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Interaction of Glutamate- and Adenosine-Induced Decrease of Acetylcholine Quantal Release at Frog Neuromuscular Junction

S. ADÁMEK[1], A. V. SHAKIRZYANOVA[2], A. I. MALOMOUZH[2], N. V. NAUMENKO[3], F. VYSKOČIL[4,5]

[1]Third Surgical Department, First Faculty of Medicine, Charles University, Prague, [2]Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, [3]Department of Normal Physiology, Kazan State Medical University, Kazan, Russia, [4]Department of Animal Physiology, Faculty of Sciences, Charles University, Prague, and [5]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Summary

In a frog neuromuscular preparation of *m. sartorius,* glutamate had a reversible dose-dependent inhibitory effect on both spontaneous miniature endplate potentials (MEPP) and nerve stimulation-evoked endplate potentials (EPP). The effect of glutamate on MEPP and EPP is caused by the activation of metabotropic glutamate receptors, as it was eliminated by MCPG, an inhibitor of group I metabotropic glutamate receptors. The depression of evoked EPP, but not MEPP frequency was removed by inhibiting the NO production in the muscle by L-NAME and by ODQ that inhibits the soluble NO-sensitive guanylyl cyclase. The glutamate-induced depression of the frequency of spontaneous MEPP is apparently not caused by the stimulation of the NO cascade. The particular glutamate-stimulated NO cascade affecting the evoked EPP can be down-regulated also by adenosine receptors, as the glutamate and adenosine actions are not additive and application of adenosine partially prevents the further decrease of quantal content by glutamate. On the other hand, there is no obvious interaction between the glutamate-mediated inhibition of EPP and inhibitory pathways triggered by carbacholine and ATP. The effect of glutamate on the evoked EPP release might be due to NO-mediated modulation (phosphorylation) of the voltage-dependent Ca^{2+} channels at the presynaptic release zone that are necessary for evoked quantal release and open during EPP production.

Key words

Endplate potentials • Guanylyl cyclase • Carbacholine • ATP • NO

Corresponding authors

František Vyskočil, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague-4, Czech Republic. E-mail: Vyskočil@biomed.cas.cz

and

Artem Malomouzh, Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia, E-mail: artur57@list.ru

Introduction

Changes in the number of quanta either released at rest or during nerve stimulation are a manifestation of synaptic plasticity, the phenomenon underlying the fine regulation of the activities of the nervous system at the cellular level (Vyskočil 2003). It was found that, along with a principal neurotransmitter, there are a number of other substances released from the nerve ending that can strengthen or weaken the process of neurotransmitter secretion. In the most studied model of synaptic contact, the vertebrate neuromuscular junction, the feedback mechanisms were found that regulate the release of acetylcholine (ACh). In particular, the action ACh itself, its co-transmitter ATP and ATP-hydrolyzation product adenosine were studied on quantal and non-quantal ACh (Galkin *et al.* 2001, Nikolsky *et al.* 2004, Silinsky 2004, Veggetti *et al.* 2008, Burnstock 2009, Voss 2009). In addition, the interaction of the purinergic and cholinergic pathways was described at the neuromuscular synapse of

Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

both cold- (Shakirzyanova *et al.* 2006) and warm-blooded (Oliveira *et al.* 2002) animals. Evidence has emerged that glutamate, a neurotransmitter whose synaptic role was previously demonstrated and analyzed mostly in the CNS (Baudry 2009, Hur *et al.* 2009) can participate in the regulation of ACh release in peripheral vertebrate synapses, in particular in the non-quantal release of ACh at the rodent endplate (Urazaev *et al*. 1998, Malomouzh *et al.* 2003). The aim of this work was to study the mechanism of glutamate action on quantal spontaneous and nerve-evoked endplate potentials at the frog neuromuscular junction and to assess the possible interactions of the glutamate pathway with several other modulators – carbacholine, ATP and adenosine.

