Modulation of Membrane Potential in Mesothoracic Moto- and Interneurons During Stick Insect Front-Leg Walking

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Ludwar, Björn Ch., Sandra Westmark, Ansgar Büschges, and Joachim Schmidt. Modulation of membrane potential in mesothoracic moto- and interneurons during stick insect front-leg walking. J Neurophysiol 94: 2772–2784, 2005. First published July 6, 2005; doi:10.1152/jn.00493.2005. During walking, maintenance and coordination of activity in leg motoneurons requires intersegmental signal transfer. In a semi-intact preparation of the stick insect, we studied membrane potential modulations in mesothoracic (middle leg) motoneurons and local premotor nonspiking interneurons that were induced by stepping of a front leg on a treadmill. The activity in motoneurons ipsi- and contralateral to the stepping front leg was induced by stepping of a front leg on a treadmill. The activity in motoneurons and local premotor nonspiking interneurons that were membrane potential modulations in mesothoracic (middle leg) motoneurons and local premotor nonspiking interneurons that were induced by stepping of a front leg on a treadmill. The activity in motoneurons ipsi- and contralateral to the stepping front leg was recorded from neuropilar processes. Motoneurons usually exhibited a tonic depolarization of \( \pm 5 \) mV throughout stepping sequences. This tonic depolarization depended on membrane potential and was found to reverse in the range of \( \pm 32 \) to \( \pm 47 \) mV. It was accompanied by a mean membrane resistance decrease of \( \pm 12\% \). During front-leg stepping, an increased spike activity to depolarizing current pulses was observed in 73% of contralateral flexor motoneurons that were tested. Motoneurons ipsilateral to the walking front leg exhibited phasic membrane potential modulations coupled to steps in accordance with previously published results. Coupling patterns were typical for a given motoneuron pool. Local nonspiking mesothoracic interneurons that provide synaptic drive to tibial motoneurons also contribute to the modulation of membrane potential of tibial motoneurons during front-leg walking. We hypothesize that the tonic depolarization of motoneurons during walking is a cellular correlate of arousal that usually increases effectiveness of phasic excitation in supporting motoneuron firing.

INTRODUCTION

The basic activity pattern of a motoneuron that drives the muscle of a limb during walking consists of spike bursts that alternate with bursts in antagonistic motoneurons. The rhythmic activity pattern in motoneurons results from the interaction of signals from three neural elements, central pattern generators (CPGs), sense organs associated with the leg, and signals that serve the coordination of different limbs (Cruse 1990). In addition, intrinsic membrane properties of the motoneurons shape their activity pattern (e.g., Mills and Pitman 1997, 1999; Powers and Binder 2001; Schmidt et al. 2001).

We aim to unravel the neuronal mechanisms that contribute to the coordination of different legs, that is, the respective groups of motoneurons, during walking in stick insects. Therefore it is necessary to discriminate the contribution of each of the three neural elements named in the preceding text that structure motoneuron activity. Evidence mostly from reduced preparations suggests that in the stick insect each leg joint is associated with an individual CPG (Bässler and Büschges 1998). Local nonspiking premotor interneurons have been identified that are elements of the CPG controlling movements of the femur-tibia joint (Büschges 1995). Signals from strain sensors in the cuticle (campaniform sensilla) and a movement receptor (the femoral chordotonal organ) serve the coordination of joint movements of the leg they are located in (Akay et al. 2004). A single legged preparation of the stick insect has been used to study the activity pattern of motoneurons in the absence of coordinating sensory signals from other segments. Data collected from this preparation, in which a middle leg walks on a treadmill, suggest that rhythmic activity patterns of motoneurons during walking are formed by alternating excitatory and inhibitory synaptic inputs (Schmidt et al. 2001). The results of two different sets of experiments on the stick insect indicate that only phasic inhibitory input can be assigned to CPG activity. First, in the deafferented thoracic nervous system, rhythmic activity in a motor neuron that is induced by application of pilocarpine is based on a tonic depolarization and rhythmic inhibitory input (Büschges 1998). Second, in a deafferented thoracic nervous system, alternating cycles of spike bursts in antagonistic motoneurons are evoked by tactile stimulation of the abdomen. These cycles of spike bursts appear to result from tonic depolarizing drive that is sculpted by phasic inhibitory input provided by a central pattern generating network (Büschges et al. 2004). During single-leg walking, additional rhythmic excitatory input to motoneurons is provided by sensory organs in the leg. For example, flexor motoneurons receive a rhythmic excitatory input that originates from campaniform sensilla in the leg (Akay et al. 2001). Segmental neural networks appear to be sufficient to coordinate the movements of a leg’s joints (Büschges 2005; Ekeberg et al. 2004; Foth and Bässler 1985). In the intact animal, intersegmental inputs also contribute to patterning motoneuron activity (Cruse 1990).

In a previous study (Ludwar et al. 2005), we began to examine the structure of intersegmental coordinating input by using the single-legged preparation. This preparation allows us to differentiate between a segment that sends coordinating signals and segments that receive these signals in the absence of local afferent input. When a front leg performed sequences of steps on a treadmill, extracellular recordings from motor nerves of the ipsilateral middle leg revealed increased motor activity that was patterned in bursts. These bursts were coupled
to the front-leg step cycle. Gross activity in antagonistic motoneurons was alternating. Recordings of the mesothoracic fast extensor tibiae motoneurons (FETi) during front-leg walking revealed a tonic depolarization between 4 and 11 mV that was weakly modulated correlated with front-leg stepping (Büschges et al. 2004). The present studies analyze the modulation of membrane potential of mesothoracic motoneurons during front-leg stepping to identify types of intersegmental signals and their effects. In addition, we have begun to examine the role of mesothoracic premotor nonspiking interneurons in the transfer of intersegmental signals during walking of the ipsilateral front leg.

