Fictive Locomotion in the Fourth Thoracic Ganglion of the Crayfish, *Procambarus clarkii*

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Bath application of muscarinic agonists induced rhythmic motor activity in an *in vitro* preparation of the thoracic nervous system of the crayfish, *Procambarus clarkii*. In 70% of the cases, the rhythm was organized into 1 of the 2 normal patterns: "backward" walking or "forward" walking. In the rest (30%), the ganglion produced either a series of bursts of impulses or no rhythm at all, just an increase in the tonic activity.

When it was isolated from all ascending and descending afferents, the fourth thoracic ganglion was still able to generate rhythmic motor output during bath application of muscarinic agonists. In certain motor neurons, muscarinic agonists induced plateau potentials. Under these conditions, some of these motor neurons were able to change the period of the motor pattern, which might suggest that these motor neurons were part of the central pattern generator (CPG) for locomotion. In the presence of 5×10^{-6} M TTX, the membrane potential of these motor neurons continued to oscillate with organized rhythmic membrane potential oscillations into 1 of the 2 patterns. Under these conditions, current injection into certain motor neurons demonstrated that they continued to affect the CPG.

Two classes of walking leg interneurons have been found. First, there are those with a sustained membrane potential: injection of a steady depolarizing current into some of these interneurons induced rhythmic activity in all thoracic motor nerves, even in the absence of any pharmacological activation. Second, there are those with an oscillating membrane potential: these seemed to enable silent motor neurons to be involved in an ongoing rhythm.

For several decades, it has been possible to demonstrate that rhythmic motor activity can be produced by invertebrate ganglia isolated from peripheral sensory elements (see review by Delcomyn, 1980). The concept of an autonomous central pattern generator (CPG) that is able to induce different motor behaviors within a CNS has been suggested from these experiments. A similar notion has been suggested in lower vertebrates (Roberts and Roberts, 1983; Grillner et al., 1987), and in mammals where,

through pharmacological activation, the isolated spinal cord displays rhythmic activity (Grillner, 1985). Because of the similarity between normal locomotion and motor patterns produced in isolated nervous systems, the latter patterns have been termed "fictive locomotion" (Grillner, 1981).

A large body of information has been collected concerning the generation of rhythmic motor patterns by isolated invertebrate preparations, notably leech swimming (Kristan and Calabrese, 1976), *Tritonia* swimming (Gettings and Dekin, 1985), and the crustacean stomatogastric system (Selverston and Moulins, 1987). It has been possible to identify many elements of the CPG and to describe their properties (Selverston and Moulins, 1985). Nevertheless, as indicated in Gettings's review (1986), the motor behaviors that have been effectively studied are relatively poor in terms of behavioral complexity.

In several isolated arthropod preparations, induction of rhythmic activity seemed to be associated with monoamines or peptides (Classen and Krammer, 1986; Mulloney et al., 1987). In vertebrates, it seemed to be produced by a preferential activation of NMDA receptors (Grillner et al., 1987). In the fourth thoracic ganglion (TG4) of the crayfish, Sillar and Skorupski (1986) maintained sensory input from only a single proprioceptor, the thoracicocoxal muscle receptor organ (TCMRO). They were able to obtain a spontaneously rhythmic preparation, and also to entrain the rhythm by stretching and releasing the strand of this receptor (Sillar et al., 1986). If sensory terminals are so important in the production of rhythmicity in the thoracic nervous system of the crayfish, then the sensory neurotransmitter, ACh in this case (see Florey, 1967; Barker et al., 1972; and Hildebrand et al., 1974) ought to be able to activate thoracic CPGs.

Therefore, in this paper, we would like to present a new preparation derived from that of Sillar and Skorupski (1986), where the locomotory rhythm was induced in the isolated thoracic ganglion preparation during bath application of muscarinic agonists (oxotremorine and pilocarpine). We will examine (1) the induced pattern and its relation to the activity recorded in the intact animal in order to determine whether the induced rhythmic activity can be considered as "fictive locomotion" and (2) the role of some motor neurons and interneurons in the production of the induced rhythmic activity in the *in vitro* thoracic preparation.

A preliminary report of this work has already been published (Chrachri and Clarac, 1987).

Materials and Methods

The preparation has been described in detail previously (Chrachri and Clarac, 1987; Chrachri and Clarac, 1989) and is illustrated in Figure 1.

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Figure 1. Drawing of the isolated thoracic preparation used in this study. The thoracic nervous system is isolated from the third ganglion (TG3) to the fifth ganglion (TG5). The fourth thoracic ganglion (TG4) is dissected with the main motor nerves intact. They are from anterior to posterior: the promotor nerve (*pro*), the anterior levator (*a.lev*), the proximal motor root (*pmr*) containing the depressor nerve (*dep*), the remotor (*rem*), and the posterior levator (*p.lev*), and the 2 distal roots, the anterior distal root (*adr*) and the posterior distal root (*pdr*). Black dots indicate the position of the platinum wires used to record differentially from or to stimulate the main proximal motor nerves and the 2 distal roots which innervate the distal muscles. *SA.*, sternal artery; *anas.*, anastomosis.

The thoracic nervous system was isolated from the third ganglion (TG3) to the fifth ganglion (TG5). The TG4 was desheathed for microelectrode penetration and dissected with the main motor nerves intact. They are, from anterior to posterior, the promotor nerve (*pro*), the anterior levator nerve (*a.lev*), the proximal motor root (*pmr*) containing the depressor nerve (*dep*), the remotor nerve (*rem*), and the posterior levator nerve (*p.lev*), and the 2 distal roots, the anterior distal root (*adr*), and the posterior distal root (*pdr*).

