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Endogenous adenosine prevents post-tetanic release facilitation mediated by α3β2 nicotinic autoreceptors

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Abstract

We investigated the modulatory role of endogenous adenosine on tetanic-induced (50 Hz for 5 s) nicotinic facilitation of [^{3}H]acetylcholine release (5 Hz for 50 s) from rat motoneurons. Adenosine deaminase (0.5 U/ml) and the adenosine A_{2A} receptor antagonist, 3,7-dimethyl-1-propargyl xanthine (DMPX, 30 μM), facilitated post-tetanic [^{3}H]acetylcholine release. Release inhibition caused by tubocurarine (1 μM), dihydro-β-erythroidine (1 μM) and α-conotoxin MII (0.1 μM) was attenuated after tetanic preconditioning. Nicotinic inhibitory action was fully restored after adenosine A_{2A} receptor block by DMPX or adenosine deaminase. DMPX (10 μM) caused a leftward shift of the inhibitory dose–response curves for *d*-tubocurarine (0.1–1 μM), dihydro-β-erythroidine (0.03–10 μM) and α-conotoxin MII (1–300 nM) on post-tetanic twitch amplitude. In contrast, the post-tetanic twitch depression caused by α-bungarotoxin (3–100 nM, which had no effect on transmitter release) was attenuated by DMPX (10 μM). It is concluded that activation of adenosine A_{2A} receptors by endogenously generated adenosine prevents the post-tetanic release facilitation mediated by nicotinic α3β2 autoreceptors.

Keywords: Post-tetanic facilitation; Nicotinic α3β2 receptor; Adenosine A_{2A} receptor; Adenosine endogenous; Acetylcholine release; Neuromuscular junction

1. Introduction

Prior stimulation can transiently modulate synaptic transmission depending on the balance between activation of facilitatory and of inhibitory processes, which might occur simultaneously at any particular synapse. The motor nerve stimulation pattern (e.g. frequency, number and duration of pulses) strongly influences both nicotinic (Wessler, 1989) and purinergic (Correia-de-Sá et al., 1996) modulation of acetylcholine release. Neuronal nicotinic receptors mediate a short-term positive feedback mechanism, which is terminated by rapid autodesensitisation (Wessler, 1989; Colquhoun et al., 1989). However, this mechanism may not fully explain the loss of a presynaptic inhibitory effect of tubocurarine during repetitive motor nerve activity (see Van der Kloot and Molgó, 1994). It has been argued, although hardly proven, that tetanic fade and depression of subsequent twitch responses in the presence of nicotinic antagonists, might not be due exclusively to a transient role of nicotinic autoreceptors (Bowman, 1980; Wessler, 1989; Hong and Chang, 1991; Van der Kloot and Molgó, 1994). Limitations of transmitter supply to the releasing sites or increased build-up of neuromodulators (namely adenosine) during maintained tetanic stimulation (e.g. Prior et al., 1997) have also been suggested.

Functional evidence that adenosine controls synaptic transmission through a sophisticated interplay between its own receptors (namely adenosine inhibitory A_1 and facilitatory A_{2A} receptors) and receptors for other neurotransmitters/neuromodulators (e.g. acetylcholine, glutamate, γ-aminobutyric acid, neuropeptides) has been recently provided (see for a review Sebastião and Ribeiro, 2000). Adenosine present at the neuromuscular junction results mainly from the hydrolysis of ATP released synchronously with acetylcholine (Cunha and Sebastião, 1993; Silinsky and Redman, 1996). Facilitation of acetylcholine release due to tonic activation of adenosine A_{2A} receptors becomes evident only at high levels of synaptic adenosine, such as those generated during high-intensity/high-frequency stimuli (Correia-de-Sá et al., 1996). The question then arises, of whether endogenous adenosine generated during neuronal firing might interfere with nicotinic autoregulation of neuromuscular transmission. So far, synaptic interplay between adenosine and nicotinic receptors

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has been reported when exogenous agonists were used under conditions that mimicked phrenic motoneuronal firing rate (5-Hz trains) during quiet respiration. Under these conditions, activation of pre-synaptic adenosine A_{2A} receptors accelerated the agonist-induced desensitisation of facilitatory nicotinic acetylcholine receptors, through a mechanism probably involving intracellular cyclic AMP (Correia-de-Sá and Ribeiro, 1994).

There appear to be two areas of uncertainty in our understanding of the process of nicotinic facilitation outlined above. Firstly, in light of the evidence for the control of nicotinic facilitation by exogenous adenosine A_{2A} receptor activation, it would be of interest to determine if adenosine, endogenously generated during a brief tetanus, affects subsequent nicotinic facilitation (post-tetanic facilitation) in a similar manner. For this purpose, we evaluated tension responses and [^{3}H]acetylcholine release triggered after tetanic preconditioning (50 Hz for 5 s) in the presence of selective adenosine antagonists or adenosine deaminase, the enzyme that inactivates adenosine into inosine. Furthermore, as the identity of the nicotinic acetylcholine receptor associated with this facilitatory process is unknown, it would also be of interest to determine which subtype mediates prejunctional facilitation. Neuronal nicotinic acetylcholine receptors are ligand-gated channels composed of pentameric combinations of α and β subunits, giving the potential for a great variety of receptor subtypes (Lukas et al., 1999). Regarding native neuronal systems, knowledge of the subunit composition of nicotinic acetylcholine receptors is generally lacking and only a few major subtypes have been identified (e.g. Wonnacott, 1997). In the present study, we tested a variety of different nicotinic acetylcholine receptor-blocking agents that target specific muscle or neuronal nicotinic acetylcholine receptor subunits (*d*-tubocurarine, dihydro-β-erythroidine, α-conotoxin MII, α-bungarotoxin) on nerve-induced tension responses and [^{3}H]acetylcholine release from phrenic nerve–hemidiaphragm preparations. Dihydro-β-erythroidine competitively blocks α4-containing as well as α3β2 nicotinic acetylcholine receptors (Chavez-Noriega et al., 1997). α-Conotoxin MII has a high degree of selectivity for α3β2-containing neuronal nicotinic acetylcholine receptors (Cartier et al., 1996). In addition to its ability to block muscle-type α1-containing receptors, α-bungarotoxin is also a high-affinity ligand for homo-oligomeric α7 and α8 channels (for a review, see Dwoskin and Crooks, 2001).

2. Methods

2.1. Preparation and experimental conditions

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 after stunning followed by exsanguination. Animal handling and experiments followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). The experiments were performed 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%).

