Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats

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Abbreviations: AK, adenosine kinase; AKI, adenosine kinase inhibitor; AMPK, AMP-activated protein kinase; ADO, adenosine; CADO, 2-chloroadenosine; ADA, adenosine deaminase; AR, adenosine receptor; A1R, adenosine A1 receptor; A2AR, adenosine A2A receptor; AC, adenylate cyclase; CGS 21680, (type VI, 1803Um^-1, EC 3.5.44), 2-p-(2-carboxyethyl) phenethylamino]-5´-N-ethylcarboxamido adenosine hydrochloride; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; ITU, 5´-iodotubericidin; ZM 241385, 4-(2-[7-amino-2-(2-furly)[1,2,4]triazolo[2,3-a][1,3,5]triazin5ylamino] ethyl) phenol; AICAR, 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside; q.c., quantal content; EPPs, evoked end-plate potentials; MEPPs, miniature end-plate potentials; NMJ, neuromuscular junction; NMT, neuromuscular transmission; CNS, central nervous system.
Abstract

Adenosine-induced modulation of neuromuscular transmission in young (3-4 weeks old) rats was evaluated. Inhibition of adenosine kinase with iodotubercidin (ITU, 10µM), which is known to induce adenosine release, enhanced the amplitude of evoked end-plate potentials (EPPs) recorded from innervated diaphragm muscle fibres. This facilitatory effect was transformed into an inhibitory one upon blockade of adenosine A_{2A} receptors with ZM 241385 (50nM); further blockade of adenosine A_1 receptors with the selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX,10nM) abolished that inhibition. Adenosine or 2-chloroadenosine (CADO), at submicromolar concentrations, increased the amplitude and the quantal content of EPPs, whereas at low micromolar concentrations decreased EPP amplitude. Blockade of A_1 receptors with DPCPX (10 nM) prevented both excitatory and inhibitory effects, whereas blockade of A_{2A} receptors with ZM 241385 (50nM) prevented only the excitatory effects. DPCPX and ZM 241385 also prevented the excitatory effect of the selective A_{2A} receptor agonist, CGS 21680 (10 nM). CADO (30 nM) also increased neuromuscular transmission in adult (12-16 weeks old) rats. It is suggested that at the motor nerve endings, low extracellular concentrations of adenosine activate both A_{2A} and A_1 receptors, but activation of A_{2A} receptors predominates over A_1 receptors; the activity of A_{2A} receptors might, however, require co-activation of A_1 receptors. This facilitatory action of low concentrations of extracellular adenosine upon acetylcholine release may be particularly relevant at developing neuromuscular junctions, where subtle changes in synaptic levels of acetylcholine might influence synaptic stabilization.
Introduction

It is well established that adenosine decreases the amplitude and the quantal content (q.c.) of evoked endplate potentials (EPPs) at the neuromuscular junctions (NMJ) of adult rats (Ginsborg and Hirst, 1972) and frogs (Ribeiro and Walker, 1975). It is also known that in the same motor nerve ending, both inhibitory adenosine A₁ receptors (A₁Rs) and excitatory adenosine A₂A receptors (A₂ARs) coexist, modulating the evoked release of acetylcholine (Correia-de-Sá et al., 1991). The way in which adenosine is able to achieve a balance in the control of neurotransmission depends on the extracellular concentration of the nucleoside, which subsequently depends on extracellular adenosine generation and inactivation (via cellular uptake and/or extracellular deamination) (Sebastião and Ribeiro, 1988). At the rat NMJ extracellular adenosine can be originated by transport-mediated release (Cunha and Sebastião, 1993), in parallel with its formation from released ATP (Smith, 1991).

Adenosine kinase (AK) is an intracellular enzyme that catabolizes adenosine to AMP and, therefore, its inhibition leads to enhancement of adenosine release into the extracellular space (see Arch and Newsholme, 1978). While exploring the action of endogenous adenosine on neuromuscular transmission (NMT) by using the adenosine kinase inhibitor (AKI), 5’-iodotubericidin (ITU), we observed, as reported in the present work, that instead of inhibition, ITU greatly facilitated NMT. In addition to this effect of ITU, it seemed, therefore, of interest to re-evaluate the excitatory effect of adenosine at the rat NMJ, by using the endogenous ligand, adenosine and the stable adenosine analogue, 2-chloroadenosine (CADO). CADO has about the same affinity as the endogenous ligand for adenosine receptors (see e.g. Ribeiro and Sebastião, 1985) with the advantage of not being metabolized, allowing to evaluate its action at defined concentrations.

