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Blockade of Neuronal Facilitatory Nicotinic Receptors Containing α3β2 Subunits Contribute to Tetanic Fade in the Rat Isolated Diaphragm

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KEY WORDS neuromuscular junction; acetylcholine release; safety factor; muscle relaxants

ABSTRACT Nicotinic receptor (nAChR) subtypes involved in pre- and postjunctional actions underlying tetanic fade were studied in rat phrenic-nerve hemidiaphragms. We investigated the ability of subtype-specific nAChR antagonists to depress nerve-evoked contractions and [^{3}H]-acetylcholine ([^{3}H]-ACh) release. Muscle tension was transiently increased during brief high frequency trains (50 Hz for 5 sec). The rank potency order of nAChR antagonists to reduce tetanic peak tension was α-bungarotoxin > *d*-tubocurarine ⩾ mecamylamine > hexamethonium. Reduction of maximal tetanic tension produced by dihydro-β-erythroidine (0.03–10 μM), methyllycaconitine (0.003–3 μM), and α-conotoxin MII (0.001–0.3 μM) did not exceed 30%. Besides reduction of peak tension *d*-tubocurarine (0.1–0.7 μM), mecamylamine (0.1–300 μM), and hexamethonium (30–3,000 μM) also caused tetanic fading. With α-conotoxin MII (0.001–0.3 μM) and dihydro-β-erythroidine (0.03–10 μM), tetanic fade was evident only after decreasing the safety factor of neuromuscular transmission (with high magnesium ions, 6–7 mM). The antagonist rank potency order to reduce evoked (50 Hz for 5 sec) [^{3}H]-ACh release from motor nerve terminals was α-conotoxin MII (0.1 μM) > dihydro-β-erythroidine (1 μM) ~ *d*-tubocurarine (1 μM) > mecamylamine (100 μM) > hexamethonium (1,000 μM). When applied in a concentration (0.3 μM) above that producing tetanic paralysis, α-bungarotoxin failed to affect [^{3}H]-ACh release. Data obtained suggest that postjunctional neuromuscular relaxants interact with α-bungarotoxin-sensitive nicotinic receptors containing α1-subunits, whereas blockade of neuronal α3β2-containing receptors produce tetanic fade by breaking nicotinic autofacilitation of acetylcholine release. **Synapse 49:77–88, 2003.**

INTRODUCTION

In myographic records, neuromuscular fade, tetanic fade, or Wedensky inhibition is the inability of a muscle to sustain tension during high frequency (30–80 Hz) motor nerve stimulation in the presence of muscle relaxants such as tubocurarine (*d*-TC) (Bowman, 1980; van der Kloot and Molgó, 1994). In electrophysiological experiments, rundown of endplate potentials is also observed during high-frequency stimulation trains, an effect resulting from decreases in the quantal output (Matzner et al., 1988) without significant changes in endplate resting potentials (Magleby et al., 1981). Iontophoretic pulses of acetylcholine (ACh) delivered at a frequency of 50 Hz did not produce tetanic fade either in the absence or in the presence of *d*-TC (Gibb and Marshall, 1986). Moreover, these authors demonstrated that depolarization of muscle fibers by iontophoretically applied ACh could still be observed following fading of nerve-evoked endplate potentials (Gibb and Marshall, 1984). Thus, it was suggested that twitch blockade and tetanic fade are separate and in-

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dependent actions; twitch tension depression is linked to the competitive block of postsynaptic nicotinic receptors (nAChRs), whereas endplate rundown or tetanic fade result predominantly from a prejunctional action of nAChRs antagonists (Gibb and Marshall, 1986).

ACh may enhance its own release during a period of repetitive motor nerve activity by activating nicotinic autoreceptors. The positive nicotinic feedback mechanism was suggested from studies measuring mechanical tension and electrophysiological signals in the presence of nicotinic antagonists (see for a review, Bowman et al., 1988). In addition, data from radiolabeled experiments showed that the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium markedly increased, while nicotinic antagonists decreased, the release of [^{3}H]-ACh release in response to nerve stimulation (Vizi and Somogyi, 1989; Wessler, 1989; Correia-de-Sá and Ribeiro, 1994). Nicotinic autofacilitation is clearly frequency-dependent and may operate as a temporary presynaptic amplifier to increase the safety factor for transmission, particularly when increased muscular strength is needed (Waud and Waud, 1971; Singh and Prior, 1998; Wood and Slater, 2001).

Nicotinic receptors are a heterogeneous family. Diversity in nAChR types is derived from the variability of genes encoding for receptor subunits and their pentameric subunit combinations (namely, associations between α1–α9 and β1–β4 subunits) (Lukas et al., 1999). Subunits confer distinct structural and functional properties (e.g., calcium permeability, desensitization rate, phosphorylation sensitivity) to the nAChR types that they form. Several lines of evidence indicate that neuronal and muscular nicotinic receptors present at the neuromuscular junction differ in their pharmacological profiles (e.g., Gibb and Marshall, 1984; 1986; Vizi et al., 1987). However, these findings are not yet clearly integrated with molecular definitions of the receptor subtypes that participate in pre- and postjunctional actions underlying changes of neuromuscular transmission. The muscle endplate nAChR is best characterized and has an $(\alpha1)_2(\beta1)\varepsilon\delta$ subunit configuration (Schuetze and Role, 1987; Salpeter et al., 1988). In contrast, the current tally of neuronal nAChR subunits in mammals is eleven (α2–α7, α9, α10, β2–β4) with an additional subunit, α8, identified in avian species.

Abbreviations

ACh	acetylcholine
BTX	α-bungarotoxin
CTX GIIIB	μ-conotoxin GIIIB
CTX MII	α-conotoxin MII
DH-β-E	dihydro-β-erythroidine
HEX	hexamethonium
Meca	mecamylamine
MLA	methyllycaconitine
MT-7	muscarinic toxin 7
nAChRs	nicotinic acetylcholine receptors
d-TC	*d*-tubocurarine

The present work was designed to study the role of facilitatory nicotinic autoreceptors block on tetanic fade and to investigate the type of nAChR that might be involved in its operation. For this purpose we tested the effects of several nicotinic antagonists, *d*-tubocurarine (*d*-TC), hexamethonium (HEX), mecamylamine (Meca), dihydro-β-erythroidine (DH-β-E), methyllycaconitine (MLA), α-bungarotoxin (BTX), and α-conotoxin MII (CTX MII) on tension responses and [^{3}H]-ACh release triggered by brief high-frequency trains (50 Hz for 5 sec) delivered to the rat phrenic nerve-hemidiaphragm preparations. Like Meca, HEX was also first recognized as a ganglionic nAChR-blocking agent; these agents exert their effects acting as nicotinic channel blockers and are considered noncompetitive antagonists. DH-β-E is a competitive neuronal nicotinic antagonist with a degree of selectivity for receptors containing α4β2 and α3β2 subunits (Chavez-Noriega et al., 1997). The Delphinium alkaloid, MLA, competitively antagonizes α7 nAChRs ($K_i \sim 1$ nM) and, unlike BTX, discriminates between neuronal α7 and muscle-type α1 receptors. BTX was instrumental in the isolation and purification of muscular α1-containing nAChRs, but it is also a highly potent and selective antagonist at α7 nAChRs ($K_1 \sim 1$ nM) without interacting with α/β heteromers (for a review, see Dwoskin and Crooks, 2001). CTX MII, a 16-residue polypeptide from the venom of the piscivorous cone snail *Conus magus*, has a high degree of selectivity for α3β2-containing neuronal nAChRs (Cartier et al., 1996), although it might also block receptors containing α6 subunits with a high affinity (e.g., Kuryatov et al., 2000).

