Inhibitory Component of the Resistance Reflex in the Locomotor Network of the Crayfish

MORGANE LE BON-JEGO AND DANIEL CATTAAERT
Laboratoire de Neurobiologie des Réseaux, United Mixte de Recherche 5816, Centre National de la Recherche Scientifique, Université Bordeaux I, Biologie Animale, Bât B2, 33405 Talence Cedex, France

Received 11 March 2002; accepted in final form 22 July 2002

Le Bon-Jego, Morgane and Daniel Cattaert. Inhibitory component of the resistance reflex in the locomotor network of the crayfish. J Neurophysiol 88: 2575–2588, 2002; 10.1152/jn.00178.2002. The aim of this study was to investigate the inhibitory components of a resistance reflex in the walking system of the crayfish. This study was performed using an in vitro preparation of several thoracic ganglia including motor nerves and the proprioceptor that codes movements of the second joint (coxobasipodite chordotonal organ—CBCO). Sinusoidal movements were imposed on the CBCO, and intracellular responses were recorded from levator (Lev) and depressor (Dep) motoneurons (MNs). We found that in MNs that oppose the imposed movements (e.g., the Lev MNs during the imposed downward movement), the response consists in a depolarization resulting from the summation of excitatory postsynaptic potentials (EPSPs). A movement in the opposite direction resulted in hyperpolarization during which inhibitory postsynaptic potentials (IPSPs) summed. The inhibitory pathway to each MN is oligosynaptic (i.e., composed of a small number of neurons in series) and involves spiking interneurons. Because it was blocked in the presence of a high-divalent cation solution. The IPSPs were mediated by a chloride conductance because their amplitude was sensitive to the chloride concentration of the bathing solution and because they were blocked by the chloride channel blocker, picrotoxin. Resistance reflex IPSPs related to single CBCO neurons could be identified. These unitary IPSPs were blocked in the presence of 3-mercapto-propionic acid, an inhibitor of gamma-aminobutyric acid (GABA) synthesis, indicating that they are mediated by GABA. In addition to this GABAergic pathway, electrical stimulation of the CBCO sensory nerve induced compound IPSPs that were blocked by glutamate pyruvate transaminase (GPT), indicating the presence of glutamatergic inhibitory pathways. These glutamatergic interneurons do not appear to be involved in the resistance reflex, however, as GPT did not block the unitary IPSPs. Functionally, the resistance reflex is mainly supported by movement-coding CBCO sensory neurons. We demonstrate that such movement-coding CBCO neurons produce both monosynaptic EPSPs in the MNs opposing imposed movements and oligosynaptic IPSPs in the antagonistic motoneurons. These results highlight the similarities between the inhibitory pathways in resistance reflex of the crayfish and in the stretch reflex of vertebrates mediated by Ia inhibitory interneurons.

INTRODUCTION

Stretch reflexes in vertebrates and resistance reflexes in arthropods constitute the most widespread proprioceptive feedback systems in joint motor control (Capaday 2000; Clarac et al. 2000). They result in the monosynaptic activation of the motoneurons (MNs) that oppose any imposed joint movement. During the vertebrate stretch reflex, two parallel pathways process proprioceptive information produced by imposed movement: while antagonist MNs are activated by the proprioceptors, agonist MNs are inhibited via Ia interneurons (Jankowska and Roberts 1972). This inhibitory pathway has been termed reciprocal Ia inhibition.

Inhibitory pathways in resistance reflexes have also been demonstrated in several invertebrate motor systems such as those controlling the crayfish uropod (Nagayama and Hisada 1987), leech bending (Lockery and Kristan 1990a,b), and locust joint control (Büschges 1990; Büschges and Wolf 1995). The wiring and the function of these inhibitory pathways appear to differ, however, from the reciprocal Ia inhibition described in mammals. For example, movement of the femoro-chordotonal organ in resting stick insects evokes both excitatory and inhibitory responses in extensor tibiae MNs (Bässler 1993). Moreover, the inhibitory pathways involved in resistance reflexes in insects include both spiking and nonspiking interneurons. The wiring of stretch reflexes in insects is likely responsible for the variability of postural reflexes seen in these animals. Indeed not only the amplitude but also the sign of the reflex can change (reflex reversal) (Bässler 1986). This amazing flexibility results from competition between parallel excitatory and inhibitory pathways between sensory neurons and motoneurons. Therefore it seems that the inhibitory interneurons described in the resistance reflex pathways of insects play a different role from that of vertebrate Ia interneurons, which seem to be devoted to resistance but not assistance reflexes.

Among the different animal models that have been used to examine the mechanisms involved in the proprioceptive control of movement and position, the crayfish walking system has proved to be particularly amenable and fruitful. In this system, a considerable amount of data has been obtained on the organization of a resistance reflex involving the proprioceptor of the second joint of the leg (the coxo-basipodite chordotonal organ—CBCO) and the MNs governing this same joint (Clarac et al. 2000). The organization of this resistance reflex (see Fig. 1C) appears to be closer to the vertebrate than to the insect model (Clarac et al. 2000). Such similarities were demonstrated in the case of the excitatory components of the resis-
In the present study, we demonstrate that in the resting crayfish locomotor network, imposed movements of the CBCO elicit excitatory postsynaptic potentials (EPSPs) in MNs that would oppose the imposed movement and inhibitory postsynaptic potentials (IPSPs) in antagonistic MNs. Unitary EPSPs and IPSPs were related to single CBCO sensory neurons. Inhibitory synapses on the MNs are mediated by GABA and seem to include nonspecific GABA/Glu receptors linked to chloride channels similar to those described previously in crayfish MNs (Pearlstein et al. 1994).

**Methods**

Experiments were performed on adult male and female crayfish (*Procambarus clarkii, n = 59*). The animals were purchased from a commercial supplier (Chateau Garreau, Landes, France) and maintained in indoor aquaria at 15°C.

**In vitro preparation**

The in vitro preparation (Chrachri and Clarac 1989; Sillar and Skorupski 1986) consisted of the last three thoracic (T3–T5) ganglia and the 1st abdominal ganglion (A1) dissected together with motor nerves of the proximal muscles (Pro, promotor; Rem, remotor; Lev, levator; and Dep, depressor) and the CBCO, a proprioceptor that encodes the vertical movements of the leg. A mechanical puller allowed us to reproduce physiological stimulations applied to the CBCO strand, mimicking the vertical movements (Mvt) of the leg. Single or paired intracellular recordings from CBCO sensory terminals or motoneurons were performed within the neuropile with glass microelectrodes (ME).

