Alternative Splicing Generates Two Variants of the $\alpha_1$ Subunit of the Inhibitory Glycine Receptor*

(Received for publication, September 7, 1990)

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The inhibitory glycine receptor (GlyR) in mammalian spinal cord displays pharmacological and molecular heterogeneity of its strychnine binding $\alpha$ subunit. Here, cDNAs were isolated which encode a variant ($\alpha'^{1}$) of the rat GlyR $\alpha_1$ subunit that contains eight additional amino acids in its putative cytoplasmic domain. Analysis of the corresponding genomic sequence showed that $\alpha'^{1}$ transcripts result from alternative splice acceptor site selection. S1 nuclease protection experiments, Northern blot analysis, and RNA amplification by polymerase chain reaction revealed $\alpha_1$ and $\alpha'^{1}$ mRNA in postnatal spinal cord, but not in other brain regions. Expression of synthetic $\alpha'^{1}$ RNA in Xenopus oocytes generated glycine-gated strychnine-sensitive chloride channels. These data indicate that alternative splicing contributes to GlyR $\alpha$ subunit heterogeneity in the mammalian central nervous system.

Glycine is a major inhibitory neurotransmitter in the vertebrate central nervous system (1). Glycine-mediated inhibition of neuronal firing results from an increase in chloride conductance that is antagonized by the plant alkaloid strychnine (2, 3). The inhibitory glycine receptor (GlyR) has been purified from mammalian spinal cord and shown to contain two types of subunits of molecular masses of 48 kDa (a) and 58 kDa (b) in addition to a 93-kDa receptor-associated peripheral membrane protein (4–7). Recently, the primary structures of GlyR $\alpha$ (8) and $\beta$ subunits (9) have been deduced by cDNA cloning. Both polypeptides share a common transmembrane topology and significant sequence homology with $\gamma$-aminobutyric acid and nicotinic acetylcholine receptor proteins (10, 11). It, therefore, is thought that ligand-gated ion channels form by assembly of homologous subunits which are members of an evolutionarily related protein superfamily (10, 12, 13).

Biochemical, immunological, and cDNA sequence data indicate molecular heterogeneity of the mammalian GlyR. Using monoclonal antibodies, a neonatal GlyR isoform has been identified that differs from the adult receptor in pharmacological properties and apparent molecular mass (49 kDa) of its $\alpha$ subunit (14, 15). Evidence for GlyR heterogeneity during development also comes from oocyte expression studies (16, 17). Furthermore, cDNAs encoding variants ($\alpha_1$ and $\alpha_2$) of the adult GlyR $\alpha$ subunit (now termed $\alpha_1$) have been sequenced and shown to produce functional GlyR upon expression in Xenopus oocytes (18, 19). At least two of the corresponding human genes map onto different chromosomes (18, 20); these cDNA variants thus represent individual primary transcripts. Here we report that alternative splicing of $\alpha_1$ subunit pre-mRNA further contributes to GlyR diversity in rat spinal cord. Differential splice acceptor site selection is shown to generate an eight-amino acid insertion in the putative cytoplasmic domain of this ligand binding receptor subunit.

MATERIALS AND METHODS

Isolation and Analysis of DNA Clones—Clone put20 was isolated by screening a randomly primed $\lambda$gt11 rat spinal cord cDNA library (21) under low stringency conditions with the nick-translated HindIII/EcoRI fragment of clone GR2 (8). Two additional cDNAs of 1372 and 1038 bp (see Fig. 1a) were isolated upon screening of a Xgt10 randomly primed rat brain stem cDNA library (Clontech) with a 38-mer oligonucleotide which contained the 24-bp insert of clone put20 plus seven flanking nucleotides on each side. All cDNA inserts were subcloned into pSPT18 or pBluescript, and both strands were sequenced (22) using different $\alpha_1$-specific oligonucleotide primers.

The genomic clone gGR7 was isolated from a rat liver genomic library in EMBL3 (gift of G. Schütz, German Cancer Research Center, Heidelberg, FRG) using the nick-translated clone put20 as a probe. Its 12.3-kilobase Sau3A insert was digested further with EcoRI and subcloned in pUC18. Southern blotting revealed an EcoRI/SalI fragment of 4.8 kilobases which hybridized to nick-translated put20 DNA. Further digestion yielded a 148-bp BanI subfragment that hybridized to a synthetic 24-mer oligonucleotide representing the insert of clone put20. The sequence of this genomic fragment was determined after subcloning in M13mp18.

