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

Yong Beom Kim[1] | Sangseok Lee[2] | Kyeong Chun Lee[1] | Ha Jung Kim[3] | Young Jin Ro[3] | Hong-Seuk Yang[3]

[1]Department of Anaesthesiology and Pain Medicine, Gil Medical Center, College of Medicine, Gachon University, Incheon, Korea

[2]Department of Anaesthesiology and Pain Medicine, Sangye Paik Hospital, College of Medicine, Inje University, Seoul, Korea

[3]Department of Anaesthesiology and Pain Medicine, Asan Medical Center, College of Medicine, Ulsan University, Seoul, Korea

Correspondence
Hong-Seuk Yang, Department of Anaesthesiology and Pain Medicine, College of Medicine, Ulsan University, Asan Medical Center, Seoul, Korea.
Email: hsyang@amc.seoul.kr

Summary

This study investigated the effect of muscarinic M_1 and M_2 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 M_1 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 M_2 receptor blocker, loaded at 0 μmol L^{-1} [control], 1 μmol L^{-1} [MET1], or 10 μmol L^{-1} [MET10]). The EC_{95} 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 M_1 and M_2 receptors affect the rocuronium-induced neuromuscular block as demonstrated by the reduced EC_{95} and TOF ratios (M_1 antagonist, pirenzepine) or the enhanced 50-Hz tetanic fade (M_2 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 M_1 and inhibitory M_2 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 neurotransmission 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 M_1 receptors may be primarily involved in the release of ACh via TOF stimulation because the M_1 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 M_1 receptor is inhibited by an M_1 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 M_1 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 A_{2A} receptors, which counteract M_1 receptors, and potentiates M_2 inhibitory receptors.[4] Therefore, we hypothesized that presynaptic M_2 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 M_1 and M_2 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 M_1 or M_2 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 EC_{95} of rocuronium. Furthermore, we assessed whether methoctramine (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 M_1 receptor block on TOFR and EC_{95} 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 μmol 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 EC_{95} and EC_{50} values for rocuronium in each group are shown in Table 1. The following equation was derived for T1 depression: $y=50 + 50\sin(\Omega x)$, 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: $y=1-\lambda x^2$, 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 (μmol L^{-1})	Mean EC_{95} (μmol L^{-1})	Mean EC_{50} (μmol L^{-1})	Mean Ω	95% CI	Mean λ	95% CI
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[a] (1.05)	22.37[a]	12.90[a]	0.678[a]	0.638-0.717	0.003[a]	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. EC_{50} and EC_{95} 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). [a]Denotes values that were significantly lower than the results of other groups (P<.01).

