ELSEVIER

European Journal of Pharmacology 316 (1996) 219–222

ejp

Short communication

# Nitroprusside decreases the early post-denervation depolarization of diaphragm muscle fibres of the rat

Albert Kh. Urazaev [a], Nikolay V. Naumenko [a], German I. Poletayev [a], Eugen E. Nikolsky [a], František Vyskočil [b,c,*]

[a] *Medical University, Butlerov Street 49, Kazan 420012, Russian Federation*

[b] *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Praha 4, Czech Republic*

[c] *Department of Animal Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 4, Prague 100 00, Czech Republic*

Received 11 July 1996; revised 2 October 1996; accepted 11 October 1996

**Abstract**

The application of sodium nitroprusside, which degrades to nitric oxide (NO) in solution, inhibits early post-denervation depolarization of isolated rat diaphragm fibres. The observation that 'old' solutions of sodium nitroprusside (that have been allowed to decompose) are without effect and that haemoglobin, oxadiazolo quinoxalinone (ODQ) and methylene blue can antagonize the inhibition normally produced by sodium nitroprusside suggests that the inhibitory effects of sodium nitroprusside on early post-denervation depolarization are mediated by NO and guanylyl cyclase. This is in accord with our recent observations with NO synthase activation and inhibition in the diaphragm.

*Keywords:* Muscle denervation; Nitroprusside; Hemoglobin; ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one); Nitric oxide (NO)

## 1. Introduction

Short-lived molecules of nitric oxide (NO) have a large number of actions in the nervous system, from NMDA receptor-mediated neurotoxicity (Dawson et al., 1991, 1992) to affecting memory processes (Barinaga, 1991). NO modulates synaptic plasticity (Zorumski and Izumi, 1993; Edelman and Gally, 1992) and transmitter secretion (Hirsch et al., 1993) and regulates activity-dependent gene expression in neurones (Peunova and Enikolopov, 1993; Kalb and Agostini, 1993). It has recently been shown that the NO-producing enzyme, NO synthase, is regulated by various processes such as neuronal activity (Matsumoto et al., 1993), neuronal damage (Verge et al., 1992; Wu, 1993; Solodkin et al., 1992) and different agents, e.g., nerve growth factor (Hirsch et al., 1993). Nitric oxide also controls the development of spinal cord motoneurones (Kalb and Agostini, 1993). We have recently demonstrated that NO also participates in the function of adult neuromuscular synapses where it appears to be a retrograde transmitter which is produced in the muscle as a consequence of glutamate and probably also acetylcholine release. Incubation of denervated muscles in the presence of exogenous glutamate, acetylcholine and carbachol induces $Ca^{2+}$-dependent synthesis of NO in the sarcoplasm which in turn slows down the early post-denervation depolarization (Urazaev et al., 1995a,b,1997). In the present experiments we checked the specificity of the NO effect using an exogenous source of NO, sodium nitroprusside, which releases this radical into aqueous solutions (Böhme et al., 1991).

## 2. Materials and methods

Diaphragms were isolated from male Wistar rats, 180–200 g of body weight, under ether anaesthesia. We used 3–4 mm wide strips of parallel intact muscle fibres of the diaphragm with no extramuscular nerve stump. The muscle strips were pinned with glass needles to the silicone rubber bottom of transparent glass dishes, with 12 ml of glutamic acid-free medium No. 199 (Hanks' salts to which 4.0 g/l $NaHCO_3$ was added for stabilizing pH to 7.2–7.4), and were placed in a moist atmosphere of 5% $CO_2$ and 95% $O_2$ at 37°C for 180–200 min. Standard glass microelectrodes (tip resistance 15–20 MΩ, filled with 2.5 M KCl)

* Corresponding author. Tel.: (42-2) 475-2529 or 475-2532; Fax: (42-2) 471-9517; e-mail: vyskocil@sun1.biomed.cas.cz


*PII* S0014-2999(96)00817-5

were used for rapid recording of the resting membrane potential of 25–30 superficial muscle fibres in each strip (within 5–10 min) (Urazaev et al., 1987a). In each group, 3–4 strips of several muscles were used. One-way analysis of variance showed no significant difference in mean resting membrane potential (RMP) between individual tissues and all RMPs from each experimental group were therefore pooled.

