NO- induced neuromuscular facilitation in the phrenic nerve diaphragm preparation of rats is Ca^{++} dependent

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ABSTRACT. The research was carried out to verify whether the neuromuscular effects induced by nitric oxide (NO) might depend on extracellular level of Ca^{++}. The donor of NO, sodium nitroprusside (SNP), and the analogue of cGMP, 8-Br-cGMP, increased the muscular contraction amplitude (MCA) in preparations indirectly stimulated at 0.2 Hz, but did not produce any effect when the nutrient solution was exchanged by Krebs buffer low Ca^{++}/ high Mg^{++}. SNP and 8-Br-cGMP reduced the MCA in preparations directly stimulated. Such effect was not recorded in Krebs buffer low Ca^{++}/ high Mg^{++}. Data suggest that the neuromuscular effects induced by NO or cGMP depend on extracellular level of Ca^{++}.

Key words: Calcium; nitric oxide; sodium nitroprusside; guanylate ciclase.

RESUMO. O efeito facilitatório do óxido nítrico sobre a transmissão neuromuscular de preparações nervo frênico- diafragma isolado de ratos é Ca++ dependente. A pesquisa foi conduzida para verificar se os efeitos neuromusculares induzidos por óxido nítrico (NO) poderiam depender dos níveis extracelulares de Ca++. O doador de NO, nitroprussiato de sódio (NPS), e o análogo de GMPc, 8-Br-cGMP, aumentaram a amplitude das contrações musculares (ACM) em preparações indiretamente estimuladas a 0.2 Hz, mas não produziram efeitos quando a solução nutriente (Krebs normal) foi trocada por Krebs com baixo Ca^{++}/ alto Mg^{++}. NPS e 8-Br-cGMP reduziram a ACM quando as preparações foram diretamente estimuladas, mas tal efeito não foi observado com o uso de Krebs com baixo Ca^{++}/ alto Mg^{++}. Dados sugerem que os efeitos neuromusculares induzidos por NO dependem dos níveis extracelulares de Ca++.

Palavras-chave: cálcio, óxido nítrico, nitroprussiato de sódio.

Introduction

The evoked release of acetylcholine from motor nerve ending depends on extracellular levels of Ca^{++} (Katz, 1969). In the motor nerve ending, Ca^{++} collaborates with proteins of vesicular and neuronal membranes to produce fusion of vesicles with presynaptic membrane, thereby increasing the acetylcholine output (Jahn and Südhof, 1994, Südhof, 1995).

Nitric oxide (NO) is generated from L-arginine by a Ca^{++} dependent NO- synthase (NOSn) found in both skeletal muscle and motor nerve ending (Kobzik *et al*., 1994, Chao *et al*., 1997, Ribera *et al*., 1998). It has been shown that NO released from endogenous (L-arginine) or exogenous (sodium nitroprusside, SNAP, SIN-1) sources increases or reduces the amplitude of muscular contractions when the muscle is indirectly or directly stimulated at 0.2 Hz, respectively (Ambiel and Alves-Do-Prado, 1997). The facilitatory effect of gas depend on its action on motor nerve ending increasing the acetylcholine output, thereby reducing its inhibitory action in muscle (Ambiel and Alves-Do-Prado). On the other hand, the literature offers evidences indicating that the neuromuscular effects induced by NO depend on activation of guanylate cyclase, thereby increasing the synthesis of cGMP in cells (Kobzik *et al*., 1994, Cruciol-Souza and Alves-Do-Prado, 1999). Controversially, it has been shown that NO increases the release of neurotransmitter from hippocampal synaptossomes, but such effect depends on direct action of gas with presynaptic membranes facilitating the vesicular fusion (Meffert *et al*., 1994). Therefore, since the effects induced by NO in phrenic nerve diaphragm preparations of rats depend on its interaction at pre- and postsynaptic synaptic levels (Ambiel and Alves-Do-Prado, 1997), the present work was carried out to verify whether the neuromuscular effects induced by NO might depend, or not, on extracellular level of Ca^{++}.

Material and methods

Phrenic nerve and diaphragm muscles were isolated from Wistar rats (200- 250g) and mounted according to the method described by Bülbring (1946). Each muscle was immersed in a 20mL

chamber containing Krebs buffer (mM, 110.0 NaCl, 4.7 KCl, 3.0 $CaCl_{2*}$ $2H_2O$, 1.3 $MgCl_{2*}$ $6H_2O$, 25.0 $NaHCO_3$, 1.0 KH_2PO_4, 11.0 glucose). The phrenic nerve was stimulated with a bipolar platinum electrode using a supramaximal rectangular pulse (0.2 Hz, 0.05 ms) and muscle contractions were recorded on an Ugo Basile polygraph. The isolated muscle were indirectly stimulated at 0.2 Hz until a steady amplitude of muscular contraction was obtained, then sodium nitroprusside (SNP) or 8-Br-cGMP were added in the bath. When the experiments were performed with Krebs buffer at low Ca^{++} / high Mg^{++} (mM, 110.0 NaCl, 4.7 KCl, 1.5 $CaCl_{2*}$ $2H_2O$, 2.8 $MgCl_{2*}$ $6H_2O$, 25.0 $NaHCO_3$, 1.0 KH_2PO_4, 11.0 glucose), the same previous experimental design was performed after a new steady amplitude of muscular contraction was reached. In the last experimental condition, the responsiveness (increase on MCA) of preparations was tested with addition of neostigmine (10 μM) in the bath (not shown). The lowest concentration of SNP and 8-Br-cGMP capable of producing maximal effects were determined. The maximal amplitude of muscular contraction recorded after addition of different agents was taken as percentage of that obtained with drug- free Krebs buffer (normal Ca^{++} or low Ca^{++}/high Mg^{++}). The similar experimental design was performed with preparations previously paralyzed by d-tubocurarine (100 μM) and directly stimulated. Data were submitted to Anova with the level of significance set at $P< 0.05$.

