



ACTIVITY PATTERNS OF INHIBITORY MOTONEURONES AND THEIR IMPACT ON LEG MOVEMENT IN TETHERED WALKING LOCUSTS

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Summary

A preparation is described which permits intracellular recording of neural activity in tethered walking locusts. Minimal dissection is required, leaving the animal nearly intact.

In this preparation, the activities of the three common inhibitory motoneurones (CIs) supplying the muscles of the middle leg were recorded. CIs were completely or almost completely silent in quiescent locusts but often produced a tonic spike discharge during walking activity. This tonic discharge was modulated in the step cycle, bursts of action potentials being generated in conjunction with the swing phase of the leg movement. The bursts peaked at around the start of leg protraction. They were remarkably similar in all three CIs and in a variety of different walking situations (forward and backward walking, searching, etc.). The only notable difference was that bursts of CI₁ normally peaked some 40 ms before those of CI₂. These results indicate that CI activity is timed according to the muscle group innervated but that no further functional specializations exist.

Activity of CI₁ was manipulated by current injection. For example, decreasing CI spike discharge by the application of hyperpolarizing current reduced the velocity of leg protraction in the walking animal. This demonstrates that CI₁ plays an important role in determining the speed of the swing movement.

Introduction

In a variety of invertebrate animals, muscles are innervated by inhibitory motoneurones in addition to their normal supply by excitatory axons. The fact that these neurones counteract depolarizations elicited by excitatory input, and in this way reduce amplitude or duration of resulting muscle contractions, allows them to be defined as inhibitory. These inhibitory motoneurones have been studied most extensively in crustaceans (review by Wiens, 1989) and insects (review by Pearson, 1973). Common inhibitors (CIs), which innervate several muscles, often with

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different and even antagonistic functions, are distinguished from specific inhibitors, which have a single target muscle. Specific inhibitors are absent in insects.

In the crab, a single common inhibitory neurone innervates all intrinsic and extrinsic muscles of a walking leg (Rathmayer and Bevington, 1986; Wiens, 1989). The locust, in contrast, has three common inhibitors in each hemisegment. They innervate most of the intrinsic and extrinsic musculature of the respective limb. CI_1 supplies 11 muscles which move the proximal leg segments, coxa and trochanter, and the extensor of the tibia, while CI_2 and CI_3 both innervate three muscles which control the tarsal segments plus the flexor of the tibia (Hale and Burrows, 1985). The presence of three different CIs in the locust — as opposed to the single inhibitor of the crab — suggests that they are specialized with regard to their function.

Analysis of CI discharges in a variety of behavioural situations can reveal possible functional specializations. This was accomplished by the recording of inhibitory motoneurone activity in walking locusts. Previously, action potentials of CI_1 have been monitored by means of neurogram electrodes in intact animals (Burns and Usherwood, 1979). Neurogram recordings are inappropriate in the present case, however. Besides the general problems of CI spike identification (Burns and Usherwood, 1979), CI_2 and CI_3 are difficult to record and impossible to distinguish in neurograms since their axons run *via* the same nerves and have very small diameters (Hale and Burrows, 1985). A preparation was therefore developed that allows intracellular recording of inhibitory motoneurons in nearly intact, walking animals.

It is known from electrophysiological studies that CIs predominantly innervate those fibres of a muscle supplied by a slow excitatory motoneurone (Hale and Burrows, 1985; Wiens *et al.* 1988). Combined electrical stimulation of excitatory and inhibitory motoneurons in nerve-muscle preparations (Usherwood and Runion, 1970; Burns and Usherwood, 1979) or isolated limb preparations (Ballantyne and Rathmayer, 1981) further demonstrated that CI activity facilitates rapid contraction and relaxation cycles. This is accomplished by reducing or preventing the activation of tonic muscle fibres *via* pre- and postsynaptic inhibition (Rathmayer and Erxleben, 1983) and by accelerating their relaxation (Pearson, 1973). These results clearly suggested that the function of CI is to promote rapid leg movements in the walking animal by disabling tonic muscle fibres in the sparsely and polyneurally innervated arthropod leg musculature. However, a demonstration of the actual behavioural relevance of CI activity during walking and a quantitative assessment of the possible impact on leg movement are lacking.

The intracellular penetration of motoneurons in tethered locusts not only permitted morphological identification of the impaled inhibitor by dye injection, but also the manipulation of CI discharges by current injection. In this way it was demonstrated that activity of CI_1 has a significant influence on the speed of leg protraction in the walking animal.

Preliminary results of the present investigation have appeared in abstract form (Wolf, 1989; Wolf and Schmidt, 1990).

Materials and methods

Animals

Male and female *Locusta migratoria* aged between 2 and 4 weeks after the imaginal moult were taken from a crowded colony at the University of Konstanz. The animals were not fed for 1 or 2 days prior to experiments to improve their motivation for walking. No differences were noted with respect to gender.

Experimental apparatus

The locusts were glued (beeswax/violin resin mixture 1:2) into a fork-shaped holder by their thoracic pleurae and placed inside a pair of treadwheels in an inverted position. Each treadwheel supported the legs on one side of the body and the thoracic sterna were accessible through the slit between the two wheels (Fig. 1). The treadwheels' axis was suspended on a balance lever so that the load to be carried by the tarsi could be adjusted to match the animal's body weight. During experiments, the treadwheels were locked on the axis because animals were much less inclined to walk when the wheels were uncoupled. Wheels and spokes were made from lightweight plastic foam (Rohacell) and Perspex, respectively. The wheels had a diameter of 16 cm and their total mass amounted to 13 g. The inertial momentum to be accelerated by the animal was about 8 g, corresponding to 300–400 % of a locust's body mass.

The animals adapted quickly to the treadwheels and displayed normal walking behaviour after a few minutes. Motivation to perform longer-lasting episodes of walking activity (up to 20 s without interruption) in response to tactile stimulation of thoracic sterna, abdomen or cerci was increased by covering compound eyes and ocelli with black lacquer.

Preparation and recording technique

The general procedure for recording intracellularly from central neurones in tethered, nearly intact locusts was adapted from Wolf and Pearson (1987). After the animal had been tethered and placed inside the treadwheels, a small piece of sternal cuticle was excised above the mesothoracic ganglion. The ganglion was exposed by displacing and partly removing overlying tracheal sacs, fatty tissue and salivary glands. The ganglion was supported on a steel platform (Fig. 1A) and kept submerged in saline (composition according to Clements and May, 1974). Somata and, rarely, neuropile processes of the three common inhibitory motoneurones (CI₁–CI₃; Hale and Burrows, 1985) were impaled with glass microelectrodes. A preliminary identification of the penetrated neurone was performed according to physiological criteria (Schmidt and Rathmayer, 1988). The electrodes were filled with a 5 % solution of Lucifer Yellow in distilled water and had resistances of 30–100 M Ω . The neurones were routinely filled with dye by injection of hyper-

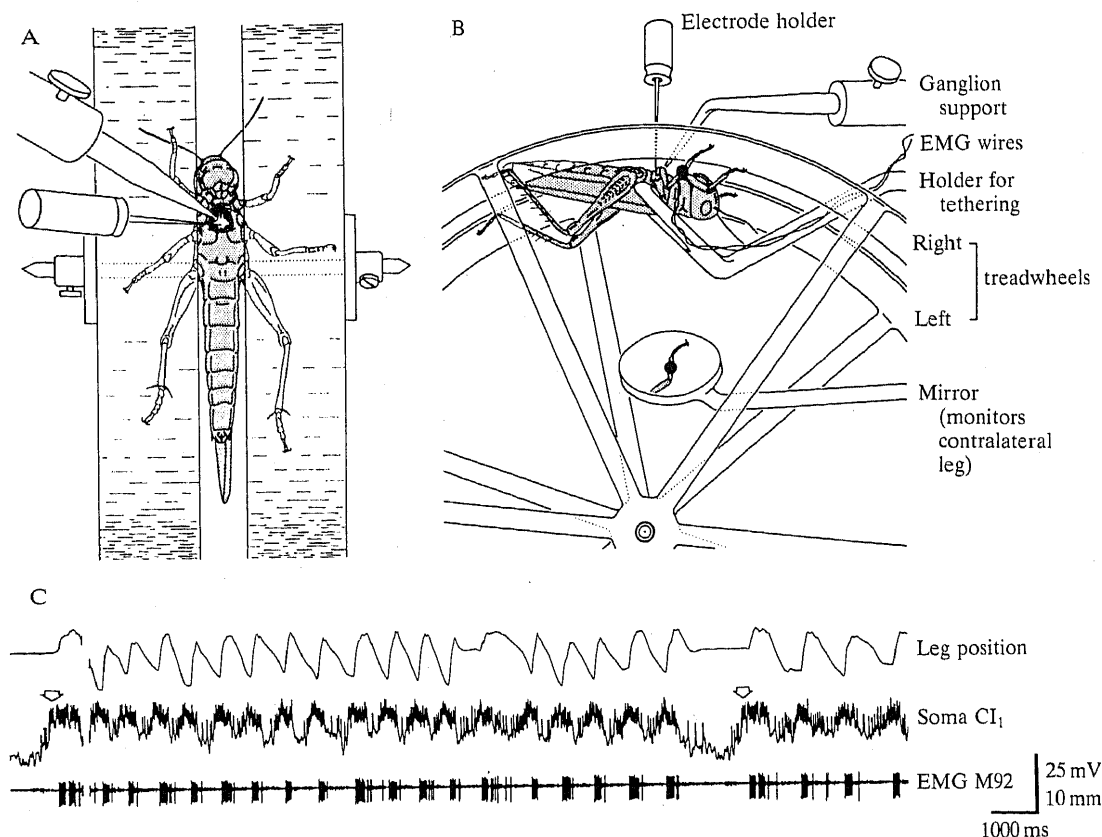


Fig. 1. Intracellular recording of neural activity in tethered, walking locusts. The experimental arrangement is shown, viewed from above (A) and from the side (B); for explanation see text. (C). Sample recordings of leg position (top trace, anterior is to the top), CI_1 activity (middle trace) and anterior rotator coxae EMG (bottom trace; M92 after Snodgrass, 1929). Walking was initiated by tactile stimulation of the abdomen (open arrows).

