The Journal of Experimental Biology 203, 3595–3602 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2917

MODULATORY EFFECTS OF NITRIC OXIDE ON SYNAPTIC DEPRESSION IN THE CRAYFISH NEUROMUSCULAR SYSTEM

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Accepted 8 September; published on WWW 2 November 2000

Summary

A characteristic physiological property of the neuromuscular junction between giant motor neurones (MoGs) and fast flexor muscles in crayfish is synaptic depression, in which repetitive electrical stimulation of the MoG results in a progressive decrease in excitatory junction potential (EJP) amplitude in flexor muscle fibres. Previous studies have demonstrated that L-arginine (L-Arg) modulates neuromuscular transmission. Since L-Arg is a precursor of nitric oxide (NO), we examined the possibility that NO may be involved in modulating neuromuscular transmission from MoGs to abdominal fast flexor muscles.

The effect of a NO-generating compound, NOC7, was similar to that of L-Arg, reversibly decreasing the EJP amplitude mediated by the MoG. While NOC7 reduced the amplitude of the EJP, it induced no significant change in synaptic depression. In contrast, a scavenger of free radical NO, carboxy-PTIO, and an inhibitor of nitric oxide synthase, L-NAME, reversibly increased the EJP amplitude mediated by MoGs. Synaptic depression mediated by repetitive stimulation of MoGs at 1 Hz was partially blocked by bath application of L-NAME. Bath application of a NO scavenger, a NOS inhibitor and NO-generating compounds had no significant effects on the depolarisation of the muscle fibres evoked by local application of L-glutamate.

The opposing effects on EJP amplitude of NOC7 and of carboxy-PTIO and L-NAME suggest that endogenous NO presynaptically modulates neuromuscular transmission and that it could play a prominent role at nerve terminals in eliciting MoG-mediated synaptic depression in the crayfish *Procambarus clarkii*.

Key words: neuromodulation, synaptic depression, L-arginine–NO pathway, motor neurone, nitric oxide, fast flexor muscle, crustacean, crayfish, *Procambarus clarkii*.

Introduction

Nitric oxide (NO) is thought to modulate neurotransmitter release and has been implicated in synaptic plasticity in the central nervous system of mammals (Schuman and Madison, 1994). It has both a potentiating (Guevara-Guzman et al., 1994) and a depressing (Kilbinger and Wolf, 1994) action on neurotransmitter release in vertebrates. NO is thought to diffuse through the cell membrane of target cells and activate soluble guanylate cyclase, which results in an increase in the level of the second messenger cyclic GMP (cGMP; Bredt and Snyder, 1989). In invertebrates, NO is also thought to act as a neuromodulator. In molluscs, NO mediates procerebral oscillations by modifying patterns of neuronal activity (Gelperin, 1994), while it modulates the synaptic efficacy of cholinergic neuro-neuronal synapses in *Aplysia californica* (Mothet et al., 1996). Nevertheless, little is known of its neuronal function.

Short-term plasticity of transmitter release, such as synaptic depression and facilitation, has been investigated using the crayfish neuromuscular system (e.g. Atwood and Cooper, 1996). One of the major characteristics of the neuromuscular junction of the abdominal postural system of crayfish is a depression of excitatory junctional potentials (EJPs) of the flexor muscles in response to repetitive stimulation of giant motor neurones (MoGs). Non-giant fast flexor motor neurones, however, elicit synaptic facilitation, even though they innervate the same muscle fibres as a MoG (Kennedy and Takeda, 1965). Since the MoG and non-giant fast flexor motor neurones use the same transmitter, L-glutamate (L-Glu) (Aonuma et al., 1998), the different responses of the flexor muscle to stimulation of these motor neurones are likely to be the result of different mechanisms of transmitter release. The MoG may be regulated to decrease transmitter release, thus producing synaptic depression, while the non-giant fast flexor motor neurones may be regulated to increase transmitter release during synaptic facilitation.

Many studies have analysed MoG-mediated synaptic depression in crayfish (e.g. Kennedy and Takeda, 1965; Zucker and Bruner, 1977), and it is thought to be induced by certain

metabolic factors and to be dependent on the availability of Ca^{2+} in the neuromuscular terminals (Czternasty and Bruner, 1980). Cooper et al. (2000) also suggest that synaptic structural complexity is an important factor regulating Ca^{2+}-mediated transmitter release at the neuromuscular synapses of crayfish walking legs. Aonuma et al. (1999) showed that L-Arg inhibited transmitter release from MoGs but that it facilitated transmitter release from non-giant fast flexor motor neurones. L-Arg is known to inhibit synaptic transmission in *Aplysia californica* through the formation of NO (Mothet et al., 1996). Endogenous NO is generated from L-Arg by nitric oxide synthase (NOS) (Moncada et al., 1991), and recent immunocytochemical and NADPH-diaphorase staining studies have revealed the presence of NOS in the central nervous system of both vertebrates (e.g. Bredt and Snyder, 1992; Southam and Garthwaite, 1993) and invertebrates (e.g. Dow et al., 1994). In crayfish, putative NOS-containing cells have been demonstrated in the cerebral ganglion by NADPH-diaphorase staining (Johansson and Carlberg, 1994; Talavera et al., 1995).