A brief report on some of the results has been presented (Pousinha et al., 2007).
Methods

The experiments were performed on isolated preparations of the phrenic nerve-diaphragm from male 3-4 weeks old Wistar rats obtained from Harlan Interfauna Iberia, SL (Barcelona). In some experiments and as mentioned, 12-16 weeks old rats were used. The animals, handled according to European Community guidelines and Portuguese Law on animal care, were killed by decapitation under halothane anaesthesia. A strip of the left hemidiaphragm was isolated together with the phrenic nerve and was mounted in a 3 ml Perspex chamber through which the solutions flowed continuously at a rate of 3 ml/min via a roller pump. Except otherwise indicated, the solutions were at room temperature (22-23°C). In some experiments the perfusion solutions were warmed up to the physiological temperature (36-37°C). The bath volume was kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from one flask to another. This involved a minimum of disturbance to the preparation and allowed prolonged recording from the same fibre with many solution changes. The change-over times in the figures of this paper indicate the times at which the inlet tube of the pump was transferred to a new solution. Whenever testing the effect of a drug in the presence of an adenosine receptor (AR) antagonist, superfusion of the antagonist started at least 45 minutes before addition of another drug to the solution. In all cases, the superfusion of the drugs was only initiated when the recordings were considered stable by online analysis of the results.

Evoked end-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) were recorded in the conventional way (Fatt and Katz, 1951), with intracellular electrodes filled with KCl (3M) and 10-20MΩ resistance, inserted in the motor end-plate. The reference electrode was an Ag-AgCl pellet. The nerve was stimulated supramaximally (rectangular pulses of 20 µs duration applied once every 2s) through a suction electrode. Individual EPPs, as well as the resting membrane potential of the
muscle fibre, were continuously monitored. Muscle fibers with a resting membrane potential less negative than -60mV, were rejected. Throughout the experiment, EPPs and the resting membrane potential were continuously monitored and digitally stored on a personal computer with the Clampex® programme (pCLAMP 10 Axon Instruments, Foster City, CA). The results were later analysed off-line and each 60 consecutive EPPs were averaged. MEPPs were sampled and stored before the application of the drugs at 16 and 30 min and in some experiments 46 min, after starting drug perfusion. Later, the samples were analysed off-line. MEPPs were detected by an event detection protocol. The threshold for detection of MEPPs was set at the level of 0.35 mV of amplitude and 2 ms of duration to prevent the contamination of the signal with electric noise. Mean MEPP frequency was measured by counting the number of MEPPs acquired in gap free mode for 100 sec periods, at the following times: -10 min (before adding the drug), 0 min (immediately before addition) and 30 min after starting drug perfusion. The values were only considered for posterior analysis whenever MEPP frequency at -10 min and 0 min did not differ by more than 5%. The mean amplitude of MEPPs was calculated considering the average of the mean amplitude of 100 consecutive MEPPs. In the calculation of the mean amplitude of the MEPPs, the occasional “giants” (above 1mV) were neglected, since it has been shown that they do not contribute to the components of evoked release (Menrath and Blackman, 1970). A change in MEPP frequency that was not accompanied by a change in MEPP amplitude was interpreted as a change in spontaneous transmitter release (Katz, 1969). Quantal contents were estimated as the ratio of the average evoked response to the average amplitude of the MEPPs recorded during the same period.

The bathing solution contained (mM): NaCl 117, KCl 5, NaHCO₃ 25, NaH₂PO₄ 1.2, glucose 11, CaCl₂ 2.5, MgCl₂ 1.2. It was continuously gassed with 95% O₂ and 5% CO₂ (pH 7.4) and kept at room temperature (22-25°C). Muscle twitches were prevented
by a submaximal concentration (1.5μM) of tubocurarine, except where otherwise indicated. In experiments designed to record MEPPs the muscle twitches were prevented by increasing the bath concentration of Mg2+ to 19mM.

Drugs used: adenosine, 2-chloroadenosine (CADO), adenosine deaminase (ADA) (type VI, 1803U/ml, EC 3.5.44), 2-p-(2-carboxyethyl) phenethylamino]-5’-N-ethylcarboxamido adenosine hydrochloride (CGS-21680), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 5’-iodotubericidin (ITU) and d-tubocurarine chloride were obtained from Sigma-RBI (St. Louis, MO); 4-(2-[7-amino-2-(2-furlyl][1,2,4]triazolo[2,3-a][1,3,5]triazin5ylamino] ethyl) phenol (ZM241385) and 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR) was supplied by Tocris Cooksonhem (Ballwin, MO). Stock solutions (5mM) of CGS21680, DPCPX, ZM241385 and ITU were prepared in DMSO. Aliquots of these stock solutions were kept frozen at -20°C until used. The stock solution of ITU was kept in the dark to prevent photodecomposition. The maximum concentration of DMSO applied to the preparations (0.02% v/v) was devoid of effect on EPPs.

The data are expressed as mean ± SEM from n experiments. To allow comparisons between different experiments, the EPP amplitude, as well as the MEPP frequency or amplitude and the quantum content, were normalised, with 100% determined as the averaged values obtained during 10 min immediately before applying the test drug. The significance of the differences between means was evaluated by Student’s t test, when only two means were compared, or by one-way ANOVA followed by the Tukeys test whenever multiple comparisons were made. Values of \( P<0.05 \) were considered to represent statistically significant differences.
Results

1. Endogenous adenosine

1.1 Effects of 5´-iodotubericidin

Fig. 1 illustrates the effect of the AKI, 5´-iodotubericidin (ITU, 10µM) on the amplitude of EPPs. As shown, 40 min after its application, ITU enhanced the amplitude of EPPs by 25 ± 6.0% (n=4, Fig. 1B) while being virtually devoid of effect on the resting membrane potential of the muscle fibre (Fig. 1A). This effect was sustained during the period of application of ITU and EPPs amplitude did not return to the baseline within 30 min after reperfusion with the normal bathing solution (Fig. 1A). At lower concentration (1 µM) ITU caused a smaller (15 ± 1.8%, n=3) increase in the amplitude of EPPs.