**Solutions**

The saline used contained (in mM) 195 NaCl, 5 KCl, 13 CaCl2, and 2 MgCl2. In some experiments, the thoracic ganglia were superfused with saline in which the concentration of divalent cations was increased ([in mM] 34 CaCl2, 6.4 MgCl2, with the sodium concentration reduced accordingly) to raise the spiking threshold of the central neurons without affecting the coding properties of the CBCO sensory neurons. The use of this altered saline allowed us to identify the monosynaptic reflex responses (Berry and Pentreath 1976). The composition of saline with low levels of chloride was (in mM) 195 Na Propionate, 5 KCl, 13 CaCl2, and 2 MgCl2. Saline solutions were buffered with 3 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and the pH adjusted to 7.65 at 15°C.

**Drug applications**

Small amounts of y-amino-butyric acid (GABA, 10 mM) or L-glutamate (10 mM) dissolved in saline were pressure-ejected at the same site within the neuropile through a double-barrelled micropipette using a Picospritzer (General Valve, Fairfield, NJ). The tip of the

FIG. 1. Sensory-motor organization of the 2nd leg joint. **A**: location of the coxo-basipodite chordotonal organ (CBCO) in the crayfish walking leg. **B**: the in vitro preparation of the crayfish thoracic locomotor system consists of thoracic ganglia 3–5 (T3–T5) and the 1st abdominal ganglion (A1) dissected together with motor nerves of the proximal muscles (Pro, promotor; Rem, remotor; Lev, levator; and Dep, depressor) and the CBCO, a proprioceptor that encodes the vertical movements of the leg. A mechanical puller allowed us to reproduce physiological stimulations applied to the CBCO strand, mimicking the vertical movements (Mvt) of the leg. Single or paired intracellular recordings from CBCO sensory terminals or motoneurons were performed within the neuropile with glass microelectrodes (ME). **C**: schematic diagram of the sensory-motor pathways involved in the resistance reflex. During downward movements imposed to the leg (1), stretch-sensitive CBCO neurons (filled circles) monosynaptically excite the levator motoneurons (Lev MNs); the connection is represented by the thick black line. Similarly, upward movements (2) imposed to the leg, activate the release-sensitive CBCO neurons (open circles), which monosynaptically excites the depressor motoneurons (Dep MNs); the connection is represented by the thick white line. In this report, we describe a new pathway (dashed elements) in the resistance reflex triggered by CBCO sensory neurons in Dep and Lev MNs. During imposed downward leg movements (1), which activate the Lev MNs, this new oligosynaptic pathway inhibits the Dep MNs. LEV, levator muscle; DEP, depressor muscle.
ejection pipette usually was positioned close to the site of intracellular recording. Picrotoxin (PTX, 40 μM), 3-mercaptopropionic acid (3-MPA, 5 mM), and glutamate pyruvate transaminase (GPT, 30 U in 100 ml), were freshly dissolved in saline and bath applied. All chemicals were obtained from Sigma (St. Louis, MO).

Electrodes and recordings

Extracellular recordings were performed using stainless steel pin electrodes contacting the nerves and isolated from the bath with Vaseline. Recorded signals were amplified by differential AC amplifiers (Grass, Quincy, MA, gain of 10,000–100,000 times). Intracellular recordings from MNs and CBTs were performed within the ganglion, using micropipettes filled with potassium-acetate (2 M, 30–40 MΩ). An “Axoclamp 2B” amplifier (Axon Instruments, Foster City, CA) was used in current-clamp mode. The level of the saline was kept as low as possible to reduce the micropipette capacitance. The MNs and the CBTs were identified using the following criteria: 1) the spike evoked by electrical stimulation of an identified nerve could be recorded by the intracellular microelectrode; 2) there was a one-to-one correlation between the intracellular spikes and extracellular spikes recorded from the corresponding nerve during spontaneous activity; and 3) intracellular injection of depolarizing current into the intracellularly recorded neuron evoked spikes that were correlated one-to-one with extracellular spikes recorded in the corresponding nerve. An eight-channel stimulator (AMPI, Jerusalem, Israel) was used for intracellular stimulation of MNs during the identification procedure. All signals were monitored on a four-channel digital oscilloscope (Yokogawa DL 1200, Tokyo, Japan) and digitized at 15 kHz on a PC-based computer through an A/D interface (CED 1401PLUS, Cambridge Electronic Design, Cambridge, UK) and written to disk.

Analysis

Physiological signals were recorded and analyzed with the CED SPIKE2 program. The SPIKE2 spike sorting tool was used to identify extracellularly recorded CBCO neurons. Spikes in the CBCO nerve were discriminated according to their waveform based on a template matching protocol. After the completion of this protocol, each CBCO unit was assigned an arbitrary number. Subsequently, it was possible to determine which sensory neuron connected a given intracellularly recorded levator motoneuron (Lev MN) or depressor MN (Dep MN; see Figs. 4, 6, 7, 9, 11, and 12).

The results are given as mean values ± SE. Two-way ANOVA was used to assess the statistical significance of the effects of drugs on the relationship between injected current and IPSP amplitude (Fig. 8). In other cases, a Tukey’s multiple comparison test following a one-way ANOVA was used to assess the significance of the effect of different conditions (control, GPT, 3-MPA, wash, etc.) on a single variable (PS amplitude). The Student’s t-test was used to assess statistical significance when only two conditions were compared.

RESULTS

The results presented here are based on intracellular recordings from 281 identified MNs and 11 identified CBTs and 59 extracellular recordings of the CBCO sensory nerve.