The preparation was pinned out in a Sylgard-lined dish and bathed continuously with oxygenated saline (Van Herreveld, 1936) at 13–15°C with a Peltier-effect cooler. Oxotremorine or pilocarpine was added to give the final bath concentrations in the range of 10^{-6} – 10^{-5} M. In some experiments, *d*-tubocurarine or atropine was bath-applied at concentrations of 10^{-5} – 10^{-4} M in order to characterize which ACh receptors were involved. In order to study the induced fictive locomotion in the absence of spikes, TTX was added at a bath concentration of 5×10^{-6} M in some experiments.

Platinum wires in contact with the thoracic nerves and insulated from the bath with vaseline were used to record differentially from or to stimulate these nerves. Intracellular recordings were made with glass microelectrodes filled with 3 M KCl and having tip resistances of 20–40 Ω . A conventional bridge circuit was used for recording and passing current pulses with a single microelectrode. It is not possible to record electrical events within the cell body in the crayfish (Reichert and Krenz, 1986), although in the large part of the neurite (10–20 μ m diameter) it is possible to record not only action potentials but also postsynaptic potentials (PSPs).

Motor neurons were identified using the following criteria: (1) one-to-one correlation between intracellularly recorded action potentials with spike recorded in one of the motor nerves (the position of the extracellular electrodes is illustrated in Fig. 1); (2) stimulation of the appropriate motor nerve causes an antidromic action potential, which can be recorded as an all-or-none action potential in the motor neuron. Interneurons were characterized as not having a one-to-one correlation between their intraneurite action potentials and any action potential recorded extracellularly in the 6 motor nerves. This physiological identification was usually confirmed anatomically by staining the recorded neuron with Lucifer yellow (Stewart, 1978).

In all our preparations we removed all proximal sensory inputs, including the TCMRO and CB chordotonal organ (Bush and Laverack, 1982) but retained all distal sensory inputs by leaving the distal leg segments intact (meropodite, carpopodite, propodite, and dactylopedite). Most of the distal sensory elements, including those from the dactyl travel through the *pdr* (Clarac and Chrachri, 1989).

Physiological data presented in this paper were collected from over 60 experiments performed in the *in vitro* thoracic preparation.

In order to characterize the fundamental walking pattern of the decapod Crustacea, we complemented the isolated thoracic nervous preparation with experiments on intact rock lobster, *Jasus lalandii*, as described in detail by Clarac and Chrachri (1989). EMGs were recorded from the 4 proximal muscles of the fourth walking leg using the same techniques as Ayers and Clarac (1978). Walking behavior was driven using a treadmill in the way described by Clarac and Chasserat (1983).

Results

Walking pattern in intact animals

Crustacea-like crayfish, lobster, and the rock lobster can walk in all directions forwards, backwards, and sideways (Ayers and Davis, 1977; Evoy and Ayers, 1982). The 2 proximal joints are fundamental to the control of forward and backward walking (Evoy and Ayers, 1982). The first joint, the thoracocoxal (T-C) moves anteroposteriorly by the action of the 2 antagonist muscles (promotor and remotor). The next joint, the coxobasal (C-B), operates in a perpendicular plane to the first one through the action of 2 levator muscles (anterior and posterior levators) and 2 depressor muscles (anterior and posterior depressors). In order to discuss the characteristics of the 2 patterns studied in the *in vitro* crayfish preparation, we performed some experiments on the intact rock lobster *Jasus lalandii*, typically used as a crustacean walking model (Clarac and Chasserat, 1983). The walking patterns presented in Figure 2 were obtained on the treadmill at a belt speed of 10 cm/sec, which corresponds to the normal walking speed of the animal. At this speed, walking is very stereotyped with regular bursts in each muscle. Figure 2*A* illustrates backward walking: the leg levates and remotes (this corresponds to the return stroke, RS) and then depresses and promotes (this corresponds to the power stroke, PS). Figure 2*B* illustrates forward walking, the leg levates and promotes during the RS and then depresses and remotes during the PS.

Figure 2 shows that forward and backward walking patterns differ from each other by a switch within the muscular synergies.

Figure 2. Simultaneous electromyographic recordings from the 4 proximal muscles of the fourth walking leg during driven walking of the rock lobster, *Jasus lalandii*. *A*, During backward walking, the promotor (*PRO*) and depressor (*DEP*) are active in phase during the power-stroke phase (*PS*), and in opposition to the remotor (*REM*) and the levator (*LEV*), which are active during the return-stroke phase (*RS*). *B*, During forward walking, the promotor is active in phase with the levator during *RS* and in opposition with the remotor and the depressor, which are active during *PS*.

For example, the promotor muscle is activated in phase with the depressor muscle during backward walking (Fig. 2*A*) but in opposite phase during forward walking (Fig. 2*B*). Therefore, in *Macrura* it is enough to record the activity of 2 proximal muscles, promotor or remotor and levator or depressor, to know whether the walking pattern is forward or backward. All the other muscles are, of course, active during walking, but they play only a secondary role in the kinematics of the walking leg during forward or backward walking (Ayers and Davis, 1977; Ayers and Clarac, 1978).