2.2. 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 fibres. Supramaximal intensity rectangular pulses with 40 μs duration and a current strength of 8 mA were used to achieve synchronization of phrenic motoneuron firing, thus reducing the number of silent units that might make interpretation of data difficult. Tetanic preconditioning consisted in the application of a brief high-frequency train (50 Hz for 5 s) preceding a phasic component (5 Hz for 50 s). This pattern mimicked physiological motoneuronal firing during episodes of intense muscular work (Waud and Waud, 1971) and recovery from inspiratory resistive loading (Road et al., 1995). In some of the experiments, the tetanic train (50 Hz for 5 s) was applied immediately after the subtetanic component (5 Hz for 50 s) for comparison. Pulses were generated by a Grass S48 (USA) stimulator coupled to a stimulus isolation unit (Grass SIU5, USA) operating in a constant current mode. The stimulation parameters were continuously monitored on an oscilloscope (Meguro, MO-1251A, Japan).

2.3. Isotope experiments

The procedures used for labelling the preparations and measuring the evoked [^{3}H]acetylcholine release have been described (Correia-de-Sá et al., 1991) and were used with minor modifications. Experiments were performed in the absence of cholinesterase inhibitors to prevent unphysiological extracellular accumulation of acetylcholine. 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 labelled 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 labelling period, the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation was 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, France) coupled to a peristaltic pump (Gilson, Minipuls3, France)-programmed

device. Aliquots (0.5 ml) of the incubation medium were added to 3.5 ml of Packard Insta Gel II (USA) scintillation cocktail. The 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 sample tritium content. The radioactivity was expressed as DPM/g of wet weight of the tissue determined at the end of the experiment. After the loading and washout periods, the preparation contained $5542 \pm 248 \times 10^3$ DPM/g and the resting release was $132 \pm 12 \times 10^3$ DPM/g 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 sample collected. [^{3}H]Acetylcholine release was evoked by electrical stimulation of the phrenic nerve as described earlier. 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 only the release of [^{3}H]acetylcholine in a Ca^{2+}- and tetrodotoxin-sensitive manner (Correia-de-Sá et al., 2000), while the output of [^{3}H]choline remained unchanged during the stimulation periods (Wessler and Kilbinger, 1986). Therefore, evoked [^{3}H]acetylcholine release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (cf. Correia-de-Sá et al., 1991).

Test drugs were added 15 min before S_2 and were present up to the end of the experiments (see e.g. Fig. 1). In some experiments, the incubation time with α-bungarotoxin was prolonged to 45 min, therefore S_2 was postponed to 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]acetylcholine release during the second stimulation period (in the presence of the test drug) and the evoked [^{3}H]acetylcholine release during the first stimulation period (without the test drug). Percentage values shown in tables and figures correspond to percentage changes in S_2/S_1 ratios as compared with the S_2/S_1 ratio in control experiments using the same stimulation design (Table 1); Zero percent represents identity between ratios; positive and negative values represent facilitation and inhibition of evoked [^{3}H]acetylcholine release, respectively. When the ability of any drug to modify the effects of nicotinic receptor antagonists was tested, the drug was applied since the beginning of the release period (time zero). This was done in order to affect in a similar manner both control (S_1) and test (S_2) stimulations. S_2/S_1 ratios determined in the presence of these drugs were not significantly different from controls (Table 1). None of the compounds studied modified the basal tritium outflow in a measurable way.

2.4. Muscle contraction recordings

When tension responses were recorded, the innervated diaphragm strips were mounted in a 10-ml capacity isolated organ bath chamber. The preparations were superfused (5 ml min^{-1}, 37 °C, pH 7.4) with gassed (95% O_2 + 5% CO_2) Tyrode's solution. The pattern of nerve stimulation, consisting in the application of a brief tetanus (50 Hz for 5 s, tetanic preconditioning) followed by a train of 250 pulses delivered at 5-Hz frequency, was repeated once every 15 min. Tension responses were recorded isometrically at a resting tension of 50 mN with a force transducer and displayed on a Hugo-Sachs (Germany) recorder. These experimental conditions allowed a well-preserved contraction pattern for several hours after the initial stabilization period in the absence of test drugs. The solutions were changed by 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 the solutions had been changed and 5 ml min^{-1} until the next changeover of solutions. Test drugs were allowed to be in contact with the preparations for at least 12 min before tetanic preconditioning; incubation time with

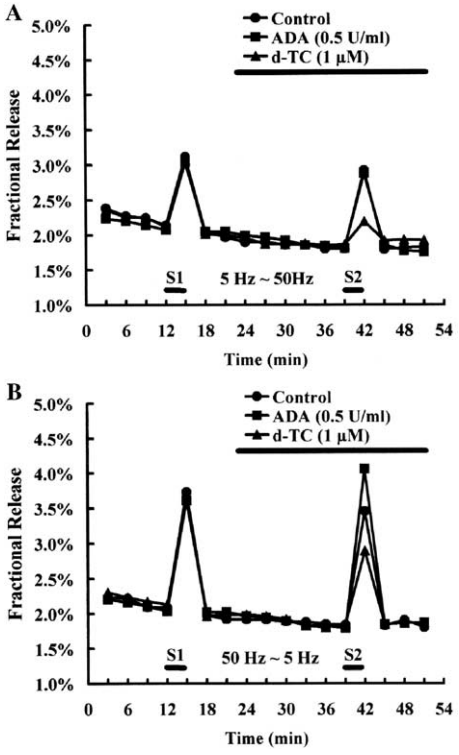

Fig. 1. Effects caused by adenosine deaminase and *d*-tubocurarine (*d*-TC) on post-tetanic facilitation of [^{3}H]acetylcholine release from motor nerve terminals. Shown is the time course of tritium outflow from rat hemidiaphragms taken from typical experiments. Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period. The abscissa indicates the times at which the samples were collected. After the labelling and washout periods (zero time), [^{3}H]acetylcholine release was elicited by electrically stimulating the phrenic nerve with 500 supramaximal intensity pulses at the indicated times (S_1 and S_2). In (A), a brief tetanus (50 Hz, 250 pulses) was applied following the 5-Hz train (250 pulses), whereas a reverse stimulation order was used in (B). Note that under control conditions (circles), the total amount of [^{3}H]acetylcholine released during S_1 was higher when tetanus preceded the subtetanic component (50 → 5 Hz) (see also Table 1 for details). Adenosine deaminase (0.5 U/ml, squares) and *d*-tubocurarine (1 μM, triangles) were applied 15 min before S_2 (horizontal bars). None of these drugs changed spontaneous tritium outflow.