Evidence for an inhibitory pathway participating in the resistance reflex

The sensory-motor organization of the pathways involved in the resistance reflex of the CB joint (El Manira et al. 1991a) is presented in Fig. 1C. The CBCO strand is innervated by two types of sensory neurons coding, respectively, for stretch (see Fig. 1C, filled circles) and release (see Fig. 1C, open circles) movements of the strand. In a preparation that does not display rhythmic activity, downward movements (1) imposed to the leg activate stretch-sensitive CBCO neurons (Fig. 1C, filled circles) which monosynaptically excite the Lev MNs (this pathway is represented by the thick black line). Similarly, upward movements (2) imposed to the leg, activate the release sensitive CBCO neurons (Fig. 1C, open circles) which monosynaptically excite the Dep MNs (the connection is represented by the thick white line). In this report, we describe an additional oligosynaptic pathway between the CBCO sensory neurons and the Dep and Lev MNs that is part of the resistance reflex (see dashed elements). This pathway is activated during imposed downward leg movements (1) and inhibits the Dep MNs. A similar pathway inhibits the Lev MNs during upward movements imposed to the leg (not represented).

This resistance reflex is presented in Fig. 2. During sinusoi-
dual mechanical stimulation of the CBCO strand, dual intracellular recordings from a Dep MN and a Lev MN (Fig. 2A) displayed resistance reflex responses (Fig. 2B). Stretching the CBCO strand elicited depolarization in the Lev MN, whereas releasing the CBCO strand evoked depolarization in the Dep MN. Between two depolarizing responses both of the MNs repolarized. Indeed during the repolarizing phase, the MNs were hyperpolarized (the resting membrane potential before application of movement is indicated with a dashed line). This hyperpolarization was due to inhibitory postsynaptic potentials (IPSPs; see following text). The resting membrane potentials of the Lev and Dep MNs presented in Fig. 2B were, respectively, −62 and −68 mV in the absence of movement.

The existence of inhibitory events in the resistance reflex was apparent in phasic response MNs (i.e., MNs which respond exclusively during imposed movements) (Le Ray et al. 1997a) when ramp-and-hold protocols were used (Fig. 3A). During release ramp movements of the CBCO, intracellular recordings from a Dep MN displayed depolarizing reflex responses (Fig. 3A, *), whereas during stretch ramp movements, the Dep MN was hyperpolarized (see Fig. 3A, ●). After each ramp movement, the membrane potential rapidly returned to its resting level (−62 mV, see Fig. 3, dashed line).

In some intracellular recordings from the MNs, IPSPs were not observed in response to either ramp-and-hold or sinewave protocols. Figure 3B shows such an intracellular recording from a Dep MN that displayed depolarizing reflex responses when the CBCO strand was released and repolarized during CBCO stretch. During stretch movements, the Dep MN did not display any hyperpolarization below its resting membrane potential before application of movement (see Fig. 3B, dashed line). In this case, the resting membrane potential before the application of movement was −77 mV. This value is close to the equilibrium potential for chloride ions (see Fig. 7B) and therefore the occurrence of IPSPs could have been masked. This hypothesis was tested by injecting depolarizing current (+5 nA) into the Dep MN. During the injection of depolarizing current into the Dep MN (Fig. 3C), the positive deflection of membrane potential due to a volley of EPSPs during release of the CBCO strand persisted. Such EPSPs are particularly pre-

![Fig. 3. Demonstration of the existence of an inhibitory pathway in the resistance reflex. A: resistance reflex response to a ramp-and-hold movement imposed to the CBCO recorded intracellularly in a Dep MN. The Dep MN is depolarized during the release ramp (*) and hyperpolarized during the stretch movement (●). The horizontal dashed line indicates the resting membrane potential in the absence of movement (−62 mV). B: an intracellular recording from a Dep MN displays resistance reflex depolarizing responses during release of the CBCO strand and repolarizes during CBCO stretch. During both responses only depolarizing postsynaptic potentials were observed (see enlarged views 1–3 on the right corresponding to onset of release, onset of stretch, and end of stretch, respectively). *, excitatory postsynaptic potentials (EPSPs); ●, depolarizing inhibitory postsynaptic potentials (dIPSPs). C: when a continuous depolarizing current (+5 nA) was injected in the Dep MN, the amplitude of its response to imposed movement was increased. This increase was mainly due to the occurrence of large IPSPs during the end of the stretch movement (see 3, right, ●). Those IPSPs were also present in B but were reversed (see B, 3, ▲). During the onset of the release movement, (1) EPSPs (+) persisted during the injection of depolarizing current and were analogous to those observed in the absence of current injection (see B, 1). During the onset of the stretch movement, both EPSPs and IPSPs were present (2).]
ponderant at the beginning of the release movement (1) (see * in Fig. 3, right). In addition, the amplitude of the repolarizing phase (during stretch movements) was increased (Fig. 3C) and was clearly a hyperpolarization below the resting potential (see dashed line). This hyperpolarization of membrane potential was due to the presence of IPSPs (see Fig. 3C, right, ●), which were the only events present during the last part of the stretch movement (Fig. 3C, 3). In the absence of current injection in the Dep MN, these IPSPs occurred as depolarizing events (dIPSP; see Fig. 3B, right, △). Therefore during the application of a sinusoidal mechanical stimulation to the CBCO, the MN response consisted of an alternation of EPSPs and IPSPs. We conclude that in this MN IPSPs occurred as depolarizing events in the absence of current injection (see Fig. 3C, right, ●) due to the resting membrane potential of this MN being very hyperpolarized at rest ($V_m = -77$ mV). Note that the early part of the stretch movement response (Fig. 3C, 2) consists of a mixture of EPSPs and IPSPs.

To find a relationship between the IPSPs and firing activity in the CBCO nerve, we used the following procedure. The different sensory spikes in the CBCO nerve were identified and classified according to their size and shape using the Spike 2 spike-sorting program. Subsequently, we attempted to correlate each spike shape (unit) in the CBCO nerve with IPSPs in the intracellularly recorded MN. The result of one such analysis is shown in Fig. 4. In this experiment, it was possible to precisely identify two CBCO units that evoked an IPSP in an intracellularly recorded Dep MN (see Fig. 4A, top). One large unit (Fig. 4A, left) evoked a $-0.168 \pm 0.010$ (SE) mV IPSP (see mean trace), and a smaller unit (Fig. 4A, right) evoked a $-0.197 \pm 0.014$ mV IPSP. Both of these units were activated during stretch of the CBCO strand (Fig. 4B). The IPSPs evoked in the Dep MN during downward movements would therefore contribute to the resistance reflex.