Results

SNP (0.2 to 1.5 mM) and 8-Br-cGMP (18 μM) increased the amplitude of muscular contractions when the preparations were indirectly stimulated at 0.2 Hz in experiments performed with Krebs buffer. The preparations were responsive to addition of neostigmine (10 μM) in the bath when the nutrient solution was exchanged by Krebs buffer low Ca^{++}/ high Mg^{++}. However, results show that addition of SNP and 8-Br-cGMP did not produce any effects when that nutrient solution was exchanged by Krebs buffer low Ca^{++}/ high Mg^{++} (Figures 1 and 2). SNP and 8-Br-cGMP reduced the amplitude of muscular contraction in neuromuscular preparations previously paralyzed by d-tubocurarine (100 μM) and directly stimulated at 0.2 Hz. The reduction on amplitude of muscular contraction muscular recorded with SNP and 8-Br-cGMP in preparations directly stimulated was not different of that obtained with Krebs buffer low Ca^{++}/ high Mg^{++} - drug free (Figure 2B).

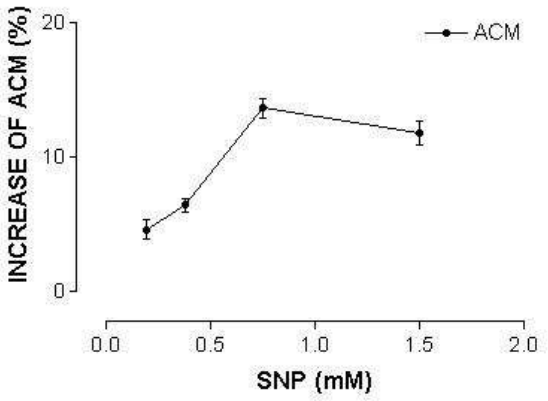

Figure 1. Percentage of increase dose- dependent of amplitudes of muscular contractions (AMC) induced by sodium nitroprusside (SNP; 0.2 to 1.5 mM) in phrenic nerve diaphragm preparations of rats indirectly stimulated at 0.2 Hz. Abscissas and ordinate represent concentration (mM) and increase of AMC, respectively. Points are mean (± SEM) of 6 experiments.

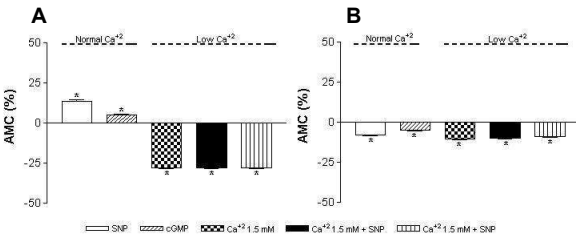

Figure 2. Percentage of increase and reduction of amplitudes of muscular contractions (AMC) induced by sodium nitroprusside (SNP; 0.75 mM) and 8-Br-cGMP (cGMP; 18 μM) in phrenic nerve diaphragm preparations of rats indirectly (A) and directly (B) stimulated at 0.2 Hz in Krebs (Normal Ca^{++}) and Krebs at low Ca^{++}/high Mg^{++} (Low Ca^{++}). The height of column represents mean (± SE) of 6 to 8 experiments. *P<0.05 compared to control (drug free Krebs buffer) taken as 100% (0%) (Anova).

+ cGMP

Discussion

Confirming a previous report with other NO donors (Ambiel and Alves-Do-Prado, 1997), our study showed that SNP (0.2 to 1.5 mM) and 8-Br-cGMP (18 μM) increased the amplitude of muscular contractions when the preparations were indirectly stimulated at 0.2 Hz in experiments performed with Krebs buffer. On the other hand, SNP and 8-Br-cGMP did not produce any effects when that nutrient solution was exchanged by Krebs buffer low Ca^{++}/ high Mg^{++}. SNP and 8-Br-cGMP reduced the amplitude of muscular contraction in neuromuscular preparations previously paralyzed by d-tubocurarine and directly stimulated at 0.2 Hz. The results with SNP resemble those obtained in other studies using similar preparations and methods (Ambiel and Alves-Do-Prado, 1997). However, the reduction on amplitude of muscular contraction muscular recorded with SNP and 8-Br-cGMP in preparations directly stimulated was not different of that obtained with Krebs buffer low Ca^{++}/ high Mg^{++} - drug free. It has been shown that the neuromuscular facilitation

induced by NO depend on its action on motor nerve ending increasing the acetylcholine output, thereby reducing its inhibitory action in muscle (Ambiel and Alves-Do-Prado, 1997). Thus, if only the presynaptic action of NO was dependent on extracellular level of Ca^{++}, a reduction on amplitude of muscular contraction (postsynaptic effect) should be expected when the preparations were indirectly stimulated in Krebs buffer at low Ca^{++}/ high Mg^{++}. Controversially, the results showed that both the increase and reduction on amplitude of muscular contraction induced by NO donor or 8-Br-cGMP were affected by reduction of Ca^{++} concentration in the bath. Therefore, the data suggest that NO does not act directly on vesicular fusion, as such mechanism has been described in hippocampal synaptossomes as not dependent on Ca^{++} ions (Ribera *et al*., 1998). Since the preparations were responsive to neostigmine (increase on MCA) when low Ca^{++}/high Mg^{++} condition was utilized, it is possible to admit that the neuromuscular effects induced by NO or cGMP at 0.2 Hz seem depend on extracellular level of Ca^{++}.

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