Cholinergic Currents in Leg Motoneurons of Carausius morosus

Eugênio E. Oliveira,1,2 Andreas Pippow,1,2 Vincent L. Salgado,3 Ansgar Büschges,1 Joachim Schmidt,1 and Peter Kloppenburg1,2

1Institute for Zoology, Bioscenter, and 2Center for Molecular Medicine Cologne (CMMC) and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany; and 3BASF Agricultural Products, BASF Corporation, Research Triangle Park, North Carolina

Submitted 2 November 2009; accepted in final form 11 March 2010

Oliveira EE, Pippow A, Salgado VL, Büschges A, Schmidt J, Kloppenburg P. Cholinergic currents in leg motoneurons of Carausius morosus. J Neurophysiol 103: 2770–2782, 2010. First published March 17, 2010; doi:10.1152/jn.00963.2009. We used patch-clamp recordings and fast optical Ca2+ imaging to characterize an acetylcholine-induced current (IACh) in leg motoneurons of the stick insect Carausius morosus. Our long-term goal is to better understand the synaptic and integrative properties of the leg sensory-motor system, which has served extremely successfully as a model to study basic principles of walking and locomotion on the network level. The experiments were performed under biophysically controlled conditions on freshly dissociated leg motoneurons to avoid secondary effects from the network. To allow for unequivocal identification, the leg motoneurons were backfilled with a fluorescent label through the main leg nerve prior to cell dissociation. In 87% of the motoneurons, IACh consisted of a fast-desensitizing (IACh1) and a slow-desensitizing component (IACh2), both of which were concentration dependent, with EC50 values of 3.7 × 10⁻⁵ and 2.0 × 10⁻⁵ M, respectively. Ca2+ imaging revealed that a considerable portion of IACh (~18%) is carried by Ca2+, suggesting that IACh, besides mediating fast synaptic transmission, could also induce Ca2+-dependent processes. Using specific nicotinic and muscarinic acetylcholine receptor ligands, we showed that IACh was exclusively mediated by nicotinic acetylcholine receptors. Distinct concentration–response relations of IACh and IACh2 for these ligands indicated that they are mediated by different types of nicotinic acetylcholine receptors.

INTRODUCTION

Insect motor systems serve as very successful models for the investigation of basic principles of walking and locomotion, e.g., cockroaches, locusts, and stick insects (Burrows 1996; Orlovsky 1999; Ritzmann and Büschges 2007). For about 25 yr, the stick insect leg motor system has been a prominent model for the study of the neuronal basis of walking and locomotion (Bässler 1983; Bässler and Büschges 1998; Büschges 2005; Büschges et al. 1995). The underlying neural networks adapt their output to the environmental and behavioral requirements by integrating centrally generated activity with local afferent, intersegment coordinating, and neuromodulatory inputs. In the end, the motor pattern is shaped by 1) the synaptic properties and connectivity and 2) the intrinsic electrical properties of the component neurons. To better understand the synaptic and integrative properties of the component neurons within the locomotor network, we asked which transmitters and receptors mediate the synaptic input to the leg motoneurons. Across insects, acetylcholine (ACh) and nicotinic acetylcholine receptors (nAChRs) are the primary candidates for mediating fast synaptic input to the leg motoneurons (see following text for references). However, there is also evidence that leg motoneurons might possess muscarinic acetylcholine receptors (mAChRs): 1) besides fast synaptic input, the motoneurons also receive tonic depolarizing input during walking that is partially blocked by the muscarinic antagonist atropine (Ludwar et al. 2005; Westmark et al. 2009); and 2) pilocarpine, a muscarinic agonist, activated a rhythmic motor pattern that is based on tonic depolarization of the motoneurons (Büschges 1998; Büschges et al. 1995).

nAChRs are ligand-gated ion channel receptor complexes that mediate fast cholinergic synaptic transmission. They are among the most thoroughly studied molecules in nervous systems. In vertebrates, the neuronal nAChRs consist of pentameric ligand-gated cation channels, formed by the assembly of multiple α (α2–α10) and β (β2–β4) subunits. Different subunit combinations result in distinct receptor subtypes with distinctive pharmacological profiles (Tomizawa and Casida 2001, 2003). In insects, >10 nAChR genes have been cloned and posttranslational modification of subunit mRNAs has been demonstrated (Grauso et al. 2002; Lansdell and Millar 2002), suggesting the existence of many nAChR subtypes. Although the identification and pharmacological profiling of nAChR subtypes is limited, many studies have reported fast, nAChR-mediated inward currents in various insect neurons (Albert and Lingle 1993; Barbara et al. 2005, 2008; Beadle et al. 1989; Campusano et al. 2007; Cayre et al. 1999; Courjaret and Lapied 2001; Courjaret et al. 2003; David and Pitman 1993; Déglise et al. 2002; Goldberg et al. 1999; Grollée et al. 1996; Gu and O’Dowd 2006; Hermsen et al. 1998; Jepson et al. 2006; Lapied et al. 1990; Nauen et al. 2001; Salgado and Saar 2004; Su and O’Dowd 2003; Suter and Usherwood 1985; van den Beukel et al. 1998; Van Eyseren et al. 1998; Wüstenberg and Grünewald 2004). Although nAChRs are associated with fast synaptic transmission, it has been shown in vertebrates and insects that a fraction of the current through nAChRs can be carried by Ca2+ (Barbara et al. 2008; Burnashev 1996; Dani 2001; Fucile 2004; Goldberg et al. 1999; Oertner et al. 1999; Rathouz et al. 1996; Vermuelen et al. 1994). This is of interest because synaptic activity could generate intracellular signals that act as a second messenger (Berridge et al. 2000; Bootman et al. 2002) and might, for example, activate Ca2+-dependent ion channels that induce sustained changes in membrane potential and/or excitability.

In addition to nAChRs, acetylcholine can also stimulate mAChRs. In vertebrates, mAChRs have been shown to activate various second messenger pathways (Caulfield 1993; Caulfield
and Birdsall 1998; Felder 1995; Gregory et al. 2007; Lanzafame et al. 2003), which can modulate ion channels or receptors in the membrane to regulate membrane properties (Hasselmo 2006; Jones 1993; Klink and Alonso 1997; Lanzafame et al. 2003). Whereas many binding (Abdallah et al. 1991; Honda et al. 2007; Liu and Casida 1993; Onai et al. 1989; Orr et al. 1991; Qazi et al. 1996; Shapiro et al. 1989), immunohistochemical (Blake et al. 1993; Bu et al. 2008; Harrison et al. 1995; Shirai et al. 2001), and systemic electrophysiological (Büschges 1998; Corronc and Hue 1993; Johnston and Levine 2002; Trimmer 1995; Trimmer and Weeks 1989; Westmark et al. 2009) studies on insect neurons suggested the expression of mAChRs, only very few studies have reported direct mAChR-mediated effects on membrane conductance in isolated neurons (Benson 1992; Lapied et al. 1992; Van Eyseren et al. 1998).

In this study, we examine the cholinergic receptors of leg motoneurons of the stick insect Carausius morosus. The experiments were performed under biophysically controlled conditions on freshly dissociated, unequivocally identified leg motoneuron somata, to avoid secondary effects caused by interaction within the nervous system. The study addresses two important issues: do leg motoneurons possess nicotinic and/or muscarinic ACh receptors, and are these ACh receptors permeable to Ca\(^{2+}\)?

**METHODS**

**Animals and materials**

Carausius morosus were obtained from breeding colonies at the University of Cologne. Animals were reared in crowded colonies at a temperature of 20 ± 2°C, under an artificial 12-h:12-h light/dark photoperiod regimen and a relative humidity of 70 ± 5%, on an unrestricted diet of blackberry leaves and water. The experiments were performed with adult females. All chemicals, unless stated otherwise, were obtained from Applichem (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany) in “pro analysis” purity grade.

