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Presynaptic M1, M2, and A1 receptors play roles in tetanic fade induced by pancuronium or cisatracurium

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Abstract
Purpose. We investigated whether presynaptic facilitatory M1 and/or inhibitory M2 muscarinic receptors contributed to pancuronium- and cisatracurium-induced tetanic fade.
Methods. Phrenic nerve-diaphragm muscle preparations of rats were indirectly stimulated with tetanic frequency (75 ± 3.3 Hz; mean ± SD). Doses of pancuronium, cisatracurium, hexamethonium, and d-tubocurarine for producing approximately 25% fade were determined. The effects of pirenzepine and methoctramine, blockers of presynaptic M1 and M2 receptors, respectively, on the tetanic fade were investigated.
Results. The concentrations required for approximately 25% fade were 413 μM for hexamethonium (26.8 ± 2.4% fade), 55 nM for d-tubocurarine (28.7 ± 2.5% fade), 0.32 μM for pancuronium (25.4 ± 2.2% fade), and 0.32 μM for cisatracurium (24.7 ± 0.8% fade). Pirenzepine or methoctramine alone did not produce the fade. Methoctramine, 1 μM, attenuated the fade induced by hexamethonium (to 16.0 ± 2.5% fade), d-tubocurarine (to 6.0 ± 1.6 fade), pancuronium (to 8.0 ± 4.0% fade), and cisatracurium (to 11.0 ± 3.3% fade). However, 10 nM pirenzepine attenuated only the fades produced by pancuronium (to 5.0 ± 0.1% fade) and cisatracurium (to 13.3 ± 5.3% fade). Cisatracurium (0.32 μM) showed antiacetylcholinesterase activity (in plasma, 14.2 ± 1.6%; in erythrocytes, 17.2 ± 2.6%) similar to that of pancuronium (0.32 μM). The selective A1 receptor blocker, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 2.5 nM), also attenuated the fades induced by pancuronium and cisatracurium.
Conclusion. The tetanic fades produced by pancuronium and cisatracurium depend on the activation of presynaptic inhibitory M2 receptors; these agents also have anticholinesterase activities. The fades induced by these agents also depend on the activation of presynaptic inhibitory A1 receptors through the activation of stimulatory M1 receptors by acetylcholine.

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Introduction

"Neurotransmission fade", "tetanic fade", "fade", "neuromuscular fade", "tetanic fade in neuromuscular transmission", or "Wedensky inhibition", is a poorly sustained contraction that follows a fast muscular contraction of high amplitude when a motor nerve is electrically stimulated with high frequencies (100 to 200 Hz) [1–3]. Fade may also occur at tetanic frequencies of stimulation that normally induce sustained muscular contraction (40 to 80 Hz) if the neuromuscular preparation is treated with antinicotinic or anticholinesterase agents [2]. Transmission fade is a consequence of the gradual reduction of the amplitude of end-plate currents (e.p.cs) [1] generated by a progressive reduction of the release of acetylcholine from the motor nerve terminal [4].

It has been shown that the fade induced by d-tubocurarine, hexamethonium, or neostigmine is antagonized by atropine [2]. It has thereby been suggested that the tetanic fade induced by d-tubocurarine or hexamethonium would not only be produced by a blockade of the facilitatory nicotinic receptors on the motor nerve [2] but it would also depend on the activation of the presynaptic inhibitory muscarinic receptors (M2) by acetylcholine released from the terminal [2]. Although the neostigmine-induced fade also depends on the presynaptic activation of inhibitory muscarinic receptors by acetylcholine released from the motor nerve terminal, the neostigmine-induced fade also involves other complex mechanisms. Facilitatory nicotinic receptors ($\alpha 3\beta 2$-containing receptors) on the motor nerve terminal [5,6] have been shown to be rapidly desensitized by acetylcholine released from the terminal [5].

