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LILIAN MARTINS CASTELLÃO SANTANA

A AUTORREGULAÇÃO E A NEUROMODULAÇÃO DO TERMINAL NERVOSO MOTOR SÃO DEPENDENTES DO TRANSPORTE DE COLINA PARA O TERMINAL E DA LIBERAÇÃO DE ADENOSINA A PARTIR DAS CÉLULAS DE SCHWANN

Maringá
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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Wilson Alves do Prado
Coorientadora: Profa. Dra. Celia Regina Ambiel da Silva

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APRESENTAÇÃO

Esta tese de doutorado é composta por dois artigos científicos. O artigo já publicado teve como objetivo investigar se as ativações dos receptores M_1 e A_{2A} poderiam atenuar a fadiga tetânica induzida por HC-3 em preparações neuromusculares. No segundo artigo, a ser publicado, investigou-se se a adenosina poderia agir como um gliotransmissor mediando a inibição da liberação de acetilcolina via ativação de receptores alfa 7 em células de Schwann.

O trabalho é apresentado de acordo com as regras do Programa de Pós-Graduação em Ciências Biológicas, e os artigos foram redigidos de acordo com as regras de submissão de trabalhos exigidas pela revista *Pharmacology* e *Journal of Neuroscience.*

Lilian Martins Castellao-Santana, Priscila YumiAbiko, Celia Regina Ambiel, Ana Rita Peixoto, Jose Bernardo Noronha-Matos, Paulo Correia-de-Sá, Wilson Alves-Do-Prado. Tetanic Facilitation of Neuromuscular Transmission by Adenosine A_{2A} and Muscarinic M_1 Receptors is Dependent on the Uptake of Choline via High-Affinity Transporters. **Pharmacology**, v. 103, p. 38 – 49, 11 2019.

Lilian Martins Castellão-Santana, José Bernardo Noronha-Matos, Laura Oliveira, Ana Rita Peixoto, Liliana Almeida, Célia Regina Ambiel, Paulo Correia-de-Sá, Wilson Alves-do Prado. Nicotinic α7 receptor-induced adenosine release from perisynaptic Schwann cells controls acetylcholine spillover from the rat motor endplate during high frequency nerve firing. **Journal of Neuroscience**.

RESUMO GERAL

Introdução: A acetilcolina (ACh) é o neurotransmissor liberado dos terminais nervosos colinérgicos. Após ser liberada para a fenda sináptica, a ACh pode interagir com receptores presentes no terminal nervoso motor (TNM), na musculatura esquelética, ou ainda, ser hidrolisada a acetato e colina pela ação da enzima acetilcolinesterase. Interagindo com os receptores nicotínicos da placa motora, a ACh determina a abertura de um canal central em cada receptor levando, como consequência, ao aumento do influxo de íons sódio e a saída de íons potássio. Esse aumento de condutância iônica pode atingir um nível limiar crítico, o qual determinará a ocorrência do potencial de placa terminal (PPT) que, por sua vez, induzirá a contração da musculatura esquelética. Interagindo com os receptores do TNM a ACh pode aumentar ou reduzir sua própria liberação, ativando receptores nicotínicos neuronais (Nn) que expressam as subunidades $\alpha_3\beta_2$ (facilitatórios), muscarínicos do subtipo M_1(facilitatórios) e muscarínicos do subtipo M_2 (inibitórios). A transmissão neuromuscular também é modulada por receptores de adenosina presentes no TNM. Tais receptores podem aumentar (A_{2A}) ou reduzir (A_1) a liberação de ACh, dependendo da demanda à qual o nervo está submetido. A adenosina que ativa tais receptores é proveniente da coliberação da ACh com ATP. Este último é degradado à adenosina por uma sequência de reações catalisadas por ectonucleotidases presentes na fenda sináptica. A adenosina também pode ser transportada do interior do TNM, por meio dos transportadores NBT1, para a fenda sináptica. As ativações dos receptores M_1 e A_1 são preferenciais quando baixas frequências (~5,0 Hz) de estimulações estão sendo aplicadas sobre o nervo motor, já que, em tais condições, há a presença de pequenas quantidades de adenosina na fenda sináptica. Por outro lado, há uma predominância da atividade dos receptores M_2 e A_{2A} quando o TNM passa a receber frequências de estimulação mais elevadas ($\geq$ 50,0 Hz), uma vez que, em tais condições, o nível de adenosina na fenda sináptica está aumentado. Conversas cruzadas (*cross-talking*) entre os receptores facilitatórios M_1 e A_{2A} e inibitórios M_2 e A_1 ocorrem no TNM. Demonstrou-se que a ativação de M_1 reduz a atividade inibitória de M_2. Os receptores M_2 podem ter sua atividade reduzida pela ativação dos receptores A_1 via *down-regulation*. Por outro lado, a atividade de M_1, por sua vez, é reduzida quando os receptores A_{2A} estão plenamente ativados (frequência de estímulos $\geq$50 Hz), podendo assim, levar a uma intensificação da atividade inibitória dos receptores M_2 do TNM. Concomitantemente a estas interações acima descritas, também está ocorrendo o transporte de moléculas de colina para o interior do TNM, para que tal substrato possa participar na síntese de novas moléculas de ACh. Na membrana pré-sináptica existem dois tipos de transportadores de colina. Os transportadores de alta afinidade (sódio dependente, inibidos seletivamente pelo *hemicholinium-3*) e de baixa afinidade (sódio independente). Os transportadores de colina são tão importantes para o controle da transmissão neuromuscular, que qualquer ineficiência em suas atividades determina grave redução na quantidade de moléculas de ACh liberada dos TNM, à qual, por sua vez, pode culminar no aparecimento de síndromes miastênicas. Sendo assim, modelos de fadiga tetânica com a utilização do *hemicholinium*-3 (HC-3) podem ser úteis para investigar as adaptações neuroquímicas pré-sinápticas que ocorrem nessas síndromes. Com base no exposto, o primeiro artigo desta tese teve como objetivo investigar se as ativações dos receptores M_1 e A_{2A} poderiam atenuar a fadiga tetânica induzida por HC-3 em preparações neuromusculares. Adicionalmente, tem sido verificado que moléculas de colina, por si mesmas, poderiam interagir com receptores nicotínicos do subtipo α_7(RCNn-α_7). Desde que a presença de receptores RCNn-α_7 foi demonstrada em células de *Schwann* (CS) presentes nos axônios motores, levantamos a hipótese de que a colina poderia ter um papel modulador da transmissão neuromuscular, além da sua bem demonstrada participação na etapa de síntese das moléculas de ACh do TNM. Esta hipótese poderia ter um impacto relevante sobre o uso de agentes anticolinesterásicos na prática clínica, uma vez que tais fármacos, como a neostigmina, são comumente utilizados para melhorar a transmissão neuromuscular em pacientes miastênicos, ou para reverter o bloqueio da transmissão neuromuscular em pacientes tratados com relaxantes musculares não despolarizantes. Além disso, não há registros da natureza química do mediador que seria liberado das CS após a ativação dos receptores RCNn-α_7 presentes em tais células. Desta forma,

no segundo artigo apresentado nesta tese, foi investigado se a adenosina poderia ser o gliotransmissor que faria a mediação da inibição da liberação de ACh após ativação de RCNn-α7 em CS e como tal via neuroquímica poderia estar ligada aos efeitos clínicos de agentes anticolinesterásicos.

Metodologia: O comitê de ética de experimentação animal da Universidade Estadual de Maringá aprovou (nº 7227300915/ CEUA-UEM) todos os procedimentos utilizados nesta tese. Os experimentos realizados no Instituto de Ciências Biomédicas Abel Salazar (ICBAS)/ Universidade do Porto seguiram as recomendações da Convenção para a Proteção dos Animais Vertebrados Utilizados para Experimentação e outros fins científicos. Preparações nervo frênico-diafragma isolado de ratos Wistar foram utilizadas para medir (i) contrações do músculo esquelético durante alta frequência de estimulação (50 Hz), (ii) liberação de ACh marcada com trício e, (iii) quantificação da exocitose do neurotransmissor por video-microscopia em tempo real usando, como marcador fluorescente, o FM4-64.

Para o registro e análise do perfil das contrações musculares (i), as preparações foram indiretamente estimuladas com pulsos de 0,2 Hz e, a cada 20 minutos, estímulos tetanizantes (50 Hz) foram aplicados no nervo frênico, durante 10 segundos. No primeiro artigo, o perfil das contrações encontrado após a estimulação foi analisado por meio da razão (R) entre a tensão tetânica obtida no final (B) da estimulação e tensão tetânica produzida no início (A) (R=B/A). Os valores de R obtidos após a administração das drogas foram considerados como porcentagem do valor de R controle (sem drogas). O HC-3 foi administrado 35 min após o tétano controle e os valores da razão B/A obtidos 45 min após a adição do HC-3 foram comparados. Quando fizemos associações de fármacos, tais agentes foram administrados 20 min antes da adição do HC-3. Para comparação, o efeito inibitório da neostigmina também foi testado 40 min depois do bloqueio seletivo do receptor nicotínico α3β2. No segundo artigo, colina e PNU 282987foram introduzidos no mesmo instante do HC-3. Ainda no segundo artigo, um cateter de polietileno foi introduzido na veia cava inferior para injeção retrógrada de 0,5 µM de ACh a fim de verificar os efeitos pós-sinápticos de drogas.

A quantificação direta da ACh (ii) foi avaliada somente no segundo artigo. O protocolo experimental baseou-se em quatro períodos, sendo eles, equilíbrio (30 min), marcação (cloreto de colina marcada com trício - 1 Hz - 40 min), lavagem (HC-3 - 60 min) e liberação (S1 e S2). Os fármacos foram adicionados 15 minutos antes do S2 e estavam presentes até o final do período de liberação. Os efeitos dos mesmos foram avaliados pela comparação das razões S2/S1: S2 na presença de fármacos e S1 na ausência de fármacos. Os parâmetros de estimulação (5 e 50 Hz) nos períodos S1 e S2 foram monitorizados em um osciloscópio e foram aplicados uma série de 5 tétanos, cada um com 150 pulsos, separados por um intervalo de 20 segundos sem estimulação. Sendo assim, a liberação do neurotransmissor foi determinada pela quantificação do efluxo evocado de trício, a partir das terminações marcadas com colina.

A técnica de vídeo-microscopia em tempo real (iii) teve como objetivo medir a exocitose de ACh em tempo real. A preparação passou por 4 etapas. Após o período de equilíbrio, ocorreu incubação da preparação com alfa-bungarotoxina, durante 20 min, para evitar contrações das fibras musculares, o que dificultaria a análise dos sinais de fluorescência. Em algumas preparações, a paralisia do diafragma foi realizada com µ-conotoxina, uma toxina que bloqueia os canais de sódio dependentes de voltagem do músculo esquelético, mas não os do nervo. A preparação passou por uma lavagem e recebeu o corante fluorescente FM4-64, durante 10 min. Após esse período de incubação com o corante, o nervo foi estimulado com 250 pulsos de intensidade supramáxima (duração de 0,04 ms, 8 mA) aplicados na frequência de 50 Hz. Após um período de 10 minutos de repouso da preparação, já na presença do corante, a fluorescência do FM4-64 foi lavada vigorosamente durante 30 minutos. Após a lavagem do corante, a taxa de exocitose vesicular foi medida a partir do decaimento resultante no sinal de fluorescência (hotspots) obtida após a estimulação.

Resultados e discussão: Nos experimentos para registros miográficos do primeiro artigo, a ativação dos receptores A_{2A} e M_1 com CGS21680 e McN-A-343c respectivamente, aumentou os valores de R. Efeitos facilitatórios similares foram obtidos com a ativação da proteína kinase C (PKC) e A (PKA) com forskolin (FSK) e forbol 12-miristato 13-acetato (PMA), respectivamente. Por outro lado, a administração de HC-3 causou uma redução nos valores da razão B/A, diminuiu a exocitose medida por vídeo microscopia e impediu a facilitação do aumento da razão B/A causada por CGS21680, McN- A-343c e FSK, com um efeito menor sobre o PMA. A neostigmina também diminuiu a exocitose de ACh, cujo efeito foi impedido por HC-3. Estes resultados indicam que a facilitação tetânica da transmissão neuromuscular causada pela ativação de receptores A_{2A} e M_1 é dependente da captação de colina pelo transportador de colina de alta afinidade, e que a fadiga tetânica da neostigmina pode ser atenuada pelo bloqueio do transportador de colina.
No segundo artigo, nos experimentos de miografia e de quantificação direta de ACh marcada, a colina e o agonista seletivo dos RCNn-α7, PNU282987, diminuíram a capacidade da transmissão neuromuscular em manter uma adequada e sustentada contração muscular. Um efeito inibitório similar foi encontrado na técnica de vídeo microscopia. Por outro lado, o antagonista RCNn-α7 metilcaconitina (MLA) e o fluoroacetato foram capazes de prevenir os efeitos inibitórios do PNU282987, bem como da neostigmina na exocitose em tempo real. Esses resultados sugerem que as CS participariam ativamente da redução da liberação de ACh do TNM, naquelas situações em que as moléculas de ACh na fenda sináptica atingissem concentrações suficientes para ativar os RCNn-α7. Nestas condições, tais receptores estariam envolvidos no controle negativo da liberação de ACh. A remoção de adenosina endógena com adenosina desaminase (ADA), a inibição da liberação de adenosina pelo transportador ENT1 com S-(4-nitrobenzil)-6-tioinosina (NBTI) e o bloqueio dos receptores A_1 com 1,3-dipropil-8-ciclopentilxantina (DPCPX) bloquearam a inibição da exocitose de ACh em tempo real por PNU282987. Estes dados indicam que a adenosina é o gliotransmissor envolvido no controle da liberação de ACh, via ativação de receptores A_1. Por outro lado, infelizmente, não conseguimos prevenir o efeito inibitório do agonista PNU 282987 na quantificação direta de ACh marcada na presença do inibidor seletivo de PLC, U73122, bem como obtido com o inibidor do receptor IP3, 2-APB, o que dificultou a investigação das vias moleculares que envolvem a liberação de adenosina via RCNn-α7.

Conclusão: No primeiro artigo, os dados indicam que a facilitação neuromuscular pelos receptores de adenosina A_{2A} e colinérgicos M_1 é altamente dependente da atividade do transportador de colina de alta afinidade. No segundo artigo, os resultados sugerem que RCNn-α7 das CS controlam a liberação de ACh da sinapse neuromuscular, favorecendo a saída de adenosina via ENT1 e ativando receptores inibitórios pré-sinápticos A_1. No entanto, é necessário elucidar quais determinantes moleculares estariam envolvidos na ativação dos RCNn-α7 e na liberação de adenosina para o meio extracelular pelas CS. Além disso, os dois trabalhos jogam um novo foco sobre os efeitos gerados por agentes anticolinesterásicos na transmissão neuromuscular, posto que, após estes estudos, não seria mais possível afirmar que os efeitos de tais agentes estão vinculados somente ao acúmulo de ACh na fenda sináptica, já que os níveis de colina também reduzem em tais circunstâncias. Assim, os baixos níveis de colina também poderiam ser responsáveis pelos efeitos dos agentes anticolinesterásicos sobre a transmissão neuromuscular.