Materials and Methods

Experiments were performed on isolated neuromuscular preparations of the sartorius muscle of the frog *Rana ridibunda*. The preparation, pinned to the bottom of a translucid sylgard chamber, was superfused continuously at 20 °C with Ringer solution containing (in mM) NaCl 115.0, KCl 2.5, $CaCl_2$ 1.8, $NaHCO_3$ 2.4; pH=7.2-7.4 (Novotný *et al.* 1962). Suprathreshold stimuli of 0.1 ms duration were applied to the nerves at 2 s intervals *via* a pair of platinum electrodes located in a small adjacent moist chamber (Samigullin *et al.* 2003). This arrangement minimized the stimulus artifact. Muscle resting potentials and endplate potentials were recorded in the muscle fibre synaptic zone by standard intracellular microelectrodes filled with 2.5 M KCl (tip resistance 8-15 MΩ) under visual control by dissection microscope (x200).

To record the nerve-evoked endplate potentials (EPP), a modified solution with a lower concentration of Ca^{2+} (0.45 mM) and higher content of Mg^{2+} (6 mM) was used to decrease the quantal content of the EPP. This Ca^{2+}/Mg^{2+} ratio lowered the number of quanta and size of EPP below the muscle spike threshold depolarization and eliminated the muscle contraction. The quantal content was less than 10 under this Ca^{2+}/Mg^{2+} ratio and therefore no correction for the non-linear summation of quanta was used (McLachlan and Martin 1981). EPP and miniature end-plate potentials (MEPP) were recorded 10-15 min prior and during 30-60 min of drug application using the standard intracellular microelectrode technique.

The quantal content of the EPP was assessed by dividing the average EPP amplitude by the average amplitude of MEPP recorded in the same muscle fiber. For MEPP and EPP analysis, at least 200 events both before (control) and after drug application were captured in the frequency band 0-3000 Hz in each fiber as well as at least 50 EPP for calculating the quantal content, where the mean EPP amplitude was divided by the mean MEPP (McLachlan and Martin 1981). Spontaneous quantal secretion was analyzed as an average frequency of the MEPP (Kovyazina *et al.* 2003). All drugs and chemicals used were from Sigma (St. Louis, MO, USA) with the exception of (S)-a-methyl-4-carboxyphenylglycine (MCPG) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), which were purchased from Tocris Cookson Inc (Ballwin, MT, USA). Hemoglobin was reduced with sodium dithionate, dialyzed and kept frozen in aliquots at –20 ^{o}C (Martin *et al.* 1985, Mukhtarov *et al.* 2000). The results were expressed as the mean ± S.E.M. of experiments on at least three endplates from different preparations. Statistical significance was evaluated using Wilcoxon's non-parametric test at a probability level of $p<0.05$.

Results

Before glutamate or other drugs were applied, the mean MEPP frequency was 0.87±0.12 s^{-1} (n=14) and quantal content was 5.7±1.9 (n=15). Glutamate at concentrations of 20, 100 and 500 μM had no significant effect on the resting membrane potential (control value –82.4±1.3 mV n=155, inside minus, and –81.0±1.6 mV, n=52 in the presence of 500 μM glutamate) but resulted in a concentration-dependent and reversible (within 30 min wash) reduction of the spontaneous MEPP frequency and decrease in the quantum content of the evoked EPP (Fig. 1). The maximal decrease in the MEPP frequency was found in the presence of 100 μM glutamate, where the average MEPP frequency declined to 67.1±7.9 % (n=7, $p<0,05$) without any changes in amplitude, rise time and decay time of the MEPP (Fig. 1B). The observed drop in evoked EPP amplitude was therefore due to presynaptic reduction of the quantal content, i.e. a lower number of quanta released by every nerve impulse. EPP amplitude depression had a maximum at 500 μM glutamate when quantal content was decreased to 75.7±5.8 % of the control (Fig. 1B).

Metabotropic glutamate receptors (mGluR) have been recently found in the frog endplate (Pinard *et al.* 2003). To verify whether these mGluRs could be mediating the drop in quantal release by glutamate, muscles were pretreated with 500 μM MCPG, an