To evaluate if this excitatory effect could be attributed to activation of A2ARs by released adenosine, ITU (10µM) was perfused in the presence of the A2AR selective antagonist, ZM241385 (Poucher et al., 1995). In these conditions, the excitatory effect of ITU was transformed into an inhibitory effect (-28 ± 2.4%, n=3; Fig. 1C), which can be related with A1R activation by released adenosine, since it was prevented by the adenosine A1R selective antagonist, DPCPX (10nM). Surprisingly, in the presence of DPCPX, ITU (10µM) failed to enhance NMT (1 ± 1.8%, n=6; Fig. 1D) indicating a possible interplay between high affinity A1Rs and A2ARs. To further evaluate the interaction between these ARs, we investigated if the A1Rs antagonist, DPCPX (10nM), could prevent the effect of the A2AR selective agonist, CGS21680 (Jarvis et al., 1989). As illustrated in the Fig. 2, this agonist enhanced EPP amplitude (23 ± 4.4%, n=7), an effect completely abolished (0 ± 3.2%, n=6) by the A1R antagonist, DPCPX. As expected the A2AR antagonist, ZM241385 (50nM), also abolished the facilitatory action of CGS21680 (10nM) on EPP amplitude.
To further confirm that the action of ITU on NMT are due to changes in the extracellular levels of adenosine, experiments were performed in the presence of both adenosine A1R and A2AR antagonists, DPCPX (10nM) and ZM241385 (50nM), which were added together to the preparations before addition of ITU (10 μM). Under these conditions, and as expected, ITU did not modify the amplitude of EPPs (Fig. 1E).

The above described effects of ITU upon NMT suggest that by enhancing extracellular adenosine it is possible to induce a long lasting facilitation of NMT due to A2AR activation. This long lasting facilitatory effect is not reverted by inactivation of extracellular adenosine with adenosine deaminase (ADA, 2U/ml), an enzyme that converts adenosine into inosine (see Arch and Newsholme, 1978); thus, in four experiments where ADA (2U/ml) was perfused after the full excitatory effect of ITU (10μM), the amplitude of EPPs did not decrease towards pre-ITU levels. However, when ADA (2U/ml) was applied before ITU (10μM), a time lag of about 10 min occurred before any facilitation of NMT started to be observed. After this time lag, the amplitude of EPPs started to increase probably because the rate of adenosine production started to be faster than the rate of adenosine deamination by the concentration of ADA used (2U/ml). Higher concentrations of ADA were not tried to avoid the risk of non-enzymatically related actions (Cunha et al., 1996).

When applied alone, ADA (2U/ml), caused only a slight decrease in the amplitude of EPPs (-6.8 ± 1.8%, n=5; P<0.05). The selective A2AR antagonist, ZM241385 (50nM) or the selective A1R antagonist, DPCPX (10nM), did not modify the amplitude of EPPs (3 ± 6.7%, n=5; 0 ± 5.5%, n=7, respectively).

ITU has been reported to inhibit AMP-activated protein kinase (AMPK) (Musi et al., 2001). To evaluate if inhibition of this enzyme could mimic the effect of ITU on NMT, we tested the AMPK activator, 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR). However, this compound even at a lower concentration (300
μM) than that usually used to activate AMPK (1 mM, see Giri et al., 2004), caused a marked hyperpolarization (by about 10 mV), and therefore any changes in EPP amplitude were masked by this postsynaptic effect. When used at 1mM, AICAR caused a similar (~10 mV) membrane hyperpolarization. Since this marked hyperpolarization was not mimicked by any adenosine receptor agonist, neither by ITU, no further protocols were designed with AICAR. Membrane hyperpolarization caused by submilimolar concentrations of AICAR is probably due to changes in membrane K+ conductance (Klein et al., 2009)

2. Facilitation by exogenous adenosine

As seen in Fig. 3, adenosine (300nM) enhanced NMT by 38 ± 6.8% (n=5), an effect that was not diminished in the next 30 minutes after stopping adenosine perfusion (Fig. 3A). This excitatory effect of a submicromolar concentration of adenosine was consistently observed (Fig. 3B) and was antagonized by the selective A2AR antagonist, ZM241385 (50nM, Fig. 3C). When concentrations of adenosine above 10µM were applied to the preparations, the amplitude of EPPs clearly decreased, in accordance with what has been previously described (see Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). To evaluate if adenosine A2ARs were fully activated in the presence of adenosine concentrations that induced a decrease in the amplitude of EPPs, we applied the selective A2AR agonist, CGS21680, to the bath solution after the full inhibitory effect of adenosine (25µM) was observed. In these conditions CGS 21680 induced an increase in the amplitude of EPPs (Fig. 3D), indicating that adenosine (25µM ) might not be able to completely activate A2ARs or that CGS 21680 is more efficient than adenosine itself to attenuate A1R functioning. Since CGS 21680 has higher selectivity and affinity for A2ARs than adenosine itself, it is plausible that it more efficiently attenuates A1R
mediated inhibition (Cunha et al., 1994) than the endogenous agonist, adenosine, which activates both A₁ and A₂A receptors. Thus, A₂A receptors at the neuromuscular junction seem to start to be activated by very submicromolar adenosine concentrations, but maximal recruitment of A₂A receptors by adenosine seems to be achieved only with very high adenosine concentrations, which hardly occur under physiological conditions. Indeed, under physiological conditions neuromodulatory receptors are usually not fully recruited by their endogenous ligands.