polarizing current (approx. 10 nA for up to 30 min) on completion of physiological experiments. The ganglia were removed from the animal, dehydrated and cleared according to standard procedures (Robertson and Pearson, 1982) and examined as wholemounts under an epifluorescence microscope. The inhibitors were identified by morphological criteria (Hale and Burrows, 1985).

Electromyographic (EMG) recordings were taken bilaterally from muscles 92 (anterior rotator coxae, numbering according to Snodgrass, 1929) and served as a reference for walking activity. EMG wires (20 μ m steel wires insulated except for the cut end) were inserted through prepared holes in the sternal cuticle at the muscle attachment sites (sternellar lobe of mesosternum; Snodgrass, 1929) and secured with wax resin.

Movements of the middle legs were monitored with an optoelectronic camera, modified after von Helvesen and Elsner (1977). A small piece of scotch-light tape

(3M type 7610) was glued to the most proximal part of the mesothoracic tibia. The reflective tape was illuminated through a semi-reflecting mirror and its image projected (*via* the same mirror) onto a position-sensitive diode (TS optoelectronics, type TS-PXY-10). Only the longitudinal (back-and-forth) component of the leg movement was recorded.

Experiments were performed at 21–25°C. Step frequencies above 3 Hz were rarely observed at these temperatures, but heating of the experimental chamber resulted in rapid deterioration of the preparations.

Data acquisition and evaluation

Intracellular and electromyographic recordings and leg position signals were stored on magnetic tape (Racal store 4DS) for later display on a chart recorder (Gould ES 1000). Regular step sequences were selected for further evaluation on a desktop computer (Hewlett Packard 9000/226). Step cycle durations and corresponding CI spike discharges were digitized (digitizing pad Hewlett Packard 9874A) and time and phase histograms calculated from these data (Figs 3, 4, 5, 8). The touch-down of the tarsus after leg protraction (anterior extreme in the position record) served as reference point in the histograms, the interval between two subsequent touch-downs defining step cycle duration. Histograms were constructed to cover 1.5 step cycles, i.e. they extended from one touch-down to the next and then another 50% of the respective cycle period, to provide a comprehensive representation of CI activity. Thus, step cycles were considered only if they were followed by at least one other step of about the same duration.

The velocity of leg protraction was determined by displaying the leg position signal on a digital storage oscilloscope (Tektronix 5223). After calibration of the optoelectronic camera, immediate velocity measurements could be made by determining the slope of the position *versus* time oscilloscope trace (insets Fig. 10).

Statistical analyses of time histograms and protraction velocity distributions were performed using the two-tailed Student's *t*-test (Sachs, 1978).

Results

The three common inhibitory motoneurons supplying the locust middle leg (CI₁–CI₃; Hale and Burrows, 1985) were studied. Mesothoracic inhibitors were examined because the middle legs are the least specialized in *Locusta* (Burns and Usherwood, 1979). CIs were impaled in tethered animals walking on a treadmill (Fig. 1) and their depolarization and discharge patterns were recorded for a variety of walking situations.

Activities of common inhibitors CI₁–CI₃ during walking

In quiescent locusts, all three inhibitory motoneurons produced action potentials only sporadically or not at all. As soon as walking activity commenced, the membrane potentials of the inhibitors were depolarized and maintained, tonic

spike discharges were often produced that lasted throughout continuous walking episodes. This tonic activity is clearly seen in the recordings shown in Fig. 2. The magnitude of the maintained depolarization, and hence of the tonic spike discharge, was variable, however, and apparently correlated with the animal's state of arousal. In general, the maintained depolarization was most pronounced (up to 20 mV) during bouts of rapid locomotion and backward walking – types of behaviour that were elicited by severe disturbances (e.g. gently pinching the abdomen). A continuous spike discharge was almost or completely absent when the locust was left undisturbed and walking commenced spontaneously (see Fig. 7C). Even in this situation, however, CI membrane potentials remained significantly higher during walking than they were in the resting animal. When

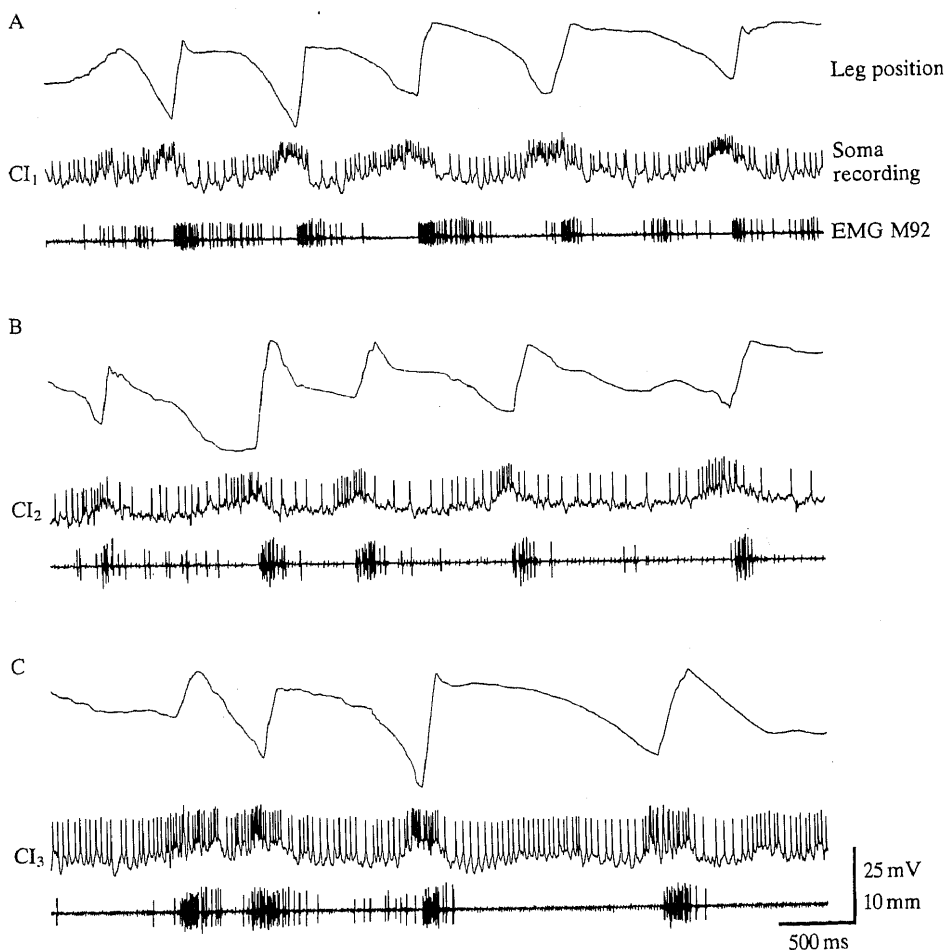


Fig. 2. Sample recordings of activity in CI_1 (A), CI_2 (B) and CI_3 (C) during walking. Top traces represent leg position signals (anterior is to the top), middle traces intracellular recordings, and bottom traces EMGs from muscle 92 (small potentials are from slow unit).

locomotion was interrupted, CI membrane potentials dropped back to the resting level. This is illustrated in Fig. 3 (see also Fig. 1C).

The maintained activity of the CI neurones during walking contrasts with the situation in most excitatory motoneurones, especially the fast axons. As a rule, fast motoneurones produce distinct, rhythmic bursts of action potentials in the step cycle and are silent in the quiescent animal. A modulated, tonic spike discharge, if present, is produced only by slow motor units. In the quiescent animal a continuous discharge of slow units is employed for the maintenance of posture (Burns and Usherwood, 1979; Bässler, 1983; compare EMGs of muscle 92 in Figs 2 and 3A).