There is pharmacological evidence of two subtypes of muscarinic receptors (M1 and M2) on the motor nerve terminal [4,5,7]. The activation of these receptors may increase (M1) or reduce (M2) the release of acetylcholine [4,5]. The selective blockade of the presynaptic facilitatory M1 muscarinic receptors by pirenzepine (10 nM) reduces the release of acetylcholine from the nerve terminal. Although pirenzepine reduces the magnitude of the initial tetanic tension, it does not produce fade when phrenic nerve-diaphragm muscle preparations of rats are indirectly stimulated with tetanizing pulses [6]. On the other hand, the activation of both M1 and M_2 presynaptic receptors by acetylcholine released from the motor nerve may be modulated by other substances released from the motor nerve (adenosine, calcitonin gene-related peptide, substance P, vasoactive intestinal peptide) or postsynaptic (arachidonic acid) sites [4,8]. Adenosine buildup from ATP catabolism has been considered a key factor in the control of the release of acetylcholine mediated by the activities of stimulatory M1 and inhibitory M2 receptors on the motor nerve terminal [4]. In this context, a decrease in the release of acetylcholine from the motor nerve terminal is mediated by inhibitory A1 adenosinergic receptors, when the presynaptic stimulatory M1 receptors are fully operative [4]. Moreover, synergism is present between the facilitatory A2A adenosinergic receptors and the inhibitory M2 receptors, which are reciprocally regulated by excitatory M1 receptors on the motor nerve terminal [4].

Although the tetanic fade induced by d-tubocurarine, hexamethonium, or neostigmine was antagonized by atropine [2], the distinct contribution of the presynaptic M1 and M2 receptors to fades is still to be evaluated. Selective blockade of the inhibitory presynaptic M2 receptors by AF-DX116(11-[(2-1L(diethyl-amino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro6 H-pyrido [2,3-b] [1,4] benzodiazepine-6-one) reduces the fade induced by tetanic stimuli [4]. Although data indicate a role of M2 receptors in the fade induced by d-tubocurarine, hexamethonium, or neostigmine, they do not explain whether the excitatory M1 receptors are involved in the fades produced by these agents. Furthermore, a comparative investigation has still to be undertaken to verify whether there is any involvement of presynaptic M1 and/or M2 receptors in the fades induced by other antinicotinic agents (pancuronium and cisatracurium), which also exhibit antimuscarinic (pancuronium and cisatracurium) and/or anticholinesterase (pancuronium) activities [9,10]. Because identification of the mechanisms through which the different antinicotinic agents produce fade could be clinically useful for the management of neuromuscular transmission in curarized patients, the effects of 10 nM pirenzepine or 1.0 μM methoctramine (concentrations that selectively block M2 receptors) [11] on the fade induced by d-tubocurarine and hexamethonium were investigated in phrenic nerve-diaphragm muscle preparations of rats and then compared with those induced by antinicotinic agents (pancuronium, cisatracurium) [10].

Materials and methods

The Ethics Committee for Experimental Studies of the State University of Maringá approved the procedures. Male Wistar rats (250 g; $n = 138$) were anesthetized with an intramuscular injection of ketamine ($27\ mg \cdot kg^{-1}$) and xylazine ($4.3\ mg \cdot kg^{-1}$). The phrenic nerve-diaphragm muscle preparations were isolated and assembled according to Bülbring [12]. Each preparation was immersed in a 20-ml chamber containing Krebs buffer (in mM: NaCl, 110.0; KCl, 4.7; CaCl2, 3.0; MgCl2, 1.3; NaHCO3, 25; KH2PO4, 1.0; and glucose, 11.1) at 37ºC, continuously bubbled with a mixture of oxygen (95%) and carbon dioxide (5%). The phrenic nerve was stimulated through a bipolar platinum electrode (supramaximal rectangular pulses, 0.05 ms). The preparations were indirectly stimulated at 0.2 Hz, and four tetanic stimuli were applied at intervals of 15 min in order to avoid the influence of previous tetanic stimulation on the fade produced by the next stimulus. The hemidiaphragm preparation was connected to a force displacement transducer (Grass FT 03; Grass Instruments Division, West Warwick, RI, USA) to record muscular contractions on Chart Software (Powerlab AD Instruments, Castle Hill, NSW, Australia). The initial tetanic tension (A) at the beginning of the tetanic stimulus and the tension (B) at the end of the tetanic stimulus (after 10 s) were obtained, and the ratio R (B/A) was calculated [2,3] (Fig. 1). The frequency of tetanic stimulation that produced a sustained tetanic muscle contraction was determined for each preparation in the absence of drugs [2]. The R and A values obtained 15 (2nd tetanic stimulus), 30 (third tetanic stimulus), and 45 (fourth tetanic stimulus) min after the first stimulus (first tetanic stimulus; control) were assayed. The drugs were administered at the same volume of 10 μl, 1 min before tetanic stimulation. The Krebs buffer solution was administered to verify whether spontaneous changes in R values could occur during the time of execution of the experiments. The doses producing approximately 25% fade of four antinicotinic agents, hexamethonium, d-tubocurarine, pancuronium, and cisatracurium were determined. Only the dose value from each agent that produced such an effect in at least four previous preparations was employed. Then the effects of pretreatments with the M1 muscarinic receptor blocker, pirenzepine, or the M2 muscarinic receptor blocker, methoctramine were investigated. Pirenzepine 10 nM or methoctramine

