Molecular Cloning of an Invertebrate Glutamate Receptor Subunit Expressed in *Drosophila* Muscle

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Insects and other invertebrates use glutamate as a neurotransmitter in the central nervous system and at the neuromuscular junction. A complementary DNA from *Drosophila melanogaster*, designated DGluR-II, has been isolated that encodes a distant homolog of the cloned mammalian ionotropic glutamate receptor family and is expressed in somatic muscle tissue of *Drosophila* embryos. Electrophysiological recordings made in *Xenopus* oocytes that express DGluR-II revealed depolarizing responses to L-glutamate and L-aspartate but low sensitivity to quisqualate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate. The DGluR-II protein may represent a distinct glutamate receptor subtype, which shares its structural design with other members of the ionotropic glutamate receptor family.

AMINO ACIDS ARE CONSIDERED PRImordial neurotransmitter substances that were used in synaptic communication before the evolution of specialized transmitters such as acetylcholine and catecholamines (*1*). Consistent with this view, L-glutamate constitutes the major excitatory neurotransmitter of the mammalian central nervous system (*2*) and has been implicated in important physiological and pathological processes, including developmental plasticity, long-term potentiation, and excitotoxic damage in ischemia and other neurodegenerative disorders (*3*). The diverse effects of glutamate are produced through a set of heterogeneous glutamate receptor subfamilies that are classified according to their pharmacological and electrophysiological properties as the *N*-methyl-D-aspartate (NMDA) subtype of glutamate-gated ion channels and the non-NMDA receptors, a category that includes the metabotropic quisqualate receptors and the L-aminophosphonobutyrate (L-AP4)–sensitive receptors as well as the kainate-AMPA ionotropic receptor subtypes (*2*). Recently, several mammalian kainate-AMPA–sensitive glutamate receptor proteins, GluR1 to GluR6 (*4–6*), a distant relative, GluR5 (*6*), and a metabotropic glutamate receptor (*7*), became accessible to pharmacological and functional analysis by cDNA cloning and are differentially expressed in mammalian brain.

Glutamate also serves as a neurotransmitter in the invertebrate central nervous system (*8*) and at insect and crustacean neuromuscular junctions (*9*). Insect muscle glutamate receptors, which constitute the functional equivalent to the nicotinic acetylcholine receptor of vertebrate muscle, are considered model glutamate-gated receptors (*10*). Cation-conducting (excitatory, D-type) and anion-conducting (inhibitory, H-type) glutamate receptors have been seen by patch-clamp techniques; both increase in density upon muscle denervation (*11*). The insect and crustacean D-type receptors are sensitive to quisqualate, but ibotenate- and aspartate-gated synaptic receptor subpopulations also occur (*12*). Some of these receptors are blocked by the competitive NMDA antagonist D-aminophosphonovalerate (D-APV) and low molecular weight toxins from wasp and spider venoms (*13*). These insect muscle proteins are therefore considered an evolutionarily distant subtype of excitatory glutamate receptors (*10*). Here we report the cloning and characterization of a *Drosophila* cDNA named DGluR-II, which is expressed in somatic musculature and encodes a distant relative of the known mammalian ionotropic glutamate receptor proteins.

The rat ionotropic glutamate receptor polypeptides (*4–6*) and the related kainate-binding proteins of chick and frog brain (*14*) share considerable sequence identity. We have exploited this homology to isolate *Drosophila* cDNAs encoding putative invertebrate glutamate receptor proteins. Glutamate receptor–related sequences from *Drosophila* genomic DNA were amplified by the polymerase chain reaction (PCR) with degenerate oligonucleotide primers (*15*) deduced from peptide sequences conserved in GluR1 (*4*) and the kainate-binding proteins (*14*). This resulted in the identification of two homologous genomic sequences, gDGluR-I and gDGluR-II (*16*). We then screened *Drosophila* embryonic and adult cDNA libraries with ^{32}P-labeled genomic probes (*17*). Several overlapping cDNA clones were thus isolated, which, for DGluR-II, had a combined size of 3214 bp, a value close to that of the mRNA determined by Northern blot analysis (see below). The polypeptide sequence predicted from the single open reading frame constitutes a presumptive DGluR-II protein of 906 amino acids, which, after cleavage of a putative signal peptide of 23 residues, has a calculated molecular mass of 101,790 daltons (Fig. 1). The cytogenetic localization of the DGluR-II gene was determined by in situ hybridization of a DGluR-II probe on salivary gland polytene chromosomes (*18*). The DGluR-II gene maps on chromosome 2L at position 25F (*19*), a region characterized by several lethal mutations (*20*).