CI activity was modulated in the rhythm of the step cycle. Periodic bursts of

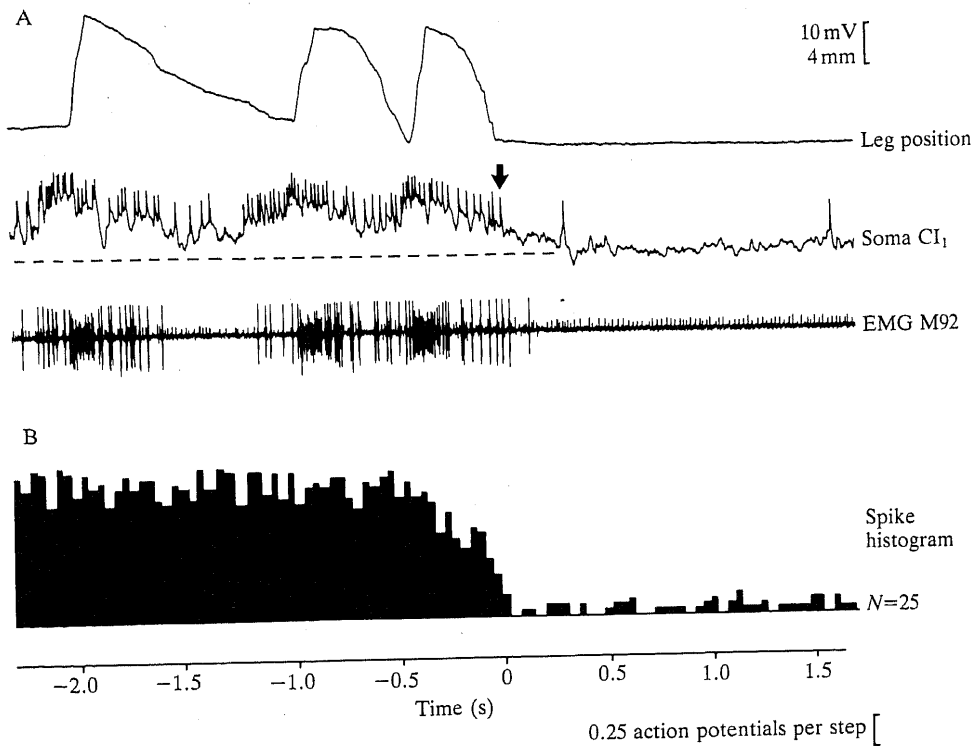


Fig. 3. Termination of walking episodes. (A) The upper three traces show the end of a selected step sequence. Top trace: leg position signal (anterior is to the top). Second trace: soma recording of CI₁. Note decrease in membrane potential after termination of the sequence (dashed reference line, compare also Fig. 1C). Third trace: EMG from M92. Small potentials are from the slow motor unit. Note its tonic activity in the quiescent locust. (B) Spikes of CI₁ recorded during 25 terminations, were evaluated. The time scale is in register with the sample recordings above. The last spike of the CI burst associated with the final swing movement (indicated by the arrow in the sample recording) was taken as a reference for calculating the histogram (bin width 40 ms). The start of walking episodes is not shown because locomotion was usually initiated by tactile stimulation resulting in additional CI excitation (see arrows in Fig. 1C).

action potentials were generated in all three inhibitory neurones (Figs 1C, 2). Depending on the magnitude of the maintained discharge, their appearance ranged from upward modulations in spike frequency (Fig. 2C) to distinct bursts of action potentials (see Fig. 7C). These spike bursts were closely associated with the swing phase of the leg movement (also termed return stroke, equivalent to leg protraction during forward walking). They peaked at about the time when the tarsus was lifted off the ground and lasted until it touched down again and often for a few milliseconds longer. Discharge patterns and rhythmic depolarizations looked similar in all three CIs (Fig. 2) but, because of the variability of both rhythmic spike bursts and maintained discharges, a detailed comparison based on individual recordings was not possible. Time and phase histograms were therefore calculated from a large number of step cycles recorded in several animals. In Figs 4, 5, and 8, data for CI₁ and CI₂ are based on recordings from 33 and 20 locusts, respectively. CI₃ was recorded only six times, a number too small for analysis of step cycle duration. Therefore, only the summary histogram in Fig. 5 is shown.

In Fig. 4, histograms are presented for the extreme ranges of step cycle periods observed during normal forward walking. Cycle periods below 500 ms were evaluated in Fig. 4A,B and those above 2000 ms in Fig. 4C,D. Two features are notable in these histograms. First, there is little change in the appearance of the spike bursts with changes in cycle period and, second, the discharges of CI₁ and CI₂ are remarkably similar – again regardless of step cycle duration. This is true not only for the extremes shown but also for intermediate step cycle periods (see Fig. 8A). For instance, with a more than fivefold increase in cycle duration, the half-widths of the spike bursts in the histograms increases by only 36 % (compare Figs 4A and C), which is close to the range of step-to-step variability when judging individual recordings. Half-widths range from 250 to 340 ms in CI₁ and from 250 to 320 ms in CI₂. A dependency on step cycle period was discernible only at cycle periods below 1000 ms. Half-widths were determined taking the baseline of the histograms as reference rather than the average level of tonic discharge. This was done because the spike bursts were apparently merged into this maintained activity rather than superimposed upon it. This is evident when comparing, for instance, the histograms in Fig. 8A and B. Note that the peak values in the histograms are independent of the level of the maintained spike discharge.

A comparison of the phase histograms in Fig. 4A,B and Fig. 4C,D again demonstrates that the activities of the inhibitory neurones bear no resemblance to a phase-constant pattern but rather are relatively time constant. The peak values of the histograms, too, remain within narrow limits throughout the observed range of cycle periods (1.9–2.5 action potentials per 40 ms bin, corresponding to peak discharge frequencies of 48–63 Hz) and are comparable in the different inhibitors (see also Fig. 5).

There was, however, a small but consistent difference with regard to the timing of the bursts of action potentials observed in CI₁ and CI₂. In all histograms, though not in all individual step cycles, the bursts peaked earlier with regard to the subsequent touch-down of the tarsus in CI₁ than they did in CI₂. A comparison of

the phase histograms in Fig. 4A,B clearly demonstrates this feature. This was also true if the two inhibitory neurones were recorded successively in the same animal.

In Fig. 5, histograms obtained from the three CIs are superimposed. Data for all step cycle durations were pooled, taking the touch-down of the tarsus as reference (zero on the abscissa). The general similarities of the CI discharges are apparent in this diagram, as is the typical delay in rising phase and peak of the spike bursts of CI_1 when compared with those of CI_2 . Since the bursts terminated at about the same time in all three inhibitory neurones, discharges of CI_2 were, on the average, slightly shorter than those of CI_1 . The spike distributions of CI_1 and CI_2 were significantly different ($P < 0.001$), their means being separated by 30 ms.

Spike distributions of CI_3 bore a slightly closer resemblance to the histograms of CI_2 than to those of CI_1 . No significant differences were observed between the histograms of CI_3 and those of either of the other two inhibitors, however, probably because of the small number of CI_3 penetrations. Apart from this, all recordings so far obtained in CI_3 were consistent with the data reported above for CI_1 and CI_2 . This is in agreement with the results of Hale and Burrows (1985), who showed that CI_2 and CI_3 have many synaptic inputs in common.

The general similarity of CI_1 and CI_2 discharges was confirmed by paired intracellular recordings in individual locusts (Fig. 6). The overall patterns of synaptic input to the two neurones were similar, although there were apparent differences in detail. Consequently, the spike discharges, too, were similar but not identical (two lower traces in Fig. 6). This agrees with the unpublished results of J. Schmidt, who observed that CI_1 and CI_2 share a large number of synaptic inputs, although these are often weighted differently in the two neurones (see also Schmidt and Rathmayer, 1988).

The remarkable stability of rhythmic CI discharges during forward walking raises the question of whether this typical pattern of CI activity is maintained during more unusual leg movements. The inhibitors were recorded in a number of different situations; during backward walking, during searching movements of the leg elicited by loss of tarsal contact, and after removal of the appendage. Sample recordings obtained from CI_1 are presented in Fig. 7 and histograms of CI_1 and CI_2 activity are compared in Fig. 8. They demonstrate the patterns of inhibitory motoneurone discharge during the different leg movements and allow a comparison with the discharges observed during forward walking (compare Figs 2, 4 and 5).

