Volume 292, number 1,2, 111–114 FEBS 10331 November 1991

ADONIS 001457939101019D

Cloning of a cDNA that encodes an invertebrate glutamate receptor subunit

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Received 1 August 1991

A full-length cDNA which encodes a putative glutamate receptor polypeptide was isolated from the pond snail *Lymnaea stagnalis*, using a short stretch of exonic sequence and two variants of the polymerase chain reaction. In this first comparison of invertebrate and vertebrate glutamate receptor sequences, the mature molluscan polypeptide, which comprises 898 amino acids and has a predicted M_r of 100 913, displays between 37% and 46% amino-acid identity to the rat ionotropic glutamate receptor subunits, GluR1 to GluR6.

cDNA cloning; Gene cloning; Glutamate receptor; Invertebrate receptor; *Lymnaea stagnalis*; Polymerase chain reaction

1. INTRODUCTION

In vertebrates, the excitatory post-synaptic effects of glutamate are mediated by ligand-gated cation channels (ionotropic receptors), the receptor types of which can be distinguished by the agonists *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate (KA); glutamate can also modulate the release of intracellular Ca^{2+} through binding to a metabotropic receptor (reviewed in [1]). In contrast, in invertebrates, L-glutamate can act both as an excitatory and an inhibitory neurotransmitter. For example, the effect of L-glutamate, when applied to molluscan neurones, is to induce either a fast depolarizing cationic current, a slow hyperpolarizing K^+ current, or a fast hyperpolarizing Cl^- current [2].

Recently, molecular biological studies have led to the isolation of 6 different vertebrate non-NMDA ionotropic glutamate receptor subunit cDNAs [3–8]. When these cDNAs were expressed singly either in *Xenopus* oocytes [3,5–8] or in mammalian cells [4], homo-oligomeric receptors were formed that were AMPA-selective (GluR1 to GluR4), KA-selective (GluR6) or responsive to L-glutamate alone (GluR5). No sequence has yet been reported for any invertebrate glutamate receptor. We describe here the isolation of genomic and cDNA sequences that encode a putative glutamate receptor polypeptide from the fresh-water snail *Lymnaea stagnalis*.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; cDNA, complementary DNA; KA, kainate; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction.

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2. MATERIALS AND METHODS

2.1. *Isolation of a molluscan glutamate receptor genomic clone*

5×10^5 clones of an amplified *L. stagnalis* genomic library, constructed in λEMBL3, were screened with an ~700 bp polymerase chain reaction (PCR)-derived cDNA fragment that encodes the first three putative membrane-spanning domains (TM1 to TM3) of the rat GluR1 subunit [3]. Hybridization was in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.5% (w/v) SDS, 5 × Denhardt's solution and 100 μg/ml yeast tRNA, at 50°C for 48 h; the final wash conditions were 1 × SSC, 0.1% (w/v) SDS at 55°C for 30 min. The library filters were then stripped and hybridized, under identical conditions, with an ~1.8 kb *Eco*RI cDNA fragment that encodes part of a locust putative glutamate receptor polypeptide (unpublished results). Only one clone hybridized to both probes; a 166 bp *Sau*3A$_I$ fragment from this was subsequently subcloned and sequenced.

2.2. *Isolation of molluscan full-length glutamate receptor cDNAs*

Full-length cDNAs were generated using two variants of the PCR. The 3′ end of the cDNA was amplified (see Fig. 1) using nested primers [9]. For this, first-strand cDNA was synthesized from *L. stagnalis* egg mass poly(A)$^+$ RNA using oligonucleotide $R_oR_idT_{17}$: 5′-ATCGATGGTCGACGCATGCGGATCCAAAGCTTGAATTCGAGCTCTTTTTTTTTTTTTTTT-3′. First-stage PCR was then performed using primers R_o: 5′-ATCGATGGTCGACGCATGCGGATCC-3′, which corresponds to part of $R_oR_idT_{17}$, and LGL1: 5′-TGTCGGGAGAATTCGTCGACTCAGTCTGGTGGTTCTTCACGCTCA-3′, which is based on exonic sequence from the 166 bp *Sau*3A$_I$ fragment. Reactions (50 μl) contained: 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25°C), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.1% (v/v) Triton X-100, 0.01% (w/v) gelatin, 1 nmol of T4 gene 32 protein (United States Biochemical), ~20 ng of first-strand cDNA, 25 pmol

of each primer, and 2.5 units of *Thermus aquaticus* DNA polymerase (Promega). Amplification was for 40 cycles of 94°C for 1 min (denaturation) and 72°C for 3 min (annealing and extension), with a final extension at 72°C for 10 min. For second-stage PCR, primers R_1: 5'-GGATCCAAAGCTTGAATTCGAGCTC-3', which also corresponds to part of $R_0R_1dT_{17}$, and LGL2: 5'-GCCGCGTTGTCGACTCTAGAGCGGATGTTGACGCCCATAGACTCG-3', which again is based on exonic sequence from the *Sau*3A$_1$ genomic fragment, were used. Reaction conditions were as described above except that 2 μl of the first-stage PCR products was substituted for the first-strand cDNA.

