Pharmacological Analysis of Tonic Activity in Motoneurons During Stick Insect Walking

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Westmark S, Oliveira EE, Schmidt J. Pharmacological analysis of tonic activity in motoneurons during stick insect walking. J Neurophysiol 102: 1049–1061, 2009. First published June 10, 2009; doi:10.1152/jn.91360.2008. Stick insect middle leg (mesothoracic) motoneurons receive tonic excitatory input during front leg stepping on a treadmill. We studied the pharmacology of this excitatory input to the motoneurons during single-legged treadmill walking (in situ). During bath application of drugs restricted to the mesothoracic ganglion, activity in motoneurons contralateral to the stepping front leg was recorded from neuropilar processes. Application of the cholinergic antagonist atropine reduced the tonic depolarization amplitude. These results were compared with findings in acutely dissociated motoneuron cell bodies (in vitro) under whole cell voltage-clamp conditions. The presence of an acetylcholine-induced current in situ was supported by the finding of an acetylcholine evoked biphasic inward current with a sustained component that could be blocked by atropine. In situ the tonic depolarization was generally increased by application of the neuro-modulator octopamine and decreased by its antagonist mianserin. In vitro, however, octopamine reduced the inward current evoked by acetylcholine application to motoneurons. Intracellular application of bis-(o-aminophenoxy)-N,N,N',N'-'tetraacetate acid (BAPTA) into motoneurons in situ revealed a dependence of the tonic depolarization on Ca2+ and application of the membrane-permeable cAMP analogue 8-bromo-cAMP increased the tonic depolarization. In contrast, 8-bromo-cAMP reduced the inward current evoked by acetylcholine application to motoneurons in vitro. We conclude that during walking, acetylcholine contributes to mediating the tonic depolarization possibly by acting on atropine-sensitive receptors on motoneurons. Octopamine that is released during walking increases the tonic depolarization. This increase, however, is not based on modulation of cholinergic action on motoneurons but rather on effects on premotor neurons. Both, Ca2+ and cAMP are likely second messengers involved in mediating the tonic depolarization, but whereas Ca2+ acts in motoneurons, cAMP does not appear to mediate a cholinergic depolarization in motoneurons.

INTRODUCTION

Activity in motoneurons that control movements of limbs for locomotion is shaped by synaptic input and intrinsic membrane properties. Rhythmic synaptic input to motoneurons has been shown to be provided by central pattern generators and leg sense organs (for review, see Büschges 2005; Büschges et al. 2008; Grillner 2003; Pearson 2004). In addition, tonic depolarizing drive was found to contribute to activation in lamprey and tadpole swimming motoneurons (Roberts et al. 1985, 1986, 2008; Wallén et al. 1985, 1993), rat walking motoneurons (Cazalets et al. 1996), and locust flight motoneurons (Hedwig and Pearson 1984). In crayfish swimmeret motoneurons, patterning appears to be based on tonic synaptic drive that is structured by cyclic synaptic inhibition (Mulloney 2003). Similarly, in the deafferented stick insect walking system bouts of spike bursts in leg motoneurons evoked by mechanical stimulation of the abdomen are based on tonic depolarizing and phasic hyperpolarizing synaptic drive (Büschges et al. 2004).

Tonic depolarizing drive of stick insect motoneurons is also present during actual stepping, and a tonic depolarization of middle leg motoneurons can be recorded intracellularly during sequences of front leg steps on a treadmill (Ludwar et al. 2005). This tonic depolarization of ~5 mV is based on a conductance increase and reverses at membrane potentials around ~39 mV, indicating a mixed inward-outward conductance as the basis of the tonic depolarization. We have shown previously that the tonic depolarization is associated with an increased responsiveness of the motoneurons to depolarizing input (Ludwar et al. 2005). Nothing is currently known about transmitters, neuromodulators or second messengers that contribute to generate this tonic depolarization. Acetylcholine (ACh) is a prime suspect as a transmitter because the muscarinic cholinergic agonist pilocarpine evokes a fictive locomotor pattern in deafferented stick insect motoneurons that is based on a tonic depolarization and phasic inhibition (Büschges 1998). Octopamine (OA) is known to have a general arousal effect on insects (Evans 1980; Orchard et al. 1993), and injection of OA into the body cavity of stick insects elicits locomotor activity (Büschges et al. 1993). This makes OA a potential modulator that could increase the tonic depolarization.

We have developed a semi-intact single leg preparation in which middle leg motoneuron activity can be studied in the presence of drugs while a front leg walks on a treadmill. In this preparation, all ganglia but the mesothoracic ganglion are bathed in normal saline while the mesothoracic ganglion is exposed to drugs. As a consequence, there is no direct chemical modulation of prothoracic neurons, which control front leg walking. In the semi-intact preparation, it is not clear whether bath-applied drugs effect mesothoracic motoneurons directly or indirectly via interneurons. Therefore drugs that affected motoneuron membrane potentials in the semi-intact preparation were tested for direct effects on acutely dissociated motoneurons under whole cell voltage-clamp conditions. We show that the tonic depolarization evoked during single leg walking and a cholinergic response in dissociated motoneurons is reversibly blocked by atropine. The tonic depolarization is modulated by OA, and Ca2+ and cAMP are involved as second messengers in mediating the tonic depolarization.
METHODS

Experiments were conducted on adult, female stick insects, Carausius morosus, from a colony maintained at the University of Cologne. All experiments were carried out under daylight conditions at temperatures between 18 and 24°C.

