ELIANE APARECIDA CAMPESATTO MELLA

EFEITOS DO ÓXIDO NÍTRICO (NO) E DA L-CITRULINA SOBRE A AUTOREGULAÇÃO NEURONAL MEDIADA POR RECEPTORES MUSCARÍNICOS E ADENOSINÉRGICOS EM PREPARAÇÕES NEUROMUSCULARES DE RATOS

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

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ORIENTADOR: PROF. DR. WILSON ALVES-DO-PRADO

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DEDICATÓRIA

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RESUMO

A Acetilcolina (Ac) é o neurotransmissor responsável pela transmissão neuromuscular e encontra-se armazenada em vesículas no terminal nervoso motor (TNM). A liberação de Ac a partir do TNM ocorre quando o potencial de ação propagado promove a abertura de canais de Ca^{2+}, determinando um aumento nos níveis intracelulares deste íon.

Uma vez na fenda sináptica, a Ac pode interagir tanto a nível pré-sináptico como pós-sináptico e/ou ser hidrolisada pela acetilcolinesterase. Ao interagir com sítios nicotínicos pós-sinápticos, a Ac altera a condutância da membrana celular para os íons Na^+, K^+ e Ca^{++} que, atingindo o limiar de despolarização, deflagra o potencial de placa terminal (ppt) iniciando, dessa forma, o processo da contração. A interação da Ac com sítios nicotínicos pré-sinápticos resulta em aumento na liberação de Ac, com o intuito de garantir a transmissão neuromuscular quando grandes demandas de neurotransmissor são necessárias. Quando os receptores muscarínicos do TNM são ativados pode ocorrer uma redução ou um aumento na quantidade de neurotransmissor liberada para a fenda sináptica, dependendo da freqüência e da duração dos estímulos elétricos aplicados no nervo motor.

Quando o nervo motor recebe estímulos elétricos com freqüências entre 30-80 Hz, as contrações musculares registradas são bem sustentadas. Entretanto, contração não sustentada, denominada fadiga de transmissão, fadiga tetânica ou inibição de Wedensky, pode ser observada quando freqüências de estimulação maiores que 100 Hz são utilizadas. A fadiga tetânica também pode ser observada com estímulos elétricos menores que 100 Hz se as preparações neuromusculares forem tratadas com agentes colinolíticos nicotínicos ou com agentes que aumentam a liberação de acetilcolina a partir do TNM.

A autoregulação neuronal mediada por receptores muscarínicos é iniciada pelo acionamento dos receptores muscarínicos estimulatórios do subtipo M_1 quando baixas freqüências de estimulação (5 Hz) são aplicadas no nervo motor. Todavia, o acionamento dos receptores M_1 é deslocado para a ativação dos receptores muscarínicos M_2 quando altas freqüências de estimulação são utilizadas sobre o nervo motor.

A Ac e o ATP são co-liberados pelo nervo motor. Ao ser metabolizado na fenda sináptica por uma cascata de ecto-nucleotidases o ATP dá origem a adenosina. Receptores adenosinérgicos inibitórios e excitatórios também estão presentes no TNM modulando a liberação de Ac. Quando o nervo motor é estimulado com pulsos de 5 Hz (freqüência que mimetiza o ritmo respiratório) ocorre o predomínio do tônus adenosinérgico inibitório, mediado por receptores do subtipo A_1. Por outro lado, quando trens de pulsos de 50 Hz (freqüência que mimetiza o movimento voluntário), com períodos de intervalos de 20 segundos, são aplicados no nervo motor, ocorre predominância do tônus A_{2A}-facilitatório sobre a liberação de ^{3}H-Acetilcolina ([^{3}H]ACh). Este mesmo efeito facilitatório (via receptor A_{2A}) pode ser evidenciado sob estímulos de baixa freqüência com a utilização de drogas que aumentam as concentrações de adenosina na fenda sináptica como o S-(*p*-nitrobenzyl)-6-tioinosina (30 µM) (NBTI), bloqueador da captação de adenosina e/ou eritro-9(2-hidroxi-3-nonil)adenina (50 µM) (EHNA), inibidor da adenosina desaminase (ADA). Assim, o balanço para a ativação dos receptores A_1-inibitório/A_{2A}-facilitatório é dependente dos níveis de adenosina na fenda sináptica. O padrão de estimulação utilizado sobre as preparações neuromusculares é que determina a maior ou menor formação deste neuromodulador.

A adenosina formada durante os disparos neuronais modula o balanço muscarínico M_1/M_2 de modo que, ao se empregar estímulos de baixas freqüências sobre o nervo motor, o tônus M_1 predomina exercendo um efeito inibitório sobre os receptores M_2. Este efeito é

sinergicamente potencializado por receptores A_1. Por outro lado, quando trens de estímulos de 50 Hz são aplicados sobre o nervo motor, o balanço neuromodulatório se altera. Em tal situação, o tônus M_2-inibitório passa a predominar, pois, além dos altos níveis de Ac na fenda sináptica atuarem preferencialmente sobre receptores M_2, a maior formação de adenosina ativa receptores A_{2A} que então exercem um efeito negativo sobre a inibição que M_2 sofre de M_1.

O óxido nítrico (NO) é um gás produzido endogenamente a partir do aminoácido L-arginina (L-arg) por uma enzima enantiomericamente específica denominada NO-sintase (NOS) e apresenta como co-produto a L-citrulina. A NOS se distribui largamente pelo organismo, nos mais diversos tipos de células, apresentando três isoformas distintas. As isoformas da NOS que estão presentes na junção neuromuscular são a NOS I e II. Ao contrário da NOS I, a NOS do subtipo II é cálcio/calmodulina-independente.

O NO, atua sobre a enzima guanilato ciclase solúvel (GC) para exercer seus efeitos biológicos através do aumento nos níveis intracelulares de guanosina monofosfato cíclica (GMPc). O NO pode atuar direta ou indiretamente sobre os tecidos biológicos. Sua ação indireta é mediada por GMPc.

Em preparações neuromusculares de ratos demonstra-se que o NO pode aumentar a amplitude de contrações musculares a 0,2 Hz ou induzir fadiga de transmissão se a freqüência de estimulação aplicada ao nervo motor for elevada para 50 Hz. Os efeitos neuromusculares do NO têm sido investigados com o uso de técnicas que envolvam registros miográficos. Contudo, os efeitos do NO sobre a transmissão não foram, até o momento, investigados com técnicas capazes de avaliar a liberação do neurotransmissor. Assim, parte do presente trabalho foi realizada para verificar os efeitos da L-Arg na liberação da [^{3}H]ACh do TNM de ratos tratados ou não com antagonista de receptores M_2 (11- [[2- 1 [(diethylamino) methyl1- 1-piperidinyl]- acetyl]- 5, 11- dihydro- 6H- pyrido [2, 3- b] [1, 4] benzodiazepine-6-one, AF-DX 116), inibidor de NOS (N^{ω}-nitro-L-arginine, L-NOARG), antagonista de receptores A_1 (1,3dipropyl-8-cyclopentylxanthine, DPCPX), adenosina deaminase (EC 3.5.44, ADA) ou antagonista de receptor A_{2A} ((4-(2-[7-amino-2- (2-furyl{1,2,4}-triazolo{2,3-a-{1,3,5}triazin-5-yl-aminoethyl)phenol, ZM241385).

L-arg (47 μM) reduziu a liberação evocada de [^{3}H]ACh em 26±6% (*n*=11) e 43±2% (*n*=5), quando o nervo frênico foi estimulado a 5 Hz e 50 Hz, respectivamente. L-NOARG (100 μM) antagonizou a redução da liberação [^{3}H]ACh induzida por L-arg a 5 Hz e 50 Hz. AF-DX 116 (10 nM) não modificou o efeito inibitório induzido por L-Arg a 5 Hz, mas promoveu um efeito excitatório a 50 Hz. ADA (0,5 U/ml) reduziu o efeito de L-arg a 5 Hz e 50 Hz. DPCPX (2,5 nM) reduziu o efeito inibitório da L-Arg a 5 Hz, mas não modificou o efeito a 50 Hz. ZM241385 (10 nM) reduziu a inibição da liberação de [^{3}H]ACh produzida por L-arg a 50 Hz, mas não modificou o efeito a 5 Hz. AF-DX-116 antagonizou o efeito inibitório produzido por oxotremorina (10 μM) na liberação de [^{3}H]ACh a 50 Hz.

A L-citrulina é um aminoácido amplamente encontrado em mamíferos, fortemente relacionado a L-Arg. Em hepatócitos a L-Citrulina é sintetizada localmente pela enzima Ornitina carbamoiltransferase (OCT) e metabolizada pela argininosuccinato sintetase (ASS) em uréia. Entretanto, na produção de NO tecidual, L-citrulina é um co-produto na biossíntese do NO pela enzima NOS. Assim, a L-citrulina pode ser facilmente convertida em L-Arg pela ação sucessiva da ASS e argininosuccinato liase (ASL), que são expressas em todas as células, incluindo neurônios. Alguns autores sugerem que a L-citrulina pode ser um precursor indireto do NO e que este aminoácido não seria simplesmente um produto da síntese do NO, mas poderia também estar envolvido na sinalização de algumas células.

Pouco se sabe a respeito da ação da L-citrulina na transmissão sináptica, isto é, no controle da liberação de neurotransmissores. Este fato levou-nos a também investigar os efeitos deste aminoácido sobre as contrações musculares e sobre a liberação evocada da [^{3}H]ACh em comparação com os efeitos causados pelo substrato de NOS, L-arg, e pelo doador de NO, 3-morpholinosydnonimine chloride (SIN-1) em preparações neuromusculares estimuladas a 5 Hz.

L-Arg (0.01-4.7 mM), concentração dependente, reduziu a liberação evocada de [^{3}H]ACh. SIN-1 (1-100 µM) e L-citrulina (0.01-4.7 mM) mimetizaram o efeito inibitório da L-arg. As concentrações exigidas para deprimir a liberação em 30% foram respectivamente 10 µM SIN-1, 47 µM L-arg e 470 µM L-citrulina. L-citrulina (0.01-47 mM) também reduziu as tensões no diafragma causadas por estimulação elétrica de forma concentração dependente. A potência de inibição ocorreu na ordem: SIN-1>L-arg>L-citrulina.

L-NOARG (100 µM, aplicado durante todo teste, incluindo S_1 e S_2) preveniu a ação depressora da L-Arg (47 µM), mas o efeito inibitório da L-citrulina (470 µM) permaneceu inalterado (P>0.05). A inativação da GC com 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 µM) atenuou parcialmente a inibição da L-arg (47 µM) e preveniu totalmente o efeito inibitório do SIN-1 (10 µM). ODQ foi incapaz de alterar a ação inibitória da L-citrulina (470 µM). L-NOARG e ODQ incrementaram a liberação evocada de [^{3}H]ACh em 22±1% (n=4) e 34±7% (n=5), respectivamente, indicando que NOS e GC são tonicamente ativados para controlar a liberação de neurotransmissores do TNM.

ADA (0,5 U/ml) ou NBTI (10 µM), atenuou os efeitos da L-arg e L-citrulina, enquanto a inibição pelo SIN-1 manteve-se inalterada. Resultados semelhantes foram obtidos com o antagonista seletivo de receptores A_1 DPCPX (2.5 nM). O bloqueio dos receptores A_{2A} com ZM241385 (10 nM) foi incapaz de modificar os efeitos inibitórios de L-Arg (47 µM), L-citrulina (470 µM) e SIN-1 (10 µM).

Os dados sugerem que:

O controle da liberação de Ac na placa motora resulta de uma complexa interação entre a síntese de mediadores intracelulares difusíveis, como o NO, e a atividade tônica de receptores pré-sinápticos operada por substâncias liberadas pelo neurônio motor. A adenosina tem um papel fundamental, pois pode exercer as suas ações diretamente através dos receptores inibitórios do subtipo A_1, ou indiretamente através do controle da atividade muscarínica (M_1-facilitatória / M_2-inibitória) pela ativação de receptores $A_{2A.}$

NOS catalisa a formação de dois produtos neuronalmente ativos, NO e L-citrulina. Enquanto NO pode agir diretamente para reduzir a liberação de neurotransmissor através da estimulação da GC, a ação inibitória da L-citrulina pode ser secundária ao induzir a liberação de adenosina que estimula os receptores A_1 inibitórios.

Os resultados obtidos desses estudos permitiram a elaboração de dois artigos científicos, que serão aqui apresentados seguindo as normas impostas pelas revistas para as quais foram enviados. Os artigos encontram-se na seguinte ordem de apresentação:

1. The reduction by nitric oxide on evoked [^{3}H]-Ach release in the phrenic nerve-hemidiaphragm preparations of rats is mediated by A_1 receptor at 5 Hz and by cross- talk between A_{2A} and M_2 receptors at 50 Hz. Artigo enviado para a revista European Journal of Pharmacology.
2. L-Citrulline inhibits [^{3}H]acetylcholine release from rat motor nerve terminals by increasing adenosine outflow channelling to A_1 receptor activation. Artigo publicado na revista British Journal of Pharmacology. Online publication, April 2, 2007; doi:10.1038/sj.bjp.0707242.

ABSTRACT

Acetylcholine (Ac) is the neurotransmitter responsible for the neuromuscular transmission and is stored in vesicles at the motor nerve terminal (MNT). The release of Ac fro the MNT occurs when the propagated action potential causes the opening of Ca^{2+} channels, determining an increase on the intracellular levels of this ion.

Once at the synaptic cleft, Ac can interact both presynaptically and postsynaptically and/or be hydrolyzed by acetylcholinesterase. When interacting with postsynaptic nicotinic sites, Ac alters the cell membrane conductance to Na^+, K^+ and Ca^{2+} which, reaching the depolarization threshold triggers the terminal plate potential (tpp) and setting into motion the contraction process. The interaction of Ac with presynaptic nicotinic sites results in increased release of Ac, with the purpose of assuring the neuromuscular transmission when large demands of neurotransmitter are needed. When the MNT muscarinic receptors are activated, a decrease or an increase in the amount of neurotransmitter released at the synaptic cleft may occur, depending on the frequency and duration of the electric stimuli applied to the motor nerve.

