



Atropine is more efficient than ZM241385 to reduce a drastic level of fade caused by cisatracurium at 50 Hz

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ABSTRACT. The effects of atropine (non selective muscarinic antagonist) and ZM241385 (A_{2A} receptors antagonist) in the cisatracurium-induced drastic (100%) level of fade at 50 Hz (10 s) (100% Fade) were compared in the phrenic nerve-diaphragm muscle preparations of rats indirectly stimulated at a physiological tetanic frequency (50 Hz). The lowest dose and the instant cisatracurium caused 100% Fade were investigated. Cisatracurium induced 100% Fade 15 min after being administered. Atropine reduced the cisatracurium-induced 100% Fade, but the administration of ZM241385 separately, or combined with atropine, did not cause any effect in the cisatracurium-induced 100% Fade. Data indicate that the simultaneous blockage of M_1 and M_2 muscarinic receptors on motor nerve terminal by atropine is more efficient than the blockage of presynaptic A_{2A} receptors for a safer recovery of patients from neuromuscular blockade caused by cisatracurium.

Keywords: tetanic fade, adenosine, muscarinic receptors, train-of-four, neuromuscular blocker.

Atropina é mais eficiente que ZM241385 para reduzir o intenso nível de fadiga causada por cisatracurium a 50 Hz

RESUMO. Os efeitos de atropina e ZM241385 (antagonistas de receptores A_{2A}) no drástico (100%) nível de fadiga (100% Fadiga) produzido pelo cisatracúrio a 50 Hz (10 s) foram comparativamente investigados em preparações nervo frênico músculo diafragma isolado de ratos indiretamente estimuladas com a frequência fisiológica tetanizante de 50 Hz. A menor dose e o instante no qual cisatracúrio causou 100% Fadiga foram pesquisados. O cisatracúrio induziu 100% Fadiga 15 min depois de ser administrado. A atropina reduziu a fadiga de 100% causada pelo cisatracúrio, mas ZM241385, ou a administração combinada de atropina com ZM241385, não causou qualquer efeito na 100% Fadiga produzida pelo cisatracúrio. Os dados indicam que o bloqueio simultâneo dos receptores muscarínicos M_1 e M_2 no terminal nervoso motor pela atropina é mais eficiente do que o bloqueio dos receptores pré-sinápticos A_{2A} no auxílio da recuperação mais segura do bloqueio da transmissão neuromuscular causada por cisatracúrio.

Palavras-chave: fadiga, adenosina, receptores muscarínicos, trem de quatro, bloqueadores neuromusculares.

Introduction

The quotient between the fourth (T_4) and first (T_1) muscular tensions generated by train-of-four (TOF) stimulus (2.00 Hz during 2.00 s) applied on motor nerve is TOF_{ratio} (T_4/T_1). TOF_{ratio} reductions are referred to as TOF_{fade} and indicate the degree of patients' curarization (Ali, Utting, & Gray, 1970; Murphy, 2006). The patients' curarization may also be monitored by assessing the ability of skeletal muscle to maintain a sustained muscular contraction (tetanic contraction) when high electrical frequencies of stimulation (tetanizing stimulus) are applied on the motor nerve. Tetanic fade (Fade) or Wedensky inhibition occurs when the tetanic

contraction is characterized by a poorly sustained muscular contraction that follows a fast muscular contraction of high amplitude. Fade is assessed by measuring the ratio (R) between the amplitude of muscular contraction obtained at the end (B) and that (A) recorded at the beginning of tetanizing stimulus ($R = B/A$). Although better reflecting the motor nerve-firing rate under physiological conditions, high frequency (e.g. 50 Hz) nerve-evoked muscle contractions produce patient discomfort in clinical practice. Thereby, TOF stimulus is clinically employed with more frequency. However, it has been shown that the values of TOF_{fade} and Fade obtained in the presence of antinicotinic agents may express different