SUMÁRIO

Tetanic Facilitation of Neuromuscular Transmission by Adenosine A_{2A} and Muscarinic M_1 Receptors is Dependent on the Uptake of Choline via High-Affinity Transporters

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Abstract

Background/Aims: In this study, we evaluated the functional impact of facilitatory presynaptic adenosine A_{2A} and muscarinic M_1 receptors in the recovery of neuromuscular tetanic depression caused by the blockage of high-affinity choline transporter (HChT) by hemicholinium-3 (HC-3), a condition that mimics a myasthenia-like condition. ***Methods:*** Rat diaphragm preparations were indirectly stimulated via the phrenic nerve trunk with 50-Hz frequency trains, each consisting of 500–750 supramaximal intensity pulses. The tension at the beginning (A) and at the end (B) of the tetanus was recorded and the ratio (R) B/A calculated. ***Results:*** Activation of A_{2A} and M_1 receptors with CGS21680 (CGS;2 nmol/L) and McN-A-343c (McN; 3 μmol/L) increased R values. Similar facilitatory effects were obtained with forskolin (FSK; 3 μmol/L) and phorbol 12-myristate 13-acetate (PMA;10 μmol/L), which activate adenylate cyclase and protein kinase C respectively. HC-3 (4 μmol/L) decreased transmitter exocytosis measured by real-time videomicroscopy with the FM4-64 fluorescent dye and prevented the facilitation of neuromuscular transmission caused by CGS, McN, and FSK, with a minor effect on PMA. The acetylcholinesterase inhibitor, neostigmine (NEO; 0.5 μmol/L), also decreased transmitter exocytosis. The paradoxical neuromuscular tetanic fadecaused by NEO (0.5 μmol/L) was also prevented by HC-3(4 μmol/L) and might result from the rundown of the positive feedback mechanism operated by neuronal nicotinic receptors (blocked by hexamethonium, 120 μmol/L). ***Conclusion:*** Data suggest that the recovery of tetanic neuromuscular facilitation by adenosine A_{2A} and M_1 receptors is highly dependent on HChT activity and may be weakened in myasthenic patients when HChT is inoperative.

Keywords: Hemicholinium・ High-affinity choline transporter ・Muscarinic receptors ・ Adenosine receptors ・Neuromuscular tetanic fade

Introduction

After being released from cholinergic nerves, acetylcholine (ACh) is rapidly hydrolyzed to acetate and cholineby acetylcholinesterase at the synaptic cleft [1]. Choline returns to the cholinergic nerve terminal through theaction of high- and low-affinity choline transporters, where it is used to recycle ACh that becomes immediately available to rapidly mobilizing synaptic vesicles [2].Under physiological conditions, choline uptake via theNa+-dependent high-affinity transporter (HChT) is the rate-limiting step for ACh synthesis [3–5]. In addition, it has been demonstrated that ACh recycling and vesicular release during high-frequency stimulation of cholinergic nerves is highly dependent on the choline uptake by HChT [6, 7]. Impairment of the HChT function causes a progressive decay in the amount of ACh released per pulse during high-frequency stimuli [8, 9], which may beacause for the prevalence of myasthenia-like syndromes [10]. Thus, mimicking the neuromuscular tetanic fade observed in myasthenics with hemicholinium (HC-3), a selective inhibitor of HChT [8, 11], may be a useful model to investigate the pre-synaptic neurochemical adaptations operating in myasthenic syndromes.

At the neuromuscular junction, nerve-evoked ACh release is also fine-tuning controlled by the interplay between pre-synaptic nicotinic, muscarinic and adenosine receptors depending on the motor nerve firing pattern. Neuronal nicotinic receptors are fast desensitizing α3β2-subunit containing receptors involved in the short-term positive feedback mechanism of ACh release [12]. Tetanic release facilitation mediated by nicotinic α3β2 auto receptors is cut-short by activation of adenosine A_{2A} receptors by endogenously generated adenosine [13] from ATP co-released with ACh [14]. Likewise, the interplay between pre-synaptic facilitatory muscarinic M_1 and adenosineA_{2A} receptors has been demonstrated [15–18].While the muscarinic M_1-positive feedback mechanism is operative, the adenosine A_{2A}-receptor-mediated facilitation is suppressed. Conversely, upon increasing the levels of adenosine generated at the synapse to levels capable of activating facilitatory A_{2A} receptors, the M_1-positive feedback is weakened and the muscarinic neuromodulatory control is shifted toward M_2 inhibition [17–20].

Muscarinic M_1 receptors typically couple via subunits of Gq/11 protein family to phospholipase C leading to inositol1,4,5-trisphosphate (IP3)-sensitive intracellular Ca^{2+}mobilization and to protein kinase C (PKC) activation [21, 22]. At the rat motor nerve terminals, facilitatory adenosineA_{2A} receptors are positively coupled to the adenylatecyclase/cyclic AMP (cAMP)/protein kinase A(PKA) transducing system [22, 23].

Evidence from our group indicates that the negative interplay between muscarinic M_1 and adenosine A_{2A} receptors operating facilitation of ACh release may be due to signal convergence to a common pathway involving PKA activation and Ca2+ recruitment through Cav1 (L-type) channels [16], which do not ordinarily participate in the release process [24–26].

Decreases in the neuromuscular transmission safety factor, defined by Wood and Slater [27] as a decline of the excess of transmitter release that normally warrants the ability of neuromuscular transmission to remain effective under various conditions, are hallmarks of myasthenic syndromes. In myasthenic conditions, the amount of transmitter released per nerve impulse during high-frequency nerve firing may become insufficient to trigger post-synaptic action potentials, resulting in progressive muscle weakness [28, 29]. Thus, understanding the features that regulate the safety factor of neuromuscular transmission, namely, the coupling between incoming action potentials and transmitter exocytosis, may be clinically relevant for devising new strategies to effectively manage myasthenia.

In healthy motor endplates, tetanic depression may be overcome by tonic adenosine A_{2A}-receptor-mediated facilitation of transmitter release [30]. However, this mechanism is impaired in rats with toxin-induced and experimental autoimmune Myasthenia gravis (MG). In these studies, no changes were detected in A_{2A}-receptors expression on motor nerve terminals by immune fluorescence confocal microscopy [31, 32]. The molecular path sunder lying these deficits in myasthenics are uncertain, but evidence point to an impairment of the adaptive shift from CaV2.1 (P/Q type) to CaV1 (L type) channels leading to tetanic failure associated with deficits in endogenous adenosine accumulation and A_{2A} receptor tone. Interestingly, this situation can be restored by the exogenous application of the adenosine precursor AMP. Moreover, methylprednisolone, a corticosteroid that is widely used to depress immune responses in MG patients, also contributes to ameliorate motor deficits bypromoting neuromuscular transmission through a mechanism that involves tonic activation of neuronal facilitatory adenosine A_{2A} receptors leading to increases in the release probability of immature recycled vesicles [33].

Thus, considering that (i) HChT is a major regulator of ACh refilling of synaptic vesicles and release during tetanic stimulus, and that (ii) inhibition of high-affinity neuronal choline uptake is associated with myasthenic-like conditions, we designed experiments to test whether the activation of facilitatory adenosine A_{2A} and muscarinic M_1 receptors on motor nerve terminals could restore tetanic depression of phrenic nerve-diaphragm preparations pre-treated

with HC-3. Results obtained using this pre-synaptic in vitro model of myasthenia will be compared to previous findings where the muscle component was predominantly affected using rats with toxin induced and experimental autoimmune MG [31, 32]. We also hypothesize that by reducing the amount of choline available for neuronal uptake, the acetylcholinesterase inhibitor, neostigmine (NEO), may mimic inhibition of HChT by HC-3 while increasing ACh accumulation at the neuromuscular junction and, hence, the cholinergic tone [34]. The influence of HC-3 the paradoxical NEO induced tetanic fade will also be evaluated for comparison.

Materials and Methods

Animals

Rats (Wistar, 150–250 g) were kept at a constant temperature (21 ° C) and a regular light (06.30–19.30 h) – dark (19.30–06.30 h) cycle for at least 10 days prior to the experiments, with food and water ad libitum. The Ethics Committee for Experimental Animals Studies of the State University of Maringa approved (ECEAS7227300915) the procedures used in the present study. All experiments performed at ICBAS/University of Porto were conducted instrict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). All experimental protocols involving animals were approved by the competent national authority Direcao Geral de Alimentacao e Veterinaria, and by the ICBAS Animal Ethical Committee (No. 224/2017). All efforts were made to minimize animal suffering and to reduce the number of animals used according to the ARRIVE guidelines.

Myographic Recordings

Phrenic nerve-diaphragm muscle preparations of rats were setup as described by Bulbring [35]. Each preparation was immersed in a 20.0 mL chamber containing Krebs' buffer solution (mmol/L,NaCl 110.0; KCl 4.7; CaCl2 3.0; MgCl2 1.3; NaHCO3 25.0; KH2PO41.0; glucose 11.1) maintained at 37.0 ° C and aerated with a mixture of O2 (95%) and CO2 (5%). The phrenic nerve was stimulated through a bipolar platinum electrode. Preparations were indirectly stimulated at 0.2 Hz and 6 tetanic stimuli (50.0 Hz) were applied at 20.0 min intervals. Each hemi-diaphragm preparation was connected to a force displacement transducer (Grass FT 03; Grass Instruments Division, West Warwick, RI, USA) to record muscular contractions on Chart

Software (Powerlab; AD Instruments, CastleHill, NSW, Australia). The initial tetanic tension at the beginning (A) of the tetanic stimulus and tension at the end (B) of the titanic stimulus (after 10.0 s; B) was recorded and the ratio (R) B/A calculated (Fig. 1a). HC-3 (4–10 μmol/L) was applied in a cumulative manner and contacted the preparations at least for 45 min before any valid myographic recording was obtained. This protocol was adopted, since HC-3 effects following each bath application required roughly 45 min to stabilize under the present experimental conditions. Test drugs (CGS21680 [CGS], McN-A-343c [McN], methoctramine [Meth], phorbol 12-myristate 13-acetate [PMA], forskolin [FSK] and NEO) were applied 20 min after starting the HC-3 application and, thus, contacted with the preparations for at least 25 min. For comparison purposes, the paradoxical inhibitory effect of NEO on tetanic tension was also tested 40 min after selectively blocking the α3β2-subunit nicotinic receptor with hexamethonium (HEX) and/or the muscarinic M_2 receptor with Meth.

Real-Time Video-Microscopy Using the FM4-64 Fluorescent Dye as a Measure of Transmitter Exocytosis

Labeling nerve terminals and measuring real-time exocytosis was performed as previously described [36–39]. Phrenic nerve hemidiaphragm preparations (4–6 mm width) were mounted on the stage of an upright epifluorescence microscope (Zeiss Axiophot,Oberkochen, Germany). The preparations were superfused (1 mL/min) in a 3-mL organ bath at 24 ° C with Tyrode's solutioncontaining (mmol/L): NaCl 137, KCl 2.7, CaCl2 1.8, MgCl2 1,NaH2PO4 0.4, NaHCO3 11.9 and glucose 11.2, which was continuously gassed with 95% O2 and 5% CO2. After a 30-min period, muscle stimulation was established at a supramaximal voltage of14.5 ± 2 V; at this point, after the equilibration period, phrenicnerve-hemidiaphragm preparations were incubated with α-BTX(4 μmol/L, during 15–20 min), which prevents nerve-evoked muscle fiber contractions, since it irreversibly blocks muscle-type nicotinic ACh receptors containing α1 subunits, but not nicotinic receptors present on motor nerve terminals [12, 40]. After a 10-minincubation period with FM4–64 (5 μmol/L) made up in Tyrode'ssolution, loading of synaptic vesicles (high-probability release pool) was achieved upon phrenic nerve trunk stimulation with 250pulses of supramaximal intensity (0.04 ms duration, 8 mA) applied at a frequency of 50 Hz, followed by an additional 10-min period of rest with the dye [31]. The excess dye was removed from the incubation fluid with a washout period of 10 min. Fluorescence images were acquired using a 63×/0.90 n.a. water-immersion objective lens (Achroplan; Zeiss). After the FM4-64

excess washout, test drugs were added to the preparation by continuous perfusion (1 mL/min) through an automatic perfusion system (ValveLink8.2,Digitimer, Welwyn Garden City, UK) connected to a fast solution heating device (TC-344B, Harvard Apparatus, March-Hugstetten,Germany). Afterwards, the phrenic nerve was stimulated (50-Hzfrequency intermittent bursts, 5 bursts of 150 pulses delivered with a 20-s interburst interval, 40 μs duration, 14.5 ± 2 V) and the FM4-64 decay (unloading of nerve terminals) monitored. Pulses were generated by a Grass S48 (Quincy, MA, USA) stimulator.

Fluorescence excitation light came from a XBO 75W Xenon ar clamp via a BP 546/12 nm excitation filter; fluorescence emission was filtered with an LP 590 nm filter. Images were acquired in the real-time mode with a high-resolution cooled CCD camera (Cool-Snap HQ, Roper Scientific Photometrics, Tucson, AZ, USA) coupled to a computer running a digital image acquisition software (MetaFluor 6.3; Molecular Devices Inc., Sunnyvale, CA, USA). Exposure time was adjusted between 200 and 550 ms (binning was adjusted to 2–3 and gain to 1–2). Regions of interest (nerve terminal areas) were manually outlined and the average intensity of the pixels inside this area was calculated. Background fluorescence was which was then subtracted from the average fluorescence measured at the interest motor endplate (for further details on the technique, see [38]. Fluorescence decay was expressed as a percentage of maximal loading considering that 100% is the fluorescence intensity at zero time. As previously described, nerve-evoked FM4-64 intensity decay reflects synaptic vesicle exocytosis at the nerve terminal [33, 36–39, 41].

Drugs and Solutions

2,2′-(4,4′-Biphenylene)bis(2-hydroxy-4,4-dimethylmorpholiniumbromide; hemicholinium-3, HC-3);2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethyl carboxamidoadenosine hydrochloridehydrate (CGS21680, CGS); (4-Hydroxy-2-butynyl)-1-trimethyl-ammonium-3-chloro-carbanilate chloride (McN-A-343c, McN); Methoctramine tetrahydrochloride hydrate (Meth); Phorbol12-myristate 13-acetate (PMA); Forskolin (FSK); Hexamethoniumbromide (HEX); 3-(N,N-Dimethylcarbamoyloxy)-N,N,N,-trimethylanilinium bromide (Neostigmine, NEO) were purchasedfrom Sigma-Aldrich (St. Louis, MO, USA). N-(3-triethylammoniumpropyl)-4-(6-[4-(diethylamino)phenyl]hexatrienyl) pyridiniumdibromide (FM4-64) and α-bungarotoxin(α-BTX) were purchased from ThermoFisher Scientific (Waltham,MA, USA). FM4-64 and PMA were made up in dimethylsulphoxide (DMSO); all

other drugs were made up in distilled water. Stock solutions were stored at 20^{o}C as frozen aliquots. Dilutions of stock solutions were made daily; solvents used at maximal concentrations were unable to change control values.

Statistics

Data are expressed as mean ± SEM from an *n* number of experiments. One animal/isolated diaphragm was used per experiment. Statistical analysis of data was carried out using one-way analysis of variance followed by Bonferroni post hoc test. For the nerve-evoked transmitter exocytosis, statistical analysis of data was carried out using a multiple *t* test method, assuming same scatter(SD) per point, followed by the Bonferroni-Dunn method. Values of $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ (adjusted *p* values)were considered to represent significant differences.

Results

Figure 1 shows myographic recording traces of nerve evoked muscle contractions obtained during short high frequency trains (50 Hz for 10 s). In control conditions, a brief facilitation (B > A) was evident during the course of tetanic (50 Hz for 10 s), that is, muscle tension transiently increased from the beginning (A) toward the end (B) of the high-frequency train delivered to the phrenic nerve trunk.

Inhibition of HChT with HC-3 (4–10 μmol/L) caused a decay of the maximal tetanic peak tension toward the end of the tetanus (B) in a concentration-dependent manner (Fig. 1a). Fading of tetanic contractile facilitation (decrease of the B value; Fig. 1a) after 45 min pre-incubation with HC-3 (4-10 μmol/L) was more pronounced than the reduction of the initial tetanic tension (A; Fig. 1c) resulting in significant decreases in R values (R = B/A), as shown in Figure 1b.

