Tetanic depression is overcome by tonic adenosine A_{2A} receptor facilitation of L-type Ca^{2+} influx into rat motor nerve terminals

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Motor nerve terminals possess multiple voltage-sensitive calcium channels operating acetylcholine (ACh) release. In this study, we investigated whether facilitation of neuromuscular transmission by adenosine generated during neuronal firing was operated by Ca^{2+} influx via 'prevalent' P-type or via the recruitment of 'silent' L-type channels. The release of [^{3}H]ACh from rat phrenic nerve endings decreased upon increasing the stimulation frequency of the trains (750 pulses) from 5 Hz ($83 \pm 4 \times 10^3$ disintegrations per minute per gram (d.p.m. g^{-1}); $n = 11$) to 50 Hz ($30 \pm 3 \times 10^3$ d.p.m. g^{-1}; $n = 5$). The P-type Ca^{2+} channel blocker, ω-agatoxin IVA (100 nM) reduced (by $40 \pm 10\%$; $n = 6$) the release of [^{3}H]ACh evoked by 50-Hz trains, while nifedipine (1 μM, an L-type blocker) was inactive. Tetanic depression was overcome ($88 \pm 6 \times 10^3$ d.p.m. g^{-1}; $n = 12$) by stimulating the phrenic nerve with 50-Hz bursts (five bursts of 150 pulses, 20 s interburst interval). In these conditions, ω-agatoxin IVA (100 nM) failed to affect transmitter release, but nifedipine (1 μM) decreased [^{3}H]ACh release by $21 \pm 7\%$ ($n = 4$). Inactivation of endogenous adenosine with adenosine deaminase (ADA, 0.5 U ml^{-1}) reduced (by $54 \pm 8\%$, $n = 5$) the release of [^{3}H]ACh evoked with 50-Hz bursts. This effect was opposite to the excitatory actions of adenosine (0.5 mM), *S*-(*p*-nitrobenzyl)-6-thioinosine (5 μM, an adenosine uptake blocker) and CGS 21680C (3 nM, a selective A_{2A} receptor agonist); as the A_1 receptor agonist *R*-N^6-phenylisopropyl adenosine (R-PIA, 300 nM) failed to affect the release of [^{3}H]ACh, the results indicate that adenosine generated during 50-Hz bursts exerts an A_{2A}-receptor-mediated tonus. The effects of ADA (0.5 U ml^{-1}) and CGS 21680C (3 nM) were prevented by nifedipine (1 μM). Blocking tonic A_{2A} receptor activation, with ADA (0.5 U ml^{-1}) or 3,7-dimethyl-1-propargyl xanthine (10 μM, an A_{2A} antagonist), recovered ω-agatoxin IVA (100 nM) inhibition and caused the loss of function of nifedipine (1 μM). Data indicate that, in addition to the predominant P-type Ca^{2+} current triggering ACh release during brief tetanic trains, motoneurones possess L-type channels that may be recruited to facilitate transmitter release during high-frequency bursts. The fine-tuning control of Ca^{2+} influx through P- or L-type channels is likely to be mediated by endogenous adenosine. Therefore, tonic activation of presynaptic A_{2A} receptors operating Ca^{2+} influx via L-type channels may contribute to overcome tetanic depression during neuronal firing.

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Multiple subtypes of voltage-sensitive calcium channels (VSCC) are colocalized in nerve terminals, where they may act synergistically to cause transmitter release (Takahashi & Momiyama, 1993; Wheeler *et al.* 1994). It is known that brief depolarizations trigger Ca^{2+} currents that seem to be different from the 'facilitatory' currents, which are normally quiescent but can be activated by repetitive depolarizations (cf. Artalejo *et al.* 1992). At mature mammalian motor endplates, activation of synaptic vesicle exocytosis is primarily accomplished by P-type Ca^{2+} channels (e.g. Atchison, 1989; Protti & Uchitel, 1993; Wessler *et al.* 1995; Correia-de-Sá *et al.* 2000*a*), which are specifically targeted into presynaptic nerve terminals (Stanley, 1997). In contrast, L-type Ca^{2+} channels,

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presumably located away from the active zones, do not ordinarily participate in the release process but they might be involved in the integration of synaptic activity (Tsien *et al.* 1988; Robitaille *et al.* 1990). Recently, we showed that long-depolarizing stimuli unmask Ca^{2+} influx through high-capacity/slow-inactivating L-type channels contributing to the facilitation of acetylcholine (ACh) release from motor nerve terminals (Correia-de-Sá *et al.* 2000*a*; see also Urbano & Uchitel, 1999). The pathophysiological significance of L-type Ca^{2+} currents has been difficult to demonstrate. They may play a role in re-establishing neuromuscular transmission (1) during development and re-innervation of motor endplates, when P-type channels are functionally immature (Katz *et al.* 1996; Sugiura & Ko, 1997), (2) during functional recovery from botulinum toxin type-A poisoning (Santafé *et al.* 2000), and (3) in Lambert–Eaton myasthenic syndrome (LEMS), where spared L-type currents can be activated by repetitive nerve stimulation to compensate for down-regulation of P-type channels (Garcia & Beam, 1996).

Understanding the fine-tuning control of Ca^{2+} influx through neuronal P- or L-type channels at various stimulation patterns, as well as the sensitivity of different VSCCs to endogenous modulators, may be of clinical relevance to increase the safety margin of neuromuscular transmission. Adenosine build-up from the catabolism of released ATP is one of such mediators (Cunha *et al.* 1996; Magalhães-Cardoso *et al.* 2003) playing a key role in adjusting the modulatory pattern of neuromuscular transmission to the stimulation conditions (Correia-de-Sá *et al.* 1996). While adenosine acts predominantly as an inhibitory signal (via A_1 receptors) under resting conditions, amplification of neuromuscular transmission due to A_{2A} receptor activation becomes evident at high levels of synaptic adenosine, such as generated during high-frequency long-lasting stimuli. At most neuro-effector junctions, adenosine A_{2A} receptors act via subtle modifications of the presynaptic inter-receptor dynamics (for review see Sebastião & Ribeiro, 2000) involving the generation of intracellular second messengers, such as cyclic AMP (Correia-de-Sá & Ribeiro, 1994) and Ca^{2+} (Correia-de-Sá *et al.* 2000*b*). The question then arises, of whether endogenous adenosine generated during neuronal firing might influence Ca^{2+} dynamics to adapt transmitter exocytosis to neuronal firing at the rat endplate.

Methods

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 gassed (95% O_2–5% CO_2) Tyrode solution (pH 7.4) containing (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.

