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ORIGINAL PAPER

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Synaptic interactions between nonspiking local interneurones in the terminal abdominal ganglion of the crayfish

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Abstract Nonspiking local interneurones are the important premotor elements in arthropod motor control systems. We have analyzed the synaptic interactions between nonspiking interneurones in the crayfish terminal (6th) abdominal ganglion using simultaneous intracellular recordings. Only 15% of nonspiking interneurones formed bi-directional excitatory connections. In 77% of connections, however, the nonspiking interneurones showed a one-way inhibitory interaction. In these cases, the presynaptic nonspiking interneurones received excitatory synaptic inputs from the sensory afferents innervating hairs on the surface of the uropods and the postsynaptic nonspiking interneurones received inhibitory synaptic inputs that were partly mediated by the inputs to the presynaptic nonspiking interneurones. The membrane hyperpolarization of the postsynaptic nonspiking interneurones mediated by the presynaptic nonspiking interneurones was reduced in amplitude when the hyperpolarizing current was injected into the postsynaptic interneurones, or when the external bathing solution was replaced with one containing low calcium and high magnesium concentrations. The role of these interactions in the circuits controlling the movements of the terminal appendages is discussed.

Keywords GABA · Graded transmission · Interneurone · Local circuit · Reflex

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Abbreviations AL:antero-lateral · epsp: excitatory postsynaptic potential · ipsp: inhibitory postsynaptic potential · PL: postero-lateral

Introduction

Local circuits are involved in the control and production of the movements of the limbs of vertebrates and invertebrates. In arthropods, nonspiking local interneurones play a crucial role in these local circuits by controlling the motor output to the muscles, through the graded and continuous release of neurotransmitter onto motor neurones (Burrows 1992; Nagayama et al. 1994; Nagayama 2002). In the terminal abdominal ganglion of the crayfish, of which we have a detailed knowledge of the neural networks that control the movements of the terminal appendages, the uropods, two distinct groups of nonspiking interneurones, postero-lateral (PL) and antero-lateral (AL) interneurones, have been described (Nagayama and Hisada 1987). These nonspiking interneurones receive monosynaptic inputs from sensory neurones and central interneurones (Namba et al. 1997; Nagayama 1997) and in turn make output connections with uropod motor neurones (Nagayama et al. 1984). The PL and AL nonspiking interneurones form opposite and parallel connections with uropod motor neurones and their activity balance is essential in forming the motor pattern regulating the movements of the uropods (Nagayama and Hisada 1987; Namba et al. 1994).

In crayfish, nonspiking interneurones receive excitatory sensory inputs directly from extero- and proprioreceptors on, and in, the tailfan (Newland and Nagayama 1993; Nagayama 1997). Furthermore, they receive excitatory inputs from ascending and descending intersegmental interneurones, and inhibitory inputs from spiking local interneurones (Nagayama and Sato 1993; Nagayama 1997; Namba et al. 1997). Although nonspiking interneurones have been characterized in many insects, e.g., locust, cricket, stick insect, and cockroach (Pearson and Fourtner 1975; Burrows and

Siegler 1976; Kobashi and Yamaguchi 1984; Büschges and Schmitz 1991) and crustaceans, e.g., crayfish, lobster, and crab (Mendelson 1971; Heitler and Pearson 1980; Takahata et al. 1981; DiCaprio and Fourtner 1988), few studies have attempted to describe their synaptic interactions. Only the pioneering work of Burrows (1979) demonstrates a one-way inhibitory interaction between nonspiking interneurones in the locust metathoracic ganglion. Recent immunocytochemical analyses have shown that many nonspiking interneurones in the crayfish and locust show GABA immunoreactivity (Nagayama et al. 1996, 1997; Wildman et al. 2002), suggesting that the nonspiking interneurones have inhibitory outputs. In the crayfish, however, the interactions between nonspiking interneurones remain to be described.

To understand the precise role of this key class of interneurone in controlling the motor output in crayfish local circuits we have, therefore, analysed the synaptic interactions between nonspiking interneurones and assess their role in motor pattern formation. Our results show that many nonspiking interneurones exhibited one-way inhibitory interactions. Interneurones that receive excitatory sensory inputs make inhibitory connections with interneurones that receive inhibitory inputs, and their inhibitory interactions are chemically mediated.