It has been often demonstrated in Crustacea that the *PS* duration is linked with walking speed. By contrast, the *RS* duration is more or less independent of walking speed. Figure 2 shows that the burst duration of the *RS* muscles is shorter than the burst duration of the *PS* muscles. If we consider the promotor and remotor muscles, their burst duration changes depending upon whether they are involved with the *PS* or *RS* phases.

The induced patterns

Motor nerves of TG4 usually displayed tonic activity. However, bath application of oxotremorine at 10^{-6} or 10^{-5} M induced rhythmic motor activity (Figs. 3, 4). Other cholinergic agents, in particular pilocarpine (10^{-6} or 10^{-5} M), also induced rhythmic activity. This rhythmicity was reversible (Chrachri and Clarac, 1987). Rhythmic activity was patterned into "forward" or "backward" motor program in 70% of the cases. In the remaining 30%, oxotremorine either induced unpatterned rhythmic activity (e.g., Figs. 11*C*, 13*A*) or increased the frequency of the tonic motor activity without eliciting any rhythmic activity (e.g., Fig. 11*A*).

The 2 induced patterns: "backward" and "forward"

When the pharmacologically induced rhythm was well organized, 2 types of relationship between motor activity were distinguished. These 2 patterns were similar to those obtained in the intact crayfish (Barnes, 1977) and rock lobster (Fig. 2), and in another *in vitro* crayfish preparation (Skorupski and Sillar,

Figure 3. The 2 induced patterns in the *in vitro* thoracic nervous system of the crayfish *Procambarus clarkii* during bath application of oxotremorine at 10^{-5} M. *A*, During the fictive backward pattern, the remotor and the anterior levator motor units are active in phase and in opposition to those of the promotor. The intracellular recording corresponds to a remotor motor neuron. *B*, During fictive forward locomotion, the promotor and the depressor are active in opposition to each other. Simultaneous intracellular recordings from one promotor and one depressor motor neurons show that these 2 motor neurons oscillate in opposition. The third intracellular record corresponds to a spiking interneuron which fired in phase with the depressor motor neuron. *REM*, remotor motor neuron; *PRO*, promotor motor neuron; *DEP*, depressor motor neuron; *INT*, interneuron. Calibration bars: horizontal, 5 sec; vertical, 20 mV.

Figure 4. Temporal characteristics of the induced fictive backward and forward locomotory patterns during bath application of oxotremorine at 10^{-5} M in 3 different *in vitro* preparations. *A* and *B*, The fictive backward pattern in 2 preparations: *A*, and *B*, are studied the synchronisms and the antagonisms of different units in the proximal motor nerves (the onset of the remotor burst is taken as reference). The mean period is faster in *B* than in *A*. *A*₂ and *B*₂, Duration of levator, depressor, remotor, and depressor bursts versus the period. *C*, Fictive forward pattern: *C*₁, the promotor and levator are active in phase and both of them are antagonistic with the remotor burst within the cycle period; *C*₂, duration of motor neuronal bursts versus the period (see text for further explanation).

1986), and they represented those seen during forward and backward walking.

Figure 3*A* illustrates a “backward” pattern. This pattern was obtained in 90% of the cases where the rhythm was clearly patterned, the other 10% representing the “forward” pattern. In the extracellular recordings, the levator motor neurons (lev) were bursting in phase with the remotor motor neurons and in opposite phase with the promotor bursts (pro). These bursts began and ended abruptly with very little overlap between them.

Figure 3*B* illustrates a “forward” pattern. The pattern showed opposite activation of promotor and depressor motor neurons. Simultaneous intracellular recordings showed opposite membrane potential oscillations between the depressor and the promotor motor neuron. The third intracellular trace showed activity of a spiking interneuron that fired in phase with the depressor motor neuron. In this example, there was a consid-

Table 1. Temporal characteristics of 3 different experiments

Exp. ^a	N ^b	Mean period ± SD (sec)	r ^c	Slope (Y in X)
A	55	21.7 ± 9.1	pro: 0.93	0.940
			rem: 0.20	0.070
			lev: 0.20	0.070
B	52	14.4 ± 3.35	rem: 0.91	0.604
			lev: 0.74	0.365
			dep: 0.69	0.365
C	36	20.8 ± 7	pro: 0.97	0.930
			rem: 0.38	0.070
			lev: 0.97	0.890

^a A and B correspond to backward patterns; C corresponds to a forward pattern.

^b Cycle number.

^c Correlation coefficient.

erable overlap between the bursts in the promotor and depressor motor nerves.

Because of the similarity between the induced patterns (Fig. 3) and the EMGs recorded in intact rock lobster (Fig. 2) and crayfish (Barnes, 1977), especially the relationship between activity of the different motor nerves, the pharmacologically induced patterns were considered to be fictive locomotion (cf. Fig. 2*A* with Fig. 3*A* and Fig. 2*B* with Fig. 3*B*). However, the induced backward pattern was 10–30 times slower than in normal crayfish walking (Barnes, 1977). Also, the period of the induced pattern was variable (see Table 1). During the same experiment, it was possible to obtain episodes of bursting at different frequencies, either “spontaneously” or by our intervention (current injection into some motor neurons).

Because of the variability of the period, we analyzed the relation between the burst duration in each motor nerve and the cycle period. In Figure 4*A*₂, it appeared that only the burst duration of the promotor nerve (pro) was correlated with the period, while the burst duration of the others (rem and a.lev) was not, as occurs during backward walking in the intact animal (Clarac and Chasserat, 1983). However, the relationship between burst duration and period was not always similar to those obtained in the intact animal (Fig. 4, *B*₂, *C*₂).