The procedures used for labelling the preparations and measuring evoked [^{3}H]ACh release were as previously described (Correia-de-Sá *et al.* 1991), with minor modifications. Phrenic nerve–hemidiaphragm preparations were mounted in 3-ml capacity Perspex chambers heated to 37°C. Nerve terminals were labelled for 40 min with 1 μM [^{3}H]choline (specific activity 2.5 μCi nmol^{-1}) under electrical stimulation at a frequency of 1 Hz (0.04 ms duration, 8 mA). The phrenic nerve was stimulated with a glass–platinum suction electrode placed near the first division branch of the nerve trunk to avoid direct contact with muscle fibres. Washout of the preparations was performed for 60 min, by superfusion (15 ml min^{-1}) with Tyrode solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was evaluated by liquid scintillation spectrometry (percentage counting efficiency, 40 ± 2%) after appropriate background subtraction using 2-ml bath samples collected automatically every 3 min. After the loading and washout periods, the preparation contained $5542 \pm 248 \times 10^3$ disintegrations per minute per gram wet weight of tissue (d.p.m. g^{-1}) and the resting release was $132 \pm 12 \times 10^3$ d.p.m. g^{-1} ($n = 8$). The fractional release was calculated to be 2.38 ± 0.14% of the radioactivity present in the tissue at the first collected sample (see e.g. Fig. 1).

[^{3}H]ACh release was evoked by two periods of electrical stimulation of the phrenic nerve, starting at the 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). Supramaximal intensity pulses were delivered at 5 Hz or 50 Hz. Either one train (750 pulses) or a series of five bursts of 150 pulses applied with a 20-s interburst interval were used when the stimulation frequency was 50 Hz (tetanus). Electrical stimulation increased only the release of [^{3}H]ACh in a Ca^{2+}- and tetrodotoxin-sensitive manner (Correia-de-Sá *et al.* 2000*a*), while the output of [^{3}H]choline remained unchanged (Wessler & Kilbinger, 1986). Therefore, evoked [^{3}H]ACh 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).

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Drug effects and pharmacological interactions

Test drugs were added 15 min before S_2 and were present up to the end of the experiments (see e.g. Fig. 1). The percentage change in the ratio between the evoked [^{3}H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. Positive and negative values represent facilitation and inhibition of evoked [^{3}H]ACh release, respectively. When we evaluated changes in the effect of tested drugs induced by a modifier, the modifier was applied 15 min before starting sample collection and hence was present during S_1 and S_2. When the same drug was present in S_1 and S_2, the S_2/S_1 ratios were not significantly ($P > 0.05$) different from controls, that is, without addition of drugs; the S_2/S_1 values were 0.79 ± 0.03 ($n = 11$), 0.81 ± 0.05 ($n = 5$) and 0.88 ± 0.03 ($n = 12$) for 5-Hz trains, 50-Hz trains and 50-Hz bursts, respectively (Fig. 1). None of the drugs changed basal tritium outflow significantly ($P > 0.05$).

Materials and solutions

Adenosine, adenosine deaminase (ADA, type VI, 1803 U ml^{-1}, EC 3.5.4.4), adenosine 5′-monophosphate (AMP), $CdCl_2$, choline chloride, ω-conotoxin GVIA (ω-CgTx GVIA), ω-conotoxin MVIIC (ω-CmTx MVIIC), ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), hemicholinium-3, nifedipine, *R*-*N*^{6}-phenylisopropyl adenosine (R-PIA) and *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI) were from Sigma, USA. ω-Agatoxin IVA (ω-AgaTx IVA) was from Peptide Institute Inc., Japan. 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamido adenosine (CGS 21680C) and 3,7-dimethyl-1-propargylxanthine (DMPX) were from Research Biochemicals Inc., USA. [Methyl-^{3}H]-choline chloride (ethanol solution, 80 Ci mmol^{-1}) was from Amersham, UK. All reagents were of the highest purity available. Nifedipine was made up in a 10 mM stock solution in ethanol, and was kept protected from the light to prevent photodecomposition. NBTI and R-PIA were made up as 10 mM and 50 mM stock solutions in dimethylsulphoxide, respectively. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were performed. The pH of the superfusion solution did not change following addition of the drugs at the maximum concentrations applied to the preparations.

Statistics

The data are expressed as mean ± S.E.M. from *n* experiments. Statistical analysis of data was carried out using paired or unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's

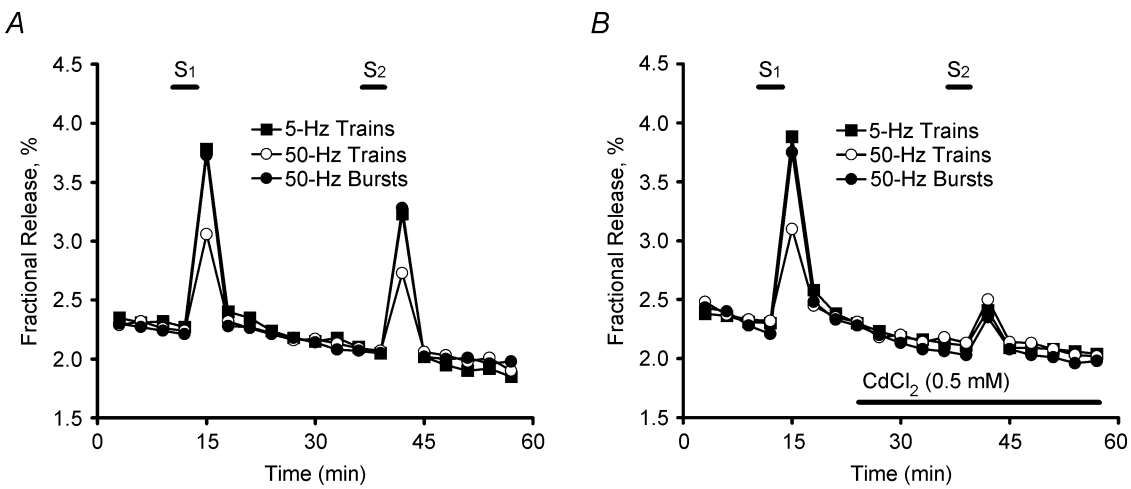

Figure 1. Time course of tritium outflow from electrically stimulated phrenic nerve terminals
Time course of tritium outflow in control conditions (*A*) and in the presence of 0.5 mM $CdCl_2$ (*B*). Tritium outflow (ordinate) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period. The abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited by stimulating the phrenic nerve trunk with 750 electrical pulses delivered with frequencies of 5 Hz (■) and 50 Hz; either one train (15 s) (○) or a series of five bursts (3 s, 150 pulses, 20-s interburst interval) (●) were delivered when 50-Hz stimulation was applied. Each period of stimulation was applied twice, starting at the 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). In (*B*), $CdCl_2$ (0.5 mM) was added to the incubation media 15 min before S_2 (horizontal bar). Note that spontaneous tritium outflow was not changed in the presence of $CdCl_2$.

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modified *t* test. Values of $P < 0.05$ were considered to represent significant differences.