Three cycles of searching movements – elicited by gently lifting the treadwheels off the animal's tarsi – are shown in Fig. 7A. The spike discharge was modulated in the cycle of the leg movement and bursts occurred in conjunction with leg protraction – similar to the situation during fast forward walking. In the recording shown in Fig. 7B, the locust produced alternating forward and backward swing movements without actual locomotion, allowing a direct comparison of CI activity during the two countercurrent movements. In both cases, the spike bursts commenced well before the start of the swing phase and outlasted the touch-down of the tarsus by several milliseconds. CI activity recorded during longer episodes

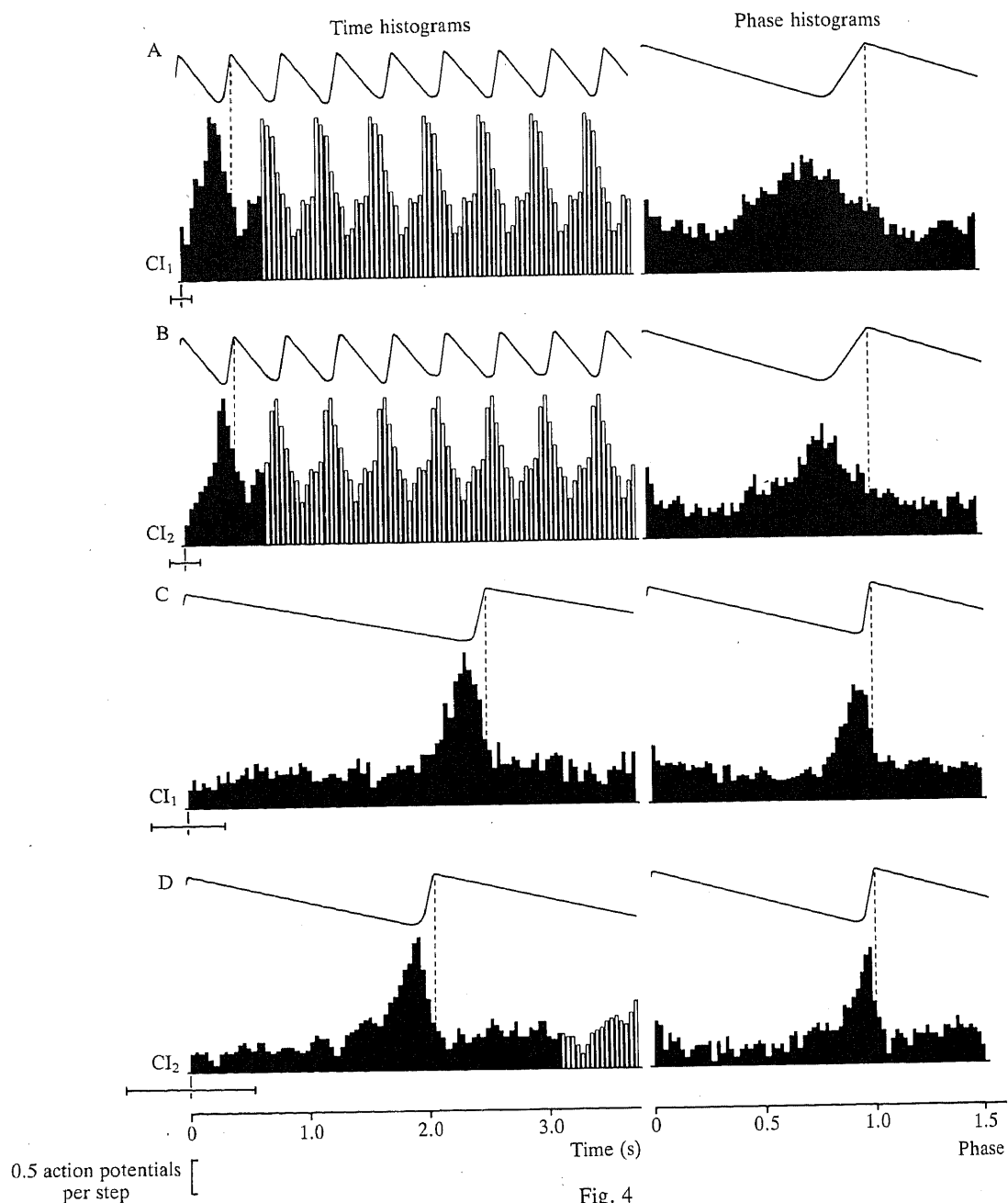


Fig. 4

of backward walking is presented in the form of histograms in Fig. 8B. For comparison, the activity observed during forward walking at similar cycle periods is shown in the upper histogram (Fig. 8A). Note the increase in the maintained spike discharge during backward as compared to forward locomotion. Apart from this enhanced tonic activity, however, the resemblance between the two sets of

Fig. 4. Comparison of CI_1 and CI_2 discharges during walking. Time (left) and phase (right) histograms of spike activity are presented for step cycle periods shorter than 500 ms (A and B) and longer than 2000 ms (C and D). CI_1 was recorded in A and C, CI_2 in B and D. Schematic leg movements are drawn above each histogram (anterior is to the top). The actual histograms cover 1.5 step cycles, i.e. CI spike occurrences were determined between two subsequent touch-downs of the tarsus and the measurement was extended into the next step cycle for 50% of the respective cycle period. The second touch-down (broken line) served as reference for calculating time histograms (see Materials and methods). Histograms cover the average cycle period in the sample (standard deviations indicated below the histograms). To illustrate the periodicity of the step cycle, the segment from 0.5 to 1.5 cycle periods is continuously repeated in A, B and D (striped area) up to the time covered by the longest histogram (C). 22 steps were evaluated in A, 43 in B, 24 in C and 17 in D. Bin widths 40 ms or 0.02 phase units. Ordinate scale is standardized in the phase histograms.

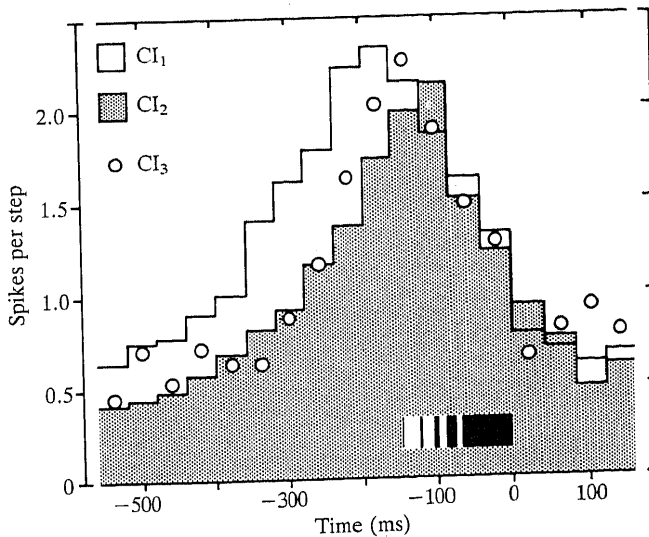


Fig. 5. Comparison of phasic spike bursts in the three inhibitors. Time histograms were constructed from 560 ms before until 160 ms after tarsus touch-down, which served as reference point (zero on abscissa). Data for CI_1 (open histogram, 154 steps), CI_2 (shaded histogram, 123 steps) and CI_3 (circles, 38 steps) are shown. Discharges from all walking episodes were superimposed, regardless of step cycle period. Swing movements lasted 50–150 ms (80 ms average) in the observed step cycles, considering all recordings of the three inhibitors. The duration of leg protraction is indicated by the bottom inset. Bin width 40 ms.

histograms is notable [the recruitment of excitatory motoneurons, by comparison, is quite different in the two situations (Bässler, 1983): the remotor of the coxa, for example, is a functional stance muscle during forward walking but active in the swing phase during backward walking]. Half-widths (260 versus 270 ms in CI_2) and peak values (2.3 versus 2.0 spikes bin^{-1}) of the CI spike distributions

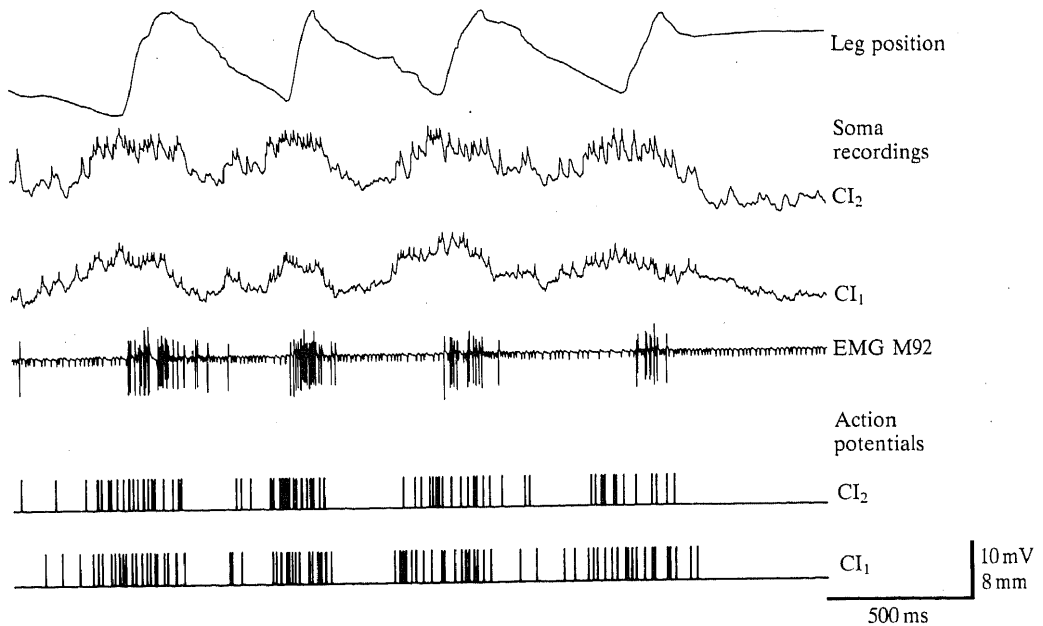


Fig. 6. Paired intracellular recording of CI_1 (third trace) and CI_2 (second trace) during walking. Top trace: leg position signal (anterior is to the top); fourth trace: EMG from M92. Action potential discharges of the two neurones are drawn separately in the fifth (CI_2) and sixth (CI_1) traces. Note general similarity and differences in detail between CI_1 and CI_2 records.