Semi-intact preparation and data acquisition

All legs except one front leg were amputated, and the animals were fixed dorsal side up on a foam platform using dental cement (Protom 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, and connective tissue was carefully removed to expose the mesothoracic ganglion. To prevent input from intact proximal sense organs, all mesothoracic lateral nerves but nervus cruris, which contains the axons of the recorded motoneurons, were crushed. A well (volume, ~100 μl) was formed by two silicone-gel (Baysilone-Paste hochviskos; GE Bayer Silicones, Leverkusen, Germany) barriers anteriorly and posteriorly of the ganglion (Fig. 1) to allow perfusion of the mesothoracic ganglion (gravity driven, flow rate: 2 ml/min) with a drug solution without affecting other parts of the CNS (isolating bath). The ganglionic sheath was mechanically removed with micro scissors to ease access of drugs. The ganglion was perfused with saline composed of (in mmol–1): 180 NaCl, 4 KCl, 5 CaCl2, 1 MgCl2, 30 saccharose, 10 HEPES buffer. pH was adjusted from a colony maintained at the University of Cologne.

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front leg stepping movements, most mesothoracic motoneurons at their resting membrane potential exhibited a tonic depolarization in membrane potential by 0.5–5 mV. The voltage difference between resting potential and the lower border of phasic modulation was taken as the amplitude in tonic shift (for details, see Ludwar et al. 2005). At least three stepping sequences were elicited for control purposes before drugs were applied. During drug application, consecutive stepping sequences (≥3) were evoked. Because the motivation for stepping was variable among animals and declined during the course of an experiment, drug application was stopped after ~10–20 min to reduce the probability of losing the cell under investigation. As a consequence, alterations in membrane potential usually did not reach steady-state values. Thus calculated means generally represent under-estimations of drug effect. We accepted not to be able to perform a classical pharmacological analysis in situ because in this study it was our goal to gather evidence for a potential role of a transmitter, modulator or second messenger in mediating the tonic depolarization in motoneurons during walking-like behavior.

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 build-in or custom-written scripts within the SPIKE 2 software. Layout editing was performed using CorelDraw X3 (Corel Corporation, Ottawa, ON, Canada), and statistical analysis and plots were rendered using Excel (Microsoft) and Microcal Origin (version 6.0, Originlab, Northampton, MA). In text and figures, N is the number of animals or cells in patch-clamp experiments, and n is the sample size. Means are given as ±SD. If not stated otherwise, a t-test was used for assessing differences between means.

Primary cell culture, drug application, and patch-clamp recordings

The responsiveness of acutely isolated motoneurons to ACh and atropine was studied using the whole cell patch-clamp technique and a fast drug application system.

Primary cell culture of motoneurons

For the identification of motoneurons that have axons in nervus cruris, all six nervi crurii were cut approximately where extracellular electrodes in semi-intact preparations were positioned. The cut ends were placed in pools that were formed with petroleum jelly and contained a tetramethylrhodamine-dextran solution (RDA; 3000 MW, anionic, lysine fixable; Invitrogen; 0.5–1.0% in distilled water) (after Kloppenburg and Höner 1998). For dye uptake, backfill preparations were kept at 4°C for 48 h. Subsequently all three thoracic ganglia were removed from the animal and treated for 10 min with collagenase (Sigma type I, 253 units/ml) and trypsin (Sigma, 8550 units/ml) in a NaOH to pH 7.2. Ganglia were then transferred into normal saline and desheathed with forceps. After another enzyme treatment for 20 min saline inflow was stopped. Cells were exposed to a constant flow of normal saline at a rate of 0.5 ml/min from the opening of a 0.5-mm ID Teflon tube placed 0.6 mm from the cell. ACh at a concentration of 100 μM in normal saline was applied using a U-tube system as described by Zhao et al. (2003). Briefly, the hole at the bottom of the U-shaped tube was positioned ~0.5 mm from the cell such that the line of test solution flow was directed at an angle of about 90° to the line of normal saline flow (Fig. 2A). Normally, the solution flows all the way through the U and out the drain, without entering the bath. For application, closure of a magnetic valve in the U-tube drain line forced the solution out of the hole, and simultaneously flow of normal saline was stopped by means of another magnetic valve as long as the test solution was applied. This system allows accurate kinetic measurements and avoidance of unintentional receptor desensitization.

Patch-clamp recordings

Whole cell recordings were performed following the methods described by Hamill et al. (1981). Electrodes (3–5 MΩ) were fashioned from borosilicate glass (Science Products, GB150-8P, 0.86 × 1.50 × 80 mm) with a temperature-controlled pipette puller (HEKA PIP5), and filled with a solution containing (in mM) 190 K-aspartate, 10 NaCl, 1 CaCl2, 2 MgCl2, 10 HEPES, and 10 EGTA adjusted to pH 7 (with KOH) and to ~415 mOsm. Whole cell recordings in voltage-clamp mode were made with a patch-clamp EPC8 or EPC9 amplifier (HEKA) controlled by Pulse software (version 8.63, HEKA). Data were sampled at intervals of 100 μs and filtered at 2.9 kHz with a Bessel-Filter. For data analysis, we used Pulse and Pulseeft software (version 8.63, HEKA) and Igor Pro 4 (Wavemetrics).