In control conditions, the activation of adenosine $A_{2A}$and muscarinic M_1 receptors with CGS (2 nmol/L) [17,23, 42] and McN (3 μmol/L) [15, 16, 43] increased (p <0.05) tetanic facilitation (R value) by 9.3 ± 1.2% (n = 4;Fig. 2a) and 4.6 ± 0.8% (n = 4; Fig. 2b) respectively. Similar facilitatory effects were obtained with FSK (3 μmol/L,11.2 ± 0.3%, n = 5; Fig. 3a) and PMA (10 μmol/L, 14.6 ± 0.5%, n = 4; Fig. 3b), which activate adenylate cyclase and PKC, respectively, resulting in increases in ACh release from stimulated motor nerve terminals [16, 20, 22, 23, 44].The effect of FSK (3 μmol/L) at the rat motor endplate seems to be specific, since 1,9-dideoxy FSK (3 μmol/L), an FSK analogue that does not activate the catalytic subunit of

adenylate cyclase, did not mimic the effect of FSK inprevious reports by the authors using similar experimental settings [23]. Likewise, PMA was used in a concentration (10 μmol/L) that facilitated evoked ACh release from rat phrenic motor nerve terminals through the activation of PKC, since its effect was prevented by the PKC inhibitor chelerythrine [20].

Pretreatment with HC-3, applied 20 min before test drugs in a concentration (4 μmol/L) previously demonstrated to be devoid of significant effect on the maximal tetanic tension to reduce blockage of muscle-type nicotinic receptors [45, 46], fully prevented (or even reversed)neuromuscular tetanic facilitation caused by CGS(2 nmol/L; Fig. 2a), McN (3 μmol/L; Fig. 2b) and FSK(3 μmol/L; Fig. 3a); the following percentage variation of R values were obtained under these experimental conditions:–9.6 ± 0.4% (n = 4), –49.3 ± 1.8% (n = 4; Fig. 2), and –4.2 ± 1.1% (n = 4), respectively. PMA (10 μmol/L)-induced tetanic facilitation (14.6 ± 0.5%, n = 4) was only slightly($p < 0.05$) attenuated (to 8.7 ± 4.6%, n = 4) after pre-treatment with HC-3 (4 μmol/L; Fig. 3b).

For comparison purposes, we tested the effect of the acetylcholinesterase inhibitor, NEO (0.5 μmol/L), a compound that is widely used in clinical practice to control safe recovery from neuromuscular block produced by antinicotinic agents and for the treatment of acute episodes of MG. Acetylcholinesterase inhibition by NEO results in ACh accumulation in the synaptic cleft while decreasing the amount of choline available for neuronal uptake, a situation that mimics the inhibition of HChT by HC-3. Despite put atively strengthening ACh-mediated effects, NEO (0.5 μmol/L) paradoxically reduced the tension of tetanic contractions toward the end to the 50Hz-frequency stimulation train by 92.7 ± 1.0% (n = 6;Fig. 4). This effect was similar to the NEO-induced trainof-4 fade that involves complex presynaptic mechanisms that regulate transmitter release, including desensitization of facilitatory α3β2-subunit containing neuronal nicotinic receptors and synaptic adenosine accumulation leading to a preferential activation of muscarinic M_2inhibitory receptors by breaking down muscarinic M_1activation [34].

As a matter of fact, NEO (0.5 μmol/L)-induced titanic fade (reduction in the R value by 92.7 ± 1.0%, n = 6) was significantly attenuated by HEX (120 μmol/L, R value decreased to 66.8 ± 1.2%, n = 5) and, to a minor extent, worsened by Meth (0.1 μmol/L, R value decreased to 97.0 ±0.0%, n = 5; Fig. 4) applied in conditions known to blockα3β2-subunit containing neuronal nicotinic receptors[12] and muscarinic M_2 receptors respectively [34].

Full prevention of NEO (0.5 μmol/L)-induced titanic rundown was obtained when HEX (120 μmol/L; 1.5 ±0.7%, n = 4) and Meth (0.1 μmol/L; 0.0 ± 0.9%, n = 4) were applied together

20 min before the acetylcholinesterase inhibitor (R value decreased to 17.7 ± 0.3%, n = 4; Fig. 4). Interestingly, the paradoxical NEO (0.5 μmol/L)-induced tetanic fade was also significantly (p <0.05) attenuated (R value decreased to 22.6 ± 0.8%, n = 4) upon the inhibition of HChT with HC-3 (4.0 μmol/L; Fig. 4).

Similar experimental findings were observed using video microscopy with the FM4-64 fluorescence dye to measure real-time transmitter exocytosis in preparations paralysed with the irreversible muscle-type nicotinic receptor blocker, α-bungarotoxin (4 μmol/L), which lacksany effect on ACh release [12]. NEO (0.5 μmol/L) significantly (p < 0.05) decreased the rate of FM4-64 fluorescence intensity decay during phrenic nerve stimulation with 50-Hz tetanic bursts (Fig. 5a). The inhibition of high-affinity choline uptake with HC-3 (4.0 μmol/L) also reduced tetanic-induced transmitter exocytosis fromFM4-64 loaded fluorescent hotspots (Fig. 5b). This results trengthens previous information indicating that ACh recycling and vesicular release during the course of a tetanus is dependent on choline uptake by HChT [6, 8, 9] and is in keeping with the theory that selective inhibition of HChT causes a myasthenia-like condition [10]. Similarly to that observed in myographic recordings, pretreatment with HC-3 (4 μmol/L) prevented the inhibitory action of NEO (0.5 μmol/L) on transmitter exocytosis evoked by50-Hz tetanic bursts (Fig. 5c).

Discussion

Data from myographic recordings and real-time videomicroscopy experiments confirm that inhibition of HChTby HC-3 causes a concentration-dependent decline of transmitter exocytosis during high-frequency (50 Hz) neuronal firing and, thus, reduces the ability of neuromuscular transmission to maintain a sustained muscular contraction in the course of a tetanus [8], resulting in a myasthenic-like condition [10]. We show here for the first time that tetanic facilitation of neuromuscular transmission caused by adenosine A_{2A} and muscarinic M_1 receptors activation is dependent on the uptake of choline via HC-3-sensitive HChT. If one considers that the impairment of HChT may be observed in pre-synaptic myasthenic conditions, the rehabilitation of tetanic neuromuscular facilitation through the activation of adenosineA_{2A} and muscarinic M_1 receptors might be endangered when this transporter is inoperative.

Given the differences in the magnitude of the blocking effect of HC-3 on tetanic neuromuscular facilitation caused by FSK and PMA, data suggest that the

adenylatecyclase/cAMP/PKA transduction system is relatively more affected by HChT inhibition than the PKC pathway. This is compatible with the reduction of the SNARE complex formation by PKA-mediated phosphorylation detected in vitro when this complex was set up by purified recombinant SNAP-25, syntaxin-1, and VAMP-2, whereas under the same experimental conditions, phosphorylation of the Ser187 residue by PKC caused an opposing effect [47]. The smaller negative impact of HC-3 on PMA induced tetanic facilitation may also be explained, considering that PKC activation may rapidly and transiently translocate HChT to the plasma membrane of motor nerve terminals, as seen in SH-SY5Y cells [5], which partially overcome transporters inhibition by HC-3.

This hypothesis, however, does not account for the dramatic reversal from facilitation (4.6%) to rundown (–49.3%) of tetanic contractions caused by the activation of M_1 receptors with McN verified in the absence or in the presence of HC-3 respectively. Reversal of M_1-induced tetanic facilitation due to HChT inhibition was much more obvious than that occurring with the adenosine A_{2A}receptor agonist, CGS, under similar experimental conditions. This is not surprising if one considers that the activation of facilitatory A_{2A} receptors prevails over M_1 facilitation during high-frequency stimulation trains delivered to the rat motor nerve terminals [15–18]. While stimulation of A_{2A} receptors leads to the activation of adenylatecyclase and production of cAMP, thereby increasing PKA activity, activated muscarinic M_1 receptor stimulates phospholipase C to release IP3 and diacylglycerol (DAG), which causes the release of intracellular Ca^{2+} and activates PKC respectively. Data from our group showed that secondary stimulation of PKA by M_1-induced PKC activation is thought to precede the augmentation of Ca^{2+}influx through Cav1 (L-type) channels and facilitation of ACh release. Therefore, coupling of muscarinic M_1 andadenosine A_{2A} receptors to a common pathway involving PKA activation and Ca^{2+} recruitment through Cav1 (Ltype) channels might explain the negative interplay between these receptors to facilitate ACh release at the rat motor endplate.

Uncoupling of the muscarinic M_1 receptor from its facilitatory pathway (secondary PKA activation and Ca^{2+}influx through Cav1 channels) may also explain the divergent effects obtained in this study comparing both the magnitude of facilitatory effects of the PKC activator, PMA, and the M_1 receptor agonist, McN, as well as their sensitivity to HChT inhibition by HC-3. The reason for the dramatic tetanic fade (49.3% less than control) obtained using McN to activate muscarinic M_1 receptors in the presence of HC-3 may be because this compound also

potently activates other muscarinic receptors, namely, the inhibitory M_2 subtype [21], when M_1 receptors are blocked or uncoupled from its intracellular second messenger pathway [15, 20, 43].

Interestingly, the involvement of Meth-sensitive muscarinicM_2, but not M_1, inhibitory receptors was shown to mediate anticholinesterase-induced fade produced by NEO in parallel to desensitization of facilitatory α3β2-subunit containing neuronal nicotinic receptors [34]. Using myographic recordings and real-time video microscopy with the FM4-64 fluorescent dye as a measure of transmitter exocytosis, we show here for the first time that the paradoxical NEO-induced tetanic fade reduction from control may be partially attenuated upon inhibition of HChT with HC-3. We surmise that our observation may be due to the fact that the inhibition of HChT leads to a significant reduction in ACh recycling and vesicular release per pulse during high-frequency trains [6, 8], as suggested by our video microscopy assay. In spite of the primary tests using high doses of HC-3 prompted for a post-synaptic neuromuscular blocking action of this drug[7], experiments elegantly designed by Elmqvist and Quastel [9] using intracellular electrophysiological recordings showed that lower doses of HC-3 (such as the one tested in our study, e.g., 4 μmol/L) exert predominant effects on ACh synthesis leading to a progressive decline of transmitter release from motor nerve terminals stimulated at high frequency rates, without affecting the postsynaptic transmitter chemosensitivity. Our findings are in keeping with this theory. As a matter of fact, HC-3 significantly decreased the rate of FM4-64 fluorescence intensity decay during phrenic nerve stimulation with 50-Hz tetanic bursts, clearly indicating that tetanic-induced transmitter exocytosis is impaired when the high-affinitycholine uptake system is inhibited. With reduced synaptic accumulation of ACh, the desensitization of facilitatory α3β2 nicotinic receptors and the activation of inhibitory M_2 receptors become less probable, thus decreasing the NEO-induced tetanic fade. The selective blockade of M_1 receptors by pirenzepine did not cause any measurable changes in NEO-induced fade, unless presynaptic adenosine A_{2A} receptors were blocked [34]. The absence of the effect of pirenzepine alone on NEO-induced fade shows that the activation of M_1 receptors located in skeletal muscle [48, 49] is highly unlikely in the presence of NEO.

Anticholinesterase-induced fade has been proposed to be modulated by ACh causing post-synaptic nicotinic receptor desensitization, but such desensitization was not observed when the function of muscle-type α1-containing receptors was evaluated during fade produced by tetanic stimulation [50]. In this regard, it is worth noting that NEO-induced fade of tetanic-induced transmitter exocytosis (unloading of FM4-64 fluorescent hotspots) was observed in

preparations paralysed with α-bungarotoxin, an irreversible muscle-type nicotinic receptor blocker that lacks any effect on ACh release[12]. Furthermore, it has been reported that atropine antagonizes the fade of neuromuscular contractions caused by HEX in the cat neuromuscular junction [43]. Here, we show that NEO-induced tetanic fade was attenuated by HEX. Thus, if one hypothesizes that tetanic fading caused by HEX and NEO is determined by interactions of both drugs with postsynaptic nicotinic receptors fading would increase, instead of decrease, when these compounds were applied together, but in fact pretreatment with HC-3 almost prevented NEO-induced fade of titanic contractions.

Corticosteroids, like prednisolone and dexamethasone, antagonize the inhibitory action of HC-3 both on the rate of choline uptake and the incorporation of choline into ACh in the rat diaphragm by interfering with Na+ influx across the plasma membrane [51, 52]. While glucocorticoids produce no changes on skeletal muscle twitches triggered by low frequency nerve stimulation, these drugs significantly enhance post-tetanic potentiation and attenuate neuromuscular block produced by antinicotinic muscle relaxants [53–55]. All these properties are mediated by non-genomic pre-synaptic actions of glucocorticoids [56, 57] and may contribute to improve neuromuscular transmission deficits in myasthenic patients besides their empirical use as immunosuppressive agents. Facilitation of the neuromuscular transmission by methylprednisolone during high-frequency motor nerve activity coincides with the predominant adenosineA_{2A} receptor tonus, which coordinates the interplay with other receptors (e.g., muscarinic) on motornerve endings to sustain ACh release that is required to overcome tetanic neuromuscular depression in myasthenics [31, 32]. Recently, we showed that the amplification of neuromuscular transmission by methylprednisolone depends on the tonic activation of presynaptic facilitatory adenosine A_{2A} receptors secondary toendogenous adenosine generated from ATP released underresting conditions [33]. The concurrent activation of facilitatory muscarinic M_1autoreceptors may also play a role. However, in contrast to the findings by Veldsema-Currie et al. [51], pretreatment with HC-3 failed to modify methylprednisolone-induced tetanic facilitation of nerve-evoked [3H]ACh release and muscle contractions (increase in R-value), thus indicating that interference with the HChT function plays a minor (if at all) role on neuromuscular transmission facilitation by the corticosteroid[33].

Taken together, these results suggest that methylprednisolone and adenosine A_{2A} (also probably muscarinicM_1) receptors synergize to amplify neuromuscular transmission during brief tetanic stimuli via an independent mechanism of the HChT, which might be beneficial to

overcome neuromuscular transmission deficits in myasthenic patients. Using video microscopy with the fluorescentFM4-64 dye and Dyngo-4a (a potent inhibitor of helicaldynamin I) to monitor endocytosis [58], we showed that corticosteroids negatively modulate synaptic vesicle turnover, thereby increasing the release probability of immature recycled vesicles via a mechanism that involves activation of adenosine A_{2A} and muscarinic M_1 receptors leading to downstream phosphorylation of synapsin I by PKA [33]. This is in line with the theory that bulk endocytosis contributes to the maintenance of neuromuscular transmission by regenerating synaptic vesicles to the readily releasable pool during high-frequency stimulation trains [59]. It remains, however, to be investigatedwhich of these mechanisms (impairment of HChT activity leading to deficits in ACh refilling of synaptic vesicles or redistribution of immature synaptic vesicles to the readily releasable pool) is the most relevant determinant of the neurochemistry deficits found in myasthenic individuals and whether those can be manipulated therapeutically with corticosteroids in association with adenosineA_{2A} and muscarinic M_1 receptor activators.

In conclusion, it was shown that tetanic facilitation of neuromuscular transmission caused by adenosine A_{2A} and muscarinic M_1 receptors activation is dependent on the uptake of choline via HC-3-sensitive HChT. Given the differences in the magnitude of the blocking effect of HC-3 on tetanic neuromuscular facilitation caused by FSK and PMA, data suggest that the adenylatecyclase/cAMP/PKA transduction system is relatively more affected by HChT inhibition than the PKC pathway.The paradoxical NEO-induced tetanic fade may be partially attenuated upon the inhibition of HChT withHC-3. This may be due to the fact that the inhibition of HChT leads to a significant reduction in ACh recycling and vesicular release per pulse during high-frequency trains.