Figure 4 summarizes the results from experiments where MEPPs and EPPs were recorded simultaneously with Mg²⁺ 19mM paralyzed preparations, to evaluate changes in the q.c. of EPPs. Adenosine (300nM) increased the frequency of MEPPs (n=3) by 40 ± 2.5%, without changing its average amplitude. The q.c. of EPPs in the presence of adenosine (300nM) was increased by 55 ± 9.7% (n=3). This facilitation caused by submicromolar concentrations of adenosine contrasts with adenosine’s inhibitory action at micromolar concentrations that inhibited both the MEPP frequencies and q.c. of EPPs (see Ginsborg and Hirst, 1972 and Ribeiro and Walker, 1975).

3. Effect of 2-Chloroadenosine (CADO).

Since adenosine is subject to uptake and enzymatic inactivation, we decided to explore the action of the stable analogue, CADO, which is not a substrate for adenosine deaminase (e.g. Daly, 1982), and has low affinity for the adenosine uptake system (Jarvis et al., 1985). The concentrations of CADO required to obtain effects similar to those of adenosine were about 10 times lower. Indeed, as observed with 300nM adenosine, CADO (30nM) increased the average amplitude of EPPs (41 ± 7.8%, n=7, Fig. 5). As observed with ITU and adenosine, the effect remained for at least 20 min after starting the washing out of CADO (Fig. 5A).
Most of the experiments now reported have been performed at room temperature (~22°C) to minimize electrical noise and to increase biological viability, compatible with long lasting protocols. However, to evaluate if the unexpected long lasting excitatory effect of low concentrations of an AR ligand, such as CADO, could be influenced by temperature, four experiments were performed at 36°C and the results compared with those obtained in similar experiments performed at room temperature with similar periods of CADO application and washout. As illustrated in Figure 5C, the facilitatory effect of CADO (30 nM) upon EPP amplitude was not influenced by the bath temperature, within the physiological limits.

The excitatory effect of CADO (30nM) was observed either in the presence of tubocurarine (1.5µM; Fig. 5A, C), i.e. when the q.c. of EPPs is high, or in the presence of high Mg²⁺ (19mM; Fig. 5D), i.e. when quantal contents are low. CADO increased the frequency of MEPPs by 39 ± 12.2% (n=3; Fig. 6) without changing by more than 2% the average amplitude of MEPPs recorded in the same period. This facilitatory effect on asynchronous release was of similar magnitude in preparations where the motor nerve was stimulated once every 2 sec (39 ± 12.2%, n=3), or in the absence of stimulation (35 ± 3.2%, n=2). CADO (30nM) did not activate maximally the A₂A Rs, since the selective agonist of A₂A Rs, CGS 21680, when applied after the full effect of CADO, caused a further enhancement of the EPP amplitude (Fig. 5D).

Fig. 7A illustrates the concentration-response curve for the effects of CADO (10nM-10µM) on EPPs amplitude, which is noticeably biphasic, with excitatory effects at concentrations between 10nM and 100nM and the maximal excitatory effect being obtained with 30nM of CADO. At the concentration of 1µM, CADO did not affect the amplitude of EPPs, probably due to a balanced Aₐ/A₂A receptor activation, whereas at a higher concentration, CADO (10µM) decreased the amplitude of EPPs.
The $A_2A$R antagonist ZM241385 (50nM) prevented the excitatory effect of CADO (10 to 100nM, n=6) but did not influence the inhibition of a higher concentration of CADO (10μM) (Fig. 7A). Interestingly, in the presence of the $A_2A$R antagonist ZM241385 (50nM, Fig. 7B) there was a clear inhibitory effect of CADO at concentrations (30-300nM) that in the absence of ZM241385 (50nM) caused a facilitation of NMT. Furthermore, in the presence of the $A_2A$R antagonist, there was a clear inhibition of EPPs caused by 1μM CADO. This indicates that at these concentrations the agonist is acting upon both $A_1$ and $A_2A$ receptors.

As shown in Fig. 7 A and C, the $A_1$R antagonist DPCPX (10nM) at a concentration only 20 times higher than its Ki value for $A_1$Rs in the same preparation (Sebastião et al., 1990) and well below its Ki value for $A_2A$Rs (see Jacobson et al., 1992), greatly attenuated both the excitatory and the inhibitory effects of CADO (10nM to 10μM).