Tryptic Digestion of GlyR Subunits and Peptide Microsequencing—GlyR isolated by affinity chromatography from rat spinal cord according to Pfeiffer et al. (4) was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel in the presence of thioglycolic acid (23). Subunit bands were isolated, subjected to tryptic digestion, and peptides dissolved in 70% formic acid and purified by HPLC as described previously (24) except that a Nucleosil C8 RP column was used. Peptide-containing peaks were collected, concentrated by lyophilization, and either directly subjected to amino acid sequence analysis on an Applied Biosystems 470A Gas Phase Sequencer or further purified by HPLC using shallow aetontitrile gradients. As complete separation of the $\alpha$ and $\beta$ subunits on preparative gels proved difficult, mixtures containing variable amounts of each polypeptide were used for tryptic cleavage. By analyzing a preparation enriched in $\alpha$ subunit, the insert peptide sequence was obtained.
S1 Nuclease Protection Experiments—The BanI fragment of clone gGR7 was cloned in a SmaI-digested pSPT18 vector (Boehringer Mannheim). After linearization of the plasmid containing the cloned fragment was obtained using a SP6 polymerase transcription kit (Pharmacia). An antisense single-stranded cDNA probe was prepared according to Weih et al. (25) as follows: the synthetic mRNA (~1.5 pmoles) was used as template for reverse transcriptase (27 units; Boehringer Mannheim) in the presence of 1 mM dGTP, dCTP, dTTP, each, and 100 µCi of [α-32P]dATP (400 Ci/mmol; Amersham Corp.), T7 primer (10 pmol), 10 mM dithiothreitol, 50 mM Tris/Cl, pH 8.0, 8 mM MgCl₂, 30 mM KCl, and 15 units RNasin (Promega). This procedure yielded a probe with a specific activity of 1.5–2.5 × 10⁶ cpm/µg. After alkaline hydrolysis of the template RNA, the DNA was extracted with phenol/ chloroform and concentrated by ammonium acetate/ethanol precipitation in the presence of 1 µg of Escherichia coli tRNA.

About 3.0–4.5 × 10⁵ cpm of radiolabeled fragment were hybridized to 2.5 µg of poly(A)⁺ RNA isolated from spinal cord or different brain regions (26) for 6–10 h at 50 °C in 400 mM NaCl, 20 mM Tris/Cl, pH 7.4, 1 mM EDTA, and 80% (v/v) formamide (27). Hybridization to GlyR α₁ RNA (17) served as a positive control. Nuclease digestion of the resulting RNA-DNA hybrids was performed in 300 αl of S1 reaction buffer (300 mM NaCl, 3 mM ZnSO₄, 60 mM sodium acetate, pH 5.2) in the presence of 10 µg of E. coli tRNA and 500 units of nuclease S1 (Boehringer) for 3–4 h at 25 °C. Control reactions were incubated in parallel without the enzyme or containing E. coli tRNA instead of poly(A)⁺ RNA fractions. Aliquots of all samples were analyzed on 8% polyacrylamide, 7 M urea gels as described (28).

Northern Blot Analysis and PCR Experiments—Spinal cord poly(A)⁺ RNA isolated from 20-day-old rats was electrophoresed (20 µg/lane) on a 1% formaldehyde/agarose gel, blotted to Hybond-N (Amersham Corp.), and, after UV-cross-linking, hybridized to a 38-mer oligonucleotide 3'-labeled by terminal transferase (6 × 10⁶ cpm/µg), which covered the insert sequence (positions 108–145 of put20, or to the nick-translated clone GR2 (2–7 × 10⁶ cpm/µg). Hybridizations were performed in 5 × SSC (0.6 M NaCl, 75 mM sodium citrate), 50 mM sodium phosphate, pH 6.5, at 37 °C in the presence of 20% (for the oligonucleotide) or 50% (for GR2) formamide. Washings were in 0.5 × SSC at 60 °C and 55 °C, respectively. After reverse transcription of 5 µg of poly(A)⁺ RNA using a DNA synthesis kit (Boehringer Mannheim), 20–50 ng of the resulting single-stranded cDNA, or 1 ng of α₁ subunit cDNA (see below), were submitted to 25–30 cycles of PCR under standard conditions (29). Two different antisense oligonucleotides (positions 115–138 and 91–114 in Fig. 1b) were used for the amplification in combination with a sense pre-Cys-Cys’ primer (positions 414–430 according to Ref. 8). After separation on a 1% agarose gel and Southern blotting, specific products were identified by hybridization to the nick-translated clone GR2 under high stringency conditions (8).