When the motor nerve receives frequency electric stimuli between 30-80 Hz, the muscle contrations recorded are well-sustained. However, a non-sustained contraction, named transmission fade, tetanic fade or Wedensky inhibition, can be observed when stimulation frequencies greater than 100 Hz are used. Tetanic fade can also be observed with electric stimuli smaller than 100 Hz if the neuromuscular preparations are treated with nicotinic cholinolytic agents or with agents that increase the release of Ac from the MNT.

The neuronal autoregulation through muscarinic receptors begins with the recruitment of M_1-subtype stimulatory muscarinic receptors when low stimulation frequencies (5 Hz) are applied to the motor nerve. Nevertheless, the recruitment of M_1 receptors is replaced for the activation of M_2 muscarinic receptors when high stimulation frequencies are used on the motor nerve.

Ac and ATP are co-released from the motor nerve. When metabolized at the synaptic cleft by a cascade of ecto-nucleotidases ATP produces adenosine. Inhibitory and excitatory adenosinergic receptors are present as well at the MNT, modulating the release of Ac. When the motor nerve is stimulated with 5Hz pulses (a frequency that mimics the respiratory rhythm) there is a predominance of inhibitory adenosinergic tonus, mediated by A_1-subtype receptors. On the other hand, when burts of 50 Hz (a frequency that mimics voluntary movement), with intervals of 20 seconds, are applied to motor nerve, there is a predominance of A_{2A} facilitatory tonus on the release of ^{3}H-acetycholine ([^{3}H]Ach). This same facilitatory effect (via A_{2A} receptor) can be seen under low-frequency stimuli with the use of drugs that increase the concentrations of adenosine at the synaptic cleft, such as S-(*p*-nitrobenzyl)-6-thioinosine (30 µM) (NBTI), a blocker of adenosine uptake, and/or erythro-9(2-hidroxi-3-nonil)adenine (50 µM) (EHNA), inhibitor of adenosine deaminase (ADA). Therefore, the balance for the activation of A_1-inhibitory/A_{2A}-facilitatory receptors is dependent on the levels of adenosine at the synaptic cleft. The stimulation pattern used on the neuromuscular preparations is what determines the greater or lesser formation of this neuromodulator.

The adenosine formed during the neuronal discharges modulates the M_1/M_2 muscarinic balance in such a way that, when low-frequency stimuli are applied to the motor nerve, the M_1 tonus predominates and exerts an inhibitory effect upon the M_2 receptors. This effect is synergistically potentialized by A_1 receptors. On the other hand, when 50 Hz (bursts) of impulses are applied to the motor nerve, the neuromodulatory balance changes. In this

situation the M_2 inhibitory tonus predominates, because in addition to the high levels of Ac at the synaptic cleft acting preferentially on the M_2 receptors, the larger formation of adenosine activates A_{2A} receptors, that in turn exert a negative effect on the inhibition that M_2 suffers from M_1.

Nitric oxide (NO) is a gas endogenously produced from the aminoacid L-arginine (L-arg) by an enantiomerically specific enzyme named NO-sinthase (NOS) and yielding L-citrulline as a by-product. NOS is widely distributed in the organism, on the most diverse cell types, showing three distinct isoforms. The NOS isoforms that are present at the neuromuscular junction are NOS I and II. Contrary to NOS I, subtype II-NOS is calcium/calmodulin-independent.

NO acts on the soluble enzyme guanilate cyclase (GC) to produce its biological effects indirectly by the increase on the intracellular levels of cyclic guanosine monophosphate (cGMP). NO can act either directly or indirectly on biological tissues. Its indirect action is mediated by cGMP.

In neuromuscular preparations of rats it has been demonstrated that NO can increase the amplitude of muscle contrations at 0.2 Hz or induces transmission fade if the stimulation frequency applied on the motor nerve is increased to values upper than 50 Hz. The effects of NO on neuromuscular transmission have been investigated using miographic record. However, the effects of NO have not been investigated using techniques that determine the release of Ac from motor nervous ending. Therefore, part of the present work was carried out to verify the effects of L-arg on the release of [^{3}H]ACh from the NMT of rats treated or not with the M_2 receptor antagonist (11-[[2-1[(diethylamino)methyl1-1-piperidinyl]- acetyl]-5,11-dihydro-6H-pyrido[2, 3- b][1, 4]benzodiazepine-6-one, AF-DX 116), inhibitor of NOS (N^{ω}-nitro-L-arginine, L-NOARG), A_1 receptor antagonist (1,3dipropyl-8-cyclopentylxanthine, DPCPX), adenosine deaminase (EC 3.5.44, ADA) or A_{2A} receptor antagonist [(4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a-{1,3,5}triazin-5-yl-aminoethyl) phenol, ZM241385].

L-arg (47 μM) decreased the evoked release of [^{3}H]ACh to 26±6% (*n*=11) and 43±2% (*n*=5), when the phrenic nerve was stimulated at 5 Hz and 50 Hz (bursts), respectively. L-NOARG (100 μM) antagonized the decrease on the release of [^{3}H]ACh induced by L-arg at 5 Hz and 50 Hz. AF-DX 116 (10 nM) did not change the inhibitory effect induced by L-arg at 5 Hz, but promoted an excitatory effect at 50 Hz. ADA (0.5 U/mL) reduced the effect of L-arg at 5 Hz and 50 Hz. DPCPX (2.5 nM) reduced the inhibitory effect of L-arg at 5 Hz, but did not change the effect at 50 Hz. ZM241385 (10 nM) reduced the inhibition of the release of [^{3}H]ACh produced by L-arg at 50 Hz, but did not change the effect at 5 Hz. AF-DX-116 antagonized the inhibitory effect produced by oxetremorine (10 μM) on the release of ([^{3}H]ACh) at 50 Hz.

L-citrulline is an aminoacid widely found in mammals, strongly linked to L-arg. In hepatocytes, L-citrulline is synthesized locally by the enzyme ornitine carbamoiltransferase (OCT) and metabolized by argininosuccinate synthase (ASS) to urea. However, in the production of tissue NO, L-citrulline is a by-product in the biosynthesis of NO by the enzyme NOS. Thus, L-citrulline can be easily converted to L-arg by the successive action of ASS and argininosuccinate liase (ASL), which are expressed in all the cells, including neurons. Some authors suggest that L-citrulline can be an indirect precursor of NO and that this aminoacid would not be simply a product of NO synthesis, but could be involved in signaling at some cells as well.

Little is known with regard to the action of L-citrulline in synaptic transmission, that is, in the control of neurotransmitter release. This led us also to investigate the effects of this aminoacid on the muscle contractions and on the evoked release of [^{3}H]ACh in comparison to the effects caused by the NOS substrate, L-arg, and by the NO donor, 3-morpholinosydnonimine chloride (SIN-1), in neuromuscular preparations stimulated at 5 Hz.

L-arg (0.01-4.7 mM) reduced the evoked release of [^{3}H]ACh in a concentration-dependent manner. SIN-1 (1-100 µM) and L-citrulline (0.01-4.7 mM) mimicked the inhibitory effect of L-arg. The concentrations demanded to depress the release in 30% were, respectively, 10 µM SIN-1, 47 µM L-arg and 470 µM L-citrulline. L-citrulline (0.01-47 mM) also decreased the tensions on the diaphragm caused by electric stimulation in a concentration-dependent manner. The potency of inhibition occurred in the order: SIN-1>L-arg>L-citrulline.

L-NOARG (100 µM, applied during the whole test, including S1 and S2) prevented the depressant action of L-arg (47 µM), but the inhibitory effect of L-citrulline (470 µM) remained unchanged ($P>0.05$). Inactivation of GC with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 µM) partially attenuated the L-arg inhibition (47 µM) and totally prevented the inhibitory effect of SIN-1 (10 µM). ODQ was incapable of changing the inhibitory action of L-citrulline (470 µM). L-NOARG and ODQ enhanced the evoked release of [^{3}H]ACh to 22±1% (n=4) and 34±7% (n=5), respectively, indicating that NOS and GC are tonically activated to control the release of neurotransmitters from the MNT.

ADA (0,5 U/ml) or NBTI (10 µM), smoothed the effects of L-arg and L-citrulline, while the inhibition by SIN-1 was unchanged. Similar results were obtained with A_1 receptors selective antagonist, DPCPX (2.5 nM). The blockade of A2A receptors with ZM 241385 (10 nM) was incapable of changing the inhibitory effects of L-arg (47 µM), L-citrulline (470 µM) and SIN-1 (10 µM).

The data suggest that:

The control of Ac release at the motor plate results from a complex interaction between the synthesis of diffusible intracellular mediators, such as NO, and the tonic activity of presynaptic receptors carried out by substances released by the motor neuron. Adenosine has a key role, because it can exert its actions directly through A_1-subtype inhibitory receptors, or indirectly through the control of the muscarinic activity (M_1-facilitatory/M_2-inhibitory) through the activation of A_{2A} receptors.

NOS catalyzes the formation of two neuronally active products, NO and L-citrulline. While NO can act directly to reduce the release of neurotransmitter through the stimulation of GC, the inhibitory action of L-citrulline may be secondary to adenosine outflow channelling to inhibitory A_1 receptors activation.

The results obtained from these studies allowed the writing of two scientific articles, which are presented here following the rules imposed by the journals to which they were sent. The articles are in the following order of presentation:

1. The reduction by nitric oxide on evoked [^{3}H]-Ach release in the phrenic nerve-hemidiaphragm preparations of rats is mediated by A_1 receptor at 5 Hz and by cross-talk between A_{2A} and M_2 receptors at 50 Hz. Article sent to the European Journal of Pharmacology.
2. L-Citrulline inhibits [^{3}H]acetylcholine release from rat motor nerve terminals by increasing adenosine outflow channeling to A_1 receptor activation. Article published at the British Journal of Pharmacology. British Journal of Pharmacology. Online publication, April 2, 2007; doi:10.1038/sj.bjp.0707242.

LISTA DE ABREVIATURAS

[^{3}H]ACh: ^{3}H-Acetilcolina

Ac: Acetilcolina

ADA: adenosina desaminase

AF-DX 116: 11- [[2- 1 [(diethylamino) methyl1- 1- piperidinyl]- acetyl]- 5, 11- dihydro- 6H- pyrido [2, 3- b] [1, 4] benzodiazepine-6-one

ASL: argininosuccinato liase

ASS: argininosuccinato sintetase

DPCPX: 1,3dipropyl-8-cyclopentylxanthine

EHNA: eritro-9(2-hidroxi-3-nonil)adenina

GC: guanilato ciclase solúvel

GMPc: guanosina monofosfato cíclica

L-arg: L-arginina

L-NOARG: N^{ω}-nitro-L-arginine

NBTI: S-(*p*-nitrobenzyl)-6-tioinosina

NO: óxido nítrico

NOS: NO-sintase

OCT: Ornitina carbamoiltransferase

ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

ppt: potencial de placa terminal

SIN-1: 3-morpholinosydnonimine chloride

TNM: terminal nervoso motor

ZM241385: (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a-{1,3,5}triazin-5-yl-aminoethyl)phenol)

The reduction by nitric oxide on evoked [^{3}H]-Ach release in the phrenic nerve-hemidiaphragm preparations of rats is mediated by A_1 receptor at 5 Hz and by cross- talk between A_{2A} and M_2 receptors at 50 Hz

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Abstract

Current work was undertaken to verify the effects of L-arginine on [^{3}H]-acetylcholine released from the motor nerve ending of rats treated or not with M_2 receptor antagonist (11-[[2- 1 [(diethylamino) methyl1- 1- piperidinyl]- acetyl]- 5, 11- dihydro- 6H- pyrido [2, 3- b] [1, 4] benzodiazepine-6-one, AF-DX 116), A1 receptor antagonist (1,3dipropyl-8-cyclopentylxanthine, DPCPX), adenosine deaminase (EC 3.5.44, ADA), or A_{2A} receptor antagonist ((4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a-{1,3,5}triazin-5-yl-aminoethyl) phenol, ZM241385). L-arginine (47 to 4700 μM) decreased evoked [^{3}H]-Ach release at 5.0 Hz. The lowest concentration (47 μM) of L-arginine able to reduce evoked [^{3}H]-Ach release at 5.0 Hz produced higher inhibition when the nerve was stimulated at 50 Hz. L-NOARG (100 μM) and AF-DX 116 (10 nM) alones increased [^{3}H]-Ach release at 5.0 Hz and 50 Hz, but the facilitatory effect of AF-DX 116 was lower at 50 HZ. L-NOARG antagonized the reduction on [^{3}H]-Ach release induced by L-arginine at 5 Hz and 50 HZ. AF-DX 116 did not modify the inhibitory effect induced by L-arginine at 5 Hz, but it changed the inhibitory effect of amino acid to facilitatory effect when the preparations were stimulated at 50 Hz- burst. ADA (0.5 U/ml) reduced the inhibitory effect of L-arginine at 5 and 50 Hz. DPCPX (2.5 nM) reduced the inhibition caused by L-arginine at 5 Hz, but did not modify the inhibitory effect of amino acid at 50 Hz-burst. ZM241385 (10 nM) reduced [^{3}H]-Ach release inhibition produced by L-arginine at 50 Hz, but did not modify the inhibitory effect of amino acid at 5 Hz. AF-DX-116 antagonized the inhibitory effect produced by oxotremorine (10 μM) on evoked [^{3}H]-Ach release at 50 Hz. Data indicate that endogenous NO has important inhibitory physiological tonic action on motor nerve terminal. The inhibitory effect of gas in the phrenic nerve-hemidiaphragm preparations of rats is mediated by A_1 receptor at 5 Hz and by cross-talk between A_{2A} and M_2 receptors at 50 Hz-burst.