**METHODS**

The experiments were conducted with adult, female stick insects, *Carausius morosus* and *C. impigra*, from colonies maintained at the University of Cologne. All experiments were carried out under daylight conditions and at temperatures between 18 and 24°C. All legs except one front leg were amputated, and the animals were fixed dorsal side up on a foam platform using dental cement (Protemp II, ESPE, Seefeld, Germany). The thorax was opened to allow access to the mesothoracic ganglion and recording from mesothoracic leg nerves. The gut was moved aside, connective tissue carefully removed to expose the mesothoracic ganglion, and the cavity filled with saline (composition according to Weidler and Diecke 1969).

Motoneuron activity was recorded extracellularly with hook electrodes (modified after Schmitz et al. 1991) from lateral nerves (nomencalature according to Graham 1985; Marquardt 1940); in addition electromyographic (EMG) recordings were performed from the walking leg’s flexor muscle. For intracellular recordings, the mesothoracic ganglion was prepared according to established procedures (Büschges 1990). Recordings from motoneurons were made using thin-walled glass microelectrodes filled with either a solution of 3 mol/l potassium acetate with 0.05 mol/l KCl or a solution of 1.5 mol/l potassium acetate (electrode resistance: 15–20 MΩ). Recordings were made from the neuropil region of the mesothoracic ganglion ipsi- or contralateral to the walking front leg. Signals were amplified by means of an SEC-10 L amplifier (NPI, Tamm, Germany). Intracellular current injections were made in discontinuous current clamp (DCC) mode (7–10 kHz switching rate). Motoneurons were identified by a one-to-one relationship of intracellularly recorded spikes with spikes in the appropriate motor nerves.

Nonspiking interneurons of type E4 were identified either by their typical morphology (*n* = 4) (based on published morphology in Büschges 1990) or according to their responses to inputs from the femoral chordotonal organ (see following text). For morphological identification, interneurons were filled with tetramethylrhodamine dextran. Therefore recordings were made using thin-walled glass microelectrodes filled with a 3.5% solution of tetramethylrhodamine dextran (3000 MW, lysine fixable, Molecular Probes, Eugene, OR) in 3 mol/l potassium acetate with 0.05 mol/l KCl. The same potassium acetate/KCl solution was used to fill the shaft of the electrodes, which had resistances between 25 and 35 MΩ. Neurons were iontophoresically injected with tetramethylrhodamine dextran using pulsed positive current (2–4 nA, 1-Hz, 500-ms duty cycle; 10–20 min). Ganglia were fixed in 4% paraformaldehyde in 0.1% phosphate buffer (pH 7.4) for 1.5 h, rinsed in a Tris-HCl solution, dehydrated in ethanol solutions of increasing strength, and mounted in methylsalicylate (Sigma, Hohenheim, Germany). Neurons were visualized using a confocal microscope (LSM 510, Zeiss, Jena) equipped with a helium/neon laser using 543 nm lines for excitation and an LP560 emission filter. For identification of other types of nonspiking interneurons, the femoral chordotonal organ (ICO) of the ipsilateral middle leg was left intact to aid in the identification of nonspiking interneurons according to their responses to chordotonal inputs (Büschges 1990; Sauer et al. 1996). See Büschges (1990) for a detailed methodological description.

A lightweight, low-friction animal-driven treadmill (Gabriel et al. 2003) was positioned under the animal’s front leg to allow the unrestricted leg to perform walking-like movements. A DC-motor attached to the treadmill served as a tachometer. The movements of the treadmill’s belt were back driving the motor and gave a reading of belt velocity. Data were analyzed with respect to the start of the front-leg stance phase, the latter being defined as the time during which the treadmill was accelerated.

All data were recorded using a MICRO 1401 A/D converter and SPIKE 2 data-acquisition/analysis software (versions 3.13–4.12, Cambridge Electronic Design, Cambridge, UK). Data evaluation was done using commercially available or custom-written scripts within the SPIKE 2 software. Layout editing was performed using Corel Draw 11 (Corel Corporation, Ottawa, ON, Canada), and statistical analysis and plots were rendered using PlotIt (Scientific Programming Enterprises, Haslett, MI). In text and figures, *N* is the number of experiments and *n* is the sample size. Means are given ± SD. For significance tests of correlation coefficients, the significance test for Pearson’s *r* was used that is computed as follows: 

\[ t = r \sqrt{\frac{n-2}{1-r^2}} \]

where *r* is Pearson’s correlation, and *n* is the number of pairs of scores that went into the computation of *r* (Sachs 1974).

**RESULTS**

Activity in mesothoracic motoneurons during walking of the ipsilateral front leg

Intracellular recordings were obtained from motoneurons to muscles of the middle leg during walking of the ipsilateral front leg. Recordings were taken from muscles at each of the major intrinsic leg joints: protractor coxae motoneurons (*N* = 3) controlling forward movements of the leg, depressor trochanteris motoneurons (*N* = 7) controlling downward movements of the leg and extensor (3 SETi, 3 FETi) and flexor (*N* = 5) motoneurons controlling flexion movements of the femur-tibia joint.

Front-leg stepping on the treadmill was evoked by briefly touching the animal’s abdomen with a paintbrush. Stimuli elicited sequences of typically 8–15 consecutive steps. Occasionally, spontaneous walking sequences occurred with no external activation of the animal. All mesothoracic motoneurons showed two distinct changes in membrane potential during stepping of the front leg (Fig. 1): a tonic shift that was usually depolarizing and a rhythmic modulation that was coupled to individual steps of the front leg.