Construction of α₁ Full-length cDNA—To obtain a full-length α₁ subunit cDNA, the insert of the previously described α₁ plasmid in pSP64 (17) was recloned in M13mp18, and 100 ng of antisense single strand DNA was used as a template for oligonucleotide-directed mutagenesis. A 64-mer oligonucleotide corresponding to nucleotides 95–158 of clone put20 served as a primer for second strand DNA synthesis by T4 DNA polymerase (30). After transformation of E. coli strain SR101, clones containing the variant sequence (α₁ns) were identified using the [32P]-labeled 64-mer oligonucleotide as a hybridization probe. After two rounds of phage purification, a fragment containing the mutated sequence was isolated by EcoRI digestion and reintroduced into the original pSP64 α₁ cDNA construct by replacing its corresponding EcoRI fragment. The resulting full-length α₁ns cDNA was isolated as a 3.4-kb EcoRI fragment of clone GR2.

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**Fig. 1. a**, restriction map of α₁ns cDNA clones. H, HindIII, M, MboII, N, NcoI; nucleotide positions are indicated in brackets. The coding region is boxed, with black regions representing transmembrane domains and a dotted one the signal peptide. In the putative cytoplasmic loop (regions 3 and 4, the 24-bp insertion is numbered on the left. c, alignment of the GlyR α₁ subunit cDNA and clone put20. The predicted protein sequence is indicated below the codons. The additional eight amino acids encoded by the 24-bp insertion of put20 are underlined. Nucleotide and amino acid positions are numbered on the left, c, alignment of the BanI fragment of the genomic clone gGR7 and the put20 cDNA. Only the corresponding regions are shown in addition to some intronic sequence (lowercase letters). Arrows indicate predicted S1 nuclease cleavage sites. Putative splice acceptor sites in front and at the end of the 24 additional nucleotides are underlined. In b and c, identical nucleotides are marked by asterisks. Continuing asterisks in c indicate identity between the genomic and the α₁ sequence. A single base difference in a third codon position probably reflects allelic variation.
cDNA was used for in vitro transcription and as a PCR template after confirming its nucleotide composition by sequencing.

*Oocyte Expression*—RNA synthesis using SP6 polymerase, injection of *Xenopus* oocytes, and voltage clamp recording were done as detailed previously (17).

**RESULTS**

**Identification of the $\alpha_1^{im}$ Subunit Variant**—We used the *Hin*II/*Eco*RI fragment of clone GR2 encoding the membrane spanning regions M1, M2, and M3 of the $\alpha_1$ subunit of the rat GlyR (8) as a probe to screen a rat spinal cord cDNA library under low stringency conditions. Several hybridizing recombinants were purified which represented sequences identical to the previously described rat $\alpha_1$ subunit cDNA. However, one clone (put20) harbored an insert of 189 bp which encoded not only part of the transmembrane segment M3 but in addition contained 24 nucleotides coding for eight additional neutral amino acids (Fig. 1, a and b). These are located in the cytoplasmic region following residue 325 and separate a contiguous stretch of 8 positively charged residues from three negatively charged ones. Screening of a rat brain stem cDNA library with a 38-mer oligonucleotide covering the 24-nucleotides insertion of clone put20 yielded two overlapping cDNAs of 1372 bp ($\alpha_1^{im}$ 21) and 1038 bp ($\alpha_1^{im}$ 18) which cover the entire coding region of the cDNA and contain the 24-nucleotide insertion of clone put20 (Fig. 1a).

Sequencing of these clones revealed no further differences to the previously published $\alpha_1$ cDNA (8) except for a silent nucleotide exchange (C → T) at a third codon position (nucleotide 464 in Ref. 8; data not shown). Thus a variant of the $\alpha_1$ mRNA is found in the rat central nervous system.

