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NITRIC OXIDE SELECTIVELY ENHANCES cAMP LEVELS AND ELECTRICAL COUPLING BETWEEN IDENTIFIED RPaD2/VD1 NEURONS IN THE CNS OF *LYMNAEA STAGNALIS* (L.)*

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The isolated CNS of the freshwater mollusc *Lymnaea stagnalis* was used as a model to study the role of cAMP in NO-mediated mechanisms. The NO donor, DEA/NO (10^{-5} – 10^{-3} M) increased cAMP concentrations in the cerebral, pedal, pleural, parietal and visceral ganglia. In contrast, in the buccal ganglia the same doses of DEA/NO decreased the level of cAMP production. The NOS inhibitor, L-NNA (10^{-4} M) increased cAMP concentrations in all areas of the CNS. L-arginine (1 mM), a metabolic precursor of NO, mimicked the action of the NO-donor. The coefficient of electrical coupling between two viscero-parietal peptidergic neurons (VD1/RPaD2) was enhanced by both DEA/NO (10^{-4} M) and 8-Br-cAMP (10^{-4} M) whereas 8-Br-cGMP (2×10^{-4} M) reduced the coupling. We suggest that cAMP-dependent mechanisms are involved in neuronal NO signaling in this simpler nervous system.

Keywords: Nitric oxide – cAMP – electrical synapses – *Lymnaea* – *Aplysia* – molluscs

INTRODUCTION

The gaseous radical nitric oxide (NO) is a versatile neuromodulatory molecule widely distributed across the animal kingdom [2, 6, 12, 16]. In the CNS of the freshwater pulmonate snail, *Lymnaea stagnalis* the presence of NO synthase (NOS) activity was demonstrated in central nervous and peripheral tissues [3, 8, 11]. Putative nitrergic neurons are mainly located in the buccal ganglia [9]; there are a relatively small number of these cells in the central ganglionic ring (i.e. in the cerebral, pedal, pleural, parietal and visceral ganglia). In *Lymnaea,* NO activates buccal motor patterns [11] and is considered a mediator of chemosensory inputs to the feeding network [4]. Although in this and other grazer gastropod molluscs *(Helix* and *Aplysia),* NO acts via cGMP-dependent pathways [4, 5, 7], cAMP-dependent mechanisms can be also involved. For example, in the predatory sea-slug *Pleurobranchaea,* NO enhanced the cAMP gated sodium currents [14, 15]. This response might be mediated by the rise of intracellular cAMP.

*Dedicated to Professor János Salánki for his 70th birthday.

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Here, we compare the action of NO-donors on endogenous levels of cAMP in two parts of the *Lymnaea*'s CNS: the buccal ganglia and the central neuronal ring. Additionally, we use a pair of multifunctional peptidergic neurons (VD1/RPaD2) to investigate the effects of NO-donors and a cAMP analog on the electrical coupling between these cells. We have shown that NO indeed increases cAMP production in selected central ganglia and, therefore, cAMP-dependent mechanisms can be involved in NO signaling in the molluscan nervous system.

MATERIAL AND METHODS

Specimens of *Lymnaea stagnalis* (2–4 g) were collected locally, kept for up to 4 weeks in tapwater at 14–16 °C and feed on the lettuce. Freshly isolated CNSs were used in both biochemical and electrophysiological tests.

NO donors [diethylamine/nitric oxide sodium complex (DEA/NO), S-Nitroso-N-acetylpenicillamine (SNAP), L-arginine], and a NOS inhibitor, N^G-Nitro-L-arginine (L-NNA) – all from RBI, were prepared immediately before use in HEPES buffered saline for *Lymnaea* (in mM: NaCl – 44, KCl – 2, $MgCl_2$ – 2, $CaCl_2$ – 4, Hepes – 10, pH = 7.8)

Endogenous cAMP levels were determined by the standard radioimmune assay technique [13]. In each experiment ten pairs of the buccal ganglia or three central ganglionic rings were incubated for 20 minutes either in 0.2 ml of control solution or in the presence of the drug tested.

Intracellular recording was performed by a conventional microelectrode technique. Glass microelectrodes (10–40 MOm) were filled with 2.5 M KCl. Central neurons were identified according to their location, size, color and electrophysiological characteristics (see the map in [1]). The coupling coefficient (CC) was measured as $CC = \Delta V_{RPaD2}/\Delta V_{VD1}$, where ΔV_{RPaD2} and ΔV_{VD1} are changes in the membrane potential of RPaD2 and VD1, respectively, and the hyperpolarizing current (0.5 nA) was injected in VD1. During the experiments the neurons were slightly hyperpolarized to prevent spontaneous spiking activity. All drugs tested were added into the experimental chamber via a perfusion system, and all concentrations indicated in the text are the final concentrations.

RESULTS

Effect of NO on cAMP production

The summary data are presented in Table 1 and Fig. 1. Incubation of the central ganglia in the presence of the NO donor, DEA/NO induced a significant increase of the endogenous cAMP in the central ganglionic ring. A precursor of NO synthesis, L-arginine, mimicked the action of the DEA/NO. It seems that even small concentrations of DEA/NO (10^{-5} M) resulted in a nearly maximal effect and higher concen-

trations of the NO donor (10^{-3} M) did not produce any further rise in the cAMP levels (Fig. 1A). In contrast, in the buccal ganglia the same small doses of DEA/NO (10^{-5} M) induced minor changes, but higher doses of the donor (10^{-3} M) markedly reduced the cAMP production (Fig. 1B).

NOS inhibitor, L-NNA (10^{-4} M) increased cAMP levels in all parts of the CNS (Table 1).