**FIG. 2.** Experimental setup for experiments in vitro. A: sketch indicates recording situation. During a whole cell patch-clamp recording of a crural motoneuron cell body plated in a dish, acetylcholine (ACh) was applied by 10.220.33.4 on November 2, 2016 http://jn.physiology.org/ Downloaded from J Neurophysiol • VOL 102 • AUGUST 2009 • www.jn.org
RESULTS

Effect of atropine in semi-intact preparations (in situ)

Application of the muscarinic ACh agonist pilocarpine onto thoracic ganglia leads to a tonic depolarization of motoneurons and subsequently to rhythmic bursting. Rhythmic bursting induced by pilocarpine is blocked by atropine (Büsches et al. 1995). To test whether muscarinic receptors play a role in mediating the tonic depolarization in middle leg motoneurons during front leg walking, atropine (500 µM) was bath-applied to the mesothoracic ganglion. Figure 3A shows the membrane potential of a middle leg crural motoneuron during a sequence of seven front leg steps. The stepping sequence was accompanied by a subthreshold ~4.1 mV tonic depolarization of the motoneuron. A 7-min bath application of atropine reduced the amplitude of the tonic depolarization in this motoneuron by ~51% to ~2.1 mV (Fig. 3B). The amplitude of the tonic depolarization partially recovered after a 9-min wash with regular saline (Fig. 3C). The change in amplitude over time in this experiment is summarized in Fig. 4A. The tonic depolarization amplitude was reduced from a control value of 4.1 ± 0.2 mV (n = 3) to ~2 mV during the fifth stepping sequence performed after 9 min of atropine application. The regression line (solid line) illustrates the progressive decline in amplitude. The mean amplitude in the presence of atropine was 2.3 ± 0.2 mV (n = 6) and thus significantly (P < 0.001) less than the control. A partial recovery in the amplitude could be observed after ~9 min of perfusion with regular saline (Fig. 4A).

Similarly decreasing amplitudes over time in the presence of atropine were observed in eight of nine experiments. Regression analysis confirmed this result (Fig. 4B, N = 8, n = 40; P < 0.01). On average, amplitudes during last stepping sequences in the presence of atropine where significantly reduced to 46.9 ± 19.1% (P < 0.01) of the control values (Fig. 4C). In four experiments, the recordings lasted long enough, and the animals still performed stepping sequences, to allow for a wash with regular saline. In these experiments, partial but significant recovery was observed after ≤15 min washing (Fig. 4C; 81.8 ± 12.2%, P < 0.05). These data indicate that ACh contributes to mediating the tonic depolarization by binding to atropine-sensitive receptors.

Effect of atropine on isolated motoneuron somata (in vitro)

From the experiments in the semi-intact preparation it is not clear whether ACh is able to affect motoneurons directly and whether such direct effects are atropine sensitive. Therefore ACh-evoked membrane currents in motoneurons were analyzed in isolated crural motoneuron somata. Cell bodies were voltage clamped at a holding potential of ~60 mV, and ACh (100 µM) was applied for 1 s (Fig. 2A). In all five motoneurons that were recorded, ACh evoked a biphasic inward current (Fig. 5). An initial peak of 503 ± 244 pA was followed by a sustained component of 277 ± 186 pA when measured at application offset. After application offset it took 1.8 ± 0.78 s (n = 5) until the current reached baseline.

In the presence of 100 µM atropine, both components of the inward current were almost completely blocked and in a reversible manner (Fig. 5). Thus the tonic depolarization observed in motoneurons during walking could be due to ACh binding directly to atropine-sensitive receptors in the motoneurons.

Modulation of the tonic depolarization by OA in semi-intact preparations (in situ)

As shown previously, the responsiveness of stick insect motoneurons is enhanced during tonic depolarization (Ludwar et al. 2005). Thus an up- or downregulation of the tonic depolarization could be a mechanism for modulating motor activity. Such regulation could be under neuromodulatory control. OA is a prime candidate for upregulation of the tonic depolarization because it elicits locomotor activity in intact stick insects for ~12 min after injection into the body cavity (Büsches et al. 1993).
To assess the appropriate OA concentration for bath application, three different concentrations were tested for their excitatory effects in extracellular recordings of motor activity in mesothoracic protractor motoneurons (nerve ni2) ipsilateral to the stepping front leg, and in crural motoneurons contralateral to the stepping front leg. Bath application of 100 μM OA for ≤20 min did not alter the activity pattern in ni2 (n = 3) and nervus cruris (n = 5) that was evoked by front leg stepping. A concentration of 500 μM OA did increase activity during stepping in ni2 in six animals and was not effective in another six animals. In nervus cruris, spike activity increased in one of six animals and was not effective in the others. A concentration of 1 mM OA increased activity in ni2 in all three animals that were tested. In nervus cruris, an increase in activity was observed in two animals and none in another eight animals. Based on these results, concentrations of 500 μM OA, as the lowest concentration that evokes effects, and 1 mM were chosen for testing OA effects in intracellular recordings in the semi-intact preparation.

Bath application of 500 μM OA onto the mesothoracic ganglion did not alter the membrane potential or spike activity in crural motoneurons in the inactive animal. During a front leg stepping sequence, however, the tonic depolarization was increased as compared with control (Fig. 6, A and B). In the experiment shown, the tonic depolarization amplitude increased from 2.5 ± 0.4 mV (n = 8) before application of OA to a significantly higher mean amplitude of 3.5 ± 0.4 mV (n = 13; P < 0.001). In addition, spike frequency was increased. The increasing depolarization amplitude over application time is summarized in Fig. 6C. A recovery was not obtained after a wash of ~15 min. In three of four experiments, the mean amplitude of the tonic depolarization was significantly increased as compared with control values (P < 0.05; data not shown). In the presence of 500 μM OA, we measured a mean increase by 139 ± 116% (n = 3; range: 40–267%). When 1 mM OA was applied (n = 6), we observed a significant increase by 172 ± 176% (range: 75–375%) in three experiments, while two experiments did not reveal a significant increase, and one experiment showed a significant decrease in amplitude by 25%.