To characterize more precisely which type of ACh receptor is involved in this induction of rhythmic activity in the thoracic nervous preparation, we used cholinergic antagonists to block the rhythm induced by oxotremorine. The nicotinic antagonist *d*-tubocurarine at 10^{-5} M (even at 10^{-4} M) did not affect the induced rhythm (Fig. 5*C*), but the muscarinic antagonist, atropine, at 10^{-5} M completely suppressed the rhythm (Fig. 5*D*).

Induction of fictive locomotion in isolated TG4

In most of our experiments, we kept the nerve cord intact from TG3 to TG5, in order to maintain the activity of the interganglionic interneurons of these 3 ganglia which might link them to each other. In order to determine whether these interneurons are absolutely necessary for the rhythm generation within TG4, we performed experiments in which TG4 was isolated from all ascending and descending afferents. Figure 6 illustrates such an example. We induced the rhythm pharmacologically using the usual 3-ganglia preparation (Fig. 6*A*), then cut the connective between TG3 and TG4 (Fig. 6*B*), and finally the connective between TG4 and TG5 (Fig. 6*C*). The results suggest that TG4 contains the elements required for generation of rhythmic ac-

Figure 5. The induced rhythmic activity in the fourth thoracic ganglion of the crayfish is suppressed during bath application of muscarinic antagonists. *A*, Tonic activity recorded in the regular crayfish saline. *B*, Induction of rhythmic activity during bath application of 10^{-5} M, oxotremorine. *C*, The nicotinic antagonist (*d*-tubocurarine) at 10^{-5} M did not affect the induced rhythmic activity. *D*, By contrast, the muscarinic antagonist (atropine) at 10^{-5} M completely suppresses the rhythm. Horizontal bar, 10 sec.

tivity, even though the rhythm is not as well organized as with a chain of 3 ganglia. It is much less stable and more variable. We therefore prefer, in this preliminary investigation, to keep not only the studied TG4 but also TG3 and TG5.

Role of certain interneurons

Most of the time, the preparations showed only tonic activity. Sometimes, however, a certain rhythmicity was detected but without a very reproducible pattern. In 5 experiments we recorded from interneurons with a sustained membrane potential. Injection of a steady depolarizing current into these interneurons induced rhythmic motor activity in TG4 motor nerves. Two examples are presented (Fig. 7). In Figure 7*A*, we recorded from such an interneuron which did not show either action potentials or membrane potential oscillations. A brief depolarizing current (6 sec) into this interneuron strongly hyperpolarized a depressor motor neuron and simultaneously activated one unit of large amplitude in the levator motor nerve (Fig. 7*A*₁). Furthermore,

Figure 6. The induced rhythmic activity of the levator and depressor motor nerves is maintained even after isolation of the fourth thoracic ganglion from all ascending and descending afferents. *A*, Rhythmic activity induced in the usual thoracic nervous preparation that contains TG3, TG4, and TG5. *B*, After the connective between TG3 and TG4 is cut (level 1), the rhythm was maintained with a preparation containing only TG4 and TG5. *C*, After the connective between TG4 and TG5 is cut, the fourth thoracic ganglion is isolated from all other thoracic ganglia (level 2). The rhythm continues to be maintained. Spikes are sometimes clipped by the recording device. The schema on the left is the preparation used in this study, from TG3 to TG5, and the successive sections, 1 and 2. Horizontal bar, 10 sec.

before the end of this pulse, a rebound was detected in the depressor motor neuron's activity, at the same time as a strong inhibition of all levator units. Applying a steady depolarizing current to this interneuron for 20–40 sec induced rhythmic activity involving opposite activation of the depressor and levator motor units (Fig. 6*A*₂). The membrane potential of a depressor motor neuron oscillated regularly. In some cases, the preparations needed to be in an active state for the full effect of this interneuron to be observable. This means that if the preparation is quiescent with a low activation (as in Fig. 7*B*₁), depolarization of this interneuron controls only the level of discharge of some motor neurons. However, if the preparation had previously been pharmacologically activated (Fig. 7*B*₂), injection of a sustained depolarizing current into this interneuron induced rhythmic activity in the different motor nerves.

Role of certain motor neurons in the induced rhythmic activity

Induction of rhythmic activity in TG4 by oxotremorine also revealed interesting properties in some motor neurons. After a brief pulse of either depolarizing or hyperpolarizing current, they exhibited voltage-dependent regenerative membrane properties or plateau potentials (Fig. 8*A*). In the absence of oxotremorine, a brief current pulse resulted in only a passive response with no plateau potentials (Chrachri and Clarac, 1987). Some of these

Figure 7. Induction of rhythmic activity in the *in vitro* thoracic preparation by interneuron stimulation. **A**, Interneuron inducing spontaneous rhythmic activity without any pharmacological activation. **A₁**, Injection of a depolarizing current (6.44 sec) into an interneuron hyperpolarizes the depressor motor neuron and excites levator motor units. Before the end of this pulse, a rebound is detected in the depressor motor neuron's activity, and the levator motor units are inhibited. **A₂**, Injection of a sustained depolarizing current into the same interneuron induces rhythmic activity in the pmr and the levator nerves. The depressor motor neuron shows regular membrane potential oscillations. **B**, Interneuron inducing rhythmic activity following bath application of oxotremorine: **1**, Injection of a depolarizing current (0.7 sec) into this interneuron depolarizes a promotor motor neuron and hyperpolarizes a depressor motor neuron; **2**, after bath application of oxotremorine, an injection of a sustained depolarizing current into the same interneuron induces rhythmic activity in the different motor nerves of the fourth thoracic ganglion. The intracellular recording shows the membrane potential oscillations of a promotor motor neuron. Calibration bars: horizontal, 5 sec (**A**), 1 sec (**B**); vertical, 5 mV and 3 nA.