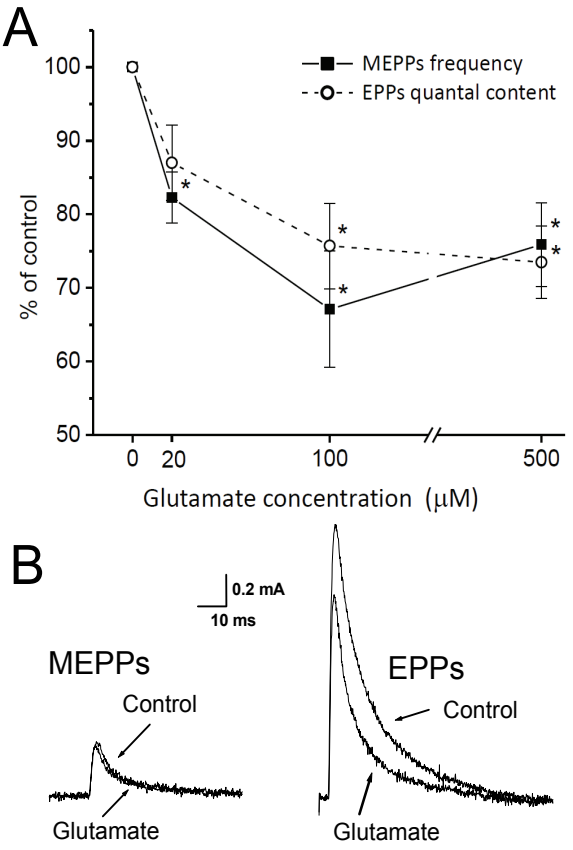

Fig. 1. Effect of 20, 100 and 500 μM glutamate (abscissa) on MEPP frequency (**A**, black squares) and on quantal content of nerve-evoked EPPs (A, empty circles). **B** – mean recordings of five MEPPs and EPPs before (Control) and 30 min after 100 μM glutamate application. Data expressed as percentage of control value before 30 min application of glutamate (ordinate). * – significant difference ($p<0.05$) between control and experiment.

inhibitor for group I metabotropic glutamate receptors ($mGlu_{1\alpha}$ and $mGlu_{5a}$) (Doherty *et al.* 1999), prior to glutamate application.

The MCPG completely abolished the inhibitory effects of 500 μM glutamate on both MEPP frequency, which was 98.8±5.6 % of the control, and EPP quantal content, which was 103.4±8.1 % of the control (n=3). This confirms the significant participation of mGluR1 in the drop in quantal ACh release (Pinard and Robitaille 2003).

The modulatory effect of glutamate is similar to the depressive action of cholinergic agonists and purines on quantal ACh release at the frog endplate (Ribeiro *et al.* 1996, Nikolsky *et al.* 2004, Shakirzyanova *et al.* 2006).

The question arises whether there are some common output pathways in quantal release regulation by these three receptor systems, as was earlier demonstrated in CNS (Ribeiro *et al.* 2003) and in the case of non-quantal ACh release (Vyskočil *et al.* 2009). The effects of three representative agonists on quantal release were therefore compared in the absence and presence of the glutamate.

10 μM carbacholine (cholinergic agonist) (Ciani and Edwards 1963), 100 μM ATP (purinergic P_2 receptors agonist) (Burnstock 2007) and 100 μM adenosine (P_1 receptors agonist) (Sawyok 2007) reduced the MEPP frequency to 54.3±3.8 %, 47.3±3.3 % and 40.8±4.0 %, and EPP quantal content to 68.3±3.9 %, 53.9±4.9 % and 55.9±4.9 % of the control values, respectively (Fig. 2). When 100 μM glutamate was added 30 min after the drugs application, this proportional drop in MEPP frequency and EPP quantal content remained unchanged with carbacholine and ATP (Fig. 2) On the other hand, the depressive effect of adenosine was significantly smaller, only to 61.0±5.2 % for MEPP frequency and to 69.4±4.7 % of the controls for the EPP quantal content. Thus the adenosine and glutamate metabotrophic pathways can at least partially interact; if the glutamate pathway is saturated, the effect of adenosine is smaller. One possible interactive pathway might be the NO cascade, NO synthase in particular (Ribeiro *et al.* 2003, Pinard and Robitaille 2008), because it was found that NO might to a certain extent mediate the suppressive effect of adenosine on the quantal secretion of ACh at the frog neuromuscular synapse (Thomas and Robitaille 2001). The NO synthase was therefore inhibited by 100 μM N-nitro-L-arginine methyl ester (L-NAME) (Hobbs and Gibson 1990).