Previous studies on the inhibitory effect of adenosine or CADO on EPPs were performed in adult rats. To allow comparisons and to evaluate whether the now described excitatory action also occurs in adult rats, we tested the effect of CADO (30nM) in 12-16 weeks old rats and, as shown in figure 8, it increased the amplitude of EPPs by 11 ± 1.0% (P<0.05, n=6). This effect was smaller (P<0.05) than the one observed in infant rats but it can also be attributed to $A_2A$R activation since it was prevented by the selective $A_2A$R antagonist, ZM241385 (50nM, Figure 8B). As it occurred in infant rats, the excitatory action of 30nM CADO was also prevented by the $A_1$R antagonist, DPCPX as well as by the $A_2A$R antagonist ZM241385 (50nM, Figure 8).
Discussion

The main findings of the present work were that submicromolar concentrations of adenosine or CADO increase the amplitude of EPPs. These effects were clearly due to $A_{2A}$R, since they were antagonized by the selective $A_{2A}$R antagonist, ZM241385. The inhibitory effects, obtained with micromolar concentrations of adenosine or CADO, were related to activation of $A_1$R and antagonized by the $A_1$R receptor antagonist, DPCPX.

The enhancement of NMT caused by adenosine or CADO results from an increase of the evoked release of acetylcholine, since they increased the q.c. of EPPs without affecting the average amplitude of MEPPs recorded concomitantly. Even in the absence of electrical stimulation, CADO increased the frequency of MEPPs, suggesting a facilitatory effect on spontaneous asynchronous release. Similar effects were observed either in experiments where the muscle twitches were prevented with tubocurarine (high q.c.) or where muscle twitches were prevented by high Mg$^{2+}$ (low q.c.). So, the initial q.c. did not influence the facilitatory effect of adenosine and precludes the possibility that the high Mg$^{2+}$ concentration influenced the action of adenosine or CADO, due to its ability to increase agonist binding (Yeung et al., 1985).

It is well established that AKIs, such as ITU, enhance extracellular adenosine, which activates ARs (Kowaluk et al., 1998). The excitatory effect of ITU was similar to that observed with nanomolar concentrations of adenosine or CADO, and the excitatory effects of these drugs were antagonized by pre-incubation with the $A_{2A}$R antagonist, ZM241385, transforming those effects into inhibitory ones. This suggests that the influence of ITU, CADO and adenosine upon NMT results from a balance between activation of adenosine excitatory $A_{2A}$Rs and inhibitory $A_1$Rs.
The reversibility of A1R-mediated inhibition of NMT, together with absence of DPCPX effect on NMT, suggests that at the NMJ of infant rats, A1Rs are not tonically activated by endogenous adenosine. In contrast, it was not possible to wash out the A2AR-mediated effects, which renders it difficult to evaluate if A2ARs were tonically influenced by endogenous adenosine. For slowly reversible actions, one would not expect that removing the endogenous ligand or preventing its binding to the receptor with a competitive antagonist added in the presence of the endogenous agonist, would result in a rapid decrease in response. Since enhancement of extracellular adenosine with ITU, or A2AR activation with the selective agonist, CGS21680, facilitates NMT, one may infer that the levels of extracellular adenosine under basal conditions should be low and not enough to fully saturate A2AR.

The facilitatory effects of adenosine, CADO or even ITU were not washed out, which was unexpected because dissociation of adenosine or CADO from its receptor is known to be fast. Adenosine is metabolically unstable, being taken up by cells and substrate for several enzymes. However, reversibility of the ligand/receptor interaction or inactivation of the ligand (as it occurs with adenosine) does not necessarily imply reversibility of the effect triggered by receptor activation. A2ARs at phrenic motor nerve endings are positively coupled to AC/cAMP/PKA transducing system (Correia-de-Sá and Ribeiro, 1994). Therefore, PKA dependent phosphorylation and its consequence upon neurotransmitter release may influence synaptic transmission, although the receptors might no longer be activated by the ligand. The possibility of a long-lasting A2AR activation is reinforced by the observation that the facilitatory action of ITU was not reversed by adding ADA after the full effect of ITU. As expected, the effect of ITU was delayed by ADA, if added before, and antagonized by pre-incubation with an A2AR antagonist. It appears that once triggered by A2AR activation, the ongoing intracellular mechanisms cause a long-lasting increase in synaptic strength, which may share some
mechanisms (e.g. cAMP/PKA-dependent phosphorylation events) with plasticity phenomena at synapses of the CNS.

Blockade of A2ARs unmasked the inhibitory actions of low CADO concentrations but did not exacerbate the inhibitory action of a high concentration. Desensitization of A2ARs cannot account for this apparent lack of effect, since the selective A2AR agonist, CGS21680, was still able to facilitate NMT when applied in the presence of adenosine (25μM). It appears that as the concentration of CADO increases (nanomolar to micromolar), there is a shift from a preponderant A2AR activation towards an apparent exclusive A1R activation. This suggests that at the motor nerve endings of infant rats, the A2AR has higher affinity and/or higher expression levels than the A1R. Once activated, A2AR might inhibit the actions of A1Rs at low A1 receptor occupancy. With high concentrations of adenosine, A1Rs would become widely activated, leading to a predominance of the inhibitory effect. Whether these results fit into an A1R/A2AR heterodimerization model (see Franco et al., 2008) with a main influence of the A2A binding site at low receptor occupancy, and a main influence of the A1 binding site at high occupancy conditions, could not be directly assessed in the present work. Whatever the molecular mechanisms behind the present results, they challenge the previous concept on predominant A1R activation at low agonist concentrations and A2AR activation at higher concentrations of adenosine (see Correia de Sá and Ribeiro, 1996; see also Franco et al., 2008).