The 5' end of the cDNA was amplified directly [10] from a *L. stagnalis* nervous system cDNA library (Stratagene), constructed in λgt10. Essentially, PCR amplification was performed on DNA from $\sim 10^7$ pfu using primers whose sequences were based on those of the vector arms (λA: 5'-CTTGAGCTCAAGTTCAGCCTGGTTAAGTCCAAGCT-3' or λB: 5'-AGAGGAAGCTTATGAGTATTTCTTCCAGGGTAAAAA-3'; see Fig. 1), in combination with LGL3: 5'-CTGGCCAGGAATTCTGTCGACTCTATGGGCGTCAACATCCGCTCCA-3', which was designed using exonic sequence from the *Sau*3A$_1$ genomic fragment. Reaction conditions were as described for 3'-end cDNA amplification. Since truncated cDNAs are amplified from the library preferentially, an additional primer, LGL4: 5'-GACTTTTTTGAATTCGTCGACAATCATTTGACCGTGACCAGTCTTG-3', was used with either λA or λB to isolate further 5' sequences. The sequence of LGL4 was based on that of partial cDNAs amplified from the library as described above.

Full-length cDNAs were amplified using primers LGL5: 5'- ATACTGTGGGATCCCCGTGTCAGATGGACACCTGTGT-3' and LGL6: 5'-TGTTATCACTCGAGATGAGCTGTGTCTTGGCAAGAGCT-3' (see Fig. 1) the sequences of which were based on parts of the 5'- and 3'-untranslated regions that were predicted from partial cDNA clones. The reaction contained 25 pmol of each primer and ~20 ng first-strand cDNA, synthesized from *L. stagnalis* egg mass poly(A)$^+$ RNA using $R_0R_1dT_{17}$. PCR was for 40 cycles of 94°C for 1 min (denaturation), 65°C for 1 min (annealing) and 72°C for 4 min (extension), followed by a final extension at 72°C for 10 min. Specific 5'- and 3'-cDNA products were cloned into M13 vectors, and full-length products were cloned into pBluescript KS+ (Stratagene), taking advantage of restriction endonuclease recognition sites incorporated into the PCR primers, and sequenced.

3. RESULTS AND DISCUSSION

DNA sequence analysis of the 166 bp *Sau*3A$_1$ fragment from a genomic clone that was isolated revealed the presence of an open reading frame that encodes a portion of a polypeptide exhibiting strong similarity (86%) to the sequence of TM3 and to part of the proposed intracellular loop of the rat GluR1 sequence [3]. Northern blot analysis of Lymnaean poly(A)$^+$ RNA (from either egg mass, dissected adult nervous systems, or adult muscle tissue) using this genomic fragment as a probe failed to detect the molluscan transcript, indicating its extremely low abundance. The exonic sequence was, therefore, used to amplify several corresponding full-length cDNAs by PCR (Fig. 1). Sequence analysis of one of these (plGluR.PCR4) revealed an open reading frame that predicts a mature polypeptide of 898 amino acids, which has a calculated M_r of 100 913, and a 19 amino-acid signal peptide [11]. Hydropathic analysis [12] of this sequence (Fig. 2) clearly reveals the presence of three strongly-hydrophobic regions (each ~20 amino acids in length), the locations of which correspond to those of TM1, TM3 and TM4 (as defined by Keinänen et al. [4]) of vertebrate ionotropic glutamate receptor subunits. Several other less-pronounced hydrophobic segments are also present. In the absence of biochemical data on this invertebrate subunit, it is difficult to draw definite conclusions about the exact number and locations of membrane-spanning domains; however, for simplicity, we assume that the topology of this polypeptide is similar to that proposed for vertebrate glutamate receptor subunits [3,4].

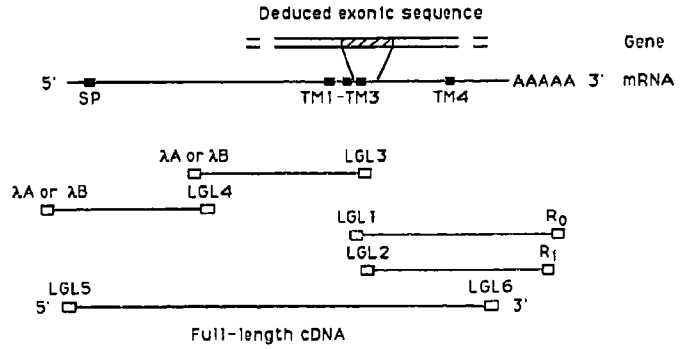

Fig. 1. Diagram showing the PCR strategy used to isolate a full-length cDNA. The relationship of the exonic sequence of the 166 bp *Sau*3A$_1$ genomic fragment (hatched box) to that of the mRNA is indicated. The relative locations of the signal peptide (SP) and the four putative membrane-spanning domains (TM1 to TM4; [4]), that are encoded by the mRNA, are shown. The details of the cloning are fully described in section 2.

