pirenzepine is generally used as a muscarinic M₁-selective blocking agent because its affinity for M₁ receptors is ~200 times more than for M₂ receptors, which indicates that it is not absolutely M₁-specific. Atropine acts as a non-specific muscarinic blocker to modulate ACh release by inhibiting both M₁ and M₂ receptors, which decreases spontaneous miniature end plate potential (mEPP) frequency and increases evoked ACh release. By modulating the spontaneous quantal release, presynaptic M₁ and M₂ receptors preserve and stabilize synaptic function. TOF fade during neuromuscular blockade is a well-known presynaptic phenomenon that is primarily mediated by the nicotinic ACh receptor (nAChR). However, in an in vivo rat study, it was found that the dihydro β-erythroidine (DHβE)-induced blockade of the presynaptic α₁β₂ nAChR did not cause any change in twitch tension or TOF fade. We speculate that, in the PZP100 group, all these factors resulting from the partial M₂ blocking effect of PZP may have influenced the results and led to reduced differences in T₁ tension depression and TOF fade.

This study also indicated that the muscarinic M₂ receptor plays an important role in tetanic fade during tetanic stimulation at 50 Hz for 5 s. The variables obtained during 50-Hz tetanic stimulation were influenced when M₂ receptors were blocked by MET. Other authors have used MET at 1 μmol L⁻¹ and reported some effects on ACh release in similar experiments. However, we did not observe any definitive changes at this concentration in the present study. Instead, we observed some unexpected changes by increasing the MET concentration to 10 μmol L⁻¹.

In the present study, because rocuronium was administered before MET, some postsynaptic nAChRs were preoccupied by rocuronium, and thus, the responses to ACh at the postsynaptic sides may have been more dependent on the amount of ACh. In such an environment, the rapid depletion of ACh due to the blockade of the M₂ receptors and the lack of accumulation of ACh at the synaptic junction could result in the failure to maintain twitch tension, making the tetanic fade more prominent in turn. In another study on frog end-plates, blockade of M₃ muscarinic ACh receptor by MET provoked a rise of quantal release and the lengthening of late releases, but a different study did not find this lengthening. This discrepancy between study findings indicates that this is a species-specific phenomenon. Another possible reason for this discrepancy was that the MET had some open channel-blocking action. Bixel et al. showed that polymethylenetetramines such as MET can bind to the transmembrane M₂ domain within the ion channel of nAChRs as non-competitive inhibitors. In their study, the MET as a non-competitive inhibitor had an IC₅₀ of 4.2 μmol L⁻¹. In the present study, the concentration of MET in the MET10 group was more than

| Parameters obtained via tetanic stimulation |
|---------------------------------------------|
| **TABLE 2** | **TABLE 2** |

| Control (n=10) | Initial | Pre-incubation | Post-incubation |
|---------------|---------|----------------|-----------------|
| A             | 61.77 (17.13) | 55.46 (21.0) | 41.62 (16.48) |
| B             | 64.25 (19.47) | 43.68 (21.65) | 19.77 (11.36) |
| R             | 1.03 (0.06) | 0.76 (0.15) | 0.45 (0.14) |
| MET1 (n=10)   | Initial | Pre-incubation | Post-incubation |
|---------------|---------|----------------|-----------------|
| A             | 63.83 (17.07) | 58.41 (19.34) | 42.20 (16.17) |
| B             | 66.92 (20.07) | 46.29 (19.06) | 20.75 (10.35) |
| R             | 1.04 (0.05) | 0.77 (0.10) | 0.47 (0.12) |
| MET10 (n=10)  | Initial | Pre-incubation | Post-incubation |
|---------------|---------|----------------|-----------------|
| A             | 61.25 (13.10) | 55.07 (13.50) | 33.56 (10.10) |
| B             | 63.71 (13.44) | 44.57 (16.39) | 10.64 (6.74) |
| R             | 1.04 (0.04) | 0.79 (0.16) | 0.29 (0.14) |