In the present work, the A2AR-mediated facilitation of NMT was antagonized not only by an A2AR-selective antagonist but also by an A1R-selective antagonist; this contrasts with the A1R mediated inhibition of NMT, which was prevented by the A1R antagonist but not by A2AR antagonist. The inhibitory effect of the A2AR agonist, CGS21680, in micromolar concentrations at both the hippocampus (Lupica et al., 1990)
and NMJ (Correia-de-Sá et al., 1991), is probably due to A1R activation. However, the presently observed excitatory effects seen with low nanomolar concentrations of CGS21680, are well below its Ki for A1R (see Jacobson et al., 1992). The observation that DPCPX displaces the specific binding of low nanomolar concentrations of CGS21680 at hippocampal membranes (Cunha et al., 1996) led to the suggestion that A2AR at the hippocampus and striatum differ slightly in pharmacological characteristics. Also, the finding that facilitation of hippocampal synaptic transmission by a low nanomolar concentration of CGS21680 requires co-activation of A1R led to the proposal that the A2AR-mediated facilitation of transmission results from attenuation of the tonic A1R-mediated inhibition (Lopes et al., 2002). Indeed, in the hippocampus, synaptic transmission is under intense tonic inhibition by adenosine that activates A1Rs. This was not observed with the NMJ (present work), where the A1R antagonist, DPCPX, did not affect transmission. Another explanation for the loss of A2AR-mediated effects upon A1R blockade is that A2ARs and A1Rs form heteromers, as it has it happens at glutamatergic terminals (Ciruela et al., 2006). So, blockade of the A1R could induce a conformational change in the heteromer that does not allow either A2AR agonist binding or effective coupling to the A2AR transducing system. Indeed, heteromerization of GPCRs may affect receptor functioning (Franco et al., 2008), since monomers and heteromers can have distinct pharmacological and signaling properties. In addition, A1R/A2AR cross talk through the intracellular transducing pathways that are downstream to receptor binding (Sebastião and Ribeiro, 2000) might also explain the need for A1R function to trigger A2AR-mediated response at the motor nerve endings. These two possibilities are not mutually exclusive.

Previous work (Correia-de-Sá and Ribeiro, 1996), also performed at the rat diaphragm NMJ, demonstrated predominance of the tonic A2AR-mediated facilitatory effect on [3H]acetylcholine release, if high frequency stimulation (50 pulses/s) is used.
Since ATP is released together with acetylcholine (e.g. Silinsky, 1975) and is quickly degraded extracellularly to adenosine (Cunha and Sebastião, 1991), extracellular accumulation of considerable amounts of adenosine is expected due to high frequency neuronal firing. Adenosine derived from high frequency-induced ATP release preferentially activates A\textsubscript{2A}Rs (Cunha et al., 1996). As we now report, at the NMJ of infant rats the concentration of adenosine that accumulates extracellularly due to AKI. We also showed that lower concentrations of adenosine are required to observe excitatory instead of inhibitory effects. This high sensitivity of A\textsubscript{2A}R, along with the consistent inhibitory effect of micromolar concentrations of the nucleoside, explains why previous studies using concentrations of adenosine above 1 µM did not detect facilitatory actions of adenosine on EPPs (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Age related differences cannot account for the results obtained, since in adult rats the facilitatory effect of CADO (30nM) predominates over the inhibitory effect.

In conclusion, the work now reported demonstrates that both A\textsubscript{1}Rs and A\textsubscript{2A}Rs at the motor nerve endings of infant rats can be activated by low extracellular concentrations of adenosine whereas at nanomolar concentrations, activation of excitatory A\textsubscript{2A}Rs predominates. This challenges previous ideas that the predominant action of adenosine at this NMJ was inhibitory at low frequencies of neuronal firing (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Facilitatory actions upon spontaneous and evoked acetylcholine release under low neuronal firing may have particular relevance at developing NMJs, where subtle changes in synaptic levels of acetylcholine and, therefore, of nicotinic receptor activation, might influence synaptic stabilisation.
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Footnotes