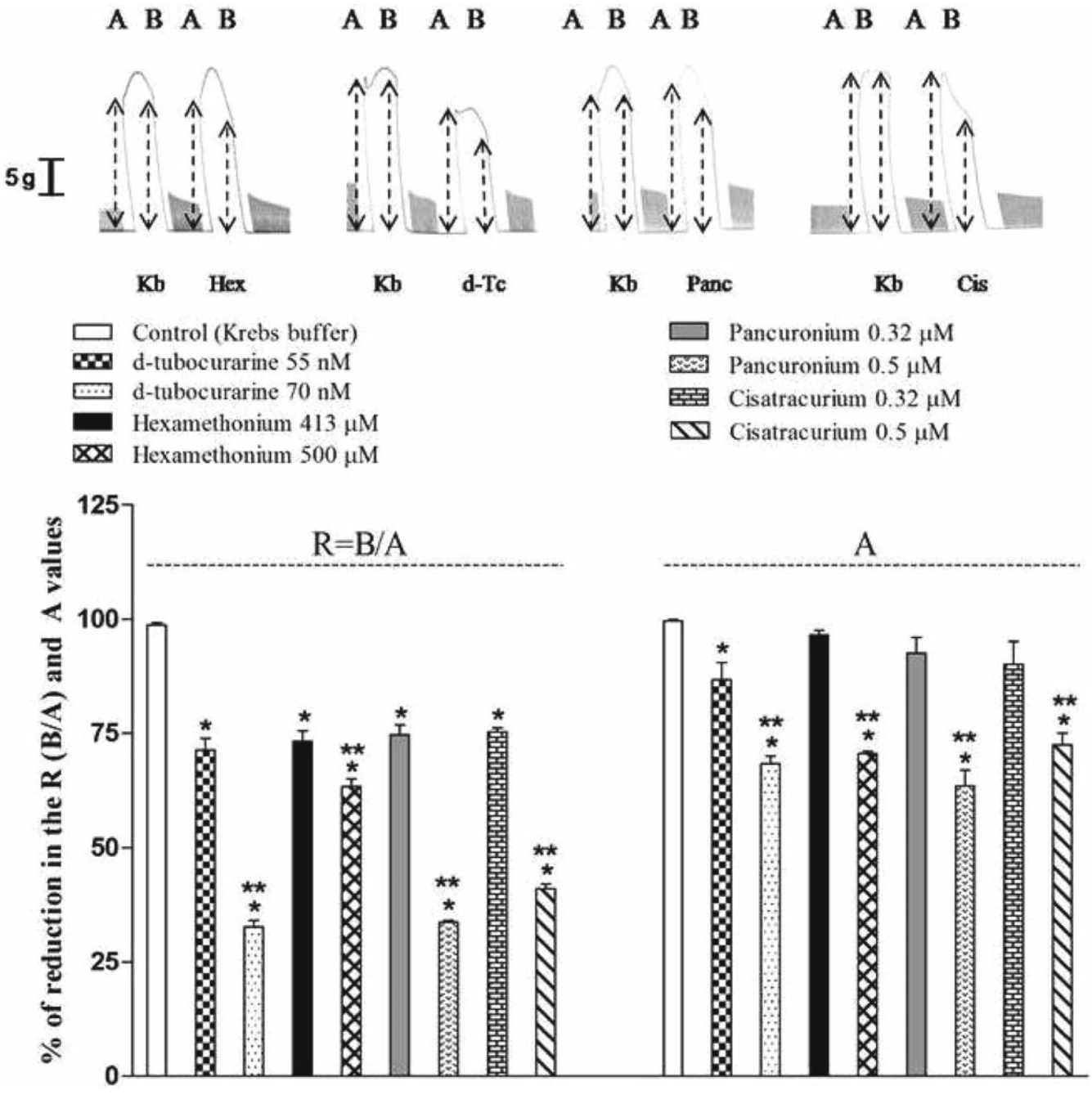

Fig. 1. Typical recording traces of nerve-evoked hemidiaphragm contractions obtained during brief tetanic stimulus (70 to 80 Hz for 10 s), in the absence (Krebs buffer; *Kb*,) and in the presence of hexamethonium (*Hex*, 413 µM at 75 Hz), d-tubocurarine (*d-Tc*, 55 nM at 70 Hz), pancuronium (*Panc*, 0.32 µM at 75 Hz), or cisatracurium (*Cis*, 0.32 µM at 80 Hz) in the bath. The equivalent tension of 5 g is indicated *on the left*. Fade was calculated as the ratio (*R*) between the tension recorded at the end (*B*) and the initial tetanic tension (*A*) at the beginning of tetanic contraction (R = B/A). The dose-effect relationship of fade (*left*) or reduction in A (*right*) values, produced by the four antinicotinic agents, is also shown by *column heights*, representing means ± SE of four experiments. **P* < 0.05 compared to control (drug-free Krebs buffer); ***P* < 0.05 vs the lowest dose of antinicotinic agent

1.0 µM was administered before the second tetanic stimulation, whereas the antinicotinic drugs were administered before the third tetanic stimulation. Further, if the fade produced by an antinicotinic agent was improved by pirenzepine, a selective blocker of A1 adenosinergic receptors, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) 2.5 nM [4] was administered before the second tetanic stimulation instead of pirenzepine.

The antiacetylcholinesterase property of cisatracurium was assessed by a colorimetric method, previously described [13], using a Shimadzu UV-visible spectrophotometer (1601 PC; Shimadzu, Kyoto, Japan). Data were compared by analysis of variance (ANOVA) followed by the Bonferroni test; P values of less than 0.05 were considered significant.

Results