Compounds and their sources were as follows: a selective inhibitor of NO-sensitive guanylyl cyclase, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, Tocris Cookson UK, USA), methylene blue (Serva, Germany), human haemoglobin $A_2$, sodium nitroprusside and the rest of the compounds (Sigma, USA). Haemoglobin was reduced with sodium dithionate, dialysed and kept frozen in aliquots at −18°C as described by Martin et al. (1985). 'Fresh' nitroprusside preparation: crystalline sodium nitroprusside was stored under a nitrogen atmosphere in the dark at 4°C and dissolved in the bath 3 min before immersion of the muscle strip. One-week-old sodium nitroprusside (Garry et al., 1994; Lindgren and Laird, 1994) was obtained either by keeping a $1 \times 10^{-2}$ M aqueous solution of sodium nitroprusside under ultraviolet light at 20°C for 7 days (solution A), by keeping a $1 \times 10^{-2}$ M aqueous solution of sodium nitroprusside in the dark at 4°C for 7 days (solution B), or by keeping crystalline sodium nitroprusside under ultraviolet light at 20°C for 7 days and dissolving it in the bath 3 min before the muscle strip was placed therein (solution C).

The SigmaStat program, version 0.1 for Windows (Jandel Corporation 1992–1994) was used for statistical analyses. Parametric analysis of variance (ANOVA) of experimental groups versus control group was made by multiple comparisons using the Bonferroni *t*-test.

## 3. Results

In the muscle strips, the average resting membrane potential measured within 10–15 min after section was −74.5 mV. After 3 h it became depolarized by about 8 mV to −66.6 mV (Table 1). In the presence of $1 \times 10^{-4}$ M fresh sodium nitroprusside for 3 h, the fall in resting membrane potential was substantially reduced (−70.0 mV, Table 1). Sodium nitroprusside therefore effectively reduced the post-denervation depolarization probably due to release of NO during sodium nitroprusside hydrolysis (Böhme et al., 1991).

Since sodium nitroprusside also breaks down into other miscellaneous by-products such as ferricyanide, we evaluated the ability of one-week-old and light-inactivated sodium nitroprusside to change the early post-denervation depolarization. Incubation of muscles with $1 \times 10^{-4}$ M sodium nitroprusside in either solution A, B or C (see Section 2) for 3 h did not prevent the drop of resting membrane potential significantly ($P < 0.05$, Table 1).

To assess whether the sodium nitroprusside-evoked resting membrane potential decrease was due to the generation of NO, new solutions of sodium nitroprusside were applied to muscles that had been pre-exposed to $2 \times 10^{-5}$ M reduced haemoglobin for 5 min. Since haemoglobin binds NO, it should prevent NO from accumulating and exerting its action (Barinaga, 1991; Hu and El-Fakahany, 1993; Meller and Gebhart, 1993). Table 1 shows that haemoglobin completely eliminated the sodium nitroprusside-induced protection of the resting membrane potential drop in muscles concurrently exposed to freshly dissolved sodium nitroprusside.

It is known that the main target of NO action is guanylyl cyclase (Moncada et al., 1991; Nathan, 1992). If NO from sodium nitroprusside acts through guanylyl cyclase, then 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a novel guanylyl cyclase-specific inhibitor (Garthwaite et al., 1995), should eliminate the sodium nitroprusside effect. Table 1 demonstrates that 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one eliminated the sodium nitroprusside-induced protection against resting membrane potential depolarization as effectively as did methylene blue which is considered as an inhibitor of both NO synthase (Meller and Gebhart, 1993) and guanylyl cyclase (Mayer et al., 1993).

1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (not shown), methylene blue and haemoglobin alone at the concentrations used together with sodium nitroprusside had no effect on resting membrane potential depolarization within 3 h.

Table 1
Resting membrane potential (mean in mV ± S.E.M., inside negative) of denervated rat diaphragm fibres with a short distal stump

| | Resting membrane potential (mV) |
|---|---|
| *10–15 min after nerve section* | −74.5 ± 0.4 (120) |
| *After 3 h incubation in a culture medium* | |
| Control | −66.6 ± 0.4 (100) |
| Fresh SNP | −70.0 ± 0.4 (65) |
| Fresh SNP + haemoglobin | −65.0 ± 0.4 (75) |
| Haemoglobin | −65.5 ± 0.3 (80) |
| Fresh SNP + ODQ | −66.3 ± 0.5 (75) |
| Fresh SNP + Methylene blue | −65.9 ± 0.4 (85) |
| Methylene blue | −66.2 ± 0.4 (75) |
| Old SNP/Solution A | −66.4 ± 0.3 (75) |
| Old SNP/Solution B | −67.7 ± 0.4 (80) |
| Old SNP/Solution C | −65.6 ± 0.4 (75) |

SNP, $1 \times 10^{-4}$ M sodium nitroprusside/$2 \times 10^{-5}$ M haemoglobin; ODQ, $1 \times 10^{-7}$ M 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one/$5 \times 10^{-5}$ M methylene blue. Numbers in parentheses indicate the number of muscle fibres recorded. For the composition of fresh and old SNP, solutions A, B and C, see Section 2. One-way analysis of variance showed significant differences in the mean values for the Control group and the Fresh SNP group (in positive direction) and in the Control group and Fresh SNP plus haemoglobin group (small negative change). Other groups vs. Control did not differ with $P < 0.05$.