Results

Influence of stimulation conditions on [^{3}H]ACh release from motor nerve endings: differential role of P- and L-type VSCCs

Figure 1 shows the time course of tritium outflow from phrenic nerve–hemidiaphragm preparations stimulated electrically. Stimulation of the phrenic nerve with 5-Hz trains (750 pulses) increased tritium outflow by an average of $83 \pm 4 \times 10^3$ d.p.m. g^{-1} ($n = 11$) above the basal tritium outflow. The average evoked [^{3}H]ACh release decreased to $30 \pm 3 \times 10^3$ d.p.m. g^{-1} ($n = 10$) upon increasing the frequency of the stimulation train to 50 Hz, keeping the number of pulses constant (Fig. 1*A*, tetanic depression). The amount of [^{3}H]ACh release recovered to $88 \pm 6 \times 10^3$ d.p.m. g^{-1} ($n = 11$) when the phrenic nerve was stimulated with 50-Hz frequency bursts (5×150 pulses, 20 s interburst interval). In all these conditions, [^{3}H]ACh release was largely attenuated by $CdCl_2$ (500 μM) indicating that transmitter exocytosis is dependent on the Ca^{2+} influx through voltage-sensitive channels (VSCCs) (Fig. 1*B*).

In order to investigate whether the influx of Ca^{2+} through different VSCCs could alternatively contribute to ACh exocytosis from electrically stimulated motor nerve terminals, we studied the effects of several type-specific blockers of VSCCs. The drugs used were: ω-agatoxin IVA (ω-AgaTx IVA), which blocks P-type channels in the nanomolar concentration range (Zhang *et al.* 1993); ω-conotoxin GVIA (ω-CgTx GVIA), which selectively blocks the N-type channels (Olivera *et al.* 1994; Dunlap *et al.* 1995); ω-conotoxin MVIIC (ω-CmTx MVIIC), a blocker of P-, Q- and N-type channels (Wheeler *et al.* 1994); and nifedipine, a blocker of L-type Ca^{2+} channels (Olivera *et al.* 1994; Dunlap *et al.* 1995). Due to the slow binding kinetics of the peptides, we performed experiments where the pre-incubation time was prolonged from 15 to 45 min and the results were not statistically different ($P > 0.05$) (see e.g. Correia-de-Sá *et al.* 2000*b*). Evoked [^{3}H]ACh release was unaffected by ω-CgTx GVIA (1 μM) or ω-CmTx MVIIC (150 nM) (data not shown), thus indicating that neither N- nor Q-type VSCCs are involved in the release process. ω-AgaTx IVA, applied at a concentration (100 nM) that selectively blocked P-type VSCCs (Dunlap *et al.* 1995), inhibited evoked [^{3}H]ACh release by $21 \pm 4\%$ ($n = 4$) and $40 \pm 10\%$ ($n = 6$) when the phrenic nerve was stimulated with 5- and 50-Hz trains, respectively (Fig. 2*A* and *C*). However, ω-AgaTx IVA (100 nM) failed to affect evoked [^{3}H]ACh release during 50-Hz bursts (Fig. 2*B* and *C*). In contrast, nifedipine (1 μM) inhibited ($21 \pm 7\%$, $n = 4$) the release of [^{3}H]ACh only when the phrenic nerve was stimulated with 50-Hz bursts, having no significant effect when 5- or 50-Hz trains were delivered (Fig. 2). These experiments demonstrate that ACh exocytosis from motor nerve terminals results mainly from the influx of Ca^{2+} through P-type channels during low frequency stimulation or high frequency trains, but L-type currents can be alternatively recruited to facilitate transmitter release during high frequency bursts. A putative role of 'resistant' R-type channels mediating transmitter release from motor nerve terminals in the presence of VSCC blockers cannot be excluded (see e.g. Lang *et al.* 2003).

Endogenous adenosine activating A_{2A} receptors shifts neuronal Ca^{2+} influx from constitutive P-type channels to facilitatory L-type channels during high frequency bursts

Previously, we showed that tonic A_1/A_{2A} receptor activation balance depends on the concentration of adenosine at the rat neuromuscular junction, which seems to be tightly regulated by the nerve stimulation conditions (Correia-de-Sá *et al.* 1996). Figure 2*C* shows that removal of endogenous adenosine tonus using adenosine deaminase (ADA, 0.5 U ml^{-1}), disinhibited (facilitated, $19 \pm 3\%$, $n = 5$) the release of [^{3}H]ACh induced by 5-Hz trains, but it significantly ($P < 0.05$) decreased ($54 \pm 8\%$, $n = 5$) transmitter release during 50-Hz bursts. The adenosine uptake blocker, *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI, 5 μM) (Paterson *et al.* 1977), which promotes extracellular adenosine accumulation, increased evoked [^{3}H]ACh release by $24 \pm 8\%$ ($n = 5$) when the bursting protocol was used (Fig. 3*B*). This contrasts with (1) the failure of NBTI (5 μM) to change [^{3}H]ACh release when only one high-frequency (50-Hz) train was delivered (Fig. 3*A*) and with (2) the situation where NBTI (5 μM) inhibited (by $18 \pm 3\%$, $n = 5$) the release of [^{3}H]ACh induced by 5-Hz trains (Correia-de-Sá & Ribeiro, 1996). These results are in keeping with the predominance of a facilitatory A_{2A}-receptor-mediated tonus during high frequency bursts (see Correia-de-Sá *et al.* 1996). Because high amounts of ATP are released by high frequency (50-Hz) trains (Magalhães-Cardoso *et al.* 2003), adenosine formation is blocked during the time needed for ATP and ADP levels to fall below the threshold for feed-forward inhibition of ecto-5′ nucleotidase (Cunha *et al.* 1996; Magalhães-Cardoso *et al.* 2003). This might explain the absence of effects of ADA (0.5 U ml^{-1}) and NBTI (5 μM) when [^{3}H]ACh release was induced by brief 50-Hz trains (Fig. 3*A*). To investigate this hypothesis further, we performed experiments in which the adenosine precursor that is inactive on ecto-5′-nucleotidase activity, AMP (100 μM), was tested in both high-frequency stimulation protocols. When only one 50-Hz train was applied,