The smaller production of NO led to an increase in MEPP frequency by 23.2±6.4 %, (Fig. 3, column 5) and partially reverted the inhibitory action of adenosine on MEPP frequency by 38.5±7.3 % (Fig. 3, columns 1 and 2). The most profound elimination of glutamate activity was observed in evoked responses. EPP amplitude and correspondingly the quantal content was 103.0±4.1 % (n=4) of the control when 100 μM L-NAME was applied 30 min before 100 μM glutamate. While our work was in progress, Pinard and Robitaille (2008) published similar data on the NO-dependent inhibition of evoked EPP by glutamate.

On the other hand, the glutamate-induced decrease of the spontaneous MEPP frequency was not significantly prevented by the inhibition of NO production (Fig. 3, columns 3 and 4). This indicates that in spontaneous quantal release, glutamate still exhibits its inhibitory effect even during a reduction in NO production.

As shown in previous papers, the main source of

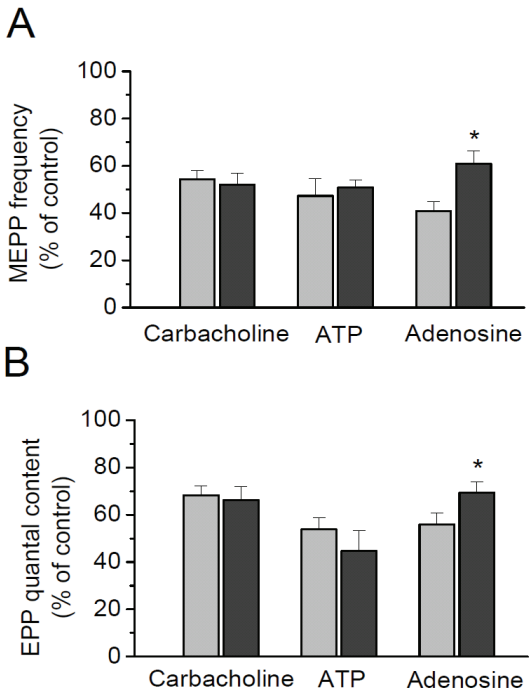

Fig. 2. Effect of 10 μM carbacholine, 100 μM ATP and 100 μM adenosine on the frequency of spontaneous (MEPP) (**A**) and stimulation-evoked EPP (**B**) recorded after 30 min of their application (gray columns), and then 30 min after their application together with 100 μM glutamate (black columns). Gray columns – EPP quantal content expressed as percentage of controls before drug application, or percentage of new level when gray columns are taken as 100 % before glutamate plus the drug application. Asterisk – difference between control and experiment ($p<0.05$). Because carbacholine depolarizes the RMP by 5-10 mV, only those experiments with carbacholine application were taken into analysis when the distribution of MEPP amplitudes in the presence of this ACh analogue still fitted by bell-shaped Gaussian function indicating that there was no loss of low-amplitude signals.

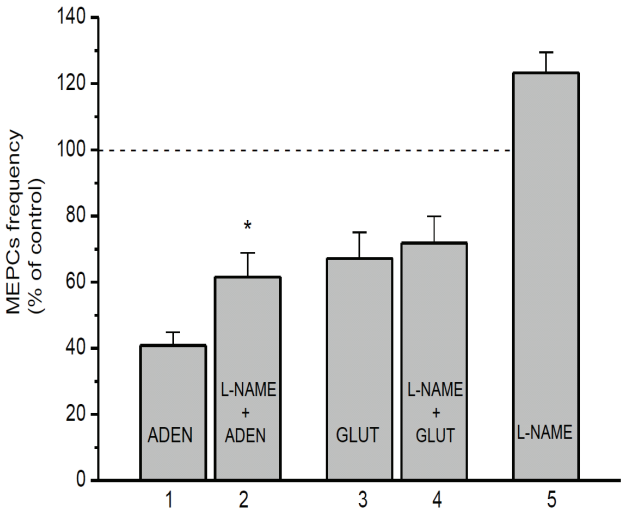

Fig. 3. Effect of NOS inhibitor 100 μM L-NAME (column 5), 100 μM adenosine (ADEN, columns 1 and 2) and 100 μM glutamate (GLUT, columns 3 and 4) in the absence (columns 1 and 3) and presence of 100 μM L-NAME (columns 2 and 4) on MEPP frequency. Data are expressed as percentage of control value before 30 min application of the drugs, either alone or together with L-NAME (ordinate). * – significant difference between control and experiment ($p<0.05$).

