Nonspiking local interneurones are widely distributed in the central nervous system of arthropods, especially insects and crustaceans (Nagayama and Hisada, 1987; Siegler and Burrows, 1979), and are one of the essential neural elements producing and controlling movements (e.g. Burrows, 1992; Nagayama et al. 1994). Some nonspiking local interneurones organize motor outputs while others process sensory information. In the crayfish, the local directionally selective interneurone (LDS) has bilateral arborizations restricted within the terminal abdominal ganglion of the crayfish Procambarus clarkii. This mean number of neurones with GABA-like immunoreactivity represents approximately 10% of the total number of neurones in the terminal ganglion. A combination of intracellular staining using Lucifer Yellow and immunocytochemical staining revealed that an identified nonspiking local interneurone (the local directionally selective interneurone, LDS) showed GABA-like immunoreactivity.

Introduction

Nonspiking local interneurones are widely distributed in the central nervous system of arthropods, especially insects and crustaceans (Nagayama and Hisada, 1987; Siegler and Burrows, 1979), and are one of the essential neural elements producing and controlling movements (e.g. Burrows, 1992; Nagayama et al. 1994). Some nonspiking local interneurones organize motor outputs while others process sensory information. In the crayfish, the local directionally selective interneurone (LDS) has bilateral arborizations restricted within the terminal (sixth) abdominal ganglion and is identifiable as a unique neurone by means of its characteristic shape and physiological properties (Nagayama and Hisada, 1988; Reichert et al. 1982). This interneurone is depolarized by headward water currents and was named the local directionally selective interneurone by Reichert et al. (1983). Branches on the soma side are input sites receiving sensory input directly from mechanosensory afferents, while branches on the opposite side of the cell body are output sites making inhibitory connections onto intersegmental ascending interneurones (Nagayama et al. 1994; Reichert et al. 1983). LDS has no measurable output effect upon the motor neurones innervating the tailfan muscles, although other nonspiking interneurones affect the activity of these motor neurones significantly (Nagayama and Hisada, 1988; Nagayama et al. 1984). Thus, LDS acts as a sensory integrator to mediate transverse lateral inhibition of the mechanosensory interneurones (Reichert et al. 1983).

γ-Aminobutyric acid (GABA) is the most widely distributed inhibitory transmitter in both the vertebrates and invertebrates (Sattelle, 1990). Immunological analyses have revealed the distribution of GABAergic neurones in the central nervous system of both insects and crustaceans (Mulloney and Hall, 1990; Watson, 1986). The somata of the inhibitory motor neurones of the lobster have far higher cytoplasmic concentrations of GABA than do the somata of excitatory motor neurones (Otsuka et al. 1967). Furthermore, pharmacological studies have been carried out to establish GABAergic inhibitory transmission in crayfish (El Manira and Clarac, 1994; Pfeiffer-Linn and Glantz, 1991; Takeuchi and Takeuchi, 1965; Vu and Krasne, 1993). We have, however, no information about inhibitory transmitters released from nonspiking local interneurones, in particular from LDS. In this paper, we show, for the first time, that LDS has GABA-like immunoreactivity by double marking using intracellular staining and immunocytochemical labelling.

Materials and methods

Crayfish Procambarus clarkii (Girard) measuring 8–12 cm in body length from rostrum to telson were used for all experiments. Details of the dissection procedure have been described previously (Nagayama et al. 1984). The isolated nerve chain including the last two (fifth and sixth) abdominal ganglia was fixed for 20 min in a primary fixative containing

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4% paraformaldehyde and 0.1% glutaraldehyde in Dulbecco’s phosphate-buffered saline (DPBS, Sigma). Before fixation, the ventral ganglionic sheath in the terminal abdominal ganglion was surgically removed. The tissue was then immersed in secondary fixative containing 0.2% picric acid and 2% formaldehyde in DPBS at 4°C. The tissue was washed for 30 min in 0.1 mol l⁻¹ glycine in DPBS, dehydrated through an ethanol series to 70% ethanol and stored at 4°C overnight. The tissue was then dehydrated through an alcohol series to 90% ethanol for 1 h, rehydrated through an alcohol series to DPBS, and preincubated in several changes of wash buffer, DPBS containing 0.3% Triton X-100 (Sigma) and 5% goat serum (Chemicon), for 6 h. In some preparations, LDS or the flexor inhibitor (FI) motor neurone was stained intracellularly by the injection of Lucifer Yellow (5–10 nA hyperpolarizing current pulses of 500 ms duration at 1 Hz for 15–30 min) into the isolated abdominal preparation. LDS showed spontaneous depolarizing and hyperpolarizing postsynaptic potentials of large amplitude and elicited no significant change of activity in the uropod motor neurones following depolarizing current injection (Nagayama and Hisada, 1988). FI was readily identified by its large-diameter cell body located near the midline (Wine, 1984). The gross morphology of LDS and FI was confirmed by in situ observations using brief blue–violet illumination from a high-pressure mercury lamp (Aonuma et al. 1996). After identification of stained neurones, the terminal abdominal ganglion was desheathed and the abdominal nerve chain was isolated for immunocytochemical staining.