In the present study, we have investigated the possible role of NO in neuromuscular transmission between the MoG and the abdominal fast flexor muscles in the crayfish. We show that a NO-generating compound suppresses neuromuscular transmission from the MoG to the flexor muscle fibres, whereas a scavenger of NO and a NOS inhibitor were found to enhance neuromuscular transmission. On the basis of these findings, we conclude that endogenous NO modulates neuromuscular transmission and that it probably plays an important role in the cellular process of synaptic depression at crayfish neuromuscular junctions.

Materials and methods

Animals and preparations

Experiments were performed on adult crayfish *Procambarus clarkii* (Girard) of both sexes with a body length of 7–10 cm from rostrum to telson. They were obtained from a commercial supplier (Sankyo Labo Service, Japan) and kept in laboratory tanks before use. There were no significant differences in results between males and females.

The crayfish neuromuscular junctions between the MoG and the abdominal fast flexor muscle fibres in the fourth abdominal segment were used in all experiments. The abdomen was isolated from a crayfish and pinned ventral side up in a Sylgard-lined chamber filled with cooled crayfish saline (van Harreveld, 1936). The swimmerets of all abdominal segments were removed, and the nerve chain was exposed by removing the sternites, soft cuticle, ventral aorta and connective tissue. The third nerve contains the fast flexor motor neurones. The slow and fast flexor abdominal muscles over the third nerve were gently removed, and experiments were carried out on the anterior oblique flexor muscle, which is innervated by the MoG and also by two non-giant motor neurones and a flexor inhibitor motor neurone (Kennedy and Takeda, 1965; Selverston and Remler, 1972). To reduce twitches of the flexor muscles due to large junctional potentials when bathed in crayfish saline, the Ca^{2+} concentration was lowered to 6.8 mmol l^{-1} (a concentration at which depression still occurred; Aonuma et al., 1999) from 13.5 mmol l^{-1} in normal saline, and the Mg^{2+} concentration was raised to 26 mmol l^{-1} (from 2.6 mmol l^{-1} in normal saline). The osmolarity was maintained by changing the Na^{+} concentration (189 mmol l^{-1} in normal saline). The temperature in most experiments was held at approximately 15 °C.

Intracellular recordings and pharmacological experiments

Intracellular recordings from muscle fibres were made with microelectrodes filled with 2 mol l^{-1} potassium acetate. Electrode resistance was approximately 20 MΩ. Intracellular recordings were usually made from a limited area of fibres at the anterior surface of the muscle. The electrode was mounted on flexible wires suspended from a micromanipulator (Larimer and Kennedy, 1969) in a floating configuration that allowed stable penetrations to be made of fast flexor muscle that twitched during stimulation of the MoG.

Motor neurone stimulation was carried out using a fine suction electrode with a tip diameter of 80–100 μm (external) on the third nerve containing only axons of fast flexor motor neurones (Kennedy and Takeda, 1965). A square pulse of 50 μs duration was delivered to the stimulating electrode. The MoG has the largest axon with the lowest threshold and usually spiked first when the stimulus intensity was increased gradually. Furthermore, the axon diameter of the MoG was large enough to be recognised under a stereoscopic dissecting microscope, and the MoG was readily stimulated selectively by placing the stimulating electrode over the axon. In some experiments, the MoG was stimulated once every 1 min to prevent synaptic depression. Alternatively, repetitive stimulation of the MoG at 1 s intervals (1 Hz) always elicits synaptic depression in the fast flexor muscle.

For bath application of drugs, a perfusion chamber (6 ml in volume) was used. Crayfish saline, with or without drugs, was supplied using a micro-tube pump (Eyela MP-3, Tokyorikakiki, Japan) at a rate of 6 ml min^{-1}. The excitatory junctional potential (EJP) amplitude evoked by electrical stimulation of a MoG axon was measured using a digital storage oscilloscope (TDS340, Tektronix). The initial EJP amplitude was defined as the control level (100 %), and the change in the amplitude of EJPs was recorded every 1 min. Drugs were perfused for 5 or 6 min, then washed out with fresh saline. Data obtained in preparations in which the original response did not recover after washing were excluded from our analyses. The following pharmacological agents were obtained from Dojindo, Japan: 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC7), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), N^{G}-nitro-L-arginine methyl ester (L-NAME), *N*-morpholino sydnomine (SIN-1) and superoxide dismutase (SOD). Just before each experiment, these drugs were dissolved in crayfish saline and made up to the required concentration.

