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Distribution of NO-Induced cGMP-Like Immunoreactive Neurones in the Abdominal Nervous System of the Crayfish, *Procambarus clarkii*

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ABSTRACT—Nitric oxide (NO) acts as a signalling molecule by activating soluble guanylate cyclase and causing accumulation of the second messenger cyclic guanosine 3',5'-monophosphate (cGMP) in target cells. In order to detect the presence of NO-cGMP signalling pathway in the crayfish abdominal nervous system, accumulation of NO-induced cGMP was investigated by anti-cGMP immunochemistry. Some preparations were incubated in a high-K^+ saline containing an inhibitor of cGMP-degrading phosphodiesterase, 3-isobutyl-1-methyxanthine (IBMX), to activate NO generating neurones, which could release NO in the ganglion, and then immunohistochemistry using an anti-cGMP antibody was performed. The other preparations were incubated in NO donor, sodium nitroprusside (SNP) saline containing IBMX before anti-cGMP immunohistochemistry was performed. The distribution of cGMP-like immunoreactive neurones in high-K^+ treated preparations was similar to that of cGMP-like immunoreactive neurones in NO donor treated preparations. About 70-80 cell bodies and many neuronal branches in the neuropilar area of the ganglion were stained, although no neurones showed immunoreactivity unless preparations were activated by either high-K^+ or the NO donor. Some of them were identical neurones, and they were intersegmental ascending interneurones and motor neurones. Sensory afferents that innervates hind gut showed strong cGMP-like immunoreactivity, although no mechanosensory afferents showed any immunoreactivity. These results strongly suggest the presence of an NO-cGMP signalling pathway that regulates neuronal events in the abdominal nervous system of the crayfish.

Key words: crustacean, cGMP, high-K^+, NO donor, immunohistochemistry

INTRODUCTION

Endogenous nitric oxide (NO) is a gaseous signalling molecule that is generated from L-arginine (L-Arg) by the activation of NO synthase (NOS), and it diffuses three-dimensionally through the membranes of target cells (Moncada *et al*., 1991). Then NO activates soluble guanylate cyclase in the target cells, which results in an increase in the level of the second messenger cyclic guanosine 3',5'-monophosphate (cGMP) (Bredt and Snyder, 1989). NO has been 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 potentiating (Guevara-Guzman *et al*., 1994) and depressing (Kilbinger and Wolf, 1994) actions on neurotransmitter release in vertebrates. NO is also thought to act as a neuromodulator in several species of invertebrate animals (Elphick *et al*., 1993). 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 (Mothet *et al*., 1996). This NO-cGMP signalling is involved in feeding behaviour (Sadamoto *et al*., 1998; Kobayashi *et al*., 2000a). NO functions as a crucial component in the motor program (Qazi *et al*., 1999, Zayas *et al*., 2000) and learning behaviour (Müller, 1997) in insects. Components of the NO-cGMP signalling pathway in crustacean animals have been identified in the neuronal networks that underlie olfaction (Johansson *et al*., 1996; Scholz *et al*., 1998; Johansson and Mellon, 1998), vision (Lee *et al*., 2000), rhythmic motor behaviours (Scholz *et al*., 1996), escape behaviour (Aonuma *et al*., 2000), sensory integration (Aonuma and Newland, 2001), and neurosecretion (Lee *et al*., 2000).

The neuronal circuits in the crayfish abdominal nervous system provide good model systems to investigate the functions of NO in nervous systems because the local circuits

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that control abdominal movements (Aonuma *et al*., 1994), escape and avoidance behaviour (Nagayama *et al*., 1994), swimmeret movement (Braun and Mulloney, 1993) and social hierarchy (Yeh *et al*., 1996) have been well investigated and a number of neurones have been identified by their morphological and physiological properties. Recent studies have demonstrated that NO has modulatory effects on neuronal transmission in the abdominal nervous systems (Aonuma *et al*., 1999, 2000; Aonuma and Newland, 2001). Putative NOS containing cells have been found in the crayfish nervous systems by NADPH-diaphorase staining (Johansson and Carlberg, 1994, Talavera et al, 1995). However, the target cells of NO in the crayfish nervous system have not been elucidated. Since NO is an unconventional molecule and diffuses three-dimensionally through the cell membrane at about 100–200 μm/sec (Philippides *et al*., 2000), most neuronal cells in the ganglion must have chance to be exposed by NO. Therefore, the target cells of NO must be identified to determine the functions of NO.

NO-induced anti-cGMP immunohistochemistry has been shown to reveal target cells of NO in the nervous systems (De Vent *et al*., 1987; Scholz *et al*., 1998; Ott *et al*., 2000). Preparations that are incubated in NO donors accumulate cGMP which are detectably stained by anti-cGMP immunohistochemistry. It has been demonstrated that high-K^+ saline depolarized the membrane potential (Oka *et al*., 1994; Oka and Ogawa, 1996) and activated NO generating cells to release NO in a ganglion (Kitamura *et al*., 2001), which in turn stimulated soluble guanylate cyclase in the target cells. In the present study, anti-cGMP immunohistochemistry is performed after stimulating soluble guanylate cyclase by incubating preparations in high-K^+ saline or NO donor saline to detect the target cells of NO in the abdominal ganglion of the crayfish. The distribution of NO donor induced cGMP-like immunoreactive cells is similar to that of high-K^+-induced cGMP-like immunoreactive cells suggesting that endogenous NO stimulates soluble guanylate cyclase in the target cells to generate cGMP, which could in turn modulate neurotransmission in the abdominal nervous system of the crayfish.

MATERIALS AND METHODS

Animals and preparations

Experiments were performed on adult crayfish, *Procambarus clarkii* (Girard), of both sexes with body lengths of 6-9 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 between results obtained from males and females.