The existence of this novel $\alpha_1$ subunit variant (termed $\alpha_1^{im}$) was also demonstrated at the protein level. Upon gas-phase microsequencing of peptides isolated by HPLC from a tryptic digest of affinity-purified GlyR subunits, one peptide was found to contain the entire insert sequence together with some flanking residues (SPMLNFQDDEGGE). Its generation is consistent with the presence of a lysine preceding the insertion in the predicted protein sequence (Fig. 1b). Thus, the put20 and $\alpha_1^{im}$ cDNAs do not represent incompletely processed transcripts or a cloning artifact, but indeed correspond to a translated mRNA.

**Localization of the Insert Sequence in the $\alpha_1$ Subunit Gene**—To characterize the gene structure surrounding the insert sequence, clone put20 was used to identify corresponding genomic DNA in a rat EMBL3 library. Two identical clones were isolated and found to contain a *Ban*I fragment of 148 bp that hybridized to a 24-mer oligonucleotide representing the insert sequence. Analysis of this fragment showed that the 24 additional nucleotides are located at an intron-exon boundary with proper splice acceptor sites in front and at the end of the insertion (Fig. 1b). Thus the two different $\alpha_1$ subunit mRNAs result from alternative acceptor site selection.

**Distribution and Developmental Accumulation of $\alpha_1^{im}$ mRNA**—The regional distribution in nervous tissue and the developmental appearance of the $\alpha_1^{im}$ mRNA was analyzed by S1 nuclease protection experiments. Use of the genomic *Ban*I fragment of 148 bp (Fig. 1b) as radiolabeled probe allowed clear distinction of $\alpha_1$ and $\alpha_1^{im}$ transcripts. RNA containing the 24-bp insert was expected to protect the entire coding region of this probe from nuclease digestion, whereas $\alpha_1$ mRNA should produce a 24-bp shorter band. Indeed, corresponding fragments were obtained with poly(A)+ RNA prepared from spinal cord (Fig. 2) and brain stem (data not shown). The existence of this novel $\alpha_1$ subunit variant (termed $\alpha_1^{im}$) was also demonstrated at the protein level. Upon gas-phase microsequencing of peptides isolated by HPLC from a tryptic digest of affinity-purified GlyR subunits, one peptide was found to contain the entire insert sequence together with some flanking residues (SPMLNFQDDEGGE). Its generation is consistent with the presence of a lysine preceding the insertion in the predicted protein sequence (Fig. 1b). Thus, the put20 and $\alpha_1^{im}$ cDNAs do not represent incompletely processed transcripts or a cloning artifact, but indeed correspond to a translated mRNA.

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of 20-day-old rats was electrophoresed, blotted, and hybridized to a cortex poly(A)+ RNA.

3'4abeled 38-mer oligonucleotide covering the insert sequence (see "Materials and Methods") and the nick-translated clone GR2 current that reverses near \(-20\) mV. The holding potentials used are indicated in millivolts.

Responses to \(\beta\)-alanine and taurine are shown as a glycinergic agonists. Responses to \(500\) pM glycine, \(10\) mM \(\beta\)-alanine, and \(10\) mM taurine; their sizes relative to the current produced by glycine (Fig. 4) were similar to those seen in \(\alpha1\) subunit expressing oocytes (17). Also the response desensitized with time constants indistinguishable from the previously reported kinetics of \(\alpha1\) subunit GlyR desensitization (Ref. 17 and data not shown). Thus, the presence of the insert sequence had no detectable effect on channel responses and pharmacology of the expressed \(\alpha1\) subunit GlyR as revealed under our voltage clamp conditions.

