Glutamate-gated chloride channels, members of the ligand-gated ion channel superfamily, have been shown in nematodes and in insects to be a target of the antiparasitic agent avermectin. Two subunits of the C. elegans glutamate-gated chloride channel have been cloned: GluCl-α and GluCl-β. We report the cloning of a Drosophila melanogaster glutamate-gated chloride channel, DrosGluCl-α, which shares 48% amino acid and 60% nucleotide identity with the C. elegans GluCl channels. Expression of DrosGluCl-α in Xenopus oocytes produces a homomeric chloride channel that is gated by both glutamate and avermectin. The DrosGluCl-α channel has several unique characteristics not observed in C. elegans GluCl: dual gating by avermectin and glutamate, a rapidly desensitizing glutamate response, and a lack of potentiation of the glutamate response by avermectin. The pharmacological data support the hypothesis that the DrosGluCl-α channel represents the arthropod H-receptor and an important target for the avermectin class of insecticides.

Glutamate-gated chloride channels were first identified in arthropods as extrajunctional glutamate receptors (H-receptors) that hyperpolarized the membrane potential of locust (Schistocerca gregaria) leg muscle (1–3) and later cloned from the soil nematode Caenorhabditis elegans (4). Glutamate-gated chloride channels are activated by the glutamate analog 60% nucleotide chloride acid and are inhibited weakly by the ligand-gated chloride channel blocker picrotoxin (1, 4–9). No glutamate-gated chloride channels have been identified in vertebrates.

Glutamate-gated chloride channels are important targets for the widely used avermectin class of anthelmintic and insecticidal compounds (10, 11). The avermectins are a family of macrocyclic lactones used throughout the world to treat parasitic helminths and insect pests of man and animals (12). Avermectins directly activate C. elegans glutamate-gated chloride channels when expressed in Xenopus oocytes (4, 9, 13) and activate a glutamate-sensitive chloride channel in locust muscle (10).

The C. elegans avermectin-sensitive glutamate-gated chloride channel (GluCl) interacts with the GenBank™/EBI Data Bank with accession number(s) US8776. To whom correspondence should be addressed: Dept. of Genetics and Molecular Biology, Merck Research Laboratories, P.O. Box 2000, Rm. 80M-213, Rahway, NJ 07065-0900. E-mail: doris_cully@merck.com.

The abbreviations used are: GluCl, glutamate-gated chloride channel; PCR, polymerase chain reaction; IVMP0a, ivermectin phosphate.
found to contain an insert of 3,958 bases, and the nucleotide sequence was determined.

Electrophysiological Recordings—Xenopus oocytes were prepared, injected (in vitro RNA 0.1–10 ng), and membrane currents recorded as described previously (9, 13). Recordings were made in standard frog saline consisting of (in mM): NaCl (115), KCl (2), MgCl₂ (1), CaCl₂ (1.8), HEPES (10) adjusted to pH 7.5 with NaOH. Pipettes were filled with 3 M KCl and had resistances between 1.0 and 3.0 megohm. Avermectin analogs were prepared as 1 mM stock solutions in dimethyl sulfoxide and diluted into frog saline. A water-soluble avermectin derivative, ivermectin phosphate (22,23-dihydroavermectin B₁a-4phosphate, monosodium salt, IVMPO₄), was used for most of the experiments because it is easily washed out of the perfusion system (11, 13). Picrotoxin (Sigma) was made as a 50 mM stock solution in dimethyl sulfoxide. Concentration-response curves were fit to a modified Michaelis-Menten equation:

\[ I_{\text{max}} = \frac{1}{1 + \left( \frac{[D]}{EC_{50}} \right)^n} \]  
(Eq. 1)

where \( I_{\text{max}} \) is the maximal response, \([D]\) is the drug concentration, \(EC_{50}\) is the drug concentration for half-maximal effect, and \(n\) is the Hill coefficient. All data in text and figures are means ± S.E. Methods for obtaining concentration effect curves for IVMPO₄ can be found in Ref. 11.

Current voltage (I/V) relationships were determined using 1-s voltage ramps from −110 to 80 mV in the presence and absence of IVMPO₄. Drug-sensitive currents were generated by subtracting drug-free from drug-containing data. Ion substitutions were accomplished by replacing NaCl in standard frog saline with KCl, choline chloride, or sodium isethionate as indicated. The bath was connected to the ground through a 3 M KCl agar bridge for experiments in which external chloride was replaced. To limit contributions from calcium-activated chloride currents to the I/V relationship, oocytes were preincubated in frog saline containing 10 μM BAPTA-AM (11).