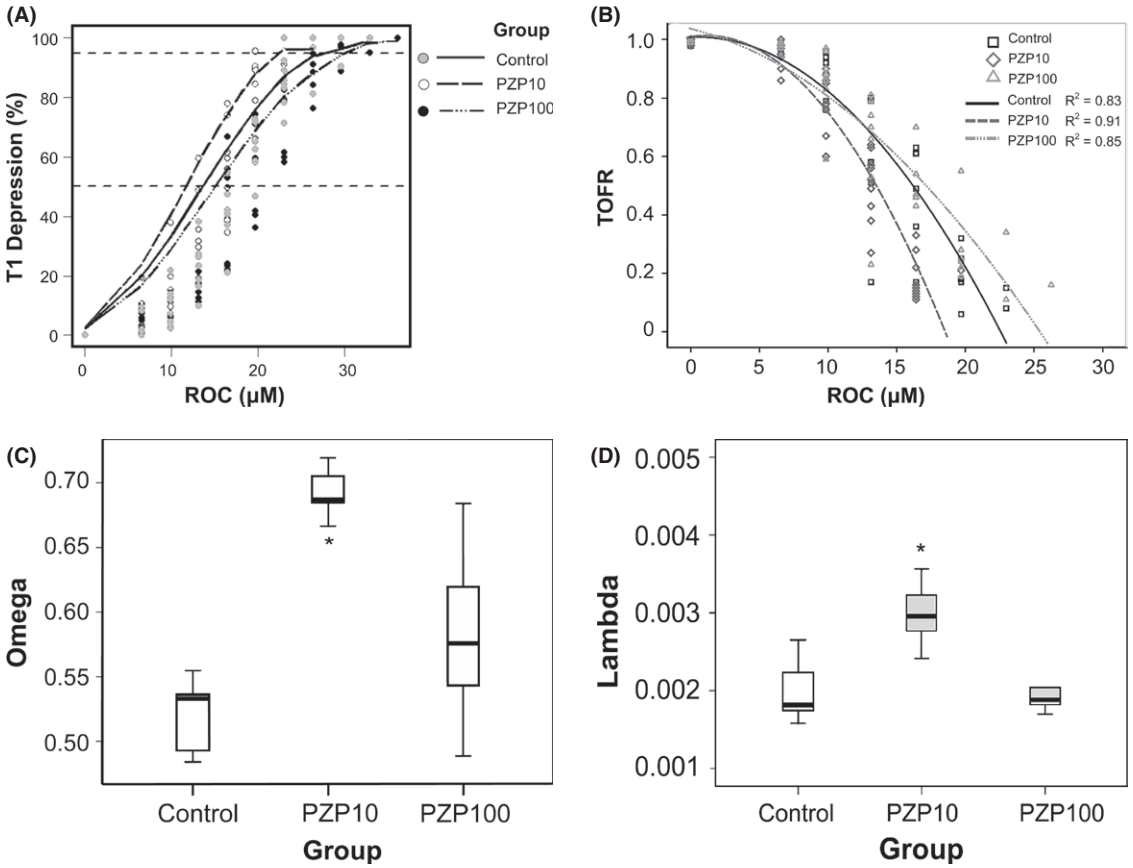

FIGURE 1 Actions of rocuronium on rat phrenic nerve/hemidiaphragm tissues. (A) Dose-response curves of the effect of rocuronium on T1 depression and (B) train-of-four ratio (TOFR) values in the control, PZP10, and PZP100 groups. The R^2 values of each regression line were > 0.8. (C) The regression equation of T1 depression: $y = 50 + 50\sin(\Omega x)$, where y represents the T1 depression, x represents the concentration of rocuronium, and Ω represents the slope of the regression curve. The dose-response curves were shifted to the left in the PZP10 group (large mean Ω, P=0.001). (D) The regression equation for TOFR: $y = 1 - \lambda x^2$, where y and x represent TOFR and concentration of rocuronium, respectively, and λ represents the slope of the regression curve. TOF fade was detected more rapidly in the PZP10 group than in the control (P=0.001). However, changes in these variables in the PZP100 group did not differ significantly from those observed in the other groups.

0.364 ± 0.121; P=.02) (Figure 2). However, no significant differences were found between the PZP100 group and the control group for any of the variables examined.

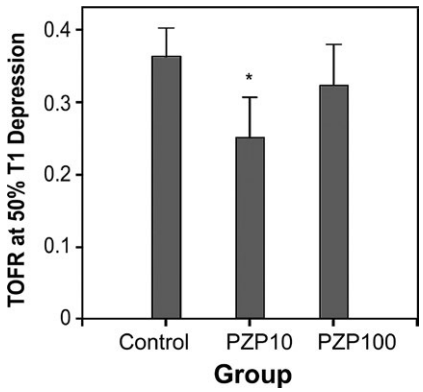

FIGURE 2 TOFR values at 40%-50% of T1 twitch tension depression. The TOFR value in the PZP10 group was significantly lower than that in the control group (*P=.02). However, the corresponding value in the PZP100 group did not differ significantly from that in the control or PZP10 groups

2.2 | Effect of muscarinic M_2 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=.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 MET1 groups (P=.005) (Table 2, Figure 3A). The percentage changes in *R* values ($\%R=100\times R_{\text{post-incubation}}/R_{\text{pre-incubation}}$) 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 M_1 receptors were found to play an important role in the rocuronium-induced decrease in twitch tension and TOF fade. The EC_{50} and EC_{95} 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 M_1 receptor is functional when the phrenic nerve is subjected to TOF stimulation; however, the findings obtained from the PZP100 group did not suggest this.

