Dorsal Unpaired Median Neurons of *Locusta migratoria* Express Ivermectin- and Fipronil-Sensitive Glutamate-Gated Chloride Channels

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INTRODUCTION

Glutamate is the main excitatory amino acid neurotransmitter in the CNS of vertebrates, where it interacts both with ionotropic receptors, i.e., ligand-gated ion channels, and metabotropic or G-protein–coupled receptors (Hogner et al. 2002). In vertebrates, ionotropic glutamate receptors are permeable to cations and can be subdivided into three groups according to their respective main agonist: N-methyl-D-aspartate (NMDA), α-aminooxy-5-hydroxy-4-isoxazolepropionic acid (AMPA), and kainate receptors.

In invertebrates, L-glutamate can also act as an excitatory transmitter at the neuromuscular junction (Ikeda et al. 2003; Wafford and Sattelle 1989). However, a biphasic response to L-glutamate is usually observed, consisting of a depolarization mediated currents by 87 and 39%, respectively. Furthermore, 1 μM ivermectin produced a persistent chloride current, suggesting the expression of ivermectin-sensitive GluCl α subunits. A degenerate PCR/RACE strategy was used to clone the full-length *L. migratoria* LmGluCl subunit. Finally, RT-PCR experiments demonstrated the presence of LmGluCl transcripts in locust DUM neurons. Our results provide the first direct evidence of a functional ivermectin-sensitive GluCl channel on the cell surface of DUM neurons of *L. migratoria*.

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At the molecular and functional level, GluCls remain poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. Until now, cloning studies led to the identification of four subunits were identified. In nematode. In poorly characterized. 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standard intracellular solution (SIS) and standard extracellular solution (SES) was \(-18\) mV, between high Cl\(^{-}\)-IS and SES was \(-21.8\) mV, between low Na\(^{+}\)-ES and low K\(^{+}\)-high Cl\(^{-}\)-IS was \(-6.7\) mV and between low Na\(^{+}\)-ES and low K\(^{+}\)-low Cl\(^{-}\)-IS was \(-19.2\) mV. The experimental protocols and data acquisition were carried out using an EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by the program Pulse (HEKA Elektronik) running on a personal computer (equipped with a Pentium III processor). Capacitive and leak currents were compensated and residual capacitances and leak currents were eliminated, by means of a P/6 protocol (Moyer and Brown 2002). The series resistance was \(1.5\) M\(\Omega\) and was compensated \(\pm 53\%\). The recording of whole cell current was started 5 min after rupture of the cell membrane to allow adequate equilibration of the cell interior with the pipette solution.

Data acquisition and analysis

Data were filtered at 2.9 kHz and sampled at 20 kHz. They were stored on a computer hard disk and analyzed with the program PulseFit 8.77 (HEKA Instruments). Further analyses, including non-linear regression, were carried out using Origin 6.0 Professional (Microcal Software, Northampton, MA).

Current–voltage relationships were fitted with the Goldman–Hodgkin–Katz (GHK) equation

\[
I_x = \frac{z^2F^2}{RT} P_x V_n \left\{ \frac{[X] - [X]_0 e^{-\frac{zF}{RT}v} e^{-\alpha V_{NCl} \frac{RT}{zF}}}{1 - e^{-\alpha V_{NCl} \frac{RT}{zF}}} \right\}
\]

Desensitization kinetics of individual glutamate-evoked currents was assessed by fitting the desensitizing phase with the following equation

\[
I = I_0 e^{-\alpha v}
\]

Except where stated otherwise, results are expressed as means \(\pm\) SE, where \(n\) is the number of experiments. Statistical comparisons were made using the Student’s \(t\)-test or one-way ANOVA followed by Dunnett’s posttests. Differences were considered significant for \(P \leq 0.05\).

Chemicals

Monosodium glutamate (Sigma–Aldrich Chemie, Steinheim, Germany) and avermectin B1A (Sigma–Aldrich) were dissolved in triply distilled water. Stock solutions of fipronil (PESTANAL) and picrotoxin (PTX), both from Sigma–Aldrich (Seelze, Germany), were made in ethanol (EtOH) and dimethylsulfoxide (DMSO), respectively. These stock solutions were diluted into the extracellular solutions immediately before the start of each experiment. The final concentrations of EtOH and DMSO in the solutions were \(\leq 0.1\%\), at

FIG. 1. Glutamate-induced membrane potential changes in locust metathoracic ganglion dorsal unpaired median (DUM) neurons, recorded with the whole cell patch-clamp technique in current-clamp mode. A: in the presence of standard intracellular and extracellular solutions (SIS–SES; see inset), application of 1 mM glutamate for 1 s hyperpolarized the DUM neuron membrane potential from \(-35\) to \(-61\) mV. B: when using almost identical chloride concentrations in the intracellular and extracellular solutions (high Cl\(^{-}\)-IS–SES; see inset), glutamate generated a depolarization from \(-55\) to \(-19\) mV.