## 4. Discussion

Recently we observed that the early post-denervation depolarization (Bray et al., 1976) is substantially reduced in denervated rat diaphragms bathed with L-glutamate, *N*-methyl-D-aspartate (Urazaev et al., 1995a), acetylcholine or carbachol (Urazaev et al., 1995b,1997). The effects were not apparent in the presence of nitro-arginine methyl ester (NAME), an inhibitor of NO synthase. This indicates that the NO synthase system might be involved in the regulation of the membrane potential in muscle fibres. Such a function of the NO system is consistent with the evidence that the neuronal isoform of NO synthase is present in rat skeletal muscles (Nakane et al., 1993; Kobzik et al., 1994) and in particular at the neuromuscular junctions, where it might be of neural origin (Oliver et al., 1996). It is known that short-lived NO molecules might function not only as neurotransmitters but also as free radicals passing through cell membranes and thus transferring information between cells (Barinaga, 1991; Bredt and Snyder, 1992). In the present study, the effect of NO extracellularly released from the freshly prepared sodium nitroprusside on early post-denervation depolarization was successfully demonstrated: haemoglobin, which is impermeable and binds NO molecules in the extracellular space, neutralized their action. The early post-denervation depolarization is apparently due to activation of the chloride inward transport (Betz et al., 1986; Urazaev et al., 1987b) which is furosemide-sensitive (unpublished observation). It can be hypothesized that the hyperpolarizing effect of NO formed from sodium nitroprusside in the present experiments, on the early post-denervation depolarization is due to its potency to activate soluble guanylyl cyclase and the production of cGTP which in turn activates specific protein kinase(s). Eventually $Cl^-$ transporter protein may be phosphorylated and modulated.

## Acknowledgements

We thank Dr. P. Hník, Dr. Viktorie Vlachová and Dr. Jan Krůšek for their comments on the manuscript. This research was supported by the Grant Agency of the Czech Academy of Sciences, A 7011502, the Physiological Society, London, Foreign Programme 1995, and INTAS Grant 93-3405.

## References

Barinaga, M., 1991, Is nitric oxide the 'retrograde messenger'? Science 255, 1296.

Betz, W.L., J.R. Caldwell and G.L. Harris, 1986, Effects of denervation on a steady electric current generated at the end-plate region of rat skeletal muscle, J. Physiol. 373, 97.

Böhme, G.A., C. Bon, J.M. Stutzmann, A. Doble and J. Blanchard, 1991, Possible involvement of nitric oxide in long-term potentiation, Eur. J. Pharmacol. 199, 379.

Bray, J.J., M.J. Hawken, J.I. Hubbard, S. Pockett and L. Wilson, 1976, The membrane potential of rat diaphragm muscle fibers and the effect of denervation, J. Physiol. 255, 651.

Bredt, D.S. and S.H. Snyder, 1992, Nitric oxide, a novel neuronal messenger, Neuron 8, 3.

Dawson, V.L., T.M. Dawson, E.D. London, D.S. Bredt and S.H. Snyder, 1991, Nitric oxide mediates glutamate neurotoxicity in primary cortical culture, Proc. Natl. Acad. Sci. USA 88, 6368.

Dawson, T.M., V.L. Dawson and S.H. Snyder, 1992, A novel neuronal messenger molecule in brain: the free radical, nitric oxide, Ann. Neurol. 32, 297.

Edelman, G.M. and J.A. Gally, 1992, Nitric oxide: linking space and time in the brain, Proc. Natl. Acad. Sci. USA 89, 11651.

Garry, M.G., J.D. Richardson and K.M. Hargreaves, 1994, Sodium nitroprusside evokes the release of immunoreactive calcitonin gene related peptide and substance P from dorsal horn slices via nitric oxide-dependent and nitric oxide-independent mechanisms, J. Neurosci. 14, 4329.

Garthwaite, J., E. Southam, C.L. Boulton, E.B. Nielsen, K. Schmidt and B. Mayer, 1995, Potent and selective inhibition of nitric oxide-sensitive quanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, Mol. Pharmacol. 48, 184.