Tonic membrane potential changes during front-leg walking

Most ipsilateral mesothoracic motoneurons exhibited a tonic shift in membrane potential at the onset of walking movements of the front leg. These shifts from resting membrane potential by −0.5–5 mV were usually depolarizing (*N* = 14; Fig. 1) and, in few motoneurons, hyperpolarizing (*N* = 3). In four motoneurons, no tonic shift in membrane potential was obvious at their resting membrane potential. Figure 1B shows activity in a slow extensor motoneuron during a rare spontaneous-walking sequence with no external activation. The characteristics of activity (time course and amplitude) did not appear to be different from walking sequences evoked by stimulation. The tonic voltage shift was dependent on membrane potential as shown for a depressor motoneuron, the membrane potential of which was manipulated by current injection (Fig. 2). The tonic
A shift in membrane potential was depolarizing at a membrane potential of $-77\text{ mV}$, hyperpolarizing at $-45\text{ mV}$, but greatly reduced at the resting membrane potential of $-64\text{ mV}$. Figure 3 shows the dependence of the tonic shift on membrane potential for all motoneurons that were recorded. Measurements of tonic voltage shifts were taken at different membrane potentials in current-clamp mode usually in the first half of a stepping sequence because in some recordings the tonic response declined in the second half of the stepping sequence (e.g., Figs. 1C and 2A). For depolarizing shifts, the voltage difference between resting potential and the lower border of phasic modulation was taken as the amplitude in tonic shift (see Fig. 3, inset). For hyperpolarizing shifts, the voltage difference between resting potential and the upper border of phasic modulations was measured. Measurements at different membrane potentials from all motoneurons of a given type were lumped together and data were fitted using linear regression. The coefficient of correlation ($r$) was significant at the 5% level for depressor and flexor motoneurons.

Slopes of regression lines (Fig. 3) as indicated by coefficients of regression (Table 1) were not significantly different between different motoneuron types. Response reversals as calculated from graph fitted equations were found between $-32$ and $-47\text{ mV}$, but again, these values were not significantly different. Mean reversal potentials in ipsilateral mo-

**FIG. 1.** Mesothoracic motoneurons depolarize tonically and show membrane potential oscillations during a stepping sequence of an ipsilateral front leg. Top right: sketch indicates the recording site in respect to the stepping leg. First trace: treadmill velocity ('tacho'). A rising tachometer trace, indicating treadmill acceleration, was used to define the stance phase of the leg. Second trace: electromyographic (EMG) recording of the stepping leg's flexor tibiae muscle. A, 3rd trace: extracellular recording from nerve nI2 that contains the axons of protractor motoneurons. Fourth trace: intracellular recording of a protractor motoneuron (MN). B, 3rd trace: extracellular recording from nerve nI3 that contains the axons of extensor motoneurons. Fourth trace: intracellular recording of a slow extensor tibiae motoneuron (SETi). C, 3rd trace: extracellular recording from nervus cruris that contains the axons of flexor motoneurons. Fourth trace: intracellular recording of a fast flexor tibiae motoneuron. - - -, resting membrane potential as given by numbers.

**FIG. 2.** Reversal of the tonic depolarization in a depressor coxae motoneuron. A: from a membrane potential of $-45\text{ mV}$, a stepping sequence of a front leg induced a slight tonic hyperpolarization in an ipsilateral mesothoracic depressor motoneuron. B: tonic response of the motoneuron on front-leg stepping is slightly depolarizing at its resting potential of $-64\text{ mV}$. C: tonic depolarization amplitude is increased at a membrane potential of $-77\text{ mV}$. Phasic membrane potential changes in mesothoracic depressor motoneurons during front leg stepping are less distinct than in other motoneuron types.
coupled to movements of the front leg (Fig. 1). The coupling in ipsilateral motoneurons

Phasic membrane potential changes during front-leg walking based on a mixed inward-outward conductance. Voltage shift in motoneurons during a stepping sequence is mean reversal potential around 

mean decrease in input resistance by 12.6

Contamination by phasic synaptic input. In six motoneurons, a decrease in input resistance and the decrease in input resistance and the mean reversal potential around −56 ± 6.8 mV (N = 21) in all motoneurons.

Motoneurons were 10–21 mV more positive than mean resting potentials (Table 1).

The depolarizing response in motoneurons appears to be associated with a decrease in input resistance. In nine motoneurons, the input resistance was measured during stepping activity of the front leg by injection of short hyperpolarizing current pulses (Fig. 4). Measurements of input resistance were taken between and not during actual stepping movements to avoid contamination by phasic synaptic input. In six motoneurons, a mean decrease in input resistance by 12.6 ± 2.3% was measured; in three other motoneurons, no change in input resistance was detected. The decrease in input resistance and the mean reversal potential around −38 mV indicate that the tonic voltage shift in motoneurons during a stepping sequence is based on a mixed inward-outward conductance.

**Phasic membrane potential changes during front-leg walking in ipsilateral motoneurons**

Rhythmic membrane potential changes in middle leg motoneurons during a front-leg stepping sequence were usually coupled to movements of the front leg (Fig. 1). The coupling pattern was typical for each motoneuron pool. Depending on membrane potential, phasic depolarizations often caused a discharge of one to about five spikes (Figs. 1, 5, and 6).

**Protractor coxae motoneurons**

Rhythmic membrane potential changes in protractor coxae motoneurons (N = 3) had amplitudes of 0.5–3 mV peak to peak. The minimum depolarization of the membrane potential was at the onset of stance in the front leg. The potential then depolarized during stance and reached a peak −150 ms after the end of the legs stance phase (Fig. 5A, same neuron as Fig. 1A). Thus spike activity in protractor motoneurons appears to be associated with front-leg swing phase.