1 mM glutamate

\(-35\) mV

1 mM glutamate

\(-55\) mV

[Image 79x341 to 534x721]
which they had no effect on glutamate-induced currents (data not shown). L-Glutamate and ivermectin were applied topically through a valve-controlled tubing system positioned laterally above the cell at a distance of about 50–100 μm. The antagonists fipronil and PTX were applied to the bath.

Degenerate PCR and RACE

To obtain the full-length sequence of the *L. migratoria* GluCl channel, a Marathon RACE library was constructed using a mixture of several tissues (including muscle and neuronal tissues) as described by the manufacturer (Clontech, Germany). Degenerated oligonucleotides were designed and synthesized based on the predicted amino acid sequences of the conserved regions of the glutamate-activated chloride alpha channel (GluClα) after alignment of the GluClα from *Drosophila melanogaster* and *Caenorhabditis elegans*: DEG-F:5'AGGCTCGATCAGGGT-3' DEG-R: 5'TC(TG)CTAGCAAGCTCCAGAATAAGTCC-3'. The thermocycler was programmed for 35 cycles at 94°C for 30 s, 41.5°C for 45 s, and 72°C for 45 s using the *L. migratoria* RACE library as a template and the Advantage II polymerase mixture (Clontech) as polymerase. The resulting band of 193 bp was directly sequenced and the following primers were designed for rapid amplification of cDNA ends (RACE): GSP-F1:5'GGCTCGATCAGGGT-GCCGGTC-3' GSP-F2:5'-CCGTTCCGGCAGAGTGTCC-3' GSP-R1:5'-TCGAGAATTGCCCCGAATACA-3' GSP-R2:5'-ATGT-TAGGCAGCAGCCGGTGCTCA-3'.

The first RACE PCR was performed for 35 cycles at 94°C for 30 s and 68°C for 45 s using the *L. migratoria* RACE library as a template and the Advantage Taq II polymerase mixture and GSP-F1 (3'-RACE) or GSP-R1 (5'-RACE) combined with adaptor primer 1 (AP1). A second nested PCR was performed with GSP-F2 or GSP-R2 and AP2 primer. The PCR fragments (1,000–2,000 bp) obtained were cloned into the pGEM-T vector (Promega, Karlsruhe, Germany) and sequenced until start and stop codons were identified.

RNA isolation from DUM neurons and RT-PCR

DUM neurons of *L. migratoria* were isolated as described earlier with slight modifications. After the collagenase/disperse treatment, the cells were plated on a NUNC petri dish and incubated for ≥15 min at 28°C. The cells were picked up using a borosilicate patch pipette, then washed with ice-cold PBS (pH 7.4) and transferred into an RNase-free microtube.

Total RNA was prepared from the material obtained from about 40 DUM neurons with the PicoPure RNA Isolation kit (Arcturus Bioscience, Westburg, Leusden, The Netherlands) with an additional DNase treatment (Rnase-free DNase set, Qiagen) following the instructions of the suppliers. In addition, RNA from a mixture of several neuronal tissues (abdominal and thoracic ganglia, brain) was prepared. First-strand cDNA was synthesized using the Promega RT system (Promega Benelux, Leiden, The Netherlands).

The cDNA of isolated locust DUM neurons and locust neuronal tissues was used as a template for a PCR using *Taq* DNA polymerase (Applied Biosystems). A fruitfly cDNA was used as a positive control. PCR primers DEG-F and DEG-R were used for amplification during 40 cycles at 94°C for 30 s, 41.5°C for 45 s, and 72°C for 45 s. PCR products were then separated on a 2% high-resolution agarose gel. Several distinctive bands were observed. The band of interest (about 190–200 bp) was extracted from the gel using the QiagQuick gel extraction kit (Qiagen), purified, and, in the case of isolated DUM neurons, subjected to a second PCR amplification using the same primers and incubation conditions. The amplified PCR fragments were either directly sequenced or cloned into pGEM-T for sequencing.