For local application of L-Glu (Sigma) to muscle fibres,

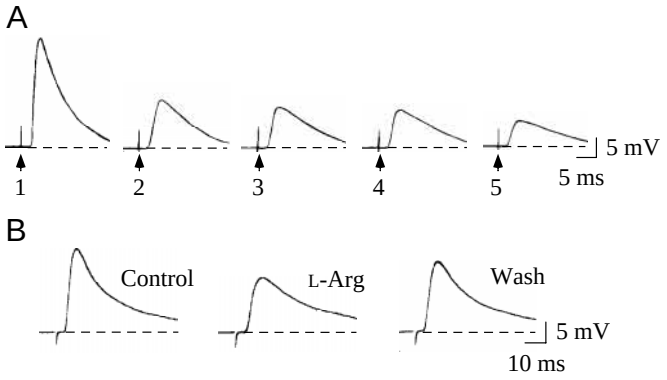

Fig. 1. Responses of abdominal fast flexor muscle fibres to stimulation of the fast flexor motor neurones and the effects of L-arginine (L-Arg) on evoked excitatory junction potentials (EJPs). (A) The response of a muscle fibre to five (1–5) stimulations of the giant motor neurone (MoG). Repeated stimulation at 1 Hz induced synaptic depression in the flexor muscle. (B) Effects of L-Arg on the MoG-evoked response of the flexor muscles. The amplitude of the EJP in a muscle fibre decreased during bath application of 200 μmol l^{-1} L-Arg.

micropipettes with a tip diameter of 15–18 μm (external) were prepared by breaking the tip under a binocular microscope. L-Glu (200 μmol l^{-1}) was applied through the micropipette *via* pressure microinjection into the anterior region of the muscle fibre close to the site of the intracellular recording electrode. Small quantities of L-Glu were ejected from a micropipette using a N_2 gas pressure controller (WPI pneumatic pico pump, PV 820) at 138 Pa for 100 ms. To produce constant-amplitude depolarisation by L-Glu in normal crayfish saline, the ejected drug was quickly washed out with a continuous flow of saline. When the response of the muscle fibres to local application of L-Glu was smaller than 5 mV in amplitude, it was judged that the micropipette tip was positioned too far from synaptic sites, and the micropipette was repositioned.

All recordings were stored on a digital tape recorder (DTR-1801, Biologic) for later analysis and displayed on an analogue storage oscilloscope (R5103N, Tektronix).

Results

Synaptic depression at the neuromuscular junction between the MoG and the abdominal fast flexor muscles is a characteristic feature of repetitive MoG stimulation. Stimulation of a MoG at 1 Hz always induces synaptic depression (Fig. 1A) (Aonuma et al., 1998). Bath application of L-Arg decreased the amplitude of the excitatory junctional potential (EJP) of the fast flexor muscles in response to stimulation of the MoG (Fig. 1B) (Aonuma et al., 1999) at low frequencies of stimulation. Furthermore, 200 μmol l^{-1} L-Arg had no significant effects on the glutamate-evoked depolarisation in the muscles fibres of the abdominal fast flexor muscles (Aonuma et al., 1999). Since L-Arg is the substrate of nitric oxide synthase (NOS), which synthesises NO, we examined the possibility that NO modulates neurotransmission and affects synaptic depression mediated by repetitive stimulation of the MoG.

Effects of exogenous NO on the EJP evoked by the MoG

The effect of NO on neuromuscular transmission was examined by bath application of the NO-generating compound NOC7, which releases NO into the crayfish and increases the level of exogenous NO. Bath application of 200 μmol l^{-1} NOC7 decreased the amplitude of the EJP of the flexor muscle elicited by stimulation of the MoG to approximately 70 % of the initial amplitude without any change in resting potential (Fig. 2A). The inhibitory effect of NOC7 reversed gradually after washing for approximately 10 min (Fig. 2B). Repetitive stimulation of the MoG at 1 Hz elicited synaptic depression. We stimulated the MoG using a series of five stimuli (1 Hz), each series being separated by 1 min without stimulation. The amplitude of the EJP evoked by repetitive stimulation of the MoG decreased to approximately 50 % of that of the first EJP (*N*=5) (Fig. 2C). During bath application of NOC7, the amplitude of EJPs mediated by the MoG was depressed to approximately 70 %, while the degree of synaptic depression was not changed (Fig. 2D, middle traces).

It is well-known that NOC7 generates NO spontaneously. The half-life of NOC7 is 5 min at 25 °C, and no additional function and no toxicity of NOC7 have been reported (Hrabie et al., 1993). In the present study, the temperature of the saline was approximately 15 °C and, since the half-life of NOC7 increases as the solution temperature decreases, the half-life of NOC7 must have been more than 5 min. To confirm that NOC7 itself has no inhibitory effects on neurotransmission, 200 μmol l^{-1} degassed NOC7 was prepared at room temperature (25–27 C) and left for approximately 30 min, then cooled to 15 °C and bath-applied. Although this solution could have contained a small amount of NO, it would have much less than in the freshly prepared NOC7 solutions used in our experiments. Bath application of degassed NOC7 solution had no clear effect on the EJPs elicited by the MoG (*N*=3) (Fig. 2E). The amplitude of EJPs during application of degassed NOC7 was almost the same as when control saline was used.

