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LILIAN MARTINS CASTELLÃO SANTANA

O BLOQUEIO DE RECEPTORES PARA ADENOSINA (A_{2A} E A_1) IMPEDE O EFEITO INIBITÓRIO CAUSADO POR HEMICOLÍNIO EM PREPARAÇÕES NEUROMUSCULARES SUBMETIDAS A ESTÍMULOS ELÉTRICOS TETANIZANTES

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Dissertação 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 Mestre em Ciências Biológicas.

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

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BIOGRAFIA

Lilian Martins Castellão Santana nasceu em Dourados/MS em 23/09/1991. Possui graduação em Ciências Biológicas pela Pontifícia Universidade Católica de São Paulo (2014). Atualmente é aluna do curso de pós-graduação em Ciências Biológicas da Universidade Estadual de Maringá. Tem experiência na área de Neurofarmacologia, Biologia Celular e Microbiologia, atuando principalmente no seguinte tema: Neurofarmacologia das transmissões colinérgicas periféricas.

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APRESENTAÇÃO

Esta dissertação de mestrado é composta por um artigo científico no qual se investigou quais seriam as influências da atividade dos receptores muscarínicos (M_1 e M_2) e de adenosina (A_1 e A_{2A}) sobre os efeitos causados pelo hemicolínio em preparações neuromusculares indiretamente estimuladas à frequência de 50 Hz. O trabalho é apresentado de acordo com as regras do Programa de Pós-Graduação em Ciências Biológicas, e o artigo foi redigido de acordo com as regras de submissão de trabalhos exigidas pela revista *Clinical and Experimental Pharmacology and Physiology*.

Lilian Martins Castellão Santana, Priscila Yumi Abiko, Celia Regina Ambiel, Paulo Correia-de-Sá, Wilson Alves do Prado. The inhibitory effect caused by hemicholinium is prevented by blockade of A_{2A} and A_1 adenosine receptors in the neuromuscular preparations submitted at tetanizing frequency.
Clinical and Experimental Pharmacology and Physiology.

RESUMO GERAL

INTRODUÇÃO

O controle da liberação de acetilcolina (ACh) do terminal nervoso motor pode ser modulado por substâncias liberadas pelo próprio nervo motor e/ou de fontes pós-sinápticas, tais como moléculas de adenosina. A adenosina é formada a partir da catálise do ATP e encontra-se presente nas vesículas sinápticas, juntamente com a ACh. Desta forma, há sempre uma co-liberação de ACh e ATP para a fenda sináptica. O músculo esquelético em atividade pode ser considerado uma fonte adicional de adenosina.

As vesículas sinápticas estão distribuídas no interior do terminal em duas frações principais: uma, mais próxima à membrana pré-sináptica, conhecida como fração imediatamente utilizável (FIU); a outra, mais dispersa pelo citoplasma do terminal nervoso, denominada fração de depósito (FD). As vesículas da FD podem ser mobilizadas para a periferia para repor os estoques liberados da FIU.

Receptores pré-sinápticos colinérgicos e para adenosina estão presentes nos terminais nervosos motores. Tais receptores podem autorregular, caso dos colinérgicos, ou modular, caso dos receptores de adenosina, a liberação da ACh/ATP a partir dos nervos motores. Os receptores colinérgicos do terminal motor têm sido identificados como sendo dos subtipos nicotínico (N) neuronal (expressando $\alpha 3\beta 2$) ou muscarínicos (M) dos subtipos M_1 (facilitatório) e M_2 (inibitório). Os receptores de adenosina (A), por sua vez, são dos subtipos A_1 (inibitório) e A_{2A} (facilitatório).

As atividades dos receptores pré-sinápticos colinérgicos e de adenosina pré-sinápticos são influenciadas pela atividade do nervo motor e, portanto, dependem da frequência de estimulação e da duração dos pulsos elétricos que são aplicados sobre o nervo. Ativações dos receptores M_1 e A_1 são preferenciais quando baixas frequências (~5,0 Hz) de estimulação estão sendo aplicadas sobre o nervo motor, já que em tais condições os receptores nicotínicos neuronais são rapidamente dessensibilizados e 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 terminal nervoso motor passa a receber frequências de estimulação mais elevadas ($\geq$ 50,0 Hz), já que os níveis de adenosina em tais circunstâncias estão aumentados.

Conversas cruzadas (*cross-talking*) entre os receptores facilitatórios M_1 e A_{2A} e inibitórios M_2 e A_1 podem ocorrer. A ativação de M_1 diminui a atividade de M_2. A atividade de M_1, por sua vez, é reduzida quando os receptores A_{2A} são plenamente ativados (frequência de estímulos $\geq$50 Hz) pela adenosina. Adicionalmente, os receptores M_2 podem ter sua atividade reduzida pelos receptores A_1 via *down regulation*.

Concomitantemente a estas complexas interações entre moléculas de ACh e adenosina com seus receptores específicos, também ocorre a hidrólise das moléculas de ACh na fenda sináptica. Tais moléculas são metabolizadas a acetato e colina pela ação da acetilcolinesterase. A colina é recaptada pelo terminal colinérgico por meio dos transportadores de colina (ChT) que podem ser classificados em ChT de alta (HChT, sódio dependente, inibido pelo hemicolínio-3) e de baixa afinidade (LChT, sódio independente). Estes ChTs são tão importantes para o controle da transmissão neuromuscular, pois a falta de eficiência em suas atividades determina graves reduções nas quantidades de ACh liberadas dos terminais colinérgicos.

OBJETIVO

O presente estudo teve como objetivo investigar se existiriam influências, ou controles, dos receptores colinérgicos e de adenosina pré-sinápticos sobre os HChTs em preparações neuromusculares indiretamente estimuladas com frequências de estimulações tetanizantes (50 Hz).

MÉTODOS

O comitê de ética de experimentação animal da Universidade Estadual de Maringá aprovou (no 7227300915/ CEUA-UEM) os procedimentos utilizados. As preparações nervo frênico diafragma isolado de ratos (Wistar, machos, 200-220g) foram montadas de acordo com o método proposto por Bülbring (1946). Após a toracotomia e isolamento do hemidiafragma esquerdo, juntamente com nervo frênico, cada preparação foi imersa em uma câmara de vidro de 30 mL contendo tampão de Krebs (composição em mmol/L) (NaCl 110.0; KCl 4.7; CaCl2 3.0; MgCl2 1.3; NaHCO3 25.0; KH2PO4 1.0; glicose 11.1) a 37ºC e continuamente aerado com um mistura de oxigênio (95%) e dióxido de carbono (5%). O nervo frênico foi estimulado por pulsos elétricos retangulares (pulsos supramáximos com duração de 0,05 ms) através de um eletrodo bipolar de platina conectado a um estimulador GRASS®. As preparações foram estimuladas com frequência de 50 Hz (durante 10 segundos), intercalada, a cada 20 min, com a frequência de 0,2 Hz. As tensões musculares produzidas no início (A) e no final (B) do estímulo de 50 Hz foram avaliadas, juntamente com o valor da razão B/A. O inibidor do HChT, hemicolínio-3, foi administrado 35 min após o tétano controle e os valores das tensões musculares e da razão B/A obtidos 45 min após a adição do hemicolínio-3 foram tomados como % dos controles. Os agonistas dos receptores M_1, A_1 e A_{2A} (McN-A-343c, R-PIA, CGS21680, respectivamente) e os antagonistas dos receptores M_2, A_1 e A_{2A} (metoctramina, DPCPX e ZM241385, respectivamente) foram administrados 20 min antes da adição do hemicolínio-3. ANOVA, seguida pelo pós-teste de Bonferroni, foi utilizada, com nível de significância $P<0,05$, para comparação dos dados.

