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NITRIC OXIDE REGULATES THE LEVELS OF cGMP ACCUMULATION IN THE CRICKET BRAIN*

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Cricket brains were incubated in a saline containing nitric oxide (NO)-donor and phosphodiesterase inhibitor IBMX, which could activate soluble guanylate cyclase (sGC) to increase cGMP levels in the targets of NO. The increase of cGMP was detected by immunohistochemistry and enzyme linked immunosorbent assay. NO-induced cGMP immunohistochemistry revealed that many cell bodies of cricket brain showed cGMP immunoreactivity when preparations were treated with a saline containing 10 mM NO-donor SNP and phosphodiesterase inhibitor IBMX, but only a few cell bodies showed immunoreactivity when preparations were incubated without NO-donor. The concentration of cGMP in cricket brains were then measured by using cGMP-specific enzyme linked immunosorbent assay. Cricket brains were treated with a saline containing 1 μM of NO-donor NOR3 and 1 mM IBMX. The cGMP levels in the brain were increased about 75% compared to control preparations that was treated with a cricket saline containing IBMX. The level of cGMP decreased about 40% when preparations were incubated NOR3 saline containing sGC inhibitor ODQ. These results indicate that NO activates sGC and increases the levels of cGMP in particular neurons of the cricket brain and that the level of cGMP would be kept a particular level, which might regulate synaptic efficacy in the neurotransmission.

Keywords: Insect brain – cricket – soluble guanylate cyclase – nitric oxide – cGMP

INTRODUCTION

Nitric oxide (NO) is unconventional molecule in the nervous systems, which propagates signals three-dimensionally to the target cells by diffusing from NO generating cells. It is thought to modulate neurotransmitter release and has been implicated in synaptic plasticity in the central nervous system in vertebrate [1]. Nitric oxide signal is also important factor in invertebrate nervous systems to regulate a variety of neuronal events. In mollusks, NO is released from procerebral lobe [2] and it modifies procerebral oscillation pattern of neuronal activities [3]. In crustacean, NO modu-

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lates synaptic efficacy in the central nervous system [4, 5]. It also regulates synaptic depression that is the principal component to elicit escape behavior in crayfish [6].

Endogenous NO is generated from L-arginine by the activation of NO synthase (NOS) [7], and it diffuses through the cell membrane of target cells to activate soluble guanylate cyclase (sGC), which results in an increase in the level of second messenger, cyclic guanosine 3',5'-monophosphate (cGMP) [8]. NO-induced anti-cGMP immunohistochemistry has been shown to reveal target cells of NO in the nervous systems [9, 10, 11]. Nevertheless, there is little known of the level of NO induced cGMP accumulation in the central nervous systems of arthropod animals. In this study, we have investigated the accumulated levels of cGMP that is generated by the activation of sGC in the cricket brain by using cGMP-specific enzyme linked immunosorbent assay.

MATERIAL AND METHODS

Animals

Crickets (*Gryllus bimaculatus* DeGeer) were reared in plastic cases (80 × 45 × 20 cm) on a 14 h : 10 h light and dark cycle at 27 °C. They were fed a diet of insect food pellet, chopped carrot and water. Freshly molted or up to 2 weeks old male crickets were used in this study.

Cyclic GMP-like immunohistochemistry

The detail of staining methods with whole-mount preparation was previously described [11]. In short, cricket brain was dissected out in the cold cricket saline and preincubated in the saline containing 1 mM 3-isobutyl-1-methyxanthine (IBMX, Sigma) for 30 min to block endogenous phosphodiesterase activity. To stimulate NO-sensitive sGC, the preparations were then exposed to NO donor SNP in the saline containing IBMX for 15 min. Incubation in the NO donor was followed by fixation in 4% paraformaldehyde. For control, preparations were incubated in saline containing IBMX without stimulating sGC, before fixing. The preparations were washed in phosphate buffer saline containing 0.2% Triton X-100 (pH 7.4, PBSTX). They were preincubated with 5% normal donkey serum in PBSTX and then incubated in the anti-cGMP antibody (1 : 20000, gift from Dr. de Vent) for 3 days at 4 °C. They were washed in PBST and incubated for 2–3 h in horseradish peroxidase-conjugated secondary anti-sheep IgG antibody (1 : 500; Jackson ImmunoResearch Laboratories). They were washed in PBSTX, and antibody binding was made visible with diaminobenzidine (Wako, Japan).