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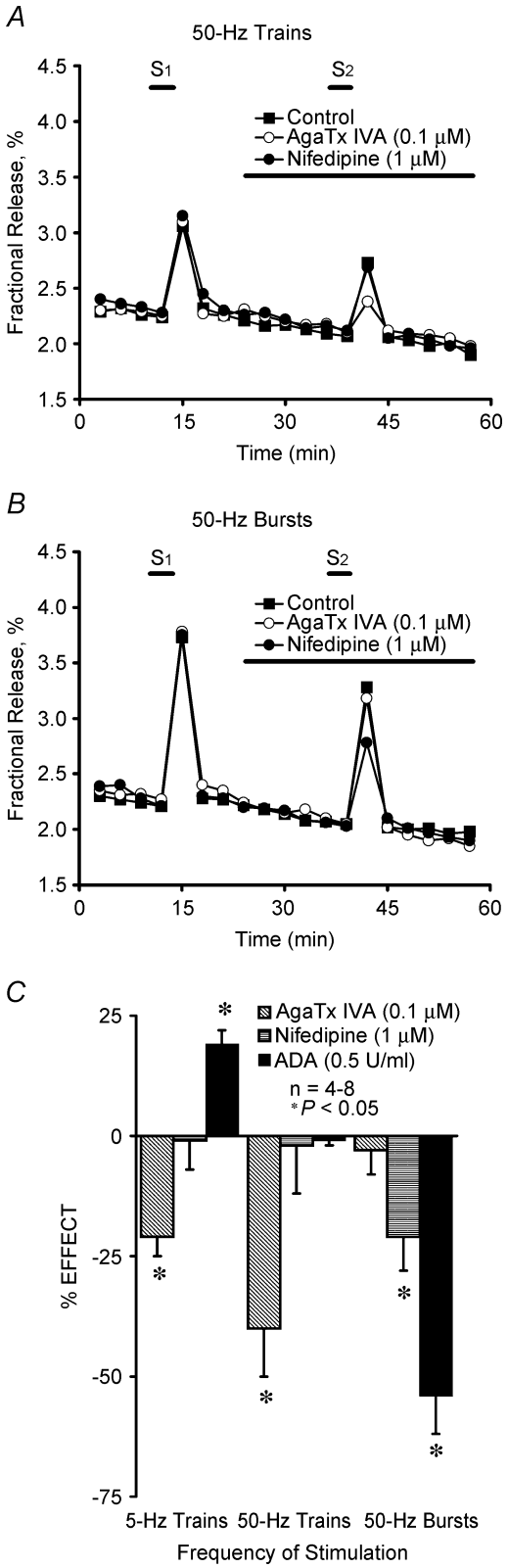

Figure 2. Influence of the stimulation parameters on the effects of P- and L-type Ca^{2+} channel blockers and endogenous adenosine on evoked [^{3}H]ACh release from motor nerve terminals

A and *B*, show the time course of tritium outflow from electrically stimulated phrenic nerve terminals taken from typical experiments in the absence (Control, ■) and in the presence of ω-Agatoxin IVA (ω-AgaTx IVA, 0.1 μM, ○) and nifedipine (1 μM, ●). Tritium outflow (ordinate) 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 samples were collected. [^{3}H]ACh release was elicited twice (S_1 and S_2) by stimulating the phrenic nerve trunk with 750 electrical pulses delivered with frequencies of 5 Hz and 50 Hz; either one train (15 s) (*A*) or a series of five bursts (3 s, 150 pulses, 20-s interburst interval) (*B*) were delivered when 50-Hz stimulation was applied. ω-AgaTx IVA (0.1 μM), nifedipine (1 μM) and adenosine deaminase (ADA, 0.5 U ml^{-1}) were applied 15 min before the end of S_2, as represented by the horizontal bars. Note that spontaneous tritium outflow was not significantly modified in the presence of the drugs. *C*, the ordinates are percentage change in S_2/S_1 ratio in the presence of test drugs as compared with the S_2/S_1 ratio in control experiments using a similar stimulation protocol. Zero percent represents identity between the two ratios. Each column represents pooled data from $n = 4$–8 individual experiments. The vertical bars represent S.E.M. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) when compared with controls obtained in the absence of test drugs.

AMP (100 μM) facilitated [^{3}H]ACh release by $26 \pm 1\%$ ($n = 5$) (Fig. 3*A*). The facilitatory effect of AMP (100 μM) was significantly ($P < 0.05$) enhanced ($44 \pm 6\%$, $n = 5$) when the release of [^{3}H]ACh was induced by five 50-Hz bursts (Fig. 3*B*). The exogenously applied adenosine (100–500 μM) significantly ($P < 0.05$) increased the release of [^{3}H]ACh in a concentration-dependent manner, independent of the protocol used to stimulate the phrenic nerve; for instance, adenosine (500 μM) facilitated [^{3}H]ACh release, respectively, by $44 \pm 3\%$ ($n = 4$) and by $49 \pm 16\%$ ($n = 5$) when nerve stimulation was delivered as 50-Hz trains and 50-Hz bursts. Likewise, the selective adenosine A_{2A} receptor agonist, CGS 21680C (3 nM), consistently facilitated (~60%) the release of [^{3}H]ACh induced by high-frequency stimuli (Fig. 3*C* and *D*, see also Correia-de-Sá *et al.* 1996). It is worth noting that *R*-N^6-phenylisopropyl adenosine (R-PIA, 300 nM), an adenosine A_1 receptor agonist, failed to modify transmitter release (Fig. 3*C* and *D*), indicating that during high-frequency nerve stimulation inhibition of [^{3}H]ACh release operated by adenosine A_1 receptors becomes less effective, as previously shown for other inhibitory presynaptic receptors (Duckles & Budai, 1990). Consistent with this hypothesis is the finding that the enzymatically stable adenosine analogue, R-PIA (300 nM), was still unable to modify [^{3}H]ACh release evoked by high-frequency stimuli in the presence of ADA (0.5 U ml^{-1}) (data not shown). Using 50-Hz bursts, a situation where ACh exocytosis depends mainly on Ca^{2+} influx through L-type channels (see above), both $CdCl_2$ (500 μM) and nifedipine (1 μM) significantly ($P < 0.05$) reduced CGS 21680C-induced (3 nM) excitation ($64 \pm 14\%$, $n = 4$) (Fig. 4). These results suggest that facilitation of [^{3}H]ACh release by A_{2A} receptor activation depends on neuronal L-type Ca^{2+} influx. Likewise, blockade of L-type channels with $CdCl_2$

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(500 μM) or nifedipine (1 μM) abolished the inhibitory effect of ADA (0.5 U ml^{-1}) (Fig. 4), further indicating that Ca^{2+} influx through L-type VSCCs is required to facilitate transmitter release by endogenous adenosine.

In view of the putative interactions between synaptic adenosine generation and Ca^{2+} influx through distinct VSCCs, we tested whether Ca^{2+} recruitment through P- or L-type channels could be modified during neuronal firing upon blocking tonic activation of A_{2A} receptors with either ADA or 3,7-dipropyl-1-propargylxanthine (DMPX, an adenosine A_{2A} receptor antagonist). As shown in Fig. 5, both ADA (0.5 U ml^{-1}) and DMPX (10 μM) switched the pharmacology of Ca^{2+} channels coupled to [^{3}H]ACh release during 50-Hz bursts; i.e. the action of nifedipine (1 μM) was completely abolished, while ω-AgaTx IVA (100 nM)-induced inhibition recovered to levels observed in conditions where adenosine had no tonic effect (e.g. 50-Hz trains). Therefore, the fine-tuning Ca^{2+} influx through either P- or L-type VSCCs can be modulated by synaptic accumulation of endogenous adenosine.