Effects of a NO scavenger and a NOS inhibitor on the EJPs

To examine whether NO is generated in the neuromuscular system of the crayfish, a NO scavenger and a NOS inhibitor were bath-applied. Carboxy-PTIO acts as a scavenger of newly synthesised free radical NO (Yoshida et al., 1993) so that endogenous NO in the neuromuscular preparation would be removed, at least partially, by bath application of carboxy-PTIO. Before bath application of 200 μmol l^{-1} carboxy-PTIO, the MoG-mediated EJPs of the flexor muscle were consistent in amplitude at an interstimulus interval of 1 min. During bath application of carboxy-PTIO, the amplitude of the EJPs increased gradually without any change in resting potential (Fig. 3A). The peak amplitude of the EJP reached approximately 120 % (*N*=5) of the initial amplitude. The EJP amplitude gradually recovered to its original level after washing for approximately 10 min with fresh saline (Fig. 3B).

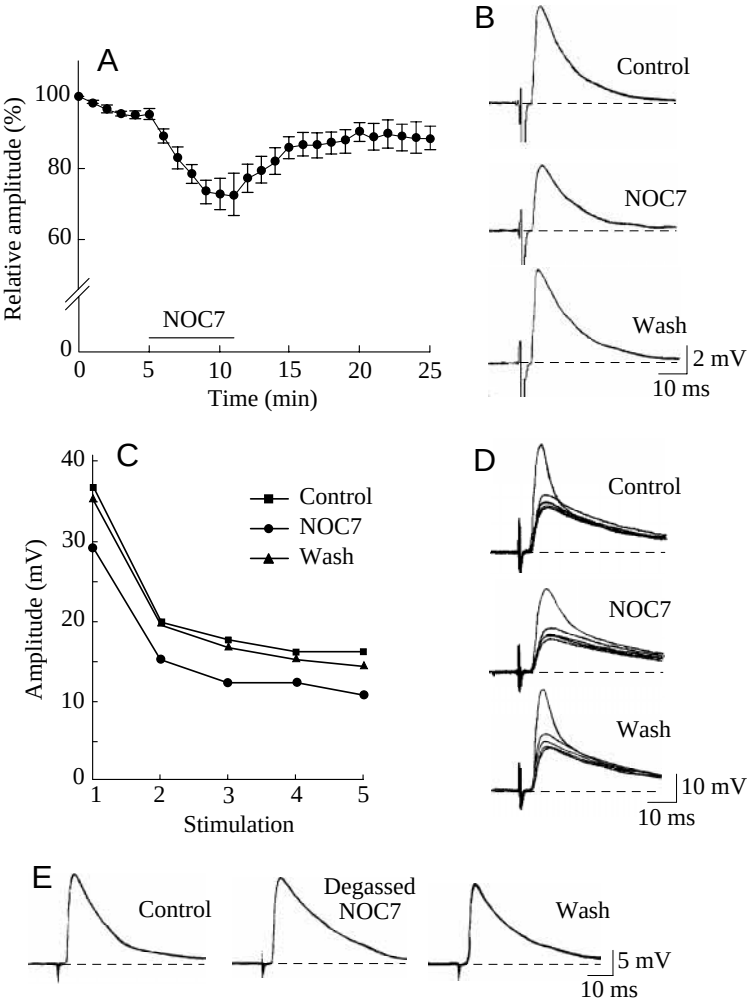

Fig. 2. Effects of the NO-generating compound NOC7 on giant motor neurone (MoG)-mediated excitatory junction potentials (EJPs). (A) Relative change in EJP amplitude of the muscle fibre mediated by stimulation of the MoG plotted at 1 min intervals. The EJP amplitude decreased during bath application of 200 μmol l^{-1} NOC7. Each point represents the mean ± S.E.M. from six different crayfish. (B) The response of a muscle fibre to stimulation of the MoG in saline (top trace). The amplitude of the EJP mediated by stimulation of the MoG decreased during bath application of 200 μmol l^{-1} NOC7 (t=10 min, middle trace). The amplitude had almost recovered after a 10 min wash (t=20 min, bottom trace). (C) The response of the muscle fibre to repeated stimulation of the MoG at 1 Hz with a series of five stimuli (1–5) and with an interseries interval of 1 min. The EJP amplitude is plotted under control conditions, in the presence of NOC7 (t=10 min) and after washing in crayfish saline. (D) The amplitude of the EJP mediated by the MoG decreased and synaptic depression was still observed during bath application of 200 μmol l^{-1} NOC7 (t=10 min; five sweeps are superimposed). (E) The effect of degassed NOC7 on MoG-mediated EJPs. The MoG was stimulated every 1 min. Degassed NOC7 (200 μmol l^{-1}) was bath-applied for 5 min after recording control responses for 5 min, and no significant change in the amplitude of the EJP evoked by stimulation of the MoG was observed (t=10 min, middle trace).

Since endogenous NO is generated by NOS from L-Arg, the response of muscle fibres to MoG stimulation was examined under bath application of the NOS inhibitor L-NAME. Inactivation of NOS by bath application of L-NAME enhanced MoG-mediated EJPs at an interstimulus interval of 1 min. For example, bath application of 10 mmol l^{-1} L-NAME caused no change in the resting potential but caused an increase in the EJP amplitude to approximately 110 % (N=8) compared with the initial amplitude of the EJP (Fig. 4) in response to the first stimulation of the MoG. The amplitude of the EJPs recovered soon after washing.