**Cell identification**

Under cold-induced anesthesia, the animal’s legs were cut distad of the coxa and the animal was mounted ventral side up in a plastic foam platform. The thorax was opened in the regions of the thoracic ganglia with scalpels and forceps. Fat and connective tissue were manually removed from the nerve’s cut end was placed in a Vaseline trough (PZN2761298; Medical Pharma, Bremerhaven, Germany) filled with tetramethylrhodamine-dextran (0.5–1.0% in H\(_2\)O, MW 3,000, D3308; Invitrogen). To prevent evaporation, the coxa and the animal was mounted ventral side up in a plastic foam (Neofluar (LSM 510, Carl Zeiss, Göttingen, Germany), equipped with Plan-Neofluar ×10 (0.3 numerical aperture [NA]) and Plan-Apochromat ×20 (0.75 NA) objectives. Tetramethylrhodamine-dextran was excited with He–Ne laser at 543 nm and emission was collected through a 560 nm LP filter. Scaling, contrast, and z-projections were performed using ImageJ v1.35d with the WCIF plug-in bundle (www.uhrresearch.ca/facilities/wcif/). The final figure was prepared with Photoshop and Illustrator CS2 (Adobe Systems, San Jose, CA).

**Cell culture**

After backfilling, the thoracic ganglia were dissociated at room temperature (RT) and neurons were cultured according to modified protocols from previous work (Alix et al. 2002; Husch et al. 2008; Salgado and Saar 2004; Westmark et al. 2009; Zhao et al. 2003). Three thoracic ganglia with labeled motoneurons were removed and immersed in cold, sterile “normal” extracellular saline (modified from Husch et al. 2008; Schmidt et al. 2001), containing (in mM): 180 NaCl; 4 KCl; 5 CaCl\(_2\); 1 MgCl\(_2\); 10 HEPES; and 48 sucrose, adjusted to pH 7.2 (with NaOH) and to 430 mOsm (with sucrose). First, the ganglionic sheath was manually removed. Before manual removal of the underlying perineurium, the ganglia were treated (10 min, RT) with a mixture of collagenase (250 units·ml\(^{-1}\), C0130; Sigma-Aldrich) and trypsin (8,550 units·ml\(^{-1}\), T8003; Sigma-Aldrich) in Hanks’ Ca\(^{2+}\)- and Mg\(^{2+}\)-free buffered salt solution (GIBCO 14170-070), containing (in mM): 10 HEPES and 135 sucrose, adjusted to pH 7.2 (with NaOH). The ganglia were transferred to normal extracellular solution and the sheath was manually removed. To facilitate dissociation of the cell bodies, the ganglia were enzyme-treated for a second time (20 min, RT). After rinsing at least five times with normal extracellular saline (RT), the ganglia were dissociated by gentle trituration through a series of pipettes of decreasing tip diameter. The neurons of one ganglion were plated in one to two culture dishes. Dishes with glass bottoms were custom-made according to modified protocols from previous reports (Hayashi and Hildebrand 1990; Hayashi and Levine 1992). A 20 mm diameter hole was drilled in the bottom of a 35 mm culture dish (627160; Greiner Bio-One, Fritzenhausen, Germany). A glass coverslip (BBO22022A1; Thermo Fisher Scientific, Portsmouth, NH) was sealed with Sylgard (Dow Corning, Midland, MI) to the bottom of the culture dish. The dishes were sterilized with UV radiation for 2 h and the glass bottom was coated with concanavalin A (0.7 mg·ml\(^{-1}\) dissolved in H\(_2\)O, C-2010; Sigma-Aldrich). Neurons were allowed to settle and adhere for 3–1 h and were used for electrophysiological experiments within 8 h. For recording, cells were visualized with an inverted microscope (IX71; Olympus) using a ×40 water objective (UAPO ×40, 1.15 NA, 0.25 mm working distance [WD]; Olympus) with phase contrast and fluorescence optics. Motoneurons could be unequivocally identified by their fluorescence and diameter.

**Whole cell recordings**

Whole cell recordings were performed at 24°C, following the methods described by Hamill et al. (1981). Electrodes with tip resistances between 3 and 5 MΩ were fashioned from borosilicate glass (GB150-8P, 0.86 mm inner diameter [ID], 1.5 mm outer diameter [OD]; Science Products, Hofheim, Germany), with a temperature-controlled pipette puller (PIPS; HEKA Elektronik, Lambrecht, Germany), and filled with a solution containing (in mM): 190 K-aspartate; 10 NaCl; 1 CaCl\(_2\); 2 MgCl\(_2\); 10 HEPES; 10 EGTA; 3 ATP; and 3 GTP, adjusted to pH 7.2 (with KOH), resulting in an osmolarity of about 415 mOsm. During the experiments, if not stated otherwise, the cells were superfused constantly with normal extracellular saline solution.

Whole cell voltage-clamp recordings were made with an EPC9 patch-clamp amplifier (HEKA Elektronik) that was controlled by the program Pulse (version 8.63, HEKA Elektronik) running under Windows. Electrophysiological data were low-pass filtered at 2 kHz with a four-pole Bessel filter and sampled at intervals of 100 μs (10 kHz).
Compensation of offset potential and capacitance were performed using the “automatic mode” of the EPC9 amplifier. The liquid junction potential between intracellular and extracellular solutions of 15.8 mV (calculated with Patcher’s Power Tools plug-in from http://www.mpibpc.gwdg.de/abteilung/140/software/index.html for IGOR Pro 6, WaveMetrics, Portland, OR) was also compensated. Voltage errors due to series resistance ($R_S$) were minimized using the $R_C$-compensation of the EPC9. $R_S$ was compensated between 30 and 70%, with a time constant (τ) of 2 μs. If not stated otherwise, the cells were held at −60 mV, which is near their normal resting potential (Schmidt et al. 2001).

**Ligand application**

The neurons were continuously superfused with normal extracellular saline (0.5 ml-min⁻¹) through a Teflon tube (483 μm ID) placed 600 μm from the cell. Ligands were applied with a U-tube system as described previously (Westmark et al. 2009; Wu et al. 2004; Zhao et al. 2003). The U-tube consisted of a Teflon tube (254 μm ID, 508 μm OD). Two synchronized computer-controlled pinch valves activated the U-tube and simultaneously stopped superfusion with normal extracellular solution. The U-tube hole (50 μm diameter) was positioned near the cell (~500 μm), with its flow direction approximately perpendicular to the flow direction of the bath. If not stated otherwise, the ligands were applied for 1 s.

**Ligands**

Except α-bungarotoxin (α-BGTX, 630-075-M001; Alexis Biochemicals, Lausen, Switzerland), all ligands were purchased from Sigma-Aldrich (Taufkirchen, Germany): acetylcholine (ACh, A2661), (-)-nicotine (MUSC, M1014), oxotremorine (OXO, O100), and pilocarpine (PILO, P6503). IMI was dissolved in dimethyl sulfoxide (DMSO, D8418), with a maximum final DMSO concentration of 0.1% (vol/vol). All other ligands were dissolved in normal extracellular saline.

**Concentration–response relation**

To determine concentration–response relations, each concentration was applied at least three times. Ligand applications arrived ≥2 min apart. If appropriate, concentration–response relations for each cell were fit with the Hill equation

$$ I / I_{\text{max}} = \frac{[\text{ligand}]^{nH}}{k^n + [\text{ligand}]^{nH}} \quad (1a) $$

for agonists or

$$ I / I_{\text{max}} = 1 - \frac{[\text{ligand}]^{nH}}{k^n + [\text{ligand}]^{nH}} \quad (1b) $$

for antagonists, where $I_{\text{max}}$ is the maximum relative current amplitude, $k$ is the EC₅₀ (concentration that activates half of the maximal current) or IC₅₀ (concentration that blocks the current by 50%), and $n_H$ is the Hill coefficient. Data were scaled as a fraction of the calculated maximal current and refit.