Anti-cGMP immunohistochemistry

Staining methods were modified from De Vent *et al*. (1987) and Scholz *et al*. (1998). An abdomen was isolated from a crayfish and pinned ventral side up in a Sylgard-lined chamber filled with cooled HEPES buffered crayfish saline (NaCl: 205.3 mM, KCl: 5.36 mM, $CaCl_2$: 13.54 mM, $MgCl_2$: 2.62 mM, $NaHCO_3$: 2.38 mM, HEPES 5 mM, pH 7.4). 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. Crayfish abdominal ganglia were dissected out and they were preincubated in 1 mM 3-isobutyl-1-methyxanthine (IBMX; Sigma, St. Luis, MO) in the saline for 30 min at 4°C to block endogenous phosphodiesterase activity. Incubation in high-K^+ saline could depolarize membrane potential of all neurones (Oka *et al*., 1994; Oka and Ogawa, 1996) including NO generating neurons, which release NO in the ganglion (Kitamura *et al*., 2001). Preparations were then incubated in high-K^+ crayfish saline (NaCl: 5.36 mM, KCl: 205.3 mM, $CaCl_2$: 13.54 mM, $MgCl_2$: 2.62 mM, $NaHCO_3$: 2.38 mM, HEPES 5 mM, pH 7.4) containing 1 mM IBMX for 15 min at 20°C to detect NO-related increase in cGMP. For further investigation, NO-sensitive soluble guanylate cyclase was stimulated by an NO donor, sodium nitroprusside (SNP; Sigma). The tissue was exposed to 10 mM SNP in the saline containing 1 mM IBMX for 15 min at 20°C after incubation of 1 mM IBMX saline. Incubation in the high-K^+ saline or SNP saline was followed by fixation in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4) overnight at 4°C. After fixation, the ganglia were washed in PBS containing 0.2% Triton X-100 (PBST) 3 times for 15 min each and preincubated with 5% normal donkey serum in PBST (PBST-NDS) overnight at 4°C with agitation. Next, they were incubated with an anti-cGMP antibody (1:20000 in PBST-NDS) at 4°C for 3 days. They were then washed in PBST and incubated in horseradish peroxidase-conjugated secondary anti-sheep IgG antibody (diluted 1:500 in PBST-NDS; Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C overnight. They were washed in PBS, and antibody binding was made visible with diaminobenzidine. Then they were washed in distilled water, dehydrated in an ethanol series, cleared in methyl salicylate, and mounted in Bioleit (Oken, Tokyo, Japan). For controls, some preparations were incubated in IBMX without stimulating NO-sensitive soluble guanylate cyclase using high-K^+ and SNP saline and some preparations were processed without the anti-cGMP antibody. The antiserum was a gift from Dr. Jan De Vente (Rijsuniversiteit Limburg, The Netherlands; see Tanaka *et al*., 1997 for its specificity and characterization).

Some preparations were cut horizontally or vertically using a cryostat (CM 3000, Lica, Germany) or vibratome (DTK-1000, Dosaka E.M.I, Kyoto, Japan) and processed for immunohistochemistry. For frozen sectioning, the tissue was treated with NO donor saline, fixed in the paraformaldehyde, cryoprotected in PBS containing 20% sucrose overnight at 4°C, embedded in Tissue-Tech O.C.T. (Sakura Fineteck, Torrence, CA), frozen, and cryosectioned at 25–30 μm using a cryostat. For vibratome sectioning, the tissue was treated with NO donor saline, fixed in paraformaldehyde, embedded in 3% agarose in PBS and sectioned at 50 μm. Both types of sections were collected on silane coated slides and air-dried. After the sections were rehydrated in PBS, they were preincubated with PBST-NDS for 1 hr at 25°C. The sections were incubated with the anti-cGMP antibody (1:20000 diluted in PBST-NDS) overnight at 4°C. They were then washed in PBST and incubated at 25°C for 2–3 hr in horseradish peroxidase-conjugated secondary anti-sheep IgG antibody diluted 1:500 in PBST-NDS. They were washed in PBS, and antibody binding was made visible with diaminobenzidine. They were then washed in distilled water, dehydrated in an ethanol series, cleared in methyl salicylate, and mounted in Bioleit. Preparations were observed under a light microscope (BX51, Olympus, Tokyo, Japan). All images were recorded using an Olympus digital imaging system (C-3040 ADU) and stored as TIF format files for later analysis.

RESULTS

It has been shown that NO regulates the efficacy of neurotransmission in the crayfish abdominal nervous sys-

tem (Aonuma *et al*., 2000, Aonuma and Newland; 2001), but it is not known which neurones are the targets of NO. In the present study, immunohistochemistry with an antibody against cGMP was performed on the abdominal terminal ganglion of the crayfish to determine the target cells of NO. Anti-cGMP immunohistochemistry followed by stimulation of soluble guanylate cyclase can demonstrate NO-dependent cGMP accumulation because NO activates soluble guanylate cyclase to elevate the cGMP level in the target cells.

Accumulation of cGMP caused by high-K^+ stimulation

Accumulation of cGMP in the terminal abdominal ganglion was detected by anti-cGMP immunohistochemistry in wholemount preparations which were previously incubated in high-K^+ saline in the presence of non-specific inhibitor of the cGMP-degrading phosphodiesterase, IBMX (1 mM). High-K^+ saline activates NO generating neurones to generate endogenous NO in ganglion (Oka *et al*., 1994; Oka and Ogawa, 1996; Kitamura *et al*., 2001). Anti-cGMP immunohistochemistry in wholemount preparation revealed that about 75 cell bodies (76±16, mean±SD, n=6) and many neuronal branches were strongly stained in the terminal abdominal ganglion (Fig. 1A), although no neurones showed immunoreactivity when preparations were not incubated in high-K^+ saline (Fig. 1B). Cyclic GMP-like immunoreactive cell bodies were located in the rostra-lateral region, medial region and posterior region. Several intersegmental axons were also detectably stained in the connective between the 5th and terminal abdominal ganglia. Some axons of motor neurones were detectably stained in nerve roots through which they innervated tailfan muscles (Fig. 1C). For example, a motor neuron whose axon ran through the 3rd nerve