Materials and methods

Freshwater crayfish, *Procambarus clarkii* (Girard) of 7–10 cm in body length from rostrum to telson were obtained from a commercial supplier and used for all experiments. The abdomen was isolated from the thorax and pinned ventral side-up in cooled van Harreveld's (1936) solution. The swimmerets were removed and the terminal (6th) abdominal ganglion exposed by removing the 6th sternite and peeling off the surrounding soft cuticle and the ventral aorta. The terminal ganglion was then stabilized on a silver platform and treated with protease (Sigma type XIV, Sigma, St. Louis, Mo., USA) for 30 s to soften the ganglionic sheath to aid penetration with intracellular electrodes.

To monitor the activity of uropod motor neurones, a suction electrode was placed over the cut end of either the nerve root 2 or 3 motor bundle. Either the closer, reductor motor neurone (Red MN no.1) was recorded at the bifurcation to the reductor and adductor exopodite muscles, or the opener motor neurones were recorded at the bifurcation to the ventral rotator and the abductor exopodite muscles (Nagayama et al. 1983, 1984; Nagayama 1999). To stimulate the sensory neurones innervating hairs on the surface of exopodite, a second suction electrode was placed over the cut end of the nerve root 2 sensory bundle. The remaining nerve roots were cut or pinched to prevent unwanted inputs. Simultaneous intracellular recordings were carried out from the left half of the terminal ganglion neuropil with glass microelectrodes filled with either a 3% solution of lucifer yellow CH in 0.1 mol l^{-1} lithium chloride (electrode resistance range 100–200 MΩ) to confirm neurone structure, or 2 mol l^{-1} potassium acetate (electrode resistance range 30–40 MΩ) to record synaptic events. For intracellular staining, presynaptic nonspiking interneurones were firstly stained by iontophoretic injection of lucifer yellow (using 1- to 7-nA hyperpolarizing current pulses of 500 ms duration at 1 Hz for 3–5 min) following physiological analysis. The gross morphology and cell body position of the presynaptic neurone was confirmed by in situ observation using blue-violet light illumination. Subsequently, the lucifer yellow dye was injected into the postsynaptic interneurones, and their gross morphology was also confirmed by in situ observation. The terminal ganglion was then removed from the abdomen and fixed in 10% formalin for 15 min, dehydrated in an ascending alcohol series and cleared in methyl salicylate. According to the position of cell bodies and their gross morphologies, these pre-motor nonspiking interneurones have been divided into two major groups of AL and PL types (Nagayama and Hisada 1987). The PL interneurones have cell bodies that are located in a posterior region of the terminal ganglion and extend main branches anteriorly. The AL interneurones have cell bodies that are located either in an antero-lateral or antero-medial portion of the ganglion, and have an arched configuration. The penetrated neurones were identified physiologically as nonspiking local interneurones according to criteria described elsewhere (Takahata et al. 1981). All physiological recordings were stored on a PCM data recording system for later analysis and display.

Results

Overview of synaptic interactions between nonspiking local interneurones

Synaptic interactions between nonspiking local interneurones were characterized in 13 pairs of successful simultaneous intracellular recordings in 55 crayfish. A further 2 pairs of the nonspiking interneurones were recorded but showed no significant synaptic interactions. Table 1 describes the types of interactions that occurred between these nonspiking local interneurones. Of the 13 recordings, two pairs of nonspiking interneurones formed excitatory connections, in which the postsynaptic interneurones were depolarized by the passage of depolarizing current injected into the presynaptic interneurones. The remaining 11 pairs of nonspiking interneurones formed inhibitory connections (11 out of 13 pairs) in which depolarizing current injected into the presynaptic nonspiking interneurone caused a hyperpolarization in the postsynaptic nonspiking interneurone. In the pairs of interneurones showing excitatory connections the interactions were bi-directional,

Table 1 Summary of synaptic interactions between nonspiking local interneurones

Output connection	One-way pathway	Bi-directional pathway
Excitatory	0	2 pairs AL ⇄ AL ? ⇄ ?
Inhibitory	10 4 PL → PL 2 PL → AL PL → ? AL → AL AL → PL ? → ?	1 pair PL ⇄ ?

AL antero-lateral, *PL* postero-lateral

whereas the interactions between interneurones with inhibitory connections were predominantly one-way (10 out of 11 pairs). Moreover, 12 presynaptic and 8 postsynaptic nonspiking interneurones were characterised by their morphologies following intracellular staining with lucifer yellow after physiological characterization. In the presynaptic interneurones, all PL interneurones ($n = 8$) had inhibitory outputs while 2 out of 4 AL interneurones had excitatory outputs (Table 1).