were comparable in both situations, as was the timing of the discharges with regard to the swing phase. But even the more subtle differences in the timing of the spike bursts of the two inhibitors, as reported for forward walking in Fig. 5, were also observed during sequences of backward locomotion. That is, on average, bursts of CI_2 peaked some 40 ms after those of CI_1 .

Indeed, rhythmic CI activity during walking appeared to be a particularly robust feature, since it persisted even after removal of the respective leg (Figs 7C,D, 8C). The EMG from muscle 92, an extrinsic leg muscle located in the thorax, served as a reference for rhythmic efferent activity in this case. Sometimes the spike discharge patterns were almost identical before (Fig. 7C) and after (Fig. 7D) leg removal. More often, though, the appearance of the spike bursts was altered and a tonic spike discharge was decreased or absent (compare histograms in Fig. 8A,C). In Fig. 8C, half-width (280 ms) and peak discharge ($2.0 \text{ spikes bin}^{-1}$) are about normal only for CI_1 . One very constant feature, however, was the timing of the spike bursts with regard to the 'fictive leg movement' (dotted line in Fig. 8C) reconstructed from the EMG pattern.

Impact of CI activity on leg movement

Through intracellular penetration of inhibitory motoneurones in tethered

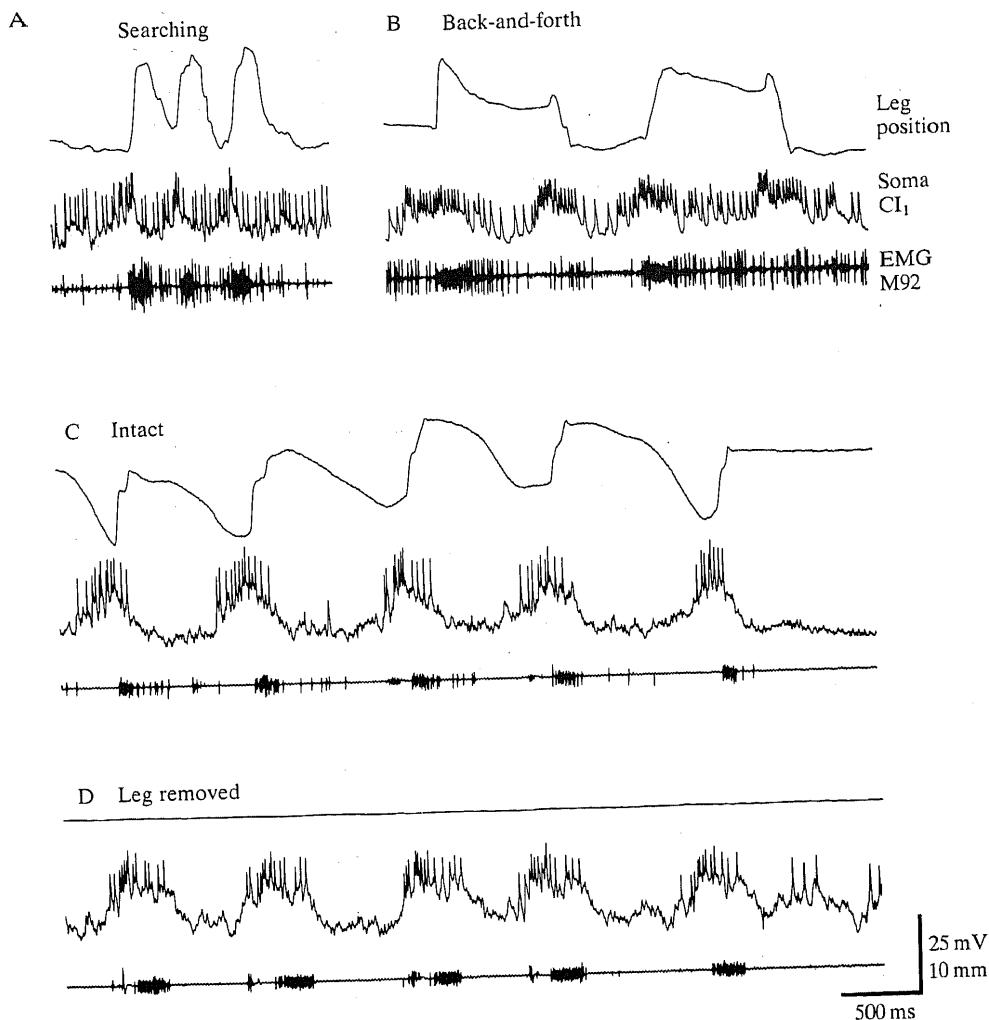


Fig. 7. Unusual walking situations, sample recordings. Top traces are leg position recordings (anterior is to the top), middle traces soma recordings from CI_1 , and bottom traces EMGs from M92. (A) A sequence of searching movements elicited by removal of the substratum. (B) Back-and-forth stepping without actual locomotion was observed in ambiguous stimulus situations (pinching the antennae after a sequence of forward walking). (C) A normal sequence of forward walking (note absence of tonic spike discharge). The sequence in D was recorded a few minutes later (same penetration), after the removal of the ipsilateral middle leg.

walking locusts it was possible not only to monitor CI activity but also to manipulate it by current injection and to observe the effects on the leg movement.

The result of such an experiment is presented in Fig. 9. During the walking episode shown, CI_1 was hyperpolarized after the third step, current injection into the soma (approx. 10 nA) was maintained during the following five steps (bottom

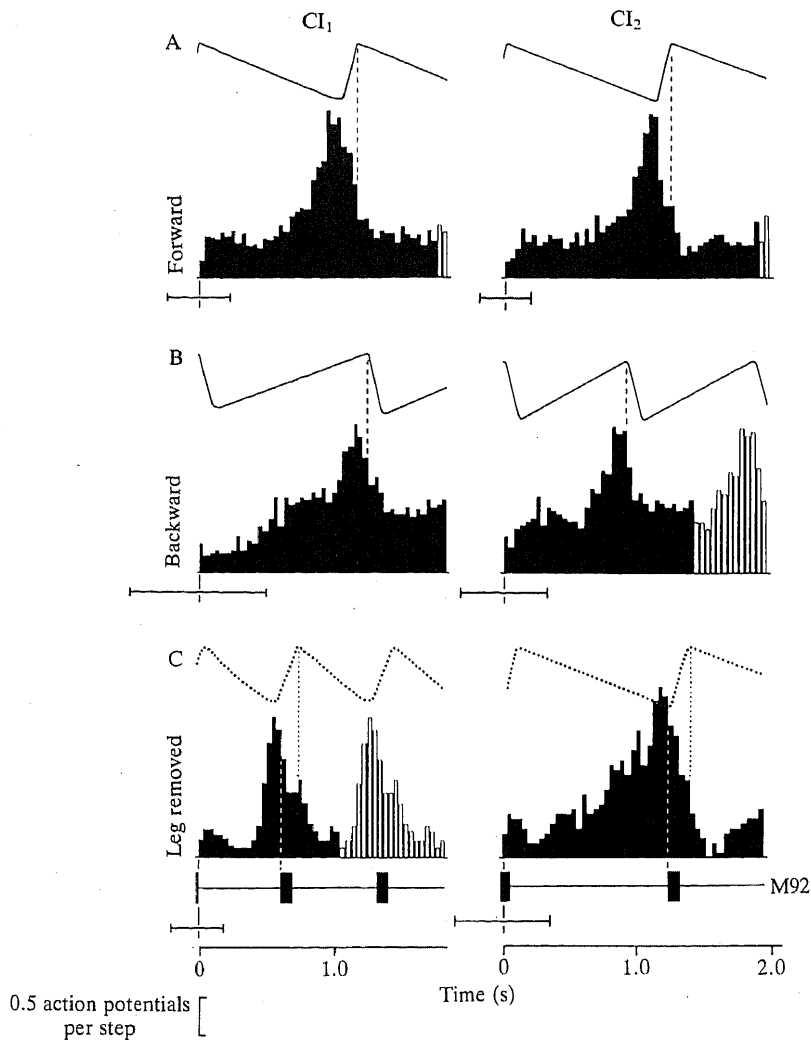


Fig. 8. Unusual walking situations, time histograms. Activities of CI_1 (left) and CI_2 (right) are compared. Histograms were constructed as in Fig. 4. Forward walking is shown in A (cycle durations between 500 and 1000 ms evaluated), backward walking in B, and the spike pattern observed after leg removal in C (M92 EMG outlined below histogram, start of EMG bursts served as reference for evaluation, broken line). Leg movements are outlined above each histogram (fictive leg movement reconstructed from EMG discharge in C, dotted line). Note that the anterior extreme position served as reference point in A and B, corresponding to the end of swing in forward walking and to the start of swing in backward walking. 52, 31 and 39 steps were evaluated for CI_1 and 46, 28 and 21 steps for CI_2 in A, B and C, respectively.