**Fluorimetric Ca²⁺ measurements**

Intracellular Ca²⁺ concentrations were measured with fura-2, a ratiometric Ca²⁺ indicator suitable for determining absolute intracellular Ca²⁺ concentration once calibrated (Gryniewicz et al. 1985; Poenie 1990). The imaging setup for fluorometric measurements consisted of an Imago/SensiCam charge-coupled device camera with a 640 × 480 chip (Till Photonics, Gräfeling, Germany) and a Polychromator IV (Till Photonics) that was coupled via a light guide into an inverted microscope (IX71; Olympus), equipped with a ×40 water objective (objective: UAPON ×40, 1.15 NA, 0.25 mm WD; Olympus). The camera and the polychromator were controlled by the software Vision (version 4.0, Till Photonics) running under Windows. The neurons were loaded with fura-2 via the patch pipette (0.2 mM in the pipette) and illuminated during data collection with 340 and 380 nm light from the polychromator that was reflected onto the cells with a 410 nm dichroic mirror (DCLP 410; Chroma, Rockingham, VT). Emitted fluorescence was detected through a 440 nm long-pass filter (LP440). Data were acquired as 80 × 60 pixel frames using 4 × 4 on-chip binning for fast kinetic measurements. Images were recorded in AVD units (ADUs) and stored and analyzed as 12 bit grayscale images. For all calculations of kinetics, the mean values of ADU within regions of interest (ROIs) from the center of the cell bodies were used. ROIs were adjusted to each cell.

**CALIBRATION**

The free intracellular Ca²⁺ concentrations were determined as in Gryniewicz et al. (1985)

$$ [\text{Ca}^{2+}]_i = \frac{F_{380,\text{min}} - R}{F_{380,\text{max}} - R} \quad (2) $$

[Ca²⁺]ᵢ is the free intracellular Ca²⁺ concentration for the background-subtracted fluorescence ratio $R$ from 340 and 380 nm excitation. $R_{\text{min}}$ and $R_{\text{max}}$ are the ratios at a Ca²⁺ concentration at virtually 0 M (i.e., ideally no fura-2 molecules are bound to Ca²⁺) and at saturating Ca²⁺ concentrations (i.e., ideally all fura-2 molecules are saturated with Ca²⁺), respectively. $K_{d,Fura}$ is the dissociation constant of fura-2. $F_{380,\text{min}}/F_{380,\text{max}}$ is the ratio between the emitted fluorescence of Ca²⁺ free dye and the emitted fluorescence of Ca²⁺ saturated dye at 380 nm excitation, reflecting the dynamic range of the indicator.

The term $K_{d,Fura}$ is dependent on the dye concentration and is substituted with the effective dissociation constant $K_{d,Fura,eff}$, which is independent of the dye concentration and specific for each experimental setup (Neher 1989)

$$ K_{d,Fura,eff} = \frac{[\text{Ca}^{2+}]_i}{(R_{\text{max}} - R)/(R_{\text{min}} - R)} \quad (3) $$

We used in vitro calibration (in solution). For calibration, $K_{d,Fura,eff}$ was determined by measuring fura-2 fluorescence ratios for $R_{\text{max}}$, $R_{\text{min}}$, and $R = R_{\text{def}}$. $R_{\text{def}}$ is the ratio for a defined [Ca²⁺]ᵢ, which was set to 0.22 μM (see following text for the preparation of the solutions). $K_{d,Fura,eff}$ was then calculated from Eq. 3. To account for environmental differences between the cytoplasmic milieu and in vitro conditions, we used a correction factor ($P$) for $R_{\text{max}}$ and $R_{\text{min}}$ as suggested by Poenie (1990; see also Pippow et al. 2009)

$$ K_{d,Fura,eff} = \left[\frac{[\text{Ca}^{2+}]_i}{(R_{\text{max}}P - R)/(R_{\text{min}}P - R)}\right] \quad (4) $$

$P$ was determined as described by Poenie (1990). First, the fluorescence (peak) at 340 nm excitation was divided by that at 380 nm excitation from voltage-induced intracellular calcium transients ($R_{\text{dcalc}}$). Second, the ratio ($R_{\text{dvitro}}$) from pairs of calibration solutions ($R_{\text{dcalc},\text{min}}$ and $R_{\text{dcalc},\text{max}}$) was determined by dividing ($F_{380,\text{max}} - F_{380,\text{min}}$)/$F_{380,\text{max}}$. The correction factor $P$ is the fraction of $R_{\text{dcalc}}/R_{\text{dvitro}}$.

The fluorescence ratio $R$ of an intracellular transient can then be converted to [Ca²⁺]ᵢ, using

$$ [\text{Ca}^{2+}]_i = K_{d,Fura,eff} \frac{(R_{\text{min}}P - R)}{(R_{\text{max}}P - R)} \quad (5) $$

**CALIBRATION SOLUTIONS**

The free Ca²⁺ concentrations of the calibration solutions were adjusted by using appropriate proportions of Ca²⁺ and the Ca²⁺ chelator EGTA. The ability of EGTA to bind calcium is highly dependent on the environmental conditions such as ionic strength, temperature, pH, and the concentrations of other metals.
that compete for binding (Harrison and Bers 1987, 1989). In theory, the necessary amount of Ca$^{2+}$ and EGTA to set the free Ca$^{2+}$ concentration for the experimental conditions can be computed (Patton et al. 2004). However, small variations in the parameters such as pH, temperature, impurities of chemicals, pipetting, or weighing errors can lead to considerable errors in the estimate of the free Ca$^{2+}$ in EGTA-buffered Ca$^{2+}$ solutions (McGuigan et al. 2007). To account for such variations, we determined the free Ca$^{2+}$ concentrations in our calibration solutions by using a Ca$^{2+}$ selective electrode, following the guide from McGuigan et al. (1991) as described in the Supplemental Methods.\footnote{The online version of this article contains supplemental data.}

ANALYSIS OF RELATIVE CA$^{2+}$ INFLUX. After establishing the whole cell configuration, neurons were voltage-clamped at their resting potentials (approx. −60 mV) and dye was loaded into cells for ≈10 min. Imaging was not started until the fluorescence reached a constant level (measured at 360 nm excitation). After loading, the cells were first superfused with normal extracellular saline and stimulated with 10$^{-5}$ M ACh (1 s). Then the cells were superfused with a solution containing specific ion channel blockers (10$^{-5}$ M tetrodotoxin (TTX), 4 × 10$^{-5}$ M 4-aminopyridine (4-AP), and 20 × 10$^{-3}$ M tetraethylammonium (TEA)) to isolate $I_{Ca}$, as described in Husch et al. (2008) and Pippow et al. (2009). $I_{Ca}$ was induced by stepping the membrane potential for 1 s to between −30 and −20 mV. To remove uncompensated leakage and capacitive currents, a p/6 protocol was used (Armstrong and Bezanilla 1974). The voltage steps were adjusted so that the resulting $I_{Ca}$ was in the same range as $I_{ACh}$. To monitor the induced increases of intracellular Ca$^{2+}$ concentrations ratiometrically, pairs of images at 340 nm (4 ms exposure time) and 380 nm (2 ms exposure time) excitation were acquired at 10 Hz for 20 s. Before the whole cell configuration was established (break-in), an image was obtained in on-cell mode for each excitation wavelength. This “background fluorescence” was subtracted from each image of the time series.

