Physiol. Res. 47: 291–295, 1998

RAPID COMMUNICATION

Carnosine and Other Imidazole-Containing Compounds Enhance the Postdenervation Depolarization of the Rat Diaphragm Fibres

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Received April 24, 1998
Accepted June 3, 1998

Summary

In the presence of carnosine, anserine, histidine, imidazole and 7-nitro indazole, the early postdenervation depolarization of muscle of about 8 mV was significantly increased by 2.15–4.8 mV. The presence of the imidazole ring in the molecule is apparently necessary for this effect. These compounds also eliminated an NO-mediated protective effect of L-glutamate and carbachol on the depolarization of membrane potential. The presence of imidazole, 7-nitro indazole, carnosine and anserine did not significantly change the effect of an external NO donor, sodium nitroprusside. The structural and functional similarity between imidazole derivatives and the known NO synthase inhibitor, 7-nitro indazole suggests that imidazole, carnosine and anserine might act by inhibiting NO production which is stimulated by glutamate and carbachol.

Key words

Muscle denervation – 7-nitro indazole – Imidazole – Carnosine – Anserine – Carbachol – Glutamate – Nitric oxide

Carnosine is present in relatively high amounts in the skeletal muscles of many vertebrates including man (Imamura 1939, Crush 1970). Carnosine, together with other related histidine containing compounds, have been considered to be potent pH and osmotic buffers (Burton 1983a,b, Hitzig *et al.* 1994), myosin ATPase activators (Parker and Ring 1970) and powerful natural antioxidants (Boldyrev 1994). The levels of carnosine are elevated in muscles of sprinters and rowers (Parkhouse *et al.* 1985). Two related histidine dipeptides, carnosine and anserine (both with the imidazole ring), are known to enhance the neuromuscular transmission at tetanized and curarized muscles (Severin *et al.* 1970). Both dipeptides were localized at the neuromuscular junction (Poletayev 1970). Carnosine is also present in other neuronal structures such as olfactory glutamatergic neurons where it might play a modulatory role (Sassoe-Pognetto *et al.* 1993) or can apparently function as a co-transmitter (Margolis 1974).

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In the present report, we investigated the extent to which the imidazole-containing peptides and imidazole itself can modulate the effects of glutamate and carbachol (Ciani and Edwards 1963) on the early postdenervation depolarization of skeletal muscles. It has been shown previously that denervated muscle fibers of the rat diaphragm kept in a glutamate-free tissue culture medium are depolarized by about 8–10 mV (10–12 %) within three hours after denervation (Bray *et al.* 1976, Urazaev *et al.* 1987a, 1995). This early postdenervation depolarization was effectively prevented by acetylcholine and its non-hydrolysable analogue carbachol (Urazaev *et al.* 1987b, 1997) and by glutamate (Urazaev *et al.* 1995, 1998) which was recently found to be present in motoneurones and in their axonal endings (Meister *et al.* 1993, Waerhaug and Ottersen 1993). These compounds most probably act on early postdenervation depolarization *via* the Ca^{2+}-dependent NO synthase system, because their protective effect vanishes when NO synthase is inhibited or Ca^{2+} entry is prevented (Urazaev *et al.* 1995, 1996, 1997, 1998).

Diaphragms were isolated from male Wistar rats, 180–200 g body weight, under ether anaesthesia. We used 3–4 mm wide strips of parallel intact muscle fibers of the diaphragm with either a long phrenic nerve stump (10–20 mm) or with no extramuscular nerve stump (considered as a short nerve stump preparation). The muscle strips were pinned with glass needles to the silicon rubber bottom of a transparent glass dish filled with 12 ml of glutamic acid-free medium 199 (Sigma Catalogue 1998 No. M 7653, with Hank's salts, without L-glutamine and containing total 22 mM $NaHCO_3$ for stabilizing pH at 7.2–7.4), and placed in a moist atmosphere of 5 % CO_2 and 95 % O_2 at 37 °C for 180–200 min. Imidazole (Reanal, Hungary), carnosine, anserine, 7-nitro indazole, L-glutamate, carbachol and histidine (all from Sigma, USA) were used. "Fresh" sodium nitroprusside (SNP, Sigma, USA), an exogenous source of NO (Böhme *et al.* 1991), was prepared as follows: crystalline SNP 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. Standard glass microelectrodes (tip resistance 15–20 MΩ, filled with 2.5 M KCl) were used for rapid recording of the resting membrane potential (RMP) of 25–30 superficial muscle fibres of each strip within 5–7 min. RMPs from 3–4 strips were compared and all values of RMP were pooled and analyzed.