To examine whether L-NAME itself modulates neurotransmission from the MoG, NOC7 was applied during the inhibition of NOS, since L-NAME may inhibit pathways other than L-Arg–NO pathways. The amplitude of EJPs was depressed by simultaneous application of NOC7 (200 μmol l^{-1}) and L-NAME (10 mmol l^{-1}). The amplitude of the EJPs was 36–37 mV under control saline. Bath application of NOC7 with L-NAME decreased the EJP amplitude to 29–30 mV (Fig. 5). A similar effect was observed in three animals. The effect was reversible, with the amplitude recovering almost to control levels.

To examine further the action of NO on the neuromuscular system, the MoG was repeatedly stimulated during application of L-NAME. When the MoG was stimulated at 1 Hz with an interstimulus interval of 1 min (a series of five stimuli at 1 Hz repeated at 1 min interval), the response of the flexor muscle to the second stimulus was depressed to approximately 55 % of the initial EJP amplitude (N=8) under control saline (Fig. 6A upper traces). The amplitude of the EJPs remained at a decreased level on subsequent stimulation, but recovered almost completely within 1 min. After application of L-NAME, the responses of muscle fibres to repetitive stimulation were enhanced (Fig. 6A second traces). Although the second stimulation of the MoG caused a decrease in EJP amplitude to approximately 65 % of the control level, the EJP amplitude gradually increased upon subsequent stimulation of the MoG (Fig. 6B, filled circles) to reach almost 90 % of the initial level after the fifth stimulus. The effect of L-NAME disappeared soon after washing with normal crayfish saline (Fig. 6A bottom traces), and the flexor muscle then showed a depression in response to repetitive stimulation of the MoG at 1 Hz similar to that in the control saline (filled triangles in Fig. 6B).

NO acts on a presynaptic site

To confirm that the NO-generating compound and the NOS

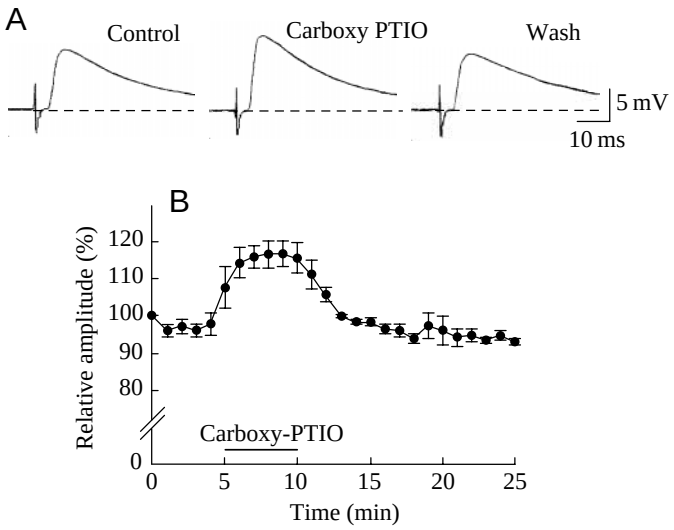

Fig. 3. Effects of the NO scavenger carboxy-PTIO on neuromuscular transmission from the giant motor neurone (MoG) to the fast flexor muscles. (A) The response of the muscle fibre to MoG stimulation. The amplitude of excitatory junction potentials (EJPs) elicited by stimulation of the MoG increased during bath application of 200 μmol l^{-1} carboxy-PTIO (*t*=10 min, middle trace). (B) Relative change in excitatory junction potential (EJP) amplitude of the muscle plotted every 1 min. The amplitude of the EJP increased during bath application of 200 μmol l^{-1} carboxy-PTIO and recovered after a 10 min wash. Each point represents the mean ± S.E.M. of responses from five different crayfish.

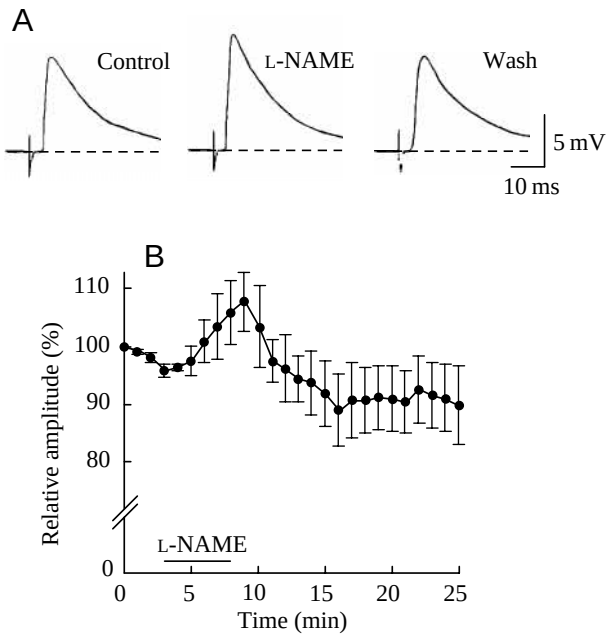

Fig. 4. Effects of the NOS inhibitor L-NAME on neuromuscular transmission from the giant motor neurone (MoG) to the fast flexor muscles. (A) The response of the muscle fibre to MoG stimulation. The amplitude of excitatory junction potentials (EJPs) elicited by stimulation of the MoG increased during bath application of 10 mmol l^{-1} L-NAME (*t*=9 min, middle trace). (B) The relative change in EJP amplitude of the muscle fibre mediated by stimulation of the MoG plotted every 1 min. Each point represents the mean ± S.E.M. of responses from eight different crayfish.