conditions of neuromuscular transmission, as different patterns of stimulation applied on motor nerve in the absence or in the presence of drugs, may modify the autoregulatory cholinergic (facilitatory-nicotinic receptors expressing $\alpha 3\beta 2$ subunits, facilitatory- M_1 and inhibitory- M_2 subtypes of muscarinic receptors) and the modulatory (facilitatory- A_{2A} and inhibitory- A_1 adenosine receptors) mechanisms on the terminal (Bornia, Bando, Machinsky, Pereira, & Prado, 2009; Bornia, Sá, & Prado, 2011; Pereira, Bornia, Sá, & Prado, 2011). Therefore, the Fade caused by antinicotinic (hexamethonium) agent may be severe ($\sim 89\%$) when the concentration of such agent is causing 25% TOF_{fade} (Pereira, Sá, & Prado, 2012). In contrast, the difference between the percentages of Fade ($\sim 17\%$) and TOF_{fade} ($\sim 25\%$) caused by an antinicotinic agent may not be so drastic if the antinicotinic (cisatracurium) agent also inhibits the acetylcholinesterase activity ($K_i \sim 0.32 \mu\text{mol L}^{-1}$) (Pereira et al., 2012).

In clinical practice, atropine, a nonselective muscarinic receptor blocker, is employed to prevent the parasympathomimetic effects induced by neostigmine when the anticholinesterase agent is clinically used to reverse the blockage of neuromuscular transmission caused by non-depolarizing muscular relaxants (Barrio, Miguel, García, & Pelegrín, 2007). Nevertheless, it has been shown that atropine alone already improves the recovery of Fade induced by antinicotinic (hexamethonium, D-tubocurarine) agents in the anterior tibial muscle nerve preparation of cats (Prado, Corrado, & Prado, 1987). This effect of atropine might be determined by the ability of the drug to reduce the action of acetylcholine on the presynaptic inhibitory- M_2 muscarinic receptors when the motor nerve is being stimulated at tetanizing frequencies (Prado et al., 1987). On the other hand, it has been shown that cisatracurium, an antinicotinic muscular relaxant agent that exhibits anticholinesterase activity in its molecules at clinical concentration (Bornia et al., 2009), also causes Fade in the phrenic nerve diaphragm muscle preparations of rats, but the Fade caused by the agent is not mediated by presynaptic inhibitory- M_2 receptors. In fact, Fade caused by cisatracurium seems to depend more on the activation of facilitatory- M_1 and facilitatory- A_{2A} adenosine receptors than on activation of presynaptic inhibitory- M_2 receptors on motor nerve (Bornia et al., 2009; Bornia et al., 2011; Pereira et al., 2012). Contrastingly, it has been reported that the cisatracurium-induced TOF_{fade} occurs mainly by the activation of M_2 and A_{2A} receptors caused by acetylcholine released from

motor nerve and from the adenosine respectively released from neuronal and muscular sources (Pereira et al., 2012).

Since the fades (TOF_{fade} and Fade) caused by non-depolarizing agents exhibiting anticholinesterase activity in their molecules may be determined by mechanisms different from those involved with the fades causing muscular relaxants without anticholinesterase activity (Pereira et al., 2012), and taking into account that atropine is the muscarinic antagonist used in anesthesiology practice (Barrio et al., 2007), current study comparatively tested the protective effects of atropine and ZM241385, the latter being a blocker of A_{2A} receptors (Pereira et al., 2012) against the cisatracurium inducing drastic levels of fades (83% TOF_{fade} , 100% Fade) in the phrenic nerve diaphragm muscle of rats.