The frequency of tetanic stimulation that produced a sustained tetanic muscle contraction (R = 1.0 ± 0.02; mean ± SE) was 75 ± 3.3 Hz (mean ± SD; n = 138). The concentrations capable of producing approximately 25% reductions in R values were 413 μM for hexamethonium (reduction in R value = 26.8 ± 2.4%; mean ± SE; n = 6), 0.32 μM for pancuronium (reduction in R value = 25.4 ± 2.2%; mean ± SE; n = 8), 0.32 μM for cisatracurium (reduction in R value = 24.7 ± 0.8%; mean ± SE; n = 8), and 55 nM for d-tubocurarine (reduction in R value = 28.7 ± 2.5%; mean ± SE; n = 6). These doses of hexamethonium, pancuronium, or cisatracurium did not change the values of the initial tetanic tensions (A) (Figs. 1, 3, 5, and 6), but the dose of d-tubocurarine produced a 13.4 ± 3.3% (mean ± SE; n = 6) reduction in the A value (Figs. 1 and 4). Pirenzepine (10 nM) alone did not modify the R value (n = 6), but reduced the A value (12.5 ± 0.1%, mean ± SE; n = 6; Fig. 2). Higher doses of all agents studied caused significant reduction in A values with more profound fades (Fig. 1). The pretreatment with pirenzepine did not modify the R values of hexamethonium (n = 8; Fig. 3), or d-tubocurarine (n = 8; Fig. 4), but attenuated the R values of pancuronium, from 25.4 ± 2.2% to 8.1 ± 4.0% (n = 8; Fig. 5) and cisatracurium, from 24.7 ± 0.8% to 11.0 ± 3.3% (n = 6; Fig 6). Pirenzepine significantly reduced the A values of hexamethonium (25.4 ± 1.6% reduction; mean ± SE; n = 8; Fig. 3), d-tubocurarine (30 ± 1.6% reduction; mean ± SE; n = 8; Fig. 4), pancuronium (22.2 ± 0.8% reduction; mean ± SE; n = 8; Fig. 5), and cisatracurium (27.3 ± 0.8% reduction; mean ± SE; n = 6; Fig. 6). Methoctramine (1 μM) alone (n = 6) did not change R and A values (Fig. 2), but improved the fades produced by hexamethonium (n = 6), d-tubocurarine (n = 6), pancuronium (n = 8), and cisatracurium (n = 6) (Figs. 3–6). Blockage of the A1-inhibitory receptors by DPCPX (2.5 nM, n = 4) alone did not change the R or A values (Fig. 2), but improved the fade induced by pancuronium (n = 8; Fig. 5) and cisatracurium (n = 6; Fig. 6). Cisatracurium 0.32 μM showed significant anticholinesterase activity (n = 5; Fig. 7).