**RESULTS**

Isolation of a GluCl cDNA from D. melanogaster—Alignment of the C. elegans GluCl proteins with the GABAA and glycine receptor subunits highlighted areas of conservation as well as regions of diversity (4). Degenerate oligonucleotide primers were designed which, in theory, would generate a PCR product related to the GluCl family and not the GABAA or glycine gene families. Several PCR clones were obtained which contained inserts representing the region spanning the M1–M3 domains of the GluCl proteins. A Drosophila cDNA clone (DrosGluCl-a) was obtained by hybridization screening of a cDNA library with one of the M1–M3 PCR products. The nucleotide sequence of DrosGluCl-a (3,958 base pairs) revealed an open reading frame that predicted a protein of 456 amino acids which contained features common to ligand-gated anion channels, such as a large amino-terminal putative extracellular domain and four putative transmembrane domains (Fig. 1).

The DrosGluCl-a cDNA contains a 5′- and 3′-untranslated region of 404 and 2186 base pairs, respectively. It is missing a 3′-poly(A) tract and is presumed to be less than full length. Alignment of the DrosGluCl-a protein with the C. elegans GluCl-a and -b and Drosophila rdl proteins revealed 48, 43, and 37% identity and 67, 62, and 58% similarity, respectively (Fig. 1). Drosophila rdl sequences that are not homologous to any of the GluCl sequences are omitted but are indicated by nₙ, where n is the number of amino acid residues deleted from the figure.
and the glycine receptors (17). Two of these cysteines are absent in the Drosophila rd1 GABA-sensitive receptor and in all other GABA receptors subunits. DrosGluCl-α is most closely related to the α subunit of the glycine receptor family, showing 57% identity. Phylogenetic analysis of all ligand-gated channels indicates that DrosGluCl-α is present on a separate branch along with the C. elegans GluCl-α and -β (data not shown).

Functional Expression of DrosGluCl-α—Glutamate evoked a rapidly activating inward membrane current in Xenopus oocytes injected with in vitro RNA transcribed from Dros-GluCl-α (Fig. 2A). The EC\textsubscript{50} for glutamate activation of current was 23 μM, and the Hill coefficient was 2.0, suggesting that binding of more than one glutamate was necessary to gate the channel. Glutamate-sensitive current desensitized rapidly in the continued presence of glutamate (Fig. 2A). The rate and magnitude of desensitization were concentration-dependent. In the presence of 100 μM glutamate, the time constant for desensitization was 2.5 ± 0.3 s, and current desensitized 87 ± 2% of the peak value in 15 s (n = 5). With 10 μM glutamate the time constant for desensitization was 6.5 ± 1.2 s, and the current desensitized 24 ± 7% of the peak current (n = 5). We did not systematically measure the time course for recovery from desensitization, but applications of 300 μM glutamate separated by 1 min had identical amplitudes, demonstrating that recovery from desensitization was complete within 1 min.

Ibotenic acid, a structural analog of glutamate known to activate glutamate-gated chloride channels (1, 5–9), maximally activated glutamate-sensitive current with desensitization kinetics similar to glutamate (Table I). L-Aspartate (1 mM) activated 5% of the maximal current, whereas other glutamate analogs such as kainic acid, quisqualic acid, and N-methyl-D-aspartic acid were inactive. Agonists known to activate vertebrate and invertebrate chloride channels such as glycine, GABA, and histamine were also inactive.

IVMPO\textsubscript{4} slowly and irreversibly activated current in oocytes injected with DrosGluCl-α (Fig. 2B). Washing with drug-free frog saline for 10 min after application of IVMPO\textsubscript{4} failed to return the current to base line. The EC\textsubscript{50} for IVMPO\textsubscript{4} was 41 nM.
Glutamate and IVMPO₄-sensitive currents, respectively, were activated with IVMPO₄ (Table I). Ivermectin, the commercially used insecticide abamectin, and the avermectin analog L-648,548, all maximally activated current at 500 nM (Table I). The maximal current elicited with IVMPO₄ was 35% of the maximal current elicited with 300 μM glutamate.

Avermectins potentiate the glutamate response of C. elegans GluCl channels (4, 9, 11). Pretreatment of oocytes expressing C. elegans GluCl channels with concentrations of avermectin below the threshold for direct activation increases glutamate-sensitive currents 300–800% (4, 9, 11). In contrast, the glutamate (10 μM) response of DrosGluCl-α-injected oocytes was potentiated only 24 ± 5% (n = 7), after a 2-min pretreatment with 1 nM IVMPO₄ (Fig. 3). The peak glutamate-sensitive current elicited with 300 μM glutamate was not altered significantly. When the IVMPO₄ concentration during the pretreatment was increased to 10 nM, we failed to observe any significant potentiation of the glutamate response and found a 49 ± 5% (n = 3) reduction in the maximal glutamate response. In addition, 10 nM IVMPO₄ slowed the rate of desensitization of the glutamate response (data not shown).