Table 1
Influence of tetanic preconditioning on the amount of [^{3}H]acetylcholine release (S_1 values) from the rat motor nerve terminals

Stimulation conditions in S_1	S_1 ($10^3 \times$ DPM/g)	S_1 (DPM/g/pulse)	Control (S_2/S_1)
5 Hz, 250 pulses	23 ± 2 (10)	92 ± 8 (10)[a]	0.83 ± 0.06 (4)
50 Hz, 250 pulses	37 ± 1 (10)[b]	148 ± 4 (10)[a,b]	0.82 ± 0.03 (4)
5 Hz, 250 pulses → 50 Hz, 250 pulses	56 ± 2 (8)	112 ± 3 (8)[a]	0.86 ± 0.05 (4)
50 Hz, 250 pulses → 5 Hz, 250 pulses	96 ± 3 (14)[c]	192 ± 6 (14)[c]	0.86 ± 0.04 (4)

Phrenic nerve was stimulated at the 12th (S_1) and 39th (S_2) min after the end of the washout period with supramaximal intensity rectangular pulses delivered at frequencies of 5 and 50 Hz. Tetanic preconditioning was performed by applying a high-frequency tetanic train immediately before the subtetanic period of stimulation (50 Hz, 250 pulses → 5 Hz, 250 pulses). In some of the experiments, similar tetanic stimuli were applied after 5-Hz frequency trains (5 Hz, 250 pulses → 50 Hz, 250 pulses) for comparison. Data represent the average (± S.E.M.) evoked [^{3}H]acetylcholine release during the first period of stimulation (S_1, $10^3 \times$ DPM/g of wet tissue); the amount of tritium outflow evoked per stimulation pulse (S_1, DPM/g/pulse) was also calculated. Number of experiments is shown in parenthesis. S_2/S_1 is the ratio between the evoked [^{3}H]acetylcholine release during the second period of stimulation and the evoked [^{3}H]acetylcholine release during the first period of stimulation.

[a] $P<0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared with the average evoked [^{3}H]acetylcholine release per stimulation pulse after tetanic preconditioning (50 → 5 Hz).

[b] $P<0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared with evoked tritium outflow obtained with trains of 5 Hz (250 pulses) applied alone.

[c] $P<0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared with evoked tritium outflow obtained with trains of 5 Hz (250 pulses) followed by a 50-Hz tetanus (5 → 50 Hz).

α-bungarotoxin was prolonged to 45 min in some of the experiments.

2.5. Materials and solutions

Adenosine deaminase (type VI, 1803 U/ml, EC 3.5.4.4), α-bungarotoxin, choline chloride, dihydro-β-erythroidine hydrobromide, hemicholinium-3, mecamylamine, methyllycaconitine citrate, *d*-tubocurarine chloride (Sigma, USA); α-conotoxin MII (Tocris Cookson, UK); 3,7-dimethyl-1-propargylxanthine (DMPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemicals Int., USA); [methyl-^{3}H] choline chloride (ethanol solution, 80 Ci mmol^{-1}) (Amersham, UK). DPCPX was made up as a 5-mM stock solution in 99% dimethyl sulphoxide/1% NaOH 1 M (v/v). All stock solutions were stored as frozen aliquots at −20 °C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. The pH of the superfusion solution was not changed by addition of the drugs at the maximum concentrations applied to the preparations.

2.6. Statistics

The data are expressed as means ± standard error of the mean, from n observations. Statistical significance of experimental results was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's modified t-test. $P<0.05$ was considered to represent significant differences.

3. Results

3.1. Post-tetanic facilitation of [^{3}H]acetylcholine release from motor nerve terminals

The data presented in Table 1 show that there is a frequency-dependent facilitation of transmitter release from rat motor nerve endings. This was evidenced because the average evoked [^{3}H]acetylcholine release per stimulation pulse (calculated as desintegrations per min (DPM)/gram of wet tissue/pulse) increased from 92 ± 8 ($n=10$) to 148 ± 4 ($n=10$) as the stimulation frequency changed from 5 to 50 Hz, keeping the number of pulses (250) constant. Moreover, upon delivering trains of 50- and 5-Hz frequency pulses in close succession, we demonstrated that tetanic preconditioning (50 Hz for 5 s) further enhanced the [^{3}H]acetylcholine release evoked during a 50-s period where the phrenic nerve was stimulated at 5-Hz frequency (post-tetanic facilitation). Under such conditions, the total amount of [^{3}H]acetylcholine released after tetanic preconditioning ($96 \pm 3 \times 10^3$ DPM/g, $n=14$) was significantly ($P<0.05$) higher than one would have expected from simple summation of the two frequency components, 50 Hz (37 ± 1 DPM/g, $n=10$) and 5 Hz (23 ± 2 DPM/g, $n=10$) (Table 1). Likewise, the average evoked [^{3}H]acetylcholine release calculated per stimulation pulse was maximal (192 ± 6 DPM/g/pulse, $n=14$) after tetanic preconditioning. These results gain further relevance since application of the two stimulation trains in reverse order, i.e. the subtetanic (5 Hz, 250 pulses) component preceding the high-frequency train (50 Hz, 250 pulses), failed to facilitate the evoked [^{3}H]acetylcholine release ($56 \pm 2 \times 10^3$ DPM/g, $n=8$) (Table 1).

3.2. Endogenous adenosine modulates [^{3}H]acetylcholine release after tetanic preconditioning

Adenosine deaminase (0.5 U/ml), the enzyme that inactivates endogenous adenosine into inosine, failed to modify the [^{3}H]acetylcholine release evoked by trains of 250 pulses, regardless of whether the frequency of stimulation was 5 or 50 Hz (Table 2). Adenosine deaminase (0.5 U/ml) was also virtually devoid of effect when a brief tetanus was applied immediately after the subtetanic component (5 → 50 Hz). It appears that this stimulation design was inappropriate for generating endogenous adenosine to levels required for activating presynaptic receptors, since neither of the adenosine receptor antagonists tested, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 2.5 nM—a selective adenosine A_1 receptor antagonist) or 3,7-dimethyl-1-propargylxanthine (DMPX, 30 μM—a preferential adenosine A_{2A} receptor antagonist), modified evoked transmitter release (Table 2).

Table 2
Tonic adenosine influence on evoked [^{3}H]acetylcholine release after tetanic preconditioning

Conditions of stimulation	Adenosine deaminase (0.5 U/ml)	DMPX (30 μM)	DPCPX (2.5 nM)
5 Hz, 250 pulses	0 ± 3% (5)		
50 Hz, 250 pulses	− 1 ± 1% (4)		
5 Hz, 250 pulses → 50 Hz, 250 pulses	+ 1 ± 3% (4)	+ 1 ± 5% (3)	0 ± 4% (3)
50 Hz, 250 pulses → 5 Hz, 250 pulses	+ 14 ± 5% (5)[a]	+ 33 ± 7% (4)[a]	− 19 ± 5% (6)[a]

Phrenic nerve was stimulated at the 12th (S_1) and 39th (S_2) min after the end of the washout period with supramaximal intensity rectangular pulses delivered at frequencies of 5 and 50 Hz. Tetanic preconditioning was performed by applying a high-frequency train immediately before the subtetanic period of stimulation (50 Hz, 250 pulses → 5 Hz, 250 pulses). In some of the experiments, tetanic stimulation was preceded by a 5-Hz train (5 Hz, 250 pulses → 50 Hz, 250 pulses) for comparison. Adenosine deaminase (0.5 U/ml), 3,7-dimethyl-1-propargylxanthine (DMPX, 30 μM), or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 2.5 nM), were applied 15 min before S_2. Data are percentage changes (± S.E.M.) in S_2/S_1 ratios as compared with the S_2/S_1 ratio in control experiments performed in the absence of test drugs using the same stimulation paradigm. Zero percent represents identity between the two ratios; positive and negative values represent facilitation and inhibition of the evoked [^{3}H]acetylcholine release, respectively. Number of experiments is shown in parenthesis.