Discussion

The present work shows that hexamethonium-induced and d-tubocurarine-induced tetanic fades are attenuated by the inhibition of M2 presynaptic receptors, although pancuronium and cisatracurium-induced fades are reversed by both M1 and M2 inhibition. These data indicate that the fades produced by all the agents tested depend on the activation of inhibitory presynaptic M2 receptors by acetylcholine released from the motor nerve terminal, but the fades induced by pancuronium and cisatracurium would also be influenced by M1 receptors. Although pancuronium and cisatracurium can exhibit antimuscarinic activities, their antimusca-

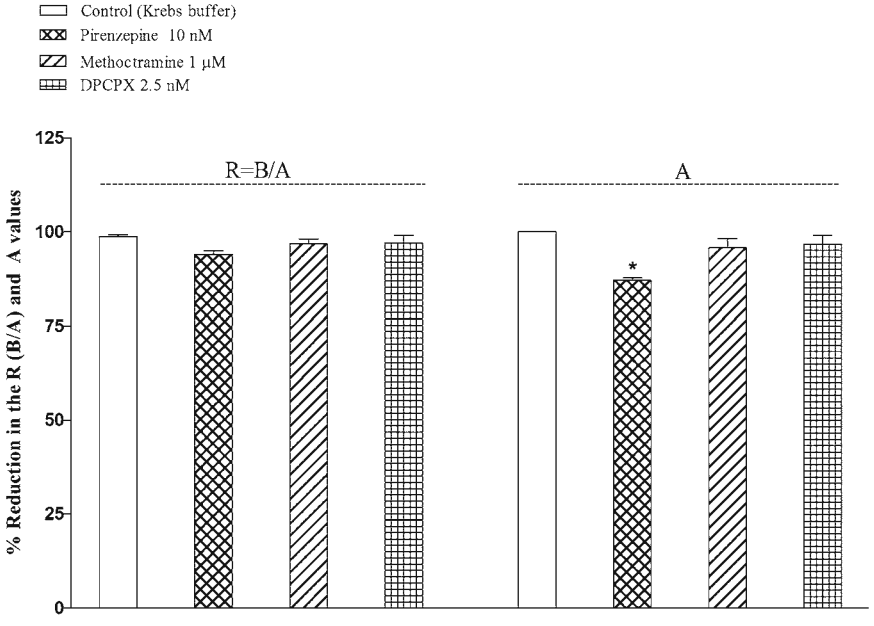

Fig. 2. Absence of effects on R (*left*) and A (*right*) values induced by methoctramine or 8-cyclopentyl-1,3-dipropylxanthine (*DPCPX*) alone in the phrenic nerve-diaphragm muscle preparations of rats indirectly stimulated at 75 ± 3.3 Hz (mean ± SD). The initial reduction of tetanic tension (*A; right*) and absence of effects on R values (*left*) induced by pirenzepine are also displayed. *Column heights* represent means ± SE of four to six experiments. *P < 0.05 compared to control (drug-free Krebs buffer)