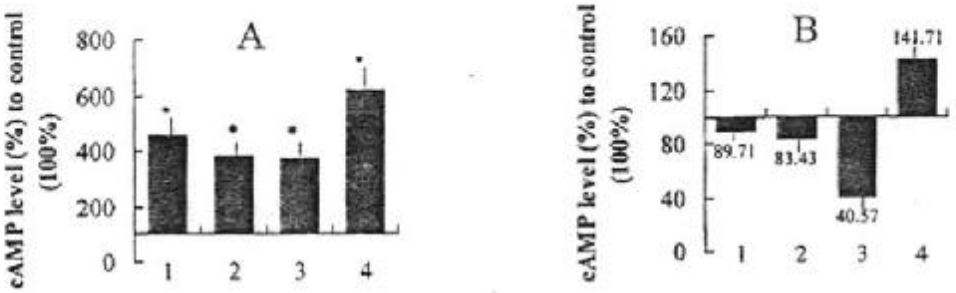

Fig. 1. The effects of NO donors and NOS inhibitors on cAMP concentrations in the different parts of the *Lymnaea*'s CNS. A) Central ganglionic ring (n = 4); B) Buccal ganglia (n = 4). *P < 0.05. 1 – L-arginine (1 mM); 2 – DEA/NO (10^{-5} M); 3 – DEA/NO (10^{-3} M); 4 – L-NNA (10^{-4} M)

Table I

The effects of NO donors and inhibitors of NO synthase on cAMP levels in the different parts of the CNS of *Lymnaea stagnalis*

	cAMP concentration in the central ganglionic ring (fmol/ml)	cAMP concentration in the buccal ganglia (fmol/ml)
Control (0.2 ml HBS)	64.0 ± 14.46	17.5 ± 2.50
L-arginine (10^{-3} M)	292.0 ± 78.48*	15.7 ± 1.70*
DEA/NO (10^{-5} M)	246.7 ± 91.97*	14.6 ± 0.83*
DEA/NO (10^{-3} M)	237.7 ± 56.51*	7.1 ± 1.35*
L-NNA (10^{-4} M)	395.7 ± 94.09*	24.8 ± 1.42*

Data present mean values SEM. *P < 0.05 vs control (Student's *t*-test)

Effect of NO and cyclic nucleotide analogs on the identified electrical synapse

Both DEA/NO (Fig. 2) and SNAP at 10^{-4} M increased the electrical coupling coefficient between VD1 and RPaD2 by about 20%. L-arginine (10 mM) induced no detectable changes in the coupling coefficient (n = 3), whereas 10^{-4} M of 8-Br-cAMP enhanced the electrical coupling between these two neurons by 31.6% (n = 4). These concentrations of the cAMP analog also caused a steady-state hyperpolarization of

VD1 (13.2 ± 2.6 mV, n = 4) and RPaD2 (8.3 ± 1.8 mV, n = 4). Interestingly, the cGMP analog, 8-Br-cGMP, at a similar concentration range (2×10^{-4} M) reduced the coefficient of electrical coupling by 31.1% (n = 5), and strongly depolarized both VD1 (43.2 ± 5.6 mV, n = 4) and RPaD2 (10.7 ± 2.8 mV, n = 4) neurons. All effects were reversible after 15 minutes of washing in the control Ringer solution.

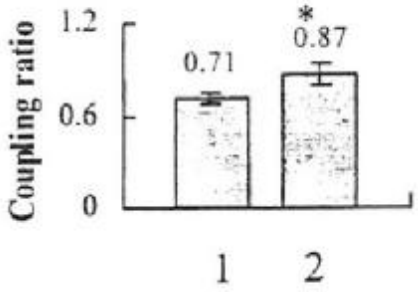

Fig. 2. Effects of NO-donors on the coupling ratio between VD1/RPaD2. 1 – control; 2 – DEA/NO (10^{-4} M). Data present mean values SEM. *P < 0.05 vs control (Student's *t*-test), n = 5

DISCUSSION

Although preliminary observations indicate the involvement of cAMP-dependent pathways in neural NO signaling, the detailed mechanisms are unknown. The suppression of cAMP production in the buccal ganglia in the presence of NO donors is correlated to the very opposite changes in cAMP levels in the rest of the CNS. The NO-induced enhancement of cAMP levels in the major central ganglia of *Lymnaea* is an unusual phenomenon requiring additional studies. On the other hand, both NO and the cAMP analog acted alike, increasing the coefficient of electrical coupling between visceral (VD1) and parietal (RPaD2) neurons. In contrast, at a similar concentration 8-Br-cGMP reduced the coupling, suggesting that the action of NO was not cGMP-dependent and can be connected to the increase of intracellular cAMP.

Elphick et al. (1995) [4] suggested that in the buccal ganglia of *Lymnaea* NO acts by enhancement of cGMP synthesis. Thus, in this system the decrease of the cAMP levels might reflect the reciprocal relationships found between cAMP and cGMP-dependent pathways. Moreover, in the buccal ganglia (but not in the central ganglionic ring) the effect of the NOS inhibitor was opposite to those induced by NO donors, suggesting that the L-NNA can suppress tonic enzymatic NO production. However, in the ganglionic ring L-NNA has an action similar to that described for NO. One possible explanation can be a potential non-enzymatic release of NO from this L-arginine analog under certain reduced conditions [10]. Since cAMP production in the ganglionic ring can be enhanced by relatively low concentrations of NO, even a small non-specific release of NO from L-NNA may contribute to the rise of the intraganglionic cAMP.

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