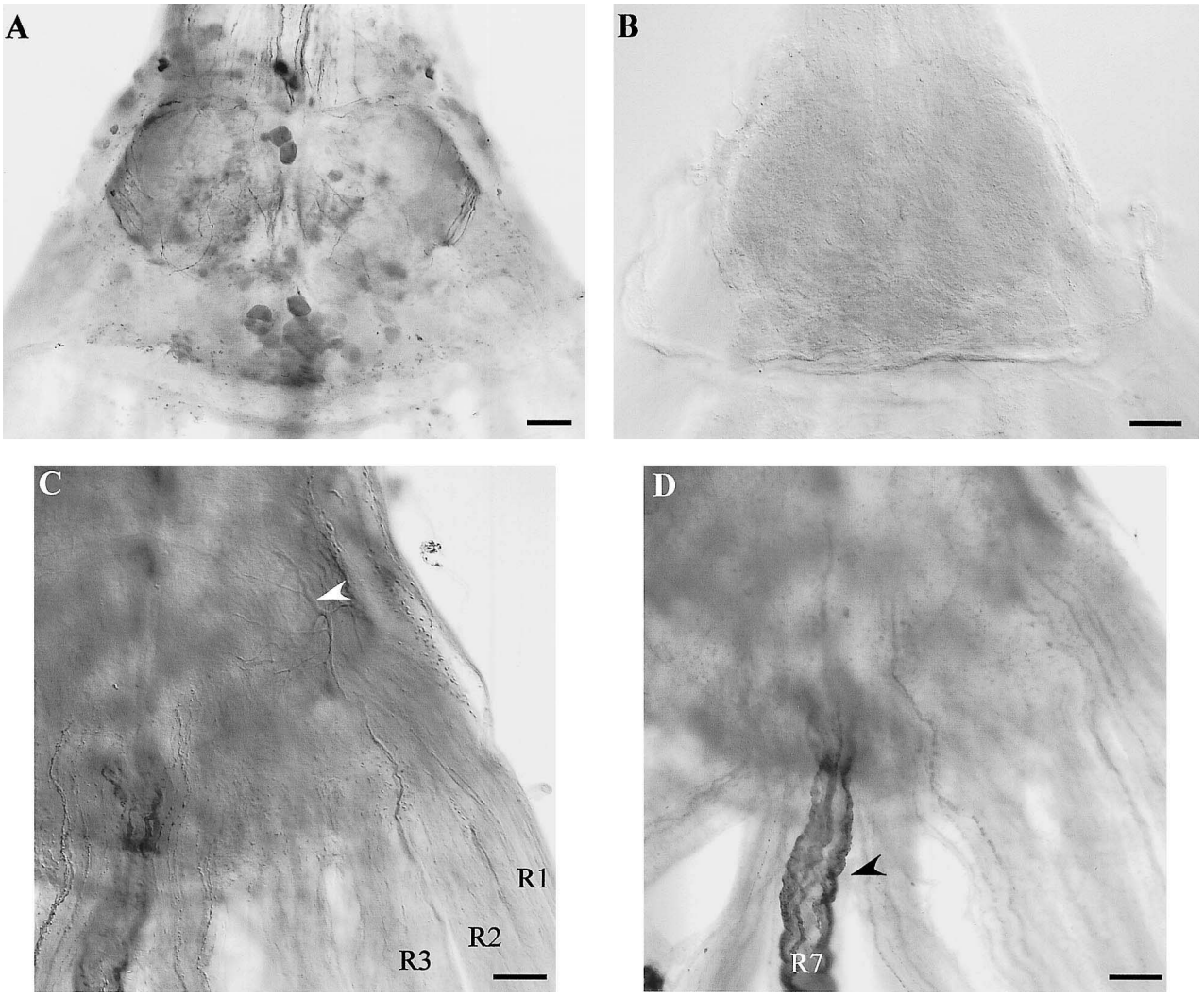

Fig. 1. Cyclic GMP-like immunoreactivity in a terminal abdominal ganglion. **A:** Wholemount preparation of the terminal abdominal ganglion. About 70–80 cell bodies and neuronal branches were observed in the ganglion. **B:** Control preparation. Neither cell bodies nor neuronal branches in the terminal abdominal ganglion were stained when anti-cGMP immunohistochemistry was performed without incubation in high-K^+ saline. **C:** The arrowhead indicates a motor neurone that innervates the endopodite through the 3rd nerve root. **D:** Sensory neurones that innervated the hind gut (indicated by arrowhead) were detectably stained in the 7th nerve root. R1-7: 1st-7th nerve roots. Scale bar = 100 μm.