Introduction

The acetylcholine released from the motor nerve ending regulates its own output activating the nicotinic and muscarinic presynaptic receptors (See Bowman, 1980). The activation of such receptors increases (nicotinic and M_1 muscarinic receptors) and reduces (M_2 muscarinic receptors) the acetylcholine output, depending on frequency and duration of pulses applied on motor nerve (Somogyi *et al*., 1987; Wessler *et al*., 1988). It has been shown that the neuronal nicotinic receptors mediate a short-term positive feedback mechanism is terminated by rapid autodesensitization (Wessler *et al*., 1986; Colquhoun *et al*., 1989). However, the liberation of acetylcholine may be regulated by others substances released from motor nerve terminal (adenosine, calcitonin gene-related peptide, substance P, vasoactive e intestinal peptide) or postsynaptic (arachidonic acid, nitric oxide) sites (Van Der Kloot and Molgó, 1994). Adenosine buildup from ATP catabolism during neuronal firing plays a key role in adjusting the modulatory pattern of neuromuscular transmission to stimulation conditions. While adenosine acts as an inhibitory signal (via A_1 receptors) under resting conditions, amplification of neuromuscular transmission depends on crosstalk between A_{2A} receptors and receptors for other neurotransmitters and neuromodulators (Correia-de-Sá *et al*., 1997). When the muscarinic M_1 positive feedback loop is fully operatives at 5 Hz, both M_2 and A_{2A} receptors functions are suppressed, and acetylcholine overflow is controlled by endogenous formation of small amounts of adenosine acting via inhibitory A_1 receptors. On the other hand, the shift on muscarinic neuromodulation may be evidenced during high frequency bursts (50 Hz), as the activation of A_{2A} receptors by adenosine generates from catabolism of adenine nucleotide promotes M_2-autoinhibitory action by braking counteraction mediated by muscarinic M_1 receptors (Oliveira *et al*., 2002). Thus, it has been proposed that the M_1-positive feedback mechanism predominates over M_2-inhibition when the motor nerve is stimulated with pulses (5Hz) close to the firing rate of phrenic motor neurons during quiet ventilation of rats (Oliveira *et al*., 2002, Monteiro and Ribeiro, 1987). Nevertheless, such predominance is shifted to activation of M_2 presynaptic muscarinic receptor if the stimulations pulses are increased to 50 Hz (Oliveira *et al*., 2002).

Miographic studies have shown that skeletal muscles respond with a well-sustained contraction when the motor nerve is being electrically stimulated at 30 to 100 hz (Bowman,

1980). However, tetanic fade may be recorded even at 100 Hz if the neuromuscular preparations are treated with endogenous (L-arginine) or exogenous (nitric oxide gas, sodium nitroprusside, SIN 1, SNAP) nitric oxide donors (Silva *et al.*, 1999). Additionally, it has been shown that intra-arterial injections of atropine or a blocker of NO-synthase (L-NOARG), antagonize L-arginine-induced fade in neuromuscular preparations of cats. Thus, it has been proposed that fade induced by NO might be determined by previous increment on acetylcholine output, thereby stimulating the inhibitory presynaptic muscarinic receptors, then reducing the liberation of acetylcholine from the motor nerve terminal (Cruciol-Souza and Alves-do-Prado, 1999). Although data obtained from miographic record offer indirect evidences to presynaptic action of NO modifying the acetylcholine release from motor nerve ending, the effects of gas on release of acetylcholine have not been investigated. The current work was undertaken to verify the effects of L-arginine on [^{3}H]-acetylcholine released from the motor nerve ending of rats treated or not with M_2 receptor antagonist (11- [[2- 1 [(diethylamino) methyl1- 1- piperidinyl]- acetyl]- 5, 11- dihydro- 6H- pyrido [2, 3- b] [1, 4] benzodiazepine-6 -one, AF-DX 116), A_1 receptor antagonist (1,3dipropyl-8-cyclopentylxanthine, DPCPX), adenosine deaminase (EC 3.5.44, ADA) or A_{2A} receptor antagonist ((4-(2-[7-amino-2- (2-furyl{1,2,4}-triazolo{2,3-a-{1,3,5}triazin-5-yl-aminoethyl)phenol, ZM241385). The preparations were particularly stimulated with pulses of 5 Hz or 50 Hz, as motoneurones are firing at these frequencies during voluntary movements (Fischbach and Robbins, 1969, Grimby and Hannerz 1977; Grimby *et al*., 1979).

Materials and Methods

The left phrenic nerve-hemidiaphragm preparations (6-8 mm width) of Rats (Wistar, 150-200 g) were previously described (Correia-de-Sa *et al*., 1991). The preparations were mounted in 2 ml chambers, with direct oxygenation (95 % O_2 and 5% CO_2), kept at 37 ^oC, and superfused (3 ml min –1) with gassed Tyrode solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, and glucose 11.2.The nerve was drawn into a suction electrode for stimulation. The preparation was allowed to equilibrate, under superfusion with gassed Tyrode solution, for 30 min. The procedures used for labelling the preparations and measuring evoked [^{3}H]acetylcholine ([^{3}H]-ACh) release have been previously described (Correia-de-Sá *et al.*, 1991; 1996). Experiments were performed in the absence of cholinesterase inhibitors to prevent non-physiological extracellular accumulation of acetylcholine that might exaggerate cholinergic neuromudalation. After a 30 min equilibration period, the superfusion was stopped and the nerve endings were labelled for 40 min with 1 μM [^{3}H]-Ach (specific activity 2.5 μCi nmol^{-1}) under electrical stimulation at 1 Hz. After the end of labelling period, the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation stopped. From this time, hemicholinium-3 (10 μM) was present to prevent uptake of choline. After a 60 min period of washout, bath samples (2 ml) were automatically collected every 3 min by emptying and refilling the organ bath with the solution in use, using a fraction collector (Gilson, FC203B, France) coupled to a peristaltic bump (Gilson, Minipuls 3, France) programmed device. Aliquots (0.5 ml) of incubation medium were added to 3.5 ml of Packard Insta Gel II (USA) scintillation cocktail. Tritium content of samples was measure by liquid scintillation (counting efficiency of 40 ± 2 %) after appropriate background subtraction, which did not exceed 5% of the tritium content of the samples. The radioactivity was expressed as disintegrations per minute (DPM) per gram wet weight of the tissue determined at the end of the experiments. [^{3}H]-Ach releases were evoked by electrical stimulating the phrenic nerve two times, at the 12th (S1) and 39th (S2) min after the end of the washout period (zero time). Drugs were added in the bath 15 min before S2. Their effects on transmitter release were expressed as ratios S2/S1 and compared as percentage of ratio control (without drugs). Statistical significance of experimental results was evaluated by one-way ANOVA ($P< 0.5$) followed by Dunnett's.

Drugs

L-Arginine, D- arginine, N^G-nitro-L-arginine (L-NOARG), adenosine deaminase (EC 3.5.44, ADA), choline chloride, hemicholinium- 3 and oxotremorine sesquifumarate (Sigma, USA). (11- [[2- 1 [(Diethylamino) methyl1- 1- piperidinyl]- acetyl]- 5, 11- dihydro- 6H- pyrido [2, 3- b] [1, 4] benzodiazepine- 6 -one, AF- DX 116) and ((4- (2- [7- amino- 2- (2- furyl {1, 2, 4}- triazolo {2, 3- a- {1, 3, 5} triazin- 5- yl- aminoethyl) phenol, ZM241385) (Tocris Cookson Inc., UK). (1, 3 Dipropyl- 8- cyclopentylxanthine, DPCPX) (Res. Biochem. Inc., USA).

Results

L-arginine (47 to 4700 μM) in a concentration-depend manner decreased evoked [^{3}H]-Ach release at 5.0 Hz (Figure 1). The effect of amino acid at lower concentration (47 μM) able to reduce (26 ± x%) evoked [^{3}H]-Ach release at 5.0 Hz produced higher inhibition (43 ± 2%) on evoked [^{3}H] Ach release when the nerve was stimulated at 50 Hz (Figure 2). L-NOARG (100 μM) and AF-DX 116 (10 nM) alones increased [^{3}H]-Ach release at 5.0 Hz and 50 Hz, but the facilitatory effect of AF-DX 116 was lower at 50 HZ (Figure 3). L-NOARG (100 μM) antagonized the reduction on [^{3}H]-Ach release induced by L-arginine at 5 Hz and 50 HZ (Figure 4). However, it was verified that AF-DX 116 (10 nM) did not modify the inhibitory effect induced by L-arginine at 5 Hz, but it changed the inhibitory effect of amino acid to facilitatory effect when the preparations were stimulated at 50 Hz- burst (Figure 4). The treatment of preparations with ADA (0.5 U/ml) reduced the inhibitory effect of L-arginine at 5 and 50 Hz (Figure 4). On the other hand, the treatment of preparations with DPCPX (2.5 nM) reduced the inhibition caused by L-arginine at 5 Hz, but did not modify the inhibitory effect of amino acid when the preparations were stimulated at 50 Hz-burst (Figure 4). Oppositely, it was verified that ZM241385 (10 nM) reduced [^{3}H]-Ach release inhibition produced by L-arginine at 50 Hz, but did not modify the inhibitory effect of amino acid recorded at 5 Hz (Figure 4). Oxotremorine (10 μM) reduced the evoked [^{3}H]-Ach release at 50 Hz, but such effect was antagonized by previous treatment of preparations with AF-DX-116 (10 nM) (Figure 5).

Discussion

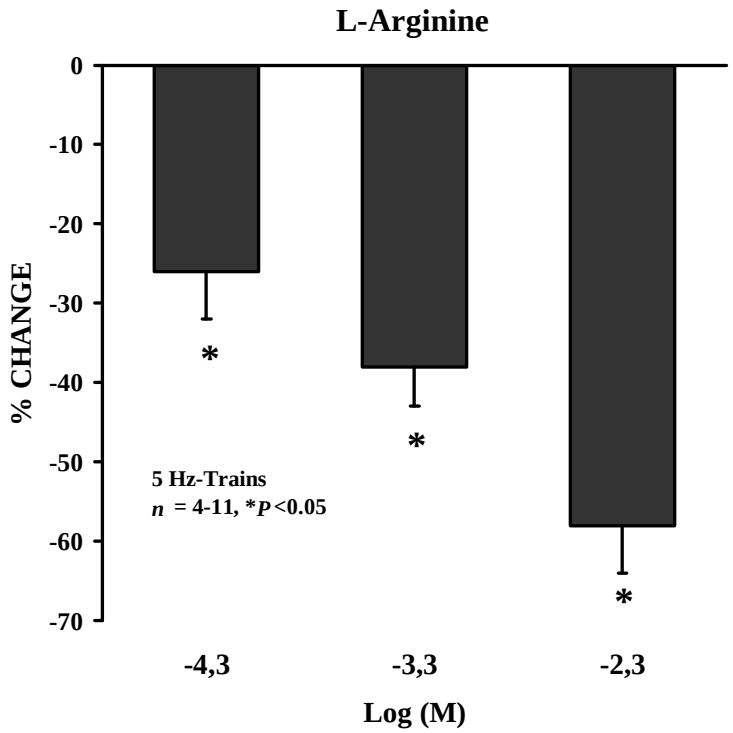
L-arginine in a concentration-depend manner decreased evoked [^{3}H]-Ach release at 5.0 Hz. The lowest concentration of amino acid that produced such effect at 5.0 Hz determined a higher inhibition on evoked [^{3}H]-Ach release when the preparations were stimulated at 50 Hz. In contrast, d-arginine did not produce any change on evoked [^{3}H]-Ach release, but the inhibitory effects of amino acid at 5.0 and 50.0 Hz were antagonized by previous treatment of preparations with L-NOARG. These data indicate that the inhibitory effect induced by L-arginine in the phrenic nerve-hemidiaphragm preparations of rats depend on frequency of stimulations applied on motor nerve, and is determined by metabolism of L-arginine to NO through action of NOS (Cruciol-Souza and Alves-do-Prado, 1999). On the other hand, it was verified that the inhibitory effect induced by NO at 50.0 Hz was impaired by treatment of preparations with AF-DX 116, but such agent did not modify the inhibitory effect produced by NO at 5.0 Hz. These results indicate that while the inhibitory effect produced by NO at 50.0 Hz depend on activation by acetylcholine of inhibitory M_2 receptors, the effect of gas at 5.0 Hz is not produced by activation of such receptors. Such hypothesis is reinforced by data that showed that the inhibitory effects induced by NO and oxotremorine at 50 Hz were qualitatively similar and impaired by treatment of preparations with AF-DX 116. On the other hand, it was verified that the inhibitory effect induced by NO at 50 Hz also depended on release of adenosine from motor nerve terminal activating facilitatory A_{2A} presynaptic receptors, as the treatment of preparation with ADA or ZM241385, but not with DPCPX, reduced the effect of gas at such frequency of stimulation. Thus, data indicate that the inhibitory effect induced by NO at 50 Hz depends on cross- talk between the presynaptic facilitatory A_{2A} receptors and the presynaptic inhibitory M_2 receptors. In contrast, the inhibitory effect induced by NO at 5.0 Hz seems have depended on activation by adenosine of inhibitory presynaptic A_1 receptors, as the treatment of preparations with ADA or DPCPX, but not with AF-DX 116 or ZM241385, reduced the effect of gas at 5.0 Hz.