ESTIMATING CA$^{2+}$ FLUXES. The net charge influx through ACh-activated channels was given by $\int f_{ACh} \mathrm{d}t = Q_{ACh}$, and the charge influx through voltage-activated Ca$^{2+}$ channels during a voltage pulse was given by $\int f_{Ca} \mathrm{d}t = Q_{Ca}$, respectively. The quantities $Q_{ACh}$ and $Q_{Ca}$ were determined by integrating the current traces. From the amplitudes of the Ca$^{2+}$ transients ($\Delta[Ca^{2+}]_i$) and the net charge influxes ($\dot{Q}$), the fraction $f$, which has been defined by Neher (1995), was determined

$$f_{ACh} = \frac{\Delta[Ca^{2+}]_{ACh}}{Q_{ACh}}$$

and

$$f_{Ca} = \frac{\Delta[Ca^{2+}]_{Ca}}{Q_{Ca}}$$

When the Ca$^{2+}$ binding ratio of fura-2 (exogenous buffer) outcompetes the endogenous buffers, the fraction of Ca$^{2+}$ contributing to the ACh-induced ion flux can be estimated with

$$P_f = \frac{f_{ACh}}{f_{Ca}}$$

The fractions $f_{Ca}$ and $f_{ACh}$ are the slopes from linear fits ($Y = \beta_0 + \beta_x \cdot X$) using the “R function” lm (R Development Core Team 2008) of their $\Delta[Ca^{2+}]_i$ vs $\dot{Q}$ relations (Eqs. 6 and 7). To estimate the variance of the slope ($f_{ACh}$), we used the bootstrap method (Efron 1979) implemented in the boot library in R (fixed-x resampling, 1,000 bootstrap samples, boot: Bootstrap R Functions, R package version 1.2–27). This resulted in bootstrap distributions (1,000) for $f_{Ca}$ and $f_{ACh}$. The ratio $f_{ACh}/f_{Ca}$ ($P_f$) provided an estimate of the proportion of Ca$^{2+}$ contributing to an ACh induced current (Vernino et al. 1994; Zhou and Neher 1993).

To ensure that the exogenous Ca$^{2+}$ buffer outcompetes the endogenous Ca$^{2+}$ buffer, high concentrations of Ca$^{2+}$ indicator are normally used (Fucile et al. 2000, 2006; Ohyama et al. 2000; Tempia et al. 1996; Vernino et al. 1994; Zhou and Neher 1993). However, high indicator (exogenous Ca$^{2+}$ buffer) concentrations reduce the amplitude of the free Ca$^{2+}$ concentration, during transient Ca$^{2+}$ influx. For small Ca$^{2+}$ influxes, the signal can be reduced to the range of the signal noise. In this study we used 0.2 mM fura-2 and $I_{ACh}$ (at 10$^{-4}$ M ACh) induced a clearly detectable free Ca$^{2+}$ signal. The linear relationship between the amplitudes of the Ca$^{2+}$ transients ($\Delta[Ca^{2+}]_i$) and the charge influxes ($\dot{Q}$) confirmed that fura-2 was the main buffer (Vernino et al. 1994).

Data analysis

Electrophysiological and imaging data were analyzed with the software Pulse (version 8.63; HEKA), IGOR Pro 6 (WaveMetrics; including the Patcher’s Power Tools plug-in, http://www.mpibpc.gwdg.de/abteilungen/140/software/index.html) and Sigma Stat (Systat Software, San Diego, CA) for analysis of electrophysiological data. All calculated values are expressed as means ± SD. All calculations for the determination of EGTA purity, its dissociation constant, and the free Ca$^{2+}$ concentrations in the calibration solutions were performed in R (R Development Core Team 2008, http://www.R-project.org). To determine differences in means, t-tests were performed. A significance level of 0.05 was accepted for all tests.

RESULTS

Using whole cell voltage-clamp recordings, we investigated the ionic currents induced by exogenously applied ACh ($I_{ACh}$) and related ligands, on isolated, identified leg motoneurons of stick insect motoneurons.

**Table 1. Properties of cholinergic currents in stick insect motoneurons**

<table>
<thead>
<tr>
<th>Cholinergic Agonists</th>
<th>EC$_{50}$, µM</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine ($n = 14$)</td>
<td>37.00 (30–51)</td>
<td>1.20 ± 0.09</td>
</tr>
<tr>
<td>ACh$_{C6}$</td>
<td>20.50 (18–31)</td>
<td>1.10 ± 0.08</td>
</tr>
<tr>
<td>Nicotine ($n = 5$)</td>
<td>35.00 (31–67)</td>
<td>1.50 ± 0.10</td>
</tr>
<tr>
<td>ACh$_{C4}$</td>
<td>~10.0</td>
<td>—</td>
</tr>
<tr>
<td>Imidacloprid ($n = 4$)</td>
<td>49.70 (44–63)</td>
<td>1.40 ± 0.10</td>
</tr>
<tr>
<td>ACh$_{C6}$</td>
<td>~10.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Blocks of $I_{ACh}$ IC$_{50}$, nM | $n_H$ |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Bungarotoxin ($n = 6$)</td>
<td>Hill model</td>
</tr>
<tr>
<td>ACh$_{C6}$</td>
<td>Linear fit</td>
</tr>
<tr>
<td>ACh$_{C4}$</td>
<td>Hill model</td>
</tr>
<tr>
<td>ACh$_{C6}$</td>
<td>Linear fit</td>
</tr>
<tr>
<td>Atoptine ($n = 5$)</td>
<td>ACh$_{C6}$</td>
</tr>
<tr>
<td>ACh$_{C4}$</td>
<td>Linear fit</td>
</tr>
</tbody>
</table>

Values are means ± SD. Asterisks indicate inverted-U shaped concentration–response relations. EC$_{50}$ is the concentration that activates the half-maximal current. IC$_{50}$ is the concentration that blocks half of the maximal current. 95% confidence intervals of EC$_{50}$ and IC$_{50}$ values are presented in parenthesis. $n_H$ is the Hill coefficient.
the adult stick insect (*C. morosus*). The pharmacological properties of *I*\(_{ACh}\) are summarized in Table 1. An important prerequisite for this project was an unequivocal identification of the motoneurons, which was achieved by retrograde tracing with tetramethylrhodamine-dextran via the main leg nerve (*nervus cruris*). In the thoracic ganglion, retrograde tracing via the *nervus cruris* for 48 h labeled 25 flexor tibia motoneurons (Goldammer 2008; Storrer et al. 1986), eight motoneurons of the tarsal muscle and four motoneurons of the retractor unguis muscle (Godden 1972) (Fig. 1, A and B). All of these somata were located in the anterior-lateral region (most of them are ventrally located) of the thoracic ganglion and had their dendritic fields in the dorsal-intermediary part of the thoracic ganglion. The soma size of these motoneurons was 40 \(\mu m\). In addition, the staining revealed up to two common inhibitor (CI) motoneurons and up to six dorsal unpaired median (DUM) neurons (Goldammer 2008; Mentel et al. 2008) (Fig. 1, A and B). Both neuron types had soma diameters of 50 \(\mu m\) (Debrodt and Bässler 1989; Goldammer 2008; Storrer et al. 1986).

Our study was based on 208 whole cell patch-clamp recordings from acutely dissociated neurons of the thoracic ganglion that were identified as leg motoneurons in culture by their fluorescence and soma size (Fig. 1C). To characterize *I*\(_{ACh}\) here, the neurons were superfused with normal extracellular saline and clamped at −60 mV. The resting membrane potential measured immediately after establishing the whole cell configuration was −63 ± 2.5 mV (n = 208) and ranged from −55 to −70 mV. ACh was applied for 1 s via a U-tube system. Over 99% of the investigated neurons generated an inward current on application of a 1 s ACh puff (10\(^{-7}\)–10\(^{-3}\) M, Fig. 2A).

### Acetylcholine-induced current (*I*\(_{ACh}\))

ACh induced a fast-desensitizing and a slow-desensitizing component (*I*\(_{ACh1}\) and *I*\(_{ACh2}\), respectively; Fig. 2, A and B). In 88% (173/197) of ACh-responsive neurons, both components were apparent (type 1; Fig. 2A). A comparison of the current profiles, however, revealed that the ratio between the components was highly variable. In 10% (20/197) of the neurons *I*\(_{ACh}\) consisted of *I*\(_{ACh2}\) only (type 2; Fig. 2A). Neurons showing only *I*\(_{ACh1}\) were observed in 2% (4/197) of the recorded neurons (type 3; Fig. 2A). Since type 2 and type 3 responses were rare, only type 1 responses were analyzed in detail during this study. The absolute amplitude and the ratio between *I*\(_{ACh1}\) and *I*\(_{ACh2}\) were variable between different motoneurons. *I*\(_{ACh}\) was reproducible during repeated ACh applications in a given neuron for >2 h (Fig. 2C).