traces in Fig. 9), and then the neurone was released from hyperpolarization (last two steps in upper traces). The slopes of the leg position record – corresponding to the velocity of leg movement – were determined for mid-protraction and are

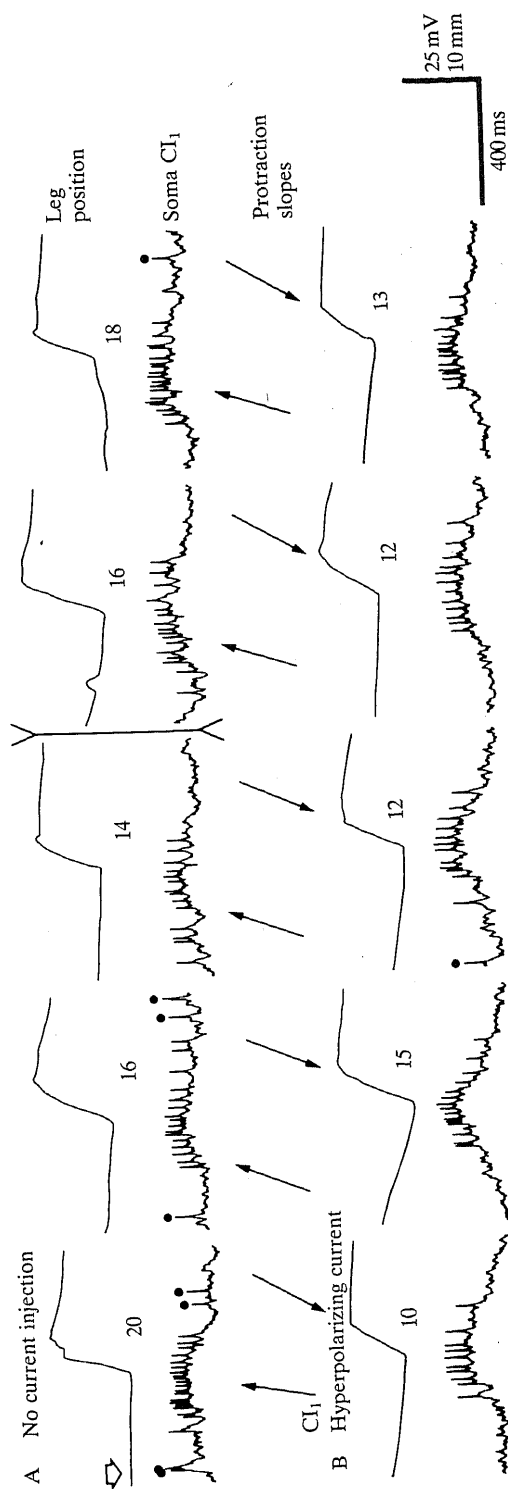


Fig. 9. Manipulating Cl₁ discharges. Top traces show leg position signals, bottom traces a Cl₁ soma recording. From a selected step cycle sequence, the protraction movements were extracted and enlarged. Steps recorded without current injection are arranged in the upper set of recordings. Cl₁ was hyperpolarized during the step cycles shown in the lower set. Step cycles are presented in chronological order, but the lower set would have to be inserted between the third and fourth leg protraction of the upper set (marker). The sequence was initiated by abdominal stimulation (open arrow). For comparison, mid-protraction slopes are drawn between the two sets of recordings, arrows indicating the respective position record. Numbers represent spike counts for phasic Cl₁ discharges (action potentials not considered are marked by dots).

shown between the two sets of recordings. Protraction velocities were variable but a general correlation between slower velocities and smaller numbers of CI action potentials in the burst associated with the swing movement is apparent. With one exception (second step in bottom record), all swing velocities recorded while CI₁ was hyperpolarized were lower than those observed without current injection.

A quantitative evaluation of the walking sequences recorded in this animal is presented in Fig. 10. Without current injection, the impaled CI₁ discharged between 7 and 14 action potentials in the burst associated with leg protraction (filled histogram and circles). A 200 ms interval prior to tarsus touch-down, covering most of the burst, was evaluated (bars in inset to Fig. 10). This was done to obtain an unequivocal burst definition in case of fluctuations in the maintained spike discharge and since an effect on swing velocity, if present, could reasonably

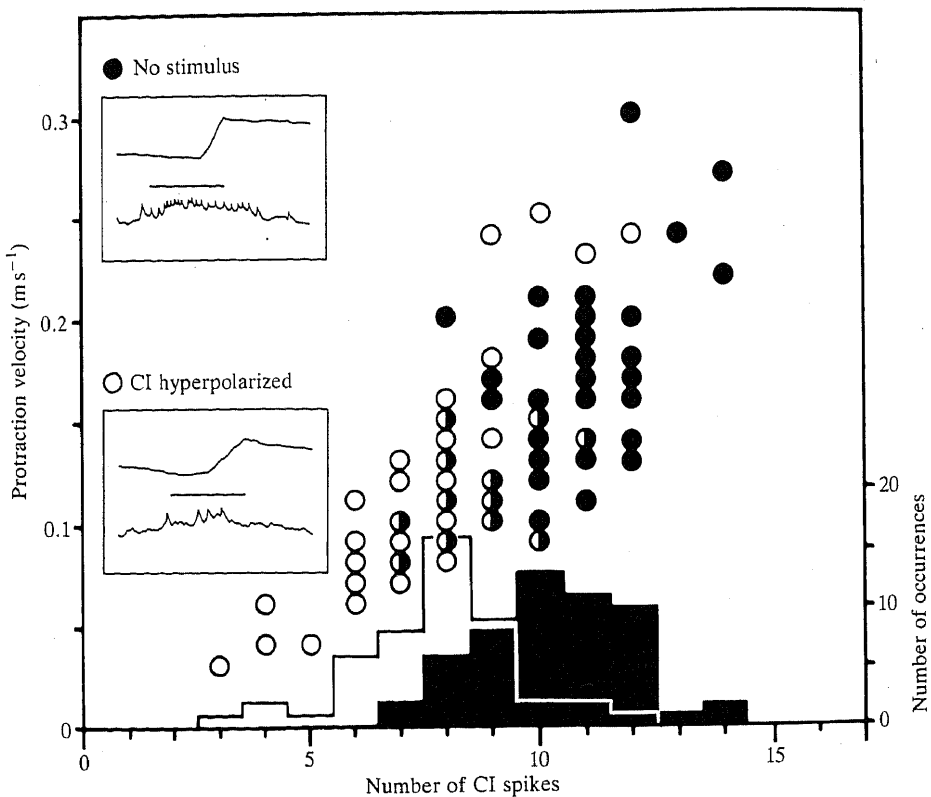


Fig. 10. Velocity of leg protraction depends on CI₁ discharge. The velocity of the protraction movement (left ordinate) was determined in steps performed with (open circles and histogram) and without (filled circles and histogram) hyperpolarization (10 nA) of CI₁. CI spikes were counted (abscissa) for a 200 ms interval preceding touch-down of the tarsus (indicated by bar in inset figures: top inset without, bottom inset with current injection). Data points often represent several steps. Hence, the occurrence of CI discharges with a certain spike number is plotted as a histogram (right ordinate).