Pirenzepine is generally used as a muscarinic M_1-selective blocking agent because its affinity for M_1 receptors is ~200 times more than for M_2 receptors,[11] which indicates that it is not absolutely M_1-specific. Atropine acts as a non-specific muscarinic blocker to modulate ACh release by inhibiting both M_1 and M_2 receptors, which decreases spontaneous miniature end plate potential (mEPP) frequency and increases evoked ACh release.[2,12] By modulating the spontaneous quantal release, presynaptic M_1 and M_2 receptors preserve and stabilize synaptic function.[12] TOF fade during neuromuscular blockade is a well-known presynaptic phenomenon that is primarily mediated by the nicotinic ACh receptor (nAChR).[13-18] However, in an in-vivo rat study, it was found that the dihydro β erythroidine (DHβE)-induced blockade of the presynaptic $\alpha_3\beta_2$ nAChR did not cause any change in twitch tension or TOF fade.[19] We speculate that, in the PZP100 group, all these factors resulting from the partial M_2 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 M_2 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 M_2 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.[4,6,20,21] 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 M_2 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,[22] blockade of M_2 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.[23] 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 M_2 domain within the ion channel of nAChRs as non-competitive inhibitors.[24] In their study, the MET as a non-competitive inhibitor had an IC_{50} of 4.2 μmol L^{-1}. In the present study, the concentration of MET in the MET10 group was more than

TABLE 2 Parameters obtained via tetanic stimulation

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

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. [a]Denotes decreases that were significant versus the initial values within the group (P<.01). [b]Denotes decreases that were significant versus the pre-incubation value within the group (P<.01). [c]Denotes values that differed significantly from those of the control group (P<.01). [d]Denotes values that differed significantly from the MET1 values (P<.01).

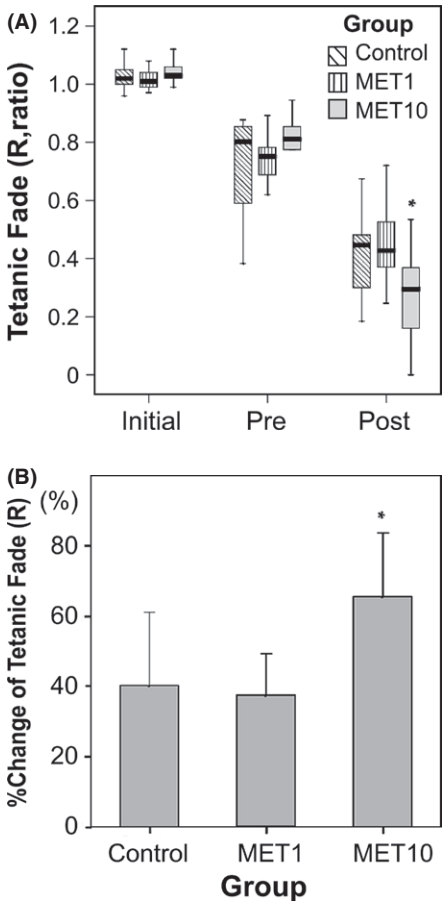

FIGURE 3 (A) Comparison of the changes in the tetanic fade ratio after incubation with MET ($R_{post\text{-}incubation}/R_{pre\text{-}incubation}$). During the initial and pre-incubation (Pre) periods, tetanic fade ratios did not significantly differ between the groups. However, the tetanic fade ratios in the post-incubation (Post) period in the MET10 group decreased more than in the other groups (*P=.005). (B) Comparison of the changes in the percentage of the tetanic fade ratio (%R) after incubation with MET ($R_{post\text{-}incubation}/R_{pre\text{-}incubation}$). During the initial and pre-incubation periods, tetanic fade ratios did not significantly differ between the groups. However, the %R was significantly larger in the MET10 group than in the other groups (*P=.005)

twice the IC_{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[24]—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-μmol L^{-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_1 and M_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 presynaptic muscarinic and coupled serine threonine kinases have been performed by other authors who explained the function of presynaptic mAChRs in ACh release under various conditions,[19-21] but most of these studies used different recording methods and muscles. These studies also reported that neuromuscular presynaptic mAChRs may have an overall conservative effect of presynaptic 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 anatogonists.[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^{-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.[27,28] The ACh receptor described so far in skeletal muscle is nicotinic.[29] In addition to the presynaptic muscarinic cholinoreceptors and nAChRs, presynaptic purinergic A_1 and A_{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.[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,[31,32] but another report found that submicromolar concentrations exerted the opposite effect.[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_1 and A_{2A} at the neuromuscular junction was similar.