MATERIALS AND METHODS

Rats (Wistar, 150–200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06.30–19.30 h) dark (19.30–06.30 h) cycle with food and water ad libitum. The animals were killed by stunning followed by exsanguination. Animal handling and experiments carried out at ICBAS followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). The experiments were carried out in vitro on left phrenic nerve-hemidiaphragm preparations (4–6 mm width). Each muscle was superfused with Tyrode's solution (pH 7.4) with the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2, and choline 0.001, at 37°C. This solution was continuously gassed with a mixture of O_2 (95%) and CO_2 (5%).

Nerve stimulation conditions

The left phrenic nerve was stimulated with an extracellular glass-platinum suction electrode placed near its first division branch, to avoid direct stimulation of muscle fibers (indirect stimulation). To evaluate drug

effects on muscle contractile properties, direct stimulation of muscle fibers was delivered through a pair of platinum electrodes placed at each side of the diaphragm near its costal insertion (field stimulation). Supramaximal intensity (current strength of 8 mA), rectangular pulses of 0.04 ms (indirect stimulation), or 1-ms (field stimulation) duration were used to achieve firing synchronization, thus reducing the number of silent units (motoneurons and/or muscle fibers) that might make interpretation of data difficult. The pulses were delivered by a Grass S48 (Quincy, MA, USA) stimulator coupled to a stimulus isolation unit (Grass SIU5) operating in a constant current mode. The stimulation parameters were continuously monitored on an oscilloscope (Meguro, MO-1251A, Japan) and were within the same range used in previous studies with this preparation (e.g., Wessler and Kilbinger, 1986; Correia-de-Sá et al., 2000).

Muscle tension recordings

When recording tension responses, the innervated diaphragm strips were mounted vertically in a conventional 10-ml capacity isolated organ bath chamber. Tetani (5 sec long) delivered with a frequency of 50 Hz were applied once every 15 min. Direct- and nerve-induced tetanic responses were recorded isometrically at a resting tension of 50 mN with a force transducer and displayed on a Hugo-Sachs (Germany) recorder. After the initial stabilization period, these experimental conditions allowed a well-preserved tetanic pattern for several hours in the absence of test drugs. Solutions were changed transferring the inlet tube of the peristaltic pump (Gilson, Minipuls3, France) from one flask to another. The flow rate was 20 ml min^{-1} during the first min after changing the solutions and 5 ml min^{-1} until the next changeover of solutions. Test drugs were allowed to contact with the preparations at least 12 min before tetanus; incubation time with BTX was prolonged to 45 min in some of the experiments. The tension produced at the beginning of tetanic stimulation (a) was compared with that obtained at the end of tetanic stimulation (b) (cf. Silva et al., 1999). The ratio R (R = b/a) obtained after drug addition was taken as a percentage of that observed before any drug administration. Zero percent represents equality between ratios. Positive and negative values represent increment and fading of the tetanic tension, respectively. In order to reduce the safety margin of neuromuscular transmission (Paton and Waud, 1967; Wood and Slater, 2001), $MgCl_2$ (6–7 mM) was added to the bath in some of the experiments. Osmolarity was maintained by equimolar substitution of NaCl. Elevation of magnesium ions to 6 and 7 mM decreased the amplitude of nerve-evoked tetanic responses by 36 ± 5% (n = 5) and 74 ± 6% (n = 4), respectively.

Isotope experiments

The procedures used for labeling the preparations and measuring evoked [^{3}H]-ACh release were previously described (Correia-de-Sá et al., 1991) and used with minor modifications. Experiments were performed in the absence of cholinesterase inhibitors to prevent unphysiological extracellular accumulation of ACh. Phrenic nerve-hemidiaphragm preparations were mounted in Perspex chambers of 3 ml capacity through which solutions flowed. After a 30-min equilibration period, the perfusion was stopped and the nerve endings were labeled for 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi $nmol^{-1}$) under electrical stimulation at 1 Hz frequency. After the end of the labeling period the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation stopped. From this time onwards, hemicholinium-3 (10 μM) was present to prevent uptake of choline. After a 60-min period of washout the perfusion was stopped. Bath samples (2 ml) were automatically collected every 3 min by emptying and refilling the organ bath with the solution in use, using a fraction collector (Gilson, FC 203B) coupled to a peristaltic pump (Gilson, Minipuls3) programmed device. Aliquots (0.5 ml) of the incubation medium were added to 3.5 ml of Packard Insta Gel II (Meriden, CT, USA) scintillation cocktail. Tritium content of the samples was measured by liquid scintillation spectrometry (% counting efficiency: 40 ± 2%) after appropriate background subtraction, which did not exceed 5% of samples tritium content. The radioactivity was expressed as DPM g^{-1} of wet weight of the tissue determined at the end of the experiment. After the loading and washout periods, the preparation contained 5,542 ± 248 × 10^3 DPM g^{-1} and the resting release was 132 ± 12 × 10^3 DPM g^{-1} in 3 min (n = 8). When the fractional release was calculated, this value proved to be 2.38 ± 0.14% of the radioactivity present in the tissue at the first collected sample. [^{3}H]-ACh release was evoked stimulating the phrenic nerve with brief high-frequency trains (50 Hz for 5 sec, 40 μs pulse width). Two stimulation periods were used: at 12 min (S_1) and at 39 min (S_2) after the end of washout (zero time). Electrical stimulation of the phrenic nerve increased the release of [^{3}H]-ACh in a Ca^{2+}- and tetrodotoxin-sensitive manner (Correia-de-Sá et al., 2000), while the output of [^{3}H]-choline remained unchanged (Wessler and Kilbinger, 1986), thus indicating that ACh comes mainly from vesicle exocytosis from depolarized nerve terminals. It is unlikely that nonquantal ACh release (Katz and Miledi, 1977) account for the total amount of ACh released upon electrical stimulation of the phrenic nerve. This assumption is based on findings indicating that the spontaneously releasable neuronal pool of ACh is not labeled with [^{3}H]-choline nor is it released by electrical nerve stimulation (Molenaar et al., 1987), and it is completely exhausted (within minutes) in the presence of hemicholinium-3

(Nikolsky et al., 1991). Therefore, evoked [^{3}H]-ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during each stimulation period (Correia-de-Sá et al., 1991). Test drugs were added 15 min before S_2. In some experiments, incubation time with BTX was prolonged to 45 min and S_2 was delivered at the 69th min after the end of washout. Drug effects were expressed by the ratios S_2/S_1, i.e., the ratio between the evoked [^{3}H]-ACh release during the second stimulation period (in the presence of the test drug) and the evoked [^{3}H]-ACh release during the first stimulation period (without the test drug). Percentage values shown in figures correspond to percentage changes in S_2/S_1 ratios as compared with the S_2/S_1 ratio in control experiments (0.83 ± 0.06, $n = 6$); zero percent represents identity between ratios. Positive and negative values represent facilitation and inhibition of evoked [^{3}H]-ACh release, respectively. None of the drugs used significantly ($P > 0.05$) changed basal tritium outflow.