RESULTADOS E DISCUSSÃO

A administração de hemicolínio-3 (4,0 a 10,0 μM) causou uma redução nos valores da razão B/A. Concentrações maiores de hemicolínio-3 (8 a 10 μM) induziram redução nos valores de A, provavelmente por uma ação pós-sináptica mais concentrada que a queda observada com o uso de 4 μM de HC-3. A redução dos valores na razão B/A obtida com hemicolínio-3 (4,0 μM) foi revertida pela administração prévia dos antagonistas dos receptores de adenosina do subtipo A_{2A} (ZM241385 - 10,0 nM) e A_1 (DPCPX - 2,5 nM), e reduzida pelos agonistas desses receptores, A_{2A} (CGS21680 - 2,0 nM) e A_1 (R-PIA - 0,1 μM). O antagonista do receptor M_2 (Metoctramina 0,1 μM) também foi capaz de reduzir o efeito inibitório do hemicolínio-3 (4,0 μM) sobre os valores da razão B/A. Embora um efeito facilitatório na razão tenha sido registrado com a administração isolada de McN-A-343c (3,0 μM), a administração do McN-A-343c (3,0 μM), um agonista do receptor M_1, potencializou o efeito inibitório causado pelo hemicolínio-3 (4,0 μM) sobre os valores da razão B/A.

A inibição do HChT por hemicolínio-3 (4,0 μM) reduziu a capacidade da transmissão neuromuscular para manter uma adequada e sustentada contração muscular (redução nos valores da R) quando o nervo motor foi estimulado com pulsos de 50 Hz. Esta afirmação poderia ser

explicada por meio da redução da liberação de ACh/ATP a partir do nervo motor, a qual foi bloqueada ou reduzida por condições experimentais que aumentaram a liberação de ACh/ATP a partir do nervo motor. Tais condições foram: bloqueio dos receptores A_{2A} por ZM241385 (permitindo a inibição dos autorreceptores M_1 sobre os M_2); bloqueio dos receptores inibitórios A_1 por DPCPX; ativação dos receptores facilitatórios A_{2A} por CGS21680; ativação do receptor A_1 por RPIA (indução de um *down regulation* dos receptores M_2) e bloqueio dos autorreceptores inibitórios M_2 por metoctramina.

É improvável que a redução do efeito inibitório do hemicolínio-3 sobre os valores de R causada pela administração do agonista A_{2A} (CGS21680) tenha sido mediada pelo *"cross talking"* entre os receptores A_{2A} / M_1 e M_2, uma vez que, por meio tal mecanismo, a ativação dos receptores A_{2A} por CGS21680 levaria a uma redução da atividade de M_1, diminuindo assim a atividade inibitória de M_1 sobre os receptores M_2, o que resultaria em uma diminuição da liberação de ACh pelo nervo motor. Portanto, CGS21680 deveria ter intensificado o efeito inibitório do hemicolínio-3 e não reduzido, como foi o efeito observado.

O *"cross talking"* entre os receptores A_{2A} / M_1 e M_2 estaria de fato envolvido no impedimento do efeito inibitório do hemicolínio-3 evidenciado com a inibição do receptor A_{2A} por ZM241385, pois, quando tal fármaco é administrado isoladamente, as reduções nos valores de R são mais intensos e quando em conjunto com hemicolínio são menos intensos.

O tratamento de preparações com hemicolínio-3 em presença do agonista M_1 (McN-A-343c) poderia afetar o "*cross talking*" entre os receptores M_1 / M_2, já que em tal situação, estaria ocorrendo um prejuízo na reposição da FIU a partir de um deslocamento da FD. Essa hipótese foi aventada após ter sido observado que a administração do McN-A-343c foi capaz de intensificar o efeito inibitório do hemicolínio-3. Possivelmente, o aumento na liberação de ACh a partir da ativação do receptor M_1 induziria uma redução da FIU e, concomitantemente, uma ativação dos receptores M_2 inibitórios pela ACh liberada na fenda sináptica. Ademais, tem sido descrito que a ativação do receptor M_2 interfere negativamente no deslocamento das vesículas da FD para a FIU. Assim, embora o receptor M_1 esteja ativado, o mesmo não consegue impedir a ativação do receptor M_2 inibitório, cuja atividade, acrescida da redução da FIU, resulta em um aumento do efeito inibitório do hemicolínio-3.

CONCLUSÃO

As reduções na atividade dos HChT por HC-3 parecem diminuir a quantidade de ACh e adenosina na fenda sináptica, uma vez que, nestas condições, haveria uma redução na liberação de ACh / ATP a partir do terminal nervoso motor. Portanto, procedimentos terapêuticos, como o bloqueio dos receptores pré-sinápticos A_1 e A_{2A} na junção neuromuscular, poderiam contribuir para o tratamento de patologias que reduzem a liberação de ACh / ATP no terminal nervoso motor, como é o caso de algumas síndromes miastênicas e da doença de Alzheimer.

GENERAL ABSTRACT

INTRODUCTION

Control of acetylcholine release (ACh) from the motor nerve terminal can be modulated by substances released by the motor nerve itself and / or post-synaptic sources, such as adenosine molecules. Adenosine is formed from ATP catalysis and is present in synaptic vesicles along with ACh. In this way, there is always a co-release of ACh and ATP into the synaptic cleft. Active skeletal muscle is an additional source of adenosine.

The synaptic vesicles are distributed within the terminal in two main pools: one, closer to the presynaptic membrane is known as immediately available pool (IAVP); and the other, one more dispersed in the cytoplasm of the nerve terminal, is called deposit pool of vesicles (DPV). The DPV can be mobilized to the periphery to replenish IAVP stocks.

Pre-synaptic cholinergic receptors and receptors for adenosine are present in the motor nerve terminals. Such receptors may self-regulate, in the case of cholinergics, or modulate, in the case of receptors for adenosine, the release of ACh / ATP from the motor nerves. Cholinergic receptors have been identified as the nicotinic (N) neuronal (expressing $\alpha 3\beta 2$) or muscarinic (M) subtypes M_1 (facilitatory) and M_2 (inhibitory). Adenosine receptors (A), on the other hand, are of the A_1 (inhibitory) and A_{2A} (facilitatory) subtypes.