In conclusion, the overall function of neuromuscular presynaptic 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_1 receptors can modulate the rocuronium-induced EC_{95} and TOF fade after PZP

administration. Indirect stimulation at <5 Hz (the most commonly applied TOFR) is often used to monitor neuromuscular blockade, but the TOFR may be altered when the M_1 receptor is blocked by PZP because the facilitatory effect of ACh release on repeated stimulation is inhibited. Our present study findings indicated that patients taking medications targeting the muscarinic M_1 receptor (eg PZP) before or during surgery may exhibit modulations or distortions in the values that are representative of recovery from neuromuscular blockade. In addition, although the M_2 receptor has an inhibitory effect on ACh release when activated, its antagonism by MET may waste the ACh released by evoked stimulation, in turn affecting the tetanic tension of the hemidiaphragm. As such, our findings emphasize that neuromuscular monitoring findings in patients treated with a muscarinic cholinoreceptor antagonist (eg PZP) must be interpreted with care.

4 | METHODS

4.1 | Basic study design and sample preparation

The study protocol was approved before study initiation by the Ethics Committee of the Laboratory of Animal Research, Asan Institute of Life Science (Seoul, Korea) on 1 December 2012 (protocol no. 2012-14-255, 2015-14-076). All animals were raised at a constant temperature of 22°C and maintained under a regular diurnal cycle with food and water supplied ad libitum. The size and weight of the phrenic nerve/hemidiaphragm tissue specimens were obtained, and the lengths of their base widths (diaphragm width attached to the thoracic wall) are described in Table 3. To ensure tissue viability throughout the experiments, tissue specimens were immersed in Krebs buffer solution (118 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $CaCl_2$, 4.7 mmol L^{-1} KCl, 1.2 mmol L^{-1} $MgSO_4$, 1.4 mmol L^{-1} KH_2PO_4, 25 mmol L^{-1} $NaHCO_3$, 11 mmol L^{-1} α-$_D$-glucose) maintained at 35°C with 95% O_2/5% CO_2 with continuous bubbling. Pirenzepine and MET were purchased from Sigma-Aldrich Korea (Gyeonggi Do, Republic of Korea). Two different PZP stock solutions and two different MET stock solutions were prepared and stored at 4°C in a refrigerator. These stock solutions were prepared such that the same volumes were used to achieve different concentrations of PZP or MET. The stock solutions were discarded 2 weeks after preparation.

4.2 | TOF fade study protocol

Twenty-four male Sprague-Dawley rats, with an average weight of 223.02 ± 19.98 g (range, 196.59-266.92 g), were used in the study. Each rat was intraperitoneally anaesthetized with 5-μg/g thiopental sodium. The phrenic nerve/hemidiaphragm tissues were immediately obtained and fixed to frames with electrodes. The tissue specimens were then mounted in an organ bath and immersed in 75-mL oxygenated Krebs buffer solution. The tendinous portion of the diaphragm of each specimen was attached to a Grass FT03 Force Transducer (Grass Instruments, West Warwick, RI, USA), and 40-mN resting tension was applied. The phrenic nerve was attached to a bipolar electrode and stimulated using a Grass S88 Stimulator (Grass Instruments). Supramaximal stimulation with a square wave pulse of 0.2 ms was administered every 30 s at a TOF time of 2 Hz. All waveforms were acquired, displayed using the PowerLab 4/26 Data Acquisition System (AD Instruments, Sydney, NSW, Australia), and stored on an offline personal computer system using LabChart 7 software (AD Instruments, Colorado Springs, CO, USA).