The delay between a sensory spike in the neuropile and its triggered IPSP recorded from the MN was measured by performing paired intracellular recordings from a CBT and a Dep MN. In one such experiment (Fig. 5), the intracellularly recorded CBT was activated during CBCO stretch, whereas the intracellularly recorded Dep MN displayed a typical resistance reflex (the neuron was activated during release of the CBCO strand and repolarized during stretch—Fig. 5A). The bursts of CBT spikes therefore occurred during the hyperpolarization of the Dep MN produced during the stretch movement (Fig. 5A).

Moreover, it appeared that CBT spikes were associated with IPSPs in the Dep MN (Fig. 5B). In such experiments, we used the following procedure to measure the synaptic delay. The CBT spikes were used as a trigger to perform spike trigger averaging of the Dep MN intracellular recording (Fig. 5C, average of 50 traces). A nonlinear regression procedure was then applied to the first 10 ms of the averaged IPSP (1 exponential decay), and the intersection between this curve and the baseline was used to define the onset of the IPSP. In the experiment presented in Fig. 5, the delay between the onset of the CBT spike and the onset of the Dep MN IPSP was 3.86 ms, suggesting that this inhibitory pathway of the resistance reflex was not monosynaptic and most likely involved only one intermediate interneuron.

**Evidence that the resistance reflex inhibitory pathway is polysynaptic**

As demonstrated previously (El Manira et al. 1991a), electrical stimulation of the CBCO nerve evokes complex responses in walking leg MNs, consisting of an early (3- to 5-ms delay, corresponding to spike conduction along the nerve) compound EPSP (see Fig. 6A, *) that is monosynaptic and a late (5- to 15-ms delay) compound IPSP (see Fig. 6A, ●) that appears to be polysynaptic. We have confirmed that the late compound IPSP is polysynaptic by using a high-frequency stimulation test (Fig. 6A). The frequency of the paired pulses applied to the CBCO nerve was progressively increased until the second IPSP began to fail. The MN responded one-to-one to the stimulus $\lesssim 83$ Hz (see Fig. 6A, ▼). At this frequency, however, some failures were observed for the second stimulus (▲) but not for the first stimulus (●) of the pair. Further increases in the frequency of the stimuli resulted in the complete failure of the second stimulus. This result indicates that the compound IPSP evoked by electrical stimulation of the CBCO sensory nerve is polysynaptic. This result was confirmed in experiments in which the ganglion was perfused with...
a high divalent cation saline (Fig. 6B). In such experiments, the compound IPSP evoked in the MN by the electrical stimulation of the CBCO nerve was suppressed.

The preceding results indicate that the compound IPSPs evoked in a MN by electrical stimulation of the CBCO nerve are polysynaptic. This suggests that the IPSPs involved in the resistance reflex are also polysynaptic. This was confirmed by an analysis of resistance reflex unitary IPSPs. In all experiments in which IPSPs were correlated with an identified CBCO unit recorded from the CBCO nerve (see METHODS and Fig. 4), the delay between the sensory spike and the corresponding IPSP varied. For example in Fig. 6C, six intracellular recordings from a Dep MN triggered by an identified CBCO spike (↑) are superimposed. In all six traces the CBCO spike evoked an IPSP in the Dep MN. The latencies of the IPSPs, however, fluctuated between 4.4 and 5.6 ms (see Fig. 6C, inset).

In conclusion, the resistance reflex inhibitory pathway is not monosynaptic. The pathway is most likely oligosynaptic as the delay between the spike in the CBCO nerve and the evoked IPSP fluctuates within a narrow range (over all experiments the amplitude of these variations was <1.2 ms) and IPSPs evoked by electrical stimulation of the CBCO nerve follow-up to a rather high-frequency (>80 Hz).

** Ionic mechanisms of the resistance reflex IPSPs**

To estimate the reversal potential of the resistance reflex IPSPs, the amplitude of these IPSPs recorded from the postsynaptic depolarization of the target MN was measured between the onset of the CBT spike and the onset of the IPSP with an arrow. The depolarization of the target MN was suppressed by 10.22 ± 0.33 mV.

** FIG. 5.** Paired intracellular recordings from a CBCO terminal and a Dep MN involved in an inhibitory resistance reflex pathway. A: schematic representation of the 5th thoracic ganglion showing the position of the 2 intracellular electrodes recording from a CBCO terminal (CBT) and a Dep MN. During these recordings, sinusoidal movements (Mvt) were imposed to the CBCO strand. The CBCO terminal was activated during the stretch movement (downward movement of the leg in intact animal) and silent during the release movement. The Dep MN was activated during the release (upward movement of the leg in the intact animal) and therefore participated in a resistance reflex. During the stretch movement, the Dep MN was slightly hyperpolarized. Its resting membrane potential in the absence of movement (~59 mV) is indicated with an arrow. B: enlarged traces of the intracellular recordings of the Dep MN and CBT showing that each spike in the CBT was associated with an IPSP in the Dep MN. C: spike trigger averaging (n = 40) from the CBCO terminal and the Dep MN presented in B and demonstrating the existence of an IPSP evoked in the Dep MN by the CBCO spike. A nonlinear regression procedure (1 exponential decay: $\tau = 2.63 \text{ ms}; r^2 = 0.987$) was applied to the 1st 10 ms of the IPSP to estimate precisely the onset of the IPSP, defined as the intersection between the baseline and the fitted curve. The synaptic delay (3.86 ms) was then measured between the onset of the CBT spike and the onset of the IPSP in the Dep MN.