Material and methods

The procedures used in current assay were approved by the Committee for Ethics in Experimental Studies of the State University of Maringá, Maringá, Paraná State, Brazil. Male Wistar rats (250 g) were anaesthetized with an intramuscular injection of ketamine (40 mg kg^{-1}) and xylazine (8 mg kg^{-1}). Phrenic nerve diaphragm-muscle preparations were isolated and assembled according to methods by Bülbbring (1946). Each preparation was immersed in a 20 mL chamber with Krebs' buffer (composition in mmol L^{-1} : NaCl 110.0; KCl 4.70; CaCl_2 3.0; MgCl_2 1.3; NaHCO_3 25.0; KH_2PO_4 1.0; glucose 11.1) at 37°C , continuously gassed with a mixture of oxygen (95%) and carbon dioxide (5%). Hemidiaphragms were connected to an isometric force transducer (FT10; Grass Instruments Division, West Warwick, RI, USA). Muscle contraction responses were recorded continuously at a resting tension of 50 mN with a PowerLab data-acquisition system (Chart Software; ADInstruments, Castle Hill, NSW, Australia). The phrenic nerve was stimulated with a bipolar platinum electrode (supramaximal rectangular pulses, 0.05 ms). The preparations were stimulated indirectly at 0.2 Hz for 15 min (equilibration protocol). Afterward, the phrenic nerve was supramaximally stimulated with TOF stimuli delivered at 2 Hz, which was repeated once every 15 s over a period of 7.3 min (TOF protocol). The above sequence was repeated three times. The muscular tension produced at the beginning of each train (T1) was compared with the muscular tension obtained at the end the train (T4). TOF_{ratio} , the quotient of these two values (T4/T1), was taken as a measure of drug-induced neuromuscular transmission failure (TOF_{fade}). TOF

stimulus obtained at instants 15 and 30 min after the cisatracurium administration (Figure 1a) was systematically used for comparison. Preparations were stimulated indirectly with 10 s-duration high-frequency (50 Hz) tetanic trains applied at 15 min intervals so that the fading of tetanic contractions could be investigated (Figure 1c). This interval was selected to avoid the possible influence of previous tetanic stimulation on fade induced by subsequent stimuli. Initial tetanic tension at the beginning of the tetanic stimulus (A) and the tension at the end of the tetanic stimulus (B) were recorded and the B/A ratio (Fade_{ratio}) was calculated (Figure 1d). A, B and Fade_{ratio} values were obtained at the same instants used to assess the TOF_{fade}, i.e., in the absence of drugs (control, one record of Fade), after atropine (0.2 μ M)

or 4-(2-[7-amino-2-(2-furyl)[1,2,4] triazol [2,3-a][1,3,5] triazin-5-ylamino] ethyl) phenol] (ZM 241385; 10 nmol L⁻¹), separately (two records of Fade) and in combined administration of drugs (atropine or ZM 241385 followed by administration of cisatracurium). The cisatracurium concentration (2.2 μ M) used was that previously tested capable of causing 25% TOF_{fade} after 3 min from its administration in Krebs buffer (Pereira et al., 2012). Muscarinic and adenosine receptor antagonists were applied to the bath solution at a constant volume of 10 μ L 1 min before the second sequence of TOF or tetanic stimulation, with the antinicotinic muscle relaxing agents, then applied 1 min before the third sequence of TOF or tetanic stimulation, respectively (Figure 1a and c).