SigmaStat version for Windows 0.1 (Jandel Corporation 1992-1994) was used for the statistical evaluation. The comparison of the experimental groups with the control group was made by one-way analysis of variation (ANOVA) and Bonferroni t-test.

10–15 min after dissection, the average resting membrane potential (RMP) of the muscles with a long nerve stump was −74.5±0.4 mV (inside negative, mean ± S.E.M, number of fibres n=75 pooled from 4 diaphragms). This value did not change substantially during the following 3 h when it was −74.2±0.5 mV (n=100 from 4 diaphragms). In the muscle strips with the short stump, the average RMP measured within 10–15 min after dissection was −74.6±0.4 mV (n=75). After 3 h the RMP depolarized by about 8 mV to −66.5±0.4 mV (Table 1).

The presence of $1x10^{-4}$ M carnosine, $1x10^{-4}$ M anserine, $1x10^{-4}$ M histidine or $1x10^{-4}$ imidazole for 3 h increased the postdenervation depolarization of RMP significantly by 2.0–4.7 mV. The incubation of muscle strips with $1x10^{-4}$ 7-nitro indazole also augmented the postdenervation depolarization by about 3 mV. A lower concentrations of these drugs ($1x10^{-5}$ M) had no significant effect or their effect was marginal ($P>0.05$). This suggests that the activity of these compounds was probably due to the presence of the imidazole ring in the molecule (cf. Severin *et al.* 1970). Therefore imidazole and 7-nitro indazole were used in the following experiments.

It has been shown that the early postdenervation depolarization is substantially smaller in rat diaphragms bathed with L-glutamate and N-methyl-D-aspartate (Urazaev *et al.* 1995, 1998) and also with acetylcholine and carbachol (CCh) (Urazaev *et al.* 1996) in concentrations mimicking the non-quantal release (Vyskočil *et al.* 1983). The effects of these drugs are mediated by Ca^{2+} entering the muscle fibre and by subsequent activation of NO synthase, the formation of NO from L-arginine and activation the guanylyl cyclase system (Urazaev *et al.* 1995, 1996, 1997, 1998). In the present study, L-glutamate and CCh decreased the postdenervation depolarization ($P>0.001$), the RMPs were −71.3 mV and −72.2 mV, respectively. Imidazole ($1x10^{-5}$) M completely eliminated this effect of glutamate and the RMP dropped again to −65.1 mV. This value was not significantly different ($P>0.05$) from the RMP of fibres bathed for 3 h with $1x10^{-5}$ M imidazole only.

Imidazole also prevented the hyperpolarizing effect of CCh (by 5.6 mV) as documented by the RMP value of which was −65.3 mV in the presence of both drugs, $5x10^{-8}$ M CCh and $1x10^{-5}$ M imidazole. This RMP did not differ ($p>0.05$) from RMP in fibres incubated with $1x10^{-5}$ M imidazole only (−64.3 mV). 7-nitro indazole, a specific blocker of Ca^{2+}-calmodulin-dependent NO synthase (Moore *et al.* 1993, Mulsch *et al.* 1994), acted in a similar way. In muscles bathed with freshly dissolved $1x10^{-4}$ M sodium nitroprusside (SNP), the RMP was depolarized much less than in the controls (−70 mV), resembling glutamate and CCh action (Urazaev *et al.* 1997). The presence of imidazole, 7-nitro indazole, carnosine and anserine did not change the SNP effect in a significant manner ($P>0.05$, Table 1).

Table 1. Resting membrane potential (inside negative) of acutely denervated rat diaphragm fibres with a short distal stump

	Resting membrane potential (mV)
10–15 min after strip dissection	
Control 1 (no drugs)	−74.5±0.5 (120)
Carnosine $1x10^{-4}$ M for 10–15 min	−74.2±0.6 (120)
7-nitro indazole $1x10^{-4}$ M for 10–15 min	−73.7±0.7 (100)
After 3 h incubation in a culture medium	
Control 2 (no drugs)	−66.5±0.4 (100)
Carnosine $1x10^{-5}$M	−64.5±0.5 (75)
Carnosine $1x10^{-4}$ M**	−61.8±0.4 (75)
Anserine $1x10^{-5}$ M**	−63.9±0.6 (75)
Anserine $1x10^{-4}$ M**	−63.2±0.4 (75)
Histidine $1x10^{-5}$ M	−66.9±0.6 (75)
Histidine $1x10^{-4}$ M**	−63.5±0.4 (75)
Imidazole $1x10^{-5}$ M**	−64.3±0.4 (75)
Imidazole $1x10^{-4}$ M**	−62.6±0.4 (75)
7–nitro indazole $1x10^{-5}$ M	−67.1±0.4 (60)
7–nitro indazole $1x10^{-4}$ M**	−63.1±0.4 (100)
Glutamate $1x10^{-3}$ M*	−71.3±0.3 (75)
Glutamate $1x10^{-3}$ M + Imidazole $1x10^{-5}$ M	−65.1±0.3 (75)
Glutamate $1x10^{-3}$ M + 7-nitro indazole $1x10^{-5}$M	−64.9±0.3 (60)
Carbachol $5x10^{-8}$ M*	−72.2±0.4 (150)
Carbachol $5x10^{-8}$ M + Imidazole $1x10^{-5}$ M	−65.3±0.4 (75)
Carbachol $5x10^{-8}$ M + 7-nitro indazole $1x10^{-5}$ M	−67.1±0.3 (60)
SNP $1x10^{-4}$ M*	−70.0±0.4 (75)
SNP $1x10^{-4}$ M + 7-nitro indazole $1x10^{-4}$ M*	−70.9±0.4 (60)
SNP $1x10^{-4}$ M + Imidazole $1x10^{-4}$ M*	−68.9±0.6 (75)
SNP $1x10^{-4}$ M + Carnosine $1x10^{-4}$ M*	−71.0±0.5 (75)
SNP $1x10^{-4}$ M + Anserine $1x10^{-4}$ M*	−70.2±0.4 (75)