Discussion

The present results support the hypothesis that VSCCs other than prevalent P-type become involved in ACh exocytosis during high frequency bursts; in particular,

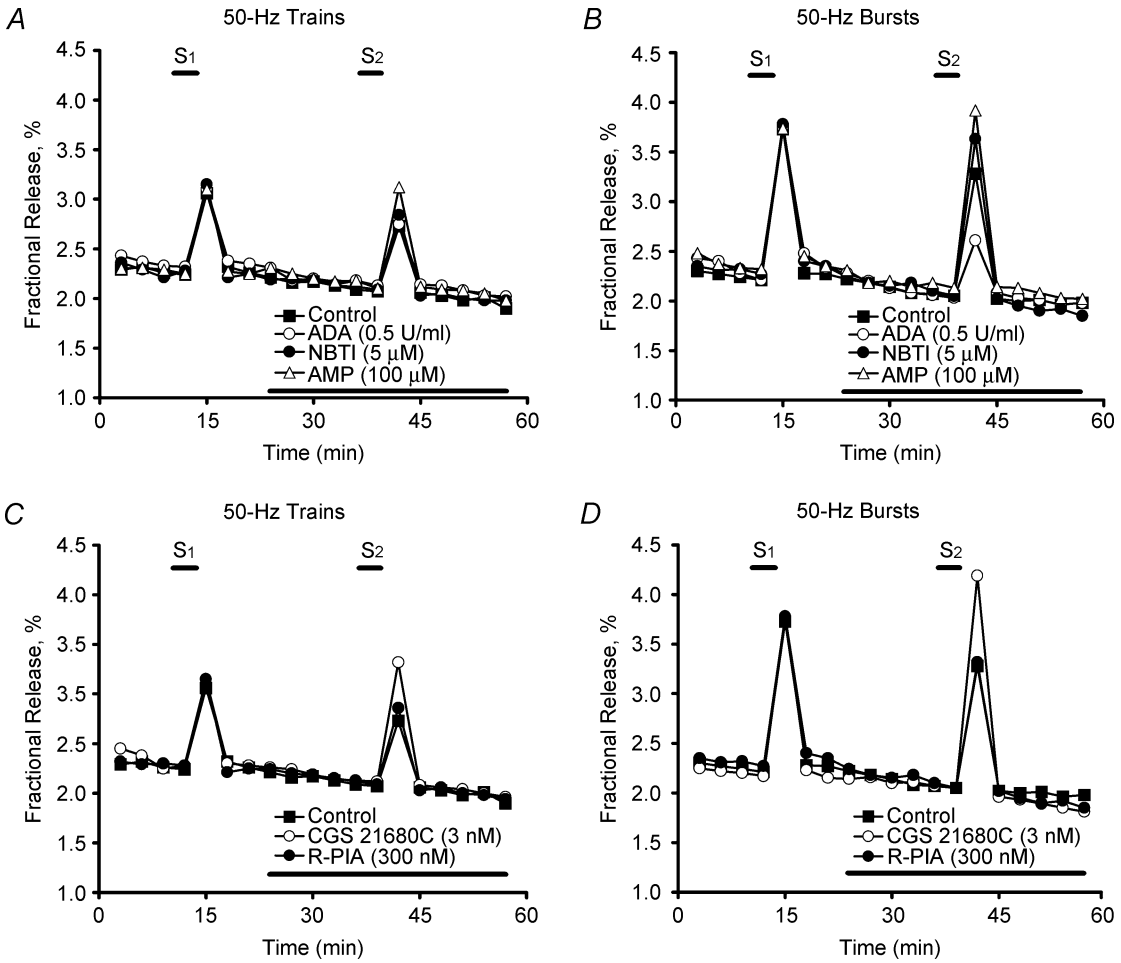

Figure 3. Comparison of adenosine modulation of [^{3}H]ACh release induced by high-frequency (50 Hz) nerve stimulation delivered as continuous trains or intermittent bursts

The time course of tritium outflow is shown from electrically stimulated phrenic nerve terminals taken from typical experiments. Tritium outflow (ordinate) 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 samples were collected. [^{3}H]ACh release was elicited twice (S_1 and S_2) by stimulating the phrenic nerve trunk with 750 electrical pulses delivered at a frequency of 50 Hz; either one train (15 s) (*A* and *C*) or a series of five bursts (3 s, 150 pulses, 20-s interburst interval) (*B* and *D*) were applied. Adenosine deaminase (ADA, 0.5 U ml^{-1}), *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI, 5 μM), adenosine 5′-monophosphate (AMP, 100 μM), CGS 21680C (3 nM), and *R*-*N*^{6}-phenylisopropyl adenosine (R-PIA, 300 nM) were added to the incubation media 15 min before S_2 (horizontal bar). Note that spontaneous tritium outflow was not changed in the presence of the drugs.

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our results suggest that motor nerve terminals possess L-type Ca^{2+} channels that may be recruited to facilitate transmitter release under this condition. The fine-tuning control of Ca^{2+} influx through either P- or L-type channels is likely to be mediated by endogenous adenosine activating presynaptic A_{2A} receptors. Data also indicate that tetanic depression of ACh release may be overcome during intermittent neuronal firing due to tonic activation of A_{2A} receptors operating additional Ca^{2+} recruitment via L-type channels (see Fig. 6).

Neurotransmission failure may result from three processes: failure of action potential conduction along the axon; diminished release of ACh from the presynaptic terminal; and reduced excitability of the endplate due to ACh receptor desensitization. Neuromuscular junction endplate potentials decrease greatly and quickly during continuous 5- to 100-Hz stimulation without a significant change in the size of miniature endplate potentials (MEPPs) (Moyer & Van Lunteren, 1999). Our results measuring [^{3}H]ACh release fully agree with the interpretation that rundown of endplate potentials during high frequency nerve stimulation is due to rapid changes in the magnitude of neurotransmitter release rather than to axonal block or postsynaptic receptor desensitization. Previous estimates of the susceptibility of neurotransmission failure are based almost entirely on protocols in which motor nerves are stimulated continuously without interruption (e.g. Wilson, 1979; Hong & Chang, 1991; Fournier *et al.* 1991; Bazzy, 1994). For some muscles, especially those used for