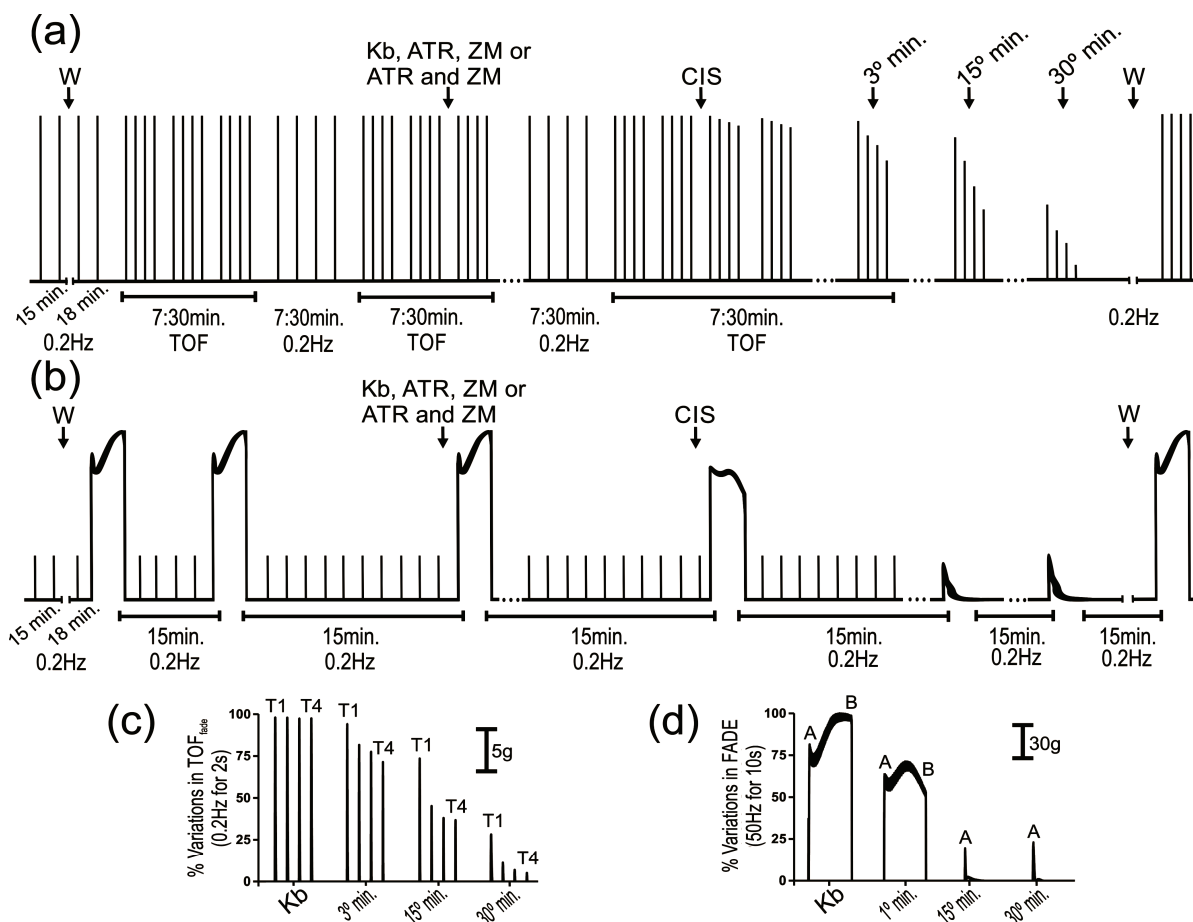


Figure 1. Schematic representation of experimental protocol used to investigate the effects of atropine and ZM241385A2A in the TOF_{fade} (a) or Fade (b) induced by cisatracurium (CIS) in the phrenic nerve diaphragm muscle preparations of rats indirectly stimulated at 0.2 Hz, TOF stimulus (2.0 Hz for 2 s) or tetanic frequency (50 Hz for 10 s). In (a) and (b) are indicated instant to addition of Krebs buffer (Kb) solution or atropine (ATR), ZM241385 (ZM) or the combined (ATR and ZM) administration of these agents in the bath 1 min before TOF stimulation (a) or tetanic frequency (b) sequences. The instant for the administration of CIS alone or in presence of ATR and/or ZM is indicated in (a) and (b). W indicates nutrient solution (Krebs buffer, Kb) exchange. Typical trace recording of nerve-evoked hemi-diaphragm contractions obtained during TOF stimulation (c) or tetanic stimulation (d) in the presence of 2.20 μ M CIS is also displayed. The equivalent tension of 5.00 g for TOF stimulus (c) and 30.00 g for tetanic stimulation (d) are also shown. TOF_{fade} was calculated as the ratio (R) between the fourth tension recorded at the end (T4) and the initial tension (T1) at the beginning of TOF stimulation (c). Fade was evaluated by the ratio between the final (B) and the initial (A) tetanic tensions obtained at 50 Hz (d).

Statistical analysis

Data are expressed as mean \pm SEM of *n* individual experiments. The statistical significance of experimental results was analyzed by two-sided one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test ($p < 0.05$, Prism 3.02; GraphPad, San Diego, CA, USA).