[a] $P<0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared to zero percent.

However, after tetanic preconditioning (50 → 5 Hz) both adenosine deaminase (0.5 U/ml) and the adenosine A_{2A} receptor antagonist, DMPX (30 μM), increased the evoked [^{3}H]acetylcholine release, whereas the adenosine A_1 receptor antagonist, DPCPX (2.5 nM), had a small inhibitory action (Table 2, see also Fig. 1). Comparing the magnitude of effects produced by each antagonist on post-tetanic facilitation, the results suggest that an inhibitory adenosine tonus mediated by adenosine A_{2A} receptors (revealed by DMPX facilitation, + 33 ± 7%, $n=4$) predominates over tonic A_1 facilitation (revealed by DPCPX inhibition, − 19 ± 5%, $n=6$). Since the net tonic action of endogenous adenosine revealed with adenosine deaminase depends on the A_1/A_{2A} receptors activation balance, one can easily explain why the adenosine deaminase (0.5 U/ml)-induced facilitation of [^{3}H]acetylcholine release (+ 14 ± 5%, $n=5$) was less than that caused by DMPX (30 μM). In addition, the present data clearly show that control of neuromuscular transmission by endogenous adenosine requires a threshold concentration of the nucleoside, which might be influenced by the stimulation paradigm (cf. Correia-de-Sá et al., 1996).

3.3. Tetanic preconditioning attenuates α3β2 nicotinic facilitation of [^{3}H]acetylcholine release

The nicotinic receptor antagonist, *d*-tubocurarine (1 μM), reduced the [^{3}H]acetylcholine release evoked by trains of 250 pulses delivered at 50 Hz (− 61 ± 5%, $n=6$) and 5 Hz (− 53 ± 10%, $n=4$) frequencies, without significantly affecting spontaneous transmitter release (data not shown). These results agree fully with previous findings (e.g. Bowman et al., 1988; Vizi and Somogyi, 1989; Wessler, 1989), indicating that acetylcholine mediates a positive feedback mechanism enhancing its own release during periods of repetitive nerve stimulation. However, it remains to be elucidated which is the mechanism that regulates temporary nicotinic amplification of transmitter outflow necessary to avoid overstimulation of motor nerve terminals. Data presented in Fig. 1 show that facilitation of [^{3}H]acetylcholine release becomes less sensitive to nicotinic modulation after tetanic preconditioning (50 Hz for 5 s). Following a brief high-frequency train (50 → 5 Hz), the release inhibition caused by *d*-tubocurarine (1 μM) was significantly attenuated (32 ± 4%, $n=5$) as compared to the situation where tetanus was preceded by the subtetanic (5 → 50 Hz) component (Fig. 1, see Fig. 2 for details). In this latter situation, *d*-tubocurarine (1 μM) reduced the evoked [^{3}H]acetylcholine release by 69 ± 7% ($n=4$), which did not significantly differ from the magnitude of inhibition detected when each frequency component (5 or 50 Hz) was tested separately (see above).

To investigate the nicotinic receptor subtype mediating prejunctional facilitation of [^{3}H]acetylcholine release, we compared the effects of several subtype-specific nicotinic antagonists (Fig. 2). When tetanus (50 Hz for 5 s) was applied immediately after the subtetanic component (5 Hz for 50 s), incubation with low concentrations of dihydro-β-erythroidine (1 μM, a competitive blocker of α4-containing as well as of α3β2 nicotinic acetylcholine receptors) and α-conotoxin MII (100 nM, a preferential nicotinic α3β2 receptor antagonist) reduced the evoked tritium outflow by 56 ± 9% ($n=4$) and 40 ± 6% ($n=4$), respectively. However, tetanic preconditioning (50 → 5 Hz) significantly ($P<0.05$) attenuated the magnitude of release inhibition caused by dihydro-β-erythroidine (1 μM, 28 ± 6%, $n=4$) and α-conotoxin MII (100 nM, 8 ± 4%, $n=5$). Neither methyllycaconitine (3 μM, data not shown), a preferential nicotinic α7 receptor antagonist, nor prolonged (45 min) application of α-bungarotoxin (300 nM, Fig. 2), a high-affinity ligand for homo-oligomeric α7 and α8 neuronal nicotinic receptors and muscle-type α1-containing receptors, significantly ($P>0.05$) modified [^{3}H]acetylcholine release, regardless of the stimulation pattern used. The antagonist rank potency order (α-conotoxin MII>*d*-tubocurarine ~ dihydro-β-erythroidine) strongly indicates that transient facilitation of [^{3}H]acetylcholine release following repetitive nerve stimulation is mediated by prejunctional nicotinic receptors expressing α3β2-subunits.

3.4. Endogenous adenosine negatively regulates post-tetanic α3β2 nicotinic autofacilitation through the activation of presynaptic A_{2A} receptors

Since A_{2A}-adenosine tonus gains functional relevance after tetanic preconditioning in parallel with the partial loss