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Disclosure Statement

The authors declare that they have no competing interests to disclose.

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Nicotinic α7 receptor-induced adenosine release from perisynaptic Schwann cellscontrols acetylcholine spillover from the rat motor endplate during high frequency nerve firing

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Running title: Nicotinic α7 receptors trigger adenosine outflow from terminal Schwann cells

Abstract

Acetylcholine (ACh) spillover from the motor endplate region may occur after long nerve firing bursts. It may also appear in the presence of cholinesterase inhibitors that are commonly used to improve the neuromuscular transmission in patients with *Myasthenia gravis* or to reverse the residual neuromuscular blockade in the context of general anesthesia. Despite nicotinic α7 receptors (α7 nAChR) localized on perisynaptic Schwann cells (PSCs) can sense and control ACh spillover from the neuromuscular synapse the mechanisms underlying communication between PSCs and the nerve terminal are not entirely understood. Here, we investigated whether adenosine could be the gliotransmitter mediating inhibition of transmitter release following α7 nAChR activation. Rat phrenic hemidiaphragms were used to measure nerve-evoked (i) myographic recordings, (ii) [^{3}H]ACh release, and (ii) transmitter exocytosiswith the FM4-64 fluorescent dye. The selective α7nAChR agonist,PNU282987, decreased tetanic (50 Hz-bursts)-induced muscle contractions. This effect, which was mimicked by the cholinesterase inhibitor neostigmine, derives from inhibition of transmitter exocytosis detected as decreases on [^{3}H]ACh release and FM4-64 dye unloading. The α7 nAChR antagonist, methyllycaconitine, and the gliotoxin, fluoroacetate, prevented the inhibitory effects of neostigmine and PNU282987. Removal of endogenous adenosine with adenosine deaminase (ADA, 2.5 U/ml), inhibition of adenosine release via ENT1 with S-(4-nitrobenzyl)-6-thioinosine (NBTI, 10 μM), and blockade of A_1 receptors with 1,3-dipropyl-8-cyclopentylxanthine, all prevented inhibition of ACh exocytosis by PNU282987. Data suggest that α7 nAChR controls tetanic-induced ACh spillover from the neuromuscular synapse by favoring adenosine outflow from PSCs via NBTI-sensitive ENT1 transporters and activation of presynaptic A_1 inhibitory receptors.

Keywords: Nicotinic α7 receptors; Acetylcholine release; Equilibrative nucleoside transporter 1 (ENT1); Adenosine A_1 receptor; Cholinesterase inhibitors; Neuromuscular junction; Perisynaptic Schwann cells.

1. Introduction

In addition to its stimulatory action on muscle fibers, acetylcholine (ACh) also acts pre-synaptically to regulate its own release at the rat neuromuscular junction (Bowman et al., 1988). Neuronal nicotinic autoreceptors containing $\alpha_3\beta_2$ subunits (Faria et al., 2003) mediate a short-term positive feedback mechanism, which is terminated by rapid receptors autodesensitization. Motor nerve terminals are also endowed with facilitatory M_1 and inhibitory M_2 muscarinic cholinoceptors (Oliveira et al., 2002; Santafé et al., 2003; 2006; 2015). Our group demonstrated that the M_1 / M_2 activation balance is fine-tuning regulated by endogenous adenosine (Oliveira et al., 2009), which can be released as such via equilibrative nucleoside transporters (Correia-de-Sá and Ribeiro, 1996; Barroso et al., 2007) or originated from the extracellular catabolism of ATP co-released with ACh upon motor nerve stimulation (Correia-de-Sá et al., 1996; Magalhães-Cardoso et al., 2003). The way adenosine builds its influence to control cholinergic neurotransmission at the motor endplate is highly dependent on the nerve firing pattern, both in health (Correia-de-Sá et al., 1996; Oliveira et al., 2004) and in disease conditions, like *Myasthenia gravis* (Noronha-Matos et al., 2011; Oliveira et al., 2015b) conditions.

While strict synaptic mechanisms are crucial to rapidly adapt the amount of transmitter release and, thereby, the safety margin of the neuromuscular transmission to the pattern of neuronal activation, little information is available about the neuromodulatory role of perisynaptic Schwann cells (PSCs). Using electrophysiology and imaging techniques, it was demonstrated that PSCs can sense and negatively modulate the excess of ACh spilling over from the motor endplate region after long nerve firing bursts in the presence of cholinesterase inhibitors through the activation of nicotinic receptors containing the α7 subunit (α7 nAChR)(Petrov et al., 2014). This

finding may be clinically relevant since cholinesterase inhibitors are commonly used to improve the neuromuscular transmission in patients with *Myasthenia gravis* or to reverse the residual neuromuscular blockade in the context of general anesthesia. Moreover, paradoxical reductions of neurotransmitter release (e.g. train-of-four fade, TOF_{fade}) have been observed with widely used cholinesterase inhibitors, like neostigmine, even in the presence of atropine (Alves-do-Prado et al., 1989; de Paula-Ramos et al., 2014). The neostigmine inhibitory effect was also reproduced by anti-(α1)nicotinic muscular relaxants exhibiting significant cholinesterase activity, like cis-atracurium (Bornia et al., 2009; 2011; Pereira et al., 2011). Adjustments of α7 nAChR activation by butyrylcholinesterase (BChE) anchored to the surface of PSCs has been demonstrated in the mouse neuromuscular junction, which might partially explain some of the paradoxical effects of cholinesterase inhibitors lacking selectively for acetylcholinesterase (AChE), as this enzyme subtype is clustered in the synaptic cleft basal lamina and therefore its inhibition is more prone to facilitate instead of inhibit the transmitter release (Petrov et al., 2014). The fact that choline, resulting from the cholinesterase breakdown of ACh, is a well-known activator of α7 nAChR (Papke et al., 1996) in physiological concentrations (Costa and Murphy, 1984; Gusev and Uteshev, 2010), also supports the functional link between α7 nAChR and BChE at the surface of PSCs.

There is, however, an area of uncertainty regarding the nature of the signal mediator underlying the control of ACh spillover from the neuromuscular synapse operated by the α7 nAChR sensor localized on PSCs. Our working hypothesis is that adenosine may play a key role in this phenomenon, because (1) activated PSCs can release the nucleoside in sufficient amounts to activate both A_1 and A_{2A} receptors localized on motor nerve terminals (Auld & Robitaille, 2003a,b; Oliveira et al., 2015a),

and (2) adenosine is a fine-tuning regulator of nerve-evoked ACh release by changing the activation balance of other presynaptic players (e.g. nicotinic, muscarinic, peptidergicreceptors) (Ribeiro et al., 1996). In this study, we tested whether adenosine is the gliotransmitter mediating inhibition of ACh release following α7 nAChR activation on PSCs focusing on the presynaptic component of the cholinergic neuromuscular transmission. To this end, we used the rat isolated hemidiaphragm to measure nerve-evoked (i)radiolabeled ACh release, (ii) transmitter exocytosis by video-microscopy with the FM4-64 fluorescent dye, and (iii) skeletal muscle contractions during high-frequency (50 Hz) stimulation bursts in the presence of a selective α7 nAChR agonist, PNU 282987, or after cholinesterase inhibition with neostigmine.

2. Material and methods

2.1. Animals

Rats (Wistar, 150–250 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30–19.30 h)–dark (19.30–06.30 h) cycle for at least ten days prior to the experiments, with food and water *ad libitum*. The animals were killed after stunning followed by exsanguination. Animal handling and experiments were conducted in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), Directive 2010/63/EU and Portuguese rules (DL 113/2013). All experimental protocols involving animals performed by the Portuguese group were approved by the competent national authority DireçãoGeral de Alimentação e Veterinária, and by the Animal Ethical Committee of ICBAS (No. 224/2017). The Ethics Committee for Experimental Animals Studies of the State University of Maringá approved (ECEAS 7227300915) all the procedures done by

the Brazilian group.All efforts were made to minimize animal suffering and to reduce the number of animals used according to the ARRIVE guidelines.

2.2. Quantification of [^{3}H]ACh release from phrenic nerve terminals

The experiments were performed using either the left or the right phrenic nerve-hemidiaphragm preparations (4-6 mm width). The procedures used for labeling the preparations and measuring evoked [^{3}H]ACh release were as described previously (Correia-de-Sá et al., 1991; 2013; Correia-de-Sá and Ribeiro, 1996; Noronha-Matos et al., 2011; Oliveira et al., 2015b) with minor modifications. Briefly, the preparations were mounted in 3-ml capacity Perspex chambers and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001, at 37°C. Nerve terminals were labelled for 40 min with 1 μM [^{3}H]choline (specific activity 2.5 μCi/nmol) under electrical stimulation at a frequency of 1 Hz (0.04 ms duration, 8 mA). The phrenic nerve was stimulated with a glass-platinum suction electrode placed near the first division branch of the nerve trunk, to avoid direct contact with muscle fibres. Washout of the preparations was performed for 60 min, by superfusion (15 ml/min) with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was measured in a Perkin Elmer TriCarb 2900TR scintillation spectrometer (% tritium efficiency: 58±2%), after appropriate background subtraction, using aliquots of 2-ml bath samples collected automatically every 3 min.

[^{3}H]ACh release was evoked by two periods of electrical stimulation of the phrenic nerve, starting at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). Supramaximal-intensity rectangular pulses (0.04 ms duration, 8 mA) were delivered at 5 or 50 Hz frequency. A series of five bursts of 150 pulses applied with a 20-s interburst interval were used when stimulation frequency was 50 Hz (tetanic

stimulation) (Correia-de-Sá *et al.*, 1996; Oliveira *et al.*, 2004; 2015b; Noronha-Matos *et al.*, 2011). Pulses were generated by a Grass S48 (Quincy, MA, USA) stimulator coupled to a stimulus isolation unit (Grass SIU5, USA) operating in current constant mode. Electrical stimulation of the phrenic nerve increased the release of [^{3}H]ACh in a Ca^{2+}- and tetradotoxin-sensitive manner (Correia-de-Sá et al., 2000), while the output of [^{3}H]choline remained unchanged (Wessler and Kilbinger, 1986). Therefore, evoked [^{3}H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (*cf.* Correia-de-Sá et al., 1991). Test drugs were added 15 min before S_2 and were present up to the end of the experiments. The 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 drug effects.

2.3. Real-time video-microscopy using the FM4-64 fluorescent dye as a measure of transmitter exocytosis

To follow real-time transmitter exocytosis from stimulated phrenic motor nerve terminals, the preparations were mounted on the stage of an upright epifluorescence microscope (Zeiss Axiophot, Oberkochen, Germany) and thereafter incubated as for the release of [^{3}H]ACh, except no [^{3}H]choline was added to the Tyrode's solution. After a 30-min equilibration period, the preparations were incubated with α-bungarotoxin (4 μM during 15-20 min, an irreversible blocker of α1-containing muscle-type nicotinic receptors) to prevent nerve-evoked muscle fibre contractions, which would otherwise complicate the analysis of fluorescence signals. In some of the preparations, paralysis of the diaphragm was performed with μ-conotoxin GIIIB (1.2 μM), a toxin that abolishes muscle action potentials by blocking voltage-gated Na^+ channels of skeletal muscle

(Cruz et al., 1985; Hong et al., 1991; Filatov and Rich, 2004) but not those of nerve (see e.g. Faria et al., 2003; Noronha-Matos et al., 2011). This alternative was used in order to bypass the putative blockage of α7 nAChR on PSCs by α-bungarotoxin, notwithstanding the fact that in contrast to the brain no staining overlap between fluorescent α-bungarotoxin and α7 nAChR immunolabeling was observed at the neuromuscular junction (Petrov et al., 2014).

The procedures used to load synaptic vesicles with the membrane-selective FM4-64 fluorescent dye were as previously described (Perissinotti *et al.*, 2008), and used with minor modifications (see e.g. Noronha-Matos *et al.*, 2011; Noronha-Matos and Correia-de-Sá, 2014). After a 10-min incubation period with FM4-64 (5 μM) made up in Tyrode solution, loading of synaptic vesicles was achieved by stimulating the phrenic nerve trunk with 250 pulses of supramaximal intensity (0,04 ms duration, 8 mA) applied at 50 Hz frequency. Following a 10-min period with the dye at rest, the FM4-64 fluorescence was washed vigorously during 30 min. Upon vigorous washout of the dye (not taken up by nerve terminals) from the incubation fluid, dissipation of FM4-64 fluorescence hotspots during electrical stimulation (*e.g.* 50 Hz bursts, see above) can be taken as a measure of synaptic vesicle exocytosis (*cf.* Betz *et al.*, 1992; Perissinotti *et al.*, 2008; Noronha-Matos *et al.*, 2011; Noronha-Matos and Correia-de-Sá, 2014). Test drugs were added to the superfusion fluid via an automatic perfusion system (ValveLink8.2; Digitimer, Welwyn Garden City, UK) connected to a fast solution heating device (TC-344B; Harvard Apparatus, March-Hugstetten, Germany).

Fluorescence images (excitation filter: BP 546/12 nm; emission filter: LP 590 nm) were acquired using a 63x/0.90 n.a. water-immersion objective lens (Achroplan, Zeiss, Germany). Images were acquired in the real-time mode with a high-resolution cooled CCD camera (CoolSnap HQ, Roper Scientific Photometrics, Tucson, AZ, USA).

Absolute fluorescence measurements were converted to a percentage of the maximum fluorescence detected after staining, by the following equation: $\%F(t) = 100 \times [F(t) - F^{N-V}]/[F^{MAX} - F^{N-V}]$, where $F(t)$ is the absolute fluorescence at time t, F^{MAX} is the absolute fluorescence after maximum loading, and F^{N-V} is the non-vesicular fluorescence background (*i.e.* fluorescence remaining at the end of the stimulation) (Betz et al., 1992; Noronha-Matos et al., 2011; Perissinotti et al., 2008; Oliveira et al., 2015b).

2.4. Myographic recordings

Phrenic nerve-diaphragm preparations were isolated and mounted according to Bülbring (1946). Each preparation was immersed in a 20-ml chamber containing gassed (95% O_2 and 5% CO_2) Krebs's solution containing (mM): NaCl 110, KCl 4.7, $CaCl_2$ 3, $MgCl_2$ 1.3, $NaHCO_3$ 25, KH_2PO_4 1, and glucose 11.1, at 37ºC. Hemidiaphragms were connected to an isometric force transducer (Grass FT 03, Grass Instruments Division, West Warwick, RI, USA). Muscle contraction responses were continuously recorded at a resting tension of 50 mN with a PowerLab data acquisition system (Chart Software; AD Instruments, Castle Hill, NSW, Australia). The phrenic nerve was stimulated through a bipolar platinum electrode (supramaximal rectangular pulses, 0.05 ms). The preparations were indirectly stimulated at 0.2 Hz for 15 min (equilibration protocol). From this time onwards, the phrenic nerve was stimulated six times with 50 Hz tetanic trains of 5 s duration, which were applied at 20 min intervals. This interval was selected to avoid the influence of the previous tetanic stimulation on tetanic facilitation or fade. The tension at the beginning (A) and at the end (B) of each tetanus was recorded, and the ratio (R) A/B was calculated (Fig. 1A). Values of A, B and R were obtained for the second and third tetanic train delivered 20 and 40 min after the first tetanic stimulus

(control), respectively. Test drugs were added after the first tetanic stimulus and were present up to the end of the experiments.