The activities of pre-synaptic cholinergic receptors and adenosine receptors are influenced by motor nerve activity and therefore depend on the frequency of stimulation and the duration of the electrical pulses that are applied to the nerve. Activations of M_1 and A_1 receptors are preferencial at low stimulation frequencies (~ 5.0 Hz) applied to the motor nerve, since in such conditions neuronal nicotinic receptors are rapidly desensitized and there are low levels of adenosine in the synaptic cleft. On the other hand, there is a predominance of M_2 and A_{2A} receptors when the motor nerve terminal receives higher stimulation frequencies ($\geq$ 50.0 Hz), under which conditions there is an increase in the amount of adenosine present in the synaptic cleft.

Cross-talk between the M_1 and A_{2A} facilitatory and M_2 and A_1 inhibitory receptors may occur. The activation of M_1 reduces the activity of M_2 receptors. The activity of M_1, in turn, can be reduced when A_{2A} receptors are being fully activated (frequency of stimuli $\geq$50 Hz) by adenosine. Additionally, M_2 receptors may have their activity reduced by A_1 receptors via down regulation.

Concomitantly to these complex interactions between ACh and adenosine molecules with their specific receptors, hydrolysis of ACh molecules in the synaptic cleft is also occurring. These molecules are metabolized to acetate and choline by the action of acetylcholinesterase. Choline is uptake to the cholinergic terminal by choline transporters (AChT) which can be classified into high affinity (HAChT, sodium dependent, inhibited by hemicholinium-3) and low affinity (LAChT, independent sodium). AChTs are too important for the control of neuromuscular transmission that their lack of efficiency determines severe reductions in the quantities of ACh released from the cholinergic terminals.

AIMS

The present study aims to investigate whether there would be influences, or controls, of presynaptic cholinergic receptors and adenosine receptors on the HAChT in neuromuscular preparations indirectly stimulated with frequencies of tetanizing (50 Hz) stimulation.

METHODS

The Ethics Committee of Animal Experimentation at the State University of Maringá (no 7227300915 / ECEAS-SUM) approved the present study. The diaphragm nerve preparations isolated from rats (Wistar, male, 200-220g) were assembled according to Bülbring methods (1946). After thoracotomy and isolation of the phrenic nerve left hemidiaphragm, each preparation was immersed in a 30 mL glass chamber containing Krebs buffer (composition in mmol/L) (NaCl 110.0, KCl 4.7, CaCl2 3.0, MgCl2 1.3, NaHCO3 25.0; KH2PO4 1.0, glucose 11.1) at 37°C and continuously aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The phrenic nerve was stimulated by rectangular electrical pulses (supramaximal pulses lasting 0.05 ms) through a bipolar platinum electrode connected to the GRASS® stimulator. The preparations were stimulated with a frequency of 50 Hz (for 10 seconds) and were inserted every 20 min at a frequency of 0.2 Hz. The muscle tensions produced at the beginning (A) and at the end (B) of the stimulus 50 Hz were evaluated along with the B/A ratio value. The hemicholinium-3, the HAChT inhibitor, was administered 35 min after the control tetanus and the values of the muscle tensions and the B/A ratio obtained 45 min after addition of the hemicholinium-3 were taken as % of the controls. The M_1, A_1 and A_{2A} receptors agonists (McN-A-343c, R-PIA, CGS21680, respectively) and the M_2, A_1 and A_{2A} receptors antagonists (metocramine, DPCPX and ZM241385, respectively) were administered 20 min before addition of the hemicholinium-3. ANOVA, followed by the Bonferroni post-test, was used, with significance level $P < 0.05$, for comparison of the data.

RESULTS AND DISCUSSION

Administration of hemicholinium-3 (4.0 to 10.0 μM) caused a reduction in B/A ratio values. However, higher concentrations of hemicholinium-3 (8 to 10 μM) induced a reduction in A values, probably due to a postsynaptic action more concentrated than the decrease observed with the use of 4 μM HC-3. Reduction of B/A ratio by hemicholinium-3 (4.0 μM) was inhibited by previous administration of A_{2A} (ZM241385-10.0 nM) and A_1 (DPCPX - 2.5 nM) adenosine receptor antagonists, and improved by agonists of these receptors, A_{2A} (CGS21680 - 2.0 nM) and A_1 (R-PIA - 0.1 μM). The M_2 receptor antagonist (Methoctramine 0.1 μM) was also able to reduce the inhibitory effect of hemicholinium-3 (4.0 μM) on the B/A ratio values. Although a facilitatory effect on the ratio has been recorded with the isolated administration of McN-A-343c (3.0 μM), an M_1 receptor agonist, potentiated the inhibitory effect caused by hemicholinium-3 (4.0 μM) on B/A ratio values.

The inhibition of HAChT (4.0 μM) reduced the ability of neuromuscular transmission to maintain adequate and sustained muscle contraction (reduction in R values) when the motor nerve was stimulated with 50 Hz pulses. This result could be explained by reducing the release of ACh/ATP from the motor nerve, since such an effect has been blocked or reduced by experimental conditions that are able to increase the release of ACh/ATP from the motor nerve. Such conditions were: the blockage of A_{2A} receptors by ZM241385, allowing the inhibition of

M_1 autoreceptors on M_2; inhibition of A_1 inhibitory receptors by DPCPX; the activation of A_{2A} facilitatory receptors by CGS21680; the activation of the A_1 receptor by RPIA, which induces a M_2 receptors down regulation and the blockage of M_2 inhibitory autoreceptors by metoctramine.

The reduction of the inhibitory effect of hemicholinium-3 on the R values caused by administration of the A_{2A} agonist CGS21680 is unlikely to have been mediated by cross-talk between A_{2A}/M_1 and M_2 receptors, since, by such mechanism, A_{2A} receptors activation by CGS21680 would lead to a reduction of M_1 activity, thereby decreasing the inhibitory activity of M_1 on M_2 receptors, which would result in a decrease in ACh release by the motor nerve. That is, CGS21680 should have intensified the inhibitory effect of hemicolinium-3 and not reduced as it was observed.

The cross-talk between A_{2A} / M_1 and M_2 receptors would indeed be involved in the antagonism of the inhibitory effect of hemicholinium-3 evidenced with inhibition of the A_{2A} receptor by ZM241385, since, the treatment of preparation with ZM241385 separately, the R values are worsened and when it was combined administrated with the hemicholinium-3 the R values are improved.

The treatment of preparations with hemicholinium-3 in the presence of the M_1 agonist (McN-A-343c) could affect the cross-talk between the M_1/M_2 receptors since, in such a situation, a difficult would occur in the IAVP replacement from the DPV. This hypothesis was raised after it was observed that administration of McN-A-343c was able to enhance the inhibitory effect of hemicholinium-3. Possibly, increased release of ACh from activation of the M_1 receptor would induce a reduction of IAVP and, concurrently, activation of inhibitory M_2 receptors by ACh released in the synaptic cleft. In addition, it has been described that the activation of the M_2 receptor interferes negatively in the displacement of the vesicles of the DPV to the IAVP. Thus, although the M_1 receptor is activated, it can not prevent the inhibitory M_2 receptor activation, whose activity, plus the reduction of IAVP, results in an increase in the inhibitory effect of the hemicholinium-3.