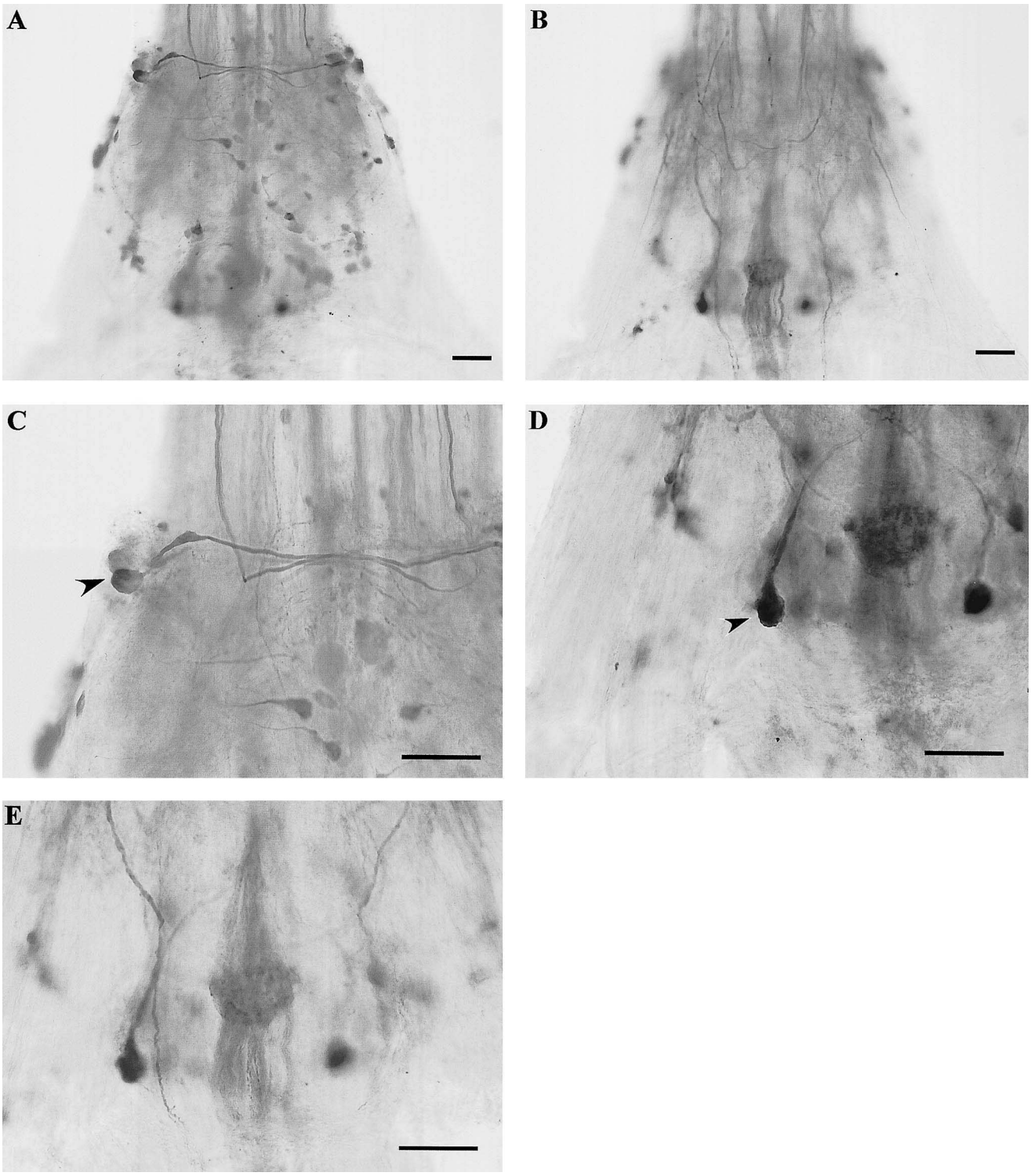

Fig. 2. NO-induced cGMP-like immunoreactivity in the crayfish terminal abdominal ganglion. **A:** Ventral view of the terminal abdominal ganglion stained by anti-cGMP immunohistochemistry. Cyclic GMP-like immunoreactive neurones were detected in a ganglion which was previously incubated in saline containing an NO donor, SNP. Anti-cGMP immunohistochemistry revealed the presence of NO-induced cGMP-like immunoreactive neurones that could be the targets of NO in the abdominal terminal ganglion. **B:** Ventral view of the ganglion. Many neuronal branches in the ganglion were stained. Sensory axons that innervated the hind gut were strongly stained. **C:** Cell bodies of ascending interneurones (indicated by arrowhead), primary neurites and ascending axons were stained. **D:** Cyclic GMP immunoreactivity was also detected in the cell body of another ascending interneurone (arrowhead). **E:** Sensory neurones (indicated by arrowhead) that innervated the hind gut were strongly stained. Scale bar = 100 μm.

root to innervate the endopodite was also detectably stained (Fig. 1 C). Many axons of sensory neurones that innervate the hind gut through the 7th nerve root were strongly stained (Fig 1. D). Cell bodies of the hind gut sensory neurones were also detectably stained in the posterior region of the ganglion.

Distribution of NO-induced cGMP-like immunoreactive neurones

High-K^+ treatment could possibly stimulate an NO-unrelated cGMP generating pathway in the terminal abdominal ganglion. It was therefore necessary to distinguish NO-related cGMP accumulation from NO-unrelated cGMP accumulation. In order to detect NO-related accumulation of cGMP in the terminal abdominal ganglion, soluble guanylate cyclase in the target cells of NO was directly stimulated using the NO donor SNP. Anti-cGMP immunohistochemistry in wholemount preparations revealed that about 70 cell bodies (67±9, mean±SD, n=14) and many neuronal branches were strongly stained in the terminal abdominal ganglion, which were previously incubated in 10 mM SNP saline containing 1 mM phosphodiesterase inhibitor IBMX (Fig. 2A, B). This immunoreactivity was eliminated when neither IBMX nor SNP was applied (Fig. 1B). Preparations without apply-