Results and discussion

Cisatracurium (2.2 μ M) causes 25% TOF_{fade} (2 Hz) 3 min after its administration in the Krebs buffer (Pereira et al., 2012). In these conditions, cisatracurium determines 17% of Fade (50 Hz) (Pereira et al., 2012). Current study shows that the fades (TOF_{fade} and Fade) caused by 2.2 μ M cisatracurium are progressively worsened (TOF_{fade} = $52.73 \pm 7.2\%$, *n* = 4, at *T* = 15 min and $82.74 \pm 6.35\%$, *n* = 4, at *T* = 30 min; Fade = $100 \pm 0.0\%$, *n* = 4, at *T* = 15 or 30 min), when the parameters (*T*₄/*T*₁ and *R* = *B*/*A*) used to verify levels of fades in neuromuscular transmission were analyzed 15 min after the instant of administration of neuromuscular relaxant (Figure 2a and d). Atropine (0.2 μ M) and the antagonist of A_{2A} receptors ZM241385 (10 nM) separately, or in combination, reduced the TOF_{fade} caused by 2.2 μ M cisatracurium (from 82.74% to $\sim 48\%$) at *T* = 30 min (Figure 2a). However, there was no significant difference ($p > 0.05$) between these treatments (Figure 2a). Atropine (0.2 μ M) alone, but not ($p < 0.05$) ZM241385 (10 nM) or the combined administration of atropine with ZM241385, expressed a significant ($p < 0.01$) reduction (from $100 \pm 0.0\%$, *n* = 4 to $81.83 \pm 3.64\%$, *n* = 4) in the cisatracurium-induced 100% Fade at *T* = 15 min (Figure 2d). This effect caused by atropine (0.2 μ M) was determined by significant ($p < 0.05$) improvements in *A* (from $77.8 \pm 2.78\%$, *n* = 4 to 59.27 ± 4.63 , *n* = 4) and *B* (from $100 \pm 0.0\%$, *n* = 4 to $85.21 \pm 2.78\%$, *n* = 4) values (Figure 2e and f).

The blockage of presynaptic A_{2A} receptors by ZM241385, or the blockages of M₁ and M₂ muscarinic receptors on the motor nerve by atropine, reduces ($\sim 48\%$) TOF_{fade} caused by cisatracurium (*T* = 30 min). However, no significant ($p > 0.05$) difference has been detected between the effect caused by the blockade of presynaptic A_{2A} and blockade of presynaptic M₁ and M₂ receptors by ZM241385 and atropine, respectively, in TOF_{fade} (2 Hz) caused by

cisatracurium. Neither the combined administration of ZM241385 with atropine produces ($p > 0.05$) any reduction in the cisatracurium-induced TOF_{fade} (2 Hz) better than that caused by ZM241385 or atropine. In contrast, it has been verified that reductions of M₁ and M₂ presynaptic muscarinic receptors activity by atropine decreased ($\sim 18\%$) the cisatracurium-induced Fade (50 Hz, *T* = 15 min). However, Fade caused by such non depolarizing neuromuscular relaxant was not affected by treatment of preparations with ZM241385, or by combined administration of ZM241385 and atropine.

It has previously been shown that the anticholinesterase activity of cisatracurium (Bornia et al., 2011; Pereira et al., 2012) plays a key role in TOF_{fade} and Fade caused by this agent, since the mechanisms that regulate acetylcholine release via presynaptic muscarinic and adenosine receptors are differently affected by TOF and tetanic stimulations conditions (Bornia et al., 2011; Pereira et al., 2012). In fact, the activations of inhibitory-M₂ and facilitatory-A_{2A} receptors by acetylcholine and adenosine, respectively, are involved with TOF_{fade}-caused cisatracurium when this agent causes 25% TOF_{fade}, as the blockades of M₂ or A_{2A} receptors by methoctramine or ZM241385, respectively, improved 25% TOF_{fade} caused by cisatracurium (Pereira et al., 2012). Nevertheless, the blockages of M₁ or M₂ muscarinic receptors by pirenzepine or methoctramine, respectively, worsen Fade (50 Hz) induced by cisatracurium when this neuromuscular relaxant causes 17% Fade (50 Hz) (Pereira et al., 2012). Current study shows that the mechanisms involved with 25% TOF_{fade} (Pereira et al., 2012) and drastic (82.74%) TOF_{fade} caused by cisatracurium seem to be similar, i.e., they involve presynaptic inhibitory-M₂ and facilitatory-A_{2A} receptors activations by acetylcholine and adenosine, respectively. However, the mechanisms involved with cisatracurium-induced 17% Fade (Pereira et al., 2012) seem to be different from those involved with cisatracurium which induces 100% Fade. In fact, in this last condition, only atropine, but not ZM241385, was able to cause a small ($\sim 18\%$), but significant ($p < 0.05$) improvement in the cisatracurium-induced 100% Fade (50 Hz).