Mianserin acts as an antagonist at OA receptors (Bischof and Enan 2004; Evans 1981; Maqueira et al. 2005; Nickisch-Rosenegk et al. 1996). We therefore applied mianserin to test for an OA-induced increase in tonic depolarization due to the endogenous presence of OA. Figure 7A shows an experiment in which a tonic depolarization of ~2.9 mV decreased to

![Figure 4](Image 371x157 to 515x185)

**FIG. 4.** Tonic depolarization amplitude in crural motoneurons over time during application of atropine. A: open circle on left indicates mean tonic depolarization amplitude (±SD, n = 3 stepping sequences) in a crural motoneuron during perfusion with normal saline (control condition). Filled circles indicate amplitudes of tonic depolarizations measured during stepping sequences while atropine (500 μM) was applied. Data points were fitted by linear regression. Open symbols on right indicate amplitudes of tonic depolarizations measured during stepping sequences during wash with normal saline. B: tonic depolarization amplitudes over time from 9 experiments. Open symbols on left indicate mean tonic depolarization amplitudes (±SD, n = 2–8 stepping sequences) in crural motoneurons under control conditions. Linear regression lines through 3–8 data points (individual data points are not shown) indicate decreasing amplitudes of tonic depolarizations in the presence of atropine (500 μM). Black solid lines: significance level of regression coefficient ±5%; dashed lines: regression coefficient not significant. Gray solid line: regression analysis for n = 8 of 9 experiments where a decrease in tonic depolarization amplitude over time was observed; regression coefficient ±1%. Open symbols on right indicate amplitudes during wash. Same symbols indicate data points from same experiment. C: normalized amplitudes of tonic depolarization show a decrease to 47.6 ± 20.0% (n = 8; P < 0.01) and a partial recovery (82.5 ± 14.9%, P < 0.05; n = 4).

![Figure 5](Image 371x217 to 515x233)

**FIG. 5.** Atropine blocks inward current evoked by application of ACh (100 μM) to a crural motoneuron in vitro. Top: sketch indicates recording situation. During a whole cell patch-clamp recording of a crural motoneuron cell body ACh was applied by means of a fast U-tube application system. During ACh application, saline inflow was stopped. Holding potential (V_h) was −60 mV. First trace: application of ACh evokes a biphasic response that consists of a fast activating and a sustained component. Second trace: the cholinergic response is blocked in the presence of atropine (100 μM) in the bath. Third trace: recovery of the response in normal saline.

![Figure 6](Image 371x261 to 515x307)

**FIG. 6.** Tonic depolarization amplitude in crural motoneurons during wash with normal saline. A: control trace: recovery of the response in normal saline. B: tonic depolarization amplitudes during wash. Open symbols on right indicate amplitudes during wash. Same symbols indicate data points from same experiment. C: normalized amplitudes of tonic depolarization show a decrease to 47.6 ± 20.0% (n = 8; P < 0.01) and a partial recovery (82.5 ± 14.9%, P < 0.05; n = 4).
Application of mianserin to a significantly lower mean amplitude of tonic depolarization (mean ± SD, motoneuron over time during application of OA. Open symbol on left indicates amplitudes of tonic depolarizations measured during stepping sequences during OA (500 µM). Filled circles indicate amplitudes of tonic depolarization measured during stepping sequences during wash.

Decrease in amplitude over time in this experiment is summarized in Fig. 7C illustrates the decline in amplitude over time for all six experiments (n = 55, P < 0.001). On average, amplitudes during the last stepping sequences in the presence of mianserin where significantly reduced to 31.1 ± 11.2% (n = 6) as compared with control amplitudes (Fig. 7D). In five experiments, a wash could be performed, and partial but significant recovery was observed (Fig. 7D; 61.0 ± 28.2%, P < 0.01). The reduction in amplitude over time shows the effectiveness of mianserin as a blocker and may indicate that OA is indeed released during walking and modulates the tonic depolarization.

Effect of OA on cholinergic responses in isolated motoneuron soma (in vitro)

From the experiments in the semi-intact preparation, it is not clear whether the OA effect is due to an upregulation of the cholinergic responses in motoneurons or to secondary effects. Therefore we evoked inward currents in isolated motoneurons by ACh application (100 µM) in the presence of increasing concentrations of OA in the bath. Otherwise, conditions were as described in the preceding text. In all five motoneurons that were recorded, bath application of OA at concentrations between 1 and 1000 µM did not evoke membrane currents (V_m = −60 mV) but reduced the peak ACh current in a concentration-dependent manner (Fig. 8, A and B). The half-maximal inhibitory concentration of OA was 158.7 ± 34.8 µM (Fig. 8B). The sustained component of the ACh current was not significantly reduced up to an OA concentration of 100 µM but was completely blocked at a concentration of 1000 µM OA (Fig. 8A).

These experiments indicate that the increase in tonic depolarization increase in motoneurons in the semi-intact preparation in the presence of OA is not due to an upregulation of the motoneurons’ response to ACh.

Dependence of tonic depolarization on intracellular calcium concentration (in situ)

The effectiveness of muscarinic cholinergic receptors, which are atropine sensitive, and the action of OA both depend on second messengers. Calcium and cAMP have been found to be second messengers in cascades that are triggered by cholinergic agonists and OA (see Discussion for details).