Materials and solutions

Chemicals used were: α-Bungarotoxin (BTX), choline chloride, dihydro-β-erythroidine hydrobromide (DH-β-E), hemicholinium-3, hexamethonium bromide (HEX), mecamylamine (Meca), methyllycaconitine citrate (MLA), pirenzepine dihydrochloride, *d*-tubocurarine chloride (*d*-TC) (Sigma, St. Louis, MO, USA); α-conotoxin MII (CTX MII) (Tocris Cookson, UK); μ-conotoxin GIIIB (CTX GIIIB), muscarinic toxin 7 (Peptide Institute, Japan); [*methyl*-^{3}H]-choline chloride (ethanol solution, 80 Ci mmol^{-1}) (Amersham, UK). Aqueous stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate controls were done. The pH of the superfusion solution did not change by the addition of drugs in the maximum concentrations applied to the preparations.

Statistics

The data are expressed as mean ± SE, from *n* experiments. Statistical significance of experimental results was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's modified *t*-test. $P < 0.05$ was considered to represent significant difference.

RESULTS

Blockade of muscle-type nicotinic receptors containing α1-subunits reduce tetanic tension

Changes in the amplitude of tetanic peak tension produced by nicotinic receptor antagonists was taken as a measure of the postjunctional activity of these compounds. Figure 1 shows that BTX (0.003–0.1 μM), *d*-TC (0.1–0.7 μM), Meca (0.1–300 μM), and HEX (10–3,000 μM) decreased tetanic peak tension in a concentration-dependent manner. Failure to detect nerve-evoked diaphragm contractions in response to tetanic trains (50 Hz for 5 sec) was observed with BTX (0.1 μM), *d*-TC (0.7 μM), Meca (300 μM), and HEX (3,000 μM). As BTX exhibits slow binding kinetics and its action is essentially irreversible, we performed experiments where the preincubation time was prolonged from 12 to 45 min. Reduction of tetanic peak tension following a 45-min contact with BTX (0.003–0.1 μM) was not statistically different ($P > 0.05$) from that obtained using a 12-min incubation period (data not shown). Depression of tetanic peak tension with DH-β-E (0.03–10 μM), which blocks preferentially α4β2- and α3β2-containing receptors, MLA (0.003–3 μM), a preferential α7-receptor antagonist, and CTX MII (0.001–0.3 μM), a selective α3β2-receptor antagonist that also blocks receptors containing the α6 subunit, did not exceed 30%. Thus, reduction in tetanic peak tension has an antagonist profile with a rank order of potency of BTX > *d*-TC ⩾ Meca > HEX. This is in agreement with previous studies suggesting that nicotinic receptors localized on skeletal muscle contain α1-subunits (Schuetze and Role, 1987; Salpeter et al., 1988).

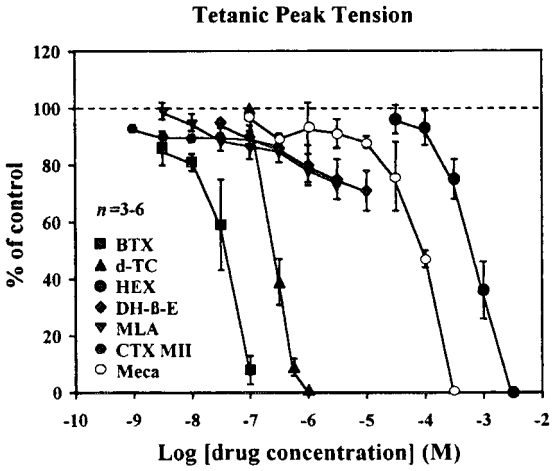

Fig. 1. Concentration–response curves for the effects of nicotinic receptor antagonists on the amplitude of nerve-induced tetanic peak tension. Tetanic responses were elicited once every 15 min by stimulating the phrenic nerve with brief high-frequency trains (50 Hz for 5 sec, 40 μs pulse width). *d*-Tubocurarine (*d*-TC), hexamethonium (HEX), mecamylamine (Meca), dihydro-β-erythroidine (DH-β-E), methyllycaconitine (MLA), α-bungarotoxin (BTX), and α-conotoxin MII (CTX MII) were applied in a cumulative manner and contacted the preparation at least 12 min before recordings. The ordinates are percentage of maximal tetanic peak tension (100%) observed in control conditions (in the absence of nicotinic antagonists). The vertical bars represent ±SE of 3–6 experiments (for each curve) and are shown when they exceed the symbols in size.

nAChR underlying tetanic fade possess a distinct antagonist profile from the muscular receptor type

Figure 2A shows pen-recorder traces of nerve-evoked muscle contractions obtained during short high-frequency trains (50 Hz for 5 sec). In control conditions, a

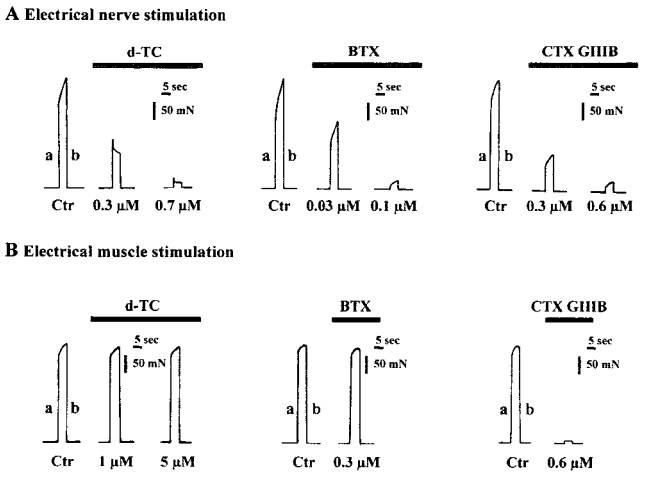

Fig. 2. Comparison between the effects of two nicotinic receptor antagonists (*d*-tubocurarine and α-bungarotoxin) and μ-conotoxin GIIIB, a sodium channel blocker in skeletal muscle, on tetanic muscle tension induced by high-frequency (50 Hz for 5 sec) stimulation trains delivered to the phrenic nerve (indirect stimulation, **A**) or to diaphragm muscle fibers (direct stimulation, **B**). **A:** Typical recording traces of nerve-evoked hemidiaphragm contractions obtained during brief tetanic trains (50 Hz for 5 sec), in the absence (Ctr) and in the presence of *d*-tubocurarine (*d*-TC, 0.3 and 0.7 μM), α-bungarotoxin (BTX, 0.03 and 0.1 μM), and μ-conotoxin GIIIB (CTX GIIIB, 0.3 and 0.6 μM). The small horizontal lines indicate the duration of tetanic stimulation (5 sec); vertical calibration: 50 mN. **B:** Note that *d*-TC (1 or 5 μM) and BTX (0.3 μM), applied in concentrations above those required to cause complete neuromuscular block were virtually devoid of effects on the contractile responses induced by direct muscle stimulation. In contrast, CTX GIIIB (0.6 μM) abolished contractions caused by direct muscle depolarization.