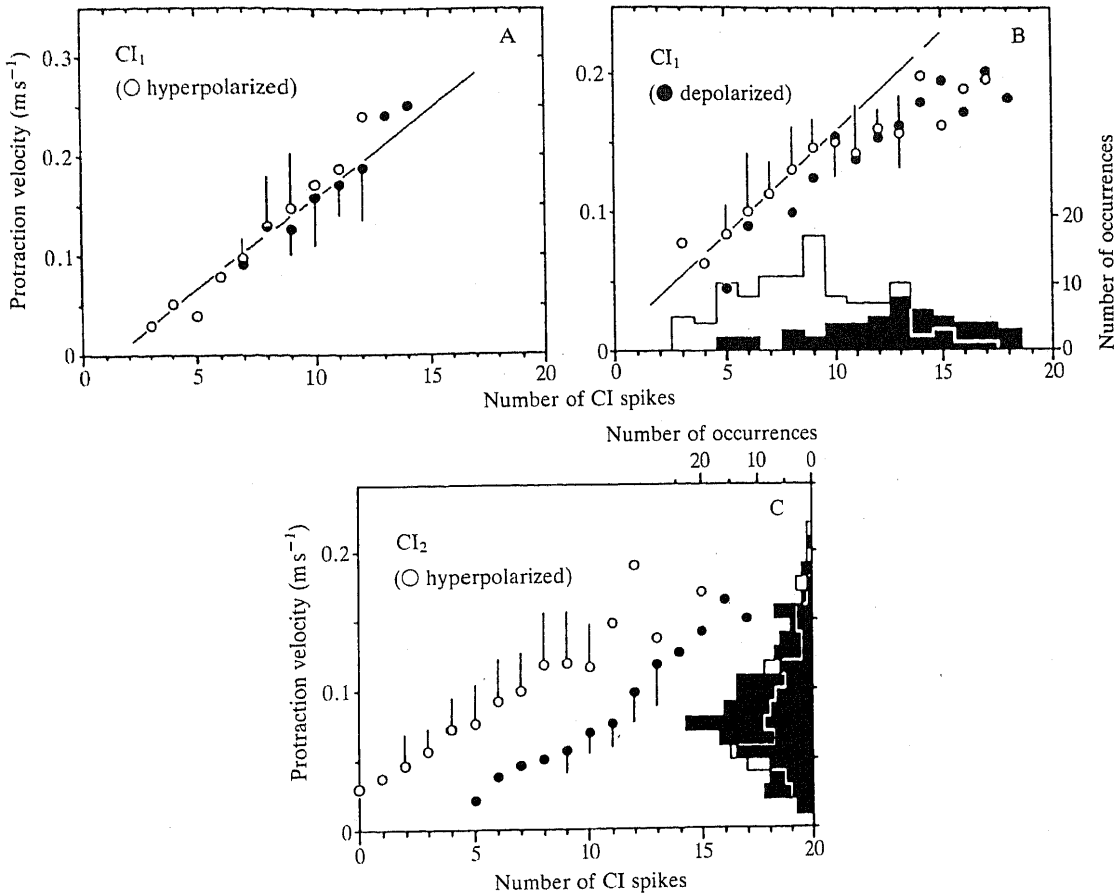


Fig. 11. Velocity of leg protraction depends on CI_1 discharge. Diagrams are constructed as in Fig. 10 but data were pooled with regard to CI spike numbers on the abscissa (bars indicate standard deviations for data points representing more than six steps). (A) Hyperpolarization of CI_1 . The same set of data was evaluated as in Fig. 10 (100 steps); open circles, with current injection; filled circles, without. Best fitting line for all data points is indicated. (B) Depolarization of CI_1 . Data are from a different animal (173 steps); filled circles and histograms, with current injection; open circles and histograms, without. Best fitting line for data points below 11 CI spikes is indicated. Histograms show distribution of CI spike numbers in phasic discharges. (C) Hyperpolarization of CI_2 . CI_2 was recorded in a third locust (261 steps); open circles and histograms, with current injection; filled circles and histograms, without. Histograms show distribution of swing velocities.

be expected for this interval (and not, for instance, after touch-down of the tarsus). Although the corresponding swing velocities showed considerable scatter, there was a clear correlation with the CI discharge. Large spike numbers were associated with faster protraction movements (regression line in Fig. 11A).

Hyperpolarization of CI_1 shifted the spike distribution to lower numbers – to between 3 and 12 action potentials (open histogram and circles). It also shifted the

swing velocities to slower movements, which proves that CI activity is indeed involved in determining protraction velocity and that the dependency observed without current injection is not merely a correlation. This result was confirmed in five other animals: there was always a clear correlation between CI_1 discharge and swing velocity (slopes between 1.0 and $1.8 \times 10^{-2} \text{ m s}^{-1} \text{ spike}^{-1}$) and hyperpolarization of CI_1 reduced the number of action potentials and shifted the velocity distribution to lower values. In all cases, the velocity distributions determined with and without current injection were significantly different ($P < 0.001$).

Injection of depolarizing current increased the CI_1 spike discharge (spike frequency histograms on the abscissa of Fig. 11B) and shifted the velocity distribution to higher values (graph in Fig. 11B). The results were less clear, however, because swing velocity apparently began to saturate above 11–13 CI spikes per burst (compare regression line calculated for data below 11 CI spikes, and Fig. 11A).

In Fig. 11C, CI_2 instead of CI_1 was impaled and recorded (filled circles). Again, hyperpolarizing current injection (open circles) reduced its spike discharge. The distribution of swing velocities remained unchanged, however ($P < 0.75$ in two-tailed *t*-test), which is illustrated by the histograms of the velocity distributions (Fig. 11C, right ordinate). In other words, protraction velocity, although correlated with CI_2 discharge as a result of the similar activity patterns in all three CIs, was actually independent of the inhibitor's activity. In the diagram this is shown by the fact that hyperpolarization displaced the graph to the left by an amount equal to the shift in CI spike distribution, instead of shifting the data points down along the slope of the graph, with swing velocity being dependent on CI discharge, as was the case in Fig. 11A.

This result can be explained by the different innervation patterns of the two CIs. Whereas CI_1 supplies extrinsic, coxal and trochanteral muscles moving the proximal segments of the leg and the extensor of the tibia, the target area of CI_2 (and probably CI_3) is restricted to muscles moving tarsal segments, plus the flexor of the tibia (see Introduction). In the present investigation, movements of the femur–tibia joint and of tarsal articulations were not recorded, since the reflective tape for position monitoring was attached to the most proximal part of the tibia (see Materials and methods). Instead, movements of the whole limb were monitored. These movements are produced in the basal articulations of the leg, between femur, trochanter, coxa and thorax. Since the muscles controlling movements of these joints are innervated by CI_1 , only manipulation of the activity of this inhibitor should appear in the position recording.

This experiment further indicates that current injection specifically affected only the impaled CI and that a change in protraction velocity resulting from CI_1 hyperpolarization was not caused, for instance, by current spread to neighbouring moto- or interneurons. This interpretation presumes, of course, that CI neurones do not influence inter- or motoneurons *via* electrical synapses and in this way elicit the observed effects. Electrical coupling was not observed in any of the previous studies (e.g. Hale and Burrows, 1985).

Discussion

Intracellular recording in tethered walking locusts

The primary method for determining motoneuronal discharge patterns in active insects is the recording of electromyograms (EMGs, e.g. Elsner, 1975). This method is inapplicable, however, if the activity of inhibitory motoneurons is to be analyzed, since inhibitory junctional potentials, and often even the excitatory potentials of slow motor units, are not discernible in EMGs. Neurogram recordings thus provide a more accurate picture of the motor command (Burns and Usherwood, 1979; Ballantyne and Rathmayer, 1981; Parsons *et al.* 1983). This technique has the invaluable advantage that the animals remain almost unrestrained and behave normally (e.g. Wolf, 1985). However, spike identification is difficult if several axons of similar diameters run in the same nerve, and the recognition of small action potentials is compromised by simultaneous discharges in fibres of large diameter because of spike superimposition. Both arguments apply in the case of the locust CI neurones (Burns and Usherwood, 1979; Hale and Burrows, 1985). Also, the analysis of central nervous events is impossible. In such cases, intracellular recording in more restricted but nearly intact preparations is a suitable alternative (Wolf and Pearson, 1987).

A preparation is described here for the intracellular analysis of neural activity in the thoracic ganglia of tethered, walking locusts (Fig. 1). The only other locust walking preparation which allows intracellular recording in the thoracic nerve cord is that of Ramirez and Pearson (1988). In this preparation, access to the nervous system is from the animal's dorsal side. The advantages of this approach are easy impalement of neurones located dorsally in the ganglion and freedom of movement for the animal, which is placed on a floating styrofoam ball. The consequences of the extensive dissection necessary, however, represent a considerable drawback. For example, all bifunctional leg muscles, i.e. those involved in both flight and walking, and some of the postural muscles attached to sternal apodemes are severed or displaced and the thoracic cuticle is distorted (discussed in Ramirez and Pearson, 1988). With the present preparation, the muscles, thoracic mechanics and leg mechanoreceptors remain intact and apparently unaffected. Disadvantages of the present preparation concern constraints imposed by the treadmill rather than artefacts caused by the operation. Rotational movements, in particular walking in curves, and lateral 'peering movements' (Burns, 1973) are prevented. And, although the load carried by the tarsi and the inertial momentum of the treadwheels are adjusted to match the locust's body weight, up-and-down movements of the body must be performed against the unnaturally high inertial momentum of treadmill counterbalance and balance lever. In summary, a dorsal preparation may be advantageous for the study of gross features of rhythm generation and walking control where the integrity of the walking system is not required, whereas the present preparation appears appropriate for more detailed and possibly quantitative analyses related to the fine control of movement.