Inhibitory connections between nonspiking interneurones

One-way interactions

Figure 1 shows typical recordings of inhibitory connections between nonspiking interneurones using glass microelectrodes filled with 2 mol l^{-1} potassium acetate. Depolarizing current injected into the presynaptic nonspiking interneurone (int1 in Fig. 1) caused a sustained hyperpolarization of the membrane potential of the postsynaptic interneurone (int2 in Fig. 1A, panel 1). The inhibitory interactions between these nonspiking interneurones were graded and depended on the amplitude of depolarizing current injected into nonspiking int1 (Fig. 1A, panel 2). The greater the current injected into the presynaptic int1, the greater the amplitude of the response in the postsynaptic int2 (cf. Fig. 1A, panels 1 and 2). Neither depolarizing (Fig. 1B, panel 1) nor hyperpolarizing (Fig. 1B, panel 2) current injected into the postsynaptic int2 had any significant effect upon the membrane potential of the presynaptic int1. Ten out of 11 pairs of recordings showed similar one-way interactions. The remaining pair of interneurones showed bi-directional inhibitory interactions in which 1-nA depolarizing current injected into the presynaptic interneurone was sufficient to cause a hyperpolarization of the membrane potential of the postsynaptic interneurone. A much higher intensity of depolarizing current (more than 6 nA) injected into the postsynaptic interneurone was necessary to cause a change in the membrane potential of the presynaptic interneurone (not shown) suggesting an indirect effect from the post- to the presynaptic interneurone.

Chemical nature of inhibitory connections

The hyperpolarization in nonspiking interneurones (int2 in Fig. 2A) induced by depolarizing current injected into the presynaptic nonspiking interneurone (int1 in Fig. 2A) decreased in amplitude during the passage of

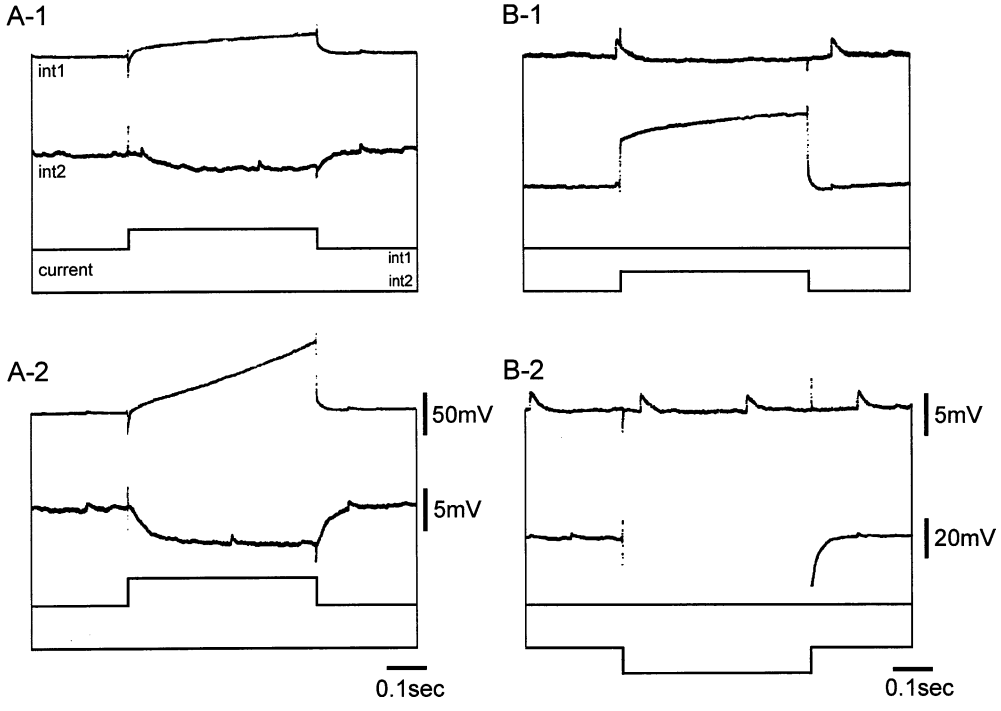

Fig. 1A, B Inhibitory connections between nonspiking interneurones. **A** Effect of current injection into nonspiking interneurone 1 (*int1*). Depolarizing current (3 nA in *panel 1* or 5 nA in *panel 2*) injected into the nonspiking interneurone 1 (*int1*) caused a membrane hyperpolarization of nonspiking interneurone 2 (*int2*). The amplitude of hyperpolarization of int2 depended on the intensity of current injected into int1. **B** Effect of current injection into nonspiking interneurone 2 (*int2*). Neither depolarizing (3 nA in *panel 1*) nor hyperpolarizing current (5 nA in *panel 2*) injected into nonspiking interneurone 2 (*int2*) caused any obvious change in membrane of nonspiking interneurone 1 (*int1*). Intracellular recordings were made with glass microelectrodes filled with 2 mol l^{-1} potassium acetate