**Depressor trochanteris motoneurons**

During a stepping sequence of a front leg, we found a general increase in synaptic input to ipsilateral mesothoracic depressor trochanteris motoneurons (Figs. 2, B and C). But step-coupled membrane potential oscillations were rather weakly expressed compared with those observed in protractor motoneurons (Fig. 5B1, same recording as in Fig. 2). Only when spikes were generated, spontaneously or due to injection of positive current, inhibition of activity some 80 ms before and after the end of stance phase was unmasked in six of seven recordings (Fig. 5B, 1 and 2). No step-coupled modulation of spike activity was detected in the seventh recording.

**Extensor tibiae motoneurons**

Similar to protractor motoneurons, membrane oscillations were usually well expressed in slow (SETi) and fast (FETi) extensor tibiae motoneurons. Peak-to-peak amplitudes were 0.5–4 mV in SETi (Fig. 6A; n = 3) and ≤6 mV in FETi (n = 3). Phasic depolarization started −100 ms before onset of front-leg stance phase and peaked near the end of stance phase. (Fig. 6A, 1 and 2). Spike activity ceased or was largely reduced after stance phase ended. No obvious difference in membrane potential modulation was detected between FETi and SETi motoneurons.

**Flexor tibiae motoneurons**

In four of five flexor motoneurons, membrane oscillations with peak-to-peak values of ≤4 mV were coupled to front-leg steps. From a most hyperpolarized potential around the onset

![FIG. 3. Dependence of the tonic membrane potential shift, which was induced by front-leg stepping, in 4 different mesothoracic motoneuron types on membrane potential. Inset: the tonic shift (ΔV) was measured as the offset between voltage baseline before onset of a stepping sequence and the lower edge of the voltage oscillations during a stepping sequence. Tonic shifts in depressor trochanteris (Dep), protractor coxae (Pro), flexor tibiae (Flex), and extensor tibiae motoneurons. The arrow indicates the mean resting membrane potential of −56 ± 6.8 mV (N = 21) in all motoneurons.](http://jn.physiology.org/)

**TABLE 1. Dependence of tonic membrane voltage shifts in mesothoracic motoneurons on membrane potential during stepping sequences of the ipsi- and contralateral front leg (Figs. 3, 7C)**

<table>
<thead>
<tr>
<th>Motoneuron Type</th>
<th>Resting Membrane Potential, mV</th>
<th>Regression Coefficient b</th>
<th>Correlation Coefficient r</th>
<th>Reversal Potential, mV</th>
<th>N/ln</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsi lateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Depressor MNs</td>
<td>−57 ± 8.9</td>
<td>−0.061</td>
<td>0.560*</td>
<td>−47</td>
<td>7/22</td>
</tr>
<tr>
<td>Protractor MNs</td>
<td>−51 ± 6.2</td>
<td>−0.100</td>
<td>0.800</td>
<td>−32</td>
<td>3/5</td>
</tr>
<tr>
<td>Extensor MNs</td>
<td>−56 ± 6.6</td>
<td>−0.182</td>
<td>0.522</td>
<td>−35</td>
<td>6/8</td>
</tr>
<tr>
<td>Flexor MNs</td>
<td>−57 ± 4.2</td>
<td>−0.137</td>
<td>0.607*</td>
<td>−39</td>
<td>5/12</td>
</tr>
<tr>
<td>Contra lateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexor MNs</td>
<td>−57 ± 5.1</td>
<td>−0.075</td>
<td>0.578*</td>
<td>−38</td>
<td>20/31</td>
</tr>
</tbody>
</table>

Data points were fitted by linear regression. Regression coefficients (b), correlation coefficients (r), and reversal potentials were taken from data-fitted equations. Values are means ± SD. N, number of animals; n, number of samples. *r values at least significant on a 5% level.

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of front-leg stance, the membrane depolarized (Fig. 6, B and C). In contrast to extensor motoneurons, peak values were gained after stance phase ended. Correspondingly, spiking that was observed in two cells was mainly associated with front-leg swing (Fig. 6B). In a fifth flexor motoneuron, no step coupled phasic modulation could be detected.

Activity in mesothoracic flexor motoneurons during walking of the contralateral front leg

In walking stick insects and crayfish, it was shown that coupling between contralateral legs is weaker than between ipsilateral legs (Cruse 1990; Cruse and Knauth 1989). Weaker coupling should result in less pronounced phasic modulation. Hence, the quality of analysis of a tonic membrane potential shift would be enhanced. From a mean resting potential of $-77 \pm 5.1$ mV ($N = 20$), the membranes tonically depolarized by $1.5 \pm 1.0$ mV (Fig. 7, A and B). A constant depolarizing drive can most clearly be seen in Fig. 7B because the membrane potential does not hyperpolarize between steps of the walking sequence. In this recording, the membrane potential repolarization to resting value took some 6 s after the last step was performed. In two recordings, the time for repolarization after the stepping sequence was even longer, taking $\sim 30$ s. Again, the amplitude of the tonic depolarization decreased with a more positive initial membrane potential. The coefficient of correlation ($r = 0.58$) was significant on the 5% level when data were fitted by linear regression (Fig. 7C). Response reversal as calculated from the graph fitted equation was found to be at $-38.3$ mV. Thus the calculated reversal potential is $-19$ mV more positive than the mean resting potential. As in ipsilateral motoneurons, tonic depolarizations were associated with a decrease in input resistance, here by $12.4 \pm 3.5\%$ ($N = 7$).

In 15 of 20 contralateral flexor motoneurons, the membrane potential was phasically modulated, coupled to the steps of the contralateral front leg (Fig. 8). In 55% of the recordings, a modulation similar to the modulation in extensor motoneurons ipsilateral to the walking leg was observed (Fig. 8A). The onset of a membrane depolarization was $\sim 0.13$ s before onset of front-contralateral stance phase. The depolarization reached a maximum during stance phase and began to decline at the end of stance phase. Mean peak-to-peak amplitudes were $2.9 \pm 1.6$ mV. Figure 8B shows a different coupling pattern with similar peak-to-peak amplitudes that was observed in 20% of the recordings. The membrane potential began to decline from a maximum some 100 ms before onset of stance and reached a minimum during the first half of the stance phase. In the other 25% of the recordings, no phasic modulation of the membrane potential coupled to front-leg steps was observed.