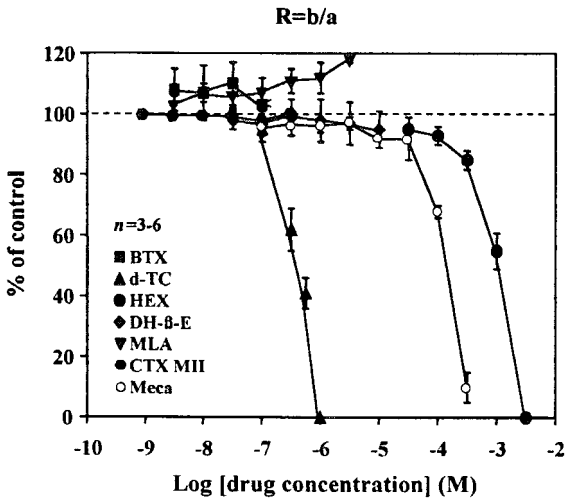

Fig. 3. Effects of nicotinic receptor antagonists on tetanic fading induced by high-frequency (50 Hz for 5 sec) stimulation trains delivered to the phrenic nerve. Tetanic fade was calculated as the ratio (R) between the tensions recorded at the end (b) and at the beginning (a) of the tetanic response (R = b/a) (see Fig. 2). *d*-Tubocurarine (*d*-TC), hexamethonium (HEX), mecamylamine (Meca), dihydro-β-erythroidine (DH-β-E), methyllycaconitine (MLA), α-bungarotoxin (BTX), and α-conotoxin MII (CTX MII) were applied in a cumulative manner and contacted the preparation at least 12 min before recordings. On the ordinate, ratio (R) is expressed as a percentage of that obtained in control (Ctr) conditions (in the absence of nicotinic antagonists), taken as 100%. The vertical bars represent ±SE of 3–6 experiments (for each curve) and are shown when they exceed the symbols in size.

brief facilitation (b > a) was evident during the course of tetani (50 Hz for 5 sec), i.e., muscle tension was transiently increased when high-frequency repetitive pulses were delivered to the nerve. Increasing concentrations of *d*-TC (0.1–0.7 μM, Fig. 2A), Meca (0.1–300 μM), and HEX (10–3,000 μM) caused a very intense fade (b < a) of tetanic contractions (Fig. 3) in parallel with a significant reduction of the maximal tetanic tension (cf. Fig. 1). Neither BTX (0.003–0.1 μM) nor MLA (0.003–3 μM) produced tetanic fade (Fig. 3), albeit BTX (0.003–0.1 μM) strongly depressed the maximal tetanic tension (Figs. 1, 2A).

To investigate the possibility of a direct action of the nicotinic antagonists on muscle contractile properties, we studied the effect of *d*-TC on tetanic tension induced by direct muscle stimulation. When applied in concentrations above those that caused complete neuromuscular block, *d*-TC (1 and 5 μM) and BTX (0.3 μM) were virtually devoid of effect on muscle tension induced by tetanic field stimulation (50 Hz for 5 sec) (Fig. 2B). In addition, measurements of tetanic tension and fading may depend on the threshold for activation of muscle action potential, which would be misinterpreted as being due to an indirect presynaptic effect. To evaluate this possibility we compared the depression of contractile responses caused by the nicotinic receptor antagonists with the effect of μ-conotoxin GIIIB (CTX GIIIB), a sodium channel blocker in skeletal muscle with no action on channels present in axons and motor nerve terminals (Cruz et al., 1985). CTX GIIIB (0.01–0.6 μM) decreased tetanic peak tension in a concentration-dependent manner with no obvious fading phenomena being associated (Fig. 2). Muscular paralysis was obtained with CTX GIIIB (0.6 μM) when tetanic trains (50 Hz for 5 sec) were delivered either indirectly, to the phrenic nerve trunk, or directly, to the muscle fibers. Data from direct muscle stimulation protocols and the absence of tetanic fading in the presence of CTX GIIIB clearly contrast with the results obtained with *d*-TC (see Fig. 2), further indicating that a presynaptic action might be involved.

Neuronal α3β2 nAChR block reduces [^{3}H]-ACh release triggered by high-frequency trains

To address the role of neuromuscular blocking agents on prejunctional nicotinic receptors, we compared their ability to produce tetanic fade and to decrease [^{3}H]-ACh release evoked by high-frequency stimulation trains (50 Hz for 5 sec). Figure 4A illustrates the time course of tritium outflow in experiments where CTX MII (0.1 μM), *d*-TC (1 μM), and BTX (0.3 μM) were applied 15 min before S_2. As can be seen from these typical experiments, evoked [^{3}H]-ACh release was decreased in the presence of CTX MII (0.1

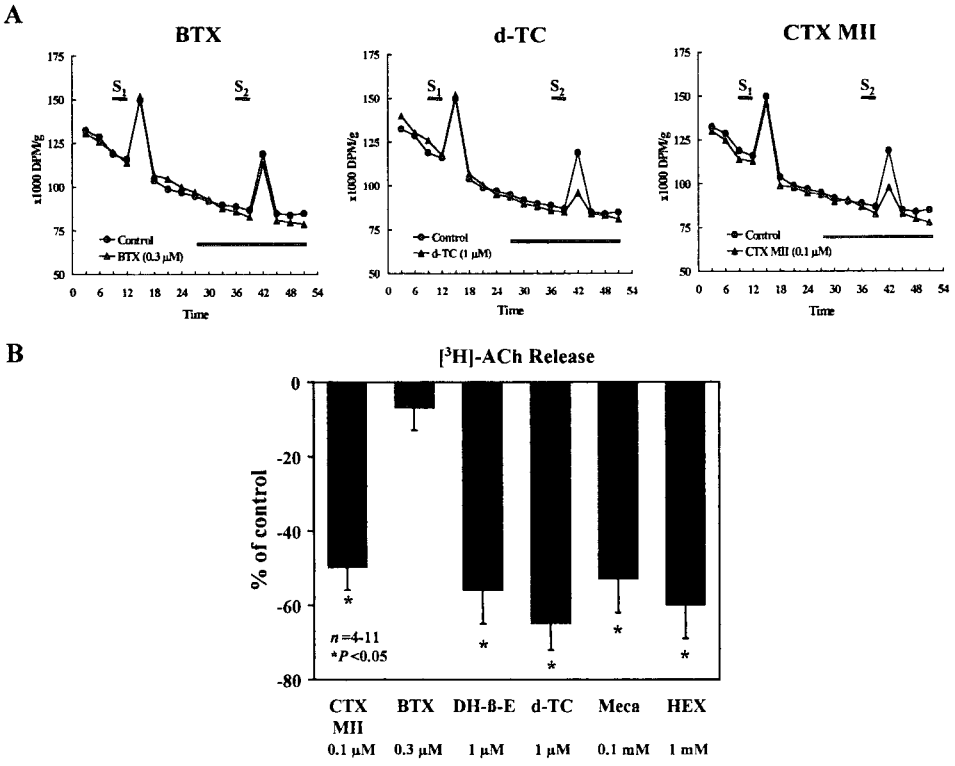

Fig. 4. Effects of nicotinic receptor antagonists on [^{3}H]-ACh release from the rat motor nerve endings evoked by high-frequency (50 Hz for 5 sec) trains. **A:** Time course of tritium outflow from rat phrenic nerve terminals in the absence (Control, ●) and in the presence (▲) of α-bungarotoxin (BTX, 0.3 μM), *d*-tubocurarine (*d*-TC, 1 μM), and α-conotoxin MII (CTX MII, 0.1 μM). After the labeling and washout periods (zero time), [^{3}H]-ACh release was elicited by stimulating the phrenic nerve twice (S_1 at the 12th min and S_2 at the 39th min) at a frequency of 50 Hz for 5 sec. Tritium outflow was measured in samples collected every 3 min. The nicotinic antagonists were applied at least 12 min before S_2 (as indicated by the horizontal bars). Note that spontaneous tritium outflow was not significantly modified in the presence of the drugs. **B:** Inhibitory effects of α-conotoxin MII (CTX MII, 0.1 μM), α-bungarotoxin (BTX, 0.3 μM), dihydro-β-erythroidine (DH-β-E, 1 μM), *d*-tubocurarine (*d*-TC, 1 μM), mecamylamine (Meca, 100 μM), and hexamethonium (HEX, 1000 μM) on evoked [^{3}H]-ACh release from motor nerve terminals determined as described in (**A**). The ordinates are percentage inhibition of [^{3}H]-ACh release as compared to control, in the absence of added drugs. Each column represents pooled data from 4–11 experiments. The vertical bars represent ± SE. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t*-test) when compared with zero percent.