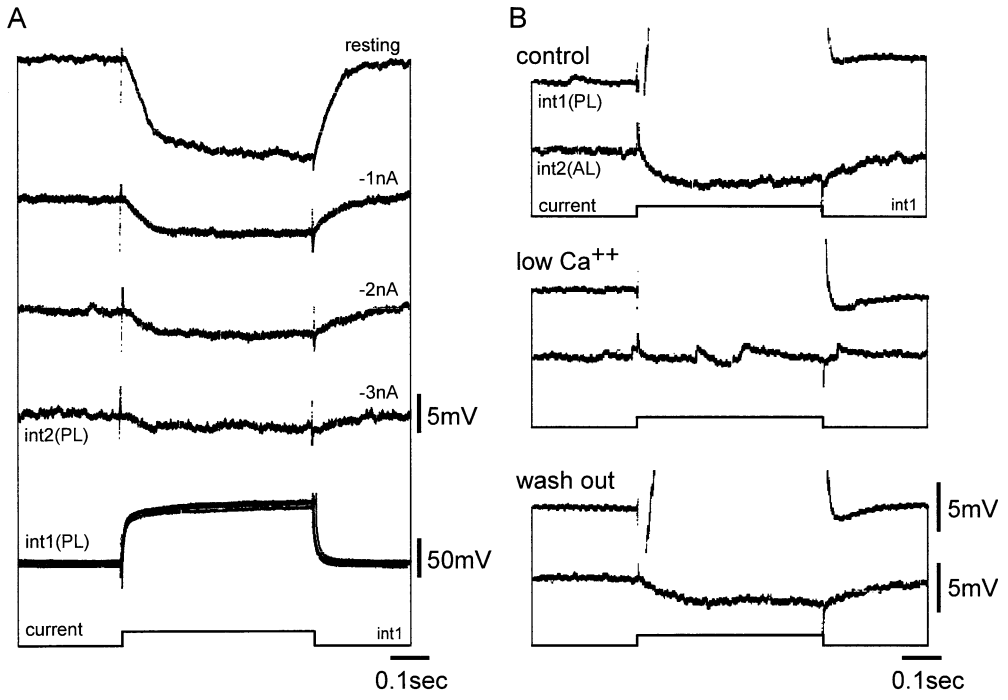

Fig. 2A, B Chemically-mediated synaptic transmission of inhibitory connections. **A** Effect of current injection. Depolarizing current (=5 nA) injected into the presynaptic PL interneurone 1 (*int1*) caused a membrane hyperpolarization of the postsynaptic PL interneurone 2 (*int2*). This membrane hyperpolarization was reduced in amplitude when the hyperpolarizing currents were injected into int2. **B** Effect of low-calcium/high-magnesium saline. Depolarizing current (=3 nA) injected into postero-lateral (PL) nonspiking interneurone 1 (*int1*) caused a membrane hyperpolarization of antero-lateral (AL) nonspiking interneurone 2 (*int2*, *top*). This int1-induced membrane hyperpolarization of int2 was abolished after 10 min in low-calcium (×1/5)/high-magnesium (×5) solution (*middle*). The response of int2 partly recovered after washing with normal saline for 15 min (*bottom*)

continuous hyperpolarizing current injected into the postsynaptic int2 (Fig. 2A). Thus when 3nA hyperpolarizing current was injected into int2, a depolarizing current injected into int1 evoked only a slight hyperpolarization of int2 suggesting that typical chemical synaptic transmission occurred between these nonspiking interneurones.

Furthermore, the membrane hyperpolarization of a nonspiking interneurone (int2 in Fig. 2B) induced by depolarizing current injected into the presynaptic nonspiking interneurone (int1 in Fig. 2B) gradually decreased in amplitude following the replacement of the external bathing solution from normal saline to one containing low-calcium (×1/5)/high-magnesium (×5) (Fig. 2B). In normal saline, int2 was hyperpolarized by the passage of 3 nA depolarizing current into int1 (top in Fig. 2B). This membrane hyperpolarization of int2 was abolished after 10 min in low-calcium solution (middle in Fig. 2B). Following washing with normal saline for 15 min, the response of int2 gradually recovered, with current injected into int1 again causing a hyperpolarization of int2 (bottom of Fig. 2B).