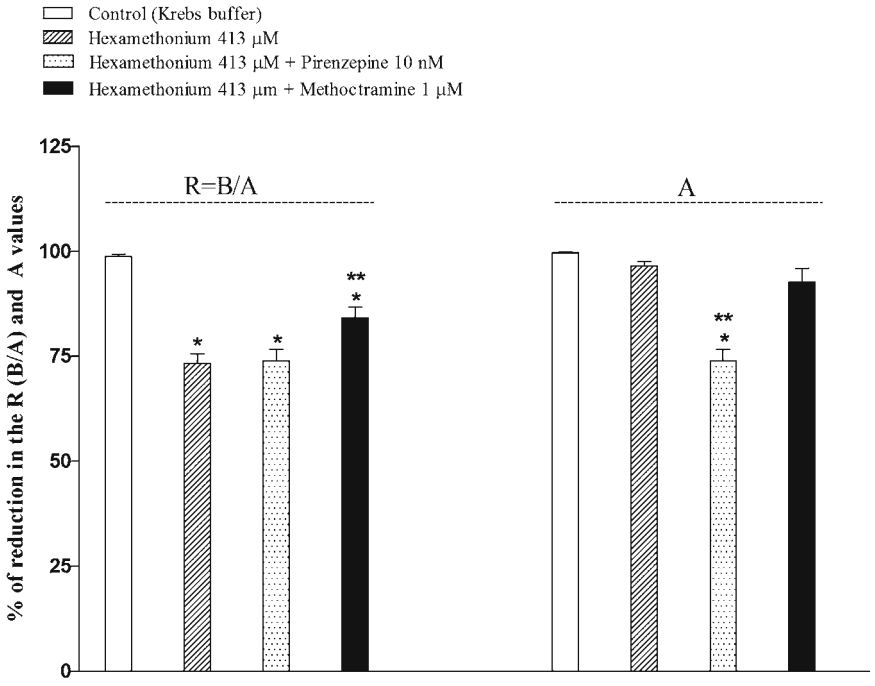

Fig. 3. Antagonism by methoctramine (percent reduction in R values; *left*) of fade induced by hexamethonium in the phrenic nerve-diaphragm muscle preparations of rats indirectly stimulated at 75 ± 3.3 Hz (mean ± SD). The reduction by pirenzepine of the initial tetanic tension (*A; right*) produced by hexamethonium is also displayed. *Column heights* represent mean ± SE of six to eight experiments. $*P < 0.05$ compared to control (drug-free Krebs buffer); $**P < 0.05$ vs hexamethonium alone

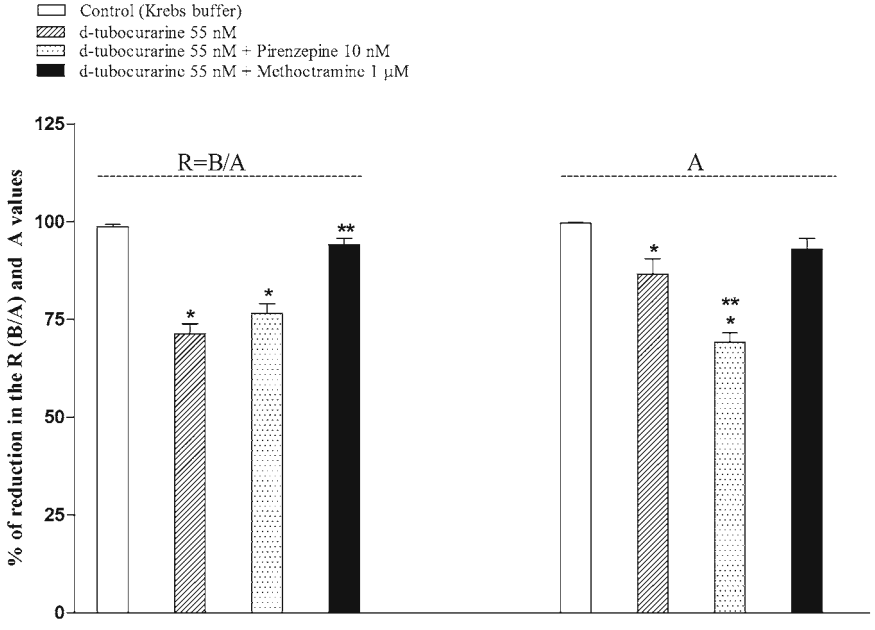

Fig. 4. Antagonism by methoctramine (percent reduction in R values; *left*) of fade induced by d-tubocurarine in the phrenic nerve-diaphragm muscle preparations of rats indirectly stimulated at 75 ± 3.3 Hz (mean ± SD) The potentiation by pirenzepine of the reduction in initial tetanic tension (*A; right*) induced by d-tubocurarine is also shown. *Column heights* represent mean ± SE of six to eight experiments. $*P < 0.05$ compared to control (drug-free Krebs buffer); $**P < 0.05$ vs d-tubocurarine alone

rinic activities do not contribute to their fades, because atropine alone (blocking presynaptic M1 and M2 receptors) [2] or the selective blockade of facilitatory M1 receptors by pirenzepine does not produce fade. On the other hand, cisatracurium exhibited anticholinesterase activities similar to those of pancuronium, as reported previously [9]. Therefore, the anticholinesterase activities of pancuronium and cisatracurium induced the activation of presynaptic inhibitory M2 receptors, as well as stimulatory M1 receptors, by acetylcholine in the synaptic cleft. Because the inhibition of acetylcholine release induced by the activation of M2 receptors is partially restrained when the stimulatory M1 receptors are fully operative, pirenzepine would be expected to potentiate the tetanic fades by blocking stimulatory M1 receptors and by enhancing inhibitory M2 receptors [4]. However, in the present study pirenzepine showed attenuation rather than potentiation of the tetanic fades induced by pancuronium and cisatracurium. We considered that the anticholinesterase activities caused activation of both M1 and M2 receptors and that M1 receptor activation also caused inhibitory A1 receptor activation,