μM) and *d*-TC (1 μM), but not when BTX (0.3 μM) was added. The antagonist rank potency order to inhibit (by about 50–70%) the release of [^{3}H]-ACh (50 Hz for 5 sec) was CTX MII (0.1 μM) > DH-β-E (1 μM) ~ *d*-TC (1 μM) > Meca (100 μM) > HEX (1000 μM) (Fig. 4B). The lack of BTX effect at the prejunctional level was observed even when the time of incubation was prolonged to 45 min and it was applied in a concentration (0.3 μM) above that necessary to cause complete muscular paralysis (data not shown). It is also worth noting that application of BTX did not significantly ($P > 0.05$) affect the basal tritium outflow (Fig. 4A; but see e.g., Apel et al., 1995). This fully agrees with data showing that failure of BTX (0.003–0.1 μM) to induce presynaptic rundown of tetanic contractions can be dissociated from its ability to decrease tetanic peak tension (see above). Thus, the present results suggest that autofacilitation of ACh release from motor nerve terminals is probably mediated by α3β2-containing nicotinic receptors sensitive to blockade by CTX MII.

Reducing the safety factor of neuromuscular transmission significantly potentiates tetanic fade caused by neuronal nicotinic receptor antagonists

In general, the amount of transmitter released per nerve impulse is greater than that required to trigger an action potential in the muscle fiber, although the transmission safety margin may become critical in pathological conditions (e.g., myasthenic syndromes). Because of the high safety factor of neuromuscular transmission, depression of nerve-evoked muscle contractions due to prejunctional acting drugs might not always reflect the magnitude of transmitter release inhibition (see for a review, see Wood and Slater, 2001). This might explain why nicotinic blocking agents like CTX MII (0.1 μM) and DH-β-E (1 μM) are more potent to inhibit (−56 ± 9%, $n = 5$ and −50 ± 6%, $n = 4$, respectively) evoked [^{3}H]-ACh release (Fig. 4) than to cause depression of tetanic contractions (Figs. 5, 6).

Increasing magnesium concentration in the bathing fluid is a useful strategy to decrease the safety factor of

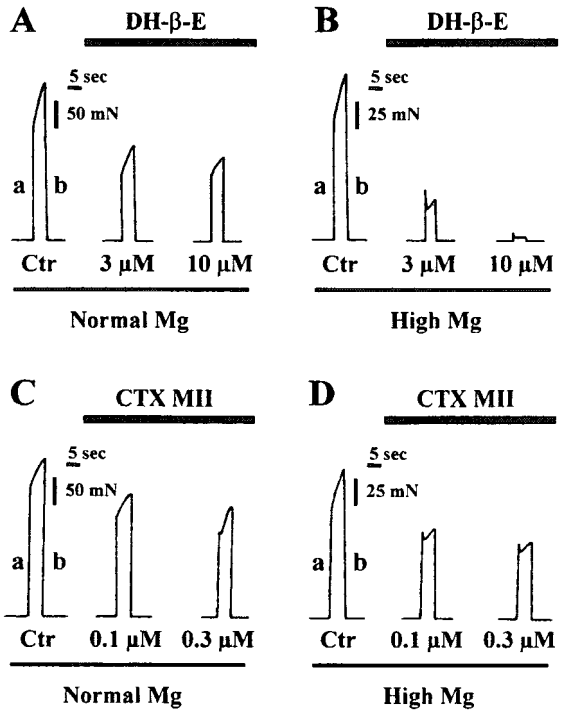

Fig. 5. Representative recordings of nerve-evoked hemidiaphragm tetani (50 Hz for 5 sec) in the presence of dihydro-β-erythroidine (DH-β-E, 3 and 10 μM) and α-conotoxin MII (CTX MII, 0.1 and 0.3 μM): influence of the safety margin of neuromuscular transmission. Recordings in **A** and **C** (normal quantal output) were obtained in normal Tyrode buffer ($MgCl_2$, 1 mM), and those in **B** and **D** (low quantal output) were obtained in high magnesium conditions ($MgCl_2$, 6–7 mM). DH-β-E (3 and 10 μM) and CTX MII (0.1 and 0.3 μM) were applied in a cumulative manner for periods of 12 min before each 50 Hz frequency stimulation train. The small horizontal line indicates the duration of tetanic stimulation (5 sec). Please note that the amplitude of traces obtained in normal (**A** and **C,** vertical calibration: 50 mN) and high (**B** and **D,** vertical calibration: 25 mN) magnesium conditions were normalized to facilitate comparisons.

synaptic transmission by reducing the amount of transmitter being released per stimulation pulse (del Castillo and Katz, 1954; Paton and Waud, 1967). We reported previously that increments of the magnesium content in the buffer (up to 8.5 mM) decreased the evoked [^{3}H]-ACh release by 53 ± 9% (n = 4), without affecting agonist-induced presynaptic nicotinic facilitation (Correia-de-Sá and Ribeiro, 1994). To probe for the apparent discrepancy existing between the magnitude of transmitter release inhibition and the fading phenomena, we reevaluated the effects of DH-β-E (0.03–10 μM) and CTX MII (0.001–0.3 μM) in conditions where the safety margin of neuromuscular transmission was partially reduced with high magnesium concentrations (6–7 mM). Figure 5 illustrates pen-recorder traces taken from representative experiments with DH-β-E (3 and 10 μM) and CTX MII (0.1 and 0.3 μM), where nerve-induced tetanic peak tension was reduced by about 50% using 6–7 mM $MgCl_2$. The amplitude of traces obtained with normal (1 mM) and high magnesium concentrations were normalized to facilitate comparison (see calibration bar in the figure). Depressions of tetanic peak tension due to both DH-β-E (0.03–10 μM) and CTX MII (0.001–0.3 μM) were significantly ($P < 0.05$) potentiated upon increasing magnesium content in the buffer (Fig. 6). A complete neuromuscular block was obtained with 0.3 and 10 μM concentrations of DH-β-E in the presence of 7 and 6 mM $MgCl_2$, respectively.