CONCLUSIONS

Taken together, the data indicate that reductions in the activity of HAChT seem to decrease the amount of ACh and adenosine in the synaptic cleft, since, under these conditions, there would be a reduction in the release of ACh/ATP from the motor nerve terminal. Therefore, therapeutic procedures, such as the blockade of presynaptic A_1 and A_{2A} receptors in the neuromuscular junction, could contribute to the treatment of pathologies that reduce the release of ACh /ATP in the motor nerve terminal, as is the case of some myasthenic syndromes and Alzheimer's disease.

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The inhibitory effect caused by hemicholinium is prevented by blockade of A_{2A} and A_1 adenosine receptors in the neuromuscular preparations submitted at tetanizing frequency.

Lilian Martins Castellão Santana[1], Priscila Yumi Abiko[1], Celia Regina Ambiel[2], Paulo Correia-de-Sá[3] and Wilson Alves-Do-Prado[1]*.

[1]Department of Pharmacology and Therapeutic, State University of Maringá, Paraná, Brazil.

[2]Department of Physiological Sciences, State University of Maringá, Paraná, Brazil.

[3]Laboratory of Pharmacology and Neurobiology/UNIB, Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal.

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*Correspondence to Dr. Wilson Alves-Do-Prado, PhD

Laboratory of Neuropharmacology of Peripheral Transmission

State University of Maringá

Colombo Avenue, 5790

87020-900 Maringá, PR, Brazil

E-mail: waprado@uem.br

ABSTRACT

Choline is taken on into the cholinergic terminal by a high-affinity choline transporter (HChT) which is selectively blockaded by hemicholinium (HC-3). The lack of efficiency in HChT reduces the amounts of acetylcholine (ACh) released from the cholinergic terminals causing severe myasthenic syndromes. As facilitatory-muscarinic (M_1), inhibitory-muscarinic (M_2), facilitatory-adenosine (A_{2A}), and inhibitory-adenosine (A_1) receptors are present on motor nerve, and taking into account that to found drugs that improved the neuromuscular transmission when the activity of HChT is diminished might useful for treatment of myasthenic syndromes; we investigated which would be the influences of the blockade and the activation of M_1, M_2, A_1 and A_{2A} receptors on the HC-3-induced inhibitory effect in the phrenic-nerve diaphragm-muscle preparation stimulated at 50 Hz. The lowest concentration of HC-3 causing reduction in neuromuscular transmission at 50 Hz frequency was 4.0 μM. The HC-3-induced inhibitory effect is prevented by blockage of A_{2A} or A_1 adenosine receptors by ZM241385 (10.0 nM) or DPCPX (2.5 nM), respectively. The HC-3-induced inhibitory effect is improved, but not prevented, by A_{2A} agonist (2.0 nM CGS21680, from -4.46± 1.04% to -0.49± 1.42%), A_1 agonist (0.1 μM R-PIA, from -4.46± 1.04% to -1.62± 0.53%) or M_2 antagonist (0.10 μM methoctramine, from -4.46± 1.04% to -2.06± 0.20). The activation of M_1 receptors by McN-A-343 worsens the inhibitory effect caused HC-3. Data suggest that the clinical utilization of antagonists of A_1 or A_{2A} receptors might be thought out as useful for treatment of myasthenic syndromes.

Keywords: Hemicholinium-3, high-affinity choline transporter, muscarinic receptors, adenosine receptors, neuromuscular junction.

INTRODUCTION

The acetylcholine (ACh) and ATP are presents in vesicles found inside of motor nerve terminal.[1,2,3] These vesicles are distributed in two main pools: one, closer of presynaptic membrane, is called immediately available vesicle pool (IAVP) and other one, more dispersed by the cytoplasm is named deposit pool (containing recycling vesicles and reserve pools of vesicles) of vesicles (DPV).[1,4] Through a process called mobilization, which is not yet fully understood, the vesicles of DPV replaced those vesicles whose contents were released from IAVP to synaptic cleft.[4,5]

In addition to its stimulatory action on muscle fibers, ACh also acts pre-synaptically to regulate its own release at the rat neuromuscular junction.[1] The release of ACh from motor nerve may be controlled by activation of neuronal (n) cholinergic (C) nicotinic (N) (expressing α3β2) receptor (R) (CnNRα3β2), C-muscarinic (M) M_1R and C-M_2R receptors present on motor nerve ending.[6] The activations of such receptors are dependent on frequency of stimulation applied on motor nerve.[7] Thus, the facilitatory-CM_1Rs are the presynaptic receptor preferentially activated when low frequency (5 Hz) of stimulation is applied on motor nerve, as in such condition occurs a fast desensitization of facilitatory-CnNRα3β2.[2,7,8]

Adenosine (A) buildup from ATP catabolism during neuronal firing plays a key role in adjusting the modulatory pattern of neuromuscular transmission.[9] Receptors for adenosine (AR), such as A_1R and $A_{2A}R$ receptors, are also presents on motor nerve terminal and their activations depend on amount of adenosine present in the synaptic cleft.[7] The presynaptic inhibitory-A_1Rs is activated by low amount of adenosine present in synaptic cleft, i.e., when low (5.0 Hz) frequency of stimulation is applied on motor

nerve.[7] On the other hand, a preferential activation of presynaptic facilitatoy-A_{2A}Rs occurs when tetanizing frequencies (50 Hz) of stimulation are applied on motor nerve, as in such circumstance there is a greater amount of adenosine in the synaptic cleft.[7] It has been shown that activation of presynaptic A_{2A}Rs reduces the facilitatory activity of CM_1Rs detected on motor nerve when 50 Hz frequency is being applied on motor nerve.[7] Since the activation of presynaptic CM_1Rs is capable, via an intraneuronal phospholipase C activation, to reduce the inhibitory activity of M_2Rs when 50 Hz frequency is applied on motor nerve; [6] the activation of A_{2A}Rs reduce the activity of facilitatory-CM_1Rs and enhances the inhibitory effect produced by CM_2Rs activation.[7] Concomitantly to these complex interactions described to action of ACh and adenosine molecules, is also occurring the hydrolysis of ACh molecules released from motor nerve terminal. In such circumstances, ACh molecules are metabolized to acetate and choline by the action of acetylcholinesterase.[10] The choline molecules are recaptured to the cholinergic terminal by the action of choline transporters (ChT).[11] The choline ChT are important for the efficiency of neuromuscular transmission that the lack of efficiency of this system determines serious reductions in the amounts of ACh released from the cholinergic terminals.[11] Additionally, it has been shown that there is a close correlation between the release of ACh from motor nerve and the activity of ChT, as occur an expressive increase the activities of ChT when the motor nerve has its activity increased.[12]

Furthermore, it has been shown that amount of ChT and hemicholinium-3 (HC-3) are increased in the presynaptic membrane of cholinergic neuron submitted at high frequency of stimulation and a reduction in activity of such transporter might decrease the mobilization of vesicles containing ACh/ATP from DPV toward IAPV pool.[11,13]

Since the efficient of choline transport system could be differentially influenced by M_1, M_2, A_1 and A_{2A} receptors activities on motor nerve, taking into account that ChT can be classified as ChT with high (sodium dependent, HChT) and low affinity (independent sodium, LChT) to HC-3[11] and considering that reduction in HChT activity cause severe mya sthenic syndromes,[14] in the present study was investigated what would be the influences of the CM_1R, CM_2R, A_1R and $A_{2A}R$ receptors activity on the effects caused by HC-3 in neuromuscular preparations indirectly stimulated with tetanizing frequency stimulation (50 Hz).