A possible role for Ca^{2+} as a second messenger in mediating the tonic depolarization in motoneurons was analyzed by loading motoneurons with BAPTA, a fast-acting Ca^{2+} chelator, to determine whether a reduction of intracellular free Ca^{2+} would reduce the amplitude of the tonic depolarization. When BAPTA (200 mM) was used in the electrode, amplitudes of tonic depolarizations that accompanied stepping sequences decreased over time as shown in Fig. 9A. In this experiment, 10 stepping sequences were evoked over a period of 31 min. Amplitudes of the tonic depolarizations decreased from 6.2 to 2.1 mV. Two original recordings of the experiment are shown in Fig. 9B. A decrease in amplitude over time was observed in seven of eight experiments (Fig. 9C). We compared the first and last amplitudes in each experiment because controls without BAPTA were not possible. This comparison revealed a significant mean reduction in amplitude by 44 ± 21% (P < 0.01, n = 8, Wilcoxon rank test). Because intracellular recordings were performed over long periods of time in this set of
experiments, we tested whether decreasing amplitudes might result from increasing leakiness of cell membranes induced by sharp microelectrode penetration. Therefore pulses of constant current were used to measure the input resistance between stepping sequences. These experiments revealed an increase in input resistance over time (Fig. 9D). Thus the decrease in amplitude is most likely related to quenching of Ca²⁺ by an increasing amount of BAPTA in the motoneuron.

Dependence of tonic depolarization on cAMP (in situ)

The AC/PKA pathway has been shown to be associated with muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) in insects (e.g., Thany et al. 2007; Trimmer 1995; Wenzel et al. 2002). In grasshoppers, mAChRs mediate excitation by activation of adenylate cyclase (AC) (Wenzel et al. 2002). We used the membrane permeable cAMP agonist 8-Br-cAMP, bath applied to the mesothoracic ganglion (Wenzel et al. 2002). In grasshoppers, mAChRs and nAChRs mediate excitation by activation of adenylate cyclase (AC) (Wenzel et al. 2002). We used the membrane permeable cAMP agonist 8-Br-cAMP, bath applied to the mesothoracic ganglion to activate protein kinase A. Application of 8-Br-cAMP (500 µM) had no effect on the membrane potential of motoneurons when animals were quiescent. However, when front leg stepping was elicited, the tonic depolarization increased in the presence of 8-Br-cAMP. Figure 10A shows an experiment in which the amplitudes of the tonic depolarization increased from 2.1 mV before application of 8-Br-cAMP to 4.2 mV after 22.5-min perfusion of 8-Br-cAMP (500 µM). The mean tonic depolarization was significantly increased to 3.4 ± 1.1 mV (n = 22, Fig. 10B) in the presence of 8-Br-cAMP in this experiment. A significant effect on mean amplitudes of the tonic depolarization could be shown in a total of five experiments (P ≤ 0.05). In three experiments, the amplitudes were increased by 65 ± 22%. In two experiments, no tonic depolarization was elicited during control, but in the presence of 8-Br-cAMP, mean amplitudes of 0.6 ± 0.4 and 0.9 ± 0.2 mV were measured.

To further analyze a role of cAMP in affecting the tonic depolarization, the AC inhibitor H-89 (100 µM) was bath applied (n = 2). In these two experiments, mean amplitudes in the presence of H-89 were reduced by 47 ± 38 and 22 ± 3.0% (data not shown).

The results gained by application of 8-Br-cAMP and H-89 indicate that cAMP is utilized to generate the tonic depolarization in motoneurons during walking.

Effect of 8-Br-cAMP on cholinergic responses in isolated motoneuron somata (in vitro)

Experiments on isolated motoneurons were performed to determine whether 8-Br-cAMP may directly upregulate depolarizing cholinergic responses in motoneurons in the semi-intact preparation. We evoked inward currents in isolated motoneurons by applying ACh (100 µM) in the presence of 8-Br-cAMP in the bath. In three motoneurons that exhibited a bi-phasic response, 8-Br-cAMP (2 µM) reversibly reduced the peak current by 46.9 ± 4.2% but did not significantly affect the sustained component (Fig. 11). Effects of 8-Br-cAMP (2 µM) on holding current (Vh: −60 mV) in the absence of ACh were not observed.

These experiments indicate that the increase of the tonic depolarization in motoneurons in the semi-intact preparation in

FIG. 7. Decrease in tonic depolarization by OA antagonist mianserin. A, 1–3, 1st trace: treadmill velocity (“tacho”). Second trace: intracellular recording of a crural motoneuron (MN). Dashed lines indicate resting membrane potentials as given by numbers. Stippled lines indicate levels of tonic depolarizations. A1: control. Tonic depolarization in a crural motoneuron. A2: reduced tonic depolarization in the presence of mianserin (100 µM, 12 min). A3: wash. Partial recovery of the tonic depolarization after 10-min perfusion with normal saline. B: tonic membrane depolarization amplitude over time during application of mianserin. Open symbol on left indicates mean tonic depolarization amplitude (+SD, n = 8 stepping sequences) in a crural motoneuron during control conditions. Filled symbols indicate amplitudes of tonic depolarizations measured during stepping sequences in the presence of mianserin (100 µM). Data points were fitted by linear regression. Open symbols on right indicate amplitudes of tonic depolarizations measured during wash. C: reduction of tonic depolarization over time by mianserin (100 µM) in 6 experiments [data points were fitted by linear regression (gray line), P < 0.001]. D: normalized amplitudes of tonic depolarization show a decrease to 30.7 ± 10.3% (n = 6; P < 0.001) and a partial recovery (59.4 ± 26.2%, P < 0.01, n = 5).
ACh as a transmitter of the tonic depolarization

The muscarinic receptor antagonist atropine blocks the tonic depolarization (Figs. 3 and 4). This indicates that ACh contributes to mediating the tonic depolarization. It is not clear whether ACh binds to typical muscarinic receptors because in locust, cockroach, and honey bee neurons have been identified that are equipped with “mixed” receptors that have both nicotinic and muscarinic pharmacological properties (see Osborne 1996 for review; locust: Benson 1992; cockroach: David and Pitman 1996; Grolleau et al. 1996; Lapied et al. 1990; honey bee: Wüstenberg and Grünewald 2004).