inhibitor modulate transmitter release from motor neurones, the effects of these chemicals on the membrane response of a muscle fibre mediated by L-Glu were examined. Local application of 200 μmol l^{-1} L-Glu for 100 ms at 20 s intervals elicited a relatively constant depolarisation of the flexor muscle fibre (Fig. 7A). Bath application of 200 μmol l^{-1} L-Arg had no significant effect on the L-Glu-induced depolarisation, while 200 μmol l^{-1} L-Arg depressed the amplitude of the EJPs mediated by MoG stimulation (Aonuma et al., 1999). Bath application of the NO scavenger carboxy-PTIO (*N*=3) had no significant effect on the resting potential and L-Glu-induced depolarisation of the flexor muscle fibre (Fig. 7A). Bath application of L-NAME also had no significant effect on the resting potential of the muscle fibres, and L-Glu-induced depolarisation of the muscle fibres was not changed significantly in response to application of L-NAME (*N*=5) (Fig. 7B). Bath application of the NO-generating compound NOC7 had no significant effect on the resting potential of the muscle fibres. L-Glu-induced depolarisation of the flexor muscle fibre did not change significantly during bath application of 200 μmol l^{-1} NOC7 (*N*=4) (Fig. 7C). Another NO-generating compound, SIN-1, releases NO and superoxide anions at the same time. Bath application of SIN-1 with superoxide dismutase (SOD) also increases NO levels in the preparation. Finally, bath application on SIN-1 with (*N*=3) (Fig. 7D) or without (*N*=4) (Fig. 7E) SOD had no significant effect on L-Glu-mediated depolarisation of the muscle fibres.

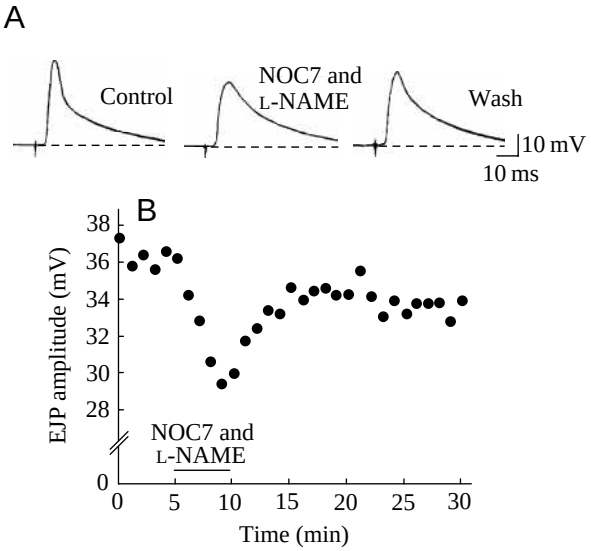

Fig. 5. Effects of exogenous NO during inhibition of nitric oxide synthase (NOS) activity. (A) Bath application of 200 μmol l^{-1} NOC7 with 10 mmol l^{-1} L-NAME depressed the amplitude of excitatory junction potentials (EJPs) mediated by stimulation of the giant motor neurone (MoG) (middle trace). (B) The amplitude of EJPs gradually decreased after bath application of NOC7 with L-NAME. The EJP amplitude gradually recovered after a 10 min wash in crayfish saline. These data are from a single experiment and are representative of three experiments.