RESULTS

Glutamate elicits changes in the membrane properties of locust DUM neurons

When recorded with solutions close to physiological conditions (SIS–SES; see METHODS and Table 1), whole cell patch- and current-clamp locust DUM neurons had a resting membrane potential of $-34.4 \pm 3.8$ mV ($n = 6$). As shown in Fig. 1A, application of 1 mM glutamate hyperpolarized the membrane to $-62.6 \pm 1.8$ mV ($n = 6$). When Cl$^-$ concentrations were identical in intracellular and extracellular solutions, DUM neurons had a resting membrane potential of $-58.8 \pm 1.4$ mV ($n = 5$) and, in that case, glutamate induced a depolarization of the membrane to $-7.9 \pm 3.4$ mV ($n = 5$; Fig. 1B).

These results suggested that glutamate could activate a glutamate-gated chloride channel (GluCl) in locust DUM neurons. Such GluClα in other species are often characterized by a...
complete desensitization under a continuous glutamate super-
fusion with a time constant on the order of hundreds of
milliseconds to seconds. To examine the kinetics of the gluta-
mate-evoked currents in DUM neurons, recordings were ob-
tained under voltage-clamp conditions using identical chloride
concentrations in the intracellular and extracellular solutions.
Under those conditions, application of 1 mM glutamate at a
holding potential (V_H) of −70 mV elicited an inward current
that reached a peak amplitude of −4.2 ± 0.7 nA (n = 7) within
150–250 ms (Fig. 2). As expected for GluCls, the current
desensitized completely in the continuous presence of gluta-
mate after a monoexponential time course (τ_{decay} = 194 ± 39
ms, n = 7).

Glutamate-induced currents are carried by chloride ions

To assess whether the main ionic species carrying the
 glutamate-induced currents was chloride, the I–V relationships
were obtained using two different intracellular solutions: one
containing nearly identical intracellular and extracellular Cl−
concentrations (low K⁺–high Cl−–IS) and a second one con-
taining a lower Cl− concentration in the intracellular solution
(low K⁺–low Cl−–IS). These solutions were also modified (see
Table 1) to reduce contaminating currents through the voltage-
dependent channels (e.g., Kᵢ, Naᵢ, Caᵢ). Currents were evoked
by repeatedly applying 1 mM glutamate for 3 s and at the same
time applying a voltage step ranging from −60 to +60 mV. In
between applications, V_H was returned to −70 mV and gluta-
mate was washed out (Fig. 3A).

Maximum peak currents were measured at each voltage step
and normalized for the cell capacitance. The reversal potential
(V_R) was calculated after fitting the data with the GHK
equation (see METHODS; Fig. 3B). Using nearly identical Cl−
concentrations in the extracellular and intracellular solutions,
glutamate-induced currents reversed in polarity at a membrane
potential of +2.6 mV (n = 4), which was close to the
calculated chloride equilibrium potential (E_{Cl} = −0.9 mV).
At lower intracellular Cl− concentrations, E_{Glu} shifted to a
more negative value (−13.6 mV; n = 3), which was in good
agreement with the expected shift in the calculated Nernst
equilibrium potential for chloride ions (E_{Cl} = −20 mV).

These results clearly demonstrate that the glutamate-induced
currents in locust DUM neurons are mainly carried by chloride
ions.

Pharmacology of glutamate-induced currents

Glutamate-induced currents in locust DUM neurons were
then tested for their sensitivity to two known blockers of
GluCls: fipronil and picrotoxin (PTX). As shown in Fig. 4, A
and B, both drugs reversibly inhibited glutamate-gated cur-
cents. Ten micromolar fipronil inhibited to 13.1%
1 mM glutamate in locust DUM neurons had a mean peak
amplitude of −2.7 ± 0.7 nA (n = 5; Fig. 4C₁). In contrast, 1
μM IVM induced a current that did not desensitize and did not
even fully reverse after the removal of IVM. IVM-induced
currents amounted to −5.9 ± 1.5 nA (n = 6; Fig. 4C₂).

These pharmacological data further support the presence of
a glutamate-gated chloride channel in efferent DUM neurons
of L. migratoria.