The block induced by bath application of atropine does not give answer to the question whether ACh acts directly on motoneurons or indirectly via premotor interneurons. ACh, applied to motoneuron cell bodies in vitro, evokes an inward current with a sustained component that is atropine sensitive (Fig. 5). Thus a direct action of ACh on motoneurons in situ is possible provided the type of receptor stimulated on the cell bodies is also located at postsynaptic sites on the dendrites. Cholinergic receptors on insect cell bodies and in the neuropil

the presence of 8-bromo-cAMP is not based on a direct upregulation of a motoneuron’s response to ACh.

DISCUSSION

We have previously shown in a semi-intact single-front-leg preparation of the stick insect that middle leg motoneurons receive tonic depolarizing drive when a front leg performs stepping movements on a treadmill (Ludwar et al. 2005). The tonic depolarization is based on a conductance increase and associated with enhanced responsiveness of the cells to depolarizing input. Here we explore the possibility of ACh mediating the tonic depolarization as a transmitter, of OA acting as a modulator and calcium and cAMP acting as second messengers in the pathway that is involved in mediating the tonic depolarization. Our focus is on the motoneurons. We use two experimental approaches, recordings from motoneurons in a semi-intact preparation under isolating bath conditions (in situ) and whole cell patch-clamp recordings from isolated motoneurons in a primary short term culture (in vitro).
may have similar pharmacological properties (e.g., Buckingham et al. 1994; Fickbohm and Trimmer 2003; Harrow and Sattelle 1983). Evidence for cholinergic receptors on insect motoneuron dendrites is provided for Manduca sexta (Trimmer and Weeks 1989) and the locust (Parker and Newland 1995).

The block of cholinergic receptors by atropine in the semi-intact preparation appears to be less effective than in the in vitro situation despite a higher concentration of atropine (500 µM) used in the semi-intact preparation. In vitro 100 µM atropine in the bath almost completely blocked an inward current evoked by application of 100 µM ACh. The likely reason for the partial block (Fig. 3) observed in the semi-intact preparation is that we did not wait for the development of a steady state (Fig. 4). We preferred to test for reversibility before establishment of a steady state because of the rather difficult recording situation (see METHODS for details). Long wash-in times indicate that although the ganglion lamella was removed by desheathing, it is generally difficult for drugs to penetrate the tissue. It might well be that the principle barrier of diffusion, the perineurium, that is a layer of cells under the lamella (Carlson et al. 2000; Schofield and Treherne 1984; Treherne and Pichon 1972) was not removed or damaged. Damage to the perineurium by desheathing appears to remove the blood-brain barrier in cockroach connectives (Lane and Treherne 1970).

Application of ACh onto motoneurons in vitro evoked inward currents that were quite variable in amplitude, mean amplitude for the initial peak was 503 ± 244 pA and the sustained component 277 ± 186 pA (n = 5). Thus the SD for the sustained component was 67% of its mean value. Different sensitivity to ACh of different motoneuron types, slow, semi-fast, and fast motoneurons, might account for response variability in vitro and in situ. Although the majority of motoneurons recorded under both conditions were flexor motoneurons (see METHODS) occasional recordings from tarsal motoneurons might also contribute to response variability. So far, different response amplitudes to ACh application in different motoneuron types in insects have not been shown. However, Parker (1996) has shown differential effects to OA between slow and fast locust flexor motoneurons and between flexor and extensor motoneurons (see following text). In this study, we neither differentiated between motoneuron types (e.g., fast and slow) nor between crural motoneurons that innervate different muscles.

What could be the source of ACh in the semi-intact preparation? So far, neurons that may provide cholinergic input to motoneurons or premotor interneurons in the stick insect have not been looked for. Input could come from mechanosensory neurons in the legs which appear to be cholinergic (Lutz and Tirer 1987, 1988). In locust, chordotonal organs associated with the middle leg that monitor movements of the thoracocoxal joint influence motoneuron activity patterns. These afferents project over all pterothoracic ganglia (Hustert 1978, 1983). If similar organs were associated with the stick insect front leg, intersegmental cholinergic input could be provided by these organs. However, this input would rather be phasic during stepping and thus unlikely to support a steady tonic depolarization between two steps that may be separated by a 2-s pause (Ludwar et al. 2005).

Locally, terminals of middle leg sensory neurons may also release ACh, although most of the middle leg sense organs were removed with leg amputation in our experiments. Such release could be subject to modulation (Torkkeli and Panek 2002). In insects, there is evidence for ACh-induced presynaptic inhibition via muscarinic receptors of sensory terminals in M. sexta (Trimmer and Weeks 1989), the locust (Judge and Leitch 1999), and cockroach (Hue et al. 1989). Consequently, in these preparations, application of atropine or other muscarinic blockers increased excitatory postsynaptic potentials in postsynaptic neurons. This mechanism does not apply to our preparation as atropine application blocks the tonic depolarization evoked by application of ACh (Fig. 11).
tion. Instead cholinergic input is possibly directly or indirectly provided by intersegmental interneurons. In locusts, such cholinergic interneurons are located in thoracic ganglia and the brain, but their number appears to be small (Lutz and Tirer 1987) and nothing is known about their connectivity.