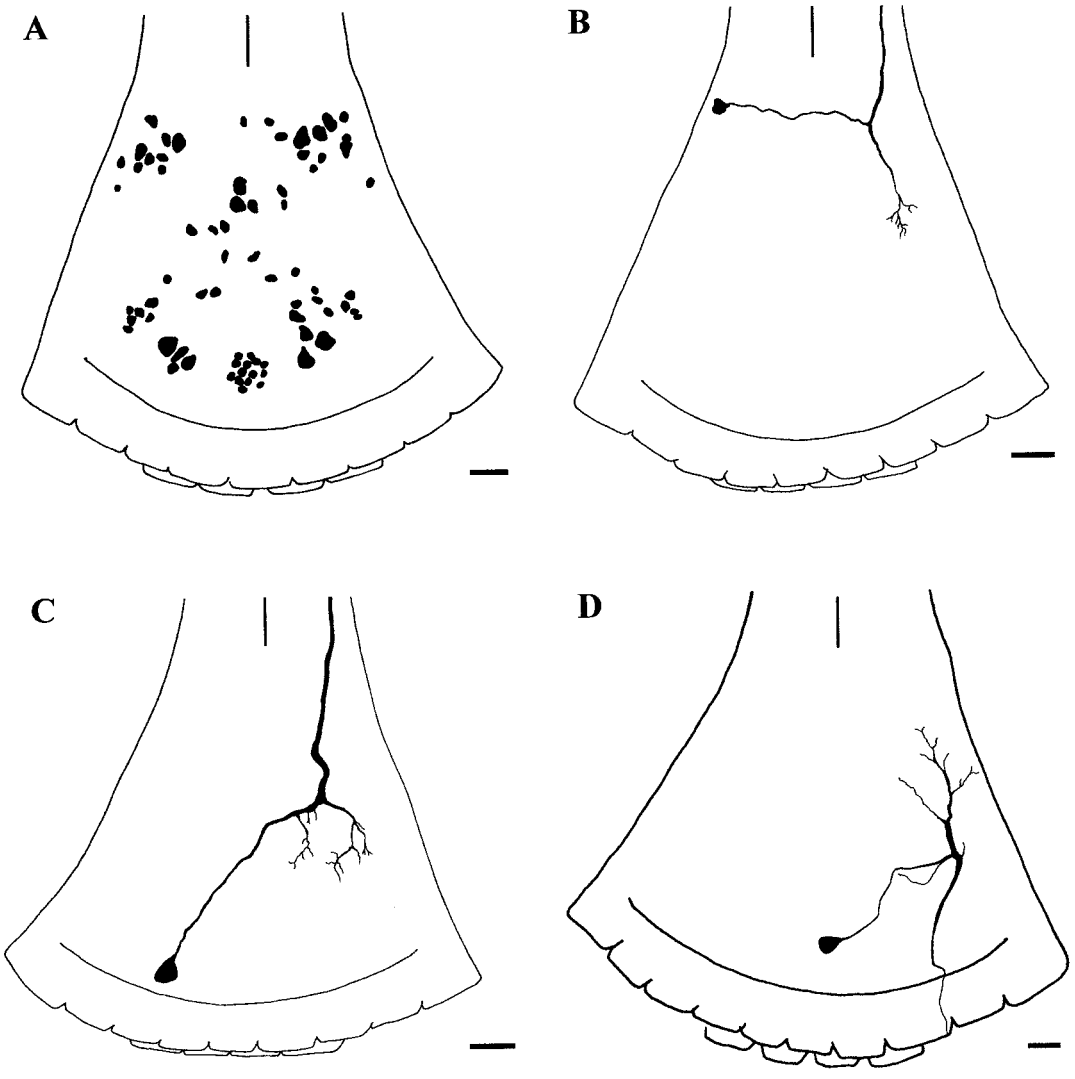

Fig. 3. **A:** Schematic drawing of the distribution of NO-induced cGMP immunoreactive cell bodies. About 70 cell bodies in the terminal abdominal ganglion were stained. The stained cell bodies were located in the rostralateral region, medial region, postero-lateral region and posterior region. **B:** One intersegmental ascending interneurone whose cell body was located in the antero-lateral region was detectably stained. This interneurone was detectably stained with a wholemount preparation and drawn by using a camera lucid. The primary neurite of the interneurone crossed the midline and expanded main branches on the opposite side of the cell body. Its axon was expanded to the anterior ganglia. **C:** One of the cell bodies stained in the posterior region was also an ascending interneurone, whose primary neurite crossed the midline and whose main neurite expanded laterally. **D:** One of the cell bodies stained in the posterior region was a motor neurone that innervated the endopodite through the 3rd nerve root and extended its main neurite laterally. This motor neurone was also stained when preparation was incubated in high-K^+ saline. Scale bar = 100 μm.

ing antiserum against cGMP also had no signals (not shown). The distributions of stained cell bodies and neuronal branches were similar to those in high-K^+ treated preparations. The stained cell bodies were located in the rostra-lateral region (Fig. 2C), medial region and posterior region (Fig. 2D). There were approximately 10 cell bodies with rather large diameters (20–50 μm) in the rostra-lateral region of the terminal abdominal ganglion (Fig. 3A). One of the stained cell bodies located in the rostra-lateral region (Fig. 2C) was an intersegmental ascending interneurone (Fig. 3B). The primary neurite of the ascending interneurone crossed the midline and expanded its branches in the neuropilar area of the terminal abdominal ganglion, and its axon ran through the connective to the anterior ganglia. There were four pairs of cell bodies with large diameters (about 50 μm) in the posterior region of the terminal abdominal ganglion (Fig. 3A). One of those neurones was also an intersegmental ascending interneurone (Fig. 3C). The primary neurite of this neurone crossed the midline and expanded it neuronal branches only on the ipsilateral side of the ascending axon. Bundle of axons were strongly stained in the caudal region of the ganglion (Fig. 2B, E). They were sensory neurones innervating the hind gut through the 7th nerve root. The cell bodies of these neurones were also stained, but the intensity of staining was not as strong as that in other stained cell bodies. Several motor neurones were strongly stained. One of those motor neurones, for example, had an axon running through the 3rd nerve root and its cell body was located in the posterior region (Fig. 3D). Several cell bodies with small diameters and fine fibres were detectably stained, but it was impossible to trace and classify them.

Sections of the terminal abdominal ganglion

Frozen sections of the preparations showed strong cGMP-like immunoreactivity in the terminal abdominal ganglion that was previously incubated in saline containing the