Both *I*\(_{ACh1}\) and *I*\(_{ACh2}\) were clearly concentration dependent. The concentration–current relation of *I*\(_{ACh}\) was determined with increasing concentration steps between 10\(^{-7}\) and 10\(^{-3}\) M (Fig. 2D). The concentration–current relations of *I*\(_{ACh1}\) (peak current) and *I*\(_{ACh2}\) (current amplitude averaged between 950 and 1,000 ms of ACh application) were fit with the Hill relation (Eq. 1a; Fig. 2D). Both components started to activate at ACh concentrations >3 × 10\(^{-6}\) M and their concentration–current curves had similar *EC*\(_{50}\) values and Hill coefficients (*I*\(_{ACh1}\): *EC*\(_{50}\) = 3.7 × 10\(^{-5}\) ± 1.9 × 10\(^{-6}\) M, *n*\(_{H}\) = 1.2 ± 0.1, *n* = 14; *I*\(_{ACh2}\): *EC*\(_{50}\) = 2.1 × 10\(^{-5}\) ± 1.5 × 10\(^{-6}\) M, *n*\(_{H}\) = 1.1 ± 0.1, *P* = 0.09, *n* = 14, paired *t*-test). The maximum amplitude of *I*\(_{ACh1}\) determined from Hill fits was 6.2 ± 2.8 × 10\(^{-4}\) nA (*n* = 14). Given a mean whole cell capacitance (*C*\(_{M}\)) of 89 ± 50 pF (*n* = 14), this corresponded to a current density (*I*\(_{ACh1}\)/*C*\(_{M}\) = *I*\(_{ACh1}\)/A*\(_{M}\)) of 8.6 ± 5.7 pA·pF\(^{-1}\) (8.6 × 10\(^{-2}\) ± 5.7 × 10\(^{-2}\) pA·\(\mu m^{-2}\)) for *I*\(_{ACh2}\). The Hill fits yielded a maximal amplitude of 3.5 × 10\(^{-1}\) ± 2.9 × 10\(^{-1}\) nA (*n* = 14), which corresponded to a current density (*I*\(_{ACh2}\)/*C*\(_{M}\) = *I*\(_{ACh2}\)/A*\(_{M}\)) of 4.5 ± 3.9 pA·pF\(^{-1}\) (4.5 × 10\(^{-2}\) ± 3.9 × 10\(^{-2}\) pA·\(\mu m^{-2}\)).

### Fluorimetric Ca\(^{2+}\) measurements

The fura-2-loaded (0.2 mM) neurons were voltage-clamped at their resting potential (about −60 mV) in normal extracel-
CHOLINERGIC CURRENTS IN MOTONEURONS

Fig. 2. Acetylcholine-induced current (I_{ACh}). The holding potential was −60 mV. ACh was applied for 1 or 5 s (10^{-4} M). A and B: I_{ACh1} and I_{ACh2} desensitized differentially under prolonged ACh application. Note the differences in ACh application time between A and B. ACh elicited a fast-desensitizing (I_{ACh1}) and/or slow-desensitizing current component (I_{ACh2}). We observed 3 principal response types: type 1: I_{ACh1} only. Type 2: I_{ACh1} and I_{ACh2}. Type 3: I_{ACh2} only. Type 1 was the most frequent response (173/197 cells). For data analysis, the peak of the transient component (I_{ACh1}) and the mean amplitude between 950 and 1,000 ms (I_{ACh2}) was determined. C: during repeated ACh application I_{ACh} was stable for >2 h. ACh applications arrived 1–2 min apart. The inset shows 3 traces of I_{ACh} that were recorded at the indicated times. Open circles represent I_{ACh1}, and filled rectangles represent I_{ACh2}. D: concentration-response curves for I_{ACh1} and I_{ACh2}. Data were calculated as fractions of the calculated maximal current. The curves are fits to a Hill relation (Eq. 1a) with the following parameters: I_{ACh1}: EC_{50} = 3.7 × 10^{-5} ± 1.9 × 10^{-6} M, n_H = 1.2 ± 0.1. I_{ACh2}: EC_{50} = 2.1 × 10^{-5} ± 1.5 × 10^{-8} M, n_H = 1.1 ± 0.1. Molecular saline (Fig. 3). First, I_{ACh} was induced by 1 s ACh puffs (10^{-4} M). I_{ACh} had a net charge influx of Q_{ACh} = 127 ± 95 pC (n = 8), whereas the free intracellular Ca^{2+} concentration increased by Δ[Ca^{2+}]_i = 2.8 ± 1.3 nM. Second, the voltage-activated Ca^{2+} current (I_{Ca}) was isolated pharmacologically (10^{-7} M TTX, 4 × 10^{-3} M 4-AP, and 20 × 10^{-3} M TEA) and the amplitude of a 1 s depolarizing voltage pulse was adjusted to set the magnitude of I_{Ca} to the range of I_{ACh}. The adjusted I_{Ca} induced a charge influx of Q_{Ca} = 177 ± 144 pC (n = 6) and increased the free Ca^{2+} concentration by Δ[Ca^{2+}]_i = 15 ± 11 nM.

The fraction f_{ACh} = 13.3 ± 3.3 M C^{-1} (n = 8, 1,000 bootstrap samples, Eq. 6), which was determined from the slope of the linear fit in Fig. 3B, indicated how much the intracellular Ca^{2+} concentration increases per coulomb (C) of I_{ACh}, whereas the fraction f_{Ca} = 72 ± 3 M C^{-1} (n = 6, 1,000 bootstrap samples, Eq. 7) indicated how much the intracellular Ca^{2+} concentration increases per coulomb of I_{Ca} (Fig. 3C). The ratio f_{ACh}/f_{Ca} = 18.4 ± 4.7% (Eq. 8) is an estimate of the proportion of I_{ACh} that is carried by Ca^{2+} (Fig. 3C). Our results show that a considerable portion of I_{ACh} is carried by Ca^{2+}.

Nicotinic ligands

Nicotine, like ACh, induced an inward current consisting of transient (I_{NIC1}) and sustained (I_{NIC2}) components (Fig. 4A, Table 1). A quantitative comparison with I_{ACh}, however, revealed significant differences in some physiologically important parameters. At the same concentration (10^{-4} M) the amplitude of I_{NIC1} was similar to that of I_{ACh1} (P = 0.14, n = 13, paired t-test, Fig. 4B), although I_{NIC2} was 50% smaller compared with that of I_{ACh2} (P = 0.017, n = 13, paired t-test, Fig. 4B). Although the nicotine concentration–response curve for I_{NIC1} showed a similar EC_{50} (EC_{50} = 3.5 × 10^{-5} ± 1.6 × 10^{-6} M) and Hill coefficient (n_H = 1.5 ± 0.1) compared with those of I_{ACh1}, the concentration–response curve for I_{NIC2} was inverted-U shaped with a similar response threshold (3 × 10^{-6} M), EC_{50} (~10^{-5} M), and a maximum at 10^{-4} M (Fig. 4C). These results suggested the presence of at least two nAChRs in stick insect leg motoneurons. Lending more evidence to our hypothesis, application of imidacloprid (IMI), a neonicotinoid insecticide known to target insect nAChRs (Barbara et al. 2008; Brown et al. 2006; Buckingham et al. 1997; Courjaret and Lupied 2001; Déglise et al. 2002; Ihara et al. 2006; Jepson et al. 2006; Nauen et al. 2001; Salgado and Saar 2004), elicited inward currents (data not shown) and showed a concentration–response relation very similar to that of I_{ACh} (Fig. 4D; Table 1). I_{MNi} showed a similar EC_{50} (EC_{50} = 4.9 × 10^{-5} ± 3.2 × 10^{-6} M) and Hill coefficient (n_H = 1.4 ± 0.1) compared with those of I_{ACh1}. The concentration–response curve for I_{MNi} was also inverted-U shaped with a response threshold at about 3 × 10^{-6} M, EC_{50} at about 10^{-5} M, and a maximum at 10^{-4} M (Fig. 4D).

