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Tuning adenosine A_1 and A_{2A} receptors activation mediates L-citrulline-induced inhibition of [^{3}H]-acetylcholine release depending on nerve stimulation pattern

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Abstract

The influence of nerve stimulation pattern on transmitter release inhibition by L-citrulline, the co-product of NO biosynthesis by nitric oxide synthase (NOS), was studied in the rat phrenic nerve-hemidiaphragm. We also investigated the putative interactions between NOS pathway and the adenosine system. L-Citrulline (10–470 μM), the NOS substrate L-arginine (10–470 μM) and the NO donor 3-morpholinylsydnoneimine (SIN-1, 1–10 μM), concentration-dependently inhibited [^{3}H]-acetylcholine ([^{3}H]-ACh) release from rat motor nerve endings. Increasing stimulus frequency from 5 Hz-trains to 50 Hz-bursts enhanced [^{3}H]-ACh release inhibition by L-arginine (47 μM) and L-citrulline (470 μM), whereas the effect of SIN-1 (10 μM) remained unchanged. NOS inhibition with N^{ω}-nitro-L-arginine (100 μM) prevented the effect of L-arginine, but not that of L-citrulline. Adenosine deaminase (2.5 U/ml) and the adenosine transport inhibitor, *S*-(*p*-nitrobenzyl)-6-thioinosine (10 μM), attenuated release inhibition by L-arginine and L-citrulline. With 5 Hz-trains, blockade of A_1 receptors with 1,3-dipropyl-8-cyclopentyl xanthine (2.5 nM), but not of A_{2A} receptors with ZM241385 (10 nM), reduced the inhibitory action of L-arginine and L-citrulline; the opposite was verified with 50 Hz-bursts. Blockade of muscarinic M_2 autoreceptors with AF-DX116 (10 nM) also attenuated the effects of L-arginine and L-citrulline with 50 Hz-bursts. L-Citrulline (470 μM) increased basal adenosine outflow via the equilibrative nucleoside transport system sensitive to NBTI (10 μM), without significantly ($P > 0.05$) changing the nucleoside release subsequent to nerve stimulation.

Data indicate that NOS-derived L-citrulline negatively modulates [^{3}H]-ACh release by increasing adenosine outflow channelling to A_1 and A_{2A} receptors activation depending on the stimulus paradigm. While adenosine acts predominantly at inhibitory A_1 receptors during 5 Hz-trains, inhibition of ACh release by L-citrulline at 50 Hz-bursts depends on the interplay between adenosine A_{2A} and muscarinic M_2 receptors.

Keywords: Neuromuscular junction; Nitric oxide; L-Citrulline; Adenosine; Muscarinic M_2 receptor; Acetylcholine release

Abbreviations: ACh, acetylcholine; ADA, adenosine deaminase; ADO, adenosine; AF-DX 116, 11-[2-1[(diethylamino)methyl-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; BAY 41-2272, 3-(4-amino-5-cyclopropyl-pyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-*b*]pyridine; CGS21680C, 2-(4-[2-*p*-carboxyethylphenylamino])-5′-*N*-etylcarboxamide adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EHNA, erythro-9(2-hydroxy-3-nonyl) adenine; L-NOARG, N^{ω}-nitro-L-arginine; NBTI, *S*-(*p*-nitrobenzyl)-6-thioinosine; NO, nitric oxide; NOS, nitric oxide synthase; OCT, ornithine carbamoyltransferase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; R-PIA, *R*-N^6-phenylisopropyl adenosine; SIN-1, 3-morpholinosydnonimine hydrochloride; ZM 241385; 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo [2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)-phenol.

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1. Introduction

Nitric oxide (NO) is a free radical with signalling functions in the central and peripheral nervous systems (Garthwaite and Boulton, 1995). It has been implicated in several processes affecting neuronal differentiation, connectivity and plasticity (Baranano et al., 2001). NO is produced by nitric oxide synthase (NOS) during equimolar conversion of L-arginine to L-citrulline, with the participation of NADPH as a cofactor (Feldman et al., 1993). Three isoforms of NOS exist, namely neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Paakkari and Lindsberg, 1995). Of the three isoforms of NOS described, nNOS is considered predominant at the neuromuscular junction and seems to be localized mainly in the cytoplasm of pre-synaptic nerve terminals and adjacent postsynaptic muscle membrane (Ribera et al., 1998; Rothe et al., 2005). Regional distribution and levels of nNOS may be altered during development and ageing (Blottner and Luck, 2001), and in some specific diseases (e.g. Duchenne muscular dystrophy, myasthenia gravis) (Yang et al., 1997; reviewed by Stamler and Meissner, 2001). Upregulation of nNOS was detected in terminal Schwann cells covering denervated neuromuscular junctions (Ribera et al., 1998). There is a close relationship between nNOS activity and depression of neuromuscular transmission (Wang et al., 1995; Thomas and Robitaille, 2001). For instance, tetanic fade may be recorded when neuromuscular preparations are incubated with the NOS substrate, L-arginine; an effect that can be prevented by application of the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NOARG), or atropine (Silva et al., 1999). Although data obtained from miographic studies offer indirect evidences for the pre-synaptic action of these compounds, their effects on the release of ACh remain elusive.