RESULTS

The HC-3 (4.0 to 10.0 μM) caused a dose-dependent reduction (-4.46± 1.04% to -12.6± 0.84%) in R-values induced by 50.0 Hz indirectly applied in neuromuscular preparations (Figure 1- left graphic). The reduction in R-values induced by HC-3 at concentrations higher than 4.0 μM was always fallowed by severe reductions (-18.5± 2.4 % at 8.0 μM and -27.7± 3.6% at 10.0 μM) in A-values (Figure 1-right graphic). The reduction in R-values obtained after administration of 4.0 μM HC-3 was prevented by ZM241385 (10.0 nM) and DPCPX (2.5 nM) blocking $A_{2A}R$ and A_1R receptors, respectively (Figure 2, A and B). The inhibitory effect caused by 4.0 μM HC-3 was improved by treating the preparations with the agonists of $A_{2A}R$ (from -4.46± 1.04% to -0.49± 1.42%) or A_1R (-4.46± 1.04% to -1.62± 0.53%) receptors CGS21680 (2.0 nM) or R-PIA (0.1 μM), respectively (Figure 3, A and B). In contrast, the McN-A-343c (3.0 μM), a M_1Rs agonist, potentiated (from -4.46± 1.04% to -15.0± 5.0) the inhibitory effect caused by HC-3 (4.0 μM) on R-values. Interestingly, M_1Rs agonist produced facilitatory (+4.60± 0.84%) effect when it was assayed separately (Figure 4A). Methoctramine (0.10 μM) alone did produced any change in R-values, but such

antagonist of inhibitory-M_2Rs improved (from -4.46± 1.04% to -2.06± 0.20) the reduction in R-value induced by 4.0 μM HC-3 (Figure 4B).

DISCUSSION

The inhibition of HChT by 4.0 μM HC-3 reduces the ability of neuromuscular transmission to maintain an adequate sustained muscular contraction (reduction in R-Values) when the motor nerve is submitted at tetanizing frequency (50 Hz). This effect muscle seems to be determined by reduction in the amount of ACh/ATP release from motor nerve, as experimental conditions previously reported an increase in ACh/ATP release from motor nerve[15] improved the reduction in R-values caused by HC-3 (4.0 μM). The experimental conditions used in current study which have been reported as able to increase the ACh/ATP releases from motor nerve were: 1- The blockage of A_{2A}Rs by ZM241385 reducing the inhibitory activity of A_{2A}Rs over facilitatory-M_1Rs on motor nerve (increase in ACh release), thereby increasing the inhibitory activity of M_1Rs on M_2Rs (increase in ACh release); 2- The blockage of inhibitory-A_1Rs on motor nerve by DPCPX; 3- The activation of stimulatory-A_{2A}Rs on motor nerve by CGS21680; 4- The activation by R-PIA of intraneuronal downregulation pathway mediated by presynaptic A_1Rs, thereby reducing the inhibitory-M_2Rs activity on motor nerve, and 5- The blockage of inhibitory-M_2Rs activity on motor nerve terminal by methoctramine.[7] It is unlike that the improvement caused by CGS21680 in reduction in R-value induced by HC-3 has been mediated by cross-talking between A_{2A}/$M_1$and M_2 receptors (activation of A_{2A}R by CGS21680 would be expected to reduce the activity of M_1R, thereby diminishing the inhibitory activity of M_1R over M_2R receptors and thus reducing the ACh release from motor nerve), as the activation of such intraneuronal

pathways determines reduction on ACh release from motor nerve and this effect should have worsened, and not improved, the reduction in R-value caused by HC-3. In contrast, it was recorded that the activation of cross-talking involving A_{2A}/M_1 and M_2 receptors seemed has a role key in the antagonism caused by ZM241385 in the reduction in R-values produced by HC-3, as it was recorded that the treatment of preparation with ZM241385 separately reduced R-Values. Such data reinforce the hypothesis that the reduction in HChT activity by HC-3 reducing the ACh/ATP release from motor nerve ending change mainly the "direct" facilitatory effect caused by A_{2A}Rs activity, as the activation and blockage of A_{2A}Rs by CGS and ZM241385 separately increases and reduces R-values, respectively.

It was also recorded that the activation of facilitatory-M_1Rs by McN-A-343c improved the ability of neuromuscular transmission to maintain an adequate sustained muscular contraction (increase in R-Values) when McN-A-343c was administered separately, but the combined administration of McN-A-343c with HC-3 caused a drastic worsening in R-values. Therefore, is suppose that treatment of preparations with HC-3 seems to affect the intraneuronal "cross-talking" between M_1/M_2 receptors when the presynaptic M_1Rs is been triggered by McN-A-343c, thereby impairing that the activation of such receptors is able to reduce the inhibitory M_2Rs activity caused by ACh. Taking into account that changes in the intraneuronal "cross-talking" between M_1/M_2 receptors appeared only when HC-3 was administered in neuromuscular preparations whose the presynaptic M_1Rs already was being previously activated by McN-A-343c, i.e. a condition with a suppose activity of presynaptic M_1Rs greater than that which is occurring in preparation treated only with HC-3, we hypothesized that in such specific experimental condition the activation of presynaptic M_1Rs by McN-A-343c caused a level of clearance of the vesicles containing ACh/ATP of the IAVP that

would be greater than the rate of mobilization of vesicles of DPV toward IAVP. In fact, the inhibitory effect caused by activation of presynaptic M_2Rs by ACh was preserved after the treatment of preparations with HC-3, as the blockage of such receptors by methoctramine improved the reduction in R-value caused by HC-3 (4.0 μM). Additionally, it is also to supposed that the activation of A_{2A}Rs on motor nerve terminal seems to be higher when the preparations are not treated with HC-3 and indirectly stimulated at 50 Hz frequency, as the blockage of A_{2A}Rs by ZM241385 seemed reduce more the ACh/ATP release mediated by A_{2A}Rs activation than effect of A_{2A}R over M_1R receptors and of this one over M_2Rs. In fact, the treatment of preparations with ZM241385 or CGS21680 separately caused reduction and increase in R-values, respectively.