Phrenic nerve/hemidiaphragm specimens were randomly allocated to one of three PZP groups: control group (PZP at 0 nmol L^{-1}), PZP10 group (PZP at 10 nmol L^{-1}), and PZP100 group (PZP at 100 nmol L^{-1}). For each sample, twitch tension was serially monitored after a 30 min stabilization time. To ensure the designated concentration of PZP was correct in each group, the same volume of Krebs solution or two different PZP stock solutions were added to the organ bath. A 30 min reaction time was allowed in each group before the addition of a 400 μg loading dose of rocuronium (Esmeron®; MSD Korea, Seoul, Korea). Subsequently, a 200 μg booster dose of rocuronium was added to the organ bath. We used a 10 min cycle until >95% depression of the T1 was achieved. The study protocol is illustrated in Figure 4A.

4.3 | Tetanic fade study protocol

Thirty male Sprague-Dawley rats, with an average weight of 243.40 ± 43.84 g (range, 193.70-325.44 g), were used in this study. Specimens were prepared as described in the TOF study protocol and allocated to one of three MET concentration groups: 0 μmol L^{-1} (control group), 1 μmol L^{-1} (MET1 group), and 10 μmol L^{-1} (MET10 group). After a 30 min stabilizing period, an initial 50-Hz tetanic stimulation

TABLE 3 Characteristics of tissue specimen

	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)	145.16 (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.