α-Bungarotoxin (α-BGTX) is a specific nicotinic antagonist in many insect systems (Albert and Lingle 1993; Campusano et al. 2007; Cayre et al. 1999; Salgado and Saar 2004). In agreement with our finding that nicotine induced an I_{ACh}-like current in the leg motoneurons, α-BGTX blocked both I_{ACh1} and I_{ACh2} with similar concentration dependences (Fig. 5, Table 1). The block started at concentrations around 10^{-11} M.
and $I_{ACh}$ was completely blocked at concentrations of $\geq 10^{-6}$ M. As shown in Fig. 5 the choice for the model to fit the $\alpha$-BGTX was not obvious. Because we waited for each $\alpha$-BGTX concentration until the steady-state level of the block was reached, this indicated the expression of more than one receptor subtype. We used a Hill model and a linear relation to fit the data. Estimated from the Hill model the IC$_{50}$ for $\alpha$-BGTX was $3.3 \times 10^{-9} \pm 1.1 \times 10^{-9}$ M ($n_H = 0.40 \pm 0.05$) for $I_{ACh1}$ and $2.5 \times 10^{-10} \pm 0.1 \times 10^{-10}$ M ($n_H = 0.44 \pm 0.06$) for $I_{ACh2}$. With the linear fit the IC$_{50}$ was $2.3 \times 10^{-9} \pm 1.0 \times 10^{-9}$ M for $I_{ACh1}$ and $3.1 \times 10^{-10} \pm 0.8 \times 10^{-10}$ M for $I_{ACh2}$. The finding that $\alpha$-BGTX was more potent against $I_{ACh2}$ (Fig. 5) suggests the existence of different subtypes of nAChR. The $\alpha$-BGTX block is relatively difficult to reverse (<50% reversibility after 45 min wash).

Muscarinic ligands

To test whether muscarinic receptors contributed to $I_{ACh}$ we applied muscarine and two “classical” muscarinic agonists (oxotremorine and pilocarpine) that have been effective in insects (Buhl et al. 2008; Corronc and Hue 1993; Okada et al. 2009; Parker and Newland 1995; Rykebusch and Laurent 1993; Trimmer 1995; Trimmer and Weeks 1989). At concentrations $\geq 10^{-8}$ M, none of these muscarinic agonists elicited any current, even when applied for 20 s (Fig. 6). These experiments showed that $I_{ACh}$ in the leg motoneuron somata, as recorded in our experimental conditions, did not contain a muscarinic component.

Despite the lack of a muscarinic component in $I_{ACh}$, we observed that the muscarinic receptor antagonist atropine blocked both components of $I_{ACh}$ (Fig. 7, Table 1) at relatively high concentrations. Atropine had an effect at concentrations of $10^{-8}$ M and nearly completely blocked $I_{ACh}$ at $10^{-4}$ M. The IC$_{50}$, estimated from linear fits of the concentration–current relation, was $1.7 \times 10^{-6} \pm 0.5 \times 10^{-6}$ M for $I_{ACh1}$ and $7.6 \times 10^{-7} \pm 2.5 \times 10^{-7}$ M for $I_{ACh2}$. Note that atropine is several orders of magnitude less potent than the nicotinic antagonist $\alpha$-BGTX. The atropine block was relatively difficult to reverse (<70% reversibility after 30 min wash).

DISCUSSION

In this study, we analyzed acetylcholine-activated ionic currents ($I_{ACh}$) in acutely dissociated leg motoneurons of the stick insect C. morosus. To ensure an unequivocal identification of the motoneurons, their somata were backfilled with a fluorescent dye prior to dissociation. Under controlled cell culture conditions, $I_{ACh}$ was characterized using whole cell patch-clamp recording and optical imaging techniques. Our data provide important insights into the ACh signaling of the stick insect’s leg sensory-motor system that has served as a very successful model to investigate basic principles of walking on the network level.
response relations for \( I_{\text{NIC}} \) with a response threshold of about 3 nM. Hill relation (EC50 3.5 M). The holding potential was +60 mV. A: nicotine, like ACh, induced an inward current consisting of a fast-desensitizing (\( I_{\text{NIC1}} \)) and slow-desensitizing (\( I_{\text{NIC2}} \)) component. A and B: at the same concentration (10^{-4} M) the amplitude of \( I_{\text{NIC1}} \) was similar to that of \( I_{\text{ACh1}} \) (\( P = 0.14; n = 13; \) paired t-test). However, \( I_{\text{NIC2}} \) (current amplitude averaged between 950 and 1,000 ms of NIC application) was 50% smaller compared with \( I_{\text{ACh2}} \) (\( P = 0.017; n = 13; \) paired t-test). C: concentration–response relations for \( I_{\text{NIC1}} \) and \( I_{\text{NIC2}} \). The data for \( I_{\text{NIC1}} \) were well fit with a Hill relation (EC50 = 3.5 \times 10^{-3} \pm 1.6 \times 10^{-3} M; n_H = 1.5 \pm 0.1). The concentration–response curve for the \( I_{\text{NIC2}} \) component was inverted-U shaped, with a response threshold of about 3 \times 10^{-6} M, an EC50 of about 10^{-5} M, and a maximum at 10^{-3} M. D: concentration–response relations for \( I_{\text{IM1}} \) and \( I_{\text{IM2}} \). \( I_{\text{IM1}} \) showed a similar EC50 (EC50 = 4.9 \times 10^{-4} \pm 3.2 \times 10^{-4} M) and Hill coefficient (\( n_H = 1.4 \pm 0.1 \)) compared with \( I_{\text{ACh1}} \) and \( I_{\text{NIC1}} \). The concentration–response curve for \( I_{\text{IM2}} \), like \( I_{\text{NIC2}} \), was inverted-U shaped, with a response threshold at about 3 \times 10^{-6} M, an EC50 of about 10^{-5} M, and a maximum at 10^{-4} M.

**Fig. 5.** Block of \( I_{\text{ACh}} \) by α-bungarotoxin (α-BTX). \( I_{\text{ACh}} \) was induced by application of 10^{-4} M ACh. α-BTX was bath-applied in the indicated concentrations. Holding potential was +60 mV. A: both components of \( I_{\text{ACh}} \) were α-BTX sensitive. The α-BTX effects reversed slowly and only in part (<50% recovery in 45 min). B: concentration–response relations of the α-BTX block for both components of \( I_{\text{ACh}} \). Current amplitudes were scaled as a fraction of \( I_{\text{ACh1}} \) and \( I_{\text{ACh2}} \) evoked by 10^{-4} M ACh without α-BTX. The model for the concentration–response relation was not obvious. From linear fits we estimated an IC50 for \( I_{\text{ACh1}} \) of 2.3 \times 10^{-9} \pm 1.0 \times 10^{-9} M and 3.1 \times 10^{-10} \pm 0.8 \times 10^{-10} M for \( I_{\text{ACh2}} \). From fits with a Hill relation (Eq. 2b) we estimated an IC50 for \( I_{\text{ACh1}} \) of 3.3 \times 10^{-9} \pm 1.1 \times 10^{-9} M (n_H = 0.40 \pm 0.05) and 2.5 \times 10^{-10} \pm 0.1 \times 10^{-10} M (n_H = 0.44 \pm 0.06) for \( I_{\text{ACh2}} \).