Results are presented as mean (SD). Pre-incubation values were obtained 20 min after administering a loading dose of rocuronium and before administering MET. Post-incubation values were obtained 30 min after administering MET. *Denotes decreases that were significant versus the initial values within the group (P<.01). †Denotes decreases that were significant versus the pre-incubation value within the group (P<.01). ‡Denotes values that differed significantly from those of the control group (P<.01). §Denotes values that differed significantly from the MET1 values (P<.01).
twice the IC\textsubscript{50} of MET on nAChR, and therefore, postsynaptic nAChR may have been affected by MET. But the nAChR used in Bixel’s experiment was comprised of the two α subunits and the β, γ, and δ subunits—that is, it was the ‘immature’ form of nAChR. Moreover, in our pilot study conducted without rocuronium, no changes in A, B, and R values were obtained by tetanic stimulation in the presence of 10\textmu mol L\textsuperscript{-1} MET. These findings suggest MET has a minimal effect on postsynaptic nAChR.

The present study had several limitations. First, this study was conducted in an ex-vivo environment, wherein the pharmacokinetic properties of each antagonist were excluded. It might be difficult to investigate this result in an in-vivo study because M\textsubscript{1} and M\textsubscript{2} mAChRs are also present in the brain, autonomic nervous system, and heart. Therefore, additional data are required to support the extension of the current results to the fine-tuned in-vivo conditions.

Second, this study was of a functional nature, and the amounts of ACh at the neuromuscular junction may have differed during each indirect stimulation among the three groups. Similar experiments on postsynaptic muscarinic and coupled serine threonine kinases have been performed by other authors who explained the function of postsynaptic mAChRs in ACh release under various conditions,\textsuperscript{19-21} but most of these studies used different recording methods and muscles. These studies also reported that neuromuscular postsynaptic mAChRs may have an overall conservative effect of postsynaptic junctions by limiting neurotransmission. As such, we did not assess the molecular differences between control, PZP10, and PZP100 tissue samples via chemical analysis. Instead, we obtained twitch tensions using the rat hemidiaphragm by indirectly stimulating the phrenic nerve. If the difference in the number of released ACh molecules was insufficient to yield differences in postsynaptic nAChR activation, no significant change in twitch tension was observed via the externally evoked indirect supramaximal stimulation. According to the pharmacology of neuromuscular blocking agents, muscle twitch tension is maintained until sufficient numbers of postsynaptic nAChRs have been blocked by neuromuscular antagonists.\textsuperscript{25,26} This so-called ‘margin of safety’ concept describes the relationship between neuromuscular antagonists, ACh, and nAChRs. We found that T1 depression was rapidly achieved, and TOF fade was exaggerated at a relatively low PZP concentration of 10 nmol L\textsuperscript{-1}, particularly in comparison to the control group.

The third limitation of this investigation arose from the fact that several types of receptors (eg mAChRs and nAChRs) are also responsible for presynaptic ACh release at the neuromuscular junction. Muscarinic cholinoreceptors may have some role postsynaptically as well, but in the smooth muscle, developing muscles, and cultured muscle.\textsuperscript{27,28} The ACh receptor described so far in skeletal muscle is nicotinic.\textsuperscript{29} In addition to the presynaptic muscarinic cholinoreceptors and nAChRs, presynaptic purinergic A\textsubscript{1} and A\textsubscript{2A} receptors modulate the spontaneous and evoked release of ACh. When ACh is released from the presynaptic terminal by spontaneous or evoked stimulation, the concentration of the endogenous purinergic agonist, adenosine, increases at neuromuscular junctions and then controls ACh release through a feedback mechanism.\textsuperscript{30} Several studies have reported conflicting results regarding the effect of presynaptic purinergic receptor activation on ACh release. In one study, micromolar concentrations of adenosine reduced the evoked quantal release or spontaneous release of ACh,\textsuperscript{31,32} but another report found that submicromolar concentrations exerted the opposite effect.\textsuperscript{32,33} As such, we tried to apply the same frequency (2 Hz for TOF and 50 Hz for tetani) in all groups to ensure that the environment of adenosine for neuromuscular purinergic A\textsubscript{1} and A\textsubscript{2A} at the neuromuscular junction was similar.