The primary antibody, rabbit anti-GABA (Sigma) at a dilution of 1:750, was preincubated with dried crayfish nerve and muscle powder (Mulloney and Hall, 1990) for 6 h at 4°C in wash buffer, 0.3% Triton X-100 in DPBS. The solution was centrifuged for 10 min at 2000g and the supernatant was collected and used. The abdominal nerve chain was incubated in the primary antibody solution for approximately 90 h at 4°C on a rotator and then washed in several changes of wash buffer for 6 h. Anti-rabbit-IgG Cy-3 (Chemicon) at a dilution of 1:50 was used as the secondary antibody. The tissue was incubated in the secondary antibody solution for approximately 40 h at 4°C on a rotator, then washed in several changes of wash buffer for 5 h. The tissue was then dehydrated in an alcohol series and cleared in methyl salicylate.

Fluorescence was detected using Zeiss or Olympus fluorescence microscopes. The light employed for excitation was passed through 450–490 nm (for Lucifer Yellow) and 510–560 nm (for Cy-3) bandpass excitation filters. The resulting fluorescence was passed through LP 520 (for Lucifer Yellow) and LP 590 (for Cy-3) barrier filters attached to the fluorescence microscope. The fluorescent images were photographed or recorded in the same focal plane using a cooled CCD video camera (Imagepoint, Photometrics) and stored on an IBM-compatible computer as files of TIF format via a parallel interface for later image analysis.

**Results and discussion**

Previous histological studies have shown that the total number of cell bodies of central neurones in the terminal abdominal ganglion of the crayfish *Procambarus clarkii* is about 650 (Kondoh and Hisada, 1986a; Reichert et al. 1982). Fig. 1 shows a typical distribution of cell bodies with GABA-like immunoreactivity in the terminal abdominal ganglion. Approximately 70 labelled cell bodies were counted on the ventral surface of the terminal abdominal ganglion (68±9; mean ± s.e.m., N=9). No labelled cell bodies were distributed on the dorsal surface. The mean number and distribution of labelled neurones in *Procambarus clarkii* were quite similar to those of the crayfish *Pacifastacus leniusculus* reported previously by Mulloney and Hall (1990). In their report, 67±17 neurones (mean ± s.e.m., N=7) showed GABA-like immunoreactivity.

To confirm the selectivity of staining against GABA, one flexor inhibitor motor neurone (FI) was stained intracellularly using Lucifer Yellow and then processed using the anti-GABA immunohistochemical procedure (Fig. 2). FI is known to possess a large cell body (approximately 100 μm in diameter) within the terminal abdominal ganglion (Fig. 2A) and has been physiologically and pharmacologically identified as a GABAergic motor neurone (Otsuka et al. 1967). The Lucifer-Yellow-filled cell body of FI (Fig. 2B) showed GABA-like immunoreactivity (Fig. 2C; see also Figs 1, 2A). The largest cell body in the terminal abdominal ganglion is a motor giant neurone (MoG), an excitatory motor neurone innervating the abdominal flexor muscle. The cell body of MoG is located just anterior to the cell body of FI and showed no measurable GABA-like immunoreactivity (arrowhead in Fig. 2C). The immunocytochemical staining used in this study thus selectively labelled GABAergic neurones.
The local directionally selective interneurone LDS is a bilateral nonspiking local interneurone in the terminal abdominal ganglion (Fig. 3A). One of the most characteristic features of LDS is its thick transverse process leading to extensive bilateral branches. In three preparations, LDS was
identified by intracellular injection of Lucifer Yellow and subsequently treated with immunocytochemical staining against GABA. The Lucifer-Yellow-labelled cell body of LDS, which ranged in diameter between 20 and 30 μm, was located on the ventral surface of the medial portion of the ganglion near the midline (asterisk in Fig. 3B). This Lucifer-Yellow-filled cell body of LDS was also labelled by immunocytochemical staining against GABA (indicated by an asterisk in Fig. 3C). In all preparations, the cell body of LDS showed GABA-like immunoreactivity.

This study, therefore, strongly suggests that LDS is a GABAergic interneuron and that its synaptic interactions are mediated by GABA. In a previous study, depolarizations of LDS caused membrane hyperpolarization in several ascending interneurons in a graded manner dependent upon the membrane potential change in LDS (Nagayama et al. 1994). Moreover, numerous synaptic vesicles have been observed on branches on the output site of LDS (Kondoh and Hisada, 1986b). These observations support the proposed chemical nature of the action of LDS. This is also supported by a preliminary study in which local injection of GABA into the neuropile of the crayfish mimicked the membrane hyperpolarization of ascending interneurons elicited by contralateral sensory stimulation (H. Miyata and T. Nagayama, unpublished data).

This study is the first to demonstrate that GABA is a neurotransmitter of an identified nonspiking local interneuron, LDS. Further pharmacological studies are now needed to characterize the GABAergic nature of the lateral inhibition from LDS to ascending interneurons.

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