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Legends for Figures

Figure 1. Adenosine kinase inhibition influence the amplitude of evoked end-plate potentials (EPPs) recorded from a muscle fibre of the rat diaphragm. In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by the adenosine kinase inhibitor iodotubericidine, ITU (10µM), in one experiment. In the lower panel in (A), are shown superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ITU (10µM). In (B) is shown the averaged change in the amplitude of EPPs caused by ITU (10µM) in 4 experiments. In (C), (D) and (E) are shown the averaged changes in the amplitude of EPPs caused by ITU (10µM), when it was applied (C) in the presence of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50nM), (D) in the presence of the selective antagonist of adenosine A_{1} receptors, DPCPX (10nM) and (E) in the presence of the co-administrated selective antagonist of adenosine A_{1} receptors, DPCPX (10nM) with the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50nM). In the ordinates, 100% represents the averaged amplitude of EPPs recorded for 10 min immediately before starting ITU perfusion, which was 3.2mV, n=1 (A); 3.9 ± 0.7mV, n=4 (B); 3.7 ± 1.2mV, n=3 (C); 3.2 ± 1.4mV, n=4 (D), 1.3 ± 0.2mV. Each point represents the averaged amplitude of 60 successive EPPs. The horizontal bars indicate the period of drugs perfusion. Resting membrane potential in all experiments at time zero (i.e. before ITU addition) ranged from -60 to -72mV. Solutions contained a submaximal concentration (1.5 µM) of tubocurarine, which decreased EPP amplitude below threshold of action potential generation therefore preventing muscle twitches in response to nerve stimulation.
Figure 2. The selective A2A receptor agonist enhances the amplitude of end plate potentials (EPPs), an action blocked by either A1 or A2A receptor selective antagonists. The time course of the changes in EPP amplitude induced by the A2A receptor agonist, CGS 21680 (10nM), when applied to preparations in the absence (□), in the presence (○) of the selective adenosine A1 receptor antagonist, DPCPX (10nM), or in the presence (●) of the selective adenosine A2A receptor antagonist, ZM241385 (50nM) is shown. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CGS 21680 (10nM): 2.2 ± 0.4 mV, n=7 (□); 2.5 ± 0.6 mV, n=4 (○); 1.6 ± 0.5 mV, n=6 (●). Resting membrane potential in all experiments at time zero (i.e. before CGS 21680 addition) ranged from -60 to -72 mV. Perfusing solutions contained tubocurarine (1.5µM).

Figure 3. At submicromolar concentrations, adenosine enhances the amplitude of evoked end-plate potentials (EPPs). From (A) to (C) are shown the time course of the changes in EPP amplitude induced by ADO (300nM) when applied to preparations in the absence (A, B) or in the presence (C) of the selective adenosine A2A receptor antagonist, ZM 241385 (50nM). In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by ADO (300nM) in one experiment. In the lower panel, in (A), are shown superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ADO (300nM). In (D) is shown the effect of the selective agonist of adenosine A2A receptors, CGS 21680, when applied in the presence of a concentration of ADO (25µM), which markedly inhibited the amplitude of EPPs. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition...
of the drugs indicated above the bars: 0.8 mV, n=1 (A); 1.7 ± 0.3 mV, n=5 (B); 3.4 ± 0.8 mV, n=3 (C); 3.0 mV, n=1 (D). Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -63 to -72 mV. Perfusing solutions contained 19 mM Mg$^{2+}$ which prevented muscle action potentials and twitches in response to nerve stimulation.

**Figure 4** – Adenosine, at a submicromolar concentration, enhances the frequency and amplitude of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs). In (A) are shown the spontaneous events recorded in gap free mode across 100 seconds before (control, upper panel) and 30 min after adenosine (ADO, 300 nM) perfusion (lower panel) in one experiment. Each point in (A) represents the amplitude of a single MEPP and each vertical trace immediately above the ordinates, indicates the occurrence of a MEPP. The resting membrane potentials were -62 mV to -61 mV throughout the experiment. In (B), the MEPP amplitude distribution recorded in the same experiment over the same period of time as in (A) is shown. The event detection was settled to signals with amplitude ranging from 0.35 to 1 mV and minimal duration of 2 ms. In (C) are represented samples of MEPPs recordings. Panels (D to F) represent the averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of adenosine (300 nM); (*P < 0.05; Student’s t test). Perfusing solutions contained 19 mM Mg$^{2+}$.

**Figure 5.** At submicromolar concentrations, 2-chloroadenosine (CADO) enhances the amplitude of evoked end-plate potentials (EPPs). In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by CADO (30 nM) in one endplate paralyzed with tubocurarine (1.5 µM). In the lower
panel in (A) are showed superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after addition of CADO (30nM). In B and E are shown the averaged changes in the amplitude of EPPs caused by CADO (30 nM) in experiments where muscle twitches were prevented by 1.5 µM tubocurarine (B) or 19 mM Mg$^{2+}$ (E). In (C) is shown the time course of the averaged changes in EPP amplitude induced by CADO (30nM) in experiments performed at room temperature (o) and at a more physiological (37ºC) temperature (●); muscle twitches were prevented with tubocurarine (1.5 µM). In (D) is shown the effect of the selective adenosine A$_{2A}$ receptor agonist when applied after the full excitatory effect of CADO (30nM); muscle twitches were prevented with tubocurarine (1.5 µM). Each point, in all panels, represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30nM): 3.7mV, n=1 (A); 2.4 ± 0.3 mV, n=7 (B); 2.0 ± 0.5 mV, n=3 (C); 2.0mV, n=1 (D); 1.1 ± 0.1 mV, n=3 (E) . Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -62 mV to -76 mV.