In spite of the extensive work that has been done on the relationship between NO and synaptic transmission, little is known about the role of L-citrulline on cell signalling. Until recently, L-citrulline attracted relative little interest, almost certainly because it is a non-protein amino acid. However, recent studies have underlined the importance of this amino acid in both cellular metabolism and in monitoring organ functionality (for a review see, Curis et al., 2005). Because L-citrulline can be easily converted into L-arginine by the successive action of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), some authors suggested that L-citrulline might be an indirect precursor of NO in NO-synthesising cells (Wu and Morris, 1998; Mori and Gotoh, 2000). Few studies revealed the possibility that L-citrulline might not be merely a by-product of the NO-synthesis, but could also play a role in the modulation of cell responses (Ruiz and Tejerina, 1998). Localization of endogenous nNOS at pre-synaptic nerve terminals prompted us to investigate the role of L-citrulline on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from the rat phrenic motor nerve terminals and how could this be modified by nerve stimulation conditions. Preliminary accounts of some of the results have already been published (Barroso et al., 2005).

Interactions between NOS activity and the adenosine system are well established in the central nervous system (Boulton et al., 1994; Broome et al., 1994; Fallahi et al., 1996; Broad et al., 2000; Rosenberg et al., 2000; Bon and Garthwaite, 2002), but there is less information on this topic in peripheral tissues (see, e.g. Nickels et al., 2007). Functional evidence that adenosine controls acetylcholine (ACh) release at the mammalian motor endplate via subtle modifications of the pre-synaptic inter-receptor dynamics, has been provided. These, involve activation of A_1-inhibitory and A_{2A}-facilitatory receptors (Correia-de-Sá et al., 1996; Oliveira et al., 2002) and generation of intracellular second messengers, such cyclic AMP (Correia-de-Sá and Ribeiro, 1994; Oliveira and Correia-de-Sá, 2005) and Ca^{2+} (Correia-de-Sá et al., 2000a; Oliveira et al., 2004). The effect of endogenous adenosine depends on the concentration of the nucleoside at the synapse (Nagano et al., 1992; Correia-de-Sá and Ribeiro, 1996). Extracellular adenosine can originate from the release of adenosine as such via equilibrative nucleoside transporters or can be formed upon the sequential extracellular dephosphorylation of ATP released synchronously with ACh (Silinsky, 1975; Magalhães-Cardoso et al., 2003; reviewed by Cunha, 2001). Interestingly, which adenosine receptor is predominantly activated is apparently determined by the differential contribution of the two main pathways leading to extracellular adenosine accumulation (Correia-de-Sá and Ribeiro, 1996; Cunha et al., 1996). Adenosine acts predominantly as an inhibitory signal (via A_1 receptors) when nerve terminals are stimulated with a frequency (5 Hz-trains) close to the firing rate of rat phrenic motoneurons during quiet ventilation (Roszek et al., 1994). The A_1 receptor is responsible for the in-vivo adenosine potentiation of the neuromuscular effects of non-depolarising blocking agents (Nitahara et al., 2005). Synaptic adenosine generated from the hydrolysis of released ATP raises drastically during high frequency (50 Hz) bursts to levels capable of activating facilitatory A_{2A} receptors (Correia-de-Sá et al., 1996). Coincidentally, the amount of evoked transmitter release in both stimulation conditions (5 Hz-trains and 50 Hz-bursts) was not significantly different (Correia-de-Sá et al., 1996), indicating that A_{2A} receptor-mediated facilitation must be balanced by an undisclosed mechanism to avoid ACh overflow during intense periods of stimulation. One hypothesis would be a co-ordinated shift on muscarinic neuromodulation during high-frequency bursts, from M_1-facilitation to M_2-inhibition of ACh output, as adenosine A_{2A} receptors activity is favoured (Oliveira et al., 2002). On the other hand, previous reports have implicated endogenous adenosine accumulation as a key player operating the inhibitory actions of NOS pathway in the nervous system (Fallahi et al., 1996; Rosenberg et al., 2000; Nickels et al., 2007). At the amphibian neuromuscular junction, it has also been demonstrated that purinergic signalling could be modulated by the production of NO during high frequency nerve stimulation (50 Hz, 30 s) by indirectly modulating perisynaptic Schwann cells activation (Thomas and Robitaille, 2001). The present work was undertaken to test the putative interactions between NOS pathway and the adenosine system at the rat motor endplate, giving particular emphasis to the neuromodulatory role of L-citrulline.