L-NOARG or AF-DX 116 alones increased [^{3}H]-Ach release at 5.0 Hz and 50 Hz, but the facilitatory effect of AF-DX 116 was lower at 50 HZ. These results indicate that both endogenous NO and acetylcholine have important physiological tonics actions on motor nerve. The lower ability of AF-DX 116 to produce a higher protection against the activation by

acetylcholine of inhibitory presynaptic M_2 receptors at 50 Hz was determined by activation of presynaptic stimulatory A_{2A} receptors to produce an increment on acetylcholine release determining a higher competition between acetylcholine and AF-DX 116 for presynaptic M_2 receptors. This hypothesis is sustained by previous study that have shown that the activation of A_{2A} receptors by adenosine generates from catabolism of adenine nucleotide promotes M_2-autoinhibitory action by braking counteraction mediated by muscarinic M_1 receptors when nerve is stimulated at 50 Hz- burst (Oliveira *et al.*, 2002). Furthermore, the M_1-positive feedback mechanism predominates over M_2-inhibition when the motor nerve is stimulated at 5Hz (Oliveira *et al.*, 2002). Thus, the highest facilitatory effect recorded with AF-DX 116 alone in neuromuscular preparations stimulated at 5.0 Hz was determined by an unbalance between activations of M_1 and M_2 receptors produced by AF-DX 116 determining a higher activation by acetylcholine of presynaptic facilitatory M_1 receptors. On the other hand, the increase on [^{3}H]-Ach release produced by inhibitor of synthesis of NO at 5.0 Hz was determined by a reduction on adenosine release to produce lower inhibition on [^{3}H]-Ach release. This hypothesis is proposed taking in count that in the current study the inhibitory effect induced by NO at 5.0 Hz was mediated by activation by adenosine of inhibitory presynaptic A_1 receptors. Controversially, the increase on [^{3}H]-Ach release produced by L-NOARG alone at 50.0 Hz might have been determined by a reduction on release of adenosine producing lower activation of facilitatory presynaptic A_{2A} receptors, thereby reducing the activation by acetylcholine of inhibitory presynaptic M_2 receptors. This hypothesis is sustained by data that showed that the inhibitory effect of NO at 50 Hz was changed to facilitatory when the preparations were treated with AF-DX 116. The hypothesis is also sustained by data that showed the inhibitory effect of NO at 50 Hz was reduced by addition of ADA or ZM241385 in the bath, but it was not modified by administration of DPCPX. It is also favorably to that hypothesis the data that showed the inhibitory effects induced by oxotremorine or NO at 50 Hz were qualitatively similar and abolished after treatment of preparations with AF-DX 116. Controversially, it was recorded that the inhibitory effect induced by NO at 50.0 Hz was transformed to facilitatory effect when the preparations were treated with AF-DX 116. Such data indicate that the cross- talk between facilitatory A_{2A} receptors and inhibitory M_2 receptors activated at 50 Hz was unbalanced by NO inducing higher activation by adenosine of A_{2A} receptors, thereby increasing the acetylcholine out put. In such situation, the activation of A_{2A}

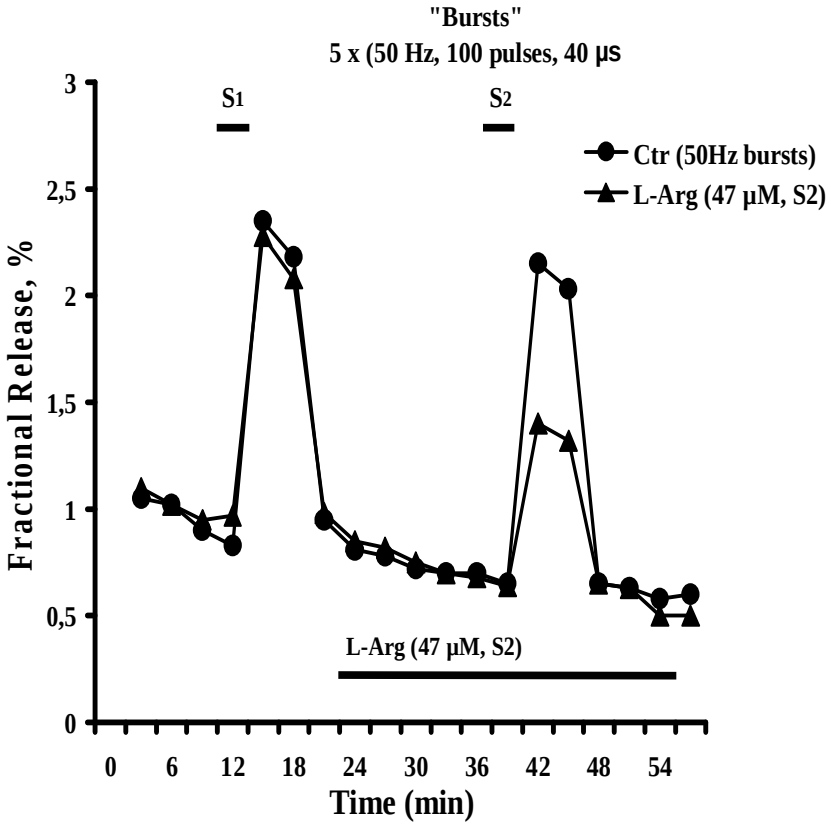
receptors induced by NO was not counteracted by inhibitory presynaptic M_2 receptors, as they were blocked by AF-DX 116.

Taken together, data show that endogenous NO has important inhibitory physiological tonic action on motor nerve terminal. The inhibitory effect of gas on evoked [^{3}H]-Ach release in the phrenic nerve-hemidiaphragm preparations of rats depend on release of adenosine and acetylcholine from motor nerve terminal, and it is mediated by A_1 receptor at 5 Hz or by a cross- talk between A_{2A} and M_2 receptors at 50 Hz-burst.



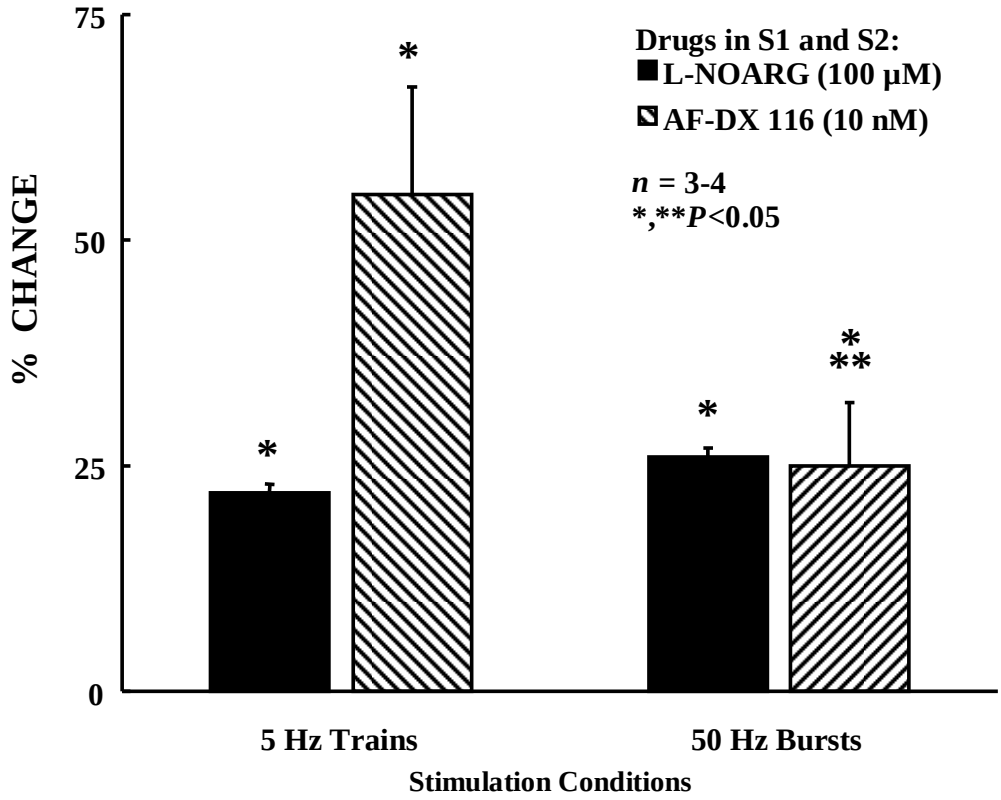
L-Arginine				
Concentration	**Log**	**s.e.mean**	**% Average**	**n**
47 μM	**-4,3**	**6%**	**-26**	**6**
470 μM	**-3,3**	**5%**	**-38**	**5**
4700 μM	**-2,3**	**6%**	**-58**	**6**

Figure 1- Reduction by L-arginine (47 to 4700 μM) on [^{3}H]-Ach release evoked by 5.0 Hz-trains indirectly applied on the left phrenic nerve-hemidiaphragm preparations of rats. Abscissas show values of logarithmic concentrations of L-arginine. Ordinates are percentage changes in S2/S1 ratios compared to control (without drugs). Height of columns indicates means ± s.e.m. of 6 experiments. * P< 0.05 (one-way ANOVA followed by Dunnett's modified *t*- test) significant differences compared to control.



	Average	s.e.mean	n
L-Arg (47 µM, S2, 50 Hz Bursts)	**-43%**	**2%**	**5**

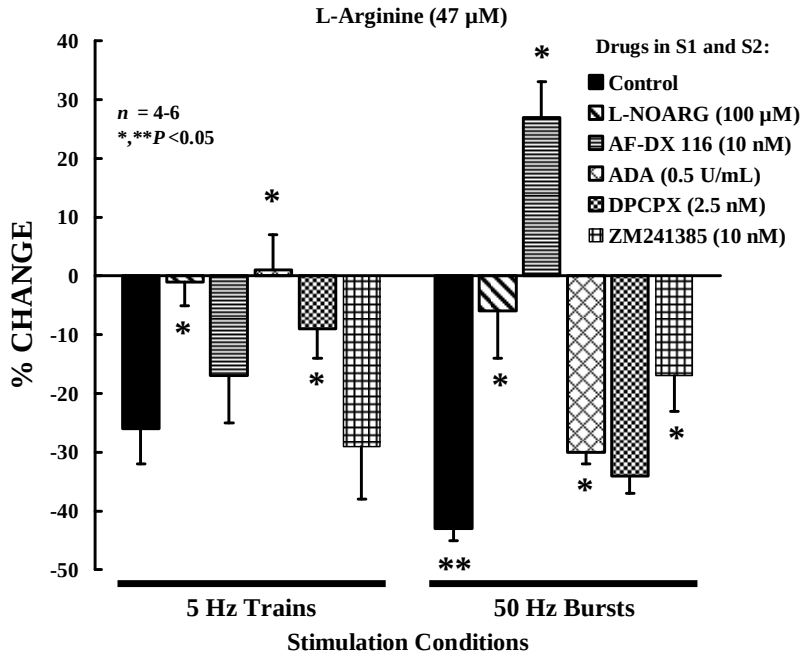
Figure 2- Reduction by L-arginine (47 µM) (horizontal bar) on [^{3}H]-Ach release from motor nerve terminals of rats stimulated at 50.0 Hz- bursts (S2, filled triangle). The graph shows the time of course of tritium outflow (ordinates) expressed as percentage of the total radioactivity present at the beginning of the collection period. Abscissa indicates time at which the samples were collected. Control curve (Ctr., filled circles) shows the fractional release induced by 50.0 Hz- bursts (S1 and S2) before addition of L-arginine. Data are means of 5 experiments.



	Average	s.e.mean	n
Effect of AF-DX 116 (10 nM, S2) 5-Hz trains	**55%**	**12%**	**4**
Effect of AF-DX 116 (10 nM, S2) 50-Hz bursts	**25%**	**7%**	**3**

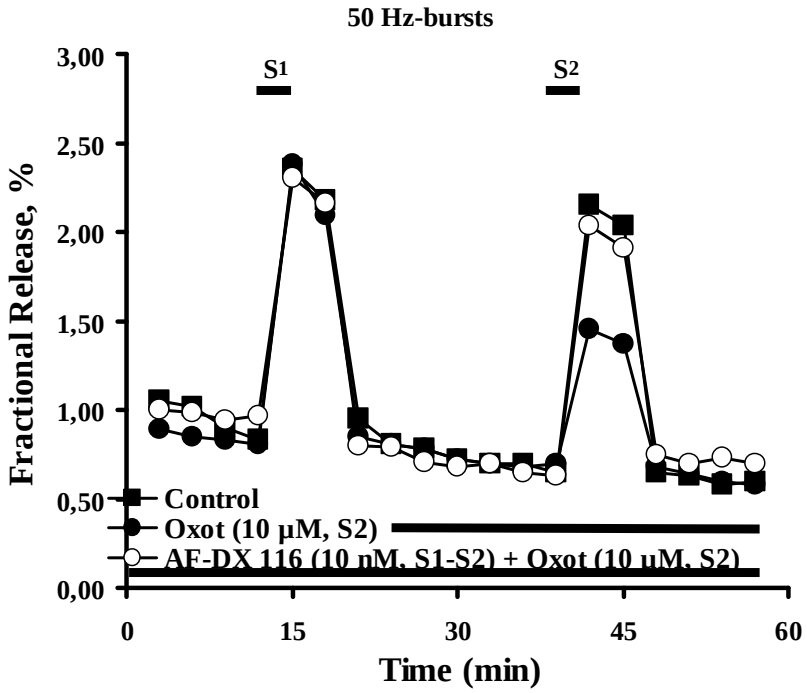
Effect of L-NOARG (100 µM, S2) 5-Hz trains	**22%**	**1%**	**4**
Effect of L-NOARG (100 µM, S2) 50-Hz bursts	**26%**	**1%**	**4**

Figure 3- Effects induced by L-NOARG (100 µM) and AF-DX 116 (10 nM) on [^{3}H]-acetylcholine release evoked by 5.0 Hz-trains or 50.0 Hz-burst indirectly applied on the left phrenic nerve-hemidiaphragm preparations of rats. Abscissa indicates stimulations conditions and ordinates shows percentage changes in S2/S1 ratios compared to control (without drugs). Heights of columns indicate means ± s.e.m. of 4 experiments. * P< 0.05 (one-way ANOVA followed by Dunnett's modified *t*- test) significant differences compared to control. ** (one-way ANOVA followed by Dunnett's modified *t*- test) significant differences compared to AF-DX 116 at 5.0 Hz- trains.