**Figure 6** – 2-Chloroadenosine, at a submicromolar concentration, enhances the frequency and amplitude of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs). In (A) are shown the spontaneous events recorded in gap free mode across 100 seconds before (control, upper panel) and 30 min after 2-chloroadenosine (CADO, 30nM) perfusion (lower panel) in one experiment. Each point in (A) represents the amplitude of a single MEPP and each vertical trace immediately above the ordinates indicates the occurrence of a MEPP. The resting membrane potentials were -63 mV to -64.5mV throughout the experiment. In (B), the MEPP amplitude distribution recorded in the same experiment over the same period of time as in (A) is shown. The event detection was settled to signals with
amplitude ranging from 0.35 to 1mV and minimal duration of 2 ms. In (C) are represented samples of MEPPs recordings. Panels (D to F) represent the averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of CADO (30nM); (*P<0.05; Student’s t test). Perfusing solutions contained 19mM Mg$^{2+}$.

**Figure 7. Biphasic influence of CADO on endplate potentials (EPPs) amplitude.** In (A) is shown the concentration-response curve for the effect of chloroadenosine (CADO) on the amplitude of EPPs. Each point is the average ± SEM of results obtained in 3-5 non cumulative experiments, except for CADO 100nM (●) where n=1, and for CADO 300 nM (○), which was applied after 30 nM CADO. *P<0.05 (Student’s t test) as compared with absence of CADO (100%). In (B) is shown the time course of the changes in EPP amplitude induced by CADO (30nM - 10µM) in one experiment where it was applied in the presence of the selective antagonist of adenosine A$_{2A}$ receptors, ZM 241385 (50nM); note that under this condition submicromolar concentrations of CADO caused an inhibition of EPPs. In (C), is shown the time course changes in EPP amplitude caused by CADO applied in the presence of the selective antagonist of A$_{1}$ receptors, DPCPX (10nM); note that under this condition CADO caused little or no effect on EPP amplitude. Averaged changes in the EPP amplitude caused by 30nM or 10µM of CADO in the presence of 10nM of DPCPX (△) or 50nM of ZM241385 (○) are shown in (A). Each point, in (B) and (C), represents the averaged amplitude of 60 successive EPPs recorded from one experiment. In all panel, 100% in the ordinates represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO: 2.29 ± 0.1mV, n=19 (A, ●); 2.21 ± 0.3mV, n=14 (A, ○); 2.43 ± 0.4mV; n=12 (A, △); 2.4mV, n=1 (B); 2.1mV, n=1 (C). Perfusion solutions contained tubocurarine (1.5µM).
Figure 8. 2-Chloroadenosine (CADO, 30nM) also enhances the amplitude of evoked end-plate potentials (EPPs) in adult (12-16 weeks old) rats. In (A) is shown the time course of the changes in EPP amplitude induced by CADO (30nM); each point represents the averaged amplitude of 60 successive EPPs. Panel (B) shows the averaged changes in EPP amplitude induced by CADO (30nM) under the experimental conditions indicated below each column. Note that the increase in EPPs amplitude caused by CADO (30nM) alone (first column from left) was prevented by the selective adenosine A$_{2A}$ receptor antagonist, ZM 241385 (50nM) (second column), and by the selective adenosine A$_{1}$ receptor antagonist, DPCPX (10nM) (third column), *P<0.05 (ANOVA one way analysis of variance followed by Tukeys multiple comparisons test) as compared with first column. 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30nM) and was 2.3 ± 0.4mV, n=6 (A and first column in B); 2.9 ± 1.3mV, n=4 (second column in B) and 2.3 ± 1.1mV, n=6 (third column in B). Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -62 mV to -75 mV. Perfusion Solutions contained 19mM Mg$^{2+}$. 
Figure 1

A

B

D

C

E

Graphs illustrating the effect of various compounds on EPPs over time.

A: ITU (10 μM)

B: ITU (10 μM)

D: ITU (10 μM)

C: ITU (10 μM)

E: ITU (10 μM)
Figure 2

EPPS (% baseline amplitude) vs. Time (min)

- CGS21680 (10nM)
- CGS21680 (10nM) + ZM241385 (50nM)
- CGS21680 (10nM) + DPCPX (10nM)
Figure 3

A

EPPs (% of change)

ADO (300nM)

60 mV

-65 mV

-20 0 20 40 60

Time (min)

B

0.5 mV

ADO (300nM)

Control

5 ms

C

EPPs (% of change)

ADO (300nM)

ZM241385 (50nM)

-20 -10 0 10 20 30 40

Time (min)

D

EPPs (% of change)

CGS21680 (10nM)

ADO (300nM)

ADO (25μM)

-20 -10 0 10 20 30 40 50

Time (min)
Figure 4

A Control

![Graph showing MEPPs Amplitude (mV) over Time (sec)]

ADO (300nM)

![Graph showing MEPPs Amplitude (mV) over Time (sec)]

B

![Graph showing MEPPs (N) against MEPPs Amplitude (mV)]

C Control

![Graph showing 0.25 mV at 50 ms]  

ADO (300nM)

D

![Bar chart showing MEPPs Frequency (N/sec)]

E

![Bar chart showing MEPPs Amplitude (mV)]

F

![Bar chart showing Quanta Content (n)]

* Significant difference