The only other insect preparation suitable for the present analysis was

developed by Godden and Graham (1984) in the stick insect *Carausius*. Because of the softer cuticle and the absence of functional wings in this species, the operation – although from the dorsal side – is much less disruptive than in *Locusta*. The 'bifunctional' leg muscles remain intact and walking performance is normal, according to the authors' kinematic analysis. Unfortunately, however, central nervous aspects of walking motor control are only beginning to be understood in the stick insect (e.g. Büschges, 1990) and little is known about the common inhibitors.

Discharge patterns of locust middle leg common inhibitors

While the common inhibitory motoneurons produced spikes only sporadically in quiescent locusts (Fig. 3), a maintained discharge of action potentials was frequently observed in walking animals (Fig. 2). This is in accordance with neurogram recordings of CI activity in crustaceans. The single CI of a crab's walking leg (see Introduction) is silent during rest but a maintained discharge of action potentials is produced during locomotion (Ballantyne and Rathmayer, 1981). This maintained discharge is weakly and irregularly modulated in the step cycle, whereas in the locust, distinct, phasic bursts of action potentials were generated in conjunction with the swing phase of the leg movement. In all three inhibitors, these bursts peaked at about the time when the tarsus was lifted off the ground.

With regard to insects, only data concerning CI₁ have been reported in the literature (Burns and Usherwood, 1979; Igelmund, 1980; Bässler, 1983; Godden and Graham, 1984). These data are in general agreement with the results of the present study. However, Burns and Usherwood (1979) failed to observe a tonic component of CI activity during walking, probably because of the small number of step cycles they evaluated (nine steps in their Fig. 5). In *Carausius*, a biphasic discharge of CI₁ (separate spike bursts at the beginning and end of the swing phase) has been reported (Bässler, 1983; Godden and Graham, 1984). Although biphasic bursts were occasionally observed in individual step cycles of *Locusta* (Fig. 1C), this was not a consistent feature and was never discernible in histograms of CI activity (e.g. Fig. 5), even those with high time resolution (10 ms bin width). The functional significance of the different CI discharge patterns in *Carausius* and *Locusta* remains unclear.

CI activity appears to be determined mainly by the walking pattern generator rather than by input from leg mechanoreceptors – despite the prominent and widespread sensory input observed in quiescent locusts (Schmidt and Rathmayer, 1988). This is suggested by the observation that the inhibitors' discharge patterns do not conform with expectations derived from known sensory connections. For instance, one would expect a clear CI discharge in response to mechanoreceptor stimulation immediately after tarsus touch-down. Such a spike burst does not occur. Indeed, the typical, phasic CI burst is terminated just after the end of the swing phase. Even removal of the respective leg – although often affecting the level of maintained activity and the general appearance of the spike bursts – had

no effect on rhythmicity and timing of the discharge (Figs 7C,D, 8C). Finally, the timing of CI activity with regard to the swing phase was maintained during both forward and backward walking (Figs 5, 8), despite the fact that patterns of afferent inflow are vastly different in the two situations (e.g. Hustert, 1985; Laurent and Hustert, 1988).

In general, the discharge patterns of all three inhibitory motoneurons were remarkably similar. Conspicuous, though, was the different timing of CI₁ and CI₂ discharges (Fig. 5). On average, the onset and peak of CI₂ bursts were delayed by about 40 ms with regard to the corresponding CI₁ bursts, while their decay was nearly simultaneous. This agrees with the overall timing of EMG activity in the muscle groups supplied by the two inhibitors. CI₁ innervates the bulk of the leg musculature (Hale and Burrows, 1985) and several of these muscles will be active at the onset and during the execution of the swing movement. This is true, for example, for the promotor/levator (muscle 94, Snodgrass, 1929) and the anterior rotator (M92) of the coxa and for the extensor of the tibia (M106) (e.g. Burns and Usherwood, 1979; Ramirez and Pearson, 1988). CI₂, in contrast, supplies only four muscles in the most distal leg segments, namely the flexor tibiae (M107), retractor unguis (M110), and depressor (M109) and levator (M108) tarsi (R. Hustert, unpublished results, and by analogy to metathoracic muscles examined by Hale and Burrows, 1985). Obviously, the first three of these muscles must be recruited at the end of the swing phase, when the tarsus has to grip the substratum, but not during leg protraction, as are several of the muscles supplied by CI₁. In fact, EMG potentials first appear in the second half or towards the end of leg protraction, in muscles 107 and 109 at least (Burns and Usherwood, 1979, and unpublished observations). The swing movement lasted about 50–150 ms (average 80 ms) in the present experiments. Considering these data, the average time delay of about 40 ms between the peak discharges in CI₁ and CI₂ appears to be tuned perfectly to the activity of the muscle groups innervated by the two neurones. This finding was unexpected considering the variable appearance of CI activity in the original records – which might merely reflect the adaptability of walking behaviour itself. The roughly simultaneous decay of the phasic discharges in all three inhibitors is obviously correlated with the termination of the rapid protraction movement.

Apart from this fine tuning, CIs apparently constitute a functional unit in the locust leg, as shown by the close similarity of their discharge patterns under all conditions examined. This makes the three common inhibitors of the locust comparable to the single inhibitory neurone innervating all the muscles of a walking leg in crabs and crayfish (Wiens, 1989). This view is supported by similarities in the discharge patterns of crustacean and insect common inhibitors mentioned above and by common physiological characteristics.

Manipulation of CI₁ discharge during walking

In crustaceans (Wiens *et al.* 1988) and insects (Pearson, 1973), common inhibitory neurones innervate mainly those fibres within a given muscle that are

also supplied by a slow motoneurone. The physiology of inhibitory muscle innervation has been studied primarily in crustaceans (Rathmayer, 1990). Within a given muscle, the group of fibres innervated by CI and slow axons covers a broad spectrum of contractile properties, from tonic to phasic (Rathmayer and Maier, 1987). CI discharges primarily affect the tonic fibres of a muscle through post- and (mainly) presynaptic mechanisms (Rathmayer and Erxleben, 1983). Postsynaptic action was found to decrease the amplitude and accelerate the relaxation of twitch contractions, while presynaptic mechanisms reduce or completely abolish tonic muscle fibre activation. In summary, it has been concluded that the main function of CI innervation is to facilitate rapid contraction-relaxation cycles in the limb musculature during fast locomotion by blocking tonic muscle fibres (Atwood, 1973; Rathmayer and Erxleben, 1983; Wiens and Rathmayer, 1985; Wiens *et al.* 1988; Wiens, 1989). This function has been demonstrated repeatedly in nerve-muscle preparations (Usherwood and Runion, 1970; Burns and Usherwood, 1979; Ballantyne and Rathmayer, 1981; Wiens *et al.* 1988).

According to these results, the maintained CI discharge observed in the locust during walking should act to relax the tonic muscle fibres. These fibres are active in quiescent animals for the maintenance of posture but would impede leg movements during walking by causing a build-up of residual tension, with a resulting increase in joint stiffness. However, manipulation of CI discharges had no observable effects on step cycle frequency or on the speed of leg retraction (stance phase). Two reasons for this are evident. First, the maintained discharge of the inhibitory motoneurons was variable and sometimes completely absent (Fig. 7C). Second, and probably more important, all legs that touch the ground support the animal and are thus mechanically coupled. Consequently, during the stance the movement of a single leg is determined not only by the muscles of that leg but also by the forces produced by the other legs. An effect of CI activity on leg retraction, although it should exist, will therefore be hard to prove.

The observation that the phasic spike bursts of the inhibitory motoneurons occur in conjunction with the rapid protraction movement is in good agreement with the functional interpretation of CI activity given above. And with regard to the swing phase, it was indeed possible to demonstrate that inhibitory input contributes to the control of leg movement, thus providing the first direct proof of the behavioural significance of common inhibitory muscle innervation. Manipulation of CI₁ discharges altered the speed of the protraction movement during normal walking behaviour (Figs 10, 11). As expected, CI discharges promoted rapid movements: hyperpolarization of the inhibitory neurone decreased and depolarization increased the average velocity of leg protraction. The influence of CI₁ is considerable (effects of CI₂ and CI₃ were not examined, since movements around the distal leg joints were not monitored in the present study). On average, one additional CI spike in the burst increased swing velocity by 0.013 m s^{-1} , i.e. by about 10 % of the normal protraction velocity. This large effect of CI₁ spikes on leg movement is remarkable. One might expect the movement to be determined predominantly by input from the excitatory motoneurons to the respective leg

muscles rather than by inhibitory control. The large scatter of the data points in Fig. 10 indicates that the influence of the excitors is predominant and, of course, these motoneurons determine the actual trajectory of the protraction movement. However, because of the scant and polyneural innervation of arthropod muscles and the high degree of internal specialization reflected in muscle fibre heterogeneity (Rathmayer and Maier, 1987) CI plays an important role in movement control.

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