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Amplification of neuromuscular transmission by methylprednisolone involves activation of presynaptic facilitatory adenosine A_{2A} receptors and redistribution of synaptic vesicles

L. Oliveira [a, b], A.C. Costa [a, b], J.B. Noronha-Matos [a, b], I. Silva [a, b], W.L.G. Cavalcante [c], M.A. Timóteo [a, b], A.P. Corrado [d], C.A. Dal Belo [e], C.R. Ambiel [f], W. Alves-do-Prado [g], P. Correia-de-Sá [a, b, *]

[a] Laboratório de Farmacologia e Neurobiologia/UMIB, Universidade do Porto, Portugal
[b] Center for Drug Discovery and Innovative Medicines (MedInUP), Universidade do Porto, Portugal
[c] Instituto de Biociências, Universidade Estadual de São Paulo (UNESP), Botucatu, São Paulo, Brazil
[d] Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Gabriel, Rio Grande do Sul, Brazil
[e] Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brazil
[f] Departamento de Ciências Fisiológicas, Universidade Estadual de Maringá, Paraná, Brazil
[g] Departamento de Farmacologia e Terapêutica, Universidade Estadual de Maringá, Paraná, Brazil

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ABSTRACT

The mechanisms underlying improvement of neuromuscular transmission deficits by glucocorticoids are still a matter of debate despite these compounds have been used for decades in the treatment of autoimmune myasthenic syndromes. Besides their immunosuppressive action, corticosteroids may directly facilitate transmitter release during high-frequency motor nerve activity. This effect coincides with the predominant adenosine A_{2A} receptor tonus, which coordinates the interplay with other receptors (*e.g.* muscarinic) on motor nerve endings to sustain acetylcholine (ACh) release that is required to overcome tetanic neuromuscular depression in myasthenics. Using myographic recordings, measurements of evoked [^{3}H]ACh release and real-time video microscopy with the FM4-64 fluorescent dye, results show that tonic activation of facilitatory A_{2A} receptors by endogenous adenosine accumulated during 50 Hz bursts delivered to the rat phrenic nerve is essential for methylprednisolone (0.3 mM)-induced transmitter release facilitation, because its effect was prevented by the A_{2A} receptor antagonist, ZM 241385 (10 nM). Concurrent activation of the positive feedback loop operated by pirenzepine-sensitive muscarinic M_1 autoreceptors may also play a role, whereas the corticosteroid action is restrained by the activation of co-expressed inhibitory M_2 and A_1 receptors blocked by methoctramine (0.1 μM) and DPCPX (2.5 nM), respectively. Inhibition of FM4-64 loading (endocytosis) by methylprednisolone following a brief tetanic stimulus (50 Hz for 5 s) suggests that it may negatively modulate synaptic vesicle turnover, thus increasing the release probability of newly recycled vesicles. Interestingly, bulk endocytosis was rehabilitated when methylprednisolone was co-applied with ZM241385. Data suggest that amplification of neuromuscular transmission by methylprednisolone may involve activation of presynaptic facilitatory adenosine A_{2A} receptors by endogenous adenosine leading to synaptic vesicle redistribution.

Abbreviations: ACh, acetylcholine; ADA, adenosine deaminase; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; Dyngo-4a, 3-Hydroxy-N'-[(2,4,5-trihydroxyphenyl)methylidene] naphthalene-2-carbohydrazide; FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)hexatrienyl) pyridinium dibromide; HC, hemicholinium-3; β,γ-imidoATP, adenosine 5′-(β,γ-imido)triphosphate; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; MP, methylprednisolone; PZ, pirenzepine; D-Tc, D-tubocurarine; ZM241385, 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol.

* Corresponding author. Laboratório de Farmacologia e Neurobiologia/UMIB and Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP), R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Tel.: +351 220428212; fax: +351 220428090.
E-mail addresses: pcorreiasa@mail.telepac.pt, farmacol@icbas.up.pt (P. Correia-de-Sá).

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1. Introduction

Corticosteroids are first line medications in the treatment of autoimmune neuromuscular transmission disorders, such as *Myasthenia gravis*, Lambert–Eaton myasthenic syndrome and neuromyotonia (reviewed in Skeie et al., 2006; Jani-Acsadi and Lisak, 2007; Vincent, 2010; Kumar and Kaminski, 2011). Besides its empirical use as immunosuppressive agents, corticosteroids may improve neuromuscular transmission deficits by acting directly on skeletal motor endplates. The mechanism(s) of action of corticosteroids at the neuromuscular junction is still a matter of debate. Anti-inflammatory and immunosuppressive effects of glucocorticoids may be mediated by cytosolic receptors (genomic mechanism) and/or by fast pathways involving non-specific interactions with cellular membranes (non-genomic mechanism) (Stahn and Buttgereit, 2008; Lösel and Wehling, 2003). While glucocorticoids produce no changes on skeletal muscle twitches triggered by low-frequency nerve stimulation, these drugs significantly enhance post-tetanic potentiation and attenuate neuromuscular block produced by anti-nicotinic muscle relaxants in both slow and fast skeletal muscles (Baker et al., 1977; Dal Belo et al., 2002; Soltész et al., 2008). Evidences for a non-genomic presynaptic action of corticosteroids at motor endplates (Makara and Haller, 2001) were corroborated by steroid-induced increases in the frequency and amplitude of miniature endplate potentials (MEPPs), without any effect on muscle resting membrane potential (Dalkara and Onur, 1987). The enhancing effect of corticosteroids on neuromuscular transmission seems to be unrelated to the blockade of phospholipase A_2 (Sen et al., 1976) and to inhibition of pro-inflammatory prostanoids (Smyth et al., 2006). Prednisolone and dexamethasone antagonize the inhibitory action of hemicholinium-3 both on the rate of choline uptake and the incorporation of choline into acetylcholine (ACh) in the rat diaphragm (Veldsema-Currie et al., 1976; van Marle et al., 1986). All these properties may contribute to the usefulness of corticosteroids in autoimmune neuromuscular transmission disorders. Therefore clarification of the underlying mechanism(s) responsible for corticosteroid-induced facilitation of transmitter release may be clinically relevant.