Application of the muscarinic agonist pilocarpine to the stick insect CNS evokes an atropine sensitive rhythmic activity pattern in leg motoneurons (Büschges et al. 1995) that is based on a tonic depolarization and phasic inhibitory input (Büschges 1998). Ludwar et al. (2005) have shown that spike activity in middle leg motoneurons during front leg walking is not only based on phasic input but also on a tonic depolarization. We have shown here that this tonic depolarization is atropine sensitive. We have also collected evidence that motoneurons are equipped with atropine-sensitive cholinergic receptors. Stimulation of these receptors evokes an inward current with a sustained component that may underlie the tonic depolarization. Therefore it seems possible that the tonic depolarization observed during front leg walking relates to the pilocarpine-induced tonic depolarization in stick insects.

Octopaminergic modulation

OA was shown to significantly increase the tonic depolarization in crural motoneurons during stepping at a concentration of 500 μM by 40–267% in three of four experiments. A concentration of 1 mM OA increased the tonic depolarization in three of six experiments. In some experiments 500 μM and 1 mM OA increased motoneuron spike activity. These findings are corroborated by the mianserin-induced decrease in tonic depolarization during stepping. Mianserin acts as an antagonist at OA receptors (Bischof and Enan 2004; Evans 1981; Maqueira et al. 2005; von Nickisch-Rosenegk 1996). These experiments indicate that OA contributes to forming the tonic depolarization during walking.

In contrast, in vitro OA reliably decreased the inward current that was evoked by ACh application to motoneuron cell bodies in a concentration-dependent manner. Therefore we assume that OA, when perfused in situ, evokes two counteracting effects. The first is an inhibition of direct motoneuron depolarization by ACh. The second is an increase in motoneuron depolarization that is not based on modulation of cholinergic action on motoneurons. Such effect could be due to an OA-mediated increase in activity in premotor interneurons that excite the motoneurons. This excitatory effect of OA on motoneurons usually prevails in situ. Counteracting effects may contribute to the lack of OA effect in some experiments. Such counteracting effects may not occur under normal conditions if actions of OA are spatially restricted due to a more local release by neurons.

OA is a well-known neuromodulator in invertebrates (Orchard 1982; Roeder 1999, 2005). In insects, OA is associated with the initiation of walking and flight activity (Claassen and Kammer 1986; Sombati and Hoyle 1984; Stevenson and Kutsch 1988; Vierk et al. 2009) but has been shown recently to be rather a modulator of flight behavior (Brembs et al. 2007; Buhl et al. 2008) than being essential for the initiation of flight. Dorsal unpaired (DUM) and ventral unpaired (VUM) neurons are known to contain and release OA in insects (Roeder 1999). DUM neurons in crickets have been found to be active during walking (Gras et al. 1990) and increased activity in mesothoracic DUM neurons in the stick insect has been shown during single middle leg walking (Mentel et al. 2008). While these DUM neurons are known to innervate leg muscles, it is unclear whether they release OA into the CNS. Injection of OA into the hemolymph of intact stick insects leads to an initial activation followed by a phase of inactivity of the animal (Büschges et al. 1993). The activity phase is compatible with the excitatory OA effects observed here. In contrast to the experiments by Büschges et al. (1993), in our experiments, the effects of OA were confined to neurons in the mesothoracic CNS, and this might account for the missing inactivity phase.

The mechanism by which OA enhances motoneuron activity in the stick insect is not known. The suppression of pathways involved in the resistance reflex of the femur-tibia control loop in stick insects (Büschges et al. 1993; Ramírez et al. 1993) probably does not account for the effect. In locust flight interneurons, OA induced plateau potentials and bursting (Ramírez and Pearson 1991). So far, there is no indication of plateau potentials in stick insect neurons. Such properties of OA will not account for the increase in tonic depolarization.

A mechanism by which OA could be effective is the modulation of synaptic transmission. For example, in our experiments an increase in tonic depolarization could be expected if OA increased the release of ACh from functional sensory terminals (see preceding text). However, the enhancing effects of OA on sensory neurons rather act peripherally than centrally (e.g., Bräunig and Eder 1998; Matheson 1997; Widmer et al. 2005). Modulation of synaptic transmission has been shown in the honey bee mushroom body and between giant interneurons and thoracic interneurons in the escape system of the cockroach where OA promotes cholinergic synaptic transmission (Casagrand and Ritzmann 1992; Oleskevich 1999). In cockroach Df motoneurons depolarizations evoked by application of ACh were reduced in the presence of OA (Butt and Pitman 2002). In locust Leitch et al. (2003) found that OA decreases inhibitory postsynaptic potentials (EPSPs) evoked by stretch receptor activity in a flight motoneuron likely by altering nicotinic receptor function.

Effects of OA can be different among different leg motoneurons in the locust (Parker 1996). EPSPs evoked by the fast extensor motoneuron in a fast flexor motoneuron were normally increased by OA. In contrast, EPSPs in slow flexor motoneurons were reduced by OA. OA reduced the input resistance in fast flexor motoneurons and depolarized them but had no influence on input resistance and membrane potential in the fast extensor motoneuron. In our experiments, although likely mixed samples of motoneuron types were used, the effects of OA in vitro were consistent. But it is possible that in our in situ experiments, the higher variability of responses to application of 1 mM OA as compared with 500 μM was due to differential effects of OA. Such effects have been shown in locusts, where 1 and 10 mM OA induced a concentration dependent increase in variability of modulation of EPSPs in fast flexor motoneurons but not in slow flexor motoneurons (Parker 1996).