Comparison of the DGluR-II amino acid sequence with the rat glutamate receptor subtypes (*4–6*) revealed overall amino acid identities between 26 and 28% (Fig. 1 and Table 1). In all of the glutamate receptor proteins analyzed, the highest conservation was found in the COOH-terminal half of the polypeptides, the putative ionotropic glutamate receptor "core" domain (residues 408 to 822 of DGluR-II in Fig. 1). Core sequence identities of 37 to 38% (Table 1) suggest that the DGluR-II protein is a distantly related member of the glutamate receptor family. Hydropathy plot analysis (*21*) resulted in a profile similar to that of rat GluR5 (*19*). Although up to seven potential membrane spanning regions may be assigned to the mature polypeptide (*6*), more precise topology predictions remain highly speculative. However, some sequence features, including the distribution (i) of charged residues around four putative core transmembrane regions, (ii) of conserved potential NH_2-linked glycosylation sites in the hypothetical extracellular non-core sequence, and (iii) of conserved cysteine residues (Fig. 1), are consistent with a four-transmembrane model proposed for the superfamily of ligand-gated ion channel proteins (*22*).

The expression pattern of DGluR-II mRNA during *Drosophila* development and its tissue distribution are shown in Fig. 2. Prominent expression of DGluR-II mRNA in the late embryo (Fig. 2A) coincided with strong labeling of the somatic musculature at embryonic stage 16 (Fig. 2B). Visualization of DGluR-II transcripts in embryos by whole-mount in situ hybridization (*23*) revealed a typical segmental organization (*24*). On serial focusing, this gave the impression of stripes laterally lining the body segments and was particularly prominent in segments t1 and a1 to a7. This staining pattern corresponds to the distribution of the somatic musculature and was never observed when

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Table 1. Homology of the DGluR-II protein and rat glutamate receptor subunits. Amino acid identities between the DGluR-II sequence and GluR1 (*4*), GluR4 (*5*), and GluR5 (*6*) were calculated (*32*) for both the core domains (upper right values) and the entire mature polypeptides (lower left values).

Receptor protein	Amino acid identity (%)			
	GluR1	GluR4	GluR5	DGluR-II
GluR1		87.9	53.2	37.4
GluR4	68.8		53.0	38.2
GluR5	38.1	36.8		38.4
DGluR-II	25.9	28.2	28.4	

we used the homeotic ultrabithorax (ubx) cDNA (*25*) or a fragment of DGluR-I (*16*) as probes. Rather, the DGluR-I mRNA was homogeneously expressed in the central nervous system of *Drosophila*, whereas ubx transcripts were segmentally distributed in the ventral cord (*25*) (Fig. 2B). The embryonic expression pattern of DGluR-II resembled that described for other mRNAs expressed in developing muscle, such as β3-tubulin, which appears after formation of the mesodermal epithelium at stage 10 (*24*). At stage 16, the final pattern of the embryonic and larval somatic musculature develops (*26*), and thereafter, in first instar larvae, we observed a decrease in transcriptional activity of the DGluR-II gene (Fig. 2A). DGluR-II mRNA expression resumed during larval growth, a result that may relate to the increasing motility of second and third instar larvae (Fig. 2A). In pupae and in adult flies, small amounts of DGluR-II transcripts were found (Fig. 2A). The temporal and spatial accumulation of DGluR-II transcripts indicates that this glutamate receptor homolog is expressed early in muscle development.

DGluR-II was transiently expressed in *Xenopus* oocytes, either by cytoplasmic injection of synthetic DGluR-II RNA or by nuclear injection of DGluR-II cDNA cloned into the eukaryotic expression vector pCIS (*27*). L-Glutamate (100 μM to 100 mM) induced inward currents of up to 500 nA (Fig. 3A) that displayed a half-maximal response at a concentration (EC_{50}) of about 35 mM and reversed at membrane potentials of about −10 mV ($n = 4$). Similar currents were also elicited by L-aspartate ($EC_{50} \approx 50$ mM) (Fig. 3D). The glutamate receptor agonists quisqualate, AMPA, and kainate induced only small inward currents (<10 nA) at a concentration of 10 mM (*19*). Control oocytes injected with glycine receptor α1 subunit cDNA (*28*) were sensitive to glycine ($EC_{50} \approx 300$ μM) but never responded to L-glutamate (100 mM) or L-aspartate (100 mM). Correspondingly, DGluR-II–injected oocytes were consistently insensitive to glycine or γ-aminobutyric acid applied at 100 mM, and the response to glutamate of these oocytes was not affected by the presence of these inhibitory neurotransmitter amino acids. Other amino acids, including β-alanine, L-serine, and L-glutamine, also had no effect up to 100 mM, and glutamate-induced currents were insensitive to the glutamate receptor antagonists (*2, 13*)