**Identification of leg motoneurons in cell culture**

Dextran conjugated with a fluorescent label have been used successfully as dyes for retro- and anterograde labeling of living neurons (Heidel and Pflüger 2006; Kloppenburg and Hörner 1998; Lakes-Harlan et al. 1998; Mentel et al. 2008; Schmued et al. 1990; Westmark et al. 2009). Here tetramethylrhodamine-dextran was used to label leg motoneurons by conventional backfilling via the *nervus cruris*. The motoneurons’ cell bodies and their major neurites were easily detected in the intact ganglion, revealing all the previously described major morphological features of these neurons (Debrödt and Bässler 1989; Goldammer 2008; Storrer et al. 1986). DUM neurons and inhibitory motoneurons that were also labeled via the *nervus cruris* could be easily differentiated from the motoneurons by their soma size. The dye did not leak out of the neurons nor did it show any significant bleaching or phototoxicity during the brief exposure to UV light for cell identification. In agreement with previous work (Heidel and Pflüger 2006; Kloppenburg and Hörner 1998; Kloppenburg et al. 1999; Westmark et al. 2009) the labeling procedure did not cause obvious changes in the electrophysiological membrane properties. Current-clamp recordings from tetramethylrhodamine-
**A** muscarinic agonist “short” application

- **ACh (10^{-4} M)**
- **MUSC (10^{-3} M)**
- **ACh (10^{-4} M)**

**B** muscarinic agonist “long” application

- **MUSC (10^{-3} M)**
- **OXO (10^{-3} M)**
- **PILO (10^{-3} M)**

---

**FIG. 6.** Effects of muscarinic agonists. The holding potential was −60 mV. A and B: short (1 s) and long (20 s) application of muscarine (MUSC), oxotremorine (OXO), and pilocarpine (PILO) at 10^{-3} M did not induce any currents.

dextran-labeled motoneurons had similar resting potentials and ACh-induced currents to unlabeled cultured neurons (Westmark et al. 2009) and they had resting potentials similar to those of leg motoneurons in the intact ganglion (compare with Schmidt et al. 2001). However, it is noticeable that our mean value of −63 mV for the resting potential had a SD of only 2.5 mV. This might indicate that our preparation is selective for a certain type of motoneuron.

**Characteristics of ACh-induced currents**

In >90% of the investigated motoneurons, ACh induced an inward current. The concentration–response parameters of \( I_{ACh} \) were well within the range reported for other insect neurons (locust: Hermsen et al. 1998; van der Beukel et al. 1998; honey bee: Barbara et al. 2005, 2008; Wüstenberg and Grünewald 2004; cockroach: David and Sattelle 1984; fly: Albert and Lingle 1993; Brown et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004). Patch-clamp recordings combined with optical Ca^{2+} imaging revealed that a considerable fraction of \( I_{ACh} \) is carried by Ca^{2+} (~18%). This Ca^{2+} influx is of great interest because Ca^{2+} can act as a second messenger (Berridge et al. 2000; Bootman et al. 2002) and could, for example, influence the cellular excitability by activation of Ca^{2+}-dependent ion channels (Catterall 1998; Klink and Alonso 1997; Lanzafame et al. 2003; Taylor and Peers 2000; Vermino et al. 1992, 1994). Such a mechanism might mediate the sustained depolarization of leg motoneurons during step sequences of foreleg as reported by Ludwar et al. (2005) and Westmark et al. (2009). The Ca^{2+} permeability observed in this study (~18%) was well within the range reported in vertebrate nAChRs (Delbono et al. 1997; Fucile et al. 2006), but was clearly lower than that in pupal Kenyon cells (Goldberg et al. 1999) and adult antennal lobe neurons (Barbara et al. 2008) of honey bees.

\( I_{ACh} \) consisted of fast-desensitizing and/or slow-desensitizing inward currents. In all, 80% of the neurons generated both the transient and sustained components, clearly demonstrating that leg motoneurons possess functional ACh receptors. Components of \( I_{ACh} \), with marked differences in desensitization time constants have been observed in whole cell recordings (honey bee: Barbara et al. 2008; cockroach: Salgado and Saar 2004; locust: Hermsen et al. 1998; van der Beukel et al. 1998) and in single channel recordings (fly: Albert and Lingle 1993; cockroach: Beadle et al. 1989).

Although the transient component was usually larger than the sustained component, their ratios were variable between cells. Although not addressed in detail in this study, our

---

**FIG. 7.** Block of \( I_{ACh} \) by atropine. \( I_{ACh} \) was induced by application of \( 10^{-4} \) M ACh. Atropine was bath-applied in the indicated concentrations. Holding potential was −60 mV. A: both components of \( I_{ACh} \) were atropine sensitive. The atropine effects reversed slowly and only in part (~70% recovery in 30 min). B: concentration–response relations of the atropine block for both components of \( I_{ACh} \). Current amplitudes were scaled as a fraction of \( I_{ACh} \) and \( I_{ACh} \) evoked by \( 10^{-4} \) M ACh without atropine. From linear fits we estimated an IC_{50} for \( I_{ACh1} \) of \( 1.7 \times 10^{-6} \) ± 0.5 \times 10^{-6} M and 7.6 \times 10^{-7} ± 2.5 \times 10^{-7} M for \( I_{ACh2} \).
findings suggest that there are subtypes of leg motoneurons with distinct ACh receptor compositions possibly resulting in distinct synaptic properties. From insect motor systems, we know that within any one, there are usually different classes of motoneurons that differ in their physiological properties (Burrows 1996; Gabriel et al. 2003; Parker 1996; Schmidt et al. 2001). Neuronal properties are largely determined by the types of ion channels and receptors expressed and by their rate of expression. It will be interesting in the future to determine, with recordings from identified motoneurons in the intact ganglion, whether different ratios of receptors can be attributed to different subtypes of leg motoneurons, possibly reflecting their different physiological properties and function.

Nicotinic ligands

The findings that nicotine and imidacloprid induced \( I_{\text{ACh}} \) like currents and that \( \alpha\)-BGTX blocked \( I_{\text{ACh}} \) suggest that \( I_{\text{ACh}} \) is mediated by nicotinic acetylcholine receptors. The nicotine and imidacloprid concentration–response relations were well within the range reported for many other insect neurons (Barbara et al. 2008; Campussano et al. 2007; Cayre et al. 1999; Déglise et al. 2002; Ihara et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004; Wüstenberg and Grünewald 2004). The following results suggest that the two components, which desensitize with different time constants, are mediated by two different nAChRs: 1) the amplitude ratios of \( I_{\text{ACh1}} \) and \( I_{\text{ACh2}} \) were variable between different motoneurons and 2) the form and EC\(_{50}\) values of the concentration–response curves for several nAChR ligands were different between \( I_{\text{ACh1}} \) and \( I_{\text{ACh2}} \).

At equal ligand concentrations we found similar amplitudes for \( I_{\text{ACh1}} \) and \( I_{\text{NIC1}} \), but observed smaller amplitudes for \( I_{\text{NIC2}} \) compared with those for \( I_{\text{ACh2}} \). These results suggest a differential affinity of nicotine to the receptor subtypes, with nicotine being a full ACh agonist for the receptors mediating \( I_{\text{ACh1}} \), but only a partial agonist for the receptors mediating \( I_{\text{ACh2}} \). Differential effects of nicotine on receptor subtypes have been described previously in several insect neuron types, in which nicotine can act as a full or better ACh agonist (Cayre et al. 1999; Courjaret and Laped 2001; David and Pitman 1993; Laped et al. 1990; Trimmer and Weeks 1989) or as a partial ACh agonist (Albert and Lingle 1993; Barbara et al. 2005; Beadle et al. 1989; Déglise et al. 2002; Van Eyseren et al. 1998). In some studies, it has been observed that nicotine acted as a partial ACh agonist, but with equal or even lower potency than that of ACh (honey bee: Barbara et al. 2008; Wüstenberg and Grünewald 2004; Drosophila: Brown et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004). Although \( \alpha\)-BGTX completely blocked both components of \( I_{\text{ACh}} \), it had a higher affinity to the nAChR that generated \( I_{\text{ACh2}} \). Differential affinity of \( \alpha\)-BGTX to nAChR subtypes has been reported by Salgado and Saar (2004) in cockroach thoracic neurons, in which \( \alpha\)-BGTX had a higher affinity to the nAChR subtype that generated a nondesensitizing \( I_{\text{ACh}} \).