Enzyme linked immunosorbent assay

An enzyme linked immunosorbent assay (ELISA) was performed to detect the accumulated levels of NO-induced cGMP in the cricket brain. Cricket brain was dissected out in the cold saline and the ganglionic sheath was removed with sharpened forceps. Optic lobe and subesophageal ganglion were also removed. Tissue was preincubated in 1 mM IBMX in the saline for 30 min at 4 °C. NO-sensitive sGC in the brain was then stimulated by NO-donor NOR3 (Dojindo, Japan) in the saline containing 1 mM IBMX at 25 °C for 15 min. For control, NOR3 was omitted from the saline. The agent, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Sigma), is an inhibitor of NO-sensitive sGC. The saline containing NOR3, IBMX and ODQ was tested to determine general levels of cGMP that might be independent from the activity of NO-sensitive sGC. Preparations were homogenized with 100 µl of the saline containing IBMX and supernatants were collected into micro tube after homogenate were centrifuged at 15,000 rpm for 10 minutes. The enzyme linked immunosorbent assay for the quantitative determination of cGMP was then performed using cGMP fluorescent assay kits (Molecular Devices Co.).

RESULTS AND DISCUSSION

The present investigation was designed to determine the accumulated level of cGMP generated by activation of NO-related sGC. In insects, putative NOS containing cells have been found in brains by NADPH-diaphorase staining [12]. Nitric oxide diffuses three-dimensionally through the membranes of target cells [13] and activates sGC, which results in an increase in the level of the second messenger cGMP [14]. Nitric oxide regulates a variety of physiological processes in vertebrate nervous systems as an anterograde transmitter [15] or as a retrograde transmitter [1]. In invertebrate, NO signaling plays important role in the peripheral and central nervous systems to regulate synaptic transmission [2, 4, 5, 6].

As the first step, we applied immunohistochemistry using anti-cGMP serum to detect the target cells of NO in the cricket brain. Nitric oxide-induced anti-cGMP immunohistochemistry revealed that cell clusters located at distal part of the protocerebrum area and antennal lobe showed strong immunoreactivity (Fig. 1). Some of local interneurons and projection neurons that cell bodies were located around antennal lobe seemed to be stained density and these neurons could be the targets of NO. These results support that NO signaling would regulate olfactory processing in insects and somehow regulate neuronal plasticity that is basis of learning and memory. In insects, olfactory learning and memory could be mediated by NO-cGMP signaling [16; Aonuma et al., in preparation].

Much of experiments have demonstrated the importance of NO during signal processing in the nervous systems [4, 5, 6]. However, we did not know much of the accumulated level of NO-induced cGMP in the insect brain. As the second step, we applied ELISA method to measure the level of accumulated cGMP that was