The reduction in input resistance that is generally observed in motoneurons throughout a stepping sequence of an ipsi- or contralateral front leg might somewhat reduce the responsiveness of the neurons to incoming depolarizing input. We tested this assumption in flexor motoneurons contralateral to the walking front leg by injecting depolarizing current pulses of 300-ms duration that evoked one or two spikes before onset of walking movements. In 73% of the flexor motoneurons that were tested ($N = 11$), the number of spikes per pulse more than doubled, indicating an increased responsiveness (Fig. 9). In two of these recordings, increased responsiveness outlasted the stepping sequence for tens of seconds. In 2 of 11 motoneurons, the responsiveness was reduced, possibly by current shunt because in both neurons the input resistance decrease was exceptionally high ($\geq 30\%$) during walking sequences. One motoneuron did not exhibit a consistent activity pattern on current injection. During four stepping sequences, the responsiveness was reduced and no tonic depolarization was observed; during one stepping sequence, the responsiveness was enhanced while the membrane potential was tonically depolarized by 1.3 mV.

Membrane potential modulation in mesothoracic local premotor nonspiking interneurons during walking of the ipsilateral front leg

Membrane potential modulation in mesothoracic motoneurons during front-leg walking could be due to synaptic drive...
from intersegmental neurons or synaptic drive from local interneurons that mediate intersegmental signals or due to a combination of both pathways. Here we attempt to explore the role of mesothoracic local nonspiking interneurons in mediating intersegmental signals to mesothoracic motoneurons during front-leg walking.

Nonspiking interneurons are important local elements in hemi-segmental networks that control leg motoneuron activity in reflex pathways (Burrows and Siegler 1976; Büschges 1990; Pearson and Fourtner 1975), and some of them are elements of CPGs (Büschges 1995). In addition, there is evidence that these neurons are involved in intersegmental information processing in a semi-intact walking preparation (Kittmann et al. 1996) and processing of intersegmental sensory signals in locusts (Lau- rent and Burrows 1989). In stick insects, nonspiking interneurons were classified as E- or I-type neurons according to their excitatory or inhibitory input to extensor motoneurons (Büschges 1990).

We investigated the influence of stepping movements of a front leg on the membrane potential of ipsilateral mesothoracic nonspiking interneurons. The membrane potential in different E- and I-type interneurons was recorded while simultaneously monitoring extensor motoneuron activity extracellularly. All nonspiking interneurons that were recorded exhibited a phasic membrane potential modulation coupled to front-leg stepping (Fig. 10). Generally, at resting membrane potential, tonic voltage shifts, if present at all, appeared to be rather small when compared with those observed in motoneurons. For example, interneuron E4 exhibited the largest tonic response observed that was a depolarization of \( \Delta V = 4 \text{ mV} \) (Fig. 10A1) in six of seven recordings from a mean resting membrane potential of \( \mu V = 55 \pm 5.3 \text{ mV} \). In one experiment, no tonic depolarization could be detected. A phasic modulation of E4 was associated with front-leg stepping with a depolarizing phase during front-leg stance (Fig. 10, A2 and B2). Both, tonic and phasic depolarization of E4 could support the activity pattern of extensor motoneurons during front-leg stepping. The supporting effect of E4 for SETi is shown in Fig. 10B1. During a stepping sequence, mean spike frequency in SETi corresponds with phasic membrane modulation in E4. Injection of negative current into E4 leads to a reduction of mean spike rate in SETi. In support of this result, depolarizing current injected into E4 leads to an increase in spike activity in SETi (Fig. 10B3).

In contrast, I1-type nonspiking interneurons when depolarized reduced SETi activity and hyperpolarization increased SETi activity (Büschges 1990). During front-leg stepping, interneuron I1 was hyperpolarized throughout the walking sequence (Fig. 10). The phasic modulation in membrane potential of \( \Delta V = 5 \text{ mV} \) peak-to-peak was coupled to front-leg stepping such that most negative values were reached when extensor motoneurons were most depolarized, close to the end of stance (compare Figs. 6A and 10C2). This modulation in membrane potential reduces inhibitory influence of I1 on extensor motoneurons during front-leg stance and therefore contributes to the observed extensor activation.

Preliminary data from other types of nonspiking interneurons (E2, E3, E7, I2, and I4; \( n = 1 \) for all neurons) provide evidence that nonspiking interneurons that control extensor motoneuron activity are generally affected by front-leg stepping movements. However, some of these initial data indicate that not all nonspiking interneurons support extensor activity as shown for E4 and I1.
DISCUSSION

In a previous study (Ludwar et al. 2005), extracellular recordings of motor activity in the deafferented mesothoracic ganglion of the stick insect revealed that walking of a single front leg is associated with increased activity in ipsilateral motoneurons of all major leg joints. This activity is modulated with individual front-leg steps. In this study, using intracellular recordings, we uncovered a tonic depolarization in these motoneurons and contralateral flexor motoneurons that was maintained throughout the stepping sequence and that was phasically modulated. Tonic and rhythmic modulation of ipsilateral motoneurons might at least in part be due to input from local nonspiking interneurons, the membrane potential of which was found to be tonically shifted throughout and phasically modulated with front-leg stepping.