In some preparations, a polyethylene catheter (dead space 50 µL) was introduced into the thoracic inferior vena cava to allow retrograde injections of 0.5 µM ACh to the muscle venous supply (Alves-Do-Prado et al., 1992); ACh was delivered as 50 µL boluses followed by the same volume of the physiological solution to washout the drug from the catheter. This procedure was used to evaluate direct effects of test drugs on ACh-induced diaphragm contractions compared to those obtained by nerve stimulation with 50 Hz tetanic trains.

2.5. Drugs and solutions

Adenosine deaminase (ADA, type VI, 1803 U/mL, EC 3.3.3.4), 2-aminoethoxydiphenylborane (2-APB), atropine sulphate, α-bungarotoxin, dihydro-β-erythroidine hydrobromide (DHβE), choline chloride, hemicholinium-3, methoctramine hydrate,methyllycaconitine citrate salt (MLA), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), neostigmine bromide, N-(3R)-1-azabicyclo[2.2.2]oct-3-yl-4-chloro-benzamide monohydrochloride hydrate (PNU 282987), sodium fluoroacetate (FlAc), S-(p-nitrobenzyl)-6-thioinosine (NBTI) (Sigma, St Louis, MO, USA); 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Res. Biochem. Inc., Natick, MA, USA); 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl) phenol (ZM 241385) (Tocris Bioscience, Bristol, UK); N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64) (Invitrogen, Barcelona, Spain; Molecular Probes, Eugene, OR, USA). Radiolabeled [methyl-^{3}H]

choline chloride (ethanol solution, 80.6 Ci mmol-1) and the scintillation cocktail (Insta-gel Plus) were obtained from Perkin Elmer (Boston, USA).

2-APB, FM4-64, NBTI, PNU 282987, U73122 and ZM 241385 were made up in dimethylsulphoxide (DMSO).DPCPX was dissolved in a 5 mM stock solution in 99% dimethylsulfoxide (DMSO) + 1% NaOH 1 M (v/v). All stock solutions were stored as frozen aliquots at -20 ºC. 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, were observed.

2.6. Statistics

The data are expressed as mean ± SEM from an *n* number of experiments. Statistical analysis of data was carried out using GraphPad Prism 7.04 for Windows software (La Jolla, USA).The statistical comparison of nerve-evoked transmitter exocytosis curves was carried out using a multiple *t*-test method, assuming the same scatter (SD) per point, followed by the Bonferroni-Dunn method. Unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multicomparisonstest with a single pooled variance were used for remaining statistical analysis. Values of $P<0.05$ were considered to represent significant differences.

3. Results

3.1. Activation of α7 nAChR reduces neuromuscular transmission during high-frequency stimulation trains

Figure 1 shows that choline (1.0 µM) and the selective α7 nAChR agonist, PNU 282987 (1.0 µM; K_i~26nM), decrease ($P<0.05$) the amplitude of myographic contractions demonstrated by the reduction of the R ratio by 8.00±1.81% (n=5) and

7.00±1.73% (*n*=5), respectively, when the phrenic nerve trunk was stimulated with 50 Hz trains (Fig. 1A), but not when lower stimulation frequencies (0.2 or 5.0 Hz) were used (data not shown).Pre-incubation with MLA (40 nM), a specific α7 nAChR antagonist, fully prevented the inhibitory effects of choline (1.0 μM) and PNU 282987 (1.0 μM) on diaphragm contractions indirectly evoked by phrenic nerve stimulation with 50 Hz trains (Fig. 1B).

The amplitude of fast twitches caused by retrograde injections of ACh (0.5 μM) to the diaphragm venous supply was not affected by PNU 282987 (1.0 μM) (Fig. 1A), thus suggesting that α7 nAChR on skeletal muscle fibers are probably not involved in the inhibition of cholinergic neuromuscular transmission caused by the α7 nAChR agonist presumably because their muscle expression is very low in healthy rats (Jones and Salpeter, 1983; Petrov et al., 2014). This prompted us to focus our subsequent experimental approach on the communication between α7 nAChR-expressing PSCs and α7 nAChR-free motor nerve terminals to control ACh release during high-frequency stimuli.

3.2. Activation of α7 nAChR significantly reduces ACh release from motor nerve terminals stimulated with high-frequency bursts

Figure 2A shows that PNU 282987 (0.3 μM) decreased by 47±4% (n=4, $P<0.05$) nerve-evoked [^{3}H]ACh release triggered by 50 Hz bursts (Fig. 2Ai and 2Aii); the α7 nAChR agonist was much less effective (-12±2%, n=4) when the phrenic nerve trunk was stimulated with 5 Hz trains keeping the number of pulses (750) and the duration of each pulse (0.04 s) constants. It is worth noting that PNU 282987 (0.3 μM) was devoid of effect on spontaneous tritium outflow under the present experimental conditions (Fig. 2Ai). The inhibitory effect of PNU 282987 (0.3 μM) on evoked [^{3}H]ACh release (Fig.

Aii) was mimicked by choline (10 µM, -28±8%, n=9; $P<0.05$) (data not shown) and by the cholinesterase inhibitor, neostigmine (0.5 µM, -58±8%, n=6; $P<0.05$) (Fig. 2Aiii), when these drugs were tested in similar experimental conditions. The inhibitory actions of choline (10 µM) and neostigmine (0.5 µM) were also significantly ($P<0.05$) attenuated when [^{3}H]ACh release was evoked by 5 Hz trains.

A similar inhibitory pattern was observed using video microscopy with the FM4-64 fluorescence dye to measure real-time transmitter exocytosis. PNU 282987 (0.3 µM) significantly ($P<0.05$) decreased the rate of FM4-64 fluorescence intensity decay along with phrenic nerve stimulation with 50 Hz bursts (Fig. 2Bi). Attenuation of transmitter exocytosis (FM4-64 unloading of hotspots) by PNU 282987 (0.3 µM) increased with the time of stimulation (Fig. 2Bii). Similarly to that observed on evoked [^{3}H]ACh release, neostigmine (0.5 µM) also mimicked the inhibitory effect of PNU 282987 (0.3 µM) on FM4-64 fluorescent dye unloading caused by 50 Hz bursts (Fig. 2Biii).

Selective blockage of α7 nAChR with MLA (20 nM) fully prevented the inhibitory effects of PNU 282987 (0.3 µM) and neostigmine (0.5 µM) on [^{3}H]ACh release (Fig. 2Aii and 2Aiii, respectively) and on the rate of FM4-64 fluorescent dye unloading (Fig. 2Bii and 2Biii, respectively) triggered by phrenic nerve stimulation with 50 Hz bursts. These results suggest that α7 nAChR are involved in the negative control of ACh overflow from motor nerve terminals stimulated with high-frequency bursts.

3.3. Perisynaptic Schwann cells (PSCs) contribute to downregulation of ACh exocytosis caused by α7 nAChR during high-frequency bursts

Previous studies elegantly demonstrated that PSCs sense and negatively modulate ACh spillover in the presence of cholinesterase inhibitors through the activation of α7 nAChR (Petrov et al., 2014). Whether this mechanism operates in more

physiological conditions remains unclear. Here, we studied the contribution of PSCs to PNU 282987 (0.3 μM)-induced inhibition of nerve-evoked [^{3}H]ACh release (Fig. 3A) and FM4-64 fluorescent hotspots unloading (Fig. 3B) using the glial cell metabolic uncoupler, sodium fluoroacetate (FlAc, 5 mM) (MacEachern et al., 2015; Vieira et al., 2017). Figure 3 shows that FlAc (5 mM) abrogated (P<0.05) the inhibitory effect of PNU 282987 (0.3 μM) on [^{3}H]ACh release (Fig. 3A) and FM4-64 fluorescent dye unloading (Fig. 3B) caused by phrenic nerve stimulation with 50 Hz bursts.

It is worth to note that, on their own, neither MLA (20 nM, Fig. 4A) nor FlAc (5 mM, Fig. 4A) affected transmitter exocytosis evoked by 50 Hz bursts in concentrations that were able to prevent inhibition of ACh release caused by the α7 nAChR agonist, PNU 282987 (0.3 μM). These results suggest that PSCs actively participate in the reduction of transmitter release from motor nerve terminals only when ACh outside the neuromuscular synapse reach high enough levels to activate α7 nAChR.

3.4. Adenosine acting via presynaptic A_1 receptors operates ACh release downmodulation triggered by α7 nAChR during high-frequency bursts

Given the uncertainty regarding the nature of the signal mediator underlying the control of ACh spillover from the neuromuscular synapse operated by α7 nAChR on PSCs, we designed experiments to investigate if endogenously generated adenosine participates in this phenomenon. Figure 5 shows that inactivation of endogenous adenosine with adenosine deaminase (ADA, 2.5 U/ml) and selective blockage of adenosine A_1 receptors with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10 nM) were both capable of preventing the inhibitory effect of PNU 282987 (0.3 μM) on [^{3}H]ACh release (Fig. 5A) and FM4-64 fluorescent dye unloading (Fig. 5B) induced by phrenic nerve stimulation with 50 Hz bursts. However unlike DPCPX (10 nM), the A_{2A} receptor

antagonist, ZM 241385 (10 nM), did not modify PNU 282987 (0.3 μM)-induced inhibition of nerve-evoked [^{3}H]ACh release (Fig. 5A) and the rate of FM4-64 fluorescent intensity decay (Fig. 5B). This was verified despite the fact that in control conditions (*i.e.* in the absence of the α7 nAChR agonist) the facilitatory A_{2A}-receptor tonus predominates over the adenosine A_1-receptor-mediated inhibition when 50 Hz-bursts are used to stimulate the phrenic nerve (Correia-de-Sá et al., 1996; Noronha-Matos et al., 2011).

Similarly to that observed with MLA (20 nM, Fig. 4A) and FlAc (5 mM, Fig. 4A), the selective A_1 receptor antagonist, DPCPX (10 nM), was also unable to affect ACh exocytosis triggered by 50 Hz bursts (Noronha-Matos et al., 2011) while being effective in preventing the inhibitory role of PNU 282987 (0.3 μM). The results strongly indicate that adenosine acting, via presynaptic A_1 receptors, is the gliotransmitter involved in the control of ACh spillover from the neuromuscular synapse resulting from α7 nAChR activation on PSCs during high-frequency stimulation bursts.

3.5. Downregulation of ACh spillover by α7 nAChR involves adenosine release from PSCs via NBTI-sensitive equilibrative nucleoside transporters

Adenosine, released as such or buildup from ATP catabolism during neuronal firing, plays a key role in adjusting the modulatory pattern of neuromuscular transmission to the stimulation conditions (Correia-de-Sá et al., 1996). While adenosine generated from released ATP activates preferentially facilitatory A_{2A} receptors at rat neuromuscular synapse (Correia-de-Sá et al., 1996; Cunha et al., 1996; Magalhães-Cardoso et al., 2003), the nucleoside released as such from non-neuronal sources, like skeletal muscle fibres and PSCs (Noronha-Matos et al., 2011; discussed in Todd and Robitaille, 2006), plays a more relevant role to control superfluous ACh release through

the activation of presynaptic inhibitory adenosine A_1 receptors (Correia-de-Sá and Ribeiro, 1996; Barroso et al., 2007). Interestingly, the negative crosstalk between muscarinic M_1 (excitatory) and M_2 (inhibitory) autoreceptors also involves endogenous adenosine outflow via S-(4-nitrobenzyl)-6-thioinosine (NBTI)-sensitive nucleoside transporters (ENT1) channeling to preferential activation of inhibitory A_1 receptors on motor nerve terminals (Oliveira et al., 2009). Taking these findings into consideration, we tested whether blockage of the ENT1 transporter with NBTI (10 μM) could reproduce the preventive effects of ADA (2.5 U/ml) and DPCPX (10 nM) on PNU 282987 (0.3 μM)-induced inhibition of transmitter release from phrenic nerve terminals stimulated with 50 Hz bursts.

Figure 5 shows that while NBTI (10 μM) failed to affect transmitter exocytosis in control conditions (*i.e.* in the absence of the α7 nAChR agonist) (Fig. 5C) it significantly ($P<0.05$) attenuated the inhibitory effect of PNU 282987 (0.3 μM) (Fig. 5D), thus partially rehabilitating the rate of FM4-64 fluorescent intensity decay observed in control conditions. These results suggest that α7 nAChR control ACh release through a mechanism involving adenosine outflow from PSCs via NBTI-sensitive ENT1 transporters.

Attenuation of the PNU 282987 (0.3 μM)-induced inhibitory role by NBTI (10 μM) on nerve-evoked ACh release was not due to the unbalance of muscarinic autoreceptors activation towards a preferential M_2 inhibition because blockage of this receptor subtype with methoctramine (100 nM) failed to attenuate PNU 282987 (0.3 μM) inhibition of [^{3}H]ACh release triggered by 50 Hz bursts (-60±8%, n=5) (data not shown).

3.6. Inhibition of ACh release by α7 nAChR might not involve metabotropic transition of the ligand-gated ion channel operating via the PLC / IP_3 signalling pathway

The α7 nAChR is a ligand-gated ion channel resulting in membrane depolarization via Na^+ influx and subsequent opening of voltage-sensitive Ca^{2+} channels in excitable cells. Recent data indicate that agonist binding to α7 nAChR fosters channel inactivation and prolonged desensitization leading to a functional transition into a metabotropic signaling state that overlaps with the desensitized channel state (Kabbani and Nichols, 2018). Coupling to $G_{(\alpha q)}$ proteins enables the α7 nAChR to convey downstream activation of intracellular signaling pathways operating phospholipase C (PLC) / protein kinase C (PKC) activation, IP_3 production and Ca^{2+} release from internal stores.

Given that the metabotropic activity of the α7 nAChR can foster translocation of adenosine to the extracellular milieu either (1) by increasing the net transport capacity (Coe et al., 2002; Fernández-Calotti et al., 2008; Mayati et al., 2017) of phosphorylated ENT1 (Reyes et al., 2011), or (2) by favoring its translocation to the plasma membrane (Hughes et al., 2015), we tested the inhibitory effect of the α7 nAChR agonist, PNU 282987 (0.3 μM), on nerve-evoked [^{3}H]ACh release in the presence of the PLC inhibitor, U73122 (3 μM). In addition, we used 2-APB(30μM), which has been instrumental to inhibit the activity of the IP_3 receptor and several TRP channels, while also affecting the function of connexin-containing hemichannels and store-operated calcium channels (SOC) under certain conditions.

Figure 6 shows that pre-incubation of phrenic nerve-diaphragm preparations with U73122 (3 μM) and 2-APB(30μM) did not significantly (P>0.05) modify the inhibitory action of PNU 282987 (0.3 μM) on [^{3}H]ACh release triggered by neuronal stimulation

with 50 Hz bursts. While one can exclude an effect of these common modulators of the α7 nAChR metabotropic state, it remains to be unraveled what are the molecular determinants coupling α7 nAChR to adenosine release via ETN1 transporters in PSCs of the rat neuromuscular junction.

4. Discussion

This study was designed to investigate if adenosine could be the gliomediator operating neurotransmitter release inhibition from motor nerve endings following ACh spillover sensed by α7 nAChR on PSCs during long high frequency neuronal bursts. Our main conclusion is that tetanic-induced α7 nAChR activation on PSCs controls ACh spillover from the neuromuscular synapse by favoring the outflow of adenosine via NBTI-sensitive ENT1 transporters and, subsequent, activation of presynaptic A_1 inhibitory receptors.

The homeostatic role of α7 nAChR expressed on PSCs functioning as sensors of ACh overflow outside the neuromuscular synapse has been evidenced in isolated mice phrenic nerve-hemidiaphragm preparations pretreated with cholinesterase inhibitors (Petrov et al., 2014). It remained, however, to be elucidated whether the sensing role of α7 nAChR on PSCs leading to a dramatic reduction of ACh release operates in more physiological conditions, like during long high frequency neuronal firing bursts. Knowledge about the chemical nature of the gliotransmitter bridging the gap between activated PSCs predominantly expressing α7 nAChR and the motor nerve terminal lacking this receptor subtype, was also missing.