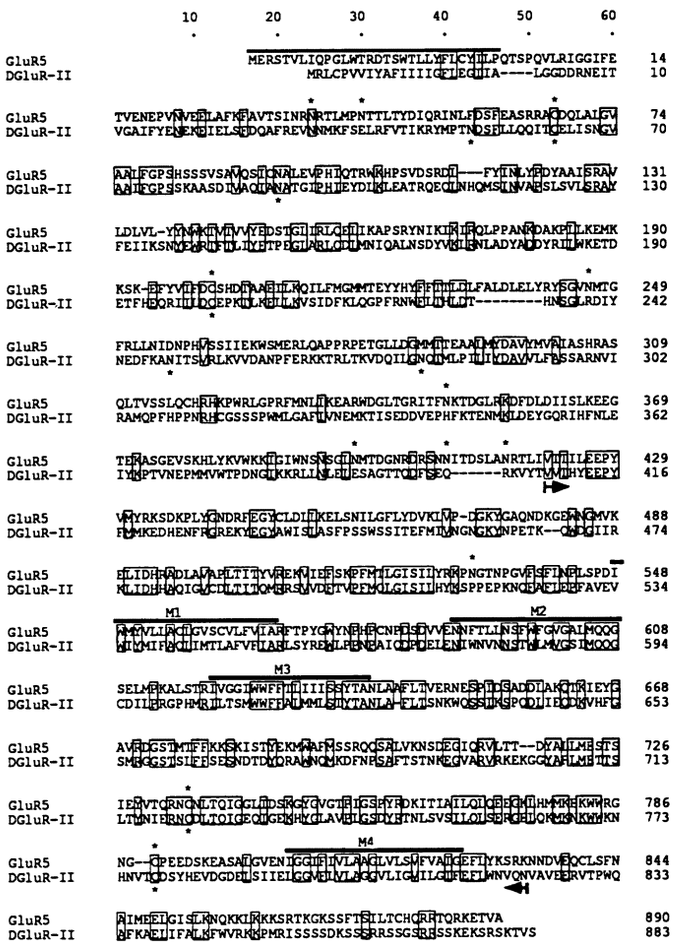

Fig. 1. Alignment of the amino acid sequences encoded by the invertebrate and vertebrate glutamate receptor cDNAs, DGluR-II and GluR5 (*6*). Numbering starts with the NH_2-terminal amino acid of the presumptive mature polypeptides. Signal peptides and putative transmembrane regions are overlined, identical residues are boxed, predicted extracellular NH_2-linked glycosylation sites and conserved cysteine residues are indicated by stars, and arrows delineate the borders of the proposed glutamate receptor core domain. Gaps were introduced to maximize homology. The complete nucleotide sequence of DGluR-II is deposited in the GenBank-EMBL database (accession number, M73271).