motor neurons, when injected by a sustained depolarizing current, were able to elicit an increase in the frequency of the rhythm (Fig. 8*B*). A sustained hyperpolarizing current not only lowered the activity of the cell below the spiking threshold (Fig. 8*C₁*, *C₂*), but also decreased or completely suppressed the rhythm. Only 1 or 2 motor neurons from each of the promotor, levator, and remotor groups were able to activate or inhibit the rhythm in this way.

Graded synaptic transmission (GST) is the common mode of synaptic function in nonspiking neurons (Pearson and Fournier, 1975; Siegler, 1984). GST can also be used by spiking neurons (Nicholls and Wallace, 1978; Raper, 1979), as demonstrated by bath blocking of spike generation by TTX. To determine if GST was used by thoracic neurons, we added TTX to the bath to give a final bath concentration of 5×10^{-6} M. This blocked spike activity in TG4 without blocking the membrane potential oscillations of the 2 recorded motor neurons (Fig. 9). Also, the

induced oscillating pattern between these 2 motor neurons remained unchanged (Fig. 9*A₂*). This result suggests that graded release may be sufficient for the regulation of phase relationships in the pattern. In another experiment, simultaneous intracellular recordings from a pair of interacting remotor motor neurons showed that, during bath application of TTX, injection of a sustained depolarizing current into one remotor motor neuron increased the frequency of membrane potential oscillations of these 2 motor neurons (Fig. 9*B₁*). Hyperpolarization of the same remotor motor neuron completely suppressed these membrane potential oscillations (Fig. 9*B₂*). This suggests that, in the presence of TTX, some motor neurons continued to have access to the CPG. Muscarinic activation was required for this property.

We found another property of this network that concerns the relationship between antagonist motor neurons. Figure 10 shows recordings from one anterior levator motor neuron and one depressor motor neuron. We changed their membrane potential

Figure 8. Importance of certain promotor motor neurons in the pharmacologically induced rhythmic activity. *A*, Some promotor motor neurons exhibit regenerative properties; a short pulse of depolarizing current into such a promotor motor neuron is enough to induce a membrane potential oscillation of this motor neuron and simultaneously induces a burst in other promotor units. *B*, A sustained depolarizing current (+2 nA) injected into a promotor motor neuron increases significantly the very slow induced rhythmic activity during bath application of oxotremorine. *C*, By contrast, during well-organized rhythmic activity, injection of sustained hyperpolarizing current (−2 nA) into a single promotor motor neuron disorganizes the rhythm completely. The square labels remotor units, while the triangle labels units of the depressor. Calibration bars: horizontal, 5 sec (*A*, *B*), 10 sec (*C*); vertical, 10 mV and 2 nA.

using sustained depolarizing or hyperpolarizing current. When both motor neurons were depolarized (Fig. 10*A*), membrane potential oscillations of one were the mirror image of the other; when the levator was activated the depressor was silent, and each change in the membrane potential of the levator was reflected in the membrane potential of the depressor. Even when the levator motor neuron was maintained at its normal membrane potential and below its spiking threshold, the membrane potential oscillations of the depressor continued to be closely linked with that of the levator motor neuron (Fig. 10*B*). When no current was injected (Fig. 10*C*), the relationship between their respective membrane potential oscillations was not as tightly linked. When the depressor was hyperpolarized (Fig. 10*D*), no relation between the 2 motor neurons was observed. The sustained membrane potential of the depressor motor neurons was sufficient to suppress the membrane potential oscillations in the levator motor neuron. This had certain consequences for the general rhythmic activity, which was still maintained but at a reduced intensity. Thus, it appeared that depolarization produced a clear opposition between antagonist motor neurons,

while hyperpolarization was able to suppress or at least disorganize the rhythm.

Modulation of the induced rhythm by stimulation of the pdr

It was possible to modify the induced rhythmic activity, especially when the rhythm was not well-organized, by pdr stimulation. This root contains both sensory and motor axons, including the dactyl sensory afferents (Clarac and Chrachri, 1989). In 30% of the experiments reported here, the induced activity was either not clearly patterned or was tonic. With different types of pdr stimulation, it was possible to produce an organized rhythm. In Figure 11*A*, the preparation was not rhythmic after bath application of oxotremorine. A sustained pdr stimulation at 1 Hz was enough to induce a bursting discharge in the promotor nerve, pmr, and the levator motor nerves. The intracellular recording showed membrane potential oscillations of one depressor motor neuron in opposition with the levator motor units. In another nonrhythmic preparation, a train of pdr stimulation simultaneously inhibited remotor and levator motor neuronal activity and excited promotor and depressor activity.