The mature Lymnaean polypeptide displays significant identity to rat [3–6,8] glutamate receptor subunits (GluR1, 43%; GluR2, 46%; GluR3, 45%; GluR4, 43%; GluR5, 37% and GluR6, 39%); in particular, it exhibits pronounced similarity to vertebrate sequences around TM3 (Fig. 3). The molluscan subunit has 13 sites for the possible addition of N-linked sugars, 12 of which are in the proposed N-terminal extracellular domain [4]; the

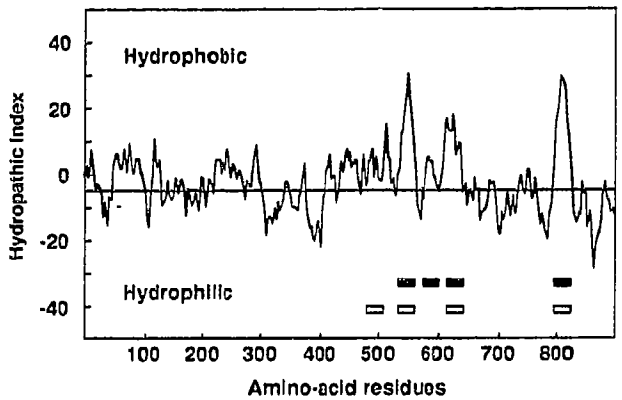

Fig. 2. Hydropathy plot of the mature molluscan polypeptide. The algorithm of Kyte and Doolittle [12] with a window size of 17 residues was used. Areas above the horizontal line are hydrophobic and those below the line are hydrophilic. Filled bars and open bars mark the relative locations of membrane-spanning domains, TM1 to TM4, as proposed by Seeburg and colleagues [4] and by Heinemann and colleagues [3], respectively, for rat glutamate receptor subunits.