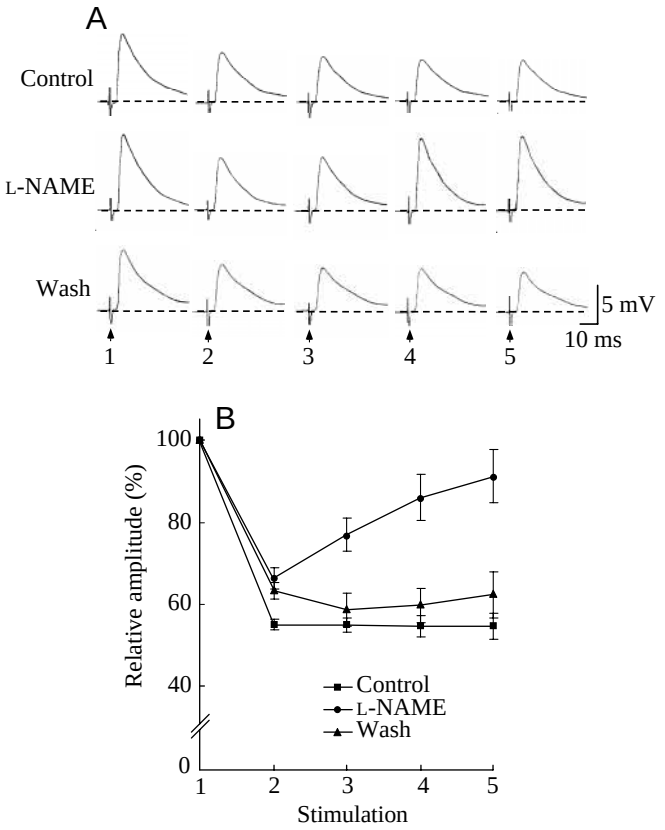

Fig. 6. Effects of L-NAME on the excitatory junction potentials (EJPs) elicited by five stimulations (1–5) of the giant motor neurone (MoG) at 1 Hz. (A) The response of the muscle fibre, showing that the amplitude of the MoG-mediated EJP increased and synaptic depression was blocked during bath application of 10 mmol l^{-1} L-NAME (middle traces). (B) Action of L-NAME on synaptic depression. The EJP amplitude in response to the first stimulation of MoG was defined as 100 %. The relative change in amplitude of the EJPs elicited by subsequent stimulation of the MoG at 1 Hz is plotted. During bath application of L-NAME, the amplitude of the EJPs increased, showing that synaptic depression induced by repetitive stimulation was partially blocked. After a 15 min wash, the amplitude of the EJPs returned to control levels and synaptic depression was apparent. Each point represents the mean ± S.E.M. of responses from eight different crayfish.

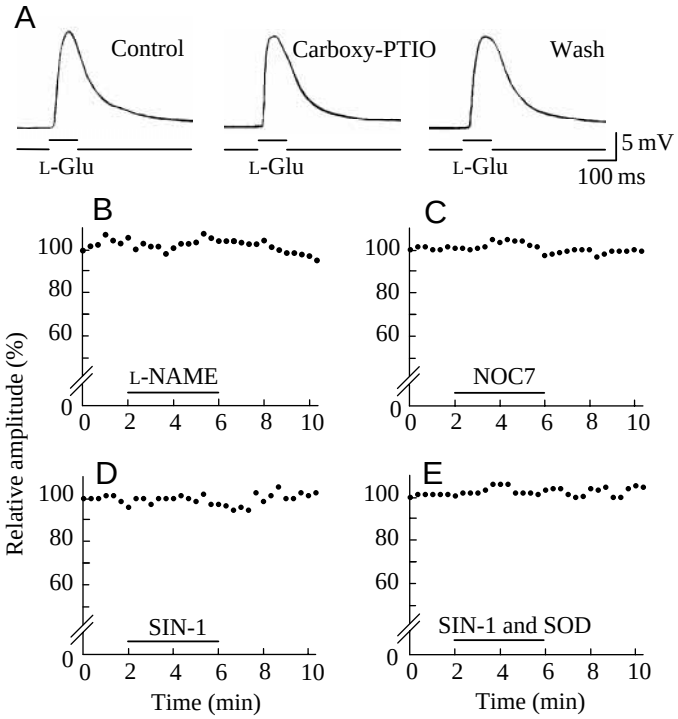

Fig. 7. Effects of NO-generating compounds and a nitric oxide synthase inhibitor on the depolarisation elicited by local application of 200 µmol l^{-1} L-glutamate (L-Glu). 200 µmol l^{-1} carboxy-PTIO (A), 10 mmol l^{-1} L-NAME (B), 200 µmol l^{-1} NOC7 (C), 200 µmol l^{-1} SIN-1 (D) and 200 µmol l^{-1} SIN-1 with superoxide dismutase (SOD) (E) had no significant effects on the L-glutamate-elicited response.

Discussion

Aonuma et al. (1999) showed that L-Arg modulates neuromuscular transmission from both MoGs and non-giant fast flexor motor neurones in different ways. The present investigation was designed to reveal the existence of an L-Arg–NO pathway, and the results strongly suggest that L-Arg, by way of NO generation, can modulate transmitter release from MoGs. Furthermore, decreasing NO activity with a NO scavenger and NOS inhibitor induced excitatory effects on the flexor muscle fibres in response to MoG stimulation. This observation suggests that NO may play a significant role in synaptic depression in the crayfish neuromuscular system.

Modulatory effects of NO on the MoG

Bath application of L-Arg, a precursor of NO, will increase the NO level in the neuromuscular system if there is an L-Arg–NO pathway present. In crayfish, it has been shown that L-Arg decreases the EJP amplitude of the abdominal fast flexor muscle fibres mediated by the MoG (Aonuma et al., 1999). Bath application of the NO-generating compound NOC7 increases NO levels in all animals tested. Like L-Arg, NOC7 decreased the EJP amplitude of the muscle fibres mediated by the MoG. In contrast, bath application of degassed NOC7 had no effect on the EJP amplitude mediated by the MoG. These results suggest that NO modulates synaptic transmission from the MoG. The NO scavenger carboxy-PTIO increased the MoG-mediated EJP amplitude. It did not, however, show clear blocking of synaptic depression, probably because of the low concentrations used. Carboxy-PTIO is a NO scavenger that reversibly removes NO without affecting enzyme activity (Yoshida et al., 1994). The presence of L-NAME also enhances MoG-mediated EJPs in the flexor muscles and blocks synaptic depression mediated by the MoG. L-NAME blocks NO-synthesis and is commonly used for analysing the activity of NOS (Moncada et al., 1991; Gelperin, 1994). The effects of carboxy-PTIO and L-NAME indicate that decreasing the NO levels enhances neuromuscular transmission. Furthermore, simultaneous application of NOC7 and L-NAME decreases the amplitude of MoG-mediated EJPs in the same way

as the application of NOC7 alone. The action of NOC7 was to release exogenous NO in the preparation during inhibition of NOS activity.