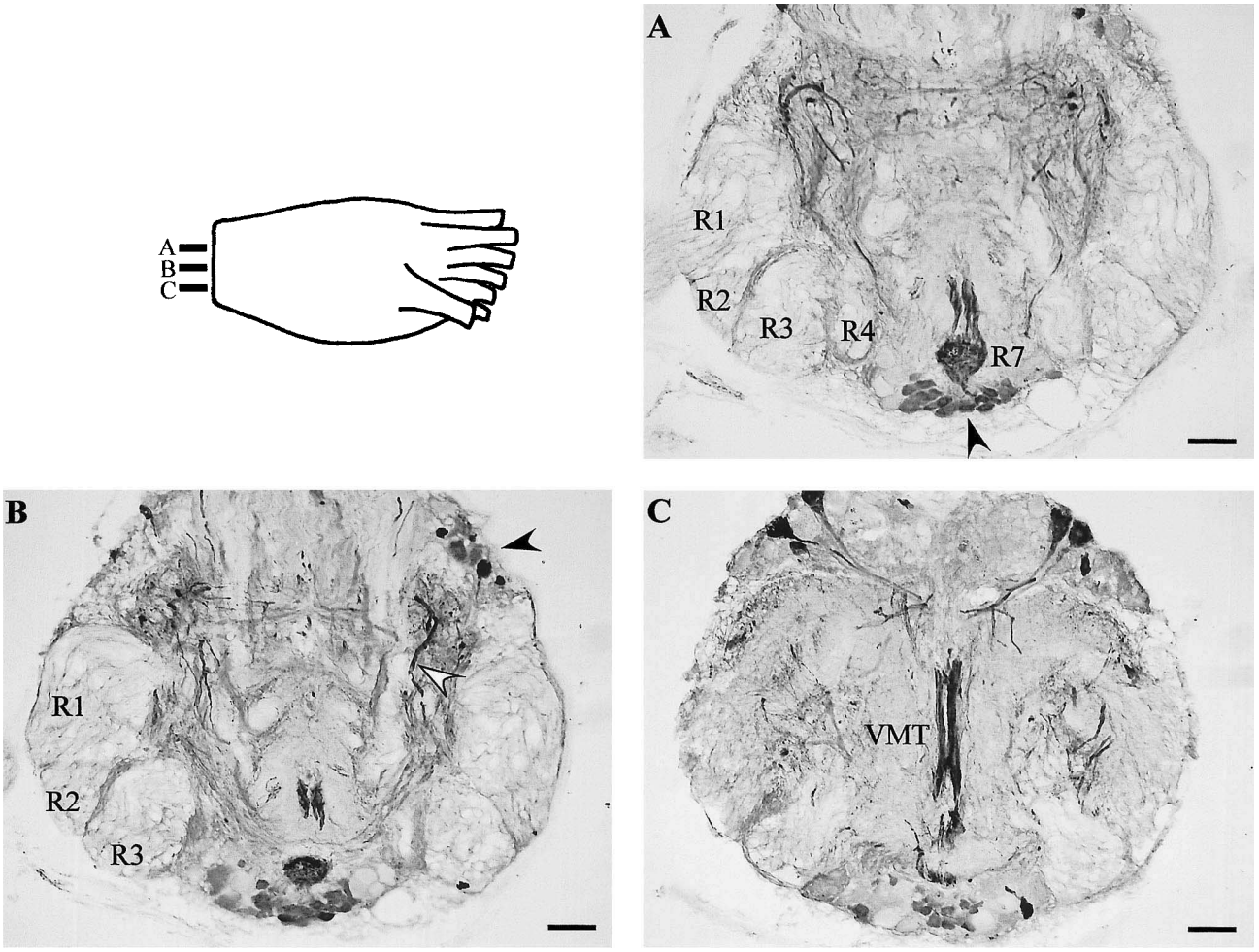

Fig. 4. Cyclic GMP-like immunoreactivity in horizontal sections of a terminal abdominal ganglion that was previously incubated in SNP saline. Frozen sections were 30 μm in thickness. **A:** Horizontal section of the dorsal part of the ganglion. Cell bodies and axons of hind gut sensory neurones were strongly stained (indicated by arrowhead). **B:** Different plane of the section. Many neuronal branches in the neuropilar area were stained. Several cell bodies in the antero-lateral region were stained (indicated by filled arrowhead). The axon indicated by an open arrowhead was that of a motor neurone that innervated the endopodite. This neurone was the same neurone as that shown in Fig. 3D. **C:** Other different plane of the section. Cell bodies with large diameter in the antero-lateral region were stained. Their primary neurite crossed midline. Several axons were stained in VMT region. VMT: ventral medial tract. Scale bar = 100 μm.

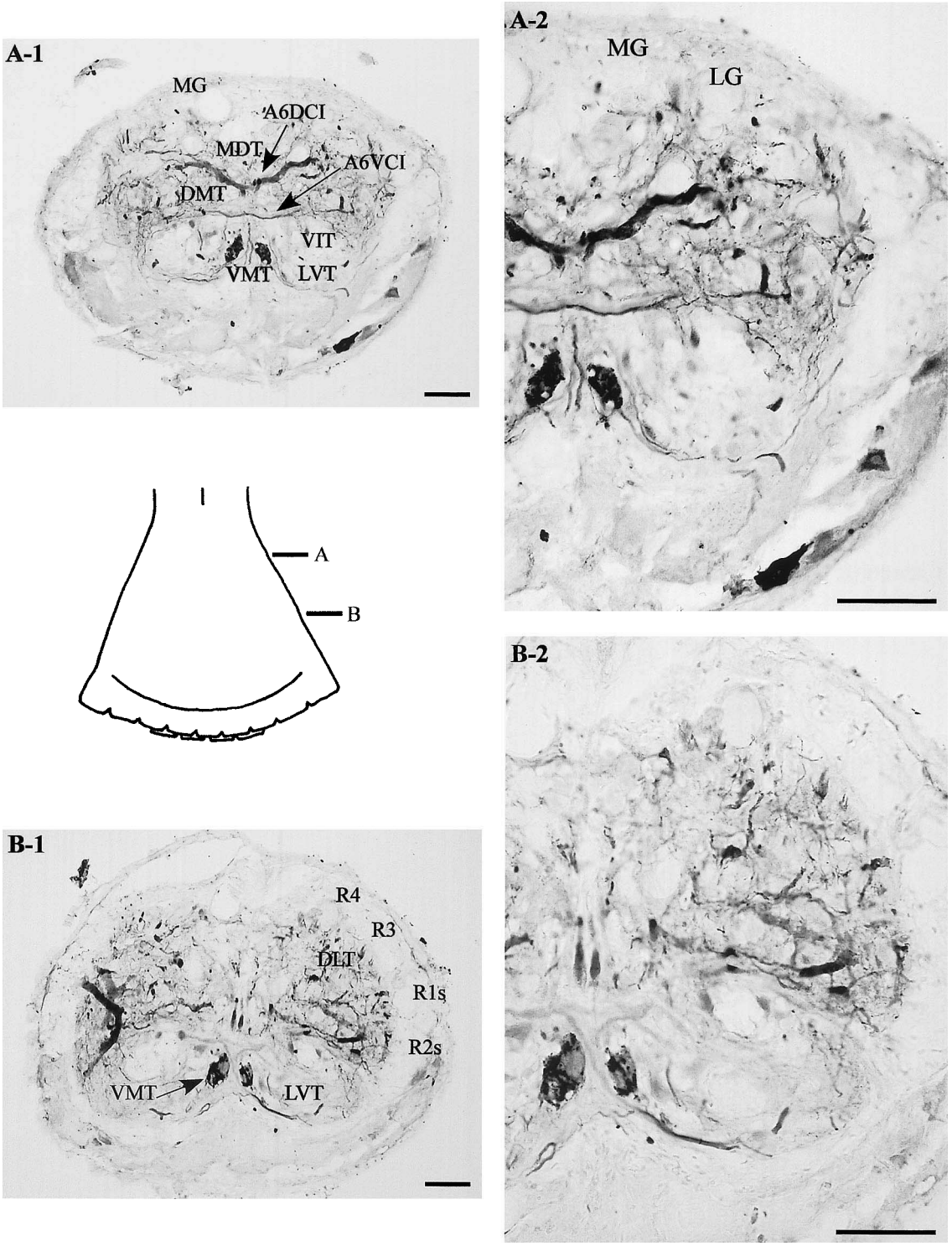

Fig. 5. Cyclic GMP-like immunoreactivity in vertical sections of a terminal abdominal ganglion which was incubated in SNP saline. Frozen sections were 25 μm in thickness. **A:** Vertical section of the anterior part of the ganglion (A-1: low magnification, A-2: high magnification). Many neuronal branches in the neuropilar area of the ganglion were stained but no neuronal branches in the VIT and LVT regions were detectably stained. Several branches in the A6DCI and A6VCI were stained. **B:** Vertical section of the medial part of the ganglion (B-1: low magnification, B-2: high magnification). The hind gut sensory neurones in the VMT region were strongly stained although no mechanosensory afferents in the nerve roots were detectably stained. Scale bar = 100 μm. A6: Sixth abdominal neuromere, DCI: dorsal comissure I, LVT: lateral ventral tract, LG: lateral giant interneurone, MG: medial giant interneurone, MDT: medial dorsal tract, R1s: 1st nerve root sensory bundle, R2s: 2nd nerve root sensory bundle, VCI: ventral comissure I, VIT: ventral intermediate tract.