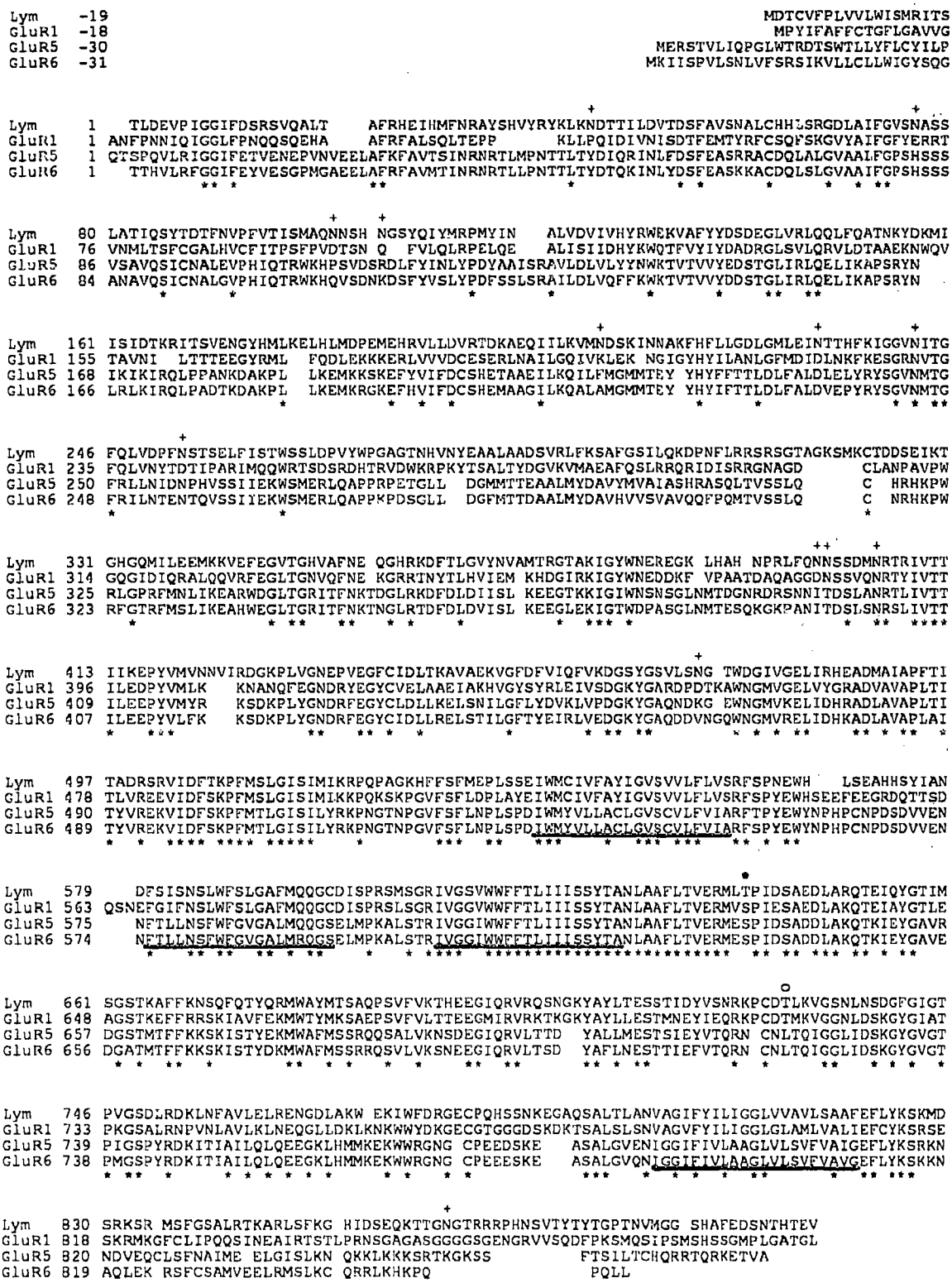

Fig. 3. Alignment of the amino-acid sequence of the molluscan polypeptide with those of three rat glutamate receptor subunits. The sequences of GluR1 (AMPA-selective; [3]), GluR5-2 (one form of GluR5 that is responsive to L-glutamate only; [6]), GluR6 (KA-selective; [8]) and that of the Lymnaean polypeptide (Lym; encoded by plGluR.PCR4) were aligned with the aid of the computer program MULTALIGN [13]. Positions at which the same amino acid is found in all four sequences are denoted by asterisks. Residues are numbered from the mature N-terminus of each polypeptide: signal peptides [11] are denoted by negative numbering. Numbers refer to the left-most amino acid on each line. Putative membrane-spanning domains (TM1 to TM4; [4]) are underlined. Sites in the Lymnaean sequence for the possible addition of N-linked sugars are indicated by crosses, and protein kinase C and calmodulin-dependent protein kinase type II consensus recognition sites [14], in the proposed intracellular domain (between TM3 and TM4), are marked by open and filled circles, respectively. The sequences of several Lymnaean, PCR-generated, full-length cDNAs have been given the EMBL accession number X60086.

other is found in the C-terminal 79 amino acids which occur after TM4, a region that has also been assigned an extracellular location in vertebrates [3,4]. In view of its predicted size, hydropathic profile and strong sequence similarity to vertebrate ionotropic glutamate receptor subunits, we conclude that this polypeptide is a subunit of a molluscan glutamate-gated ion-channel receptor.

To test whether the Lymnaean subunit is capable of forming a functional homo-oligomeric ion channel, RNA was transcribed in vitro from a full-length cDNA (plGluR.PCR4) and injected into *Xenopus* oocytes. The sensitivity of the oocytes to the application of different glutamate receptor agonists was then investigated for a period of up to 15 days. No reproducible responses could be recorded in a large number of oocytes (n>100) tested (N.S. Bhandal and P.N.R. Usherwood, unpublished). Negative results were also obtained upon injection of oocytes with RNA from three other PCR-generated full-length cDNAs, which encode polypeptides that differ from each other, and from that encoded by plGluR.PCR4, by only a few amino acids.

The electrophysiological results suggest either that the expression of the molluscan RNA in *Xenopus* oocytes is poor or, perhaps more likely, that the Lymnaean glutamate receptor subunit described here requires at least one additional subunit to form a fully-functional receptor complex. In this context, it is noteworthy that two vertebrate homo-oligomeric glutamate receptors (GluR2 and GluR5) show only very small responses to agonists when their corresponding cDNAs are expressed singly in *Xenopus* oocytes [5–7] and that co-expression studies [5,7] indicate that vertebrate glutamate receptors exist in vivo as hetero-oligomers.

Acknowledgements: We thank Dr Erno Vreugdenhil (Vrije Universiteit, Amsterdam) for his help in the initial stages of this work and for the *L. stagnalis* nervous system cDNA library, Michael D. Squire (Cambridge) for help with DNA sequence analysis, and Dr Fergus Earley (ICI, Jealott's Hill) for his interest in this work. M.L.H. is supported by an MRC Partnership Award with ICI, and R.J.H. was supported by an SERC Research Studentship.

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