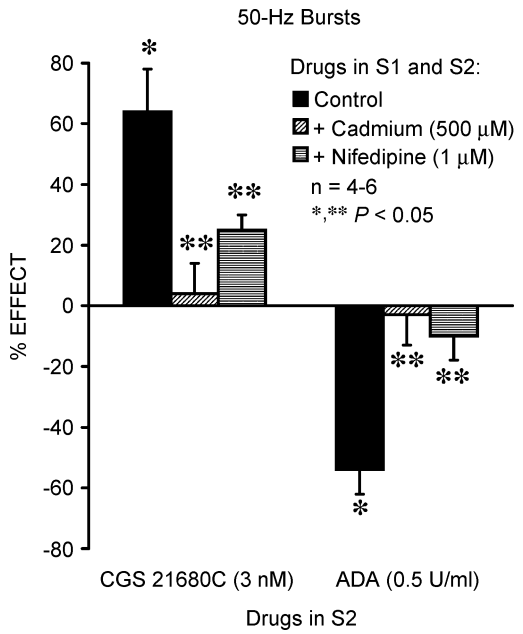

Figure 4. Tonic adenosine A_{2A}-receptor-mediated facilitation of evoked [^{3}H]ACh release from motor nerve terminals depends on Ca^{2+} influx through L-type channels
[^{3}H]ACh release was elicited twice (S_1 and S_2) by stimulating the phrenic nerve trunk with 50-Hz bursts (five bursts of 150 pulses each, delivered with a 20-s interburst interval). CGS 21680C (3 nM) and adenosine deaminase (ADA, 0.5 U ml^{-1}) were applied 15 min before the end of S_2; $CdCl_2$ (500 μM) and nifedipine (1 μM) were added to the incubation solution at 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 change in S_2/S_1 ratio in the presence of CGS 21680C (3 nM) or ADA (0.5 U ml^{-1}) as compared with the S_2/S_1 ratio in control conditions, either in the absence or in the presence of $CdCl_2$ (500 μM) or nifedipine (1 μM). Zero percent represents identity between the two ratios. Each column represents pooled data from $n = 4$–6 individual experiments. The vertical bars represent S.E.M. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represent significant differences from the control; $^{**}P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represent significant differences when compared with the effects of CGS 21680C (3 nM) or ADA (0.5 U ml^{-1}) alone.

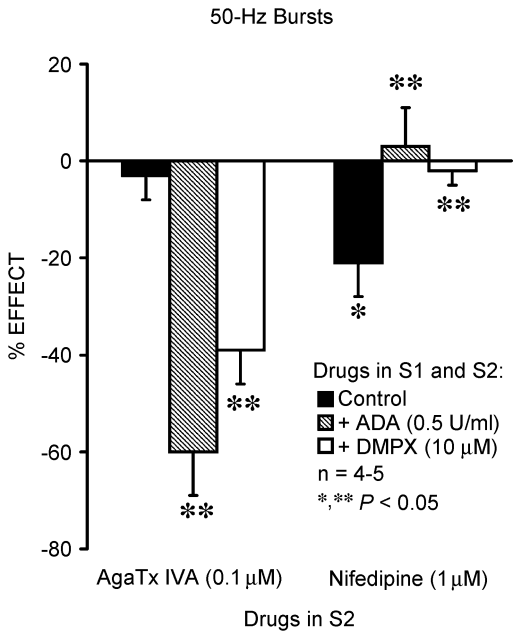

Figure 5. Activation of excitatory A_{2A} receptors by endogenous adenosine switch Ca^{2+} influx triggering [^{3}H]ACh release from prevalent P-type to silent L-type neuronal Ca^{2+} channels
[^{3}H]ACh release was elicited twice (S_1 and S_2) by stimulating the phrenic nerve trunk with 50-Hz bursts (five bursts of 150 pulses each, delivered with a 20-s interburst interval). ω-AgaTx IVA (0.1 μM) and nifedipine (1 μM) were applied 15 min before the end of S_2. Adenosine deaminase (ADA, 0.5 U ml^{-1}) and the adenosine A_2 receptor antagonist, 3,7-dipropyl-1-propargylxanthine (DMPX, 10 μM), were added to the incubation solution at 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 change in S_2/S_1 ratio in the presence of ω-AgaTx IVA (0.1 μM) and nifedipine (1 μM) as compared with the S_2/S_1 ratio in control conditions, either in the absence or in the presence of ADA (0.5 U ml^{-1}) or DMPX (10 μM). Zero percent represents identity between the two ratios. Each column represents pooled data from $n = 4$–5 individual experiments. The vertical bars represent S.E.M. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represent significant differences from the control; $^{**}P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represent significant differences when compared with the effects of ω-AgaTx IVA (0.1 μM) or nifedipine (1 μM) alone.

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postural tasks, this is a physiologically relevant activation pattern. However, many muscles (in particular thoracic muscles used for breathing) are activated intermittently, with a duty cycle as short as ∼0.25–0.35 (see Kong & Berger, 1986). This pattern of activation potentially allows decrements in neurotransmission during repetitive stimulation to recover, thereby substantially preserving synaptic transmission during intermittent compared with continuous stimulation. Recovery from tetanic depression of [^{3}H]ACh release in the course of 50-Hz bursts is in keeping with previous findings showing a complete mitigation of endplate potential drop-out during intermittent stimulation (Moyer & Van Lunteren, 1999). Although the interburst interval (20 s) used for the intermittent 50-Hz stimulation was longer than would normally occur during rhythmic activities, data suggest that recovery between trains can be sufficient to substantially improve neuromuscular junction function.

The extent to which modulation of spontaneous (asynchronous) quantal exocytosis influences neuromuscular transmission is unknown. Continuous nerve stimulation (20–50 Hz) causes slow transient increases in MEPP frequency (MEPP-hump) and intracellular free Ca^{2+} in motor nerve terminals (Narita *et al.* 1998). Such a mechanism might not contribute to facilitation of [^{3}H]ACh release during 50-Hz bursts, because short interruptions of tetanus (5–9 s) during MEPP-hump quickly reduced MEPP frequency to the basal level (Narita *et al.* 1998). Furthermore, these authors also demonstrated that resuming tetanus swiftly raised MEPP frequency to the previous or a higher level in a manner inversely dependent on pause duration (< 1 min). This situation was opposite to our findings showing that enhanced amounts of [^{3}H]ACh are released upon increasing the interburst interval (0–20 s), which may be required to generate enough endogenous adenosine capable of activating facilitatory A_{2A} receptors (Correia-de-Sá *et al.* 1996). Although it has been shown that exogenous adenosine (via A_1 receptors) may reduce spontaneous ACh release by modulating Ca^{2+} influx via L-type VSCCs in mouse motoneurones (De Lorenzo *et al.* 2004), our findings showed that adenosine A_1 receptor activation with R-PIA (300 nM) was without effect on [^{3}H]ACh release induced by high-frequency nerve stimulation. Failure of R-PIA (300 nM) to inhibit tetanus-induced [^{3}H]ACh release may not be due to occupation of A_1 receptors by endogenously generated adenosine, because (1) the K_d of R-PIA for A_1 receptors is several orders of magnitude lower (low nanomolar range) than that of adenosine (high micromolar range) (e.g. Jacobson *et al.* 1992), and