Figure 9. TTX effect on the induced rhythmic activity during bath application of oxotremorine. *A₁* and *A₂*, During bath application of oxotremorine and TTX, the promotor and depressor motor neurons continue to show membrane potential oscillations with the same relationships that they had before the addition of TTX (compare *A₁* and *A₂*). *B₁* and *B₂*, Simultaneous intracellular recordings from a pair of remotor motor neurons (arbitrarily named REM.1 and REM.2) shows that these 2 motor neurons are oscillating in phase. Injection of a sustained depolarizing current into REM.1 increases the membrane potential oscillations of these 2 motor neurons. By contrast, a hyperpolarizing current into the same motor neuron stops their membrane potential oscillations. Calibration bars: horizontal, 8 sec; vertical, 20 mV and 2nA.

The intracellular recording from a depressor motor neuron showed a strong depolarization with each train of stimuli. This particular pdr stimulation was able, artificially, to organize a “backward” pattern, as demonstrated in Figure 11*C₁*, 11*C₂*. The preparation was initially rhythmic but with very chaotic bursts, without a simple recognizable pattern (Fig. 11*C₁*). A tonic stimulation at 1 Hz significantly organized the pattern (Fig. 11*C₂*). Although the promotor units were not clearly bursting, the remotor, levator and depressor clearly showed patterned activities. This is further shown by regular oscillations of the depressor motor neuron (compare depressor motor neuron’s membrane potential oscillations in Fig. 11*C₁* and 11*C₂*). If the pharmacologically induced pattern corresponds to a “forward” pattern (promotor and depressor motor neurons are active in

Figure 10. Study of the antagonism between 2 antagonist motor neurons (levator and depressor) during successive injection of a sustained current injection. *A*, When both motor neurons are depolarized (+2 nA), the membrane potential oscillations of one are exactly the mirror image of its antagonist. *B* and *C*, When the levator and the depressor, respectively, are returned to their resting membrane potential, they are not as tightly linked as in *A*. *D*, Injection of a sustained hyperpolarizing current (−4 nA) into the depressor motor neuron disorganizes not only the membrane potential oscillations of this motor neuron but also that of the levator motor neuron. Calibration bars: horizontal, 10 sec; vertical, 10 mV and 2 nA.

opposition), a tonic stimulation at 2 Hz reversed the relationship, since the promotor and depressor became active in phase, corresponding to a “backward” pattern (cf. Fig. 12, *A* and *B*).

Modulation of the induced rhythm by certain interneurons

When the rhythm was very irregular, some interneurons seem to be important in the organization of the synchrony and antagonism between motor units. Two examples are presented. In Figure 13*A*, the rhythm was very irregular, with no clear coordination between the promotor and the levator motor units. In the depressor there were bursts of 2 different intensities. The larger burst corresponds to the complete inhibition of all levator motor neurons, the smaller burst to a partial inhibition. We recorded from one interneuron whose discharge at rest was irregular (Fig. 13*A*). Injection of a sustained depolarizing current

Figure 11. Influence of the posterior distal root (pdr) stimulation on the induced rhythmic activity during bath application of oxotremorine. *A*, Sustained stimulation of pdr at 1 Hz is able to induce rhythmic activity in the thoracic motor nerves of TG4. *B*, Phasic stimulation of pdr simultaneously inhibits remotor and levator units and excites promotor and depressor units. *C* and *D*, When the induced rhythm is not well organized, a sustained pdr stimulation (2 Hz) organizes the rhythm into the pattern characteristic of fictive backward locomotion. Calibration bars: horizontal, 10 sec (*A*, *B*), 5 sec (*C*, *D*); vertical, 15 mV.

into this interneuron organizes the induced rhythm (cf. Fig. 13, *A* and *B*). Hyperpolarization of the same interneuron disorganizes the rhythm (Fig. 13*C*). This interneuron seems to be crucial for the organization of the rhythm into a coherent pattern. Sometimes rhythmicity was detected only in the motor neurons innervating a single pair of antagonist muscles. Figure 14 shows such an example where only the levators and depressor were firing in opposition, while activity in the promotor nerve was tonic. Depolarization of one interneuron was able to produce a patterned output. At rest, the interneuron's membrane potential was flat (Fig. 14*A*), but when depolarized, its membrane potential oscillated in phase with a depressor motor neuron and also could condition the promotor tonic discharge to fire in phase

Figure 12. Electrical stimulation of the posterior distal root (pdr) is able to reverse the pattern from forward to backward fictive locomotion. *A*, The induced pattern during bath application of oxotremorine corresponds to a fictive forward pattern (promotor and depressor motor units are active in opposite phase). *B*, A tonic stimulation of pdr reverses the induced pattern from forward to backward (the promotor and the depressor motor units are active in phase). Calibration bars: horizontal, 5 sec; vertical, 10 mV.

with the levator (Fig. 14*B*). When this interneuron was depolarized, it did not change any particular relationship between the levator and depressor motor neurons but seemed to involve the promotor motor units in the rhythmic output.

In the 30% of experiments in which muscarinic agonists did not produce a well-organized pattern, some interneurons were able to organize the pattern. It could be that these interneurons were at a membrane potential below the threshold required for this effect.

Discussion

Although we have a good knowledge of the locomotory behavior of Crustacea as a result of the extensive electromyographic studies of the intact animal moving freely or on the treadmill (Clarac and Chasserrat, 1983), it is only recently that we have been able to obtain rhythmic motor output in an *in vitro* crayfish thoracic preparation (Sillar and Skorupski, 1986; Chrachri and Clarac, 1987). In this report we studied the pharmacologically induced rhythmicity in relation to that obtained in intact Crustacea. The great similarities between the patterns recorded in both conditions allow us to call the induced *in vitro* pattern "fictive locomotion" as occurs in vertebrates (Grillner, 1981).