** FIG. 6.** Demonstration that the inhibitory resistance reflex pathway is oligosynaptic. A: electrical stimuli delivered to the CBCO nerve (stim. CBCO n) evoked a composite response in the Dep MN, consisting of a fast monosynaptic compound EPSP (*) and a late compound IPSP (○). When delivered in pairs, the electrical stimuli evoked this complex response twice. The paired-pulse protocol were delivered at 3 different frequencies: 50 Hz (top), 66 Hz (middle), and 83 Hz (bottom). The 2nd electrically evoked IPSP (thick arrow) followed without failure ±66 Hz. At a higher frequency (83 Hz), failures occurred. B: IPSPs evoked in a Dep MN by electrical stimulation of the CBCO nerve (control) were suppressed in the presence of a high divalent cation solution (high Ca$^{2+}$, Mg$^{2+}$), indicating that the IPSP was polysynaptic. C: intracellular recording from a Dep MN showing unitary IPSPs triggered by a CBCO unit (see arrow) in the CBCO nerve. Superimposition of 6 traces shows that the synaptic delay was variable. The variability of the synaptic delay was estimated using the same fitting procedure as in Fig. 5C (see the 2 enlarged traces in the inset). In this experiment, the synaptic delay (Δ) varied by ±1.2 ms.
aptic MN was measured at various membrane potentials (Fig. 7, A and B). In the experiment shown in Fig. 7A, a stretch-sensitive CBCO unit (data not shown) was correlated with a resistance reflex IPSP recorded from a Dep MN. When the MN was depolarized by the injection of +6 nA, the unitary IPSP was hyperpolarizing (Fig. 7A, top), whereas the injection of −4 nA reversed the IPSP (Fig. 7A, middle). The reversal potential of unitary resistance reflex IPSPs estimated from the linear regression curve of the IPSP amplitude versus the imposed membrane potential was −72 mV (Fig. 7B). This value corresponds to the reversal potential of Cl− ions for crayfish walking MNs (Pearlstein et al. 1994) suggesting that a Cl− conductance mediated the IPSPs. This hypothesis was confirmed in experiments in which the ganglia were superfused with a low-[Cl−] saline. In the absence of Cl− ions, electrical stimulation of the CBCO nerve failed to evoke IPSPs in MNs (Fig. 7C). The IPSPs were restored after 45 min of rinsing in normal saline (Fig. 7C). Moreover, the compound IPSPs evoked by electrical stimulation of the CBCO nerve were blocked by the application of the Cl− channel blocker picrotoxin (PTX, 40 μM; Fig. 7D). The preceding results demonstrate that the IPSPs involved in the resistance reflex inhibitory pathway are mediated by Cl− ions.

Evidence that resistance reflex IPSPs are mediated by GABA

In crayfish MNs, the neurotransmitters GABA and glutamate produce IPSPs mediated by Cl− ions (Pearlstein et al. 1994). Both responses are blocked by PTX (Pearlstein et al. 1994). To determine which neurotransmitter is involved in the inhibitory pathway of the resistance reflex, we examined the effects of two different pharmacological agents: GPT (an enzyme that degrades glutamate in the synaptic cleft) and 3-MPA (an inhibitor of GABA synthesis). These two pharmacological agents have been previously used in crayfish: GPT was shown to block the retrograde effect of glutamatergic MNs onto their own CBCO sensory afferents (Le Ray and Cattaert 1999); 3-MPA blocks GABAergic postsynaptic and presynaptic inhibitory neurotransmission in crayfish opener neuromuscular synapses (Golan and Grossman 1996). The effects of these pharmacological agents on the compound IPSPs evoked in the MNs by electrical stimulation of the CBCO nerve, indicate that both GABA and glutamate were involved in the compound IPSPs. For example, in Fig. 8, A and B, in the presence of 3-MPA the compound IPSP was almost totally abolished (Fig. 8A), and the linear curve representing IPSP amplitude versus injected current (Fig. 8B) showed a marked and significant reduction of the slope (2-way ANOVA, F = 152.8, df = 1, n = 282, P < 0.0001). In the same experiment, GPT had no significant effect on the compound IPSP (Fig. 8B; 2-way ANOVA, F = 0.77, df = 1, n = 256, P = 0.38). Therefore in the experiment presented in Fig. 8, A and B, the compound IPSP was exclusively mediated by GABA. In other experiments, the compound IPSP evoked in a Dep MN by electrical stimulation of the CBCO nerve was reduced in the presence of GPT. Such an experiment is presented in Fig. 8, C and D. In this experiment, the linear curve representing IPSP amplitude versus injected current (Fig. 8D) showed a marked and significant reduction of the slope (2-way ANOVA, F = 8.73, df = 1, n = 90, P = 0.004), indicating that part of the compound IPSP was mediated by glutamate. The results obtained with these pharmacological agents therefore were found to vary between preparations. However, in most experiments in which compound IPSPs were blocked by 3-MPA (6 of 9), GPT had no effect on IPSPs, indicating that those compound IPSPs were exclusively mediated by GABA, whereas in three experiments GPT produced a limited decrease of the compound IPSP amplitude, indicating that GABA and glutamate were involved in the compound IPSPs. Moreover, contrary to the case of 3-MPA, when an effect of GPT onto compound IPSPs was observed, no effect was detected on the resistance reflex uni-

![Figure 7](http://jn.physiology.org/doi/10.1152/jn.00589.2001.supp.Plots/bfig7.png)
tary IPSPs. This last observation indicates that glutamate is most likely not involved in resistance reflex IPSPs but may participate in other inhibitory pathways that remain to be determined.

To confirm that resistance reflex inhibitory pathways are mediated by GABA, we analyzed functionally identified unitary CBCO-evoked IPSPs. Such an experiment is presented in Fig. 9. In normal saline, a unitary IPSP in a Dep MN was correlated with a CBCO unit coding for stretch of the CBCO strand (Fig. 9A, 1 and 2). In the same Dep MN, a unitary EPSP was correlated to a release-sensitive CBCO (Fig. 9B, 1 and 2). The addition of GPT (30 U/100 ml) to the bath did not change the amplitude of the unitary IPSPs (Fig. 9A3) or the unitary EPSPs (Fig. 9B3). In contrast, in the presence of 3-MPA (5 mM) the amplitude of the unitary IPSPs decreased progressively and completely disappeared after 1.5 h of exposure (Fig. 9A3). The amplitude of the unitary EPSPs remained constant in the presence of 3-MPA (Fig. 9B3), indicating that the effect of 3-MPA was specific to the inhibitory pathway. Statistical analyses of the peak amplitudes of IPSPs and EPSPs in the four conditions (control, GPT, wash, and 3-MPA) are presented in the histograms of Fig. 9C. There was no significant effect of GPT on either the IPSPs or EPSPs (Fig. 9C). In contrast, the decrease of IPSP amplitude that occurred in the presence of 3-MPA was highly significant (1-way ANOVA, Tukey’s multiple comparison test, P < 0.01). Table 1 summarizes the results from 11 unitary resistance reflex IPSPs triggered by 11 different CBCO units. Of the 11 unitary IPSPs, the amplitudes of 8 were significantly decreased in the presence of 3-MPA indicating that most of these inhibitory synapses were mediated by GABA. By contrast, the unitary IPSPs recorded from the MN in this experiment were insensitive to GPT confirming that glutamate was not involved in the resistance reflex inhibitory pathways to MNs.