Imaging studies using a polyclonal antibody directed against the α7 nAChR revealed a strong signal appearing predominantly on PSCs. This staining pattern contrasts with the weak signal identified by fluorescently-labelled α-bungarotoxin on

PSCs. α-Bungarotoxinis a α-neurotoxin that binds irreversibly to α1-containig nAChR abundantly expressed on skeletal muscle fibers, even though it can also bind to α7 nAChR in the brain (Chen and Patrick, 1997) and in inflammatory cells (Vieira et al., 2017). The lack of overlap between these two markers together with the co-localization of the α7 nAChR antibody staining with the glial cell marker, S100, indicates that α7 nAChR at the neuromuscular junction are mainly located on PSCs (Petrov et al., 2014). The presence of functional α7 nAChR has been previously shown on cultured astrocytes (Sharma and Vijayaraghavan, 2001).

Besides their existence on PSCs, low expression levels of α7 nAChR were detected on healthy skeletal muscle fibers whereas no staining was detected on motor nerve terminals (Jones and Salpeter, 1983; Petrov et al., 2014). This scenario dramatically changes after muscle denervation (Fischer et al., 1999; Tsuneki et al., 2003) or during immobilization leading to muscle atrophy (Lee et al., 2014). Under these pathological conditions, α7 nAChR overexpression on skeletal muscle fibers may contribute to approximately 20% of the twitch tension. While this finding may explain resistance to paralysis caused by clinically used muscle relaxants, such as pancuronium and atracurium, after immobilization, our results suggest that α7 nAChR on skeletal muscle fibers are probably not involved in the inhibition of cholinergic neuromuscular transmission caused by the selective α7 nAChR agonist, PNU 282987 (K_i= 29 nM) (Bodnar et al., 2005; Hajós et al., 2005), under the present experimental conditions. This was concluded by the lack of effect of PNU 282987 on the amplitude of fast twitches produced by retrograde injections of ACh to the diaphragm venous supply. The preventive effect of the glial cell metabolic uncoupler, FlAc (MacEachern et al., 2015; Vieira et al., 2017), on PNU 282987-induced inhibition of nerve-evoked transmitter

exocytosis also strengthens the theory that PSCs, via α7 nAChR activation, are the main controllers of ACh overflow during long high frequency bursts.

While we and others proved that the inhibitory effects of α7 nAChR agonists (e.g. PNU 282987, nicotine, choline) on evoked ACh release from stimulated motor nerve terminals could be prevented by methyllycaconitine (MLA) (this study; see also e.g. Balezina et al., 2006; Gaydukov et al., 2014), this selective α7 nAChR antagonist as well as α-bungarotoxin (an irreversible blocker of α1 and α7 nAChR) failed to modify the quantal content (Gaydukov et al., 2014), [^{3}H]ACh release (Faria et al., 2003) and transmitter exocytosis measured by video-microscopy in the real-time mode using the FM4-64 fluorescent dye (this study) during by high frequency neuronal bursts. These results indicate that, under the present experimental conditions, ACh overflow and/or the endogenous accumulation of its breakdown product, choline, are not enough to tonically activate α7 nAChR at the mammalian motor endplate. Yet, this situation may change upon inhibition of the activity of cholinesterases, as shown in this study using a low concentration (0.5 μM) of neostigmine (see also Petrov et al., 2014). As a matter of fact, Petrov et al (2014) elegantly demonstrated that cholinesterases, specifically the BChE anchored to the surface of PSCs, plays a relevant role in the extrasynaptic sensor system that controls ACh spillover from the neuromuscular synapse via α7 nAChR activation.

From a clinical point of view, drugs that selectively inhibit synaptic AChE over extrasynaptic BChE may be beneficial to improve the neuromuscular transmission in patients with *Myasthenia gravis* and to reverse the residual neuromuscular blockade caused by non-depolarizing muscle relaxants. Activation of extrasynaptic α7 nAChR by ACh spilling over from the neuromuscular synapse might explain the paradoxical reduction of nerve-evoked neurotransmitter release (e.g. train-of-four fade, TOF_{fade}) that

has been observed with widely used cholinesterase inhibitors, like neostigmine, leading to a partial and/or longer recovery from the neuromuscular block even in the presence of atropine (Alves-do-Prado et al., 1989; de Paula-Ramos et al., 2014). Likewise, a similar trend was also detected with muscle relaxants exhibiting significant cholinesterase activity, like cis-atracurium (Bornia et al., 2009; 2011; Pereira et al., 2011).

In a previous study, our group showed that phrenic nerve stimulation with 50 Hz-bursts led to a significant increase in extracellular adenosine accumulation in isolated rat phrenic nerve-hemidiaphragm preparations (Noronha-Matos et al., 2011). Interestingly, blockage of non-neuronal sources of adenosine, like skeletal muscle fibers and PSCs (Noronha-Matos et al., 2011; discussed in Todd and Robitaille, 2006), but not those of nerve with α-bungarotoxin (Faria et al., 2003), decreased nerve-evoked adenosine outflow by about 95% without significantly affecting (~15%) the amount of ATP in the same samples. At the rat motor endplate, adenosine released as such through NBTI-sensitive(s) nucleoside transporters (ENT1) acts predominantly on presynaptic A_1 receptors to inhibit nerve-evoked [^{3}H]ACh release (Correia-de-Sá et al., 1996; Barroso et al., 2007) and to mediate the negative crosstalk between muscarinic M_1 and M_2 autoreceptors (Oliveira et al., 2009). The A_1 receptor-mediated inhibitory action of adenosine contrasts with the amplification of transmitter exocytosis caused by adenosine build-up from the extracellular catabolism of released ATP by ecto-5'-nucleotidase/CD73, which is located in close proximity with excitatory A_{2A} receptors on rat motor nerve terminals (Cunha et al., 1996; Magalhães-Cardoso et al., 2003). Co-localization of A_{2A} receptor and ecto-5'-nucleotidase/CD73 was accordingly verified in astrocytes of the human hippocampus (Barros-Barbosa et al., 2016).

To our knowledge, this is the first study demonstrating that (1) removal of endogenous adenosine with ADA, (2) inhibition of adenosine release via NBTI-

sensitive ENT1 transporter, and (3) blockage of presynaptic A_1 receptors with DPCPX, all prevented nerve-evoked ACh release inhibition caused by α7 nAChR activation on PSCs. These findings lead to the conclusion that PSCs sense ACh spillover from the neuromuscular synapse through the activation of α7 nAChR by favoring adenosine outflow via NBTI-sensitive ENT1 transporters and, subsequent, activation of presynaptic A_1 inhibitory receptors. Despite our efforts, we failed to reach any firm conclusion about the molecular determinants coupling α7 nAChR activation to adenosine translocation to the extracellular milieu via ETN1 transporters in PSCs of the rat motor endplate.

The involvement of calcium waves at PSCs during long high frequency bursts has been observed at mammalian neuromuscular junctions (Rochon et al., 2001; Petrov et al., 2014). Nicotinic receptors containing the α7 subunit are highly permeable to Ca^{2+} (P_{Ca}/P_{Na} = 6.6-20) and, therefore, it is plausible that intracellular Ca^{2+} accumulation in PSCs may be the trigger to depress ACh release from stimulated motor nerve terminals. This could be indirectly mediated by activation of apamin-sensitive SK-type K_{Ca} channels if α7 nAChR were also localized on motor nerve terminals resulting in sustained decay of the quantal content of the evoked transmitter release (Gaydukov et al., 2014). Any result involving apamin affecting neuromuscular transmission must be interpreted carefully, because on its own the SK-type K_{Ca} channel inhibitor increased, rather than decreased, nerve-evoked tetanic fade without changing the maximal tetanic tension in healthy rats (de Matos-Silva et al., 2010). Another difficulty in exploring the Ca^{2+} wave hypothesis under the present experimental conditions is that any modification of Ca^{2+} signaling processes would also affect nerve-evoked transmitter exocytosis (Correia-de-Sá et al, 2000; Oliveira et al., 2004; Noronha-Matos et al., 2011).

Another appealing hypothesis to link α7 nAChR activation to adenosine outflow from stimulated PSCs via ENT1 transporters was raised by a recent review publication suggesting that α7 nAChR can operate in ionotropic and metabotropic modes, leading to Ca^{2+}-induced Ca^{2+} release and G protein-associated IP_3 induced Ca^{2+} recruitment from internal stores, respectively (Kabbani and Nichols, 2018). Binding of agonists, such as nicotine, choline and PNU 282987, fosters the transition of the α7 nAChR into a high-affinity ligand-bound desensitized sate supporting a metabotropic response marked by $G_{(\alpha q)}$ protein activation of phospholipase C (PLC) and downstream stimulation of protein kinase C (PKC), IP_3 production and Ca^{2+} release from internal stores. It is still uncertain if α7 nAChR operating in the metabotropic mode is able to govern adenosine translocation to the extracellular milieu via ENT1 transporters. Nevertheless, both human and mouse ENT1 are directly phosphorylated by Ca^{2+}-sensitive and -insensitive PKC and PKA (Reyes et al., 2011). Thus, post-translational modification of ENT1 by PKC-dependent phosphorylation can potentially serve as a mechanism to control transport efficacy (Coe et al., 2002; Fernández-Calotti et al., 2008). Phosphorylation-operated regulation of substrate transport can be achieved by several distinct mechanisms, including modulation of the transporter expression, either at a transcriptional (mRNA) or translational (protein) level. Kinases can also regulate the transporter localization by promoting its translocation to the plasma membrane or by facilitating its internalization (Hughes et al., 2015). Transporter intrinsic activity may be affected by kinase-mediated phosphorylation (Mayati et al., 2017).

Unfortunately, we failed to modify the inhibitory effect of the α7 nAChR agonist, PNU 282987, on nerve-evoked [^{3}H]ACh release in the presence of the selective PLC inhibitor, U73122. A similar negative result was obtained with the IP_3 receptor inhibitor, 2-APB, also taking into consideration that this drug is able to block several

TRP channels, connexin-containing hemichannels and store-operated calcium channels (SOC). The lack of effect of these common modulators of the α7 nAChR operating in the metabotropic mode attenuated our efforts to unravel the molecular pathway involving α7 nAChR in the release of adenosine from PSCs operated via ENT1 transporters. Other possibilities involving α7 nAChR coupled to G proteins may involve downstream signaling through monomeric GTPases, such as RhoA, which are intimate regulators of actin and cytoskeletal dynamics thereby regulating synaptic efficacy (King and Kabbani, 2016). While evidence has been provided that this mechanism may occur in non-excitable cells, exploring such as mechanism is beyond the scope of this study.

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Tetanic Facilitation of NeuromuscularTransmission by Adenosine A_{2A} and MuscarinicM_1 Receptors is Dependent on the Uptake ofCholine via High-Affinity Transporters

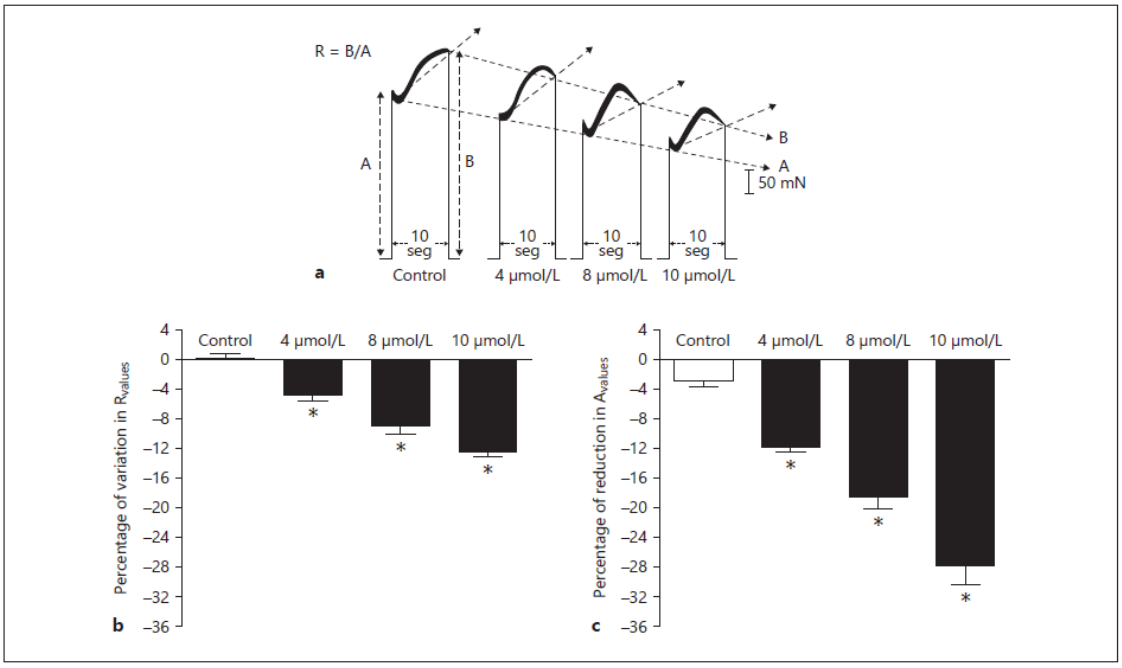

Fig. 1. Hemicholinium-3 (HC-3, 4–10 µmol/L) causes a decay of the maximal tetanic peak tension toward the end of the tetanus (B) in a concentration-dependent manner. Fading of tetanic contractions in the presence of HC-3 (4–10 µmol/L) was more pronounced than the reduction of the initial tetanic tension (A) resulting in significant decreases in R values (R = B/A). **a** Typical recording traces of nerve-evoked hemidiaphragm contractions obtained during 50-Hz tetanic trains (50 Hz for 10 s), in the absence (control) and in the presence of HC-3 (4–10 µmol/L). The small horizontaldotted lines indicate the duration of tetanic stimulation (10 s); vertical calibration bar: 50 mN. **b** Tetanic depression was calculated as decreases in the ratio (R) between the tensions recorded at the end (B) and at the beginning (A) of the tetanic response (R = B/A). **c** The percentage reduction in the amplitude of the initial tetanic tension (A). HC-3 (4–10 µmol/L) was applied in a cumulative manner and contacted the preparations at least 45 min before recordings. Ordinates represent the percentage variation of the ratio (R, **b**) and the initial tetanic tension (A, **c**) values compared to the control situation (in the absence of HC-3). The vertical bars represent SEM of 5–6 experiments. * $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicates a significant difference from the control condition (no drug added).