The neuromuscular transmission is controlled by a sophisticated interaction between the activation of presynaptic inhibitory and facilitatory receptors, which is dependent on the neuronal firing pattern, including the number, frequency and duration of each stimulus (Wessler, 1989). Adenosine, released as such or buildup from ATP catabolism during neuronal firing, plays a key role in adjusting the modulatory pattern of neuromuscular transmission to the stimulation conditions (Correia-de-Sá et al., 1996). Fine-tuning synaptic control by adenosine emerges via subtle modifications at the presynaptic interreceptor dynamics (Sebastião and Ribeiro, 2000) involving intracellular second messengers, such as cyclic AMP (Correia-de-Sá and Ribeiro, 1994) and calcium (Correia-de-Sá et al., 2000). Our group provided evidence suggesting that adenosine generated from released ATP activates preferentially facilitatory A_{2A} receptors, both at rat hippocampal and neuromuscular synapses (Correia-de-Sá et al., 1996; Cunha et al., 1996). Tonic activation of adenosine A_{2A} receptors operates a coordinated shift in Ca^{2+} channel dynamics leading to facilitation of ACh release, from the "prevalent" $Ca_v2.1$ (P-type) to the "facilitatory" Ca_v1 (L-type) channel (Correia-de-Sá et al., 2000), which may contribute to overcome tetanic depression during high-frequency neuronal firing (Oliveira et al., 2004). This adaptive mechanism seems to be severely affected in myasthenic motor endplates, but we demonstrated that it may be rehabilitated by A_{2A} receptor activation (Noronha-Matos et al., 2011).

In addition to its stimulatory action on skeletal muscle fibres, ACh may also acts presynaptically to regulate its own release at mammalia neuromuscular junctions. Neuronal nicotinic receptors expressing α3β2 subunits (Faria et al., 2003) mediate a short term positive feedback mechanism, which is terminated by rapid autodesensitization (Wessler et al., 1986; Colquhon et al., 1989). Interestingly, nicotinic-induced transmitter overflow is regulated by synchronous activation of adenosine A_{2A} receptors (Timóteo et al., 2003). Transmitter release is also controlled by presynaptic muscarinic receptors via the activation of facilitatory M_1 and inhibitory M_2 receptors (see *e.g.* Wessler, 1989; Oliveira et al., 2002). The synaptic levels of adenosine accumulated during neuronal firing fine-tuning modulate the muscarinic tonus (Oliveira et al., 2002). Muscarinic M_1 autofacilitation predominates during low stimulation frequencies (5 Hz) when small amounts of extracellular adenosine activate preferentially inhibitory A_1 receptors (Correia-de-Sá et al., 1996). During high frequency (50 Hz) stimulation bursts, there is a shift from muscarinic M_1 facilitation towards M_2-mediated inhibition, which results mainly from the activation of A_{2A} receptors by high adenosine amounts formed from the catabolism of released adenine nucleotides. Thus, impairment of adenosine accumulation in myasthenic motor endplates due to reduced skeletal muscle activity and/or to low amounts of released adenine nucleotides (Noronha-Matos et al., 2011), might contribute to tetanic failure of neuromuscular transmission reflecting a loss of the nicotinic and muscarinic autoreceptor control in myasthenics.

Considering that dominant neuromodulatory actions of corticosteroids are observed during high-frequency neuronal activity and that this situation coincides with the predominant facilitatory tonus of adenosine (via A_{2A} receptors), we tested whether these receptors are involved in the amplification effect of methylprednisolone on transmitter exocytosis triggered by tetanic stimulation of the phrenic motor nerve using myographic recordings, radioisotope neurochemistry and video microscopy with the FM4-64 fluorescent dye (see *e.g.* Noronha-Matos et al., 2011; Correia-de-Sá et al., 2013). The presynaptic involvement of muscarinic (M_1 and M_2) and adenosine A_1 receptors on corticosteroid-induced facilitation of transmitter exocytosis was also investigated.

2. Material and methods

2.1. Animals

Rats (Wistar, 150–250 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 for at least ten days prior to the experiments, with food and water *ad libitum*. The animals were killed after stunning followed by exsanguination. Animal handling and experiments were in accordance with the guidelines prepared by the Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All animal studies comply with the ARRIVE guidelines.

2.2. Experiments to measure the release of [^{3}H]ACh and ATP from phrenic nerve-hemidiaphragm preparations

The experiments were performed using either the left or the right phrenic nerve-hemidiaphragm preparations (4–6 mm width). The procedures used for labelling the preparations and measuring evoked [^{3}H]ACh release were as described previously (Correia-de-Sá et al., 1991, 2013; Correia-de-Sá and Ribeiro, 1996; Noronha-Matos et al., 2011) with minor modifications. Briefly, the preparations were mounted in 3-ml capacity Perspex chambers and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's 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. Nerve terminals were labelled for 40 min with 1 μM [^{3}H]choline (specific activity 2.5 μCi/nmol) 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) with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was measured in a Perkin Elmer TriCarb 2900TR scintillation spectrometer (% tritium efficiency: 58 ± 2%), after appropriate background subtraction, using aliquots of 2-ml bath samples collected automatically every 3 min. In some experiments, the ATP content of the samples was evaluated with the luciferin–luciferase ATP bioluminescence assay kit HS II (Roche