In conclusion, the overall function of neuromuscular postsynaptic mAChRs is to preserve or save the presynaptic function in the presence of various neuronal stimulations. This function may be affected when the balance of action between these receptors is disrupted. Moreover, we noted that presynaptic muscarinic M\textsubscript{1} receptors can modulate the rocuronium-induced EC\textsubscript{95} and TOF fade after PZP
Table 3

| Characteristic | TOF fade study | Tetanic fade study |
|---------------|----------------|-------------------|
|               | Control (n=8) | PZP10 (n=8) | PZP100 (n=8) | Control (n=10) | MET1 (n=10) | MET10 (n=10) |
| **Mwt (mg)**  | 156.78 (26.25) | 150.30 (28.69) | 149.75 (23.09) | 158.68 (35.03) | 154.16 (37.05) | 152.28 (29.20) |
| **Wwt (mg)**  | 909.15 (110.20) | 894.40 (115.57) | 899.76 (118.63) | 956.56 (97.76) | 948.61 (135.57) | 959.68 (138.54) |
| **Length (mm)** | 11.28 (1.11) | 11.43 (1.40) | 11.14 (1.57) | 11.33 (1.50) | 10.83 (1.33) | 11.33 (1.86) |
| **Width (mm)** | 22.71 (1.80) | 22.86 (1.35) | 22.71 (1.60) | 23.50 (1.38) | 24.00 (0.89) | 22.83 (1.46) |

The muscles were weighed after the experiments were complete. No significant inter-group differences were found. Results are presented as mean (SD).

Length, length of the hemidiaphragm; Mwt, weight of the hemidiaphragm only; width, width of the hemidiaphragm; Wwt, weight of the diaphragm and adjacent tissues.
was administered for 5 s. A resting time of 30 min was then allowed, until the T1 tensions recovered to levels measured just before the tetanic stimulation (reference T1 tension). After it was confirmed that the T1 tension had recovered to >95% of the reference T1 tension, rocuronium loading doses were administered. A rocuronium loading dose of 100 μg was considered to be suitable for inducing tetanic fade without T1 tension depression or TOF fade. Approximately 20 min was allowed for sufficient reaction time of rocuronium. A second 50-Hz tetanic stimulation was then administered for 5 s, and a resting time of 30 min was again permitted. After the T1 tensions returned to >95% of the reference T1 tension, MET was administered to achieve concentrations of 0, 1, or 10 μmol L⁻¹ in the organ bath. After a reaction time of 30 min, the third (final) 50-Hz tetanic stimulation was administered, and each tetanic fade parameter (A, B, and R, illustrated at the inset figure in Figure 4B) was measured and calculated. As a final step, a rocuronium dose-response study was performed 30 min after the administration of the third tetanic stimulation. This study protocol is illustrated in Figure 4B.

4.4 | Statistical analysis

The primary end-point of this study was the concentration of rocuronium required to depress T1 by >95%. To achieve this goal, the rocuronium concentration was serially increased in Krebs buffer by repeatedly adding loading and booster doses of the drug at regular intervals while recording twitch tension. The second study end-point was the mean TOFR when T1 depression was in the range of 50%-60%. In the clinical setting, the recovery index (ie the period of T1 recovery from 25% to 75%) is useful for comparing recovery rates from certain neuromuscular blocking agents, including PZP. The concentration-response relationships during this period are somewhat linear; therefore, the middle of this period was considered suitable for TOFR comparisons.

The results are expressed as means and standard deviations or means and standard errors. All doses were converted to μmol L⁻¹. The group average TOFR value at the point where T1 twitch depression was in the range of 50%-60% was estimated and is expressed as a mean and standard deviation. Graphs were plotted and statistical analyses were completed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). For T1 depression, we used the following equation: \( y = 0.832 - 0.50 \times \sin(0.3x) \), where \( y \) represents the T1 depression, \( x \) represents the concentration of rocuronium, and \( \Omega \) represents the range of the regression curve \( (R^2=0.868) \). For TOFR, we used the following equation: \( y = 0.868 - 0.50 \times x^2 \), where \( y \) represents TOFR, \( x \) represents the concentration of rocuronium, and \( \Omega \) represents the slope of the regression curve \( (R^2=0.832) \). The mean group values of \( \Omega \) and \( \lambda \) were compared using the Kruskal-Wallis test and Bonferroni’s post-hoc test. Rocuronium EC₅₀ and EC₉₀ values for twitch tension data were calculated by fitting nonlinear regression curves to group data. Statistical significance was accepted for P-values <0.05.
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DISCLOSURE

The authors have no conflicts of interest to declare in association with this work.

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