Crosstalk NO and M2/A1				
L-arginine (47 µM)				
	5 Hz Trains		**50 Hz Bursts**	
Control	**-26**	**-6**	**-43**	**-2**
L-NOARG (100 µM)	**-1**	**-4**	**-6**	**-8**
AF-DX 116 (10 nM)	**-17**	**-8**	**27**	**6**
ADA (0.5 U/mL)	**1**	**6**	**-30**	**-2**
DPCPX (2.5 nM)	**-9**	**-5**	**-34**	**-3**
ZM241385 (10 nM)	**-29**	**-9**	**-17**	**-6**

Figure 4- Effects induced by L-NOARG (100 µM), AF-DX 116 (10 nM), adenosine deaminase (EC 3.5.44, ADA, 0.5 U/mL), 1,3dipropyl-8-cyclopentylxanthine (DPCPX, 2.5 nM) and (4-(2-[7-amino-2-(2-furyl {1,2,4}-triazolo {2,3-a- {1,3,5} triazin-5-yl-aminoethyl) phenol (ZM241385, 10 nM) on [^{3}H]-Ach release evoked by 5.0 Hz-trains or 50.0 Hz-burst indirectly applied on the left phrenic nerve-hemidiaphragm preparations of rats. Abscissa indicates stimulations conditions and ordinates shows percentage changes in S2/S1 ratios compared to control (without drugs). Heights of columns indicate means ± s.e.m. of 4 to 6 experiments. * P< 0.05 (one-way ANOVA followed by Dunnett's modified *t*-test) significant differences compared to controls (black columns, 47 µM L-arginine, 5 Hz or 50 Hz). ** P< 0.05 (one-way ANOVA followed by Dunnett's modified *t*- test) significant differences compared to control at 5.0 Hz-trains condition.



	Average	s.e.mean	n
Oxot (10 µM, S2, 50 Hz Bursts)	**-35%**	**9%**	**6**
AF-DX 116 (10 nM, S1-S2) + Oxot (10 µM, S2, 50 Hz Bursts)	**-5%**	**9%**	**7**

Figure 5- Antagonism by AF-DX 116 (10 nM, open circles) of reduction by oxotremorine (Oxot, 10 µM, filled circles) on [^{3}H]-acetylcholine release from motor nerve terminals of rats stimulated at 50 Hz- bursts. The graph shows the time of course of tritium outflow (ordinates) expressed as percentage of the total radioactivity present at the beginning of the collection period. Abscissa indicates time at which the samples were collected. Horizontal dark bars indicate administration of Oxot in presence and absence of AF-DX 116. Control curve (filled square) shows the fractional release induced by 50.0 Hz- bursts (S1 and S2) before addition of drugs. Data are means of 6 experiments.

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L-Citrulline inhibits [^{3}H]acetylcholine release from rat motor nerve terminals by increasing adenosine outflow channelling to A_1 receptor activation

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Running Title: L-citrulline-induced neuronal adenosine outflow

Summary

Background and purpose. There is a close relationship between NOS activity and depression of neuromuscular transmission, but little is known about the role of L-citrulline, a co-product of NO biosynthesis, on neurotransmitter release.

Experimental approach. Muscle tension recordings and outflow experiments were performed on rat phrenic nerve-hemidiaphragm preparations stimulated electrically.

Key results. L-citrulline concentration-dependently inhibited evoked [^{3}H]ACh release from motor nerve terminals and depressed nerve-evoked muscle contractions. The NOS substrate, L-arginine, and the NO donor, 3-morpholinosydnonimine (SIN-1), also inhibited [^{3}H]ACh release with a rank potency order of SIN-1>L-arginine>L-citrulline. Co-application of L-citrulline and SIN-1 caused additive effects. NOS inactivation with N^{ω}-nitro-L-arginine prevented L-arginine inhibition, but failed to modify the effect of L-citrulline. The NO scavenger, haemoglobin, abolished inhibition of [^{3}H]ACh release caused by SIN-1, but did not change inhibition by L-arginine. Inactivation of guanylyl cyclase with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) fully blocked SIN-1 inhibition, but only partially attenuated L-arginine effect. Reduction of extracellular adenosine accumulation with adenosine deaminase or with the nucleoside transport inhibitor, S-(*p*-nitrobenzyl)-6-thioinosine, attenuated the effects of L-arginine and L-citrulline, while keeping unchanged inhibition by SIN-1. Similar results were obtained with the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine. L-citrulline enhanced the resting adenosine outflow, without changing the basal release of adenine nucleotides.

Conclusions and implications. Data suggest that NOS catalyses the formation of two neuronally active products, NO and L-citrulline. While, NO may directly reduce transmitter release through stimulation of soluble guanylyl cyclase, the inhibitory action of L-citrulline may be secondary to adenosine outflow channelling to inhibitory A_1 receptors activation.

Keywords. Neuromuscular junction – Acetylcholine release – Adenosine – A_1 receptor – Nucleoside transport – Nitric oxide (NO) – Nitric oxide synthase – L-citrulline – L-arginine – NO donor.

Abbreviations. ACh, acetylcholine; ADA, adenosine deaminase; ADO, adenosine; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; 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; 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.

INTRODUCTION

Nitric oxide synthases (NOS) are a family of enzymes capable of oxidizing the amino acid, L-arginine, to form L-citrulline and nitric oxide (NO). NO is an exceptionally membrane-permeant gas molecule with short half-life. It has been shown that in the brain NO acts as a neurotransmitter or neuromodulator-like substance during synaptic transmission (Garthwaite, 1991). In the peripheral nervous system, NO has been extensively studied and its involvement in a wide variety of functions has been demonstrated in the autonomic nervous system, though its functions in the motor system are less well defined. There is a close relationship between NOS activity and depression of neuromuscular transmission (Wang *et al.*, 1995; Thomas & Robitaille, 2001). The most common mode of action of NO is by stimulating soluble guanylyl cyclase thereby increasing neuronal levels of cyclic GMP (Garthwaite, 1991; Boulton *et al.*, 1994), but it also has several other biochemical effects of potential biological significance (reviewed by Stamler & Meissner, 2001). It has been proposed that NO acts through both cyclic GMP-dependent and -independent pathways at the amphibian neuromuscular junction, with the dominance of a particular pathway determined by the level of synaptic activity (Thomas & Robitaille, 2001).

L-citrulline, which is a ubiquitous amino acid in mammals, is strongly related to arginine (reviewed by Curis *et al.*, 2005). In hepatocytes, L-citrulline is locally synthesized by the enzyme ornithine carbamoyltransferase (OCT) and metabolised by argininosuccinate synthetase (ASS) for urea production. However, in NO producing tissues, L-citrulline is the co-product in the NO biosynthesis by NOS enzyme. Because L-citrulline can be easily converted into L-arginine by the successive action of ASS and argininosuccinate lyase (ASL), which are expressed in every cell examined including neurons (Wiesinger, 2001), some authors suggested that L-citrulline might be an indirect precursor of NO in NO-synthesising cells (Mori & Gotoh, 2000). Previously, Ruiz and Tejerina (1998) advanced the possibility that L-citrulline could be not merely a by-product of the NO-synthesis but might also play a role in cell signalling. In spite of this, little is known about the action of L-citrulline on synaptic transmission, namely in the control of neurotransmitters release. This prompted us to investigate the role of L-citrulline on nerve-evoked muscle contractions and on [^{3}H]acetylcholine ([^{3}H]ACh) release from stimulated motor nerve terminals in comparison

with the effects caused by the NOS substrate, L-arginine, and by the NO donor, 3-morpholinosydnonimine chloride (SIN-1). In order to block L-citrulline conversion to L-arginine and subsequent formation of NO, we used the NOS inhibitor N^{ω}-nitro-L-arginine (L-NOARG) (Moncada *et al.*, 1991).

The depressing action of the NO pathway on synaptic transmission in the central nervous system is, at least, partially mediated through increases in the release of endogenous adenosine acting on A_1 receptors (Fallahi *et al.*, 1996; Bon & Garthwaite, 2002). Controversy, however, exists on whether cyclic GMP is involved on NOS-dependent adenosine outflow (Boulton *et al.*, 1994; Broad *et al.*, 2000; Rosenberg *et al.*, 2000; Bon & Garthwaite, 2002). At the rat neuromuscular junction, adenosine acts as neuromodulator either inhibiting (via A_1 receptors) or facilitating (via A_{2A} receptors) the release of [^{3}H]ACh from motor nerve terminals (Correia-de-Sá *et al.*, 1991) depending on the concentration of the nucleoside at the synapse (Correia-de-Sá & Ribeiro, 1996). 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 & Ribeiro, 1996; Cunha *et al.*, 1996). Indeed, adenosine can either be released as such or can be formed upon the sequential extracellular dephosphorylation of ATP co-released with ACh in a frequency-dependent manner (Magalhães-Cardoso *et al.*, 2003). The activity of the ecto-nucleotidase pathway is critical to define the pattern of formation of extracellular ATP-derived adenosine, which activates preferentially facilitatory A_{2A} receptors (Cunha *et al.*, 1996) overtaking the A_1 receptor activation required to restrain superfluous transmitter release by low synaptic adenosine levels (Correia-de-Sá & Ribeiro, 1996).

Due to the putative interactions between NOS activity and the adenosine system, we manipulated extracellular adenosine accumulation using either adenosine deaminase (ADA), the enzyme that inactivates endogenous adenosine into inosine, or the nucleoside transport inhibitor, S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) (Correia-de-Sá & Ribeiro, 1996), to probe its role on NOS-induced depression of [^{3}H]ACh release from nerve terminals of the motor endplate. We also investigated the ability of L-citrulline to cause the outflow of adenine nucleotides and adenosine from the rat phrenic-nerve hemidiaphragm. This work is pioneering to suggest that NOS catalyses the formation of two biologically active products, NO and L-citrulline, capable of inhibiting neurotransmitter release from motor nerve terminals. Data is

presented suggesting that while NO directly reduces transmitter release probability via a soluble guanylyl cyclase-dependent mechanism; the inhibitory action of L-citrulline is guanylyl cyclase-independent requiring endogenous adenosine outflow channelling to pre-synaptic inhibitory A_1 receptors activation.

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 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 stimulation conditions

The left phrenic nerve was stimulated with an extracellular glass-platinum suction electrode placed near its first division branch, to avoid direct stimulation of muscle fibres (indirect stimulation). To evaluate drug effects on muscle contractile properties, direct stimulation of muscle fibres was delivered through a pair of platinum electrodes placed at each side of the diaphragm near its costal insertion (field stimulation). Supramaximal intensity (current strength of 8 mA) rectangular pulses of 0.04 ms (indirect stimulation) or 1-ms (field stimulation) duration were used to achieve firing synchronization, thus reducing the number of silent units (motoneurons and/or muscle fibres) that might make interpretation of data difficult. The pulses were delivered by a Grass S48 (Quincy, MA, USA) stimulator coupled to a stimulus isolation unit (Grass SIU5) operating in a constant current mode. The stimulation parameters were continuously monitored on an oscilloscope (Meguro, MO-1251A, Japan) and were within the same range used in previous studies with this preparation (*e.g.* Wessler and Kilbinger, 1986; Correia-de-Sá et al., 2000).

[^{3}H]Acetylcholine release experiments

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 1 Hz frequency. 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 (% 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 (5,542±248) x 10^3 disintegrations per minute per gram (d.p.m. g^{-1}) wet weight of tissue and the resting release was (132±12) x 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.

Unless otherwise stated, [^{3}H]ACh release was evoked by electrical stimulation of the phrenic nerve with trains of 750 supramaximal intensity pulses of 0.04 ms duration delivered at a frequency of 5 Hz. Two stimulation periods were used, starting respectively at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). Electrical stimulation increased only the release of [^{3}H]ACh in a Ca^{2+}- and tetrodotoxin-sensitive manner (Correia-de-Sá *et al.*, 2000), while the output of [^{3}H]choline remained unchanged (Wessler & Kilbinger, 1986), thus indicating that ACh comes mainly from vesicle exocytosis from depolarised nerve terminals. 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).

Test drugs were added 15 min before S_2 and were present up to the end of the experiments (see *e.g.* Figure 2). 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. When we evaluated changes in the effect of test drugs induced by a modifier (*e.g.* enzymatic inhibitor, receptor antagonist, transport inhibitor), these compounds were applied 15 min before starting sample collection and hence were present during S_1 and S_2. When present during S_1 and S_2, none of the modifiers significantly altered (P>0.05) the S_2/S_1 ratio as

compared to the S_2/S_1 ratio obtained in the absence of the modifiers (0.81±0.03%, *n*=8). None of the drugs changed significantly ($P>0.05$) basal tritium outflow.

Release of adenine nucleotides and adenosine

To follow the release of adenine nucleotides and adenosine, the preparations were incubated as for the release of [^{3}H]ACh, except that no [^{3}H]choline was added to the Tyrode's solution. The preparations were superfused (3 ml min^{-1}) for 30 min with gassed Tyrode solution containing the adenosine deaminase inhibitor, erythro-9(2-hydroxy-3-nonyl) adenine (EHNA, 0.3 µM), which was present from then on. After stopping superfusion, the preparations were incubated with 2-ml oxygenated Tyrode solution that was automatically changed every 3 min by emptying and refilling the organ bath with the solution in use. As for the release of [^{3}H]ACh, the preparations were also stimulated twice using similar nerve stimulating conditions (750 pulses of 0.04 ms duration delivered at a frequency of 5 Hz), starting respectively at 12th (S_1) and 39th (S_2) min after starting sample collection (zero time). In these experiments, only the four samples collected before stimulus application and the three samples collected immediately after stimulation were retained for analysis.