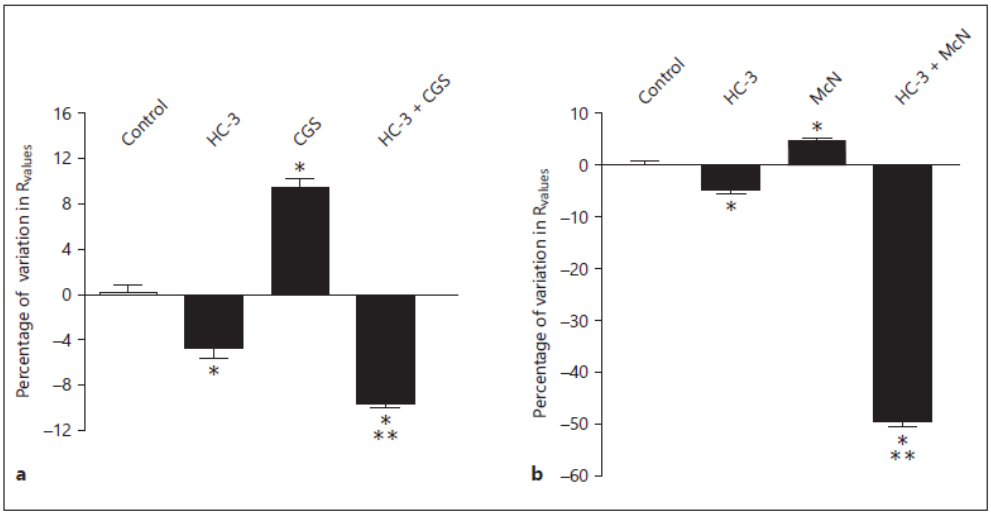

Fig. 2. Percentage (%) variations in R-values (for details, see the legend of Figure 1) caused by CGS21680 (CGS, 2 nmol/L; **a**) and McN-A-343c (McN, 3 µmol/L; **b**) in rat phrenic nerve-diaphragm preparations tested in the absence and in the presence of hemicholinium (HC-3, 4 µmol/L). The phrenic nerve trunk was stimulated with 50 Hz-frequency trains. HC-3 contacted the preparations at least for 45 min before recordings; CGS21680 and McN-A-343c were applied 20 min after starting HC-3 application and, thus, contacted with the preparations for 25 min. Heights of columns indicate mean ± SEM of 4–5 experiments. * $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicates significant difference from the control condition (Krebs buffer without test drugs). ** $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicate significantdifference from the effect of CGS (**a**) or McN (**b**) applied alone.

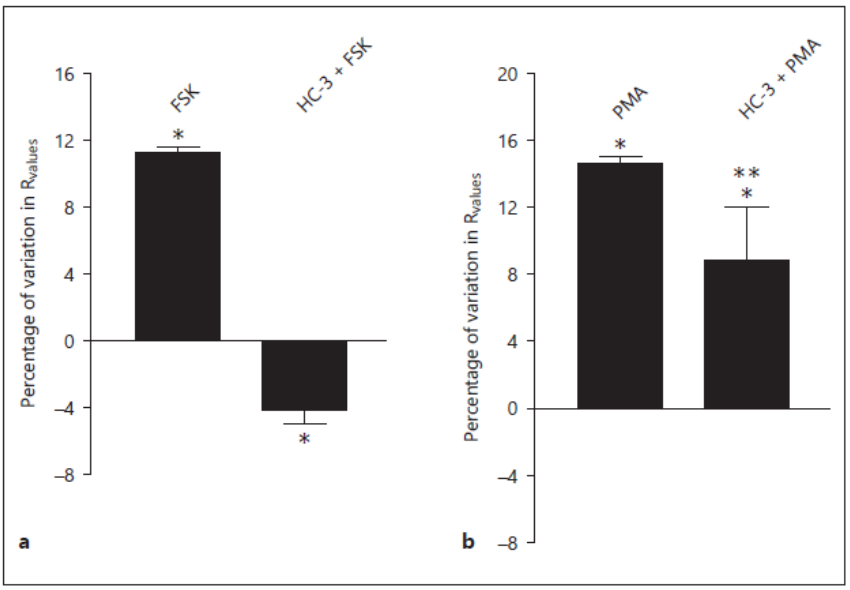

Fig. 3. Percentage (%) variations in R-values (for details, see the legend of Figure 1)caused by forskolin (FSK, 3 µmol/L; **a**) and phorbol 12-myristate 13-acetate (PMA, 10µmol/L; **b**) in rat phrenic nerve-diaphragm preparations tested in the absence and inthe presence of hemicholinium (HC-3, 4.0 µmol/L). The phrenic nerve trunk was stimulated with 50 Hz-frequency trains. HC-3 contacted the preparations at least for 45min before recordings; FSK and PMA were applied 20 min after starting HC-3 application and, thus, contacted with the preparations for 25 min. Heights of columns indicate mean ± SEM of 4 to 5 experiments. * $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicates a significant difference from the control condition (Krebs buffer without test drugs); ** $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicate significant difference from the effect of FSK (**a**) or PMA (**b**) applied alone.

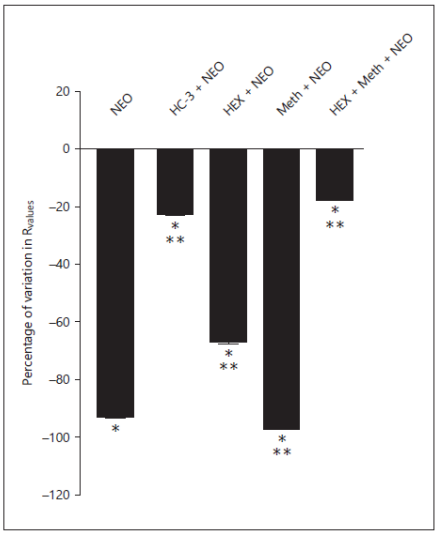

Fig. 4. Percentage (%) variations in R-values (for details, see the legend of Figure 1) caused by neostigmine (NEO, 0.5 µmol/L) in rat phrenic nerve-diaphragm preparations tested in the absence and in the presence of hemicholinium (HC-3, 4 µmol/L), hexamethonium (HEX, 120 µmol/L), and methoctramine (Meth, 0.1 µmol/L). The phrenic nerve trunk was stimulated with 50 Hz-frequency trains. HC-3, HEX, and/or Meth contacted the preparations at least for 45 min before recordings; NEO was applied to the preparations 25 min before recordings either in the absence or in the presence of HC-3, HEX and/or Meth. Heights of columns indicate mean ± SEM of 4 to 5 experiments. * $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicates a significant difference from the control condition (Krebs buffer without test drugs); ** $p < 0.05$ (ANOVA followed by Bonferroni post hoc test) indicate significant difference from the effect of NEO applied alone.

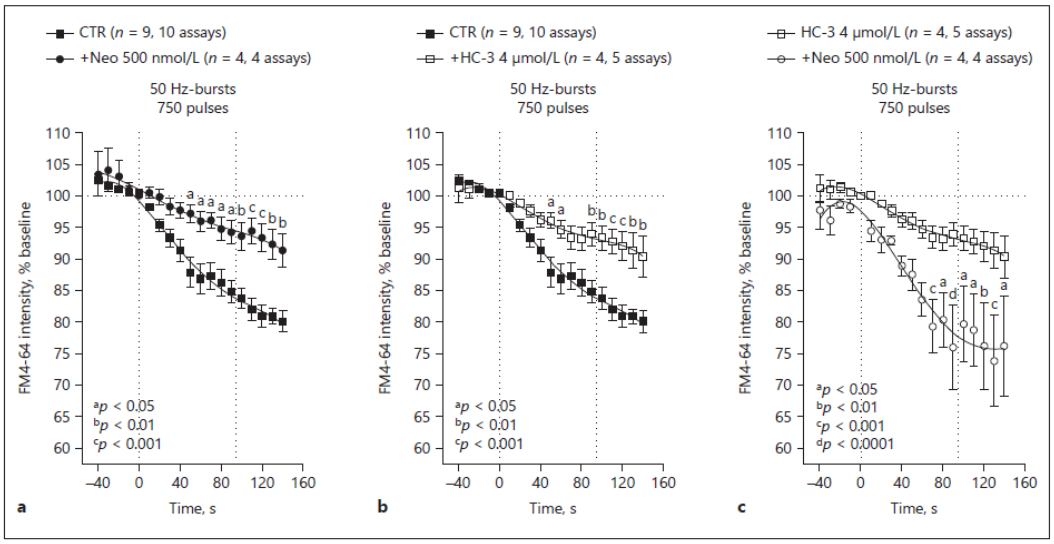

Fig. 5. Effect of neostigmine (NEO, 500 nmol/L) and hemicholinium-3 (HC-3, 4 µmol/L) on nerve-evoked transmitter exocytosis measured by real-time video microscopy using the FM4-64 fluorescent dye. Transmitter exocytosis was elicited by stimulating the phrenic nerve trunk with 50-Hz tetanic bursts (5 trains of 150 pulses applied with a 20-s interburst interval). Drugs were applied alone (**a**, **b**) or in combination (**c**) at least 15 min before test stimulus; the control situation correspond to test stimuli in the absence of any added drug. Shown is the time-course of FM4-64 fluorescence intensity decay during electrical stimulation of the phrenic nerve. The fluorescence decay is expressed as a percentage of maximal loading considering that 100% is the fluorescence intensity at zero time (dashed horizontal line). The vertical dashed lines represent starting and ending of the stimulus. Each value represents pooled data from 4 to 9 animals. The vertical bars represent ± SEM. a $p < 0.05$, b $p < 0.01$, c $p < 0.001$, d $p < 0.0001$ (multiple *t* test assuming the same scatter per point, Bonferroni-Dunn method) represent significant differences compared to the control situation or to
NEO alone.

Nicotinic α7 receptor-induced adenosine release from perisynaptic Schwann cellscontrols acetylcholine spillover from the rat motor endplate during high frequency nerve firing

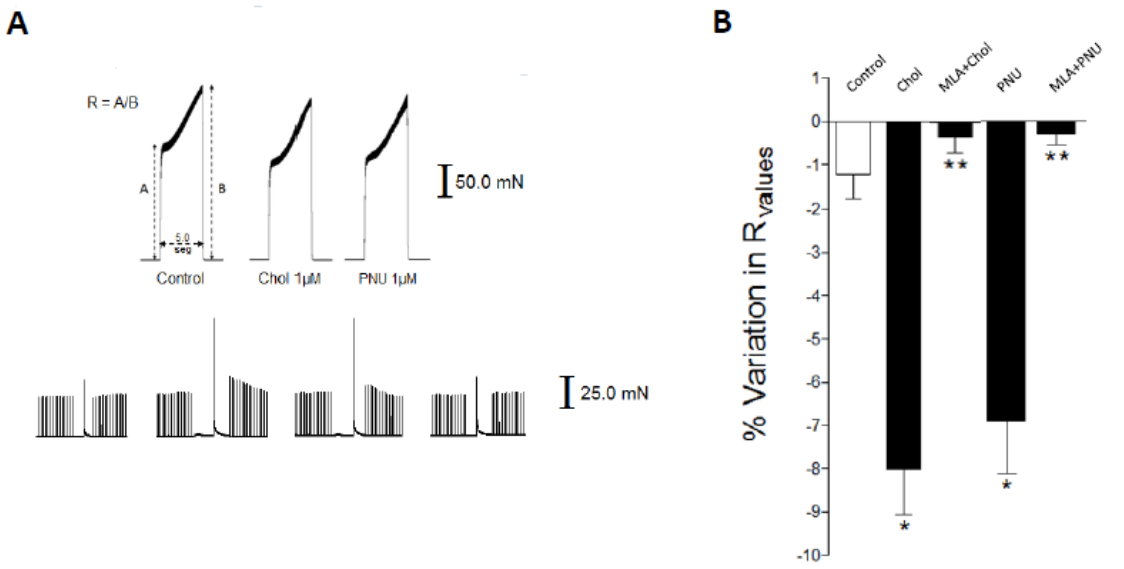

Fig. 1. Choline (Chol, 1 μM) and the α7 nAChR agonist, PNU 282987 (1 μM), decrease the amplitude of myographic recordings obtained from isolated rat phrenic nerve-diaphragm preparations during bursts of tetanic stimuli (50 Hz for 5 s) applied to the phrenic nerve trunk. Panel A, shows typical recording traces in the absence and in the presence of choline and PNU 282987 applied at least 15 min before recordings (upper tracings). Vertical calibration: 50 mN. The absence of effect of choline and PNU 282987 on muscle twitches caused by retrograde infusion of acetylcholine (ACh) into the diaphragm venous supply indicate not postsynaptic origin of inhibitory effect of drugs and is shown for comparison purposes (bottom traces); Vertical calibration: 25 mN. First and second myographic records show muscular twitches generate by retrograde administration of Kreb`s solution (100 μL) and acetylcholine (50 μL followed by 50 μL of Kreb`s solution) into thoracic inferior vena cava, respectively. The same effects is shown after addition of Chol or PNU 282987 in the bath (third and fourth muscular twitches generate by Kreb's solution and acetylcholine respectively). Panel B, shown is the percentage variation of the R value compared to the control situation (no test drugs); the R value was calculated by the ratio between the tensions recorded at the beginning (A) and at the end (B) of the tetanus (R=A/B). The vertical bars represent SEM of 5-6 experiments. *,**P<0.05 (one-way ANOVA followed by Dunnett's modified t test) when compared to the control situation or with the effect of choline or PNU 282987 alone.

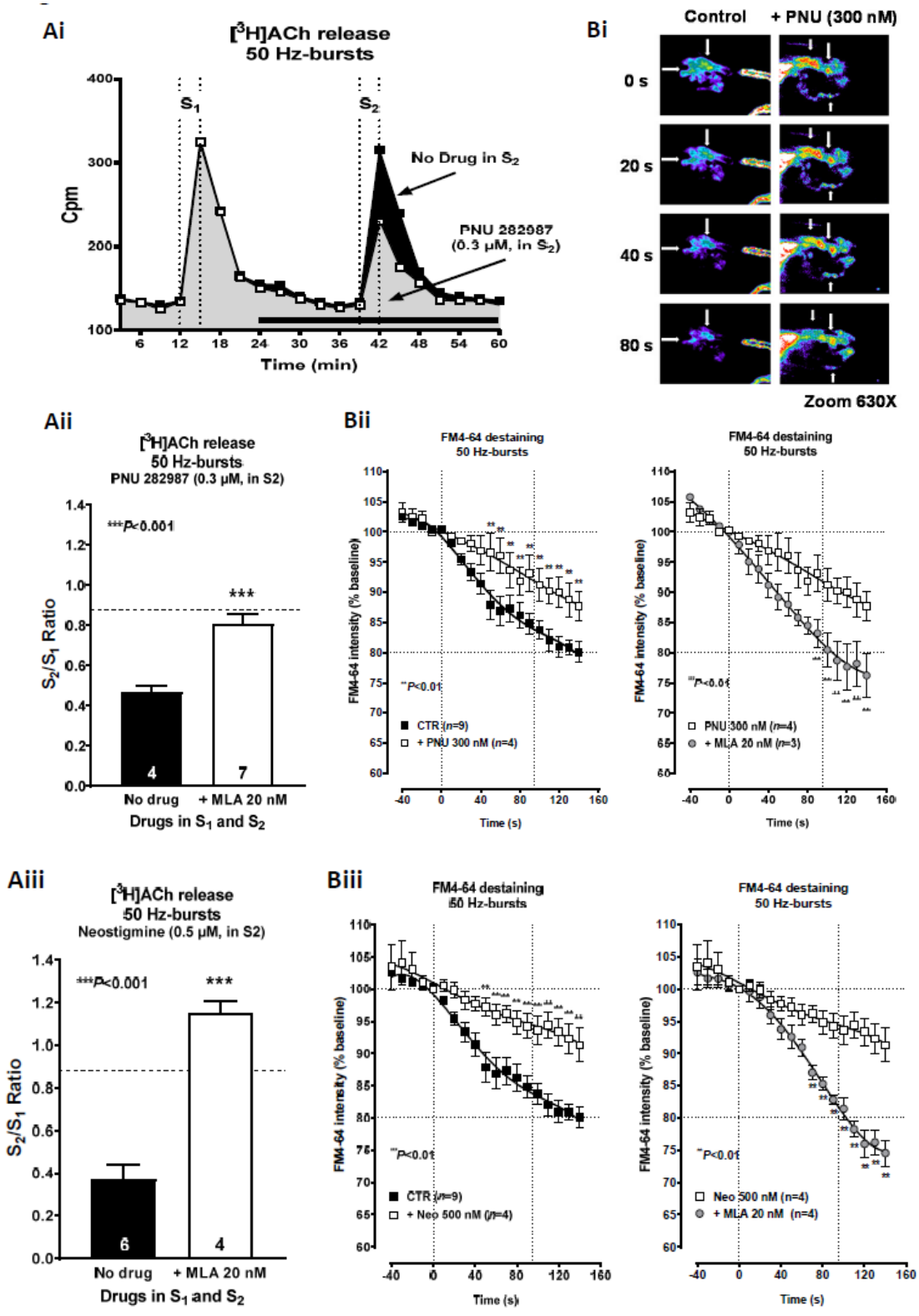

Fig. 2. Inhibitory effects of PNU 282987 (a selective α7 nAChR agonist) and neostigmine (a cholinesterase inhibitor) on nerve-evoked transmitter release from motor nerve terminals loaded with [^{3}H]choline (A) or with the FM4-64 fluorescent dye (B). Transmitter release was elicited by stimulating the phrenic nerve trunk with 50 Hz-bursts (5 trains of 150 pulses applied with a 20-s interburst interval). PNU 282987 (0.3 µM) and neostigmine (0.5 µM) either in the absence or in the presence of MLA (20 nM, a selective α7 nAChR antagonist) were applied at least 15 min before test stimulus.Panel Ai, tritium outflow (ordinates) is expressed in scintillations per minute (cpm); Abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited twice, starting at 12th (S_1) and 39th (S_2) minutes