Functional pathways of inhibitory nonspiking local interneurones

In six pairs of recordings, the responses of interneurones to electrical stimulation of the second nerve root sensory neurones were also characterized. In all cases, the presynaptic interneurones were PL interneurones that received excitatory postsynaptic potentials (epsps) from the sensory afferents. These PL interneurones made inhibitory connections with both AL interneurones (e.g., Fig. 3) and other PL interneurones (e.g., Fig. 4), and the postsynaptic interneurones received inhibitory postsynaptic potentials (ipsps) in response to sensory stimulation. For example, a depolarizing current injected into a PL interneurone (int1 in Fig. 3A) caused a membrane hyperpolarization of an AL interneurone (int2 in Fig. 3A). By contrast, depolarizing current injected into int2 induced no significant change in membrane of int1, although it excited the closer, reductor motor neurone (Fig. 3B). When the sensory bundle of the 2nd nerve root was stimulated electrically, the presynaptic PL interneurone received excitatory sensory inputs while the postsynaptic AL interneurone received a short latency excitatory inputs then received inhibitory inputs (Fig. 3C, panel 1). This short latency epsp of the AL interneurone derived from direct excitatory inputs from the sensory afferents (Nagayama 1997). When a 1 nA hyperpolarizing current was injected into the presynaptic PL interneurone in advance, the ipsps evoked in the AL interneurone, in response to the sensory stimulation, were reduced in amplitude (Fig. 3C, panel 2). Since this PL interneurone received epsps and made an inhibitory connection with the postsynaptic AL interneurone, the presynaptic PL interneurone must contribute, at least in part, to the ipsps of the postsynaptic AL interneurone.

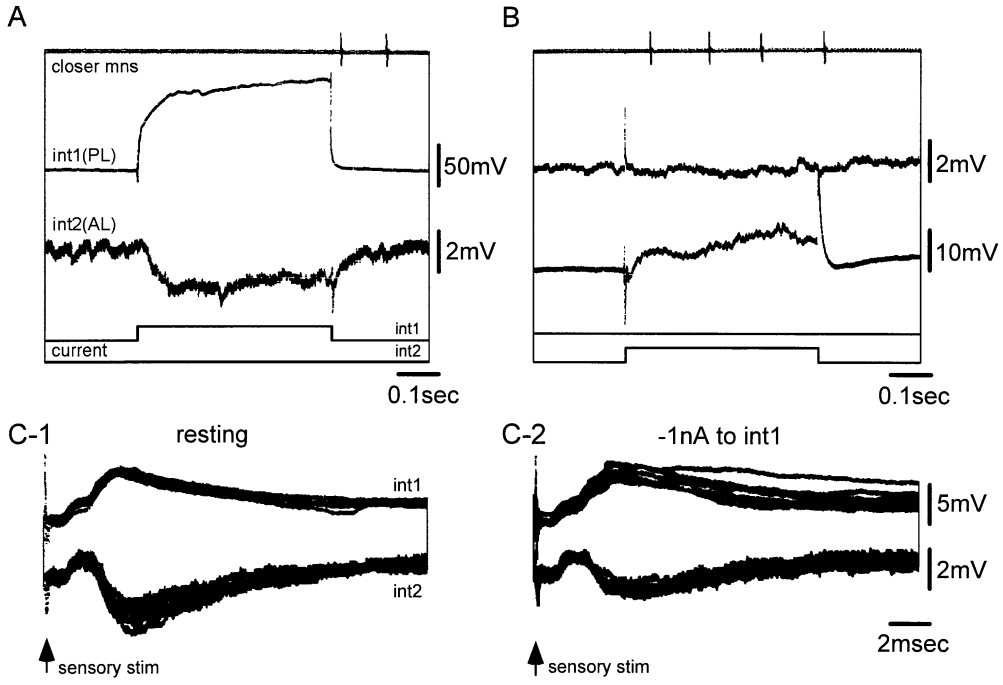

Fig. 3A–C Inhibitory connections between the PL and the AL nonspiking interneurones. **A** Depolarizing current (=2 nA) injected into a PL nonspiking interneurone (*int1*) caused a membrane hyperpolarization of an AL nonspiking interneurone (*int2*). **B** Depolarizing current (=5 nA) injected into int2 caused a spike discharge of the closer, reductor motor neurone but no obvious change in the membrane of int1. **C** Response of the nonspiking interneurones to the electrical stimulation of the sensory afferents. The PL interneurone, int1 received excitatory postsynaptic potentials (epsps), while the AL interneurone, int2 received both epsps and inhibitory postsynaptic potentials (ipsps) when the sensory afferents were stimulated electrically (*panel 1*). Sensory-evoked ipsps in int2 were reduced in amplitude when int1 was hyperpolarized, in advance, by current injection