Our preparation closely resembled that of Skorupski and Sillar (1986). These authors have studied the proximal motor neurons in relation to the TCMRO in *Pacifastacus leniusculus*. The rhythms obtained are very similar in both preparations. However, there are some qualitative differences. In their recordings, the depressor motor neurons were often not rhythmic. We maintained the distal afferents, and this has facilitated the activation of the depressors as suggested by Grote (1981). With the TCMRO kept intact, Skorupski and Sillar (1986) obtained a "forward"

Figure 13. Influence of a single interneuron on the induced rhythmic activity during bath application of oxotremorine. *A*, Nonorganized rhythm during bath application of oxotremorine. *B*, Injection of a sustained depolarizing current (+1 nA) into a single spiking interneuron organizes the rhythm. *C*, By contrast, injection of a sustained hyperpolarizing current (−1 nA) into the same interneuron disorganizes the rhythm. Calibration bars: horizontal, 5 sec; vertical, 20 mV.

rhythm more frequently than a “backward” one. In our preparation, “backward” activity was obtained more frequently. We also demonstrated that a general distal stimulation was able to “reorganize” the induced pattern or even to switch a “forward” into a “backward” pattern (Fig. 12). The pdr is quite complex because it contains both distal motor neurons and a great amount of distal afferents. All attempts to split the sensory from the motor components of this nerve were unsuccessful. Nevertheless, we hypothesize that both could play a role. It was reported by Sillar and Skorupski (1986) that antidromic stimulation of most motor nerves is able to induce rhythmic activity or to organize the rhythm. This is to be expected, as some motor neurons have access to the CPG. The switch from forward to backward could be mainly due to sensory terminals which could

Figure 14. Interneuron enabling motor neurons to be involved in an ongoing rhythm. *A*, In the control, only the levator and depressor motor units are rhythmic, the promotor units being tonically active. *B*, Injection of sustained depolarizing current (+3 nA) into a single interneuron seems to condition the promotor motor units to be involved in the ongoing rhythm (the promotor and the levator motor units are active in phase and in opposition with the depressor motor neuron). Calibration bar: horizontal, 5 sec; vertical, 10 mV.

activate some interneurons involved in the locomotory circuitry.

The differences between the 2 preparations may be due to different sensory elements being kept intact in each preparation. We maintained the distal afferents, while Sillar and Skorupski (1986) maintained the proximal ones, in particular, the TCMRO, which seems to have a very powerful action on the promotor and the remotor motor neurons.

Motor program reversals of the type we have observed have been reported in other appendages and in other arthropods. Heitler (1985) obtained spontaneous reversal of the swimmeret rhythm. Also, he demonstrated that the depolarization of certain interneurons could reverse the direction of swimmeret rhythm. The scaphognathites rhythmically beat to drive the water across the gills and periodically reverse the direction of the flow (Simmers and Bush, 1983). Bässler et al. (1985) showed that a decapitated stick insect produced spontaneous forward walking in the prothoracic and backward walking in the metathoracic legs after the interappendicular connectives were cut off.

Cholinergic effects in TG4

Spontaneous rhythmic activity was not clearly obtained in our preparation unless the TCMRO was kept intact (Skorupski and Sillar, 1986). In the absence of the TCMRO and most of the proximal sensory afferents, bath application of oxotremorine or pilocarpine was required to evoke rhythmic activity in TG4. This induction seemed to act through muscarinic receptors. The addition of *d*-tubocurarine did not abolish the rhythm. By contrast, the addition of atropine to the bath stopped the induced rhythm (Fig. 5). This strong action of cholinergic agents was not

surprising; Florey (1967) demonstrated that a direct perfusion of ACh or eserine through the crab sternal artery induced movement of the locomotory appendages. He also showed that long stimulation of sensory nerves released ACh. Furthermore, using a radiochemical assay, Hildebrand et al. (1971, 1974) and Barker et al. (1972) confirmed that sensory afferents synthesize and release ACh. Therefore, ACh plays an important role in sensory transmission.

A few experiments have recently demonstrated the role of cholinergic effects on the CPG. It has also been demonstrated in the stomatogastric system that an identified interneuron, APM, was able to alter strongly the pyloric rhythm which had been previously depressed (Nagy and Dickinson, 1983; Dickinson and Nagy, 1983). These authors explain APM's effects on the pyloric rhythm by its ability to alter plateau properties of the pyloric neurons. They also showed that APM's effect was through muscarinic receptors. In the mollusc, *Limax maximus*, it has been demonstrated that exogenous ACh applied to the cerebral ganglia was able to trigger the feeding motor program, while antagonist substances blocked it (King et al., 1987).

Characteristics of the induced rhythmic activity

The motor patterns induced in the *in vitro* preparation reflect the same patterns found in intact animals. However, the *in vitro* pattern differs from the intact pattern in several respects. The frequency in the isolated preparation was variable. In some preparations, the period was short (3–10 sec); in others, it was much longer (40–50 sec; see Fig. 4). This is also true in the locust flight system (Pearson and Wolf, 1987).

The induced rhythmic activity did not seem to be organized in the same manner from one preparation to another. A systematic study of the relation between burst duration and cycle period showed that it was often not maintained as in intact animals, where the burst duration of the PS, but not of the RS, muscles is linked to the period (Clarac and Chasserat, 1983). This is not always true in the *in vitro* preparation (Fig. 4). Clarac (1978) has reported such variability after limb autotomy in Crustacea.