It is worth noting that magnesium (6–7 mM) slightly enhanced tetanic facilitation (see R values in Figs. 6B, D). This is a well-known phenomenon seen during high-frequency repetitive nerve stimulation when quantal output is low (see e.g., van der Kloot and Molgó, 1994). Albeit, preservation of the tetanic ascendant in high magnesium solutions, simultaneous application of DH-β-E (0.03–10 μM) significantly ($P < 0.05$) enhanced fading (Fig. 6B). Tension at the end of tetani was virtually abolished when 1 and 10 μM DH-β-E was added in the presence of 7 and 6 mM $MgCl_2$, respectively. Under low quantal conditions, CTX MII (0.001–0.3 μM) significantly ($P < 0.05$) attenuated tetanic facilitation in a concentration-dependent manner (Fig. 6D). CTX MII (0.3 μM, n = 3) decreased the R ratio (b/a) from 1.50 ± 0.02 (n = 3, normal Tyrode buffer) to 1.20 ± 0.05 (n = 3) when 7 mM $MgCl_2$ was added to the incubation media.

d-TC (0.7 μM), Meca (300 μM), and HEX (3,000 μM) used in concentrations high enough to decrease the safety factor of neuromuscular transmission induced the rundown of tetanic contractions until complete neuromuscular block (see Fig. 3). These achievements did not require the raising of magnesium concentration, because these agents may simultaneously block pre- and postjunctional nicotinic sites.

Blockade of muscarinic M_1 autoreceptors reduce transmitter release without producing tetanic fade

In addition to the short-term nicotinic positive feedback mechanism, ACh may increase its own release by acting at muscarinic M_1 receptors on motor nerve terminals (see e.g., Wessler, 1989; Oliveira et al., 2002). As illustrated in Figure 7A, the muscarinic M_1 receptor antagonist pirenzepine (10 nM) inhibited (26 ± 5%, n = 4) the release of [^{3}H]-ACh induced by 50 Hz frequency trains. This inhibitory action was mimicked (37 ± 6%, n = 5) by the muscarinic toxin 7 (MT-7, 1 nM, data not shown) isolated from the venom of the green mamba (*Dendroaspis angusticeps*), which exhibits high subtype selectivity for M_1 ($pK_B \sim 9.8$) receptors (Adem and Karlsson, 1997).

Pirenzepine (1–30 nM), applied cumulatively at least 12 min before recordings, decreased tetanic peak tension in a concentration-dependent manner. Depression of tetanic contractions due to pirenzepine (1–30 nM) did not exceed 20% in normal Tyrode solution ($MgCl_2$, 1 mM) (Fig. 7B), but it was significantly ($P < 0.05$)

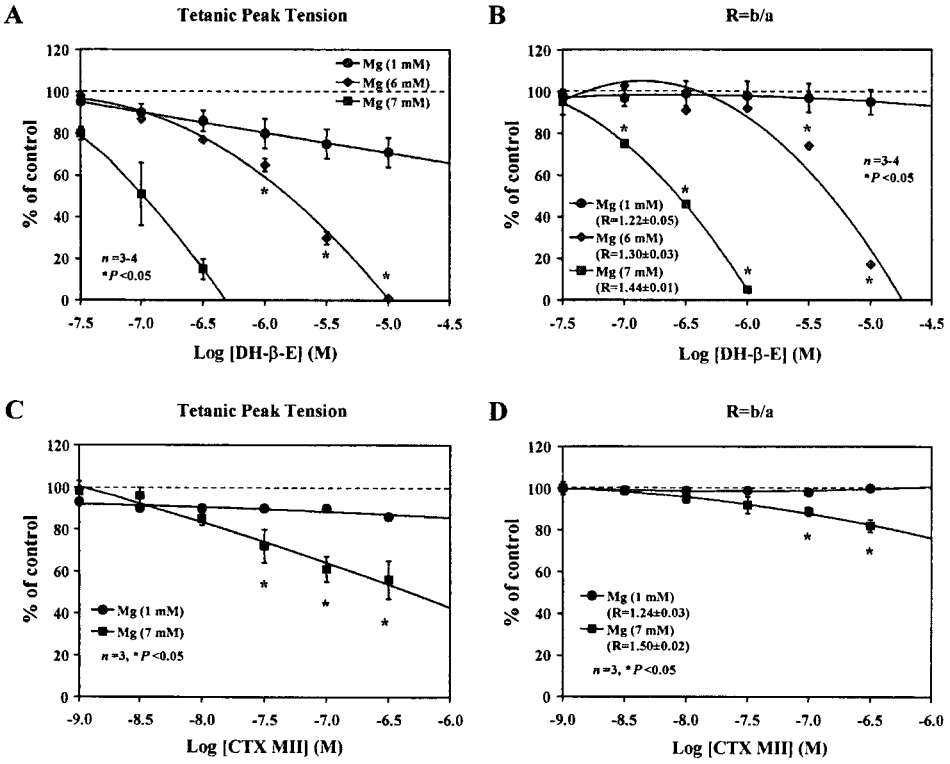

Fig. 6. Effects of dihydro-β-erythroidine (DH-β-E, 0.03–10 μM) and α-conotoxin MII (CTX MII, 0.001–0.3 μM) on tetanic peak tension and fade (R = b/a) in conditions where the safety factor of neuromuscular transmission was reduced. Tetanic responses were induced once every 15 min by stimulating the phrenic nerve with brief high-frequency trains (50 Hz for 5 sec, 40 μs pulse width). DH-β-E (0.03–10 μM, **A** and **B**) and CTX MII (0.001–0.3 μM, **C** and **D**) were applied in a cumulative manner and contacted the preparation at least 12 min before recordings. **A,C:** The ordinates are percentage of maximal tetanic peak tension (100%) determined in the absence of nicotinic antagonists. **B,D:** The ratio (R) is expressed as a percentage of that obtained in the absence of nicotinic antagonists, taken as 100%. R values for each set of experiments are indicated for comparison. The vertical bars represent ±SE of n experiments and are shown when they exceed the symbols in size. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) as compared with the effect of each nicotinic receptor antagonist in normal Tyrode buffer ($MgCl_2$ 1 mM).

potentiated (46 ± 7%, $n = 3$) when the magnesium content in the buffer was raised to 6 mM (Fig. 7C). In contrast to the findings obtained with several nicotinic channel blockers (e.g., DH-β-E, CTX MII, *d*-TC), pirenzepine (30 nM) was virtually devoid of effect on tetanic facilitation even after decreasing the safety factor of neuromuscular transmission. Under these conditions, percent variation of R ratio (b/a) was not higher than 3–7% when compared to controls obtained in normal Tyrode's solution (1.23 ± 0.07, $n = 3$) or after raising magnesium levels (1.35 ± 0.06, $n = 3$).

DISCUSSION

In this study we demonstrate that the rat neuromuscular junction is equipped with α3β2-containing neuronal nAChRs mediating facilitation of ACh release, in addition to the classical muscle-type nAChR containing the α1 subunit. Subtype-specific nicotinic antagonists (e.g., BTX, DH-β-E, CTX MII) had distinct profiles to inhibit evoked [^{3}H]-ACh release and to depress tetanic peak tension, clearly indicating that pre- and postjunctional receptors have different pharmacological properties. Unlike tetanic peak depression due to the "pure" muscular relaxing agent, BTX, fading of tetanic contractions induced by DH-β-E and CTX MII consisted primarily of the inhibition of ACh release from motor nerve terminals. This provides further support for the hypothesis that tetanic fade is due to an underlying attenuation of nicotinic autofacilitation rather than to a use-dependent block of postjunctional nicotinic receptors (Wilson and Nicholson, 1997). Due to the high safety margin of neuromuscular transmission, repercussions of the fine-tuning nicotinic modulation of transmitter release at the postjunctional level require significant decreases in the synaptic quantal content, like those observed during high-frequency trains or after increasing the magnesium content in the buffer.