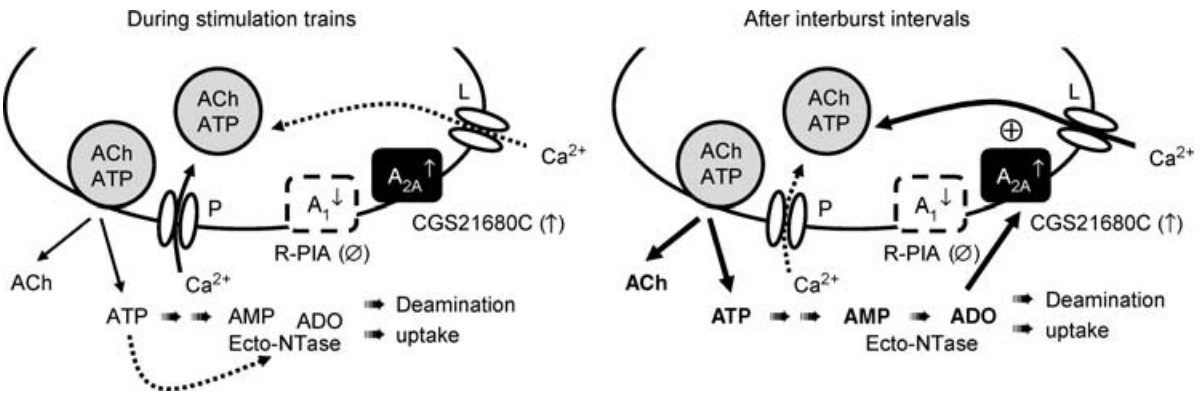

Figure 6. Fine-tuning control of Ca^{2+} recruitment through P- and L-type VSCCs by endogenous adenosine generated during motoneuronal firing

During short stimulation trains, P-type Ca^{2+} channels clustered at active zones control nerve-evoked ACh release from adult mammalian motor nerve terminals. The function of P-type channels declines rapidly as consequence of a Ca^{2+}-dependent inactivation system, which might contribute to neurotransmission tetanic failure. During intermittent high-frequency bursts, decline of transmission within a train may completely recover by the onset of the next train probably due to the recruitment of Ca^{2+} via quiescent high-capacity/slow-inactivating L-type channels located away from the active zones. The fine-tuning control of Ca^{2+} currents through P- or L-type channels is likely to be mediated by endogenous adenosine. The predominance of inhibitory A_1/excitatory A_{2A} tonus is balanced by the stimulation pattern (trains *versus* bursts), which also tightly regulates the amount of extracellular adenosine formed from released ATP. As ATP is released synchronously with ACh in a frequency-dependent manner, it can reach high enough levels during 50-Hz trains to be able to inhibit ecto-5′-nucleotidase (ecto-NTase), the rate-limiting enzyme responsible for adenosine formation from adenine nucleotides. Interburst intervals favour recovery from enzymatic inhibition, leading to a burst-like formation of adenosine and channelling to the activation of facilitatory A_{2A} receptors. In parallel, there is a co-ordinated shift in Ca^{2+} channel dynamics operating ACh exocytosis, from the prevalent P-type to the 'facilitatory' L-type channel, in a way completely reversed by blocking A_{2A} receptor activation. This mechanism represents a novel form of synaptic plasticity mediated by adenosine at the rat motor endplate and may function to overcome tetanic depression during intermittent neuronal firing. The transducing pathway mediating the crosstalk between adenosine and VSCCs at the motor endplate remains to be elucidated.

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(2) the effect of R-PIA (300 nM) was not significantly ($P > 0.05$) modified in the presence of ADA (0.5 U ml^{-1}) (see also Correia-de-Sá *et al.* 1996). Moreover, both adenosine (500 μM) and the selective A_{2A} receptor agonist, CGS 21680C (3 nM), consistently facilitated evoked [^{3}H]ACh release independently of the protocol of stimulation, without significantly changing the resting tritium outflow.

Ca^{2+} entry through presynaptic VSCCs links membrane depolarization and exocytosis of synaptic vesicles in the nerve terminal. The amount of transmitter released is steeply dependent on presynaptic Ca^{2+} concentrations (Dodge & Rahamimoff, 1967; Mintz *et al.* 1995) such that increases or decreases in Ca^{2+} influx can powerfully alter neurotransmission. Normally, P/Q-type Ca^{2+} channels control nerve-stimulated ACh release from adult mammalian motor nerve terminals (e.g. Atchison, 1989; Protti & Uchitel, 1993; Wessler *et al.* 1995; Correia-de-Sá *et al.* 2000*a*). In the present study, we showed that nifedipine had no effect on [^{3}H]ACh release induced by 5- and 50-Hz trains, but it caused a significant inhibitory action during intermittent high frequency stimulation bursts, a situation where blockade of the P-type VSCC with ω-AgaTx IVA was ineffective. Transmitter release was not modified either by the N-type channel blocker, ω-CgTx GVIA (1 μM) (Olivera *et al.* 1994; Dunlap *et al.* 1995), or by ω-CmTx MVIIC (150 nM), which blocks P-, Q- and N-type channels (Wheeler *et al.* 1994). These findings are consistent with the observation that induction of quiescent L-type Ca^{2+} currents may represent an adaptive response to a function loss of prevalent P-type Ca^{2+} channels during periods of high intensity long-lasting nerve stimulation (Correia-de-Sá *et al.* 2000*a*; see also Urbano & Uchitel, 1999). Inactivation of P-type channels may be enhanced by additional Ca^{2+} influx via nearby located VSCCs (for review see e.g. Tareilus & Breer, 1995), whereas Ca^{2+} recruitment through L-type channels requires strong depolarizations to become functionally apparent (Miller, 1987). This might happen because L-type channels are distributed away from the active zones (Tsien *et al.* 1988; Robitaille *et al.* 1990), are more resistant to inactivation in neurones, and possess a high conductance capacity (~25 pS) able to saturate intraterminal buffers which limit Ca^{2+} diffusion to the release apparatus (for review see e.g. Tareilus & Breer, 1995).