**DISCUSSION**

This report shows that a GlyR \(\alpha1\) subunit variant having eight additional amino acids inserted in its putative cytoplasmic region is expressed in postnatal rat spinal cord and results from alternative splice acceptor site selection at the corresponding exon of the \(\alpha1\) gene. Upon S1 nuclease analysis \(\alpha1^{\text{ins}}\) transcripts were protected in spinal cord and brain stem, but not in other regions of the central nervous system, and estimated to represent \(\geq30\%\) of the total \(\alpha1\) subunit mRNA. As the ratio of \(\alpha1^{\text{ins}}\) over \(\alpha1\) mRNA remained constant at all postnatal stages examined, alternate splice site selection may be stochastically controlled (31). Northern hybridization analysis and PCR data support the temporal and regional coexpression and relative abundance of both \(\alpha1\) subunit variants during late postnatal development. Isolation and sequencing of clones covering the entire coding region of the \(\alpha1^{\text{ins}}\) cDNA confirmed that the cytoplasmic insert sequence is the only structural difference between mature \(\alpha1\) and \(\alpha1^{\text{ins}}\) transcripts. Furthermore, protein microsequencing data dem-
onstrated the existence of the insert sequence in a tryptic digest of affinity-purified GlyR. The $\alpha_{1}^*$ polypeptide thus constitutes a significant fraction of the GlyR protein present in adult spinal cord.

Previous work from our laboratory has revealed an ~1 kDa difference in apparent $\alpha$ subunit molecular mass between neonatal and adult spinal cord GlyRs (14). The late developmental appearance of both $\alpha_{1}$ and $\alpha_{2}$ mRNAs excludes the alternative splicing event reported here as a cause of these differences. Also, our oocyte expression data using a synthetic $\alpha_{1}^*$ RNA show that insert-containing subunits form functional channels which closely resemble the previously characterized $\alpha_{1}$ subunit GlyR (17, 32). In particular, the $\alpha_{1}^*$ channels were blocked by nanomolar concentrations of strychnine, whereas the biochemically characterized neonatal GlyR in rat spinal cord displays only low strychnine binding affinity (14, 15). The $\alpha_{1}^*$ subunit variant thus is unrelated to the previously described developmental and pharmacological heterogeneity of this chloride channel protein. This interpretation is consistent with the recent identification of a novel rat $\alpha$ subunit variant (a?) that forms channels of low strychnine sensitivity upon expression in oocytes.

The eight amino acid insertion generated by alternative splicing occurs in a highly charged region postulated to mediate chloride binding and/or channel rectification (8). However, the current voltage relation of $\alpha_{1}$ GlyR channels expressed in oocytes was not detectably altered by the insertion. Although minor differences in channel properties cannot be excluded presently, we favor a specific role of the additional amino acids. For example, alternative splicing may alter binding of cytoplasmic receptor-associated proteins, like the previously identified 93-kDa polypeptide of the glycine receptor (7) and thus affect receptor distribution. Correspondingly, $\alpha_{1}$ and $\alpha_{2}$ receptors might sort into different regions of the neuronal plasma membrane, e.g. dendritic and somatic regions, or pre- and postsynaptic areas, respectively. On the other hand, both $\alpha$ subunit variants may coassemble in the native receptor. GlyR affinity-purified from adult spinal cord membranes is known to behave as a pentameric protein containing at least three $\alpha$ polypeptides (33). The inserted amino acids then might create novel targets for posttranslational modification. Consistent with currently accepted cyclic nucleotide-dependent protein kinase consensus sequences, the first serine residue of the insertion might serve as a potential phosphorylation site. A preliminary report indicating in vitro phosphorylation of GlyR $\alpha$ polypeptides has appeared recently (34).

Subunit variants have been identified for different neuronal ligand-gated ion channels including the human GlyR (18, 19, 35–40). Nucleotide sequence comparison indicates that this subunit heterogeneity results from transcription of different genes, for the $\alpha_{2}$ subunit of muscle and neuronal nictinamide-acyethylcholine receptors, evidence for alternative splicing variants has been reported (41–43). However, the amino acid differences generated are localized at the very C terminus or within the extracellular regions of the corresponding $\alpha$ subunits. The alternative splice event reported here creates a novel sequence within the cytoplasmic segment of the GlyR $\alpha_{1}$ subunit and thus provides a mechanism for diversifying this domain which is highly variable among subunits of ligand-gated ion channels. Interestingly, a similar alternative splice event is found in the cytoplasmic region of the dopamine D$_2$ receptor, a member of the G-protein coupled receptor family (44–46). It will be interesting to see whether analogous splicing variants also exist for subunits of other receptor protein families.

Acknowledgments—We thank H. Krischke for expert technical assistance, G. Multhaup for protein microsequencing, Y. Maulet for critical reading, and I. Baro and I. Veit-Schirmer for help during preparation of the manuscript.

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