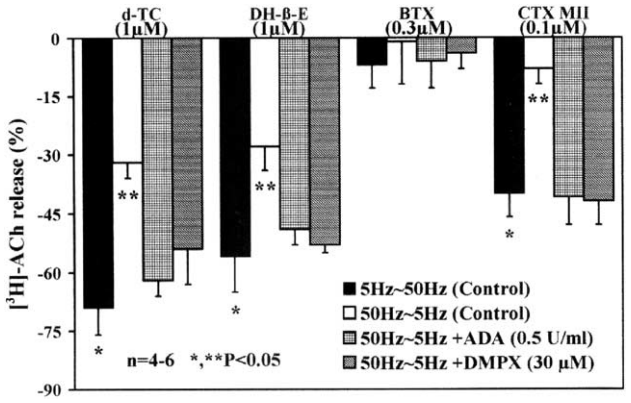

Fig. 2. Tonic adenosine A_{2A} receptor activation attenuates nicotinic autofacilitation of [^{3}H]acetylcholine release after tetanic preconditioning. Phrenic nerve was supramaximally stimulated twice (S_1 and S_2); a brief tetanic train (50 Hz, 250 pulses) was applied either immediately before (open and hashed columns) or following (filled columns) the subtetanic component (5 Hz, 250 pulses). The nicotinic receptor antagonists, *d*-tubocurarine (*d*-TC, 1 μM), dihydro-β-erythroidine (DH-β-E, 1 μM) and α-conotoxin MII (CTX MII, 100 nM), were applied 15 min before S_2 (delivered at the 39th min after washout). Due to its slow association/dissociation kinetics, incubation with α-bungarotoxin (BTX, 0.3 μM) was prolonged to 45 min and S_2 was delivered at the 69th min after washout (see Methods). Although under these latter conditions the interval between each stimulation period (S_1 and S_2) was prolonged, control S_2/S_1 ratios did not significantly differ from those shown in Table 1. Adenosine deaminase (0.5 U/ml) and the adenosine A_{2A} receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX, 30 μM), were applied since the beginning of the release period (time zero), i.e. they were present during the whole assay including S_1 and S_2. The ordinates are percentage inhibition of [^{3}H]acetylcholine release as compared to control, in the absence of added drugs. Each column represents pooled data from 4 to 6 experiments. The vertical bars represent ± S.E.M. *$P<0.05$ (one-way ANOVA followed by Dunnett's modified *t*-test) when compared with zero percent; **$P<0.05$ (one-way ANOVA followed by Dunnett's modified *t*-test) when compared with results obtained applying the 5-Hz train before tetanus (5 → 50 Hz), using the same nicotinic receptor antagonist.

of nicotinic autofacilitation, experiments were designed to investigate whether A_{2A} receptor activation by endogenous adenosine could downregulate prejunctional nicotinic acetylcholine receptor function. The magnitude of [^{3}H]acetylcholine release inhibition caused by *d*-tubocurarine (1 μM), dihydro-β-erythroidine (1 μM) and α-conotoxin MII (100 nM) after tetanic preconditioning (50 → 5 Hz) was fully restored in the presence of adenosine deaminase (0.5 U/ml) or DMPX (30 μM), which was applied to prevent A_{2A} receptor activation by endogenous adenosine (Fig. 2). Recovery of the inhibitory amplitude caused by nicotinic antagonists was evident regardless of the fact that pretreatment with both adenosine deaminase (0.5 U/ml) and DMPX (30 μM) increased the amount of [^{3}H]acetylcholine release after tetanic preconditioning (50 → 5 Hz, see Table 2). These findings indicate that saturation and/or desensitisation of presynaptic nicotinic receptors only partially explains the loss of function of *d*-tubocurarine (1 μM), dihydro-β-erythroidine (1 μM) and α-conotoxin MII (100 nM) during repetitive nerve activity (see e.g. Wessler, 1989; Van der Kloot and Molgó, 1994; Correia-de-Sá and Ribeiro, 1994). The ability of DMPX to prevent the partial loss of nicotinic autofacilitation was also observed when this compound was applied in a concentration (10 μM, data not shown) near its K_i value (9.6 μM) for selectively blocking adenosine A_{2A} receptors (see for a review Jacobson et al., 1992); pretreatment with 10 μM DMPX had no effect on evoked [^{3}H]acetylcholine release but completely antagonized the facilitatory effect of the selective adenosine A_{2A} receptor agonist, CGS 21680C, in this preparation (Correia-de-Sá and Ribeiro, 1994). In contrast, DPCPX (2.5 nM) did not significantly ($P>0.05$) change *d*-tubocurarine (1 μM) inhibition after tetanic preconditioning (data not shown), although it antagonized the inhibitory effect of the adenosine A_1 receptor agonist, *R*-N^6-phenylisopropyl adenosine, on evoked [^{3}H]acetylcholine release from phrenic motor nerve terminals (Correia-de-Sá et al., 1991) with a K_i value of the same order as that (0.54 nM) for this xanthine at adenosine A_1 receptors (Lohse et al., 1987). As expected, adenosine deaminase (0.5 U/ml) did not further potentiate the inhibitory effect of *d*-tubocurarine (1 μM, $-69 \pm 7\%$, $n=4$) when the 50-Hz train was applied immediately after the subtetanic (5-Hz train) component, using a similar number of pulses ($-65 \pm 3\%$, $n=4$). The results indicate that adenosine buildup after brief high-frequency trains may prevent nicotinic autofacilitation, due to the crosstalk between adenosine A_{2A}- and nicotinic α3β2-containing receptors at the rat motor nerve terminals.

3.5. Blockade of A_{2A}-adenosine receptors differentially affects neuromuscular relaxation caused by nicotinic antagonists acting at pre- and/or postjunctional levels

Fig. 3 shows pen-recorder traces of nerve-evoked hemidiaphragm contractions obtained by the application of a brief tetanus (50 Hz, 250 pulses) immediately followed by subtetanic twitches (5 Hz, 250 pulses). Under control conditions, post-tetanic twitch tension (50 → 5 Hz) was transiently increased for about 30 s. Brief facilitation was also observed during the course of tetani (50 Hz, 250 pulses), i.e. muscle tension was transiently increased when high-frequency repetitive pulses were delivered to the nerve. *d*-Tubocurarine (0.3 μM) decreased both tetanic peak tension (50-Hz train) and twitch amplitude (5-Hz train) by about 50%. Fading of tetanic tension was also observed in the presence of *d*-tubocurarine (0.3 μM). In view of the putative interactions between purinergic and nicotinic receptors to control neuromuscular transmission, we also investigated whether depression of contractile activity caused by *d*-tubocurarine was modified on selective blocking of presynaptic adenosine A_1 and A_{2A} receptors. Relaxation induced by *d*-tubocurarine (1 μM) was exaggerated in the presence of the adenosine A_{2A} receptor antagonist, DMPX (10 μM), but not when the adenosine A_1 receptor antagonist, DPCPX (2.5 nM), was applied (Fig. 3, see also Fig. 4 for details of DMPX interaction). Note that both adenosine

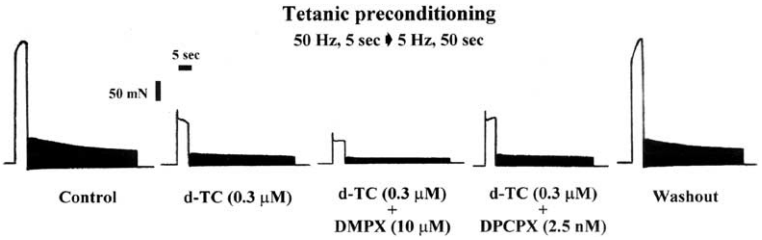

Fig. 3. Representative pen-recorder traces of nerve-evoked hemidiaphragm contractions obtained after brief tetanic (50 Hz, 250 pulses) preconditioning followed by 5-Hz frequency twitches (250 pulses), in the absence (Control and Washout) and in the presence of *d*-tubocurarine (*d*-TC, 0.3 μM). Contraction responses were recorded at least 12 min after adding *d*-tubocurarine (0.3 μM). Muscular relaxation caused by *d*-tubocurarine (0.3 μM) was also evaluated in the presence of two selective adenosine antagonists, 3,7-dimethyl-1-propargylxanthine (DMPX, which preferentially blocks adenosine A_{2A} receptors) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, a selective adenosine A_1 receptor antagonist). DMPX (10 μM) and DPCPX (2.5 nM) was in contact with the preparations for at least 45 min; these drugs were used in concentrations virtually devoid of effects on nerve-evoked muscle tension. The horizontal bar indicates a 5-s period of stimulation. Vertical calibration: 50 mN.

receptor antagonists were tested in concentrations with virtually no effects on nerve-evoked muscle contractions.