Tonic depolarization

Not only mesothoracic motoneurons ipsilateral to the walking front leg receive tonic drive; flexor motoneurons, and perhaps other motoneurons, contralateral to the walking leg also receive tonic drive. This observation supports the notion that tonic depolarizing drive to motoneurons is ubiquitous during locomotion. The tonic depolarization was associated with a decrease in input resistance by \( \sim 12\% \). The depolarization amplitudes increased when membrane potentials were hyperpolarized and reversed at more depolarized potentials, between \(-47\) and \(-52\) mV. The reversal potentials and the input resistance decrease indicate that either the tonic response is based on a nonspecific cation conductance e.g., for Na\(^+\) and K\(^+\), or it is a heterogeneous product of currents through different channels.

The decrease in input resistance potentially hinders spike generation. However, in 8 of 11 experiments on flexor motoneurons, the responsiveness throughout a stepping sequence to depolarizing input was enhanced as indicated by an increased number of spikes on depolarizing current pulses that were just suprathreshold in control. We assume that responsiveness is enhanced because the tonic depolarization brings the membrane potential closer to spike threshold, which more than compensates for current shunt that is due to the reduced input resistance. However, in two experiments, the responsiveness was reduced, and this reduction was accompanied by an exceptionally high decrease in input resistance by 30\%. One flexor motoneuron showed no enhanced responsiveness throughout four stepping sequences. In all these cases, no tonic depolarization was obvious. In another neuron, throughout a fifth stepping sequence, the membrane tonically depolarized by 1.3 mV, and responsiveness to depolarizing pulses was enhanced. The latter experiments indicate that the mechanisms that generally serve enhancement of excitability of motoneurons may allow fine tuning of motoneuron activity.

FIG. 6. Phasic membrane potential modulation in mesothoracic extensor and flexor motoneurons during stepping of the ipsilateral front leg. A1: slow extensor tibiae motoneuron (SETi) is depolarized during stance phase (indicated by boxes). A2: overlay of 12 sweeps (triggered by the onset of stance) shows that spiking is largely restricted to stance phase. First solid line and black arrowhead indicate start of stance phase, open arrowhead and 2nd solid line with dashed lines indicate mean end of stance phase \( \pm SD \), respectively. B: flexor motoneuron generates spikes during front leg stepping except for stance phases. C: average membrane potential (13 sweeps triggered by onset of stance phase) of a different flexor motoneuron (same neuron as Fig. 1C). Similar to the extensor motoneuron the flexor depolarized during stance but, in contrast, does not reach its peak depolarization during stance.

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Spike generation would also be hindered if mean reversal potentials of tonic depolarizations were more negative than spike threshold. Spike threshold in flexor motoneurons is $-51$ to $-47$ mV (Gabriel et al. 2003) and thus more negative than the mean reversal potential of $-38$ mV in these neurons. If this relationship holds for other motoneuron types, spike generation is probably not being hindered by a net outward current that is evoked at membrane potentials.

**FIG. 7.** A and B: activity in mesothoracic flexor motoneurons contralateral to the stepping front leg. **First and 2nd trace:** treadmill velocity (tacho) and EMG recording of the stepping leg’s flexor tibiae muscle. **Third trace:** extracellular recording from the contralateral mesothoracic nervus cruris that contains the axons of flexor motoneurons (MN). A, **4th trace:** flexor motoneuron shows a tonic depolarization as well as membrane potential oscillations during front-leg stepping. B, **4th trace:** flexor motoneuron shows a tonic depolarization but no apparent membrane potential oscillations during front-leg stepping. Thus the tonic depolarization appears to be largely independent of the actual stepping movement. ---, resting membrane potentials. C: dependence of the tonic membrane potential shift that was induced by front-leg stepping on membrane potential in contralateral mesothoracic flexor motoneurons. $\uparrow$, the mean resting membrane potential of $57.3 \pm 5.1$ mV ($N = 20$).

**FIG. 8.** Phasic modulation in membrane potential in contralateral mesothoracic flexor motoneurons during front-leg stepping. A–C: overlays (gray areas) and averages (solid lines) of 5 to 8 sweeps triggered by onset of stance phase (1st vertical solid lines and black arrowheads). Mean end of stance phase is indicated by open arrowheads and 2nd solid line, dashed lines indicate SD. A: depolarization of membrane potential in a flexor motoneuron starts $-200$ ms before onset of front-leg stance phase and reached its peak depolarization with onset of stance (observed in 11 of 20 experiments). The membrane potential remains depolarized during stance. B: hyperpolarization of membrane potential in a different flexor motoneuron starts $200$ ms before onset of front-leg stance phase and reached its minimum $100$ ms after onset of stance ($N = 4/20$). During stance, the membrane potential repolarized. C: in a different flexor motoneuron, there is no obvious coupling of membrane potential modulation to front-leg steps ($N = 5/20$).
potentials more negative than spike threshold. However, except for contralateral flexors, this is a conclusion with reservations because of the high variability in reversal potentials. This variability is likely to result from the variability in depolarization amplitudes that in turn is due to the circumstance that tonic responses were not evoked by a

FIG. 9. Increased membrane responsiveness during tonic depolarization of contralateral mesothoracic flexor motoneurons. Short current pulses (5th trace) that depolarized the flexor motoneurons (4th trace) just above threshold were injected. A: before and after a front leg stepping sequence (1st trace) each current pulse induced 1 or 2 spikes in a flexor motoneuron (see clipping B1). During a stepping sequence of the front leg, the number of current induced spikes increased (see clipping B2).

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standardized stimulation protocol but by the execution of a highly variable locomotor behavior.

Enhanced responsiveness of motoneurons associated with motor acts has been observed in other motor systems as well. For example, in proleg retractor motoneurons in Manduca larvae a long-lasting depolarization is evoked by stimulation of mechanosensory hairs and blocked by muscarinic receptor antagonists (Trimmer and Weeks 1993). The underlying inward current has a voltage-sensitive range where it increases with depolarization, making the current regenerative. Because this current should affect the responsiveness of motoneurons to all incoming input, it is assumed that it acts as a form of motor arousal to enable a faster and stronger response to small stimuli (Trimmer 1994). In contrast to these Manduca motoneurons, stick insect leg motoneurons do not appear to be endowed with alike regenerative properties as similar voltage sensitivity associated with the tonic response were not detected.