In contrast to the pivotal role of nAChRs in autonomic neurotransmission and to initiate muscle contraction, neuronal nAChRs are considered to exert a modulatory influence (Wonnacott, 1997). In native neuronal systems, knowledge of the subunit composition of nAChRs is generally lacking and only a few major subtypes have been identified. These include α4β2* nAChR, which is relatively abundant in the CNS (Flores et al., 1996). The other major subtype is com-

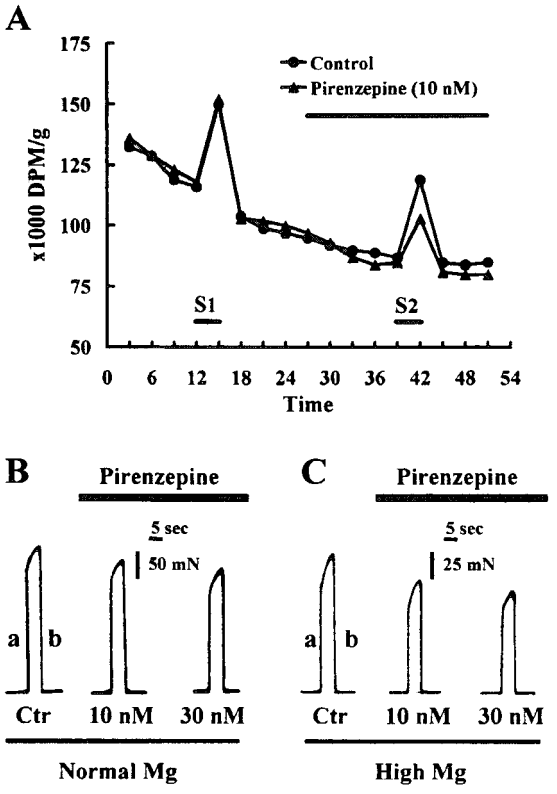

Fig. 7. Effect of the muscarinic M_1-receptor antagonist, pirenzepine, on transmitter release and muscular tension induced by high-frequency (50 Hz for 5 sec) stimulation trains delivered to the phrenic nerve. **A:** Time course of tritium outflow from rat phrenic nerve terminals in the absence (Control, ●) and in the presence (▲) of pirenzepine (10 nM), applied 15 min before S_2 (as indicated by the horizontal bar). After the labeling and washout periods (zero time), [^{3}H]-ACh release was elicited by stimulating the phrenic nerve twice (S_1 at the 12th min and S_2 at the 39th min) at a frequency of 50 Hz during 5 sec. Tritium outflow was measured in samples collected every 3 min. **B,C:** Typical recording traces of nerve-evoked hemidiaphragm contractions observed during brief tetanic trains (50 Hz for 5 sec), in the absence (Ctr) and in the presence of pirenzepine (10 and 30 nM), obtained in normal Tyrode buffer (**B,** $MgCl_2$, 1 mM) and in high magnesium conditions (**C,** $MgCl_2$, 6 mM). Pirenzepine (1–30 nM) was applied in a cumulative manner for periods of 12 min before tetani. The small horizontal line indicates the duration of tetanic stimulation (5 sec). Please note that the amplitude of traces obtained in normal (**B,** vertical calibration: 50 mN) and high (**C,** vertical calibration: 25 mN) magnesium conditions were normalized to facilitate comparisons.

prised of α7 subunits, which form homomeric receptors in both central and peripheral nervous systems (Chen and Patrick, 1997). A variety of heteromeric α3* nAChRs may also exist in the peripheral nervous system; the predominant subtype contains α3 and β4 subunits but may also assemble with α5 and/or β2 subunits (Conroy and Berg, 1995; Flores et al., 1996). Attempts to identify the subunit composition of nAChRs present at motor nerve terminals have been made using nicotinic antagonists lacking subtype selectivity (e.g., Vizi et al., 1995). In this article we show that the antagonist rank potency order to produce neuromuscular tetanic fade and to reduce the release of [^{3}H]-ACh evoked by high-frequency trains was CTX MII > DH-β-E ~ *d*-TC > Meca > HEX. Neither BTX nor MLA caused tetanic fade and/or modified the release of [^{3}H]-ACh, virtually excluding the involvement of neuronal BTX-sensitive receptors (like α7*, α8*, and α9*) in the nicotinic positive feedback mechanism. Others have found that BTX produced a disproportionately higher reduction on the amplitude of miniature endplate potentials when compared to the amplitude of endplate potentials recorded during brief tetanic trains (Domet et al., 1995). However, one cannot exclude the possibility that this transient facilitation of ACh release prior to the establishment of the neuromuscular block could be due to phospholipase A_2 activity (Fathi et al., 2001), which is a known contaminant of several commercially available snake toxins, including BTX (see e.g., Apel et al., 1995). Equipotency between DH-β-E and *d*-TC to inhibit evoked [^{3}H]-ACh release, together with the higher potency of these agents as compared to Meca, make the involvement of α4*- and α3β4-containing receptors also highly improbable (see e.g., Dwoskin and Crooks, 2001). Since, DH-β-E is a relatively weak antagonist at α3β4- (ganglionic-like) and $(\alpha1)_2\varepsilon\beta1\delta$- (muscle-type) receptors as compared to the α3β2 subtype (Chavez-Noriega et al., 1997) and evoked [^{3}H]-ACh release was highly sensitive to CTX MII (a preferential α3β2-antagonist at the nanomolar concentration range) (Cartier et al., 1996), our data indicate that facilitatory nAChRs located at the rat motor nerve terminals exhibit a α3β2 subunit configuration. Immunohistochemical studies performed in mouse diaphragms support the view that α3-containing nAChRs may exist at the prejunctional level (Tsuneki et al., 1995). The involvement of CTX MII-sensitive neuronal α6-heteroceptors (Kuryatov et al., 2000) cannot be excluded from the present data, despite the fact that in the chick retina these receptors are also blocked by nanomolar concentrations of MLA (Vailati et al., 1999).

The antagonist profile found in the release experiments clearly differs from that concerning depression of tetanic peak tension (BTX > *d*-TC ⩾ Meca > HEX), which is a well-known phenomenon resulting from the blockade of muscle-type α1-containing nAChRs (Schuetze and Role, 1987; Salpeter et al., 1988). It is not surprising that *d*-TC, Meca, and HEX could simultaneously depress tetanic peak tension, reduce evoked [^{3}H]-ACh release, and induce tetanic fade, as nonselective agents can antagonize cooperatively both neuronal and muscular nAChRs (see e.g., de Oliveira and Oliveira, 1999). In contrast, transmitter release inhibition caused by neuronal nAChR antagonists, like DH-β-E- and CTX MII, was associated with a mild fading phenomenon. This apparent discrepancy was attenuated once the safety factor of neuromuscular transmission was decreased by reducing the probability of transmitter release using high magnesium concentrations in the buffer (del Castillo and Katz, 1954; Paton and Waud, 1967; see also Correia-de-Sá and Ribeiro,