The current voltage relationship for the IVMPO₄-sensitive current was slightly inwardly rectifying (Fig. 4A). The reversal potentials of −28 ± 1 mV (n = 3) and −27 ± 1 mV (n = 4) for glutamate and IVMPO₄-sensitive currents, respectively, were close to the Nernst potential for chloride (−28 mV, assuming 40 mM intracellular chloride) (18). The reversal potential of the IVMPO₄-sensitive current showed a strong dependence on extracellular chloride with a 57 mV change for a 10-fold change in external chloride (Fig. 4B). Replacement of external NaCl with KCl or choline chloride did not shift the reversal potential or the shape of the current voltage relationship. Picrotoxin is an inhibitor of ligand-gated chloride channels which inhibits glutamate-gated chloride channels weakly (1, 4–9). The IVMPO₄-sensitive DrosGluCl-α current was resistant to picrotoxin at concentrations up to 100 μM and was blocked only 14% with 500 μM.

Analysis of DrosGluCl Transcripts—Several different size classes of RNA transcripts were identified, by Northern blot analysis, from three different developmental stages of Drosophila: embryo, larvae, and adult (Fig. 5A). Transcripts of 4.4 and 9.0 kb were found exclusively in embryo and larvae, and those...
of 9.7 and 6.0 kb were found solely in adult. The predominant 3.0-kb transcript, found in all stages, appeared to be underrepresented in larvae. Since the 4.0-kb DrosGluCl-α cDNA was isolated from an adult cDNA library it may be derived from any one of the larger transcripts detected by Northern analysis. The multiple transcripts detected in this high stringency hybridization analysis of Drosophila RNA may indicate the presence of other GluCl subunits, as has been observed in C. elegans (4). Multiple related subunits have been identified in other ligand-gated chloride channels (19).

Transcripts related to DrosGluCl-α were found in other insects: C. felifs (flea), O. moubata (tick), S. fugiperda (fall army worm), and H. zea (tomato fruitworm), but not in the arthropod T. urticae (two spotted spider mite). High stringency washes of RNA from DrosGluCl-α transcripts indicated that there was a high degree of identity between DrosGluCl-α and the major transcripts found in flea, fall army worm, and tomato fruitworm (data not shown). No C. elegans transcripts were detected, even though the DrosGluCl-α cDNA is 60% identical to the C. elegans GluCl-α and -β cDNAs. Several rat transcripts hybridized at low stringency to DrosGluCl-α but were only faintly detected following a high stringency wash (data not shown).

DISCUSSION

DrosGluCl-α encodes a glutamate-gated chloride channel with characteristics similar to those first reported for the H-receptor glutamate-gated chloride channels on locust leg muscle (1-3, 8). Insect glutamate-gated chloride channels have since been reported in Drosophila muscle (20), in several insect neuronal preparations (6, 7, 21), and have been expressed from S. gregaria leg muscle mRNA (22). DrosGluCl-α channels expressed in Xenopus oocytes are pharmacologically and kinetically similar to insect glutamate-gated chloride channels. Both the expressed and native channels desensitize rapidly in the continued presence of glutamate and are activated by the structurally constrained glutamate agonist ibotenate (1, 2, 8, 23, 24). Similarly, native and expressed channels are activated weakly with aspartate (23) and inhibited with high concentrations of picrotoxin. Finally, avermectins directly activate glutamate-gated chloride channel conductance in S. gregaria leg muscle (10, 25), as do they for the DrosGluCl-α subunit expressed in Xenopus oocytes.

The physiological relevance of the glutamate-gated chloride channels remains unknown. Excitatory glutamate-gated cation channels are localized in insect neuromuscular junctions, the site of glutamate release (26). Glutamate-gated chloride channels, on the other hand, are located extrajunctionally on the muscle and on neuronal cell bodies, both of which are distal to the glutamate synapses (1-3, 6, 8, 21, 23). It is possible that the glutamate-gated chloride channels are activated by "spillover" of glutamate from hyperpolarizing synapses to limit muscle excitability. Alternatively, the inhibitory action of the GluCl channel would act to limit calcium entry following muscle or nerve damage. Other roles could include developmental regulation and feeding behavior. Genetic analysis with Drosophila mutants defective in the DrosGluCl-α gene may resolve these issues.

Several properties of the DrosGluCl-α channel differ from the nematode C. elegans GluCl-α and -β channels. A single insect subunit is sufficient to enable avermectin and glutamate gating, whereas two nematode subunits are required (4). The DrosGluCl-α channel desensitizes rapidly and completely in the continued presence of glutamate compared with a slow and incomplete desensitization of the nematode channel (4). Finally, the DrosGluCl-α channel is less sensitive to avermectin potentiation of the glutamate response. The presence of other related genes in both C. elegans and Drosophila is evident both by Northern analysis and by PCR amplification of related genes (data not shown). It remains to be seen whether there are additional subunits in Drosophila which, like the nematode GluCl-α and -β, are uniquely sensitive to either avermectin or glutamate.

The results presented here clearly show that the DrosGluCl-α channel is a target for the avermectin class of insecticides. Avermectins also interact with insect GABA channels (25, 27, 28), therefore, multiple targets for the avermectins may be present in insects. However, since no GluCl channels have been identified in mammals, where GABA channels are ubiquitous, the GluCl channels may be the safer target for future insecticide development.

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