In the scratch-like motor network of turtles, motoneurons exhibit not only a persistent inward current but also increased excitability for tens of seconds after episodes of rhythmic activity. This increase appears to be based on a metabotropic glutamatergic facilitation of L-type Ca\(^{2+}\)-channels (Alaburda and Hounsgaard 2003). As in turtle motoneurons, hyperexcitability in stick insect motoneurons could outlast the episode of motor activity for tens of seconds, indicating that metabotropic receptors may be involved in mediating the effect. Therefore it seems possible that the tonic depolarization observed during front-leg walking relates to the tonic depolarization in stick insect motoneurons that is the basis of rhythmic bursting evoked by application of the muscarinic agonist pilocarpine (Büsschges 1998). In the deafferented mesothoracic ganglion, this tonic suprathereshold depolarization is sculpted into bursts of spikes by inhibitory input from central pattern generating networks. However, during stepping of a single front leg, spike activity in mesothoracic motoneurons was never as vigorous as during application of pilocarpine. Clearly, a tonic depolarization of \(\sim5\) mV during single front-leg walking is insufficient to provide the basis for a fully expressed bursting pattern during walking. Apart from a contribution of local nonspiking interneurons, we do not know which source provides tonic drive and whether a more complete locomotor system would cause an enhanced sustained depolarization (see following text). At least tonic drive during bursting activity in motoneurons does not appear to depend on walking per se. There is evidence that tonic drive is the basis of bursting in motoneurons that is evoked by tactile stimulation in the deafferented mesothoracic ganglion (Büsschges et al. 2004).

Tonic depolarization of motoneurons was found with rhythmic motoneuron activity in a variety of motor systems in vivo as well as in vitro. In the locust flight system, Hedwig and Pearson (1984) have shown that elevator motoneurons are tonically depolarized throughout sequences of fictive flight. Crayfish swimmeret motoneurons have been described to be tonically depolarized during execution of rhythmic activity (Mullone 2003). In the marine mollusk Tritonia, initiation and maintenance of a swim episode depends on the establishment of a long-lasting ramp depolarization in both premotor pattern-generating interneurons and the motoneurons (Getting and Dekin 1985; Lennard et al. 1980). In vertebrates, tonic excitation of spinal motoneurons is occurring during fictive locomotion, e.g., in lamprey and tadpole fictive swimming, cat and neonatal rat fictive walking (Cazalets et al. 1996; Roberts et al. 1985, 1986; Shefcyk and Jordan 1985; Wallén et al. 1985, 1993; review in Orlovsky et al. 1999). In the spinal cord, the source of the tonic depolarization can be attributed to excitatory synaptic inputs from descending reticulospinal projections, which are active during locomotion. In the insect walking system, as well, interneurons from the brain descending into the thoracic nerve cord have been recorded that are activated tonically on the initiation and generation of locomotor activity (Kien 1990). Such neurons are potential candidates for providing tonic drive to leg motoneurons.

A less likely source of tonic drive is input from sense organs in the walking leg, for example, from the femoral chordotonal organ that signals movement of the femur tibia joint or campaniform sensilla that sense strain in the cuticle. These sense organs play a prominent role in the timing and coordination of motor activity (for review see Bässler and Büschges 1998; Zill et al. 2004). But campaniform sensilla, which control the magnitude of motor output, have opposite effects for antagonistic muscles (Akay et al. 2001; Schmitz 1993; Zill and Moran 1981).

Not only walking is associated with a tonic depolarization of motoneurons but also searching movements of the front leg (Büsschges et al. 2004). Tactile stimulation of the abdomen almost always leads to a depolarization of motoneurons. As Büschges et al. (2004) have shown in a preparation in which all legs were severed, tactile stimuli evoked coactivation of antagonistic motoneurons in 75% of all trials. In these cases, subthreshold depolarizations of \(<1–10\) mV in motoneurons lasted about six times longer than the stimulus (Büsschges et al. 2004). Long-lasting depolarizations evoked by an external stimulus are not essential for expression of the typical activity pattern of middle leg motoneurons during front-leg walking. For the tonic depolarization during walking, it does not matter whether walking was induced by a stimulus (e.g., Fig. 1A and C) or generated spontaneously (Fig. 1B). Thus tonic drive to motoneurons usually associated with walking probably reflects a general rather unspecific level of excitation in the locomotor system. In the intact locomotor system, phasic excitatory input from sense organs, e.g., campaniform sensilla, in the mesothoracic leg will contribute to gain a fully expressed bursting pattern (Akay et al. 2001). We hypothesize that the tonic depolarization of motoneurons during walking is a cellular correlate of arousal that usually increases effectiveness of phasic excitation in supporting motoneuron firing. The underlying mechanisms appear to be under modulatory control because reduced excitability throughout stepping sequences was observed in few instances.