Fig. 4 shows the dose–response curves for the inhibitory effects of *d*-tubocurarine (0.1–1 μM), α-bungarotoxin (3–100 nM), dihydro-β-erythroidine (0.03–10 μM) and α-conotoxin MII (1–300 nM) on post-tetanic twitch tension induced by phrenic nerve stimulation. When applied cumulatively, the four nicotinic antagonists decreased post-tetanic twitch contractions in a concentration-dependent manner. Application of α-bungarotoxin (100 nM) and *d*-tubocurarine (1 μM) produced complete neuromuscular paralysis, whereas depression of post-tetanic twitches with dihydro-β-erythroidine (10 μM) and α-conotoxin MII (300 nM) did not exceed 30%. Thus, a reduction in post-tetanic twitch

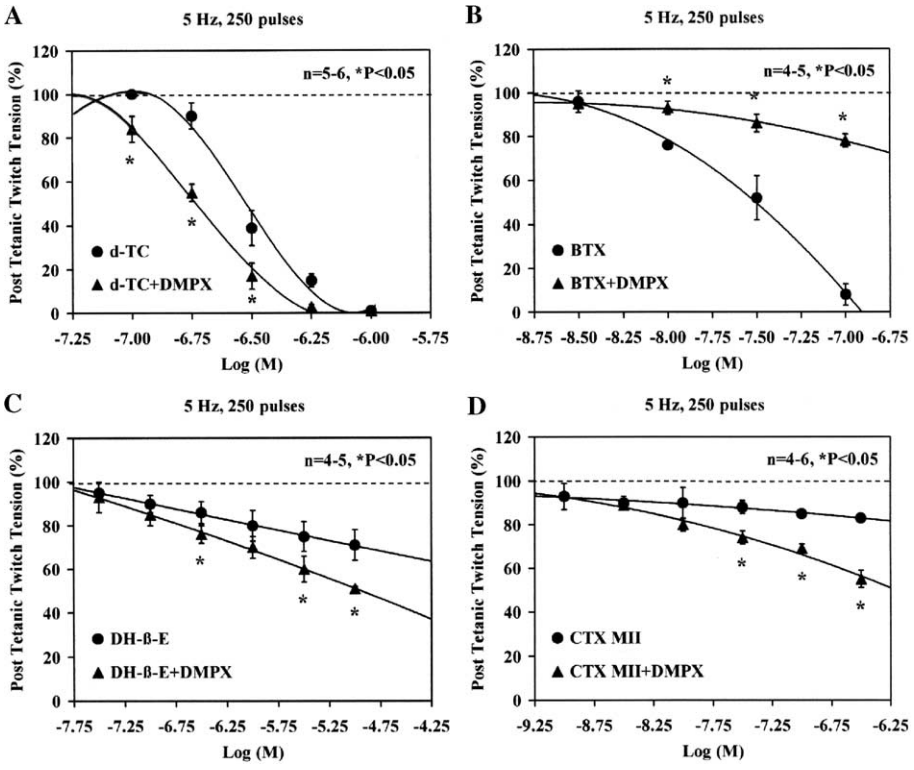

Fig. 4. Dual modification of post-tetanic twitch depression caused by nicotinic antagonists acting preferentially on pre- or postsynaptic sites in the presence of 3,7-dimethyl-1-propargylxanthine (DMPX), an adenosine A_{2A} receptor antagonist. Post-tetanic twitch responses were induced once every 15 min by electrically stimulating the phrenic nerve with a brief high-frequency train (50 Hz for 5 s) followed by a 50-s period where the stimulation frequency was 5 Hz. (A) *d*-Tubocurarine (*d*-TC, 0.1–1 μM), (B) α-bungarotoxin (BTX, 3–100 nM), (C) dihydro-β-erythroidine (DH-β-E, 0.03–10 μM) and (D) α-conotoxin MII (CTX MII, 1–300 nM), were applied in a cummulative manner and were in contact with the preparation at least 12 min before recordings. DMPX was in contact with the preparations for at least 45 min, and was used in a concentration (10 μM) virtually devoid of effect on nerve-evoked muscle tension. The ordinates are percentage of maximal post-tetanic twitch tension (100%) determined in the absence of nicotinic antagonists. The vertical bars represent ± S.E.M. of *n* number of experiments and are shown when they are larger than the symbols. $*P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t*-test) as compared with the effect of each nicotinic receptor antagonist in the absence of DMPX.

tension has an antagonist profile with a rank order of potency of α-bungarotoxin>*d*-tubocurarine≫dihydro-β-erythroidine>α-conotoxin MII, indicating the presence of nicotinic receptors containing α1 subunits in rat skeletal muscle (Schuetze and Role, 1987). Furthermore, our data demonstrate that muscular receptors have pharmacological characteristics distinct from those of nicotinic receptors involved in the control of transmitter release (which might be composed by α3β2-subunits, see above).

As demonstrated for *d*-tubocurarine (0.1–1 μM, Fig. 3, see also Fig. 4), DMPX (10 μM) also shifted to the left the dose–response curves for the inhibitory effects of dihydro-β-erythroidine (0.03–10 μM) and α-conotoxin MII (1–300 nM) on post-tetanic twitch tension (Fig. 4), while the adenosine A_1 receptor antagonist, DPCPX (2.5 nM), was virtually ineffective (data not shown). Interestingly, adenosine A_{2A} receptor blockade with DMPX (10 μM) significantly ($P<0.05$) attenuated the post-tetanic twitch depression caused by α-bungarotoxin (3–100 nM), an irreversible nicotinic receptor antagonist that has high affinity for muscle-type α1-containing receptors with virtually no effect on [^{3}H]acetylcholine release. A reduction in the relaxing power of 'pure' postsynaptic nicotinic antagonists, such as α-bungarotoxin (3–100 nM), might result from increases in the safety margin of neuromuscular transmission due to post-tetanic release facilitation in the presence of DMPX (see above). These findings demonstrate that facilitation of acetylcholine release from motor nerve terminals is balanced by tonic activation of presynaptic nicotinic and adenosine A_{2A} receptors. Such interplay may become physiologically relevant to limit neuronal overstimulation after sustained high-frequency trains due to increased adenosine generation.