Cloning of full-length LmGluClα

Because functional GluCls usually require the expression of
at least GluClα subunits (Cully et al. 1996; Semenov and Pak
1999), we developed a degenerate PCR and RACE strategy in
an attempt to clone the full-length sequence of L. migratoria
GluClα (LmGluClα). Within the isolated cDNA, prepared as
described in METHODS, a 1,362-bp open reading frame was
identified encoding for a 453 amino acid protein. Typical
features of GluCl subunits as in other members of the Cys-loop
ligand-gated ion channel superfamily were found: a large
extracellular N-terminal domain containing the ligand-binding
site, a conserved Cys-loop and four transmembrane regions (Fig. 5). Moreover, the LmGluCl subunit showed strong homology to orthologous sequences from Drosophila melanogaster (CAA05260; 82.2% identity, 87.6% similarity), Tribolium castaneum (XP 973383; 84.8% identity, 91.4% similarity), Anopheles gambiae (XP 321697; 83.8% identity, 88.6% similarity), Apis mellifera (ABG75738; Jones and Sattelle 2006; 78.3% identity, 87.1% similarity), Musca domestica (BAD16657; 81.3% identity, 86.7% similarity), and Lucilia cuprina (AAC31949; 83.7% identity, 89.1% similarity). The cDNA sequence of the LmGluCl subunit was deposited in GenBank under Accession Number DQ643254.

DUM neurons of L. migratoria express LmGluClα messengers

The expression of LmGluClα in locust DUM neurons was assessed as follows. mRNAs of isolated DUM neurons were reverse transcribed and used as template for a two-step PCR procedure. After the first PCR, a faint band was visible (Fig. 6A). This PCR band was excised, purified, and reamplified (Fig. 6B). Direct DNA sequencing confirmed the LmGluClα sequence.

DISCUSSION

In the work reported herein, we have clearly demonstrated that efferent DUM neurons of L. migratoria express functional glutamate-gated chloride channels (GluCls), using molecular biology and electrophysiological methods. In the presence of physiological solutions mimicking the hemolymph, glutamate hyperpolarized the membrane of DUM neurons as would be expected were GluCls to be present and active. Voltage-clamp experiments indicated that glutamate-induced currents in locust DUM neurons:
1) desensitize completely under continuous glutamate application, 2) are mainly carried by chloride ions, and 3) are blocked by fipronil and PTX, consistent with the properties of GluCls in other invertebrate species. Expression of GluClα subunit-containing GluCls was suggested by the sensitivity to IVM, which induced nondesensitizing persistent currents. Expression was confirmed by the RT-PCR detection of GluClα transcripts in locust DUM neurons. Finally, we also
report the full DNA sequence of the GluCl/\(H_9251\) subunit of \(L.\) migratoria.

In invertebrates, glutamate can interact with two classes of ionotropic receptors: excitatory channels, mainly permeable to cations as in vertebrates, and inhibitory anionic channels that are found only in invertebrates. When using intra- and extracellular solutions with an ionic composition close to that of the hemolymph, we observed a hyperpolarizing response on glutamate application, suggesting that locust DUM neurons express only inhibitory glutamate-activated channels (Ikeda et al. 2003; Wafford and Sattelle 1989). The chloride dependency of glutamate-induced responses was further confirmed by the shift to depolarization when \(E_{Cl}\) was changed from 79.6 to 4.1 mV and by the concordance of reversal potentials of glutamate-induced currents with chloride equilibrium potentials. Thus it appears that \(L.\) migratoria DUM neurons express inhibitory ionotropic glutamate receptors only and not their excitatory counterparts. This was also observed for DUM neurons of other insect species such as the cockroach \(Periplaneta\) americana (Washio et al. 2002) and for the stomatogastric ganglion of the lobster \(Panulirus\) interruptus (Cleland and Selverston 1998). This is in contrast to the expression of both inhibitory and excitatory glutamate receptors by muscle cells in the locust or the crayfish (Dudel and Franke 1987; Kerry et al. 1987, 1988) or by motor neurons such as the fast coxal depressor motor neuron of the cockroach thoracic ganglion (Wafford and Sattelle 1989).

In our hands, both glutamate-induced voltage and current changes were monophasic. Furthermore, under voltage-clamp conditions, glutamate-induced currents completely desensitized in about 1 s in the continuous presence of glutamate. These properties are similar to those reported for \(Caenorhabditis\) elegans.
**FIG. 6.** Expression of the GluClα channel in DUM neurons of *L. migratoria*. A: agarose electrophoresis of the first PCR using locust DUM neuron cDNA as a template (lane a2; marker = 100-bp ladder shown in lane a1). B: reamplification of the corresponding 193-bp PCR product for DNA sequencing (lane b3; marker = 50-bp ladder shown in lane b1). Positive control used was *Drosophila melanogaster* glutamate-activated chloride alpha channel (DmGluClα, lane b2).

diis evelens* GluCls expressed in Xenopus oocytes (Forrester et al. 2003). In cockroach DUM neurons, on the other hand, biphasic glutamate responses were observed, consisting of an initial transient and a subsequent prolonged phase (Heckman and Dudel 1995; Raymond et al. 2000; Zhao et al. 2004a). The desensitizing and nondesensitizing components of the glutamate-induced responses seem to be attributable to the presence of pharmacologically distinct GluCls: the former sensitive and the latter insensitive to PTX or BIDN. The desensitizing glutamate-evoked current we report in locust DUM neurons is also PTX sensitive. It therefore probably corresponds to the transient current described in cockroach CNS neurons.