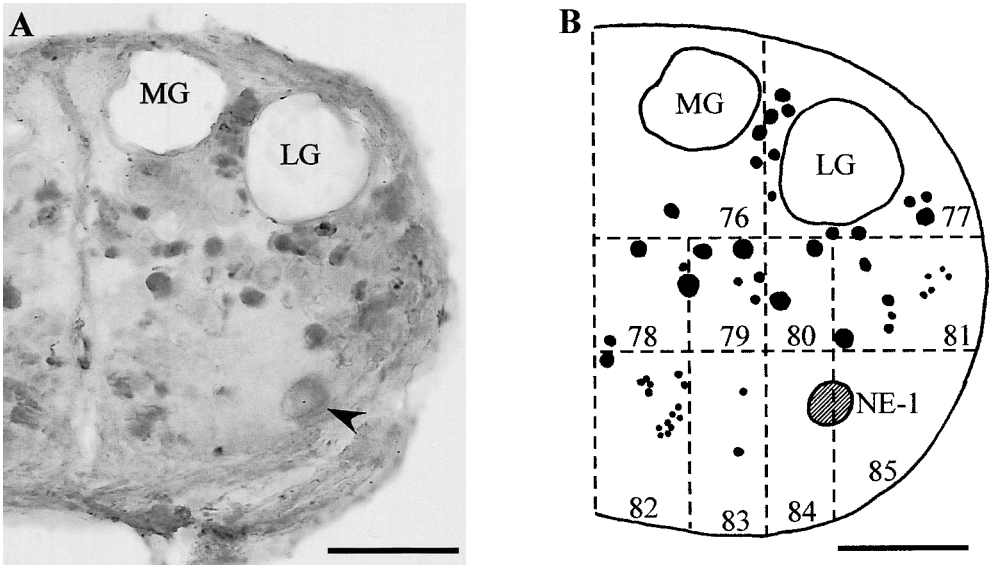

Fig. 6. Vertical section of the connective between the 5th and terminal abdominal ganglia. **A:** Vibratome section of a hemiconnective of 50 μm in thickness. About 50 axons in each hemiconnective were stained. Arrow head indicates the axon of interneurone NE-1. **B:** Schematic drawing of NO-induced cGMP immunoreactive axons in a hemiconnective. Dotted lines and numbers indicate the divisions of the hemiconnective determined by Wiersma and Hughes (1961). Landmarks are medial (MG, 76) and lateral giant (LG, 77) interneurones.

NO donor SNP in the presence of the phosphodiesterase inhibitor IBMX (Figs. 4, 5). Horizontal sections (Fig. 4) and vertical sections (Fig. 5) showed the distributions of NO-induced cGMP-like immunoreactive neuronal branches in the neuropilar area of the ganglion, where interneurones and motor neurones expand their branches. Many neuronal branches in the dorsal comissure (DC), ventral comissure (VC), medial dorsal tract (MDT) and ventral medial tract (VMT) were stained strongly (Fig. 4A, Fig. 5A, B). Several cell bodies located in the rostra-lateral region showed strong cGMP-like immunoreactivity (Fig. 4B, C). Some of the cell bodies and primary neurites of ascending interneurones showed strong immunoreactivity (Fig. 4 C). There were many stained neuronal branches in the dorsal lateral tract (DLT) region, while few neuronal branches in the ventral intermediate tract (VIT) and lateral ventral tract (LVT) region showed detectable immunoreactivity (Fig. 5). In the neuropilar region, strong immunoreactivity was observed in a thick axon of the 3rd nerve root motor neurone (Figs. 4, 5), which was the same type of neurone as that shown in Figures 1C and 3D. The cell bodies and axons of hind gut sensory neurones in the posterior region were strongly stained (Fig. 4A). Cyclic GMP-like immunoreactivity was observed in the ventral medial tract (VMT), where these hind gut sensory neurones extend their branches (Fig. 4C, Fig. 5). None of the mechanosensory neurones in the ganglion showed any detectable immunoreactivity, while hind gut sensory axons were strongly stained (Fig. 4A, B, Fig. 5).

To determine the distribution of descending and ascending axons of intersegmental neurones in the connective between the 5th and terminal abdominal ganglia, NO-induced anti-cGMP immunohistochemistry was performed with vertical vibratome sections (Fig. 6A). In vertical sections of the connective, about 50 pairs of ascending and/or descending axons showed strong cGMP-like immunoreactivity (Fig. 6 B). NO-induced cGMP immunoreactive axons in the connective were located between areas 76 and 77, where medial giant neurone (MG) and lateral giant neurone (LG) are located. Several axons with thick diameters were located areas 78, 79 and 80, where some axons of identified intersegmental ascending interneurones are located (Nagayama *et al*., 1994). In some preparations, an identically thick axon of ascending interneurone NE-1 was weakly stained. The axon of NE-1 is located between areas 84 and 85. Several fine immunoreactive axons were located in area 81 where, some intersegmental descending interneurones have been identified (Namba *et al*., 1995). There were also several fine axons stained in area 82, where intersegmental hind gut sensory neurones (Kondoh and Hisada, 1986) and some ascending interneurones (Sigvardt *et al*., 1982) are located.