Role of second messengers

The tonic depolarization in stick insect motoneurons decreased when intracellular free calcium is buffered by BAPTA. Therefore Ca$^{2+}$ might play a role as a second messenger in stick insect motoneurons. A similar modulation by Ca$^{2+}$ of
responses to stimulation of cholinergic receptors in insect neurons has been shown before. For example, in cockroach DUM neurons, stimulation of a muscarinic ACh receptor leads to a Ca\(^{2+}\) dependent up- or downregulation of currents mediated by nicotinic ACh receptors (nAChR1). Low concentrations of muscarine lead to Ca\(^{2+}\) release from internal stores that upregulate nicotinic ACh receptors. Higher concentrations of muscarine and subsequently intracellular Ca\(^{2+}\) have the opposite effect (Courjaret et al. 2003). A Ca\(^{2+}\)-dependent upregulation of nicotinic ACh receptors during a stepping sequence could contribute to the tonic depolarization and would be consistent with the reduction in amplitude observed in the presence of atropine and BAPTA. In cockroach Df motoneurons, however, stimulation of muscarinic receptors leads to an attenuation of a nicotinic receptor-mediated response triggered by an increase in intracellular calcium concentration, likely by release of Ca\(^{2+}\) from intracellular stores (David and Pitman 1996a). Such downregulation of a cholinergic response by intracellular Ca\(^{2+}\) does not fit our observation. Also in Df motoneurons, stimulation of a cholinergic receptor with a mixed pharmacological profile leads to a delayed inward current that is suppressed by intracellular injection of BAPTA (David and Pitman 1996b). This effect is proposed to be due to a reduction in voltage-dependent calcium current produced by an increase in intracellular calcium concentration, perhaps released from intracellular stores. The reduction in voltage-dependent Ca\(^{2+}\) current in turn leads to a reduction in a strong Ca\(^{2+}\)-dependent K\(^+\) current and that underlies the inward current (David and Pitman 1996b). Whether this effect applies to motoneurons in the stick insect is doubtful because the inward current in Df motoneurons is only observed at membrane potentials more positive than −40 mV and is based on a decrease in a large K\(^+\) conductance, which is considerably larger than \(I_{ca}\) (David and Pitman 1996b). We have shown, however, that the tonic depolarization in stick insect motoneurons reverses around −39 mV and appears to be based on a conductance increase (Ludwar et al. 2005). In motoneurons of the sphinx moth M. sexta, the muscarinic agonist oxo-M evokes an inward current that is atropine sensitive and carried predominantly by Na\(^+\) and is dependent on Ca\(^{2+}\) (Trimmer 1994). Whether such current plays a role in stick insect motoneurons is uncertain because it peaks near −40 mV, close to −45 mV, the normal resting potential in these neurons (Trimmer and Weeks 1989), a situation again different from the stick insect.

We found that bath application of 8-bromo-cAMP increased the tonic depolarization in stick insect motoneurons during a stepping sequence and that bath application of the PKA inhibitor H-89 reduced the amplitude of the tonic depolarization. Both results indicate a role for cAMP in upregulating the tonic depolarization. However, in the in vitro experiments, 8-bromo-cAMP (2 μM) reduced the peak inward current and did not affect the persistent component of the inward current that was evoked by ACh application to motoneuron cell bodies. Therefore an ACh-evoked inward current or depolarization in motoneurons does not appear to involve cAMP as a second messenger. More likely, in situ, cAMP is involved in up-regulating activity in interneurons that will depolarize motoneurons during stepping. Whether this upregulation is at some point associated with ACh is unclear. Such association is possible and has been shown in insects for the AC/PKA and mACHRs and nACHRs (e.g., Thany et al. 2007; Trimmer 1995; Wenzel et al. 2002). In addition, Wenzel et al. (2002) have shown a positive coupling of mACHRs to AC in the grasshopper brain that led to an increased cAMP level.

Like ACh, OA may depend on intracellular Ca\(^{2+}\) or cAMP as second messengers for being effective. Drosophila OA receptors, for example, specifically induce cAMP production or Ca\(^{2+}\) release (Balfanz et al. 2005). Cockroach OA receptors have been described that increased both cAMP levels and intracellular concentrations of calcium when stimulated (Bischof and Enan 2004). In cricket Kenyon cells, an OA induced down-modulation of an L-type Ca\(^{2+}\) channel is mediated by cAMP (Kosakai et al. 2008), and in cockroach DUM neurons, OA increased or decreased spike activity in a concentration-dependent manner by up- or down-modulation of low and high-threshold Ca\(^{2+}\)- and Ca\(^{2+}\)-activated K\(^+\) currents. In addition cAMP appears to be involved in mediating the effects of high concentrations of OA (Achenbach et al. 1997). The activity enhancing effects of OA on mechanosensory neurons in the spider C. salei can be mimicked by 8-Br-cAMP (Widmer et al. 2005). The excitatory effect of OA on neurons of the snail Lymnea is enlarged if cAMP breakdown is blocked (Pitt et al. 2004). Whether the modulatory effects of OA on motoneurons in vitro and the activity enhancing effects of OA during stick insect stepping depend on the intracellular Ca\(^{2+}\) concentration and cAMP remains to be tested.

Concluding remarks

By use of a semi-intact isolating bath preparation, we could show that the tonic depolarization that is induced during walking in motoneurons is mediated by ACh, enhanced by OA and dependent on the Ca\(^{2+}\) concentration in the motoneurons and on cAMP. We have begun to complement the experiments on behaving animals with experiments on motoneurons in vitro and could show that ACh is able to evoke sustained inward currents in motoneurons that might underlie the tonic depolarization. In addition, we collected evidence that the enhancing effects of OA and cAMP are not based on a direct upregulation of motoneuron responses to ACh. Based on these results, in vitro experiments will now allow a detailed pharmacological analysis of cholinergic and octopaminergic action and the role of Ca\(^{2+}\) and cAMP as second messengers in stick insect motoneurons. A further aim is the use of semi-intact preparations to locate the octopaminergic neurons that may increase the tonic depolarization and thereby enhance motoneuron activity.

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