Since McN-A-343c caused only facilitatory effect (increase in R-value) when it was separately investigated in neuromuscular preparation indirectly stimulated at 50 Hz frequency; it is unlikely that M_1Rs found in membranes of skeletal muscle16 might also be involved with effect inhibitory (drastic reduction in R-value) recorded in present study with the combined administration of McN-A-343c and HC-3, as the activation of such receptors increases the level of nitric oxide in skeletal muscle[16,17] and such gas reduces ACh release from motor nerve.[17]

Concentrations of HC-3 higher than 4.0 μM also caused reductions in R-values, but such reductions were always followed by expressive reductions A and B values. Since marked reductions in A- and B-values caused by HC-3 at concentrations levels higher[18] than 4.0 μM might be generated by blockage of postsynaptic nicotinic muscular receptors, the inhibitory effect induced by 4.0 μM HC-3 in the phrenic nerve diaphragm muscle preparation indirectly stimulated at 50 Hz frequency might has a postsynaptic (blockage of muscular nicotinic receptors) component of action less dramatic, as the

reduction in A-value cased by 4.0 μM HC-3 was much lower (~50% and 180 % lower) than that recorded with administrations of HC-3 8.0 and 10.0 μM.

The effects induced by HC-3 in the neuromuscular preparations indirectly simulated at 50 Hz frequency could be displaying the operating conditions of neuromuscular transmission in patients expressing deficiency in HChT, as it has been recently shown that reduction in HChT activity cause severe myasthenic syndromes.[14] Therefore, data of present study indicate that the clinical utilization of antagonists of A_1 or A_{2A} receptors might be thought out, at least a priory, as useful for treatment of myasthenic syndromes, and others diseases generate by reductions in ACh release, such as Alzheimer disease.[19,20] This hypothesis are supported by data that showed both the activations and the blockades of $A_{2A}R$ (activated by CGS21680 and blocked by ZM241385) and A_1R (activated by R-PIA and blocked by DPCPX) receptors improved the fade of transmission caused by HC-3. However, it was also recorded that the blockages of adenosine receptors by ZM ($A_{2A}R$) or DPCPX (A_1R) were more efficient than the activations of $A_{2A}R$ (CGS21680) and A_1R (R-PIA) receptors to impair the inhibitory effect induced by HC-3 in the phrenic nerve diaphragm muscle preparations indirectly stimulated at physiological tetanizing frequency (50 Hz).

Taken together, data indicate that reductions in HChT activity seem to decrease the amount of ACh and adenosine at synaptic cleft because in such conditions there would is a reduction on ACh/ATP release from motor nerve terminal. Therefore, therapeutic procedures, such as blockade of presynaptic A_1 and A_{2A} receptors at neuromuscular junction could contribute to the treatment of pathologies reducing the release of ACh/ATP from motor nerve.

METHODS

The Ethics Committee for Experimental Animals Studies of the State University of Maringá approved (ECEAS 7227300915) the procedures used in the present study. Phrenic nerve–diaphragm muscle preparations of rats were set up as described by Bülbring.[21] 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; KH2PO4 1,0; glucose 11,1) maintained at 37.0°C and aerated with 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 six tetanic stimuli (50.0 Hz) were applied at 20.0 min intervals. The 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; ADInstruments, Castle Hill, NSW, Australia). The initial tetanic tension at the beginning (A) of the tetanic stimulus and tension at the end (B) of the tetanic stimulus (after 10.0 s; B) was recorded and the ratio (R) B/A calculated (Figure. 1). The instant (T= 45.0 min) and the value of lowest concentration of HC-3 (4.0 μM) able to produce effect in R values were researched. The effect caused by HC-3 separately, or in presence of others drugs, was analyzed at T= 45.0 min. The others drugs (ZM241385, CGS21680, DPCPX, R-PIA, methoctramine and McN-A-343c) were administered 20 min prior to the administration of HC-3.

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REFERENCES

1. Bowman WC. Prejunctional and Postjunctional Cholinoceptors at the Neuromusclar Junction. *Anesth Analg*. 1980;59:935-943.

2. Wessler I, Halank M, Rasbach J, Kilbinger H. Presynaptic nicotine receptors mediating a positive feed-back on transmitter release from the rat phrenic nerve. *Naunyn Schmiedebergs Arch Pharmacol*. 1986;334:365-372.

3. Westfall TC, Westfall DP. Neurotransmission: The autonomic and somatic motor nervous system. In: Brunton LL, Chabner BA, Knollmann BC. The Pharmacogical Basis of Therepautics: McGraw-Hill; USA; 2011. p. 171-218.

4. Chamberland S, Toth K. Functionally heterogeneous synaptic vesicle pools support diverse synaptic signalling. *J Physiol*. 2016;594:825-835.

5. Van Der Kloot W, Molgo J. Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol Rev*. 1994;74:899-992.

6. Oliveira L, Timóteo MA, Correia-De-Sá P. Negative crosstalk between M1 and M2 muscarinic autoreceptors involves endogenous adenosine activating A1 receptors at the rat motor endplate. *Neurosci Lett*. 2009;459:127-131.

7. Oliveira L, Timóteo M, Correia-De-Sá P. Modulation by adenosine of both muscarinic M1-facilitation and M2-inhibition of [3H]-acetylcholine release from the rat motor nerve terminals. *Eur J Neurosci*. 2002;15:1728-1736.

8. Colquhon D, Mathie A, Mulrine NK, Ogden DC. Studies on single acetylcholine-receptor channels in muscle end-plate and sympathetic neurones. *Fernstrom Foundation Series*. 1989;13:217-234.

9. Correia-De-Sá P, Timóteo MA, Ribeiro JA. Presynaptic A_1 inhibitory/A_{2A} facilitatory adenosine receptor activation balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm. *J Neurophysiol*. 1996;76:3910-3919.

10. Soreq H, Seidman S. Acetylcholinesterase—new roles for an old actor. *Nat Rev Neurosci*. 2001;2:294-302.

11. Ferguson SM, Blakely RD. The choline transporter resurfaces: new roles for synaptic vesicles? *Mol Interv*. 2004;4:22.

12. Kuhar MJ, Murrin LC. Sodium-dependent, high affinity choline uptake. *J Neurochem*. 1978;30:15-21.

13. Iwamoto H, Calcutt MW, Blakely RD. Differential impact of genetically modulated choline transporter expression on the release of endogenous versus

newly synthesized acetylcholine. *Neurochem Int*. 2016;98:138-145.

14. Bauché S, O'regan S, Azuma Y, Laffargue F, Mcmacken G, Sternberg D, Lacène E. Impaired Presynaptic High-Affinity Choline Transporter Causes a Congenital Myasthenic Syndrome with Episodic Apnea. *Am J Hum Genet*. 2016;99:753-761.

15. Slater CR. The functional organization of motor nerve terminals. *Prog Neurobiol*. 2015;134:55-103.

16. Malomouzh AI, Nurullin LF, Arkhipova SS, Nikolsky EE. NMDA receptors at the endplate of rat skeletal muscles: precise postsynaptic localization. *Muscle Nerve*. 2011;44:987-989.

17. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev*. 2011;81:209-237.

18. Lopes G, Bazotte RB, Curi R, Alves-Do-Prado W. L-and DL-carnitine induce tetanic fade in rat neuromuscular preparation. *Braz J Med Biol Res*. 2003;36:1255-1262.