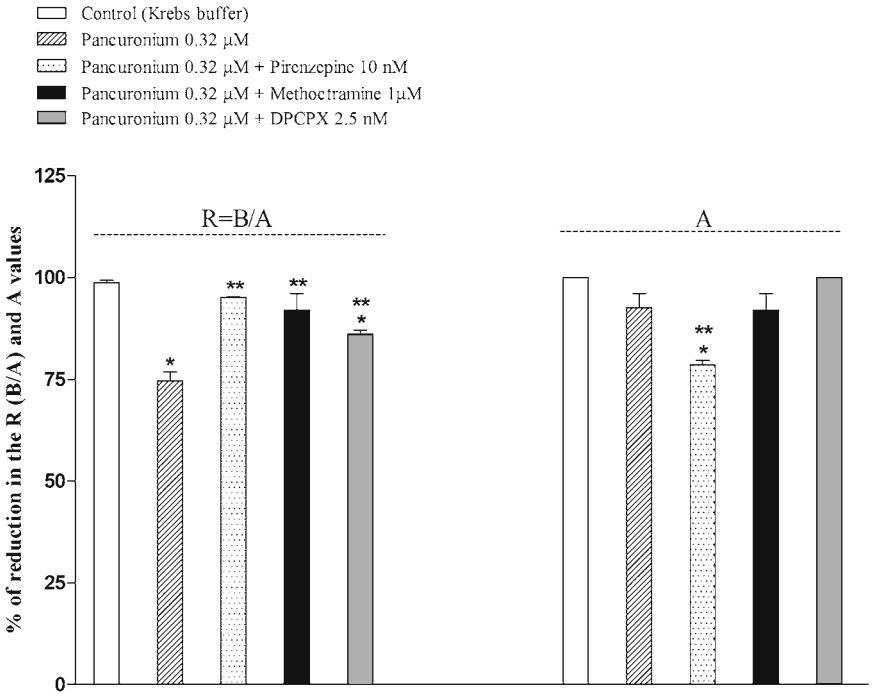

Fig. 5. Antagonism by methoctramine, pirenzepine, and DPCPX (percent reduction in R values; *left*) of fade induced by pancuronium in the phrenic nerve-diaphragm muscle preparations of rats indirectly stimulated at 75 ± 3.3 Hz (mean ± SD). The reduction by pirenzepine of the initial tetanic tension (*A; right*) produced by pancuronium is also shown. *Column heights* represent mean ± SE of eight experiments. **P* < 0.05 compared to control (drug-free Krebs buffer); ***P* < 0.05 vs pancuronium alone