Bath aliquots were frozen in liquid nitrogen immediately after collection, stored at -20°C and analysed by HPLC (see Cunha & Sebastião, 1991) within one week of collection. To measure adenine nucleotides and adenosine, we used 200 µl aliquots from collected samples. Test drugs were added 15 min before S_2 and were present throughout the assay. The effects of test drugs were expressed by the ratios S_2/S_1, *i.e.* the ratio between the evoked release of adenine nucleotides and adenosine during the second stimulation period (in the presence of the test drug) and the corresponding release during the first stimulation period (without the test drug). To evaluate the effect of test drugs on the basal outflow of adenine nucleotides and adenosine, we calculated the $B_{2\text{-}n}/B_{1\text{-}n}$ ratios (see Figure 6). $B_{1\text{-}n}$ and $B_{2\text{-}n}$ correspond to the content of adenine nucleotides and adenosine in bath samples collected *n* minutes before the first (without the test drug) and the second (in the presence of the test drug) stimulation periods, respectively.

The remaining incubation medium was used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity. The negligible activity of LDH in bath samples collected before

(0.14±0.02 mU ml^{-1}, *n*=16) and after (0.15±0.01 mU ml^{-1}, *n*=16) electrical nerve stimulation, is an indicator of the integrity of cells during the experimental procedure.

Muscle contraction recordings

When tension responses were recorded, the innervated diaphragm strips were mounted in 10-ml capacity isolated organ bath chambers. The preparations were superfused (5 ml min^{-1}, 37°C, pH 7.4) with gassed (95% O_2 + 5% CO_2) Tyrode's solution. Alternate (0.1-Hz frequency) direct- and nerve-induced responses were recorded isometrically at a resting tension of 50 mN with a force transducer and displayed on a Hugo-Sachs (Germany) recorder. After the initial stabilization period, these experimental conditions allowed a well-preserved contraction pattern for several hours in the absence of test drugs. The solutions were changed by transferring the inlet tube of the peristaltic pump (Gilson, Minipuls3, France) from one flask to another. Test drugs were allowed to be in contact with the preparations for at least 12 min. In order to reduce the safety margin of neuromuscular transmission (see *e.g.* Wood and Slater, 2001), $MgCl_2$ (6 mM) was added to the bath in some of the experiments. Osmolarity was maintained by equimolar substitution of NaCl. Elevation of magnesium ions to 6 mM, decreased the amplitude of nerve-evoked responses from a control value of 40±2 mN to 17±1 mN (*n*=6), without significantly (*P*>0.05) changing the contractions induced by direct muscle stimulation.

Materials and solutions

Adenosine deaminase (ADA, type VI, 1803 U ml^{-1}, EC 3.5.4.4), choline chloride, hemicholinium-3, haemoglobin from rat, D- and L-arginine, D- and L-citrulline, N^{ω}-nitro-L-arginine (L-NOARG), S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) (Sigma, St. Louis, MO, USA); 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), erythro-9(2-hydroxy-3-nonyl) adenine (EHNA) (Research Biochemicals, Natick, USA); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-morpholinosydnonimine hydrochloride (SIN-1), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) (Tocris Cookson Inc., U.K.); [*methyl*-^{3}H]choline chloride (ethanol solution, 80 Ci $mmol^{-1}$) (Amersham, UK). All other reagents were of the highest purity available. EHNA was made up in a 5 mM stock solution in ethanol. DPCPX was made up in a 5 mM stock solution in 99%

dimethylsulphoxide (DMSO) + 1% NaOH 1M (vv^{-1}). ZM 241385 and NBTI were made up in 5 and 50 mM stock solutions in DMSO, respectively. Other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20^{o}C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% vv^{-1}), were observed. The pH of the superfusion solution did not change following addition of the drugs at the maximum concentrations applied to the preparations.

Presentation of data and statistical analysis

The data are expressed as mean ± s.e.mean from *n* observations. 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 modified *t*-test. A value of $P<0.05$ was considered to represent a significant difference.

RESULTS

Neuronal NOS catalyses the formation of two biologically active products, NO and L-citrulline, acting independently to reduce ACh release from motor nerve terminals

When applied in the physiological concentration range (see Griffith & Stuehr, 1995), the NOS substrate L-arginine (0.01-4.7 mM) concentration-dependently decreased [^{3}H]ACh release from stimulated phrenic nerve terminals (Figure 1). The NO donor, SIN-1 (1-100 µM), and the co-product of NO biosynthesis catalysed by NOS, L-citrulline (0.01-4.7 mM), mimicked the inhibitory effect of L-arginine on [^{3}H]ACh release; the concentrations required to decrease neurotransmitter release by about 30% were 10 µM SIN-1, 47 µM L-arginine and 470 µM L-citrulline. The effects of L-arginine and L-citrulline were stereo specific, as their D-isomers (0.01-4.7 mM) were devoid of effect on [^{3}H]ACh release; in the highest concentration (4.7 mM) tested, D-arginine and D-citrulline decreased transmitter release only by 7±3% (*n*=7) and 6±1% (*n*=4), respectively. The maximal inhibitory effects of SIN-1 (100 µM, 38±5%, *n*=4) and L-citrulline (470 µM, 26±3%, *n*=6) were about half the magnitude of L-arginine (4.7 mM, 58±6%, *n*=4)-induced depression of [^{3}H]ACh release (Figure 1). Interestingly, SIN-1 (10 µM) applied 15 min before S_2 to preparations incubated with L-citrulline (470 µM) during the whole assay, including S_1 and S_2, could still reduce evoked [^{3}H]ACh release by a similar amount (27±5%, *n*=5) to that observed in control conditions (30±6%, *n*=4).

Figure 2 illustrates the time course of tritium outflow in experiments where L-arginine (47 µM) and SIN-1 (10 µM) were applied 15 min before S_2 in the absence and in the presence of the extracellular NO scavenger, haemoglobin (10 µM). As can be seen from these typical experiments, the inhibitory effect of the NO donor SIN-1 (10 µM), but not that of the NOS substrate L-arginine (47 µM), was completely prevented by haemoglobin (10 µM). It, thus, appears that the inhibitory effect of L-arginine on [^{3}H]ACh release from stimulated motor nerve terminals does not require diffusion of NO throughout the extacellular space.

In order to investigate whether changes in neurotransmitter release caused by L-citrulline (0.01-47 mM) correspond to alterations in the contraction of hemidiaphragm preparations, we studied the effect of this compound on twitch tension induced either by

phrenic nerve stimulation or by direct depolarisation of muscle fibres. Because of the high safety factor of neuromuscular transmission, depression of nerve-evoked muscle contractions due to prejunctional acting drugs might not always reflect the magnitude of transmitter release inhibition (for a review, see Wood and Slater, 2001). This might explain why L-citrulline (0.01-47 mM) was more potent to inhibit evoked [^{3}H]ACh release (Figure 1) than to cause depression of nerve-evoked muscle contractions while keeping a high transmission safety margin (data not shown). By decreasing the safety factor of synaptic transmission with high magnesium ($MgCl_2$, 6 mM), L-citrulline (0.01-47 mM) reduced diaphragm twitch tension caused by electrical nerve stimulation in a concentration-dependent manner (Figure 3). The magnitude of inhibition was significantly ($P<0.05$) higher when the contractions were induced by phrenic nerve stimulation as compared with direct muscle depolarisation, indicating that the inhibitory effect of L-citrulline on nerve-evoked contractions requires synaptic transmission rather then an action on excitation-contraction coupling or on the muscle contraction apparatus.

In order to block formation of NO by exogenous L-citrulline acting as a precursor of L-arginine, we used the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NOARG, 100 μM). As shown in Figure 4, L-NOARG (100 μM, applied during the whole assay including S_1 and S_2) completely prevented the depressing action of L-arginine (47 μM), but the inhibitory effect of L-citrulline (470 μM) remained virtually unchanged ($P>0.05$). Moreover, pre-treatment of the preparations with the soluble guanylyl cyclase inhibitor, ODQ (10 μM, applied during the whole assay including S_1 and S_2), partially attenuated L-arginine (47 μM) inhibition, but fully prevented the inhibitory effect of SIN-1 (10 μM). ODQ (10 μM) was unable to change the inhibitory action of L-citrulline (470 μM) even though this substance reduced the release of [^{3}H]ACh by a similar amount (~30%) to that caused by the two NO generating compounds, L-arginine (47 μM) and SIN-1 (10 μM) (Figure 4). L-NOARG (100 μM) and ODQ (10 μM) increased the evoked [^{3}H]ACh release by 22±1% (n=4) and 34±7% (n=5), respectively, indicating that both NOS and guanylyl cyclase are tonically activated to control neurotransmitter release from stimulated phrenic nerve terminals.

L-citrulline inhibition of [^{3}H]ACh release is secondary to activation of pre-synaptic inhibitory A_1 receptors by endogenous adenosine

Inactivation of endogenous adenosine with adenosine deaminase (ADA, 0.5 U ml^{-1}) or inhibition of the nucleoside transport system with S-(*p*-nitrobenzyl)-6-thioinosine (NBTI, 10 µM) partially attenuated the inhibitory role of L-arginine (47 µM) on [^{3}H]ACh release (Figure 5a). Interestingly, ADA (0.5 U ml^{-1}) and NBTI (10 µM) fully prevented the inhibitory effect of L-citrulline (470 µM), but kept unchanged SIN-1 (10 µM)-induced reduction of evoked [^{3}H]ACh release (Figure 5a).

We, then, investigated the adenosine receptor subtype involved in the control of [^{3}H]ACh release by NOS activity. Selective blockade of A_1 receptors with 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX, 2.5 nM) completely prevented release-depression caused by L-citrulline (470 µM) (Figure 5b). In the presence of DPCPX (2.5 nM), the inhibitory action of L-arginine (47 µM) was only partially attenuated by a similar amount to that observed in the presence of ADA (0.5 U ml^{-1}) or NBTI (10 µM) (see Figure 5a). The A_1 receptor antagonist failed to affect inhibition of [^{3}H]ACh release induced by SIN-1 (10 µM). Conversely, blockade of A_{2A} receptors with ZM 241385 (10 nM) was unable to modify the inhibitory effects of L-arginine (47 µM), L-citrulline (470 µM) and SIN-1 (10 µM) (Figure 5b).

L-citrulline increases adenosine outflow via the equilibrative nucleoside transport system

Stimulation of the phrenic nerve at a frequency of 5 Hz (750 pulses of 0.04 ms duration) led to an increased accumulation of ATP (and related adenine nucleotides) in the bath effluent from an average basal value of 7,093±25 fmol (mg tissue)$^{-1}$ to a total value of 11,363±57 fmol (mg tissue)$^{-1}$ (n=4). Nerve-evoked release of ATP (and related adenine nucleotides) was dependent on extracellular Ca^{2+} and on neuronal activity, since omission of Ca^{2+} in the Tyrode's solution or application of 1 µM tetrodotoxin essentially abolished nucleotides outflow (Magalhães-Cardoso *et al.*, 2003). Electrical nerve stimulation also led to significant (P<0.05) extracellular adenosine accumulation while the amounts of inosine and hypoxanthine remained virtually unchanged (data not shown) providing that adenosine deaminase activity is inhibited with erythro-9(2-hydroxy-3-nonyl) adenine (EHNA, 0.3 µM); nerve-evoked adenosine outflow increased from an average basal value of 2,343±26 fmol (mg tissue)$^{-1}$ to a total value of 3,525±88 fmol (mg tissue)$^{-1}$ (n=4).

L-Citrulline (470 µM) decreased the nerve-evoked release of adenine nucleotides by a similar proportion to that observed when measuring [^{3}H]ACh release, but slightly (P>0.05) increased the extracellular adenosine accumulation following phrenic nerve stimulation (Table 1). The inhibitory effect of L-Citrulline (470 µM) on the release of [^{3}H]ACh and adenine nucleotides from stimulated nerve terminals was prevented by the nucleoside transport blocker, NBTI (10 µM) (see also Figure 5a). Although these results indicate an essential role of the adenosine transport system mediating L-citrulline-induced inhibition of evoked transmitter release, they do not clearly implicate L-citrulline as a promoter of extracellular adenosine production. Therefore, we investigated in more detail the outflow pattern of adenine nucleotides and adenosine during the resting period before stimulus application. As shown in Figure 6, L-citrulline (470 µM) increased the resting adenosine outflow above the control level, without significantly (P>0.05) affecting the release of adenine nucleotides. Adenosine production caused by L-citrulline (470 µM) appears to be originated predominantly via the equilibrative nucleoside transport system, as it was blocked by NBTI (10 µM) (Griffith & Jarvis, 1996).

Discussion and Conclusions

NOS immunoreactivity has been localized in the sarcolemmal surface of muscle cells, intramuscular axons and neuromuscular synapses in a variety of vertebrate species including man. At the skeletal neuromuscular junction, constitutive neuronal NOS (nNOS) is considered to be the predominant isoform and seems to be localized mainly in the cytoplasm of pre-synaptic nerve terminals and terminal Schwann cells (Ribera *et al.*, 1998; Rothe *et al.*, 2005). Regional distribution and cellular density of nNOS may be altered during development and ageing (Blottner & Lück, 2001), and in some specific diseases (e.g. Duchenne muscular dystrophy, myasthenia gravis) (reviewed by Stamler & Meissner, 2001). Localization of endogenous nNOS and cyclic GMP-dependent protein kinase at the neuromuscular junction suggests that NO may be involved in the physiological modulation of ACh release. Alternatively, NO may act as a short-lived (milliseconds) diffusible (~500 µm) messenger originated from Schwann cells and muscle fibres to regulate synaptic activity and synapse formation in severed neuromuscular junctions (Descarries *et al.*, 1998; Thomas & Robitaille, 2001). The literature provides evidence that NO released from postsynaptic sources acts presynaptically (Moncada & Higgs, 1993), increasing the release of glutamate from neurons of the central nervous system (Garthwaite, 1991) or decreasing the release of both substance P and ACh from neurons of peripheral tissues (Gustafsson *et al.*, 1990). Although this hypothesis is feasible, we failed to modify the inhibitory effect of L-arginine by incubating the preparations with the extracellular NO scavenger, haemoglobin, while inhibition of NOS activity with L-NOARG completely prevented L-arginine action. As haemoglobin does not easily enter cells (Hakim *et al.*, 1996), but it was able to block the action of the NO donor, SIN-1 (Figure 2), the results suggest that L-arginine-induced NO formation catalysed by NOS occurs mainly inside nerve terminals directly leading to depression of [^{3}H]ACh release at the rat motor endplate.