presynaptic NO regulation of quantal and non-quantal release is muscle NO synthase (Pinard and Robitaille 2003, Urazaev *et al.* 1995). To further assess whether the glutamate-evoked presynaptic decrease in the MEPP frequency is really independent of the generation of NO in the muscle, solutions of glutamate were applied to muscles 5 min after the exposure to 20 μM reduced hemoglobin. As hemoglobin binds NO in the synaptic cleft, it should prevent extracellular NO from accumulating and exerting its presynaptic action (Barinaga 1991, Hu and El-Fakahany 1993, Meller and Gebhart 1993). Indeed, hemoglobin did not affect the 100 μM glutamate-induced decreases in MEPP frequency, which was 72.3±6.1 % (n=5, $p>0.05$), when compared with the corresponding controls without hemoglobin (70.1±3.5 %) (Fig. 1). On the other hand, hemoglobin almost completely eliminated the glutamate-induced decrease in EPP quantal content. The values were 70.0±6.0 % and 102.5±5.1 % of the control (n=5) in muscles exposed to glutamate and glutamate plus hemoglobin, respectively. Hemoglobin applied alone for 60 min caused a small but significant ($p<0.05$) increase in the EPP quantal content to 110.2±3.6 % (n=5), which indicates that there is certain resting production of NO controlling ACh-evoked quantal release. The MEPP frequency remained unchanged during the presence of 20 μM hemoglobin (98.3±7.2 % of the control). The application of guanylyl cyclase inhibitor 2 μM ODQ (Garthwaite *et al.* 1995, Urazaev *et. al*. 1996) led to the elimination of the glutamate effect on EPP but not on the MEPP frequency. The quantal content was 97.7±1.3 % of the control in the presence of 100 μM glutamate and 2 μM ODQ (n=5). Similarly as L-NAME, the ODQ slightly increased the MEPP frequency by 8.2±2.0 % (n=5). Nevertheless, other tentative phosphorylation pathways should be tested to trace the possible presynaptic action of glutamate *via* mGluR1 on the MEPP frequency. Experiments in this regard are underway.

Discussion

The application of exogenous glutamate resulted in a concentration-dependent reversible decrease in both MEPP frequency and quantal content of EPP. Because no

changes were found in MEPP amplitude, rise time and decay time constant, the inhibitory action of glutamate is presynaptic, in agreement with a previous report (Pinard and Robitaille 2008). Besides the NMDA receptors pharmacologically defined during non-quantal ACh release (Urazaev *et al.* 1995, 1998), metabotropic glutamate receptors were found in the frog endplate (Pinard *et al.* 2003). The action of glutamate was eliminated in the presence of metabotropic glutamate receptor blocker MCPG. It confirms the participation of this class of glutamate receptors in the regulation of both spontaneous and evoked quantal release.

The presynaptic effect of glutamate is similar to the effects of cholinergic and purinergic agonists (Van der Kloot *et al.* 1997, Grishin *et al.* 2005). The effects of three representative agonists – carbacholine, ATP and adenosine – on quantal release showed that carbacholine- and ATP-induced reduction of the MEPP frequency and EPP quantum content remained unchanged in the presence of glutamate, whereas the depressive effect of adenosine was significantly smaller. This means that metabotropic effect of glutamate is mostly independent of cholinergic and ATP-ergic regulation, whereas the adenosine (A1 receptors) and glutamate metabotrophic effects at least partially interact. When the glutamate pathway is saturated, the adenosine effect is much smaller. Previous studies on the presynaptic adenosine and carbacholine-evoked depression of MEPP frequency suggested that membrane-delimited cross-talk did not occur directly between A1 and M2 receptors. Rather, there should be a convergence of signals triggered by either of these metabotropic receptors to a common intracellular pathway in the nerve terminal (Shakirzyanova *et al.* 2006).

Such an interaction can be mediated by the NO cascade (Ribeiro *et al.* 2003, Pinard and Robitaille 2008). It was found that NO partly mediates the suppressive effect of adenosine on the quantal secretion of ACh at the frog and rat neuromuscular synapses (Thomas and Robitaile 2001, Galkin *et al.* 2001). Glutamate has been shown to also regulate non-quantal release in rats and mice (for a review see Vyskočil *et al.* 2009) through NMDA postsynaptic receptors and the NO cascade (Urazaev *et al.* 1995, 1998, 1999). In the experiments presented here, the inhibition of the NO synthase and lower production of NO caused an increase in MEPP frequency (Fig. 3) and partially reverted the inhibitory action of adenosine, but not glutamate, on MEPP frequency (Fig. 3). However, the EPP amplitude and EPP quantal content completely recovered when L-NAME was applied together with glutamate. This indicates that in spontaneous quantal release, glutamate still exhibits its inhibitory effect in the absence of NO production while the inhibition of evoked ACh release by glutamate strongly depends on NO synthase activity.