**Coupling of phasic membrane potential changes to front-leg steps and the role of local nonspiking premotor interneurons**

Using extracellular recordings from mesothoracic motor nerves during front-leg stepping, Ludwar et al. (2005) have shown that spike activity in ipsilateral protractor and flexor motoneurons is largely restricted to front-leg swing phase, extensor and, in most cases, depressor activity to front-leg stance phase. These coupling patterns were confirmed by the intracellular recordings performed here. As already described by Ludwar et al. (2005), the patterning of activity in mesothoracic motoneurons is inconsistent with a functional interleg
coordination during walking. This nonfunctional coordination pattern is probably best explained by missing local sensory signals. Local sensory signals from the femoral chordotonal organ (reviewed in Bässler and Büschges 1998) and from campaniform sensilla (Akay et al. 2004; Duyssens et al. 2000; Riddel et al. 2000) are known to contribute to the local control of leg movements. In stick insects, local sensory information appears to be used to gate out signals from other walking legs. The evidence is given by experiments on stick insect that walked on a tread wheel. Retractor motoneurons of a middle leg that was amputated at mid coxa level showed weak rhythmic activity in a fixed phase relation to the activity of retractor motoneurons of other walking legs provided the stump was immobile (Graham 1985). When proximal sensory organs were stimulated by moving the stump forward and backward, retractor activity was observed only if the imposed movement occurred in a certain phase window with respect to the walking movements of the ipsilateral front and hind legs (Graham 1985). The coupling of phasic membrane potential changes in mesothoracic flexor motoneurons contralateral to the stepping front leg was variable in comparison to ipsilateral flexor motoneurons. In most experiments (55%), these flexor motoneurons exhibited a phasic modulation pattern that was similar to that observed in extensor motoneurons ipsilateral to the walking front leg, that is, maximum activity during front-leg stance. Although this pattern implies antagonistic movements of both middle legs, it must be kept in mind that it is not known how motoneurons of other joints are coordinated between left and right side. Generally, a co-activation of the same motoneuron type on both sides appears possible because in 20% of the recordings contralateral flexors showed a maximum depolarization after the end of front-leg stance that is similar to the pattern observed in ipsilateral flexor motoneurons. In the other 25% of flexor motoneurons, no step-coupled phasic modulation was detected. The observed variability is in agreement with weaker coupling of contralateral legs in walking intact stick insects and crayfish (Cruse 1990; Cruse and Knauth 1989). Thus the reduced state of the walking system may not solely account for the observed variability.

In stick insects, when prothoracic CPGs were activated by bath application of pilocarpine onto the prothoracic ganglion, activity in mesothoracic motoneurons was not coupled to prothoracic rhythmic activity (Ludwar et al. 2005). This observation indicates that the phasic modulation of mesothoracic motoneuron activity is not attributable to activity in prothoracic CPGs. In line with this assumption, in stick insects, rhythmic activity in protractor motoneurons of all three thoracic ganglia showed no stereotyped or fixed coupling when evoked by application of pilocarpine onto all thoracic ganglia (Büsches et al. 1995). Thus phasic modulation of mesothoracic motoneurons appears to be coupled to front-leg movements, at least partly, based on sensory information from the front leg, namely signals from the front leg’s femoral chordotonal organ (fCO) as shown by Ludwar et al. (2005). The individual pathways that transmit this information have not yet been identified. Possible candidates are intersegmental descending interneurons in the thoracic ganglia that receive input from the fCO and other sensory afferents. Such neurons have been characterized morphologically and physiologically in the stick insect and locust (Brunn and Dean 1994; Büschges 1989; Laurent and Burrows 1989).

The transmission of signals from the prothoracic segment to mesothoracic extensor motoneurons—and probably other leg motoneurons as well—involves mesothoracic nonspiking interneurons. Of course, this indirect pathway does not exclude that leg motoneurons receive direct input from intersegmental interneurons in parallel. Interestingly, antagonistic motoneurons of all three proximal leg joints are activated in alternation during front-leg walking (Ludwar et al. 2005; this study). This observation may suggest that intersegmental signals activate mesothoracic central pattern-generating networks (CPGs) that have been proposed to control movements of individual leg joints in the stick insect (Bässler and Wegener 1983; Büschges et al. 1995). Some observations, however, are not in support of this idea. First, we have found that although their suprathreshold activity is alternating, flexor and extensor tibiae motoneurons of the mesothoracic segment are depolarized simultaneously during stance phase of the front leg (Fig. 6). Recordings from deafferented rhythmic preparations (Büsches 1998; Büschges et al. 2004) have shown that antagonistic motoneuron pools of a given leg joint always received alternating inputs and were never found to receive simultaneous synaptic drive. Second, during front-leg walking, phasic modulation of mesothoracic motoneurons was rather smooth and did not appear to be shaped by marked inhibitory input as known from motoneuron recordings in preparations in which CPGs were active (Büsches 1998; Büschges et al. 2004). Third, the nonspiking interneuron E4 has been found to be an element of leg-joint CPGs (Büsches 1995) and indeed, the membrane potential of E4 was rhythmically modulated during front-leg walking. However, the magnitude of this modulation, which was ∼4mV, is only a fraction of the amplitude known from deafferented rhythmic preparations (Büsches 1995) or from semi-intact preparations with intact middle legs (Büsches et al. 1994). We therefore assume that the rhythmic membrane potential changes in E4 are not due to oscillatory activity that was generated by a CPG that includes interneuron E4.

Our data provide evidence that local nonspiking interneurons play a role in transmitting tonic and phasic intersegmental signals to mesothoracic extensor motoneurons. In fact, spike-frequency alterations in the slow extensor tibiae motoneuron reflect membrane potential oscillations in E4 (Fig. 10B) and hyperpolarization of E4 by current injection reduced spike frequency in extensor motoneurons. All nonspiking interneurons that were recorded are also known to process sensory signals from the middle leg in local reflex pathways. Thus during walking, local and intersegmental signals appear to converge on the level of nonspiking interneurons. A similar layout in intersegmental processing of sensory signals has been shown for the locust (Laurent 1991). In nonspiking interneurons that control extensor activity, the diversity of response patterns to local sensory input suggests parallel distributed signal processing (Büsches 1990; Sauer et al. 1996). Not surprisingly, preliminary data from a variety of identified nonspiking interneurons (E3, E7, 12 and 14) indicate a similar distributed and not dedicated processing of intersegmental signals during front-leg walking.

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INTERSEGMENTAL MODULATION OF NEURONS DURING WALKING