In skeletal muscle, NO plays a role in the regulation of neural transmission (Wang *et al.*, 1995). NO appears to influence both quantal and nonquantal ACh release from pre-synaptic terminals in several types of neuromuscular junctions, including the mammalian skeletal muscle (Ribera *et al.*, 1998; Mukhtarov *et al.*, 2000). It is unlikely that nonquantal transmitter release accounts for the total amount of [^{3}H]ACh released upon electrical

stimulation of the phrenic nerve. This assumption is based on findings indicating that the spontaneously releasable neuronal pool of ACh is not labelled with [^{3}H]choline nor it is released by electrical nerve stimulation (Molenaar *et al.*, 1987), and it is completely exhausted (within minutes) in the presence of hemicholinium-3 (Nikolsky *et al.*, 1991). Whether nerve-induced glutamate secretion modulates non-quantal and spontaneous ACh release, directly via activation of neuronal metabotropic receptors or indirectly via activation of muscle N-methyl-D-aspartate (NMDA) receptors and retrograde diffusion of NO synthesized by skeletal muscle fibres, has been a matter of debate (see *e.g.* Malomouzh *et al.*, 2005). Although the glutamatergic modulation of neuromuscular transmission deserves further investigation, we failed to modify the nerve-evoked [^{3}H]ACh release in the presence of D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 50 µM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM), which antagonize respectively ionotropic NMDA and non-NMDA glutamate receptors (unpublished observations).

The mechanism underlying NO suppression of transmitter exocytosis remains unclear. NO may regulate calcium-activated potassium currents by mechanisms independent of any effect on the calcium influx through voltage-sensitive calcium channels (Certiner & Bennett, 1993). Such channels modulate the release of ACh from motor nerve terminals of frogs (Zefirov et al., 2002), where NO may regulate transmitter release via cyclic GMP-dependent and independent pathways (Thomas & Robitaille, 2001). In this work, we showed that the NO donor, SIN-1, decreased [^{3}H]ACh release from stimulated motor nerve terminals. The inhibitory action of SIN-1 was prevented by the guanylyl cyclase inhibitor, ODQ, suggesting that NO triggers activation of soluble guanylyl cyclase and thereby raises cyclic GMP levels in order to cause depression of transmitter exocytosis. Many actions of cyclic GMP are elicited by cyclic GMP-dependent (G) kinase (Moncada *et al.*, 1991). However, cyclic GMP can also directly gate certain ion channels and regulates the activity of cyclic AMP phosphodiesterase yielding to increases in intracellular cyclic AMP (Ignarro, 1991). This occurrence is highly improbable as activation of the cyclic AMP pathway increases, rather than decreases, [^{3}H]ACh release from stimulated motor nerve terminals of the rat (Correia-de-Sá & Ribeiro, 1994). At the amphibian neuromuscular junction, NO inhibition of neurotransmitter release cannot be solely explained by a reduction in calcium entry, suggesting that a regulation occur downstream calcium entrance (Thomas & Robitaille, 2001). Alternatively, NO has been

shown to mediate post-transcriptional modifications of proteins through reactions with thiol and/or transition metal centres (identified in ion channels, receptors, enzymes, transcription factors, and small G proteins) (Ignarro, 1991). These modifications may prevent normal interactions between proteins involved in synaptic vesicle-presynaptic membrane specific interactions occurring during exocytosis. The regulation via the frequency and the kinetics of vesicle fusion is, however, improbable since the frequency of miniature endplate potentials and the quantal content of neuromuscular transmission were not affected by NO donors at the rat diaphragm (Mukhtarov *et al.*, 2000). It, thus, appears that there are important species differences concerning the mechanisms underlying control of neuromuscular transmission by NO (namely between mammalian and amphibian), which may be of both structural and functional in nature (see *e.g.*, Wang *et al.*, 1995; Thomas & Robitaille, 2001).

L-arginine is a widespread amino acid involved in many physiological processes. The main importance of L-arginine is attributed to its role as a precursor for the synthesis of NO and its effects on neuromuscular transmission are not observed when L-arginine is exchanged for D-arginine (see also Silva *et al.*, 1999). Recently, data about specific targets of L-arginine action independent from NO-synthesis have emerged (Mori & Gotoh, 2000). Here, we showed that L-arginine depresses the release of [^{3}H]ACh release from stimulated motor nerve terminals with a higher efficacy than the NO donor, SIN-1, while the soluble guanylyl cyclase inhibitor, ODQ (applied in a 10 µM concentration that fully blocked the effect of SIN-1), partially preserved L-arginine inhibition. Hence, this suggests that inhibition of electrically evoked transmitter release by L-arginine involves co-operation of NO-dependent and independent mechanisms. The latter mechanism may be linked with an increased synthesis of L-citrulline, as this co-product of NO biosynthesis also reduced [^{3}H]ACh release from stimulated motor nerve terminals in a way that was additive to the inhibitory action of SIN-1. Inhibition of transmitter release by L-citrulline was independent of soluble guanylyl cyclase activity, since it was not affected by ODQ. Although it has been reported that L-citrulline is capable of sustaining maximal rates of NO production in NO-synthesising cells due to an extra supply of L-arginine (reviewed by Curis *et al.*, 2005), our results showed that the inhibitory effect of L-citrulline was virtually unchanged in the presence of the NOS inhibitor, L-NOARG (Moncada *et al.*, 1991). Thus, data suggest that NOS catalyses the formation of two biologically active products, NO and L-citrulline, which contribute independently to depress

[^{3}H]ACh release from stimulated motor nerve terminals. Re-cycling of L-arginine from an endogenous precursor, L-citrulline, might not occur at the rat motor endplate and, hence, L-citrulline acts by itself or strengthens the action of other release inhibitory compound in order to depress neuromuscular transmission.

The real novelty of the present study is the observation that endogenous adenosine mediates L-citrulline-induced inhibition of [^{3}H]ACh release at the rat motor endplate. Previous reports have implicated endogenous adenosine accumulation as a key player operating the effects of the NOS pathway in the nervous system (Fallahi *et al.*, 1996; Rosenberg *et al.*, 2000). In keeping with this hypothesis, we showed that the inhibitory effect of L-arginine was partially attenuated by reducing endogenous adenosine accumulation with ADA, the enzyme that inactivates adenosine into its inactive metabolite inosine, or with the nucleoside transport inhibitor, NBTI (Correia-de-Sá & Ribeiro, 1996). Inactivation of extracellular adenosine with ADA prevented the ability of L-citrulline to inhibit [^{3}H]ACh release, while keeping unchanged the effect of the NO donor, SIN-1. Because NBTI also blocked L-citrulline inhibition of transmitter release, as well as L-citrulline-induced adenosine outflow without causing a parallel shift in the release of ATP (and related adenine nucleotides), it seems reasonable to assume that adenosine is transported as such across the plasma membrane via the equilibrative nucleoside transport system (Griffith & Jarvis, 1996). The adenosine A_1 receptor seems to be the receptor underlying L-citrulline-induced inhibition, since pre-treatment with the selective A_1 antagonist, DPCPX, but not with the A_{2A} antagonist, ZM 241385, completely prevented the inhibitory effect of L-citrulline. It remains, however, to be elucidated whether L-citrulline-induced adenosine outflow results from inhibition of intracellular adenosine kinase, the primary metabolic pathway regulating both intra- and extracellular levels of the nucleoside (Lloyd & Fredholm, 1995), because most adenosine kinase inhibitors also compete with the nucleoside for the uptake system. Alternatively, intracellular adenosine may be generated from the hydrolysis of AMP produced as consequence of ATP consumption during the metabolism of L-citrulline catalysed by argininosuccinate synthetase (ASS, EC 6.3.4.5). Unfortunately, there are no inhibitors of this enzyme responsible for the rate-limiting step in the utilization of L-citrulline, but its activity is genetically impaired in citrullinemia, a rare autossomal recessive disorder leading to the accumulation (low millimolar range) of citrulline and ammonia in tissues and body fluids

(Curis *et al.*, 2005). Whether abnormalities of adenosine signalling contribute to the neurochemical imbalance (glutamate-mediated excitation) underlying neurological sequelae of citrullinemic patients and animal models (*e.g.* convulsions, tremor, seizures, extensor rigidity, coma, brain oedema) (Dodd *et al.*, 1992), deserve to be investigated in the future.

To our knowledge, this is the first demonstration that enzymatic conversion of L-arginine by NOS originates two neuronally active products, NO and L-citrulline, that act independently to depress evoked [^{3}H]ACh release from motor nerve endings. Physiologically, the NO pathway (and the formation of L-citrulline) is activated by nerve stimulation. While, NO may directly reduce transmitter release probability through stimulation of soluble guanylyl cyclase, L-citrulline acts by enhancing the A_1 inhibitory tonus secondary to adenosine transport into the synaptic cleft. Because of the inhibitory role of NO on evoked transmitter exocytosis, tonic activation of facilitatory A_{2A} receptors by adenosine generated from the breakdown of ATP co-released with ACh may become weaker. On the other hand, reduced formation of adenosine from ATP hydrolysis may be compensated by the action of L-citrulline generated during nerve stimulation, which facilitates adenosine release as such via equilibrative nucleoside transporters thereby changing the receptor activation balance towards the inhibitory A_1 receptor (Correia-de-Sá & Ribeiro, 1996) (see Table 1). These findings gain pathophysiological relevance since systemic L-arginine administration has been recently shown to provide therapeutic benefit in Duchenne dystrophic patients by decreasing muscle degeneration (Barton *et al.*, 2005), and adenosine-mediated changes in transmitter release, as described here, may contribute to the neurological signs of citrullinemia.

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Table 1 Effect of L-citrulline on the release of [^{3}H]ACh, adenine nucleotides (ATP) and adenosine (ADO) from stimulated motor nerve terminals in the absence and in the presence of the nucleoside transport inhibitor, S-(*p*-nitrobenzyl)-6-thioinosine (NBTI, 10 µM).

	S_2/S_1 ratios		
	[^{3}H]ACh release	*ATP release*	*ADO release*
Control	0.81±0.03 (8)	0.95±0.02 (4)	0.86±0.05 (4)
L-citrulline (470 µM)	0.59±0.03 (6)*	0.66±0.05 (4)*	0.91±0.12 (4)
L-citrulline (470 µM) + NBTI (10 µM)	0.80±0.06 (5)	0.95±0.09 (4)	0.76±0.03 (4)

The release of [^{3}H]ACh, adenine nucleotides (ATP) and ADO was elicited by two trains (S_1 and S_2) of electrical stimulation consisting of 750 pulses delivered at a 5 Hz frequency (0.04 ms pulse duration). Values for S_2/S_1 ratios are means ±s.e.m. L-citrulline (470 µM) was applied 15 min before S_2. NBTI (10 µM) was present throughout the assay, including S_1 and S_2; the S_2/S_1 ratio obtained in the presence of NBTI (10 µM) alone was not statistically different from control ratios (without any drug during S_1 and S_2). The number of experiments is between parentheses. **P*<0.05 (one-way ANOVA followed by Dunnett's modified *t*-test), significant differences from the control.

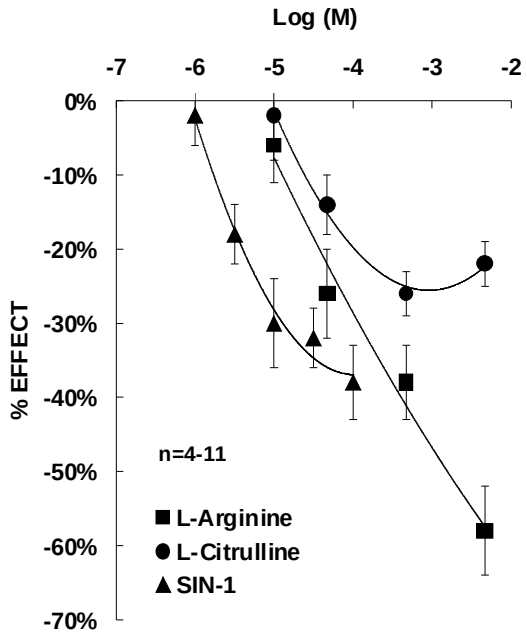

Figure 1 Concentration-response curves for the inhibitory effects of L-arginine (0.01-4.7 mM), L-citrulline (0.01-4.7 mM) and SIN-1 (1-100 µM) on [^{3}H]ACh release from motor nerve terminals stimulated with 5 Hz-trains (750 pulses). Abscissa, log of the concentration (M) of L-arginine (0.01-4.7 mM, squares), L-citrulline (0.01-4.7 mM, circles) and SIN-1 (1-100 µM, triangles), applied 15 min before S_2. Ordinate, percentage change in S_2/S_1 ratio as compared with the S_2/S_1 ratio in control experiments. Zero per cent represents identity between the two ratios, negative values indicate inhibition of evoked [^{3}H]ACh release. Each point represents the mean±s.e.mean of 4-11 experiments.