Depolarizing current injected into a different PL interneurone (int1 in Fig. 4A) caused a membrane hyperpolarization of a second PL interneurone (int2 in Fig. 4A). At the same time, the activity of the closer, reductor motor neurone increased as a consequence of this current injection (top trace in Fig. 4A). The postsynaptic PL interneurone had no obvious output effect on the presynaptic PL interneurone, but did affect the activity of the reductor motor neurone. Depolarization of int2 inhibited the reductor motor neurone (not shown), and hyperpolarization of int2 excited the motor neurone (Fig. 4B). This could be explained if we assume that int2 released inhibitory transmitter continuously at its resting level, and hyperpolarization decreased the release of transmitter. Since int1 received epsps while int2 received ipsps when the sensory afferents were stimulated electrically (Fig. 4C), and the passage of depolarizing current into int1 caused a membrane hyperpolarization of int2 (Fig. 4A), the presynaptic int1 must again contribute to the ipsps in the postsynaptic int2. Furthermore, the excitatory effect of int1 upon the reductor motor neurone could occur through an indirect interaction with int2.

Our physiological results suggest that nonspiking local interneurones occupy at least two layers in the local circuit for uropod motor pattern formation. Nonspiking interneurones within the first layer receive excitatory inputs from sensory afferents and make inhibitory connections to interneurones of a second layer that provide inhibitory inputs of sensory information.

Excitatory connections between nonspiking local interneurones

Figure 5 shows typical recordings of a excitatory connection between nonspiking interneurones. Depolarizing current injected into a presynaptic AL interneurone (int1 in Fig. 5A, panel 1) induced a membrane depolarization of a postsynaptic AL interneurone (int2 in Fig. 5A, panel 1). The interaction between these interneurones was bidirectional since a depolarization of the postsynaptic int2 also caused a membrane depolarization in the presynaptic int1 (Fig. 5B, panel 1). On the other hand, hyperpolarizing current injected into int1 caused a membrane hyperpolarization of int2 (Fig. 5A, panel 2), and similarly hyperpolarizing current injected into int2 hyperpolarized int1 (Fig. 5B, panel 2). The amplitude of the membrane depolarization or hyperpolarization in the nonspiking interneurones, induced by presynaptic current injection, was not changed significantly by the amplitude of hyperpolarizing current injected into the postsynaptic interneurones, in advance (Fig. 5A, panel 3 and B, panel 3).

Discussion

We found in this study that the majority of nonspiking interneurones recorded in the terminal ganglion formed