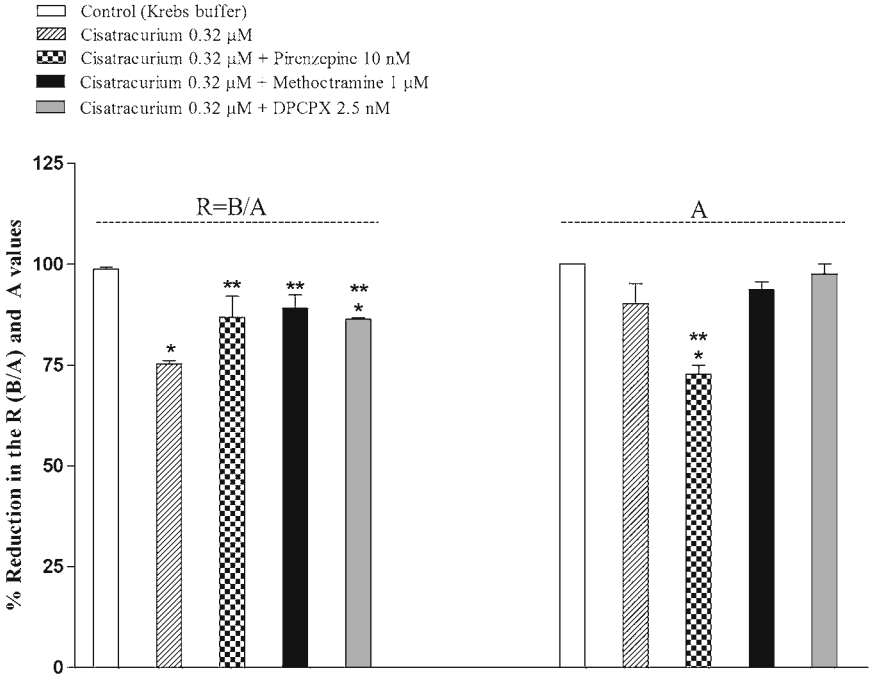

Fig. 6. Antagonism by methoctramine, pirenzepine, and DPCPX (percent reduction in R values; *left*) of fade induced by cisatracurium in the neuromuscular preparations of rats indirectly stimulated at 75 ± 3.3 Hz (mean ± SD). The reduction by pirenzepine of the initial tetanic tension (*A; right*) produced by cisatracurium is also shown. *Column heights* represent mean ± SE of six to eight experiments. **P* < 0.05 compared to control (drug-free Krebs buffer); ***P* < 0.05 vs cisatracurium alone

which reduced acetylcholine release. In fact, both pirenzepine and the A1 receptor blocker, DPCPX, attenuated the fades induced by pancuronium and cisatracurium.

Some studies have recently shown that the concentrations of hexamethonium inducing fade are those that reduce the initial tetanic tension when neuromuscular preparations are stimulated with 50-Hz pulses [6]. However, such data were obtained with parameters of stimulation different from those used in the present study. Because the lowest concentration of hexamethonium (550 μM) found in these studies as being capable of producing a 25 ± 3% (mean ± SE) reduction in R value was higher than that (413 μM) determined in the present study, it is possible that the pattern of stimulation (50 Hz, 5 s) in those studies reduced the safety

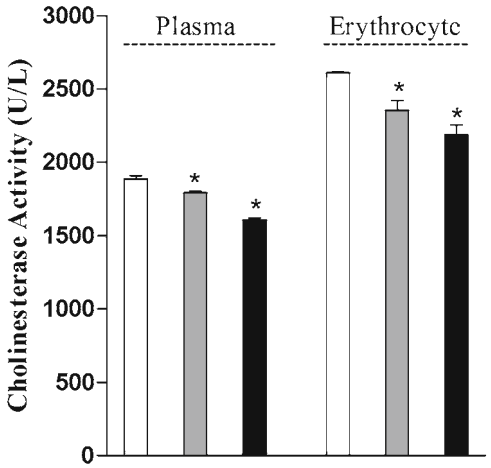

Fig. 7. Inhibition of plasma and erythrocyte acetylcholinesterase by cisatracurium at a concentration (0.32 μM) that produced a 25 ± 3% (mean ± SE) reduction in R values (R = B/A; see Fig. 1 for details). *Column heights* represent mean ± SE of four experiments. *$P < 0.05$ compared to control drug-free condition (plasma = 5, 5′-dithiobis-2-nitrobenzonic acid, plasma and acetylthiocholine; erythrocyte = 5,5′- dithiobis-2-nitrobenzonic acid, hemolyzed and acetylthiocholine) by analysis of variance (ANOVA) followed by the Bonferroni test. *White bars*, Control; *gray bars*, cisatracurium 0.1 μM; *black bars*, cisatracurium, 0.32 μM

margin of transmission to such an extent that it would not have been sufficient to obtain a myographic record that expressed only the effects produced by the presynaptic action of hexamethonium.

Because the experimental design in the present study could simulate a condition that would occur in the recovery of patients treated with pancuronium or cisatracurium [14], and taking into account that neostigmine is used clinically to reverse the blockade of neuromuscular transmission produced by these agents, the data obtained in the present study indicate that it is clinically relevant to consider not only the capacity of pancuronium and cisatracurium for blocking nicotinic receptors at both the endplate and motor nerve [10] but also the anticholinesterase properties of such agents [10]. When combined with the anticholinesterase properties of neostigmine, these properties of these agents may lead to the induction of higher levels of activation of presynaptic M1, M2, and A1 receptors, thereby reducing the efficiency of neuromuscular transmission. In this condition, control of the patient's atropinization level would avoid the emergence of this problem, because the combined administration of atropine with neostigmine, in contrast to the separate administration of neostigmine, produces a more rapid recovery of the tetanic fade produced by an antinicotinic agent [2].

In conclusion, pancuronium and cisatracurium have anticholinesterase activities. These activities activate both presynaptic inhibitory M2 and facilitatory M1 receptors. The activation of M1 receptors may activate inhibitory A1 receptors. All these effects may play roles in the tetanic fades induced by pancuronium and cisatracurium.

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