Receptors involved in the resistance reflex inhibitory response

The experiments reported in the preceding text indicate that GABA is involved in the resistance reflex IPSPs. Previous experiments in crayfish have shown that inhibitory responses in MNs may result from the activation of a mixed GABA/glutamate...
mate receptor (Pearlstein et al. 1994). We therefore designed a series of experiments to determine whether such a mixed receptor is involved in the resistance reflex inhibitory response. We used a double-barrelled micropipette to pressure-eject GABA or glutamate close to the site at which the MN was penetrated by the intracellular electrode (Fig. 10A) and subsequently analyzed how IPSPs evoked by electrical stimulation of the CBCO sensory nerve were altered by the saturation of the receptors with either of those neurotransmitters. To prevent a shift in the membrane potential of the MN due to the effect of GABA or glutamate, a DC current was intracellularly injected into the neuron. It should be noted that because we did not know where the sensory-motor synapses were located on the arborizations of the intracellularly recorded MNs, space clamp was uncontrolled at these inhibitory synaptic sites (those sites are, however, unlikely to have been electrically distant from the intracellular recording sites because it was possible to reverse sensory-evoked IPSPs by injecting current through the electrode). The problem of uncontrolled space clamp was overcome by repeating the pressure-ejection of GABA or glutamate while injecting various current injection (+5 to +8 nA). In each case, although the amplitude of the EPSP was not changed, the IPSP was almost totally abolished after the application of GABA regardless of the amount of injected current. Data presented in Fig. 10, B and C were recorded while injecting +5 nA into a MN. IPSPs evoked by CBCO sensory nerve stimulation were almost totally abolished during the application of GABA (Fig. 10B1). This reduction in the amplitude of the IPSPs was highly significant (t-test, P < 0.0001, Fig. 10B2). In addition, in the presence of glutamate the CBCO-evoked IPSPs were almost entirely abolished in the MN (Fig. 10C1), indicating that these neurotransmitters did not block the sensory spikes in the CBT. We cannot exclude, however, the possibility that these neurotransmitters may have effects on the interneurons involved in the inhibitory oligosynaptic pathway.

FIG. 9. Resistance reflex unitary IPSPs evoked by identified CBCO units are mediated by GABA. A1: histogram representing the discharge of the CBCO unit during the imposed sinusoidal movement. This CBCO unit (CBCO n, unit 15) was activated during the stretch movement. A2: extracellular recording of unit 15 from the CBCO nerve. A3: intracellular recording from a Dep MN displaying a unitary IPSP triggered by the CBCO unit presented in A, 1 and 2, in 4 situations (control, 1.5 h GPT, 1 h GPT wash, and 1.5 h 3-MPA). The occurrence of the IPSP is indicated by an ↑. In the presence of 3-MPA, the unitary IPSP disappeared (gray trace). B: data are from the same MN but the analyzed unitary EPSP were triggered by another identified CBCO unit (CBCO n, unit 20) coding for release movements (see histogram in B1). The unitary EPSP was affected neither by GPT nor by 3-MPA. C: statistical analysis of the amplitude of these unitary IPSP and EPSP recorded in the Dep MN in the 4 situations presented in a bar diagram. Vertical bars represent ±SE. C1: the amplitude of unitary IPSPs was not significantly different in control and GPT situations (P > 0.05, Tukey’s multiple comparison test after 1-way ANOVA). By contrast the decrease in amplitude of the unitary IPSP in the presence of 3-MPA was significantly different from control and wash (P < 0.01, Tukey’s multiple comparison test after one-way ANOVA). C2: the absence of effect of GPT and 3-MPA on the EPSP was confirmed by statistical analysis (P > 0.05, Tukey’s multiple comparison test after one-way ANOVA).
The blocking effect of glutamate was highly significant (ANOVA, F-test, P < 0.05; **, P < 0.01). The numbers of PSPs used for statistical analysis are represented in the last column (n).

**TABLE 1. Effect of GPT and 3-MPA on unitary IPSPs**

<table>
<thead>
<tr>
<th>Units</th>
<th>Control/GPT</th>
<th>Wash/3-MPA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NS</td>
<td>**</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>NS</td>
<td>*</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>NS</td>
<td>**</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>NS</td>
<td>*</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>NS</td>
<td>**</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>NS</td>
<td>NS</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>NS</td>
<td>**</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>NS</td>
<td>**</td>
<td>12</td>
</tr>
<tr>
<td>11</td>
<td>NS</td>
<td>**</td>
<td>27</td>
</tr>
</tbody>
</table>

Effect of glutamate pyruvate transaminase (GPT) and 3-mercaptopropionic acid (3-MPA) on unitary inhibitory postsynaptic potentials (IPSPs) from a single experiment. Tukey’s multiple comparison test: NS, non significant; *, P < 0.05; **, P < 0.01. The numbers of PSPs used for statistical analysis are represented in the last column (n).