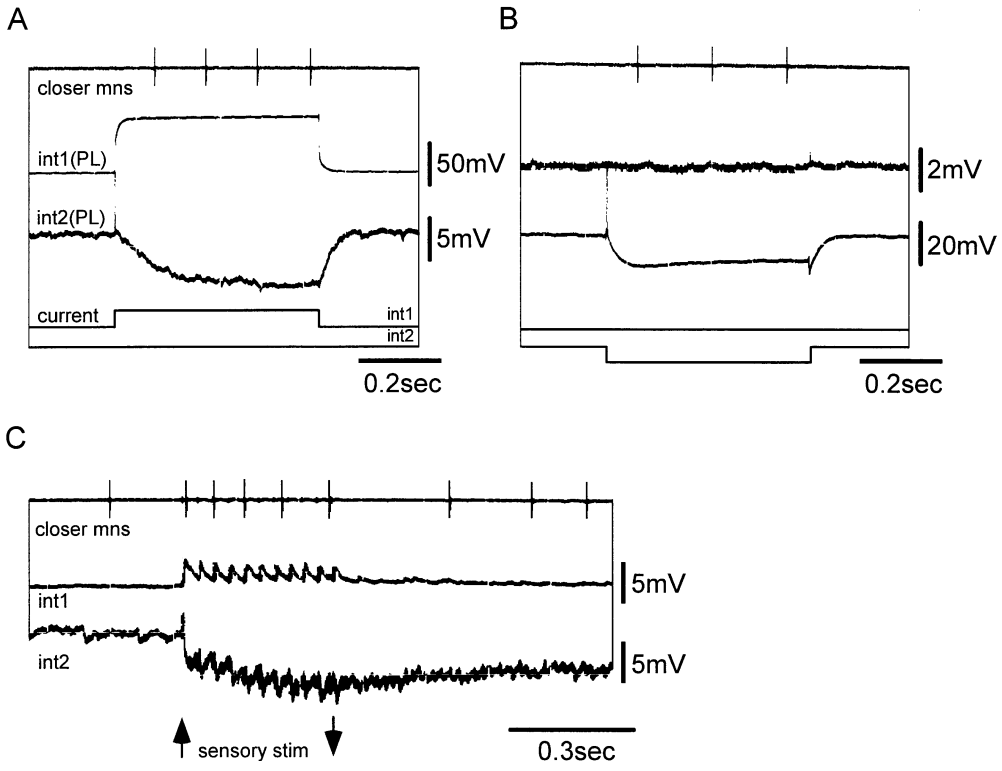

Fig. 4A–C Inhibitory connections between two PL nonspiking interneurones. **A** Depolarizing current (=3 nA) injected into the presynaptic PL interneurone, int1 caused a membrane hyperpolarization of the postsynaptic PL interneurone, int2 with an increase in the spike frequency of the closer, reductor motor neurone. **B** Hyperpolarizing current (=1 nA) injected into int2 caused a discharge of spikes of the closer, reductor motor neurone but no obvious change in the membrane potential of int1. **C** Response of the nonspiking interneurones to the sensory stimulation. The presynaptic PL interneurone, int1 received epsps while the postsynaptic PL interneurone (*int2*) received ipsps

one-way inhibitory synaptic connections with each other, although a small number of interneurones formed bi-directional excitatory connections.

Synaptic organization of nonspiking interneurones

The one-way inhibitory interactions between nonspiking interneurones were likely to be mediated by chemical synaptic transmission, since the membrane hyperpolarization mediated by a presynaptic nonspiking interneurone was reduced in amplitude when hyperpolarizing current was injected into the postsynaptic nonspiking interneurone. In addition, the membrane hyperpolarization mediated by a presynaptic nonspiking interneurone was gradually reduced in amplitude under bath application of low calcium saline. Furthermore, immunocytochemical studies have indicated that many nonspiking interneurones in the terminal ganglion are GABAergic (Nagayama et al. 1997). The hyperpolarization of the postsynaptic nonspiking interneurones occurred with a short latency following injection of a depolarizing current into the presynaptic nonspiking interneurones suggesting a direct inhibitory connection between nonspiking interneurones. These observations are consistent with the work of Burrows (1979) that demonstrated a one-way inhibitory interaction between nonspiking interneurones in the locust metathoracic ganglion.

One of the contrasts between the locust and the crayfish nonspiking interneurones was the existence of excitatory connections between crayfish nonspiking interneurones. Although the probability of encountering a excitatory connection was low (2 out of 13 connections), the depolarization of some AL interneurones caused a depolarization of another AL interneurones. The output effects were bi-directional so that a depolarization of the postsynaptic interneurones also caused a depolarization of the membrane potential of the presynaptic interneurones. Since the injection of hyperpolarizing current into the postsynaptic interneurones had no effect upon the amplitude of membrane potential change induced by the presynaptic interneurones, their synaptic interactions are not likely to be mediated by typical chemical synapses, but instead through electrical coupling. In this study, we could not characterize the synaptic interactions between excitatory nonspiking interneurones under low calcium solution since stable and long duration simultaneous intracellular recording from two nonspiking interneurones was extremely difficult. In mammalian retina, however, many retinal neurones are found to make electrical connections following the intracellular injection of biotinylated compounds, biocytin and Neurobiotin (Vaney 1991). The possibility of electrical coupling between excitatory nonspiking interneurones could, therefore be examined in the future by intracellular staining with Neurobiotin and subsequent ultrastructural analysis.

Functional pathways of inhibitory nonspiking interneurones

The finding of one-way inhibitory interactions between nonspiking interneurones suggests that nonspiking