The opposing effects of NOC7 and of carboxy-PTIO and L-NAME support the hypothesis that neuromuscular transmission is modulated by the action of NO generated from L-Arg in the neuromuscular system. Furthermore, the finding that carboxy-PTIO, L-NAME and NO-generating compounds had no effects on the muscle fibres suggests that NO must act at a presynaptic site. Since NO is generated by NOS from L-Arg, both NOC7 and L-Arg could elevate the presynaptic NO level that decreases transmitter release from MoGs, while both carboxy-PTIO and L-NAME could reduce the presynaptic NO level that increases transmitter release from MoGs. Some NO may always be present at the synaptic site, therefore, and while increasing NO levels could cause a depression of transmitter release from the MoG, decreases in NO levels would enhance transmitter release.

How can NO regulate transmitter release and contribute to synaptic depression?

NO regulates a variety of physiological processes in the mammalian nervous system as an anterograde transmitter (Bredt and Snyder, 1992) or as a retrograde transmitter (Schuman and Madison, 1994). It is synthesised from L-Arg by the action of NOS which, in turn, is activated by the generation of Ca^{2+}/calmodulin following an influx of Ca^{2+} into neurones (Moncada et al., 1991). These reactions follow the activation of *N*-methyl-D-aspartate (NMDA) receptors (Garthwaite et al., 1989; East and Garthwaite, 1991). One action of NO is to stimulate a soluble guanylate cyclase (Miki et al., 1977) to produce cGMP in target cells (East and Garthwaite, 1991). This NO–cGMP pathway is also found in insect nervous systems (Elphick et al., 1993). NO and cGMP induce transmitter release at glutamatergic neuromuscular junctions in *Drosophila melanogaster* (Wildemann and Bicker, 1999). Since the neurotransmitter of MoGs is L-Glu (Aonuma et al., 1998), it may be possible to activate the NO-synthesis process in muscle fibres. However, crayfish muscle fibres do not possess NMDA receptors (Parnas et al., 1996). At the crayfish neuromuscular synapse, L-Glu affects presynaptic autoreceptors on motor neurones (Parnas et al., 1996; Schramm and Dudel, 1997). These receptors respond to NMDA and are stained by antibodies to mammalian NMDA R1 receptors (Feinstein et al., 1998), indicating that the motor neurones have the ability to generate NO. In crustaceans, putative NOS-containing cells have been found in the crayfish cerebral ganglion using NADPH-diaphorase staining (Johansson and Carlberg, 1994; Talavera et al., 1995). At present, however, no histochemical study has been attempted to detect NOS-containing neurones, or their target cells which must contain soluble guanylate cyclase and cGMP, in the crayfish neuromuscular system.

In crayfish, MoG-mediated synaptic depression seems to be related to a reduction in Ca^{2+} concentration at nerve terminals (Czternasty and Bruner, 1980; Czternasty et al., 1980). The depression of Ca^{2+} influx as a result of the action of NO must decrease the probability of transmitter release. Although, at present, there are few studies demonstrating an action of NO on intracellular Ca^{2+} levels in the invertebrate nervous system. In vertebrate nervous systems, in contrast, a NO-induced rise in cGMP level can decrease intracellular Ca^{2+} levels; cGMP activates a cGMP-dependent protein kinase that is ultimately responsible for muscle relaxation in smooth muscle fibres (Rapoport et al., 1983) by decreasing intracellular Ca^{2+} levels (Rashatwar et al., 1987). Ca^{2+} currents are depressed by NO in ciliary neurones and by cGMP in hippocampal neurones (Doerner and Alger, 1988). It has also been shown that NO stimulates Ca^{2+} uptake from intracellular stores in macrophages *via* cGMP-dependent processes (Randriamanpita et al., 1991). If a similar type of signal transduction occurred in the nerve terminals of the MoG, transmitter release would be depressed by a decrease in intracellular Ca^{2+} levels. Further analyses using either 8-bromo-cGMP as a membrane-permeable cGMP or a specific inhibitor of cGMP phosphodiesterase, to demonstrate an enhancement of the action of synaptic depression, are now necessary to clarify the nature of synaptic depression. To do this, it will be necessary to measure quantal events from single boutons (Dudel, 1981, 1983) and directly assess quantal content to investigate further the action of NO on synaptic depression.

This work was funded in part by a Grant-in-Aid from the Japan Society for Promotion of Science to H.A. and Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to T.N. (08640856) and M.T. (07554071). We are grateful to Dr P. L. Newland for his critical reading of this article.

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