after the end of washout (zero time). Note that the spontaneous tritium outflow was not changed in the presence of PNU 282987 (horizontal bar). Panels Aii and Aiii, the ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. The S_2/S_1 ratio obtained in the absence of test drugs is represented by the dashed horizontal line (see Materials and Methods, for details). Each column represents pooled data from 4-7 individual experiments. The vertical bars represent ±SEM. ***P<0.001 (one-way ANOVA followed by Dunnett's modified t-test) when compared to the effect of PNU 282987 (Aii) or neostigmine (Aiii) alone. Panel Bi, shows the FM4-64 fluorescence intensity changes in two typical motor endplates during nerve stimulation. Images were taken at the indicated times just before (0 s) and during phrenic nerve stimulation using a 63x/0.90 n.a. water-immersion objective lens (Achroplan, Zeiss, Germany). Note that fluorescence FM4-64 hotspots (white arrows) dimmed almost to the basal level (achieved before application of the loading stimulus) as a consequence of phrenic nerve stimulation. Panels Bii and Biii, show the time–course of FM4-64 fluorescence intensity decay during electrical stimulation of the phrenic nerve. Fluorescence decay is expressed as a percentage of maximal loading considering that 100% is the fluorescence intensity at zero time. The vertical dashed lines represent starting and ending of the stimulus. Each value represents pooled data from 3-9 experiments performed in duplicate (left and right hemidiaphragms). The vertical bars represent ± SEM. **P<0.01 (multiple t-test, assuming the same scatter (SD) per point followed by the Bonferroni-Dunn method) when compared either to the control situation (in the absence of test drugs) or to the effect of PNU 282987 (Bii) or neostigmine (Biii) applied alone.

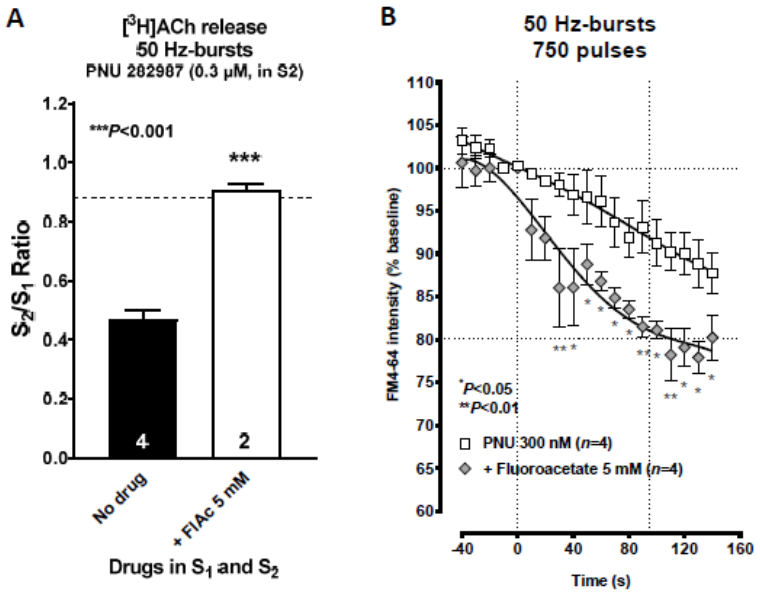

Fig. 3. The glial cell metabolic uncoupler, sodium fluoroacetate (FlAc, 5 mM), prevents the inhibitory effects of PNU 282987 (a selective α7 nAChR agonist) on nerve-evoked transmitter release from motor nerve terminals loaded with [^{3}H]choline (A) or with the FM4-64 fluorescent dye (B). Transmitter exocytosis was elicited by stimulating the phrenic nerve trunk with 50 Hz-bursts (5 trains of 150 pulses applied with a 20-s interburst interval). PNU 282987 (0.3 µM) was applied at least 15 min before test stimulus either in the absence or in the presence of FlAc (5 mM). Panel A, the ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. The S_2/S_1 ratio obtained in the absence of test drugs is represented by the dashed horizontal line (see Materials and Methods). Each column represents pooled data from 4 individual experiments. The vertical bars represent ±SEM. ***P<0.001 (one-way ANOVA followed by Dunnett's modified t-test) when compared to the effect of PNU 282987 alone. Panel B, shown is the time-course of FM4-64 fluorescence intensity decay caused PNU 282987 (0.3 µM) in the absence and in the presence of FlAc (5 mM). Fluorescence decay is expressed as a percentage of maximal loading considering that 100% is the fluorescence intensity at zero time. The vertical dashed lines represent starting and ending of the stimulus, respectively. Each value represents pooled data from 4 experiments performed in duplicate (left and right hemidiaphragms). The vertical bars represent ±SEM. *P<0.05 and **P<0.01 (multiple t-test, assuming the same scatter (SD) per point followed by the Bonferroni-Dunn method) when compared to the effect of PNU 282987 alone.

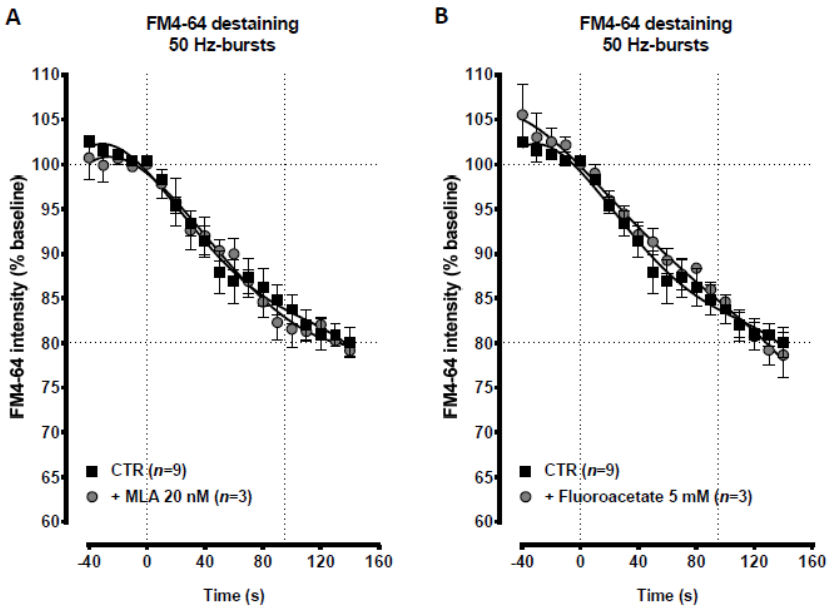

Fig. 4. Blockage of α7 nAChR with MLA (20 nM, A) and inactivation of PSCs with sodium fluoroacetate (FlAc, 5 mM, B) does not affect nerve-evoked transmitter release from motor nerve terminals loaded with [^{3}H]choline (A) or with the FM4-64 fluorescent dye (B). Transmitter exocytosis was elicited by stimulating the phrenic nerve trunk with 50 Hz-bursts (5 trains of 150 pulses applied with a 20-s interburst interval). Shown is the time-course of FM4-64 fluorescence intensity decay expressed as a percentage of the maximal loading (100% obtained at zero time) in the absence (CTR) and in the presence of MLA (20 nM, A) and FlAc (5 mM, B) incubated for at least 15 min before test stimulus. The vertical dashed lines represent starting and ending of the stimulus, respectively. Each value represents pooled data from 3-9 experiments performed in duplicate (left and right hemidiaphragms). The vertical bars represent ±SEM.

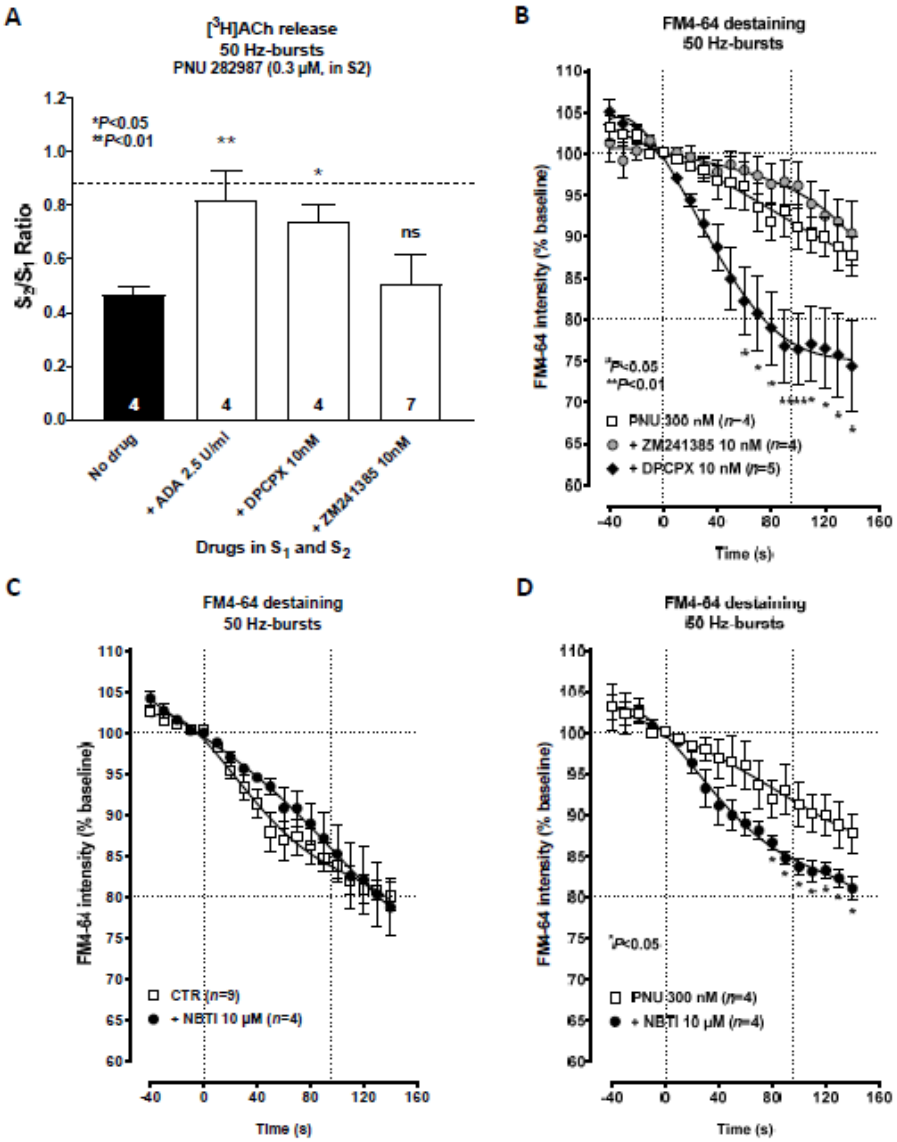

Fig. 5. Role of endogenous adenosine on α7 nAChR-mediated inhibition of nerve-evoked transmitter release from motor nerve terminals loaded with [^{3}H]choline (A) or with the FM4-64 fluorescent dye (B-D). Transmitter release was elicited by stimulating the phrenic nerve trunk with 50 Hz-bursts (5 trains of 150 pulses applied with a 20-s interburst interval). The α7 nAChR agonist, PNU 282987 (0.3 µM), was applied at least 15 min before test stimulus either in the absence or in the presence of adenosine deaminase (ADA, 2.5 U/ml, adenosine inactivating enzyme), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 nM, selective A_1 receptor antagonist) and ZM 241385 (10 nM, selective A_{2A} receptor antagonist). Panel A, the ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. The S_2/S_1 ratio obtained in the absence of test drugs is represented by the dashed horizontal line (see Materials and Methods). Each column represents pooled data from 4-7 individual experiments. The vertical bars represent ±SEM. *P<0.05 and **P<0.01 (one-way ANOVA followed by Dunnett's modified t-test) when compared to the inhibitory effect of PNU 282987 alone. Panel B, shown is the time-course of FM4-64 fluorescence intensity decay caused by PNU 282987 (0.3 µM) in the absence and in the presence of DPCPX (10 nM) and ZM 241385 (10 nM) during high frequency stimulation bursts; the effect of the ENT1 transport inhibitor, S-(4-nitrobenzyl)-6-thioinosine (NBTI, 10 µM), either alone (C) or in the presence (D) of PNU 282987 (0.3 µM) is also shown for comparison. Fluorescence decay is expressed as a percentage of maximal loading considering that 100% is the fluorescence intensity at zero time. The vertical dashed lines represent starting and ending of the stimulus, respectively. Each value represents pooled data from 4-9 experiments performed in duplicate (left and right hemidiaphragms). The vertical bars represent SEM. *P<0.05 and **P<0.01 (multiple t-test, assuming the same scatter (SD) per point followed by the Bonferroni-Dunn method) when compared to the effect of PNU 282987 alone.

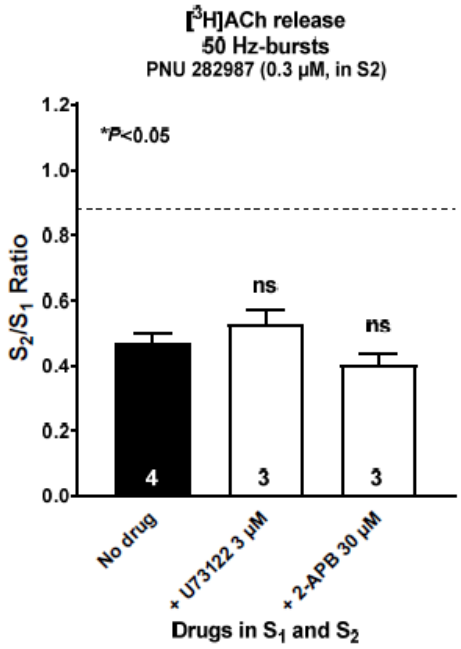

Fig. 6. Inhibition of [^{3}H]ACh release by PNU 282987 does not involve the metabotropic transition of the α7 nAChR channel operating PLC activation and IP_3-mediated Ca^{2+} recruitment from internal stores.[^{3}H]ACh release was elicited by stimulating the phrenic nerve trunk with 50 Hz-bursts (5 trains of 150 pulses applied with a 20-s interburst interval). PNU 282987 (0.3 µM) was applied at least 15 min before test stimulus either in the absence or in the presence of U73122 (3 µM, a PLC inhibitor) or 2-APB (30 µM, an IP_3 receptor inhibitor). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. The S_2/S_1 ratio obtained in the absence of test drugs is represented by the dashed horizontal line (see Materials and Methods). Each column represents pooled data from 3-4 individual experiments. The vertical bars represent ±SEM.