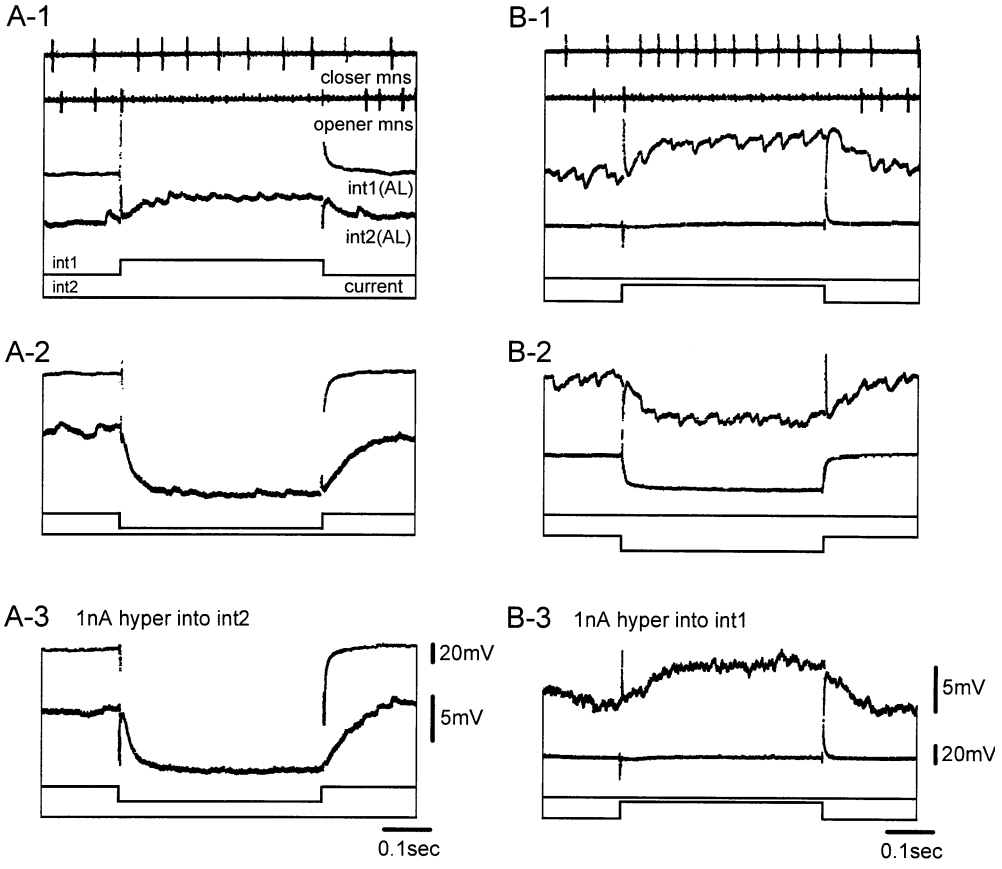

Fig. 5A, B Excitatory connections between two AL nonspiking interneurones. **A** Effect of current injection into nonspiking interneurone 1 (*int1*). Depolarizing (3 nA in *panel 1*) or hyperpolarizing current (3 nA in *panel 2*) injected into int1 caused a membrane depolarization (*panel 1*) or hyperpolarization (*panel 2*) of the nonspiking interneurone 2 (*int2*). The membrane hyperpolarization of int2, induced by 3 nA hyperpolarizing current injected into int1, was not changed significantly when 1 nA hyperpolarizing current was injected into int2 (*panel 3*). **B** Effect of current injection into nonspiking interneurone 2 (*int2*). Depolarizing (3 nA in *panel 1*) or hyperpolarizing current (3 nA in *panel 2*) injected into int2 caused a membrane depolarization (*panel 1*) or hyperpolarization (*panel 2*) of the nonspiking interneurone 1 (*int1*). The membrane hyperpolarization of int1, induced by 3 nA depolarizing current injected into int2, was not changed significantly when 1 nA hyperpolarizing current was injected into int1 (*panel 3*)

interneurones occupy at least two layers within the local circuits for uropod motor control. Nonspiking interneurones that received excitatory inputs from exteroceptive afferents always made inhibitory connections with other nonspiking interneurones. Electrical stimulation of sensory afferents always evoked ipsps in the postsynaptic nonspiking interneurones. The sensory-evoked membrane hyperpolarization of the postsynaptic nonspiking interneurones was modified in amplitude by the manipulation of presynaptic nonspiking interneurones with current injection. These results indicate that the presynaptic nonspiking interneurones act as signal inverters for the postsynaptic nonspiking interneurones. Within the terminal ganglion, spiking local interneurones are known to receive excitatory sensory inputs and make inhibitory output connections with nonspiking interneurones (Nagayama 1997). At present the functional difference between spiking local interneurones and nonspiking interneurones as signal inverters remains unclear, but one possible difference is the transmitter they would release. Although GABA is an inhibitory transmitter released from nonspiking interneurones, spiking local interneurones are not GABAergic (Aonuma and Nagayama 1999) and some of them would release glutamate as an inhibitory transmitter (Nagayama et al. 2004). Further physiological, pharmacological and neurochemical studies are therefore necessary to understand in detail the functional pathways of this local circuitry.

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