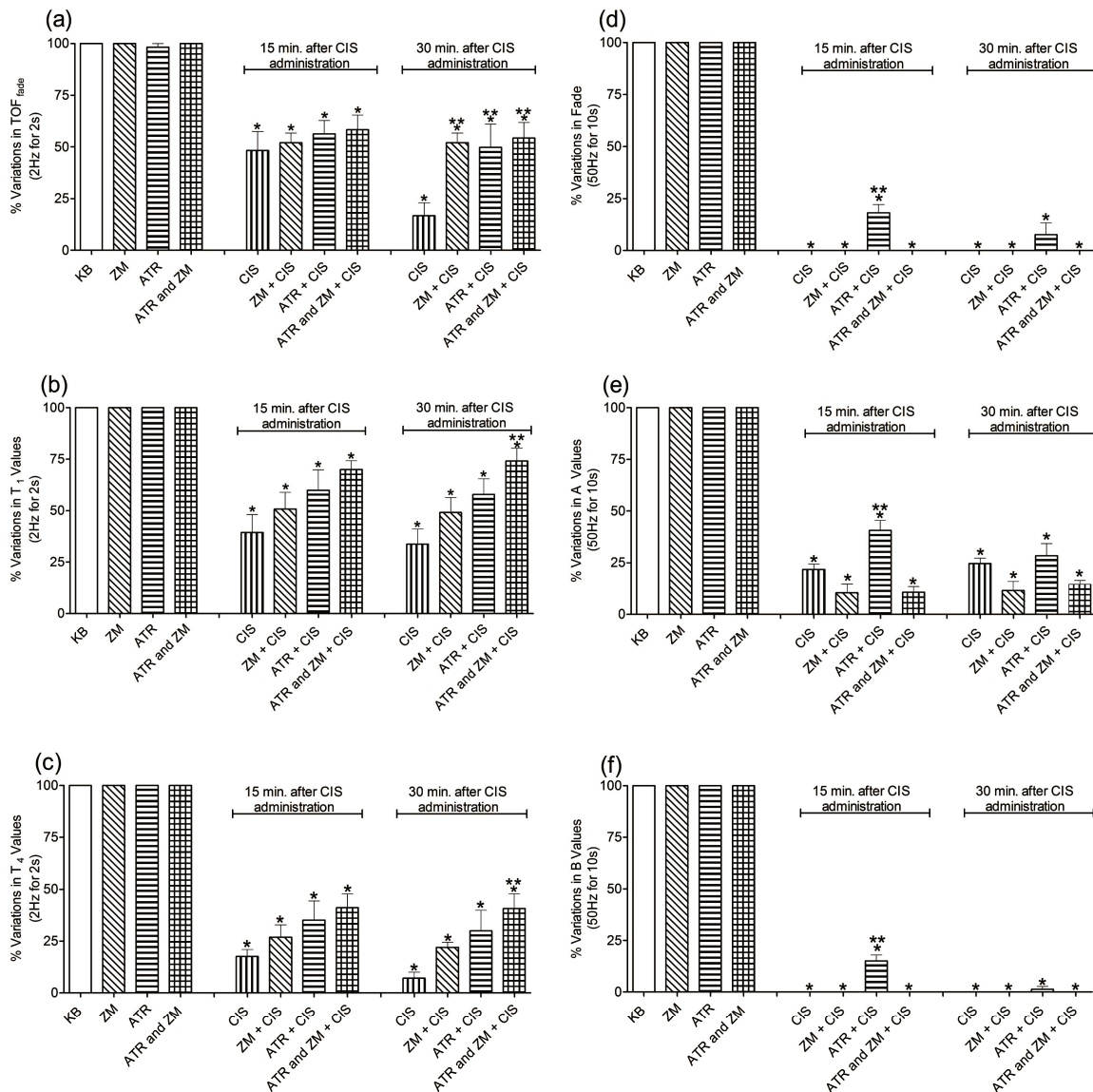


Figure 2. Percentage variation of TOF_{fade} (a) and Fade (d) caused by 2.20 $\mu\text{mol L}^{-1}$ cisatracurium (CIS) when 2 Hz train-of-four (a) and 50 Hz (d) tetanic frequency were used to stimulate the phrenic nerve trunk in the presence of atropine (ATR, 0.20 μM) or ZM241385 (ZM, 10 nmol L^{-1}) separately or in combination (ATR and ZM + CIS). The percentage variations in T₁ (b), T₄ (c), A (e) and B (f) values obtained in TOF (a) and Fade (d) conditions are displayed. 15 and 30 min after CIS indicate instants used to assess the effects of drugs. Data are the mean \pm SEM of 4 experiments per group. * $p < 0.05$ compared with the control condition in the absence of test drugs and ** $p < 0.05$ compared with CIS separately (ANOVA followed by two-sided Bonferroni post-hoc test).

The drastic (82.74%) TOF_{fade} caused by cisatracurium seems to exhibit an amount of adenosine in the synaptic cleft which might be lower than that extant during 100% Fade, as at 50 Hz there is a higher activation of presynaptic M₁ receptors by acetylcholine. Thereby, the adenosine outflow through the equilibrative transport system is increased (Oliveira, Timóteo, & Sá, 2009). Previous findings of our research group show that adenosine A_{2A} receptors play a pivotal role in motor nerves, favoring muscarinic M₂ inhibition by breaking M₁ receptor counteractions caused by downstream signaling involving activation of protein kinase A

(PKA) and Ca⁺⁺ level in the motor nerve (Oliveira & Sá, 2005). In fact, current study shows that atropine improved the Fade caused by cisatracurium by mitigating more the reduction in A than in B values caused by muscular relaxant in R (B/A) parameter. These data indicate that a higher