Hirsch, D.B., J.P. Steiner, T.M. Dawson, A. Mamnen, J. Hayek and S.H. Snyder, 1993, Neurotransmitter release regulated by nitric oxide in PC-12 cells and brain synaptosomes, Curr. Biol. 3, 749.

Hu, J. and E.E. El-Fakahany, 1993, The calmodulin antagonist calmidazolium stimulates release of nitric oxide in neuroblastoma NIE-115 cells, NeuroReport 4, 198.

Kalb, R.G. and J. Agostini, 1993, Molecular evidence for nitric oxide-mediated motor neuron development, Neuroscience 57, 1.

Kobzik, L., M.B. Reid, D. Bredt and J.S. Stamler, 1994, Nitric oxide in skeletal muscle, Nature 372, 546.

Lindgren, S.A. and M.W. Laird, 1994, Nitroprusside inhibits neurotransmitter release at the frog neuromuscular junction, NeuroReport 5, 2205.

Martin, W., G.M. Villani, D. Jothianandan and R.F. Furchgott, 1985, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta, J. Pharmacol. Exp. Ther. 232, 708.

Mayer, B., F. Brunner and K. Schmidt, 1993, Inhibition of nitric oxide synthesis by methylene blue, Biochem. Pharmacol. 45, 367.

Matsumoto, T., J.S. Pollock, M. Nakane and U. Forstermann, 1993, Developmental changes of cytosolic and particulate nitric oxide synthase in rat brain, Dev. Brain Res. 73, 199.

Meller, S.T. and G.F. Gebhart, 1993, Nitric oxide (NO) and nociceptive processing in the spinal cord, Pain 52, 127.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology, pharmacology, Pharmacol. Rev. 43, 109.

Nakane, M., H.H. Schmidt, J.S. Pollock, U. Forstermann and F. Murrad, 1993, Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle, FEBS Lett. 316, 175.

Nathan, C., 1992, Nitric oxide as a secretory product of mammalian cells, FASEB J. 6, 3051.

Oliver, L., O. Goureau, Y. Courtois and M. Vigny, 1996, Accumulation of NO synthase (type-1) at the neuromuscular junctions in adult mice, Neuroport 7, 924.

Peunova, N. and G. Enikolopov, 1993, Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells, Nature 364, 450.

Solodkin, A., R.J. Traub and G.F. Gebhart, 1992, Unilateral hindpaw inflammation produces a bilateral increase in NADPH-diaphorase histochemical staining in the rat lumbar spinal cord, Neuroscience 51, 495.

Urazaev, A.Kh., A.V. Čikin, E.M. Volkov, G.I. Poletaev and Kh.S. Khamitov, 1987a, The role of calcium ions and impulse activity in the neurotrophic control of the resting potential in rat muscle fibres, Neurofiziologija 19, 449 (in Russian).

Urazaev, A.Kh., V.A. Surovcev, A.V. Čikin, E.M. Volkov, G.I. Poletaev and Kh.S. Khamitov, 1987b, Neurotrophic control of the chloride transport in muscle fibres of mammals, Neurofiziologija 19, 766 (in Russian).

Urazaev, A.Kh., S.T. Magsumov, G.I. Poletaev, E.E. Nikolski and F. Vyskočil, 1995a, Muscle *N*-methyl-D-aspartate receptors regulate the resting membrane potential through NO-synthase, Physiol. Res. 44, 205.

Urazaev, A.Kh., S.T. Magsumov, G.I. Poletaev, E.E. Nikolski and F. Vyskočil, 1995b, Carbamylcholine prevents the denervation depolarization in muscle fibres by promoting synthesis of nitric oxide, Abstracts of the 9th Symposium on Cholinergic Mechanisms, June 7–10, 1995, Mainz, p. 26.

Urazaev, A.Kh., S.T. Magsumov, G.I. Poletaev, E.E. Nikolski and F. Vyskočil, 1997, Acetylcholine and carbachol prevent muscle depolarization in denervated rat diaphragm, NeuroReport 8, in press.

Verge, V.M.K., Z. Xu, X.J. Xu, Z. Wiesenfeld-Hallin and T. Hockfelt, 1992, Marked increase of nitric oxide synthase in RNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies, Proc. Natl. Acad. Sci. USA 89, 11617.

Wu, W., 1993, Expression of nitric oxide synthase (NOS) in injured CNS neurones as shown by NADPH diaphorase histochemistry, Exp. Neurol. 120, 153.

Zorumski, C.F. and Y. Izumi, 1993, Nitric oxide and hippocampal synaptic plasticity, Biochem. Pharmacol. 46, 777.