4. Discussion

The results indicate that activation of A_{2A} receptors by endogenous adenosine generated during high-frequency stimuli limits the post-tetanic nicotinic overstimulation of motor nerve terminals by released acetylcholine. Despite the high safety margin of neuromuscular transmission, it appears that under appropriate conditions (e.g. sustained high-frequency motor nerve firing) the interplay between presynaptic nicotinic- and adenosine A_{2A} receptors becomes functionally relevant to control synaptic transmission. In view of the current interest regarding the subtype of nicotinic receptor involved in pre- and postjunctional actions of acetylcholine that may determine the sensitivity to non-depolarizing muscle relaxants (see e.g. Paul et al., 2002), this work provided two interesting new insights. First, the pharmacological profile of the presynaptic nicotinic (α-conotoxin MII>*d*-tubocurarine ~ dihydro-β-erythroidine≫α-bungarotoxin, methyllycaconitine) clearly differed from that of the typical α-bungarotoxin-sensitive α1 containing muscle receptor, suggesting that the facilitatory receptor located on motor nerve terminals expresses α3β2-subunits. Second, the pattern of drug interaction with nicotinic antagonists used to produce muscle relaxation might be strongly influenced by their subtype selectivity. This was evidenced when application of the xanthine-derived adenosine A_{2A} receptor antagonist, DMPX, enhanced the neuromuscular block caused by nicotinic antagonists acting at the presynaptic level, whereas the relaxing effect of 'pure' muscular agents, such as α-bungarotoxin, was largely attenuated. These findings may be clinically relevant, as human nicotinic acetylcholine receptor subunits (α2–α7 and β2–β4) show 91–99% amino-acid identity to their rat homologues, and synergism between structurally distinct neuromuscular blocking agents (e.g. pancuronium and mivacurium) has been used to reduce autonomic and cardiovascular side-effects of individual compounds (Lebowitz et al., 1981).

At the neuromuscular junction, ATP is released synchronously together with acetylcholine (Silinsky and Redman, 1996) and synaptic adenosine accumulation depends on nerve stimulation frequency (Silinsky, 1975; Cunha and Sebastião, 1993). Extracellular ATP may act as a source of adenosine that fulfils a key modulatory role to control neuromuscular transmission through the activation of adenosine A_1 and A_{2A} receptors co-localized on motor nerve terminals (Ribeiro and Walker, 1975; Silinsky, 1975; Correia-de-Sá et al., 1991; Cunha et al., 1996). Adenosine formation from adenine nucleotides is tightly regulated by released ATP, which can exert feed-forward inhibition of ecto-5′ -nucleotidase, limiting hydrolysis of AMP into adenosine (James and Richardson, 1993; Cunha et al., 1996). As large amounts of ATP are released during high-frequency (50 Hz) stimulation, adenosine formation may be delayed until ATP and ADP levels fall to below the threshold value for enzymatic inhibition. This might explain why at least >250 pulses are required before adenosine tonus gains physiological relevance (cf. Malinowski et al., 1997). In addition, time-dependent synaptic accumulation of endogenous adenosine in parallel with A_1 receptor down-regulation, shifts tonic A_1/A_{2A} inter-receptor equilibrium towards the activation of adenosine A_{2A} receptors during high-frequency bursts (Correia-de-Sá et al., 1996). This is in good agreement with what we found following a 50-Hz tetanus, when the nicotinic positive feedback mechanism is still operative. Under these circumstances, the net adenosine A_{2A}-mediated tonus (revealed by the facilitatory actions of adenosine deaminase and DMPX) was responsible for preventing post-tetanic facilitation of [^{3}H]acetylcholine release, while the activation of adenosine A_1 receptors (revealed by the small inhibitory effect caused by DPCPX) partially counteracted this action.

Acetylcholine may enhance its own release during periods of repetitive motoneuronal firing by activating nicotinic autoreceptors. This positive nicotinic feedback mechanism was suggested from countless studies measuring mechanical tension, electrophysiological signals and radiolabelled acetylcholine release in the presence of nicotinic antagonists (e.g. Bowman et al., 1988; Vizi and Somogyi, 1989;

Wessler, 1989; Hong and Chang, 1991). Nicotinic autofacilitation is clearly frequency-dependent and may operate as a temporary presynaptic amplifier to increase the safety factor for transmission, reaching a maximum within a range that corresponds to the motoneurone firing rate (5–50 Hz) during voluntary movements (Wessler, 1989; Singh and Prior, 1998). At higher frequencies or during longer periods of stimulation, the nicotinic facilitation is cut short to avoid transmitter flooding and muscular overstimulation by mechanisms not clearly understood. Rapid nicotinic receptor desensitisation might serve to limit transient nicotinic autofacilitation (Colquhoun et al., 1989; Wessler, 1989; Correia-de-Sá and Ribeiro, 1994). Although autodesensitisation partially explains the loss of nicotinic-induced release facilitation following a brief 50-Hz tetanus, as indicated by attenuation of the inhibitory actions of nicotinic receptor antagonists (see also Wessler, 1989; Van der Kloot and Molgó, 1994), our data demonstrate that presynaptic A_{2A} receptor activation by endogenous adenosine may also control the nicotinic positive feedback (Fig. 2, see below).

A major feature of the present work is the pharmacological exploration of the identity of the presynaptic nicotinic receptor that facilitates acetylcholine release, using subtype selective antagonists. Nicotinic receptors are a heterogeneous family of ligand-gated ion channels composed of four homologous subunits associated pseudosymmetrically in a pentameric structure. Several lines of evidence indicate that presynaptic (neuronal) and postsynaptic (muscular) nicotinic receptors of motor endplates differ in their pharmacological profile (e.g. Gibb and Marshall, 1986; Vizi et al., 1987; Paul et al., 2002), Ca^{2+} permeability and desensitization properties (Vernino et al., 1994). However, these findings are not clearly integrated with molecular definitions of the receptor subtypes that participate in pre- and postjunctional actions underlying changes in neuromuscular transmission. The antagonist rank potency order to reduce the release of [^{3}H]acetylcholine evoked after tetanic preconditioning was α-conotoxin MII>*d*-tubocurarine ~ dihydro-β-erythroidine. In the present study, neither α-bungarotoxin nor methyllycaconitine, which selectively block α7-containing neuronal nicotinic receptors, reduced the evoked [^{3}H]acetylcholine release. The lack of effect of α-bungarotoxin could not be attributed to its slow association/dissociation kinetics, because it had no effect on evoked transmitter release even when applied during long incubation periods (45 min) at concentrations (300 nM) above that required to produce neuromuscular paralysis. Thus, the involvement of neuronal α-bungarotoxin-sensitive nicotinic receptors, namely α7*, α8* and α9*, could also be excluded by the present findings. Equipotency between dihydro-β-erythroidine and *d*-tubocurarine to inhibit the evoked [^{3}H]acetylcholine release, makes the involvement of α4-containing nicotinic receptors highly improbable (see e.g. Alexander and Peters, 2000). Furthermore, dihydro-β-erythroidine 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 the evoked [^{3}H]acetylcholine release was highly sensitive to α-conotoxin MII (100 nM) (Cartier et al., 1996). Taken together, our data indicate that nicotinic receptors at the rat motor nerve terminals have a pharmacological profile compatible with the expression of α3β2-subunits. Results of immunohistochemical studies with mouse diaphragm support our view that α3-containing nicotinic receptors may exist at the presynaptic level (Tsuneki et al., 1995). The involvement of α-conotoxin MII-sensitive neuronal α6-heteroceptors cannot be excluded on the basis of the present data although, in the chick retina, these receptors are blocked by nanomolar concentrations of methyllycaconitine (Vailati et al., 1999). The antagonist profile found in the release experiments clearly differs from that concerning depression of post-tetanic twitch tension (α-bungarotoxin>*d*-tubocurarine≫dihydro-β-erythroidine>α-conotoxin MII). Although some of these agents may act at both pre- and postsynaptic sites, this rank potency order is consistent with the blockade of typical muscular α1-containing nicotinic receptors (Schuetze and Role, 1987).