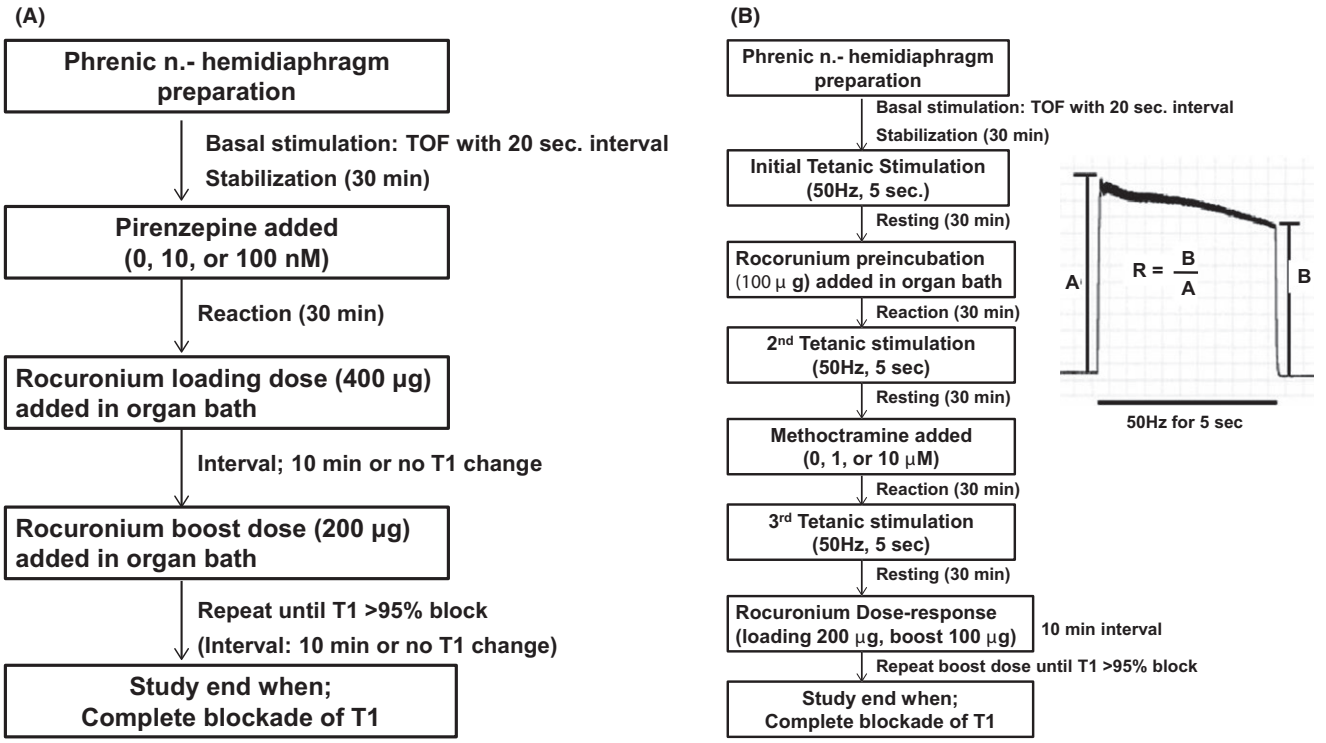

FIGURE 4 Study protocol. (A) Diagram of the TOF study. The baseline stimulation pattern is the TOF stimulation, which was repeated every 20 s. (B) Diagram of the tetanic fade study. The baseline stimulation pattern is the TOF stimulation, which was repeated every 20 s. For the tetanic fade study, we used 50-Hz tetanic stimulation for 5 s; the interval between each tetanic stimulation was 30 min. The inset figure shows twitch tension of a single tetanic stimulation. *A*, the initial tetanic twitch tension; *B*, the last tetanic twitch tension; *R*, the ratio of these twitch tensions (*R*=*B*/A)

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: $y=50 + 50\sin(\Omega x)$, where y represents the T1 depression, x represents the concentration of rocuronium, and Ω represents the slope of the regression curve (R^2=0.868). For TOFR, we used the following equation: $y=1 - \lambda x^2$, where y represents TOFR, x represents the concentration of rocuronium, and λ represents the slope of the regression curve (R^2=0.832). The mean group values of Ω and λ were compared using the Kruskal-Wallis test and Bonferroni's post-hoc test. Rocuronium EC_{50} and EC_{95} values for twitch tension data were calculated by fitting nonlinear regression curves to group data. Statistical significance was accepted for *P*-values <.05.