activation of M₁ receptors on motor nerve by acetylcholine might be occurring in the cisatracurium-induced 100% Fade. It has already been demonstrated that A-value is influenced by presynaptic M₁ receptors activity when the motor nerve is stimulated at 50.00 Hz trains (Oliveira, Timóteo & Sá, 2002). Since an improvement in the

cisatracurium-induced Fade would be the expected effect when the preparations were previously treated with ZM241385 (Oliveira et al., 2002; Pereira et al., 2012), it was supposed that the anticholinesterase activity of cisatracurium (Bornia et al., 2009; Bornia et al., 2011; Pereira et al., 2011) in the experimental condition causing 100% Fade reduced the expression of facilitatory effect induced by the blockade of A_{2A} receptors by producing a level of M_1/M_2 activation by acetylcholine higher than that determined by the separate treatment of preparations with cisatracurium. Indeed, the previous treatment of preparations with ZM241385 caused a reduction in the A-value induced by cisatracurium which was not significantly ($p > 0.05$) different from that obtained after the administration of cisatracurium. However, such apparent reduction caused by ZM241385 might be indicative of a previous increment in the acetylcholine release, thereby inducing a higher activation of presynaptic inhibitory- M_2 receptors. Moreover, it has been proposed that failure to activate A_{2A} receptors may be exaggerated by blocking A_{2A} receptors with ZM241385, mainly when the level of extracellular adenosine is reduced. This situation is expected when the postsynaptic action of cisatracurium on muscular nicotinic receptors reduces the amount of adenosine from postsynaptic source in 100% Fade condition (Matos et al., 2011).