**Functional characterization of CBCO units involved in the resistance reflex inhibitory response**

As shown in the preceding text (see Fig. 2), during the stretch of the CBCO strand (downward movement of the leg) some CBCO units evoke EPSPs in Lev MNs while others evoke IPSPs in Dep MNs. It is unclear from these experiments, however, whether some CBCO units could evoke both excitatory and inhibitory responses in antagonist MNs. Paired intracellular recordings from a Lev and a Dep MN were therefore performed during sinusoidal and ramp movements of the CBCO strand. In Fig. 11, we present an example of such an experiment in which a CBCO unit was activated during release movements of the CBCO strand (corresponding to upward movements of the leg in intact animal) and was silent during stretch movements (Fig. 11A). During application of ramp-and-hold movement protocols, this phasotonic neuron (Le Ray et al. 1997a) coded for both movement and position, displaying a high-frequency phasic discharge (47.3 ± 2.8 Hz) during release ramp and a continuous low-frequency discharge (5.2 ± 1.5 Hz) during maintained release position (data not shown). The analysis of PSPs triggered by this unit in intracellularly recorded MNs subsequently performed during application of sinewave movements (Fig. 11A) rather than ramp-and-hold movements as ramps activate many different sensory CBCO neurons at high frequency making the analysis of unitary PSPs very difficult. During sinewave release movements of the CBCO strand, each sensory spike in this CBCO unit triggered an EPSP in the Dep (monosynaptic resistance reflex). This EPSP occurred very reliably (see superimposed raw data in Fig. 11B, top) and was stable in amplitude (0.438 ± 0.009 mV). These same CBCO spikes (see Fig. 11B, bottom) also triggered IPSPs in the Lev MN (Fig. 11B, middle). The unitary IPSPs also occurred very reliably (amplitude 0.134 ± 0.006 mV). Unlike the unitary EPSPs, however, the unitary IPSPs disappeared in the presence of high divalent cation solution (Fig. 11B, right). These results were confirmed by the observation of the average traces (Fig. 11C). These data are summarized in a schematic diagram (Fig. 11D).

To gain insights into the functional importance of the inhibitory connections between CBCO units and the MNs, systematic intracellular recordings from several Lev and Dep MNs were performed while imposing sinusoidal movements on the CBCO strand. In the experiment shown in Fig. 12, we performed intracellular recordings from eight Dep MNs and four Lev MNs. In this figure, only those MNs that showed a reflex response are shown (5 Dep MNs and 2 Lev MNs). The responses of these neurons to imposed sinusoidal movements of

**FIG. 10.** Compound CBCO-evoked IPSPs are blocked in the presence of either GABA or glutamate. A: experimental procedure. A large amount of GABA or glutamate was pressure-ejected in the vicinity of an intracellularly recorded Dep MN, while the CBCO nerve was electrically stimulated (0.5 Hz). B1: during the ejection of GABA (gray trace), the Dep MN was continuously injected with +5 nA to maintain the membrane potential at −60 mV (resting potential of the control situation, black trace). In the presence of GABA, the compound CBCO-evoked IPSP disappeared. Note that during this response, the monosynaptic early EPSP persisted. B2: statistical analysis of the amplitude of the CBCO-evoked IPSP recorded in a Dep MN in control (n = 11) and in the presence of GABA (n = 23) are presented in a bar diagram. Vertical bars represent SE. The blocking effect of GABA was highly significant (t-test, P < 0.0001). C: same representation as in B in another Dep MN. C1: in the presence of glutamate, the compound IPSP disappeared. The membrane potential was maintained at −64 mV in control and glutamate conditions. C2: statistical analysis of the amplitude of the CBCO-evoked IPSP in control (n = 7) and in the presence of glutamate (n = 6) are presented in a bar diagram. The blocking effect of glutamate was highly significant (t-test, P < 0.0001).
the CBCO strand ranging from 0.15 to 1.9 mV in amplitude are presented in the top row.

In this experiments, the CBCO sensory units were discriminated as explained in METHODS. Only those CBCO units that evoked a unitary response (EPSP or IPSP) in one of the intracellularly recorded MNs are shown (16 of 25; left). The coding properties of the CBCO units were established according to their response to a ramp protocol (Le Ray et al. 1997a). Purely phasic units fire exclusively during the ramp movement, phasotonic units fire during both the ramp movement and maintained positions, while tonic units display continuous tonic activity, the frequency of which depends on the degree of stretch. With the exception of two tonic units (units 11 and 16), all of the identified CBCO units in this experiment were phasotonic. Five CBCO units evoked resistance reflex IPSPs in MNs (dark gray background). Some IPSPs were very consistent and their averaged traces displayed an abrupt initial falling phase (units 1 and 2 in Lev MN2, unit 10 in Lev MN4, and unit 12 in Dep MN5 and Dep MN7). Other inhibitory responses were more variable and the corresponding average IPSPs were therefore less distinct (unit 3 in Lev MN4). The observed EPSPs were very reliably evoked by a given CBCO unit, and their rising phases were very abrupt; this is in agreement with a previous demonstration that the early component of the excitatory pathway is monosynaptic (El Manira et al. 1991a). Four of 25 identified CBCO units evoked both a resistance reflex EPSP in a MN and a resistance reflex IPSP in the antagonistic MN (release-sensitive units 1–3 and stretch-sensitive unit 12). Nine of 25 identified CBCO units evoked resistance reflex EPSPs without IPSPs in antagonistic MNs (release-sensitive units 4–9 and 11 and stretch-sensitive units 13 and 14) and one CBCO unit (10) exclusively evoked IPSPs in Lev MN4. In addition, two CBCO units did not evoke pure resistance responses in MNs. Unit 16 evoked assistance EPSPs in Dep MN4 and assistance IPSPs in antagonistic Lev MN2 and Lev MN4. This unit behaved then as a purely assistance reflex unit. The EPSP evoked by this CBCO unit in the Dep MN4 was not monosynaptic, however. Finally unit 15 evoked mixed resistance and assistance responses in Lev MN2 and Lev MN4 respectively. In conclusion, in a majority of MNs (4 of 7 in experiment shown in Fig. 12) presenting a resistance reflex response to movement, unitary EPSPs and IPSPs sustained this resistance reflex.

The results shown in Fig. 12 are typical. In all experiments, fewer units (15.7 ± 2.8%) evoked an IPSP in a MN as compared with the number of units evoking an EPSP (23.3 ± 3.6%).

**DISCUSSION**

In this paper, we have demonstrated the existence of an inhibitory pathway in the crayfish walking system and its involvement in a resistance reflex. These IPSPs, evoked in the MN antagonistic to the imposed movement, are oligosynaptic and are mediated by GABA. However, other inhibitory sensory-motor pathways seem to be mediated by glutamate too.
How many interneurons are involved in the resistance reflex inhibitory pathway?

The resistance reflex inhibitory pathway is not monosynaptic as it is blocked in the presence of high-divalent cation saline (Fig. 11, B and C). In addition, this result indicates that the inhibitory resistance reflex involves spiking interneurons. Two criteria indicate that very few interneurons are involved in this polysynaptic resistance reflex pathway: compound IPSPs elicited by electrical stimulation of the CBCO nerve follow one-to-one paired-pulse stimuli up to 83 Hz, and the measured synaptic delays are rather short.