The observed glutamate inhibition of the evoked vesicular release, i.e. EPP quantal content, is clearly a mGluR1- and NO-dependent process and our observations are in agreement with recent data on bath-applied and stimulation-evoked release of endogenous glutamate from nerve terminals and Schwann cells (Pinard and Robitaille 2008). Glutamate action on evoked release is not only prevented by NO synthase inhibition but also by extracellular hemoglobin, an NO scavenger. This points to a muscular origin of the NO that has to cross the synaptic cleft before entering the nerve ending and activating the soluble guanylyl cyclase involved in the Ca^{2+}-dependent release of quanta.

The detailed mechanism of the influence of the NO cascade on evoked quantal release is unclear. Pinard and Robitaille (2008) simply stated that NO diffuses to the presynaptic terminal where it affects the evoked quantal release of the ACh. One possibility is that NO-triggered guanylyl cyclase-induced phosphorylation hinders the openings of the voltage-dependent Ca^{2+} channels that are linked to release sites during a nerve spike. This might diminish the necessary amount of intracellular Ca^{2+} in the active zone for quanta release (Gilmanov *et al.* 2008). A less likely possibility is that the glutamate triggers an NO- and cGMP-induced phosphorylation of proteins that are directly involved in vesicle fusion and non-quantal release (Doležal *et al.* 1983, Vyskočil *et al.* 1983). The glutamate only lowers the evoked Ca^{2+}-dependent synchronized release of quanta (EPP), not spontaneous release of quanta, despite the fact that similar scaffolds like v- and t-SNAREs apparently exist at the sites of spontaneous and evoked quanta release (Jackson and Chapman 2006). What might be different is the regulation of the spontaneous and evoked release by Ca^{2+} channels and Ca^{2+} entry. MEPP frequency is less sensitive to rapid intracellular Ca^{2+} elevation *via* voltage-sensitive Ca^{2+} channels, at least at rest, and the number of spontaneous quanta depends rather on the "tonic" or residual level of Ca^{2+} (Rahamimoff and Yaari 1973, Searl and Silinsky 2005). Thus, despite the molecular membrane machineries for spontaneous and evoked release being apparently identical, the inhibition of glutamate action was only

demonstrated here with evoked Ca^{2+}-channel-dependent EPP, indicating altered Ca^{2+} entry.

If the glutamate-induced decrease of spontaneously released quanta is not influenced by the inhibition of NO synthase, why then does L-NAME alone (and in experiments with the inhibition of soluble guanylyl cyclase as well) increase the MEPP frequency? This can be explained as an indirect consequence of the absence of NO production. We found earlier that postsynaptic resting membrane potential is regulated by a NO-dependent, furosemide-sensitive transporter (Urazaev *et al.* 1999, Vyskočil 2003). When the NO production is inhibited, e.g. by L-NAME or during the early postdenervation period, the Cl^- transporter starts to transmit Cl^- ions into the muscle fiber and the synaptic zone is depolarized by 7-10 mV (Urazaev *et al.* 1999, Vyskočil 2003). One can suppose that a similar slight depolarization might also develop in the nerve terminal when NO synthase is inhibited. Because the MEPP frequency is potential-dependent and increases with depolarization (Losavio and Muchnik 1997) the more frequent spontaneous release is observed during reduced NO production. The dissipation of the evoked and spontaneous transmitter release control was also found in central glutamatergic synapses (Pan *et al.* 1996) and does not contradict contemporary views on neuromuscular microphysiology (Holohean and Magleby 2009). Physiologically, it is important not to regulate the spontaneous subthreshold release too much, but rather the nerve-evoked quanta. The NO-dependent cross-talk between glutamate, cholinergic and purinergic postsynaptic and presynaptic receptors could take place during intense synaptic activity and thereby ensure the proper neuromuscular transmission.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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