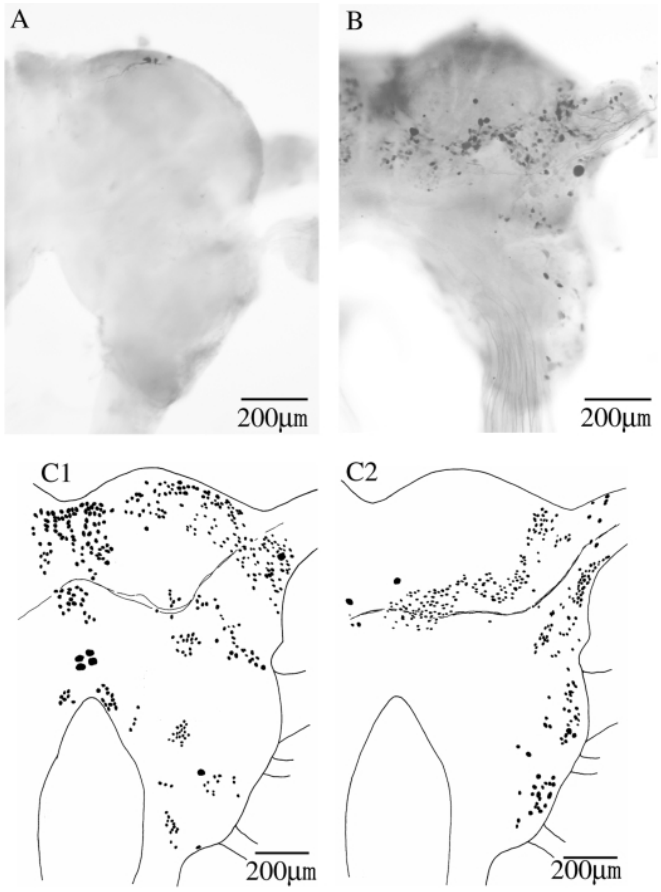

Fig. 1. Distribution of NO-induced cGMP immunoreactive neurons in the cricket brain. **A:** NO-induced anti-cGMP immunohistochemistry without stimulating sGC. Several cell bodies and their neuronal branches were stained at anterior part of the brain. **B:** NO-induced cGMP immunoreactive neurons in the cricket brain. Immunoreactive cell bodies located protocerebrum and deutocerebrum area. Several fibers in protocerebrum area and connective between brain and subesophageal ganglion showed strong cGMP immunoreactive signals. **C:** Mapping of typical distribution of cGMP immunoreactive cell bodies drawn with camera *lucida.* Frontal view of the stained brain (C1) and distal view (C2) were drawn in the same preparation

increased by stimulation of sGC using NO-donor NOR3. One μM NOR3 increased the level of cGMP about 75%, although 0.1 μM NOR3 increased about 50% (Fig. 2). The level of cGMP, on the other hand, was decreased about 40% when preparations were incubated with NOR3 saline containing sGC inhibitor ODQ at 100 μM. This indicated that NO could be always released to keep particular levels of cGMP in the insect brain and that increase or decrease in cGMP levels must be important factor during signal processing in the central nervous systems, which could regulate synap-

tic plasticity. Physiological experiments indeed demonstrated similar results. The effects of NO substrate, NO-donor and cGMP analog on the neurotransmission in crayfish were opposite effects of NOS inhibitor, NO scavenger and sGC inhibitor [4, 5, 6, 17]. Now we need further investigation to reveal accumulated levels of NO-induced cGMP in the insect brain during olfactory processing, olfactory learning and memory and so on. This must help us to understand principle role of NO in insect nervous systems.

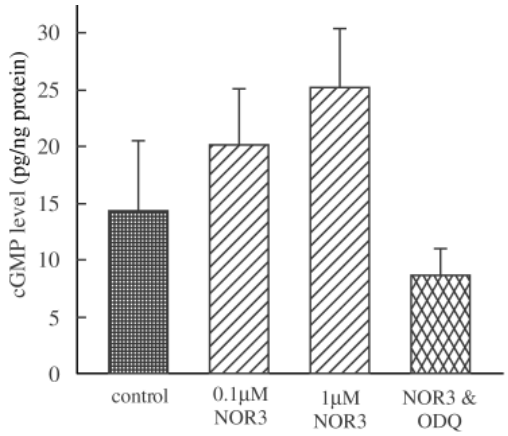

Fig. 2. The accumulation of NO-induced cGMP in the cricket brain measured by ELISA method. The level of cGMP in the cricket brain was about 15 pg/ng protein when preparations were treated with 1 mM IBMX. Cyclic GMP level in the brain increased about 50% and 75% when preparations were treated with 0.1 μM and 1 μM NO-donor NOR3 saline containing IBMX, respectively, but decreased about 40% when they were treated with NOR3 saline containing 100 μM sGC inhibitor ODQ

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