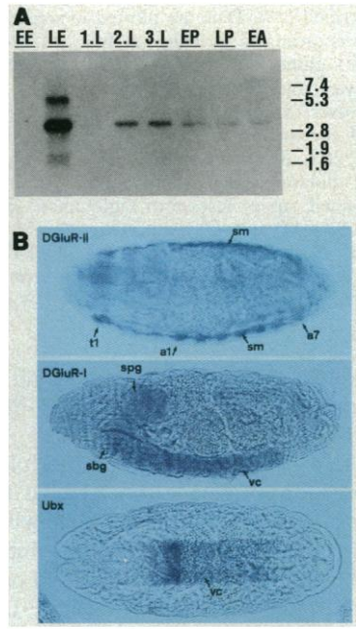

Fig. 2. Temporal and spatial accumulation of DGluR-II transcripts revealed by (**A**) Northern blot analysis and (**B**) whole mount in situ hybridization of *Drosophila* embryos. (A) The DGluR-II probe labels a 3.6-kb RNA that is regulated during development. Poly(A)$^+$ RNA isolated at different developmental stages (EE, early embryos, 0 to 4 hours; LE, late embryos, 14 to 22 hours; 1.L, first instar larvae; 2.L, second instar larvae; 3.L, third instar larvae; EP, early pupae; EA, early adult flies, 1 to 2 days after eclosion) was separated by electrophoresis (5 μg per lane), transferred to a nylon membrane, and hybridized to a ^{32}P-labeled DGluR-II cDNA fragment (1100 bp) as described (*33*). The sizes of RNA molecular weight markers (in kilobases) are given on the right. (B) Whole mount in situ hybridizations were performed as described (*23*). All embryos are oriented anterior to the left. Top and bottom, dorsal views of stage 16 embryos hybridized to the respective probes; center, lateral view of a stage 16 embryo hybridized to a DGluR-I–specific probe. An ultrabithorax (*25*) whole mount in situ hybridization performed in parallel was included as a control of specificity. Abdominal segments, a1 and a7; thoracic segment, t1; subesophageal ganglia, sbg; supraesophageal ganglia, spg; ventral cord, vc; somatic musculature, sm.

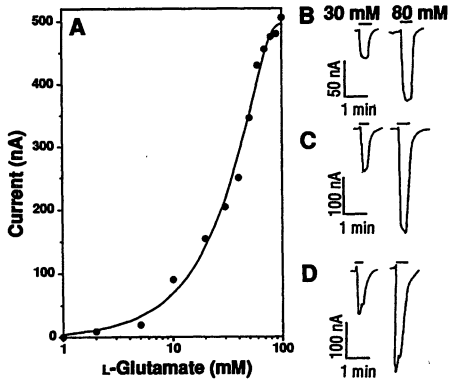

Fig. 3. Agonist-evoked currents in oocytes expressing DGluR-II polypeptide. (**A**) To investigate the dose-response relationship of L-glutamate, oocytes were injected with a DGluR-II cDNA construct (*27*) and voltage-clamped to −70 mV, and recordings were performed as described (*28*). Data are plotted in semilogarithmic coordinates showing an EC_{50} of about 35 mM L-glutamate. This result was also obtained in oocytes injected with DGluR-II cRNA which, however, exhibited generally lower agonist-generated currents. In different injection series (n > 30), 20 to 80% of the injected oocytes responded to L-glutamate. (**B** and **C**) Typical current traces obtained on application of L-glutamate (30 mM and 80 mM) and (**D**) L-aspartate (30 mM and 80 mM). Oocytes were injected with either DGluR-II cRNA (B) or DGluR-II cDNA construct (C and D). Perfusion times are indicated by bars.

argiotoxin (2 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (100 μM), 6,7-dinitroquinoxaline-2,3-dione (10 μM), and D-APV (2 mM). Hence, on heterologous expression in *Xenopus* oocytes, DGluR-II formed functional cation channels that were preferentially gated by L-glutamate and L-aspartate and thus displayed properties of previously described invertebrate glutamate receptor subtypes (*1, 12*). The low agonist sensitivity of DGluR-II as compared to in vivo recordings from *Drosophila* larval muscle (*9*) may reflect the lack of complementary subunits. Indeed, a potentiation of maximal current responses is seen on coexpression of the mammalian glutamate receptor proteins GluR1 to GluR4 (*5*). On the other hand, *Drosophila* hemolymph contains 0.9 mM glutamate (*29*); low-affinity agonist binding thus may be crucial for preventing persistent channel activation. Moreover, rapid desensitization (*10, 30*) may have masked fast events under our recording conditions.

Extensive single-channel analysis of D-type glutamate receptors in locust muscle has generated a detailed picture of this allosterically gated, relatively unselective cation channel that has at least four binding sites for glutamate (*31*). The features of DGluR-II indicate that such insect receptors are also members of the ionotropic glutamate receptor protein family. Because DGluR-II is sensitive to L-aspartate, this protein might share common domains not only with the already cloned mammalian glutamate receptor polypeptides of the kainate-AMPA subfamily (Fig. 1), but also with the elusive NMDA receptor subtype. By mutational analysis of the DGluR-II gene, the role of glutamate receptors in development, synaptic plasticity, and neuronal control of gene expression may now be approached in one of the best characterized developmental and genetic model systems, the fruit fly *Drosophila melanogaster.*

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