Role of motor neurons in the induced rhythm

Muscarinic agonists induced plateau properties in some walking motor neurons. These motor neurons showed slow regenerative potentials like some neurons of other excitable systems in both invertebrates (Gola, 1974; Russell and Hartline, 1982, 1984) and vertebrates (Ohba et al., 1975). Plateau properties in thoracic motor neurons were not exclusively caused by muscarinic agonists, because these plateau potentials can be detected in some motor neurons in spontaneously rhythmic preparation (Sillar and Elson, 1987; A. Chrachri, unpublished observations). A brief depolarizing current into thoracic motor neurons was able to induce plateau potentials in the injected motor neuron, while a brief hyperpolarizing current pulse terminated an ongoing membrane potential oscillation (see Fig. 8A and Chrachri and Clarac, 1987). This type of motor neuron was found in each of the motor neuron pools with the exception of the depressor pool. These plateau potentials might have an important role in pattern generation, because these motor neurons could control the burst timing in response to phasic inputs (Russell and Hartline, 1978; A. Chrachri et al., unpublished observations).

Some of the motor neurons that exhibit plateau properties were also able, through a sustained current injection, to increase or decrease the general rhythmic activity, demonstrating that

they have access to the CPG (Fig. 8B, C). On the other hand, other motor neurons seemed to behave like followers. They were unable to affect the induced rhythmic activity and did not exhibit plateau properties.

In Crustacea, it is not uncommon for motor neurons to be part of the CPG. In the pyloric network of the stomatogastric system, all motor neurons exhibit plateau properties and are part of the CPG (Selverston and Moulins, 1985). In the swimmeret system, Heitler (1978) was unable to demonstrate plateau properties, but he did find that current injection into some motor neurons can increase the frequency of the swimmeret rhythm. By contrast, in other preparations, motor neurons are considered only as followers, without direct or indirect access to the CPG. For example, Wallen and Lansner (1984) demonstrated in lamprey that a simultaneous depolarization of a large number of motor neurons by antidromic stimulation does not modify the fictive locomotory rhythm.

In the presence of TTX, both membrane potential oscillations and the relationship between neurons within TG4 were maintained. This reveals that, as in the stomatogastric system (Raper, 1979) and the lamprey (Grillner et al., 1987), there is a graded liberation of neurotransmitter. This graded release is necessary and sufficient for the generation of rhythmic membrane potential oscillations of the fourth walking leg. Muscarinic activation is required for this effect.

The antagonism between motor neurons in TG4 could be direct or indirect, especially for the levator and depressor motor neurons (Chrachri and Clarac, 1989). Figure 10 shows that the antagonism between these 2 motor neurons is very strong when they are both depolarized and weak when the depressor motor neuron is hyperpolarized. Also the general rhythmic activity was decreased. This can be explained by an interaction between these 2 motor neurons either direct or via one or more interneurons. Because most of the interactions among walking leg motor neurons are not spike-mediated, it is very difficult to decide whether these interactions are direct or indirect using the usual physiological test for monosynapticity (the latency to the onset of the response). However, since hyperpolarization of a single motor neuron has a dramatic effect on the rhythmic motor output, it can be suggested that rhythm generation in the thoracic nervous system is mainly carried out at the motor neuronal level.

Importance of certain interneurons on the rhythm

Two types of interneurons were studied: those which seemed to be able to induce patterned activity in TG4 and those which seemed to enable motor neurons to be involved on an ongoing rhythm.

The former did not show any membrane potential oscillations, and their depolarization was able to induce rhythmic activity even in the absence of pharmacological activation. We have presented 2 examples in Figure 7. The first interneuron was able to induce a walking motor program in TG4 in the absence of muscarinic agonists. The other interneuron was able to induce a pattern only after weak muscarinic activation. Due to their effects, the 2 interneurons appear to belong to the same class, but their action seems to depend upon the general activity of the preparation.

The second class of interneuron appears to be crucial for the organization of rhythmic activity. Such interneurons stabilize the latency between bursts of different groups of motor neurons. As shown in Figure 13, this type of interneuron seemed to play

an important role in the coordination between bursts, especially between promotor and levator. Depolarization of one of these interneurons coordinated the 2 bursts, and hyperpolarization partially disorganized the rhythm. In preparations where all motor groups were not rhythmic (Fig. 14), these interneurons were capable of involving the promotor motor units in the rhythmic motor output.

In conclusion, for a given thoracic ganglion (TG4), the network generating the locomotory activity was quite complex, and in isolation, it produced outputs which were sometimes different from the normal walking pattern (especially in frequency). This may be explained by the absence of certain sensory elements (Pearson and Wolf, 1987). Our data suggest that (1) most motor neurons were not sufficiently depolarized and the rhythm was slower; (2) phasic sensory afferents were not present to entrain the rhythm (Sillar et al., 1986); and (3) coordinating interneurons were not sufficiently active to link the timing of different bursts in a characteristic pattern.

The central activity of the *in vitro* preparation was often not robust, which may seem to be a handicap for studying the generation of motor patterns but is, in fact, a real advantage for the characterization of the role of individual motor neurons and interneurons in activation and coordination of the walking motor program. We hope by further experiments to obtain more information on the central circuitry and especially to determine how sensory information is used in such a system. This preparation is appropriate for the study of the mechanism and functional significance of a behavioral switch.

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