Adenosine present at the mammalian neuromuscular junction results mainly from the hydrolysis of ATP released synchronously with ACh (Smith, 1991; Magalhães-Cardoso *et al.* 2003). The activity of the ecto-nucleotidase pathway appears critical to define the pattern of formation of ATP-derived adenosine to allow the activation of either inhibitory A_1 or facilitatory A_{2A} receptors. As ATP is released from motor nerve terminals in a frequency-dependent manner (Silinsky, 1975; Cunha & Sebastião, 1993; Magalhães-Cardoso *et al.* 2003), it can reach levels high enough to inhibit ecto-5′-nucleotidase, the enzyme that forms adenosine from released adenine nucleotides (discussed by Cunha, 2001). This prediction was confirmed as the facilitatory effect of AMP (100 μM), the adenosine precursor that does not inhibit the enzyme, was almost doubled when 50-Hz bursts were used (see Fig. 3*A* and *B*), reaching a magnitude comparable to that observed with adenosine (500 μM) and CGS 21680C (3 nM) under similar stimulation conditions. During intermittent high-frequency stimulation, there is a delayed burst-like formation of adenosine leading to high synaptic concentrations of the nucleoside similar to those required to activate facilitatory A_{2A} receptors (Correia-de-Sá *et al.* 1996; Cunha *et al.* 1996). This might explain the absence of adenosine tonus when [^{3}H]ACh release was induced by brief 50-Hz trains (see also Malinowski *et al.* 1997). To bypass feedforward inhibition of ecto-5′-nucleotidase, we used high frequency (50 Hz) intermittent (five bursts, 150 pulses, 20 s interburst interval) nerve stimulations (cf. Correia-de-Sá *et al.* 1996). Under these conditions, inactivation of endogenous adenosine with ADA (0.5 U ml^{-1}) significantly reduced (~50%), whereas inhibition of adenosine uptake with NBTI (5 μM) facilitated the evoked [^{3}H]ACh release, indicating that adenosine exerts a predominant facilitatory tonus. Therefore, the predominance of A_1 *versus* A_{2A} tonus is balanced by the stimulation pattern. Thus, if the release of ATP is small, A_1 inhibition prevails, whereas when ATP release is high, adenosine formed in a burst-like manner preferentially activates A_{2A} receptors. In addition, the tonic facilitatory action emerging with increased stimulation frequency may also depend on the attenuation of A_1-mediated inhibition of evoked [^{3}H]ACh release (see above). Taken together these results indicate that during tetanic stimulation both time-dependent endogenous adenosine formation from released adenine nucleotides and attenuation of the inhibitory action mediated by A_1 receptors contribute to the strong adenosine facilitatory tonus operated by A_{2A} receptors on evoked [^{3}H]ACh release (see Fig. 6).

Additionally, our data showed a co-ordinated shift on Ca^{2+} channel subtypes operating ACh exocytosis from stimulated motor nerve terminals. The P-type channels are operative when the amount of endogenous adenosine is small and, hence, the nucleoside activates preferentially inhibitory A_1 receptors. During high frequency bursts, activation of A_{2A} receptors by high extracellular adenosine facilitates Ca^{2+} recruitment through quiescent L-type channels and a correspondent decrease in the proportion of P-type currents. Blocking the activation of A_{2A} receptors with ADA or DMPX could relieve down-regulation of P-type channel function. Moreover, facilitation of [^{3}H]ACh release operated by A_{2A} receptors seems to be highly dependent on L-type Ca^{2+} currents, as nifedipine

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largely, but not completely, eliminated the excitatory effect of CGS 21680C. This might be partially explained because activation of adenosine A_{2A} receptors may alternatively mobilize Ca^{2+} from thapsigargin-sensitive endoplasmic stores in the presence of nifedipine (Correia-de-Sá *et al.* 2000*b*). It is well established that the activity of presynaptic Ca^{2+} channels is modulated by a membrane-delimiting pathway involving G proteins, as well as by activation of cytoplasmic messenger molecules. For instance, high levels of intracellular cyclic AMP enhance the open probability, reduce the rundown, and recruit newly functioning L-type Ca^{2+} channels (reviewed by Carbone *et al.* 2001). Likewise, protein kinase C phosphorylation increases quantal ACh release by opening quiescent L-type Ca^{2+} channels in frog motor nerves at resting potential, without needing for terminal depolarization (Arenson & Evans, 2001). In contrast, N-type and P/Q-type Ca^{2+} channels are inhibited by modulators acting through G protein-coupled receptors through a direct involvement of $G_{\beta\gamma}$ binding to pore-forming subunits (Sandoz *et al.* 2004; for review see e.g. Jarvis & Zamponi, 2001). This supports the hypothesis that, besides the negative shift in P-type channel function, adenosine A_{2A} receptor stimulation of the adenylate cyclase/cyclic AMP transducing system (Correia-de-Sá & Ribeiro, 1994) would activate L-type Ca^{2+} currents via protein kinase A-mediated phosphorylation (see e.g. Miller, 1987; Artalejo *et al.* 1992). So, much Ca^{2+} entering through this pathway will be sensed by the release apparatus facilitating evoked [^{3}H]ACh release and, thereby, contribute to overcome neuronal tetanic depression. Thus, endogenous formation of adenosine during neuronal firing appears to operate a novel form of synaptic plasticity at the rat motor endplate involving Ca^{2+} recruitment through different Ca^{2+} channel subtypes.

The physiological significance of the expression of multiple VSCC subtypes in nerve terminals is still not completely clear. Ca^{2+} channel subtypes differ from each other in their distinct biophysical properties, differential modulation by mediators released during cell activity and variable coupling to intracellular processes (for review see Miller, 1987). The existence of different Ca^{2+} channel subtypes at motor nerve terminals would allow a more flexible and versatile regulation of ACh release, especially under non-physiological conditions. Different VSCC subtypes may play important and selective roles during development and regeneration of neuromuscular junctions (Gray *et al.* 1992; Fu & Huang, 1994; Santafé *et al.* 2001) and in pathological conditions when transmitter release is compromised. For instance, LEMS is an autoimmune disorder associated with the loss of function of motoneuronal P-type VSCCs due to high titres of anti-P/Q-type antibodies, which is characterized by a decreased release of vesicles in response to single nerve impulses (quantal content), facilitation following high frequency stimulation and normal postsynaptic responses (Lambert & Elmqvist, 1971). Following treatment with LEMS sera, down-regulation of P/Q-type channels is accompanied by a concomitant rise or unmasking of dihydropyridine-sensitive L-type, as well as in 'resistant' R-type, Ca^{2+} currents (for review see Lang *et al.* 2003). Similarly, compensatory increases in the proportion of facilitatory L-type Ca^{2+} currents become apparent following amyotrophic lateral sclerosis (ALS) IgG-treatment of murine neuromuscular junctions (Fratantoni *et al.* 2000). As under normal conditions the Ca^{2+} influx through P/Q-type VSCCs makes up > 95% of the current evoked at the mammalian neuromuscular junction, this dynamic regulation may explain why these patients are not more severely affected than in fact they are. Our results show for the first time that, under physiological conditions, endogenous adenosine generated during intermittent neuronal firing may facilitate extracellular Ca^{2+} recruitment through silent L-type channels. Specifically, the tonic A_{2A}-receptor-mediated switch of Ca^{2+} influx into motor nerve terminals, from a predominant fast desensitizing P-type component to a high-capacity/slow-inactivating L-type current, allowed for a near complete recovery of neurotransmitter release from the decline that had occurred during a previous train. On the basis of these findings, manipulation of adenosine A_{2A} receptor activation might be of clinical interest to recruit spared VSCCs in order to preserve neuromuscular transmission in compromised (low safety factor) motor endplates, particularly those involved in respiratory activity.

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