As a whole, data show that the blockage of presynaptic A_{2A} adenosine receptors by ZM241385 contributes more to recover the blockage of neuromuscular transmission produced by a non-depolarizing muscular relaxants exhibiting inhibitory acetylcholinesterase activity in their molecules when the level of acetylcholine in the synaptic cleft is not yet causing intense M_1/M_2 muscarinic receptors activity on motor nerve terminal, i.e. when the motor nerve is electrically stimulated with moderate (TOF) rate frequencies (Figure 3). In contrast, for the first time it is being shown that the simultaneous blockages of M_1 and M_2 presynaptic receptors by atropine seem to be more efficient than blockage of A_{2A} receptors to reduce the Fade caused by cisatracurium. In fact, a higher M_1/M_2 muscarinic receptors activity on motor nerve terminal seems to exist in this condition (Figure 3). Since an anticholinesterase agent is used in anesthesiology practice to induce recovery of patients from neuromuscular blockade and, since blockers of A_{2A} receptors are efficient to reduce the fades caused by cisatracurium when this agent is not causing drastic level of fades (Pereira et al., 2012), it is supposed that atropine is useful for initial recovery of patients from neuromuscular

blockade caused by cisatracurium. However, the blockage of A_{2A} receptors might contribute towards a more secure recovery of neuromuscular transmission, mainly when the muscular nicotinic receptors blockade caused by cisatracurium decreases.

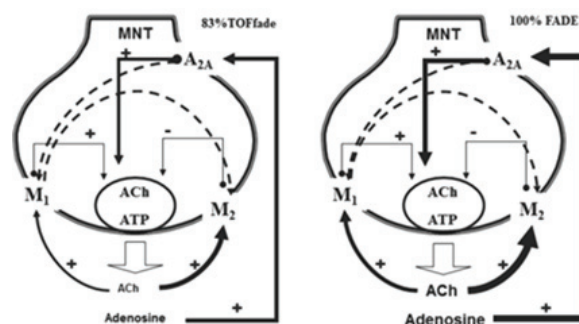


Figure 3. The role of muscarinic (M_1 and M_2) and adenosine (A_{2A}) receptors on motor nerve terminal (MNT) in the drastic TOF_{fade} (82.74%) and Fade (100%) induced by a neuromuscular blocking agent (cisatracurium) exhibiting anticholinesterase activity in the rat phrenic nerve diaphragm preparations. The different size of the letters in the adenosine and acetylcholine (ACh) words, and the different widths of the arrows indicate higher or smaller amounts of ACh or higher or lower activation of adenosine or cholinergic receptors. Blockade of muscle-type nicotinic receptors by cisatracurium reduces adenosine from skeletal muscle fibers to levels beyond those required to a full activation of facilitatory A_{2A} receptors. However, the activation of presynaptic M_1 receptors induced by acetylcholine increases the adenosine outflow through the equilibration transport system (mainly at 50 Hz frequency). Levels of extracellular adenosine and acetylcholine may be higher in the presence of a neuromuscular blocking agent exhibiting anticholinesterase activity. Fine-tuning modulation of muscarinic M_1 and adenosine A_{2A} receptors activation may change upon blocking of nicotinic autoreceptors. Decrease in the release probability of the readily releasable pool of vesicles (antinicotinic effect) coupled with the presence of adenosine, may favor unbalanced activation of muscarinic M_1/M_2 receptors. Under these circumstances, muscarinic M_1 activation may increase the amount of transmitter release per pulse leading to a higher M_2 activation, thereby inducing a poorly sustained acetylcholine release during repetitive nerve stimulation (100% Fade).

Conclusion

Blockage of presynaptic A_{2A} adenosine receptors by ZM241385 contributes more to recover the blockage of neuromuscular transmission produced by a non-depolarizing muscular relaxants exhibiting inhibitory acetylcholinesterase activity in their molecules when the level of acetylcholine in the synaptic cleft is not yet causing intense M_1/M_2 muscarinic receptors activity on motor nerve terminal. However, the simultaneous blockages of M_1 and M_2 presynaptic receptors by atropine seem to be more efficient than blockage of A_{2A} receptors to reduce the drastic level of fade caused by cisatracurium at 50 Hz.

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