Muscarnic ligands

Even high concentrations of muscarine and “classical” muscarinic agonists (oxotremorine and pilocarpine) did not induce any current under our experimental conditions, showing that mAChRs did not contribute to \( I_{\text{ACh}} \). Although many binding, immunohistochemical, and systemic electrophysiological studies on insect neurons suggested the expression of mAChR (binding studies: Abdallah et al. 1991; Honda et al. 2007; Liu and Casida 1993; Onai et al. 1989; Orr et al. 1991; Qazi et al. 1996; Shapiro et al. 1989; immunolabeling studies: Blake et al. 1993; Bu et al. 2008; Harrison et al. 1995; Shirai et al. 2001; systemic electrophysiological studies: Büschges et al. 1995; Corronc and Hue 1993; Johnston and Levine 2002; Trimmer 1995; Trimmer and Weeks 1989), our results are in agreement with many previous studies on dissociated insect neurons, in which “classical” mAChR agonists induce only very small or no ionic currents (Albert and Lingle 1993; Cayre et al. 1999; Hermsen et al. 1998; van Eyseren et al. 1998; Wüstenberg and Grünewald 2004).

Provided that the cholinergic receptors on the motoneuron cell bodies and on the dendritic regions have similar pharmacological properties (e.g., Buckingham et al. 1994; Fickbohm and Trimmer 2003; Harrow and Sattelle 1983), the finding that stick insect motoneurons do not appear to be equipped with muscarinic receptors that induce ionic currents indicates that neither the tonic depolarization observed in stick insect motoneurons during pilocarpine-induced rhythmic activity (Büschges 1998) nor the tonic depolarization observed in motoneurons during walking (Ludwar et al. 2005; Westmark et al. 2009) are related to ACh binding to motoneuron mAChRs. This would suggest that muscarinic action for generation of rhythmic activity in the stick insect locomotor system, and perhaps also in locomotor systems of other insects, is confined to premotor neurons.

However, our failure to observe muscarine-induced currents could also be due to our experimental conditions. If the mAChRs were exclusively expressed on the neurites, we would not measure their currents in our soma preparation. Because mAChRs are known to activate second messenger pathways (Caulfield 1993; Caulfield and Birdsay 1998; Felder 1995; Gregory et al. 2007; Lanzafame et al. 2003), it could be that much longer application times are necessary to activate mAChR-mediated ionic currents or that mAChRs modulate the activity of nAChRs without directly evoking a current. The reduction of \( I_{\text{ACh}} \) by atropine might point in that direction and regulation of nAChR-mediated responses by activation of mAChRs has been suggested in previous studies (Brown and Galligan 2003; Courjaret et al. 2003; Shen et al. 2009).

In any case, we found no direct evidence for “mixed” receptors as reported in cockroach DUM neurons (Laped et al. 1990) or motoneurons (David and Pitman 1993). However, note that the “mixed” receptors, observed by David and Pitman (1993), have been recorded at more depolarized holding potentials than those in this study and that the effective concentrations have to be compared with great caution given the different methods of ligand application.

The approach used in this study allowed the investigation of acetylcholine-induced currents of unequivocally identified adult leg motoneurons under biophysically controlled conditions. Whole cell patch-clamp recordings showed that somata of leg motoneurons possess functional AChRs. Although we did not attempt to determine a complete pharmacological profile, the study provides solid evidence that the motoneurons possess at least two types of nicotinic receptors that mediate current components with differing rates of desensitization. The functional roles of these receptors have yet to be verified in...
preparations with functionally intact synapses. Using quantitative Ca\(^{2+}\) imaging, we found that roughly 18% of \(I_{\text{ACh}}\) is carried by Ca\(^{2+}\). Thus \(I_{\text{ACh}}\) contributes not only to the membrane potential, but might also induce Ca\(^{2+}\) triggered processes. We consider this study to be an important prerequisite to better understand cholinergic synaptic transmission in the sensory-motor system of the stick insect, which has served very successfully as a model for walking and locomotion.

Future studies will have to focus on the analysis of transmitter receptors on the dendrites of motoneurons, as well as on premotor interneurons, to unravel network interactions underlying walking pattern generation. In addition, it will be important to unravel the possible modulation of nAChR activity by mAChR-induced intracellular signaling cascades and the functional consequences of the increase of intracellular Ca\(^{2+}\)-elicited via cholinergic inputs.

ACKNOWLEDGMENTS

We thank H. Wratil for excellent technical assistance and M. Paehler for valuable help with the confocal microscopy.

GRANTS

This work was supported by grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, the Brazilian Ministry of Education (CAPES), and the Deutscher Akademischer Austausch Dienst to E. E. Oliveira and grants from the Deutsche Forschungsgemeinschaft to J. Schmidt, A. Büschges, and P. Kloppenburg.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


Debrodt B, Bäsßler U. Motor neurons of the flexor tibiae muscle in phas- 

Dégilde P, Grünweald B, Gauthier M. The insecticide imidacloprid is a 
partial agonist of the nicotinic receptor of honeybee Kenyon cells. Neurosci 

Delbono O, Gopalakrishnan M, Renganathan M, Monteggia LM, Messi 
ML, Sullivan JP. Activation of the recombiant human alpha 7 nicotinic 
acetylcholine receptor significantly raises intracellular free calcium. J Phar- 

Efron B. 1977 Rietz Lecture: “Bootstrap Methods.” Another look at the 

Felder C. Muscarinic acetylcholine receptors: signal transduction through 

Fickbohm D, Trimmer BA. The role of acetylcholine in learning and memory. 

Hayashi J, Levine R. Calcium and potassium currents in leg motoneurons 
during postembryonic development in the hawkmoth Manduca sexta. J Exp 

Heidel E, Pflüger HJ. Ion currents and spiking properties of identified 
subtypes of locust octopaminergic dorsal unpaired median neurons. Eur J 

Hermsen B, Stetter E, Thees R, Heiermann R, Schrattenholz A, Ebbing- 
haus U, Kretschmer A, Methfessel C, Reinhardt S, Maehlke A. Neuro- 

Honda H, Tomizawa M, Casida JE. Insect muscarinic acetylcholine recep- 
tor: pharmacological and toxicological profiles of antagonists and agonists. 

Hrusc A, Hess S, Koppelburg P. Functional parameters of voltage-activated 
Ca2+ currents from olfactory interneurons in the antennal lobe of Peripla- 


Jepson JEC, Brown LA, Sattelle DB. The actions of the neonicotinoid 

Johnston RM, Levine RB. Thoracic leg motoneurons in the isolated CNS of 
adult Manduca produce patterned activity in response to pilocarpine, which 
is distinct from that produced in larvae. Invert Neurosci 4: 175–192, 2002.

Jones SVP. Muscarinic receptor subtypes: modulation of ion channels. Life Sci. 

Klink R, Alonso A. Ionic mechanisms of muscarinic depolarization in entro- 

Kropp P, Hörner M. Voltage-activated currents in identified giant 

Kropp P, Kirchhof BS, Mercer AR. Voltage-activated currents from adult honeybee (Apis mellifera) antenna motor neurons recorded in vitro 

Lakes-Harlan R, Jacobs K, Heinrich R. Identification of auditory interneu- ons in situ and in vitro by tracer injection into an afferent neuropil of 

Lanske I, Millar NS, D beta 3, an atypical nicotinic acetylcholine receptor 
subunit from Drosophila: molecular cloning, heterologous expression and 

Lanzafame AA, Christopoulos A, Mitchelson F. Activation of the recombinant human alpha 7 nicotinic acetylcholine receptor by 10.220.33.5 on May 26, 2017 http://jn.physiology.org/ Downloaded from