In locust DUM neurons, fipronil seems to be a more powerful antagonist of GluCl-mediated responses than does PTX. At 10 μM, it blocked 86% of glutamate-gated currents. This is in good agreement with the 75% block by 10 μM fipronil of the transient component of glutamate responses in cockroach DUM neurons reported by Raymond et al. (2000). To the best of our knowledge, our work is the first report of an effect of fipronil on isolated DUM neurons of *L. migratoria*. The blocking effect of fipronil on GluCls was previously proposed to be PTX-sensitive and might change the amplitude of the glutamate-evoked current described in cockroach CNS neurons.

Ivermectin (IVM) at 1 μM generated an irreversible inward current under identical chloride concentrations in DUM neurons of *L. migratoria*. At concentrations in the micromolar range, avermectin derivatives are known activators of ligand-gated chloride channels, i.e., strychnine-sensitive glycine receptors (Shan et al. 2001), GABA receptors (Adelsberger et al. 2000; Robertson 1989), and GluCls (Cleland 1996). Because glycine receptors are not found in invertebrates, the IVM-induced current observed in DUM neurons could be the consequence of the activation of either GABA receptors or GluCls— or even both, as already demonstrated in the fruit fly (Ludmerer et al. 2002). Accordingly, the amplitude of the current induced by 1 μM IVM was significantly higher than that induced by 1 mM glutamate, a potentially saturating concentration for GluCls. This could suggest that IVM activated both GABA receptors and GluCls in DUM neurons of the locust. On the other hand, avermectin-sensitive chloride currents in *Xenopus* oocytes injected with *C. elegans* mRNA were GABA insensitive, but sensitive to glutamate (Yates et al. 2003). Further characterization of IVM-induced currents is needed to solve this issue, but that is beyond the scope of this work. With respect to GluCls, it is usually accepted that the ability to irreversibly bind IVM is typically diagnostic for α-like subunits of GluCls (Cully et al. 1994; Li et al. 2002). This was previously demonstrated in several invertebrate species: locust (*Schistocerca americana*; Rohrer et al. 1994), barber pole worm (*Hemonthus contortus*; Forrester et al. 2001; Portillo et al. 2003), heartworm (*Dirofilaria immitis*; Yates et al. 2004), elegant worm (*Caenorhabditis elegans*; Dent et al. 1997, 2000; Horoszok et al. 2001), and strongylid worm (*Cooperia oncophora*; Njue et al. 2004). In contrast to GluClα subunits, GluClβ subunits were never shown to respond to IVM (Cully et al. 1994; Li et al. 2002). Note, however, in some species, splice variants of GluClα subunits were shown to be IVM resistant, e.g., DiGluClα3A from *Dirofilaria immitis* (Yates et al. 2004) or CoGluClα3 from *Cooperia oncophora* (Njue et al. 2004).

The functional expression of α-subunit–containing GluCls by locust DUM neurons suggested by their IVM sensitivity was demonstrated at the molecular level. A full-length LmGluClα channel was cloned, using a PCR/RACE strategy, and its messenger RNA in isolated DUM neurons was detected by RT-PCR. This does not rule out the expression of other LmGluClα subunits and thus does not allow any conclusion about the homo- or heteromeric nature of locust GluCls. Transfection of the cloned LmGluClα in heterologous expression systems might help to solve this question.

The functional implication of glutamate-gated chloride channels in DUM neurons of *L. migratoria* certainly deserves further attention. Because metathoracic effluent DUM neurons innervate flight and leg muscles, their modulation by inhibitory GluCls is likely to influence flight control (Groleau and Lapied 2000). Furthermore, and more generally, because GluCls have been described until now in invertebrates only, a better insight into their functioning would undoubtedly contribute to the development of novel insecticides both more selective and more potent (Raymond and Satelle 2002; Zhao et al. 2004b).

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**REFERENCES**


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