DISCUSSION

The accumulation of cGMP, which was induced by high-K^+ treatment and NO donor treatment, was investigated to determine the distribution of target cells of NO in the terminal abdominal ganglion. NO generating neurones were activated by high-K^+ treatment to detect accumulation of endogenous NO induced cGMP using anti-cGMP immunohistochemistry. Staining of about 70 cell bodies and many neuronal axons and branches was detected by anti-cGMP immunohistochemistry in wholemount preparations. Some of them in the ganglion were strongly stained, while others were weakly but detectably stained, probably because of the difference in the concentrations of induced cGMP or pene-

tration of antibodies. These stained neurones could be good candidates of the target cells of NO. High-K^+ treatment activates all neurones in the ganglion (Oka et al., 1994; Oka and Ogawa, 1996) and could activate an NO-unrelated pathway. Scholtz *et al*. (1996), for example, demonstrated that peptide neurohormones activate cGMP synthesis in the crab. In order to confirm NO-related cGMP accumulation in the ganglion, soluble guanylate cyclase was directly stimulated by an NO donor before the immunohistochemistry was performed. The distribution of high-K^+-induced cGMP-like immunoreactive neurones in the terminal abdominal ganglion was similar to that of NO-induced cGMP-like immunoreactive neurones, although the total numbers of stained cell bodies were slightly different. Most of the stained neurones detected by anti-cGMP immunohistochemistry after treatment of both high-K^+ and the NO donor could be target cells of NO, suggesting that NO-generating neurones are activated by high-K^+ treatment and release NO in the terminal abdominal ganglion, which in turn activates soluble guanylate cyclase to accumulate cGMP in the target cells. This supports the involvement of NO-cGMP signalling in the crayfish abdominal nervous system (Aonuma and Newland, 2001).

The results of this study demonstrated accumulation of NO-induce cGMP in some motor neurones in the neuromuscular system of the crayfish, suggesting that NO-cGMP signalling regulates neuromuscular transmission. Since these motor neurones innervate tailfan muscles, NO-cGMP signalling could regulate motor control of tailfan movements. Aonuma *et al*. (2000) suggested that NO reduces transmitter release from motor giant neurones (MoGs), which receive input from command neurones LGs and MGs to drive tail-flip escape by producing contraction of abdominal fast flexor muscles (Zuker *et al*., 1971; Wine, 1977). MoGs in the terminal abdominal ganglion have large cell bodies and their thick axons run though the 6th nerve root; however, no thick motor axons in the terminal abdominal ganglion were stained, suggests that NO might regulate neuromuscular transmission via an NO-cGMP-unrelated pathway. It has been shown, for example, that NO directly activates cyclic ADP ribose (cADPR) hydrolase to reduce the intracellular level of Ca^{2+} (Lee, 1994). The decrease in the Ca^{2+} level as a result of the action of an NO-dependent pathway must reduce the probability of transmitter release since Ca^{2+} is required for the vesicles to bind to releasing sites. Since NO has been shown to reduce neurotransmitter release from MoGs (Aonuma *et al*., 2000), it could drive a cGMP-unrelated signalling pathway to reduce the intracellular Ca^{2+} level. Aonuma *et al*. (1999, 2000) suggested that NO has opposite modulatory effects on neuromuscular transmission. Excitatory junctional potential was depressed by stimulation of some type of motor neurones in the presence of NO, while it was enhanced by the stimulation of other type of motor neurones. Some motor neurones in the abdominal ganglion showed cGMP immunoreactivity, while others did not. Further physiological studies in the cGMP immunoreactive motor neurones are needed to determine how NO-cGMP signalling regulates neuromuscular transmission. Further study is also needed to determine how NO regulates neurotransmission via a cGMP-unrelated pathway using MoG system. Neuromuscular system of the crayfish should provide good model system to investigate the function of NO. To compare the nature of the cGMP-like immunoreactive neurones and none immunoreactive neurones would clarify the function of NO in the nervous systems.

Sensory neurones innervating the hind gut in the terminal abdominal ganglion were strongly stained by anti-cGMP immunohistochemistry, suggesting that NO-cGMP signalling regulates hind gut systems, while no mechanosensory afferents that innervate mechanoreceptors on the tailfan or chordotonal organ showed detectable immunoreactivity. NO has been known to mediate procerebral oscillations by modifying patterns of neuronal activity through cholinergic neuro-neuronal synapses in molluscs, (Gelperin, 1994, Mothet *et al*., 1996). NO-induced cGMP immunoreactive sensory neurones that innervate hind gut would possibly regulate hind gut muscle or anus movements (Muramoto, 1985) by changing efficacy of transmitter release onto hind gut system. On the other hand, the results indicate that endogenous NO cannot induce the accumulation of cGMP in mechanosensory afferents. Ott *et al*. (2000) suggested on the basis of results of immunohistochemistry against the anti-α subunit of soluble guanylate cyclase that mechanosensory afferents are the targets of NO in insects. It is difficult to conclude that mechanosensory afferents in the crayfish are not the target cells of NO, because NO could regulate not only NO-cGMP signalling but also some other signalling (Lee, 1994; Watson *et al*., 2001). Aonuma and Newland (2001) demonstrated that NO-cGMP signalling had both excitatory and inhibitory effects on ascending interneurones in the crayfish. Since NO did not induce accumulation of cGMP in mechanosensory afferents, NO would stimulate soluble guanylate cycles in some interneurones in the ganglion so as to elicit modulatory effects on ascending interneurones. Some of the interneurones in the terminal abdominal ganglion indeed accumulated cGMP when preparations were incubated in high-K^+ or NO donor saline. The results suggest that intersegmental ascending interneurones and descending interneurones could be the target cells of NO. Cell bodies with small diameters in the ganglion were also stained, and some of them might be local interneurones. Therefore, these interneurones must be good candidates of target cells of NO and they introduce modulatory effects of NO-cGMP signalling on the ascending interneurones. The identified ascending interneurone NE-1 indicated weak but detectable cGMP immunoreactive. Therefore NO could regulate the neuronal circuit of escape behaviour because interneurone NE-1 whose activity is modulated by NO (Aonuma and Newland, 2001) has been known to form part of a disynaptic pathway from excitatory pathway from mechanosensory afferents innervating tailfan and excite the pair of LG to mediate tail flip escape (Zucker *et al*., 1971).

Evidence of NO-cGMP signalling in the crayfish nervous system was obtained in the present study by examining accumulation of cGMP induced by NO. Double labelling with Lucifer yellow and immunohistochemistry (Aonuma and Nagayama, 1999) should enable identification of other cGMP-like immunoreactive neurones and thus elucidate the target cells of NO. Immunohistochemistry against soluble guanylate cyclase (Jones and Elphick, 1999) would confirm the target cells of NO. To determine the functions of NO in nervous systems, we need to investigate, as the next step, physiological concentration of NO using NO-electrode (Kobayashi *et al*., 2000b; Fujie *et al*., 2002) or NO indicator (Kojima *et al*., 1998). Further study of the action of PKG and/or cyclic nucleotide-gated channels to understand the function of NO-cGMP signaling in nervous systems is needed in order to determine the role of accumulated cGMP in the target cells of NO.

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