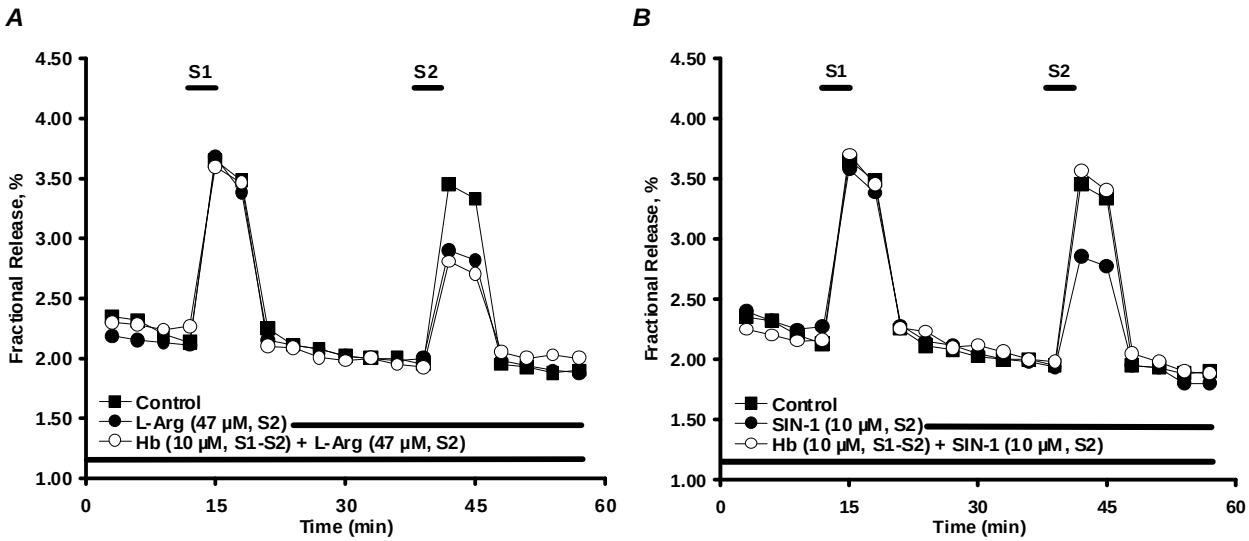

Figure 2 Effect of the extracellular NO scavenger, haemoglobin, on L-arginine- and SIN-1-induced inhibition of [^{3}H]ACh release from stimulated motor nerve terminals. Shown is the time course of tritium outflow from phrenic nerve terminals taken from typical experiments in the absence (Control, filled squares) and in the presence of (A) L-arginine (L-Arg, 47 µM) or (B) SIN-1 (10 µM), used either alone (filled circles) or in the presence (open circles) of haemoglobin (Hb, 10 µM). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 3 min. [^{3}H]ACh release was elicited by stimulating the phrenic nerve trunk with 750 pulses delivered with a frequency of 5 Hz at the indicated times (S_1 and S_2). L-arginine (47 µM) and SIN-1 (10 µM) were applied 15 min before S_2; haemoglobin (10 µM) was present throughout the assay, including S_1 and S_2 (horizontal bars). None of the drugs changed spontaneous tritium outflow.

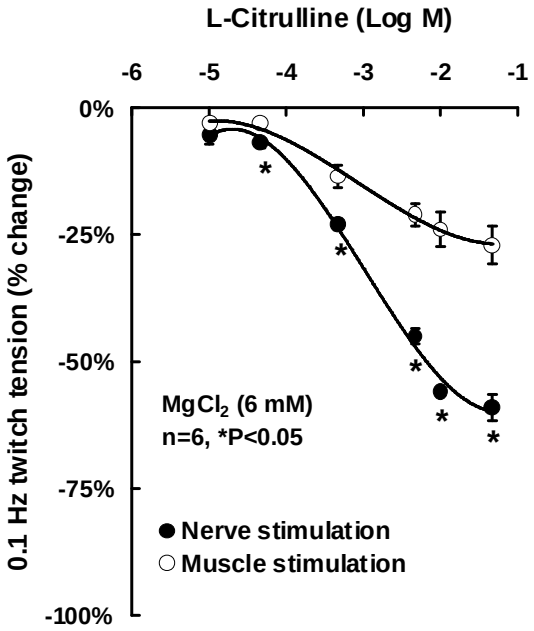

Figure 3 Inhibitory effect of L-citrulline (0.01-47 mM) on diaphragm twitch tension induced by phrenic nerve stimulation (indirect stimulation, filled circles) or by direct muscle depolarisation (direct stimulation, open circles) in conditions where the safety factor of neuromuscular transmission was reduced (high Mg^{2+}, 6 mM). Twitch responses were induced alternating stimulus application to the phrenic nerve trunk or to muscle fibres at a frequency of 0.1 Hz. L-citrulline (0.01-47 mM) was applied in a cumulative manner; each concentration contacted the preparation at least 12 min before solution changeover. Ordinate, percentage of inhibition from maximal twitches tension obtained in control conditions. Each point represents the mean±s.e.mean of 6 experiments. *P<0.05 (one-way ANOVA followed by Dunnett's modified t test) as compared with the effect of L-citrulline on twitch tension induced by direct muscle stimulation.

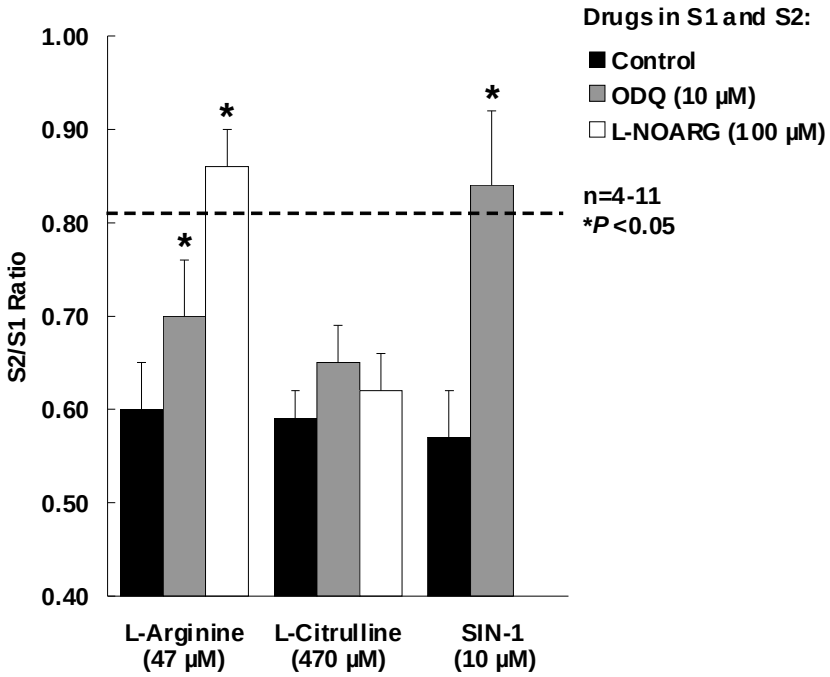

Figure 4 Influence of inhibition of soluble guanylyl cyclase (with ODQ) and NOS (with L-NOARG) on the reduction of evoked [^{3}H]ACh release caused by L-arginine, L-citrulline and SIN-1. L-arginine (47 µM), L-citrulline (470 µM) and SIN-1 (10 µM) were applied 15 min before S_2 in concentrations that caused about 30% inhibition of [^{3}H]ACh release from stimulated motor nerve terminals. ODQ (10 µM) and L-NOARG (100 µM) were present throughout the assay, including S_1 and S_2; the S_2/S_1 ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S_1 and S_2) (dashed horizontal line, see Methods section). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from 4-11 experiments. The vertical bars represent s.e.mean. *P<0.05 (one-way ANOVA followed by Dunnett's modified *t* test) when compared with the effects of L-arginine, L-citrulline and SIN-1 applied alone, respectively.

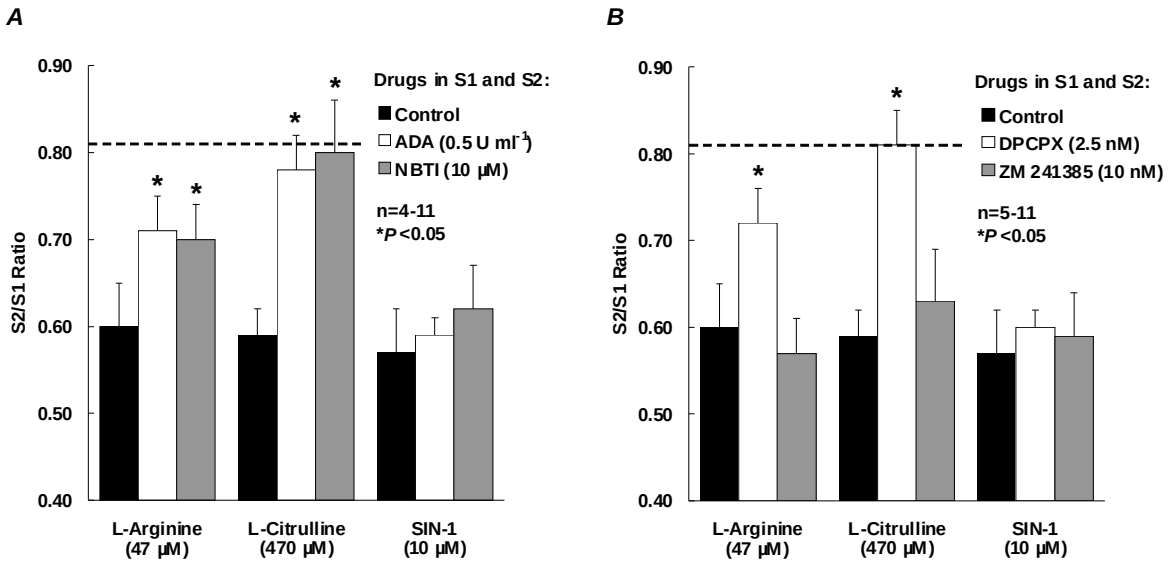

Figure 5 Role of endogenous adenosine on the inhibitory effect of L-arginine, L-citrulline and SIN-1 on evoked [^{3}H]ACh release from motor nerve terminals. L-arginine (47 µM), L-citrulline (470 µM) and SIN-1 (10 µM) were applied 15 min before S_2 in concentrations that caused about 30% inhibition of [^{3}H]ACh release from stimulated motor nerve terminals. (A) Adenosine deaminase (ADA, 0.5 U ml^{-1}), the nucleoside transport inhibitor (NBTI, 10 µM) and (B) the two adenosine antagonists exhibiting high subtype selectivity for A_1 (DPCPX, 2.5 nM) and A_{2A} (ZM 241385, 10 nM) receptors were present throughout the assay, including S_1 and S_2. The S_2/S_1 ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S_1 and S_2) (dashed horizontal line, see Methods section). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from (A) 4-11 and (B) 5-11 experiments. The vertical bars represent s.e.mean. *P<0.05 (one-way ANOVA followed by Dunnett's modified *t* test) when compared with the effects of L-arginine, L-citrulline and SIN-1 applied alone, respectively.

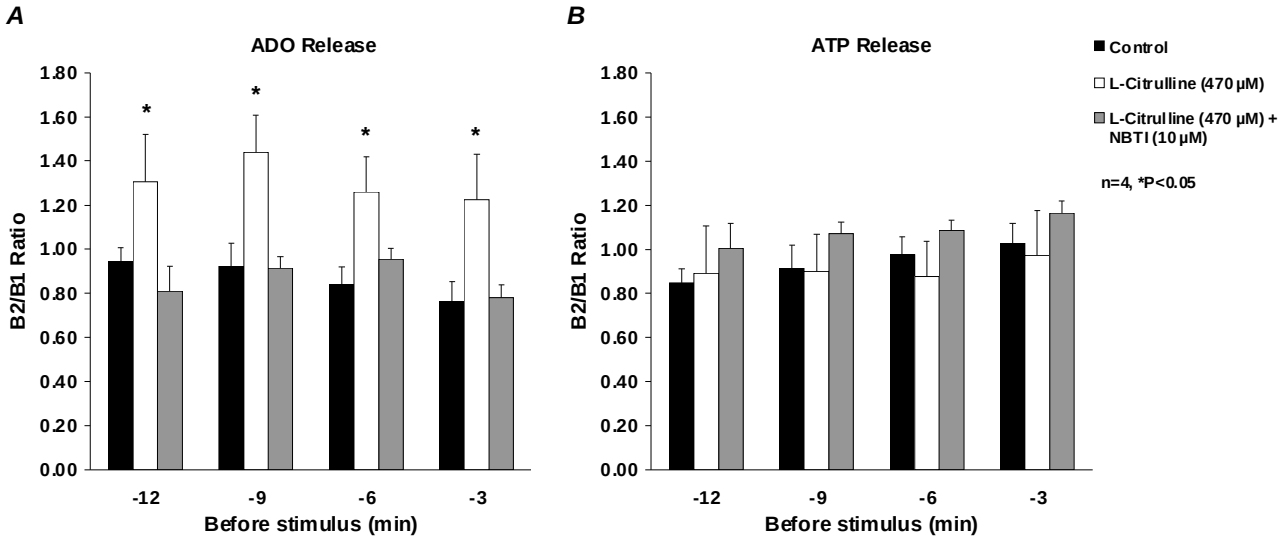

Figure 6 Effect of L-citrulline (470 µM) on the resting outflow of (A) adenosine and (B) ATP (and related adenine nucleotides) from the rat innervated hemidiaphragm in the absence and in the presence of the nucleoside transport inhibitor, S-(*p*-nitrobenzyl)-6-thioinosine (NBTI, 10 µM). L-citrulline (470 µM) was applied 15 min before S_2; NBTI (10 µM) was present throughout the assay, including S_1 and S_2. The ordinates represent the resting outflow of (A) adenosine and (B) ATP (and related adenine nucleotides) expressed by the $B_{2\text{-}n}/B_{1\text{-}n}$ ratios; $B_{1\text{-}n}$ and $B_{2\text{-}n}$ correspond to the purines content in bath samples collected *n* minutes before the first (without L-citrulline) and the second (in the presence of L-citrulline) stimulation periods, respectively. Each column represents pooled data from 4 experiments. The vertical bars represent s.e.mean. **P*<0.05 (one-way ANOVA followed by Dunnett's modified *t*-test), significant differences from the control.

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