19. Noronha-Matos JB, Morais T, Trigo, D, Timóteo MA, Magalhães-Cardoso MT, Oliveira L, Correia-De-Sá P. Tetanic failure due to decreased endogenous adenosine A2A tonus operating neuronal Cav1 (L-type) influx in Myasthenia gravis. *J Neurochem*. 2011;117:797-811.

20. Rahman A. The role of adenosine in Alzheimer's disease. *Curr Neuropharmacol*. 2009;7:207-216.

21. Bülbring E. Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br J Pharmacol Chemother*. 1946;1:38-61.

FIGURES

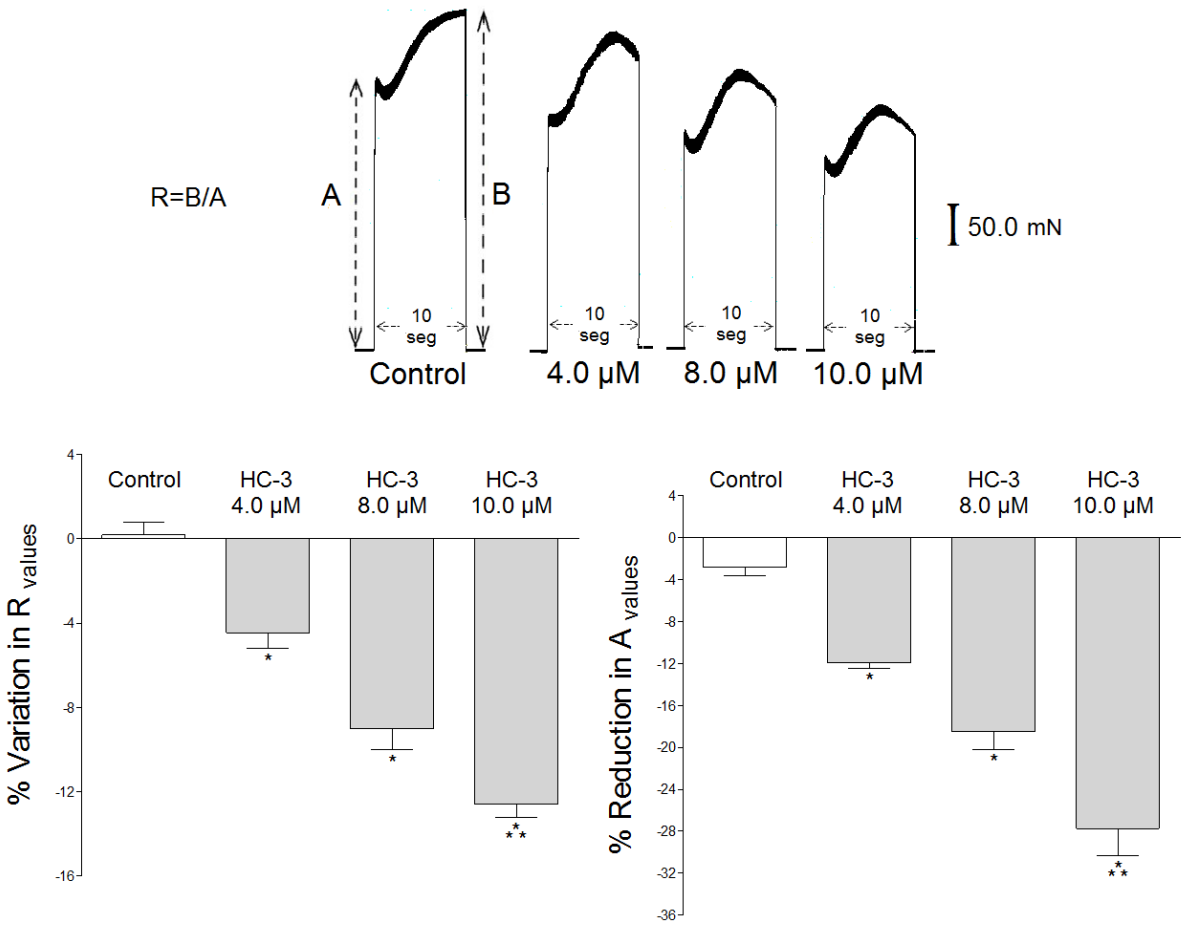

FIGURE 1 Typical trace produced by HC-3 (4.0 to 10.0 µM) in rat phrenic nerve diaphragm muscle preparations indirectly stimulated at 50.0 Hz for 10.0 s. R-value (B/A) was the parameter analyzed which was measured as shown in muscular record. Vertical line (right of typical trace) indicates force in Newton (N). The percentages (%) of variations in R-values (left graphic) and A-values (right graphic, postsynaptic action) caused by HC-3 are shown. The height of columns indicate mean ± SEM of 5 to 6 experiments. * indicate significant difference from control at P< 0.05 (ANOVA followed by Bonferroni post hoc test).

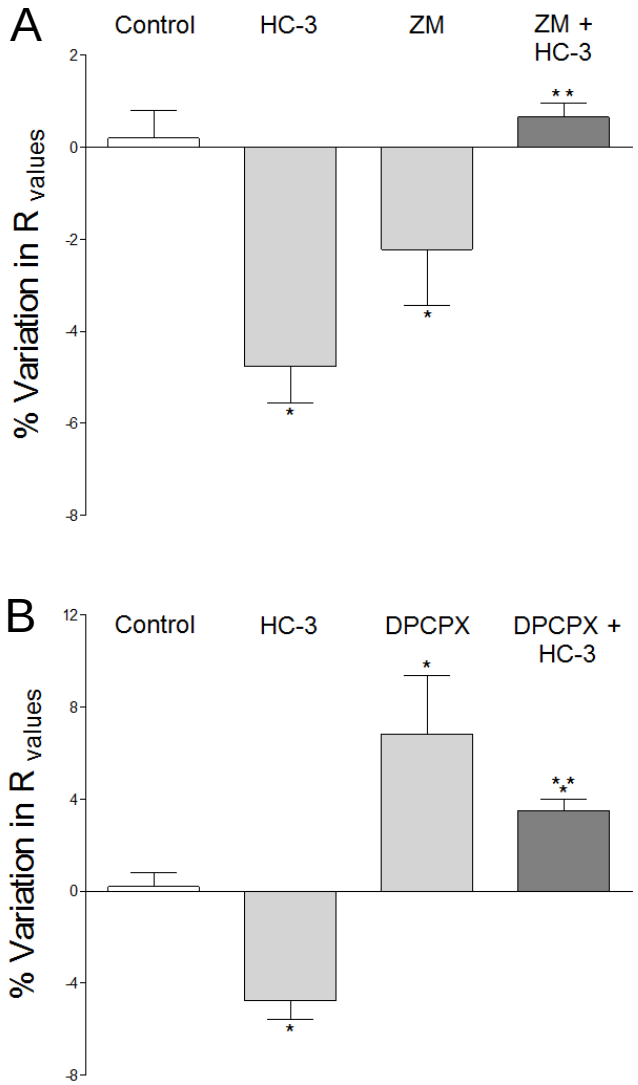

FIGURE 2 Antagonism by ZM241385 (ZM, 10.0 nM, A) and DPCPX (2.5 nM, B) of reduction in R-value (see Fig. 1) caused by HC-3 (4.0 µM, A and B) in neuromuscular preparations of rats indirectly stimulated with 50.0 Hz frequency. The percentages (%) of variations in R-values in the presence of HC-3 (4.0 µM, A and B), ZM241385 (ZM, 10.0 nM, A) and DPCPX (2.5 nM, B) separately are also displayed. The height of columns indicates mean ± SEM of 5 to 6 experiments. * indicate significant difference from control at P< 0.05 (ANOVA followed by Bonferroni post hoc test). ** indicate significant difference (P< 0.05, ANOVA followed by Bonferroni post hoc test) from HC-3 alone.