**HIGH-FREQUENCY STIMULATION.** The fact that compound IPSPs follow high-frequency stimulation of the CBCO nerve indicate that at least some of the inhibitory pathways are mediated by a small number of interneurons. One caveat of this experiment is that electrical stimulation of the CBCO nerve activates most of the CBCO neurons, which in turn may recruit interneurons that do not participate in the resistance reflex. Even so, we believe that for the most part the IPSPs are mediated by the sensory neurons of the resistance reflex because the IPSPs of the resistance reflex have a low threshold for activation (they are triggered by single sensory spikes), and the amplitude of these IPSPs is large (in the range of 0.2–0.5 mV).

**MEASUREMENT OF THE SYNAPTIC DELAY.** In the experiment shown in Fig. 6C, the unitary IPSP triggered by an identified CBCO unit in the CBCO nerve occurred 4.4–5.6 ms after the CBCO unit. This delay includes a conduction time in the nerve of at least 3 ms (El Manira et al. 1991a). Therefore the synaptic delay per se would be in the range of 1.4–2.6 ms. These values are compatible with a single relay interneuron. However, in experiments using paired intracellular recordings from a CBT and a postsynaptic MN (Fig. 5), the synaptic delay could reach up to 3.9 ms. This rather-long delay still includes the conduction time within terminal arborization of the CBCO neuron plus the conduction time in the putative spiking inhibitory interneuron itself. From these arguments, it is therefore likely that the resistance inhibitory pathways are oligosynaptic and probably mediated by a single interneuron.

**What is the neurotransmitter of the resistance reflex inhibitory interneuron?**

Among the various neurotransmitters that have been demonstrated to mediate inhibitory responses in crustacea, our results indicate that GABA is involved in the inhibitory resistance reflex. This conclusion was drawn from the finding that unitary IPSPs involved in the resistance reflex were abolished by 3-MPA (n = 8 of 11 unitary IPSPs analyzed, Table 1). By contrast, we did not find any unitary resistance reflex IPSPs that were abolished by GPT (Table 1). In some experiments, however, compound IPSPs triggered by electrical stimulation of the CBCO nerve, were partially blocked by GPT, indicating that some of these inhibitory pathways are mediated by glutamate. Those compound IPSPs that were partially blocked by GPT must therefore belong to pathways that are not related with resistance reflex. Such interneurons could be involved in the reflex reversal (i.e., the switch of a resistance reflex to an assistance reflex) (Le Ray and Cattaert 1997; Skorupski and Sillar 1986). Such interneurons have been described in the stick insect (Bässler 1986) although their neurotransmitter is not known (Bässler 1993).
What type of receptor in the MNs mediates the inhibitory resistance reflex?

Both GABA and glutamate seem to activate a Cl⁻ conductance that is blocked by the Cl⁻ channel blocker PTX (Marder and Paupardin-Tritsch 1978; Pearlstein et al. 1994). In crayfish MNs, both neurotransmitters appear to bind to the same receptor and to activate the same PTX-sensitive Cl⁻ conductance (Pearlstein et al. 1994). In the present study, we demonstrated that resistance reflex IPSPs also involve a Cl⁻ channel because IPSPs were not observed in low-[Cl⁻] saline and were blocked by PTX (Fig. 7). Moreover, in the presence of large amounts of GABA, IPSPs evoked by electrical stimulation of the CBCO nerve were usually suppressed while monosynaptic EPSPs persisted. Similarly, saturation with glutamate generally prevented CBCO-evoked IPSPs. Those results are compatible with the hypothesis that a mixed GABA/glutamate receptor on the MN is involved in the inhibitory resistance reflex. However, the fact that application of glutamate totally abolished the CBCO-evoked compound IPSP may be due to glutamate directly inhibiting the interneurons involved in the inhibitory pathway.

Contribution of a functional inhibitory pathway to the resistance reflex in crayfish walking legs

The resistance reflexes of arthropods are negative feedback reflexes resulting in the activation of MNs that oppose an imposed movement. In this paper, we have demonstrated that both excitatory and inhibitory pathways participate in this resistance reflex. In insects, such as locusts (Burrows et al. 1988) and stick insects (Bäsßler and Büschges 1998), inhibitory pathways between proprioceptive neurons from chondotonal organs and MNs have been described. These inhibitory pathways comprise nonspiking interneurons (NSIs) (Burrows et al. 1988; Büschges and Schimtz 1991). Such a disynaptic pathway involving NSIs inhibitory interneurons is unlikely to exist in crayfish as the compound IPSPs are suppressed in the presence of a high-divalent cation solution (Fig. 6B). However, we cannot exclude such a possibility as NSIs could be inserted between spiking INs and MNs in a trisynaptic pathway, resembling that proposed in the stick insect (Sauer et al. 1996).

The resistance reflex in crayfish is analogous to the stretch reflexes observed in vertebrates (Clarac et al. 2000). When a movement is imposed on a joint, the MNs that counteract this movement are activated by a monosynaptic excitatory response. In addition, in vertebrates, the antagonistic MNs are inhibited via a disynaptic pathway involving Ia interneurons (Clarac et al. 2000; Munson and Sypert 1979). This inhibitory pathway (reciprocal Ia inhibition) constitutes a key element in the functional definition of synergy (McCullum 1993) used in the analysis of movement control in mammals.

Our results indicate that an inhibitory pathway similar to that of vertebrates participates in movement control in the crayfish. This is supported by the fact that identified sensory units coding for given movements activate MNs opposing these movements and inhibit MNs supporting these movements (Figs. 11 and 12). The involved pathways are ologosynaptic because the delays between sensory spikes and the evoked IPSPs are relatively constant (Fig. 5) and the IPSPs are highly reliable.

We are grateful to Dr. P. Meyrand for laboratory facilities. We thank Drs. D. Le Ray, J. Sullivan, and A. Hill for helpful comments on the manuscript and for improving the English.

This work was supported by the Centre National de la Recherche Scientifique. M. Le Bon-Jego received a fellowship from the Ministère de l’Enseignement Supérieur et de la Recherche (MRT).

REFERENCES