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DISCLOSURE

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

REFERENCES

1. Parnas SH, Parnas I. Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction. *J Physiol*. 1999;514(Pt. 3):769-781.
2. Tomàs J, Santafé MM, Garcia N, et al. Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. *J Neurosci Res*. 2014;92:543-554.
3. Pereira MW, Bornia E, Correia-de-Sá P, Alves-Do-Prado W. Presynaptic muscarinic and adenosine receptors are involved in 2 Hz-induced train-of-four fade caused by antinicotinic neuromuscular relaxants in the rat. *Clin Exp Pharmacol Physiol*. 2011;38:764-770.
4. Oliveira L, Timóteo MA, Correia-de-Sá P. Modulation by adenosine of both muscarinic M_1-facilitation and M_2-inhibition of [^{3}H]-acetylcholine release from the rat motor nerve terminals. *Eur J Neurosci*. 2002;15:1728-1736.
5. Bornia E, Correia-de-Sá P, Alves-Do-Prado W. Presynaptic facilitatory adenosine A_{2A} receptors mediate fade induced by neuromuscular relaxants that exhibit anticholinesterase activity. *Clin Exp Pharmacol Physiol*. 2011;38:164-169.
6. Campana E, Bornia S, Bando E, Machinski M Jr, Pereira MW, Alves-Do-Prado W. Presynaptic M_1, M_2, and A_1 receptors play roles in tetanic fade induced by pancuronium or cisatracurium. *J Anesth*. 2009;23:513-519.
7. Viby-Mogensen J. Neuromuscular monitoring. In: Miller RD, ed. *Miller's Anesthesia*, 7th edn. Philadelphia, PA: Churchill Livingstone; 2010:1515-1531.
8. Viby-Mogensen J, Claudius C. Neuromuscular monitoring. In: Miller RD, ed. *Miller's Anesthesia*, 8th edn. Philadelphia, PA: Churchill Livingstone; 2014:1606-1608.
9. Viby-Mogensen J, Howardy-Hansen P, Chrammer-Jørgensen B, et al. Post tetanic count (PTC): a new method of evaluating an intense nondepolarizing neuromuscular blockade. *Anesthesiology*. 1981;55:458-461.
10. Bonsu AK, Viby-Mogensen J, Fernando PU, et al. Relationship of post-tetanic count and train-of-four response during intense neuromuscular blockade caused by atracurium. *Br J Anaesth*. 1987;59:1089-1092.
11. Caulfield MP, Birdsall NJM. International Union of Pharmacology. XVIII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev*. 1998;50:279-290.
12. Santafé MM, Lanuza MA, Garcia N, Tomàs J. Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. *Eur J Neurosci*. 2006;23:2048-2056.
13. Bowman WC. Prejunctional and postjunctional cholinoceptors at the neuromuscular junction. *Anesth Analg*. 1980;59:935-943.
14. Wessler I. Control of transmitter release from the motor nerve by presynaptic nicotinic and muscarinic autoreceptors. *Trends Pharmacol Sci*. 1989;10:110-114.
15. Hwang KH, Kim SY. Neuromuscular monitoring. In: Kim SY, ed. *Neuromuscular Pharmacology in Clinical Anesthesia*. Ryo Moon Gak: Seoul; 2011:176-180.
16. Martyn JA, Fagerlund MJ, Eriksson LI. Basic principles of neuromuscular transmission. *Anaesthesia*. 2009;64(suppl 1):1-9.
17. Jonsson M, Gurley D, Dabrowski M, Larsson O, Johnson EC, Eriksson LI. District pharmacologic properties of neuromuscular blocking agents on human neuronal nicotinic acetylcholine receptors: a possible explanation for the train-of-four fade. *Anesthesiology*. 2006;105:521-533.
18. Faria M, Oliveira L, Timóteo MA, Lobo MG, Correia-De-Sá P. Blockade of neuronal facilitatory nicotinic receptors containing α3β2 subunits contribute to tetanic fade in the rat isolated diaphragm. *Synapse*. 2003;49:77-88.
19. Nagashima M, Sasakawa T, Schaller SJ, Martyn JA. Block of postjunctional muscle-type acetylcholine receptors in vivo causes train-of-four fade in mice. *Br J Anaesth*. 2015;115:122-127.
20. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J. Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. *Eur J Neurosci*. 2003;17:119-127.
21. Santafé MM, Lanuza MA, Garcia N, Tomàs J. Calcium inflow-dependent protein kinase C activity is involved in the modulation of transmitter release in the neuromuscular junction of the adult rat. *Synapse*. 2005;57:76-84.
22. Slutsky I, Silman I, Parnas I, Parnas J. Presynaptic M2 muscarinic receptors are involved in controlling the kinetics of ACh release at the frog neuromuscular junction. *J Physiol*. 2001;536:717-725.
23. Dudel J. The time course of transmitter release in mouse motor nerve terminals is differentially affected by activation of muscarinic M1 or M2 receptors. *Eur J Neurosci*. 2007;26:2160-2168.
24. Bixel MG, Krauss M, Liu Y, et al. Structure-activity relationship and site of binding of polyamine derivatives at the nicotinic acetylcholine receptor. *Eur J Biochem*. 2000;267:110-120.
25. Pennefather P, Quastel DM. Relation between subsynaptic receptor blockade and response to quantal transmitter at the mouse neuromuscular junction. *J Gen Physiol*. 1981;78:313-344.
26. Schuh FT. On dose-response curves and the receptor interaction of nondepolarizing neuromuscular blocking drugs. *Anaesthesist*. 1981;30:321-328 (article in German).
27. Wesseler I, Kirkpatrick CJ, Raché K. The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. *Clin Exp Pharmacol Physiol*. 1999;26:198-205.
28. Reyes R, Jaimovich E. Functional muscarinic receptors in cultured skeletal muscle. *Arch Biochem Biophys*. 1996;331:41-47.
29. Duclert A, Changeux JP. Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol Rev*. 1995;75:339-368.
30. Correia-de-Sá P, Sebastião AM, Ribeiro JA. Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. *Br J Pharmacol*. 1991;103:1614-1620.
31. Adamek S, Shakirzyanova AV, Malomouzh AI, Naumenko NV, Vyskocil F. Interaction of glutamate- and adenosine-induced decrease of acetylcholine quantal release at frog neuromuscular junction. *Physiol Res*. 2010;59:803-810.
32. Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA. Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats. *J Pharmacol Exp Ther*. 2010;332:153-163.
33. Santafé MM, Priego M, Obis T, et al. Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. *Eur J Neurosci*. 2015;42:1775-1787.

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