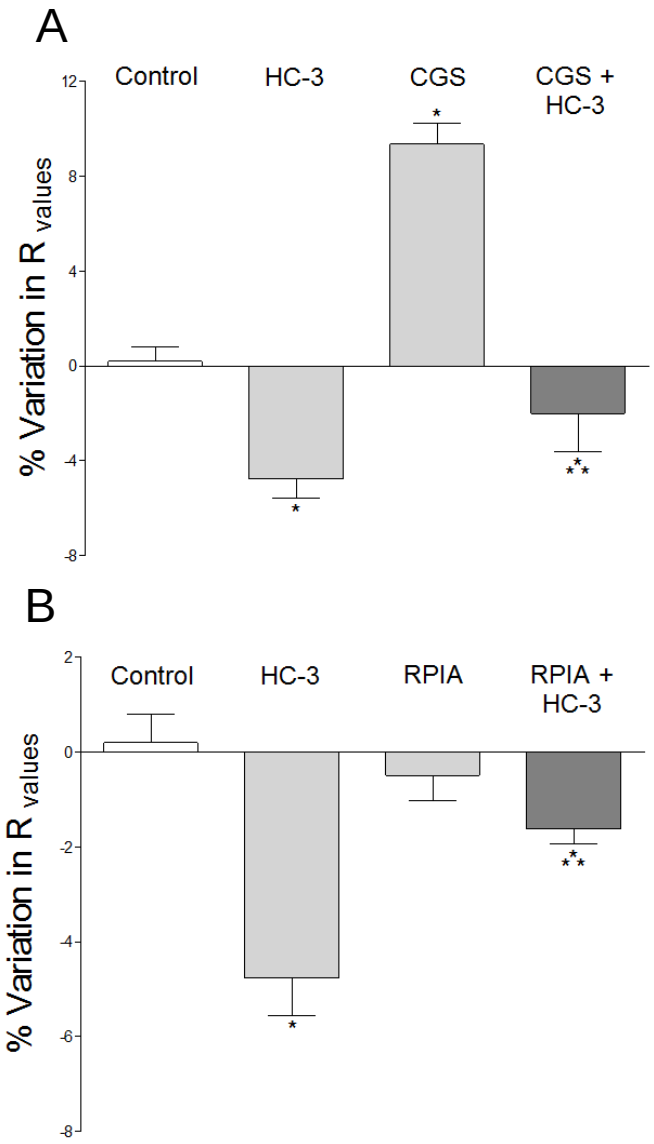

FIGURE 3 Effects caused by CGS21680 (CGS, 2.0 nM, A) and R-PIA (0.1 µM, B) on reduction in R-value (see Fig. 1) caused by HC-3 (4.0 µM, A and B) in neuromuscular preparations of rats indirectly stimulated with 50.0 Hz. The percentages (%) of variations in R-values caused by CGS21680 (A), R-PIA (B) and HC-3 (A and B) separately are also displayed. The height of columns indicate mean ± SEM of 5 to 6 experiments. * indicate significant difference from control at P< 0.05 (ANOVA followed by Bonferroni post hoc test). ** indicate significant difference (P< 0.05, ANOVA followed by Bonferroni post hoc test) from HC-3 alone.

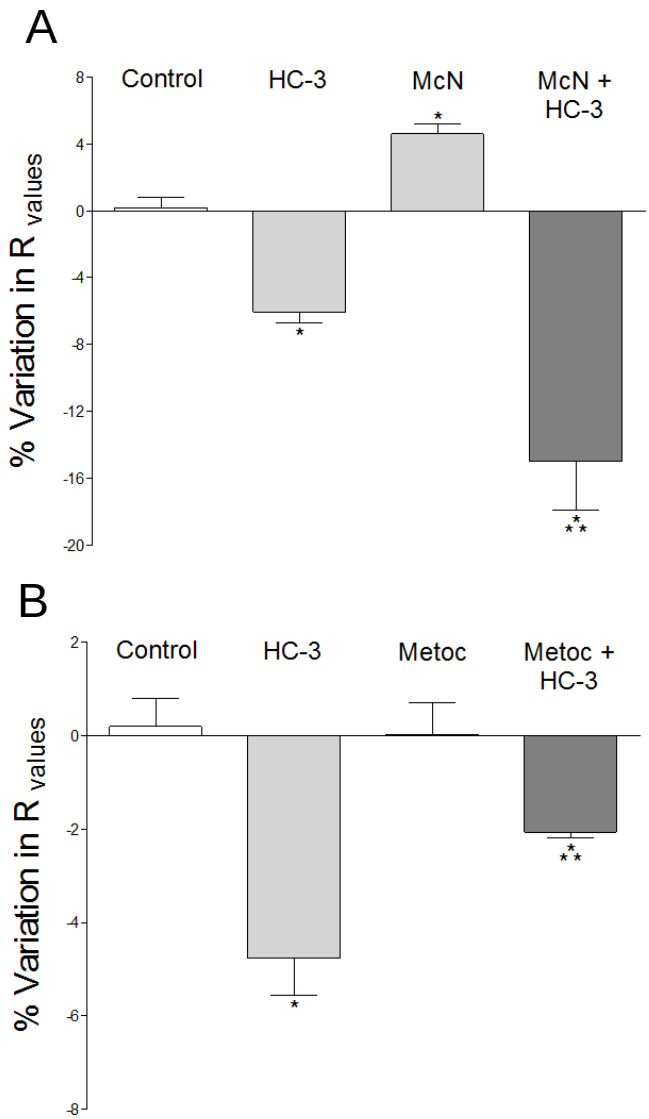

FIGURE 4 Effect caused by McN-A-343c (McN, 3.0 µM, A) and improvement by methoctramine (Metoc, 0.1 µM, B) in the inhibitory effect caused by HC-3 (4.0 µM, A e B) in neuromuscular preparations of rats indirectly stimulated with 50.0 Hz frequency. The percentages (%) of variations in R-values (see Figure 1) caused by McN (A), Metoc (B), and HC-3 (A and B) separately are also displayed. The height of columns indicate mean ± SEM of 5 to 6 experiments. * indicate significant difference from control a $P< 0.05$ (ANOVA followed by Bonferroni post hoc test). ** indicate significant difference ($P< 0.05$, ANOVA followed by Bonferroni post hoc test) from HC-3 alone.