1994). Under conditions of low quantal output, tetanic facilitation was preserved and muscular tension could reflect transmitter release changes in a more accurate manner (Wood and Slater, 2001). In addition, reducing the amount of transmitter being released per stimulation pulse may attenuate competition between added antagonists and released ACh for nicotinic receptors binding. Albeit fading and tetanic peak depression due to CTX MII were less robust than what was observed with DH-β-E, both agents inhibited tetanic-induced transmitter release with a similar efficacy. This might reflect the higher selectivity of CTX MII for neuronal α3β2 receptors (Cartier et al., 1996) as compared with DH-β-E, while the latter compound might also marginally block muscle-type α1-containing nAChRs (Chavez-Noriega et al., 1997), particularly under conditions of low quantal output. Due to the fine-tuning control of the nicotinic positive feedback loop triggered during repetitive motor nerve stimulation (e.g., respiration drive, voluntary movements), care must be taken to avoid restricted interpretations considering synaptic levels of ACh as the leading player. Nicotinic autofacilitation is cut short rapidly after high-frequency (5–50 Hz) trains to avoid transmitter flooding and muscle overstimulation by mechanisms that might involve receptor dessensitization (Colquhoun et al., 1989; Wessler, 1989) and crosstalk with endogenous mediators (e.g., adenosine) build-up during periods of intense nerve stimulation (e.g., Prior et al., 1997; Correia-de-Sá and Ribeiro, 1994). Indeed, the inhibitory actions caused by CTX MII, DH-β-E, and *d*-TC were enhanced after pretreatment with adenosine deaminase, the enzyme that inactivates adenosine, although manipulation of adenosine tonus increased ACh levels (Timóteo, Faria, and Correia-de-Sá, 2002, pers. commun.). Nevertheless, the current results obtained by manipulating the safety factor of neuromuscular transmission may help to explain several discrepancies found in the literature concerning the functional role of nicotinic autoreceptors underlying tetanic fade.

It is still a matter of debate whether tetanic fading observed with *d*-TC and related compounds reflect the blockade of presynaptic facilitatory nicotinic receptors (Bowman, 1980; Wessler, 1989; Hong and Chang, 1991). Some authors argue that rundown or tetanic fade reflects a complex (post- and presynaptic) set of phenomena that might also depend on species and stimulating conditions (Magleby et al., 1981; van der Kloot and Molgó, 1994). The possibility of a transsynaptic signal coming from muscle fibers (e.g., ATP) presumed to mediate the modulation of transmitter release (see e.g., Vizi et al., 2000) could be excluded, since complete blockade of postsynaptic receptors with BTX did not automatically change nerve-evoked [^{3}H]-ACh release. Hong and Chang (1991) showed that postjunctional nicotinic receptors did not functionally change during a period of repetitive stimulation, giving further support to the positive feedback hypothesis under physiological conditions (i.e., in the absence of cholinesterase inhibitors). ATP cotransmission generating adenosine (Silinsky and Redman, 1996; but see e.g., Malinowski et al., 1997) and transmitter mobilization changes (Foldes et al., 1989) are among the presynaptic features proposed to explain tetanic fade. In addition, it was suggested that nicotinic antagonists could bind to a different inhibitory site (Gibb and Marshall, 1986; Wilson et al., 1995) that might also bind acetylcholinesterase inhibitors and vesamicol (Pemberton et al., 1992).

Several possible mechanisms for a direct inhibitory effect of nicotinic antagonists on transmitter release have been proposed. 1) Neuronal nicotinic receptors have a higher relative permeability to Ca^{2+} compared to their muscle-type counterparts and Ca^{2+} entry accompanying receptor activation might be sufficient to facilitate exocytosis (Vernino et al., 1994). Moreover, the transient influx of Ca^{2+} through the nicotinic channel could also link nicotinic autoreceptor activation to localized second messenger pathways (e.g., protein kinase C, Ca^{2+} calmodulin-dependent kinase II), facilitating exocytosis by rapidly increasing the size of the readily releasable pool of transmitter (e.g., Singh and Prior, 1998; Soliakov and Wonnacott, 2001). Recently, intracellular Ca^{2+} stores (Tsuneki et al., 2000) and Ca^{2+}-induced Ca^{2+} release (Sharma and Vijayaraghavan, 2001) in response to nicotinic receptor stimulation have been also demonstrated. Whether blockade of these mechanisms contribute to the decreased tetanic facilitation observed in the presence of the neuronal nicotinic receptor antagonists remains to be elucidated. 2) On the other hand, depolarization of nerve terminals by Na^+ influx through nicotinic channels, namely, of the α3β2 receptor type, may increase intracellular Ca^{2+} concentration by activating voltage-sensitive Ca^{2+} channels (VSCC) (Kulak et al., 2001), which might subsequently amplify Ca^{2+} transients. However, blockade of such a mechanism by nicotinic antagonists seems unlikely to occur at the rat neuromuscular junction, since high magnesium content in the bathing fluid augmented tetanic facilitation by decreasing the release probability and enhanced fading in the presence of DH-β-E- and CTX MII. 3) The inhibition of choline uptake leading to changes in neuronal ACh synthesis also does not explain release inhibition produced by nicotinic antagonists, because their actions were observed in the presence of the fast choline uptake blocker, hemicholinium-3 (10 μM) (Nikolsky et al., 1991).

Presynaptic muscarinic facilitatory M_1- and inhibitory M_2-receptors may also be involved in the feedback modulation of ACh release and synaptic efficacy at the rat neuromuscular junction (see e.g., Wessler, 1989; Oliveira et al., 2002; Santafé et al., 2003). Differences between physiological M_1 and M_2 receptor activation

depend on the nerve stimulation pattern; while the M_1 receptor may act as a presynaptic amplifier of transmitter release during brief high-frequency trains, limitation of transmitter overflow might occur through M_2 receptor activation during long periods of stimulation (Oliveira et al., 2002; Santafé et al., 2003). Thus, we performed preliminary experiments to investigate how the M_1 receptor antagonists pirenzepine (1–30 nM) and MT-7 (1 nM) affected transmitter release and diaphragm muscle tension during brief 50 Hz frequency trains. In contrast with the findings obtained with the neuronal nicotinic receptor antagonists under similar conditions, blockade of muscarinic M_1 autoreceptors inhibited evoked [^{3}H]-ACh release and depressed tetanic peak tension, without causing tetanic fade. In addition, stimulation of nicotinic and M_1 autoreceptors seem to facilitate ACh release through mechanisms that can be activated independently, because the release-enhancing effect of the M_1-receptor agonist McN-A-343 was not significantly affected by *d*-TC (Oliveira et al., 2002). It thus appears, that i) facilitatory muscarinic M_1 receptors are not involved in the fading of tetanic contractions caused by neuronal nicotinic receptor antagonists, and that ii) different second-messenger systems might couple transmitter-release facilitation mediated by metabotropic M_1 and ionotropic nicotinic autoreceptors at the rat neuromuscular junction.

As previously suggested, understanding the features that regulate the safety factor of neuromuscular transmission is of interest at both the basic and clinical levels (van der Kloot and Molgó, 1994; Wood and Slater, 2001). Both pre- and postsynaptic components change during development and may show plasticity in response to injury or disease. Since both acquired autoimmune and inherited congenital diseases of the neuromuscular junction can significantly reduce the safety factor, understanding its modulation might be of importance for devising effective therapies. So far, most attempts to improve muscle weakness that characterize neuromuscular disorders have been to prevent ACh breakdown by blocking cholinesterase activity. In view of the present data, activation of facilitatory neuronal nAChRs containing α3β2 subunits might be beneficial to ensure an adequate safety factor of neuromuscular transmission. Such a mechanism might additionally contribute to the therapeutic action of AChE inhibitors. Although there is a lack of selective nicotinic agonists available for α3β2-containing receptors and the characterization of motoneuronal nicotinic receptors in humans has not been performed yet, the rationale to minimize functional deficits in neuromuscular transmission may be useful in the near future.

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