Mean values ± S.E.M. are given. SNP = fresh sodium nitroprusside. Numbers in parentheses indicate the number of muscle fibres recorded. One-way analysis of variance showed significant decrease of the early posdenervation depolarization (single asterisk) and significant increase (double asterisk) of the early postdenervation depolarization versus Control 2 group. Other groups did not differ from particular controls at $P<0.05$ level.

Carnosine (*N*-β-alanyl-L-histidine), which is composed of two amino acids and anserine (*N*-β-alanyl-3-methyl-L-histidine), is the methylated form of carnosine. The effects of carnosine and anserine are apparently determined by the imidazole ring in the histidine residue. The molecule of another compound, 7-nitro indazole, is composed of the phenyl and imidazolyl rings. In the imidazole molecule, there are two nitrogen atoms in position 2 and 4, whereas in 7-nitro indazole, the nitrogens of imidazole occupy position 3 and 4. 7-nitro indazole is a specific blocker of Ca^{2+}-calmodulin-dependent NO synthase in neurones (Moore *et al.* 1993, Mulsch *et al.* 1994). NO synthase catalyses the production of NO molecules

during the action of L-glutamate on neurones (Bredt and Snyder, 1989) and on diaphragm muscle fibres (Urazaev *et al.* 1995, 1998). It is therefore possible that at the neuromuscular junction, the carnosine and anserine might also act on the NO synthase as they contain an imidazole group.

The structural similarity between imidazole and the NO synthase inhibitor 7-nitro indazole (Moore *et al.* 1993, Mulsch *et al.* 1994) and the findings that glutamate and CCh regulate muscle postdenervation depolarization through NO production points to the possibility that imidazole, carnosine and anserine also inhibit NO production and hence they decrease – as 7-nitro indazole does – the hyperpolarizing effect of glutamate and CCh. If this is true, then the exogenous source of NO, sodium nitroprusside, (Böhme *et al.* 1991, Urazaev *et al.* 1996) could mimick the effect of glutamate and CCh in lowering the postdenervation depolarization and imidazole compounds would not prevent it. The experiments with SNP and 7-nitro indazole, carnosine, anserine and imidazole support this idea. Noteworthy, average resting membrane potential of the muscles either with a long nerve stump (data not given) or short nerve stump did not change significantly during the short-lasting (10–15 min) presence of any studied drugs (data for 7-nitro indazole and carnosine are in Table 1). This almost excludes their direct receptor or channel action on the muscle fibre membrane.

It can be concluded that imidazole and imidazole-containing compounds interfere with glutamate- and CCh-depolarization preventing effect as 7-nitro indazole does, apparently by inhibiting the skeletal muscle NO synthase. Because carnosine and anserine are released from the nerve during neuromuscular transmission (Poletayev 1970), one can speculate that they may have a local balancing function on muscle NO synthase during liberation of neurotransmitters at the endplate and play a role in the trophic dialogue between the nerve and muscle, besides their antioxidant function as suggested for brain and muscles (Kohen *et al.* 1988).

Acknowledgments

The authors are grateful to Dr. Charles Edwards and Dr. Pavel Hník for their comments on the manuscript and Mrs. Miloslava Kuldová for technical assistance. Supported by EU Grant "Nesting" 1997-99, VS-97099 and The Physiological Society. Cooperation was enabled by Russian Foundation for Basic Research (Grant 96-15-98120 for A.K.U. and N.V.N.) and Grants of President and Government of the Russian Federation (for N.V.N.)

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