We have now provided evidence showing that tetanic preconditioning not only reversed the normal actions of adenosine inhibitory A_1 and facilitatory A_{2A} receptors, but also reduced nicotinic autofacilitation. Crosstalk between nicotinic autoreceptors and receptors for other neuromodulators, namely adenosine, has been hypothesized (Prior et al., 1997). Using exogenously applied agonists, we had demonstrated that activation of adenosine A_{2A} receptors favoured desensitisation of presynaptic facilitatory nicotinic receptors induced by 1,1-dimethyl-4-phenylpiperazinium (Correia-de-Sá and Ribeiro, 1994). Therefore, we now aimed at investigating whether activation of A_{2A} receptors by endogenously generated adenosine could influence post-tetanic nicotinic facilitation of acetylcholine release from motor nerve terminals. Indeed, blockade of adenosine A_{2A} receptors (with adenosine deaminase or DMPX) completely restored the post-tetanic release facilitation mediated by α3β2-nicotinic receptors. It is worth noting that antagonist inhibitory actions caused by α-conotoxin MII, dihydro-β-erythroidine and *d*-tubocurarine recovered although manipulation of A_{2A} adenosine tonus increased acetylcholine levels. These results further indicate that autodesensitisation of facilitatory nicotinic acetylcholine receptors only partially explains the loss of release inhibition observed with nicotinic antagonists. Moreover, the post-tetanic twitch depression caused by neuronal nicotinic antagonists (like α-conotoxin MII, dihydro-β-erythroidine and *d*-tubocurarine) was magnified when prejunctional adenosine A_{2A} receptors were blocked with DMPX. In contrast, DMPX largely attenuated the neuromuscular relaxation produced by α-bungarotoxin. As α-bungarotoxin selectively blocks muscular α1-subunit containing nicotinic acetylcholine receptors, and, consequently, has no effect on evoked [^{3}H] acetylcholine release, it is not surprising that compounds

capable of increasing transmitter output, such as DMPX, may overcome postjunctional antagonism. While DMPX is not considered a selective adenosine A_{2A} receptor antagonist, the lack of interactions between DPCPX (a selective antagonist of adenosine A_1 receptors) and nicotinic receptor blockers, rules out the possibility of crosstalk with adenosine A_1 receptors as anticipated by others (Prior et al., 1997). Using the mouse neuromuscular junction, these authors showed that, unlike that with hexamethonium, the tetanic fade induced by vecuronium was attenuated by manipulating the A_1-adenosine tonus using either DPCPX (100 nM) or the adenosine deaminase inhibitor, erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA, 100 μM). To our knowledge, both treatments (DPCPX and EHNA) shift the A_1/A_{2A} receptor balance towards the activation of adenosine A_{2A} receptors (Correia-de-Sá and Ribeiro, 1996). It thus appears that the activation of presynaptic A_{2A} receptors increased by adenosine generated during high-frequency trains becomes physiologically relevant to prevent nicotinic transmitter flooding. High extracellular adenosine levels can be also detected during hypoxia, a situation where post-tetanic twitch potentiation at the mouse phrenic nerve–hemidiaphragm is also reduced (Nishimura et al., 1987). Therefore, the controversies in the literature regarding interpretation of effects of non-depolarizing muscle relaxants acting at either pre- and/or postjunctional levels should be re-evaluated, taking into account the possibility that tonic activation of adenosine A_{2A} receptors may control the nicotinic facilitation triggered by repetitive nerve firing.

Presynaptic ionotropic receptor-mediated pathways complement G protein transduction pathways to modulate the activity of presynaptic terminals (although on different time scales). The results presented here strongly suggest that adenosine A_{2A} receptors modulate the efficacy of facilitatory nicotinic autoreceptors in motor nerve terminals. Agonist-induced nicotinic receptor desensitisation is linked to changes in biophysical properties of the receptor (Colquhoun et al., 1989). However, a slower, but long-lasting, mechanism of desensitisation due to covalent allosteric modifications of the receptor protein has also been demonstrated in different tissues (for a review, see Léna and Changeux, 1993). Several kinases, namely protein kinase A, which can be stimulated by adenosine A_{2A} receptor activation (Correia-de-Sá and Ribeiro, 1994), may accelerate the rate of nicotinic acetylcholine receptor desensitisation without affecting ligand sensitivity or the kinetics of receptor ion channel (for a review, see Voitenko et al., 2000; but see e.g. Nishizaki and Sumikawa, 1998). Although there is some controversy about the role of phosphorylation in regulating agonist-induced nicotinic acetylcholine receptor desensitisation, previous observations suggest that downregulation of neuronal α3β2 nicotinic receptors by endogenous adenosine results from the activation of the adenylate cyclase/kinase A transducing system by adenosine A_{2A} receptors (Correia-de-Sá and Ribeiro, 1994). Further studies are needed to investigate whether modulation of P- and L-type Ca^{2+} currents and/or mobilization of intracellular Ca^{2+} by adenosine A_{2A} receptors in motor nerve terminals (Correia-de-Sá et al., 2000) might additionally regulate post-tetanic nicotinic facilitation of acetylcholine release.

In summary, neuromuscular transmission is under the tight control of synaptic adenosine, generated during repetitive neuronal firing, which may activate co-localized adenosine A_1 inhibitory and/or A_{2A} facilitatory receptors in motor nerve terminals (Correia-de-Sá et al., 1991). After tetanic preconditioning, time-dependent synaptic formation of endogenous adenosine from released nucleotides shifts tonic A_1/A_{2A} receptor balance towards the activation of adenosine A_{2A} receptors. Modifications of the modulatory pattern of neuromuscular transmission after tetanic preconditioning may result from the decline of transient α3β2-nicotinic autofacilitation, due to a rapid autodesensitisation process favoured by synchronous activation of adenosine A_{2A} receptors.

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