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Cloning and functional expression of alternative spliced variants of the ρ1 γ-aminobutyrate receptor

ATAÚLFO MARTÍNEZ-TORRES, ANA E. VAZQUEZ*, MITRADAS M. PANICKER†, AND RICARDO MILEDI‡

Department of Psychobiology, Laboratory of Cellular and Molecular Neurobiology, University of California at Irvine, Irvine, CA 92697-4550

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ABSTRACT The ρ1 γ-aminobutyrate receptor ($GABA_{\rho 1}$) is expressed predominantly in the retina and forms homomeric GABA-gated Cl^- channels that are clearly different from the multisubunit $GABA_A$ receptors. In contrast to these, $GABA_{\rho 1}$ receptors desensitize very little and are not blocked by bicuculline. In addition to $GABA_{\rho 1}$, two new variants were identified in human retina cDNA libraries. Cloning and sequence analysis showed that both variants contain large deletions in the putative extracellular domain of the receptor. These deletions extend from a common 5′ site to different 3′ sites. The cDNA with the largest deletion, named $GABA_{\rho 1}\Delta 450$, contains a complete ORF identical to that of $GABA_{\rho 1}$ but missing 450 nt. This cDNA encodes a protein of 323 aa, identical to the $GABA_{\rho 1}$, but has a deletion of 150 aa in the amino-terminal extracellular domain. $GABA_{\rho 1}\Delta 450$ mRNA injected into *Xenopus* oocytes did not produce functional GABA receptors. The second $GABA_{\rho 1}$ variant ($GABA_{\rho 1}\Delta 51$) contains a 51-nt deletion. In *Xenopus* oocytes, $GABA_{\rho 1}\Delta 51$ led to the expression of GABA receptors that had the essential $GABA_{\rho 1}$ characteristics of low desensitization and bicuculline resistance. Therefore, alternative splicing increases the coding potential of this gene family expressed in the human retina, but the functional diversity created by the alternative spliced forms is still not understood.

In the mammalian nervous system the neurotransmitter γ-amino butyric acid (GABA) exerts its inhibitory effects by binding to at least two different types of receptors. These pharmacologically, and structurally, distinct subclasses of receptors are classically divided as $GABA_A$ and $GABA_B$ (1–5). The $GABA_A$ receptor is a complex of several polypeptide subunits that form a Cl^- channel that is modulated by benzodiazepines, barbiturates, neurosteroids, and ethanol, and is blocked by bicuculline. So far, 13 members of the $GABA_A$ receptor subclass have been cloned, including 6 α subunits, 3 β subunits, 3 γ subunits, and 1 δ subunit. $GABA_B$ receptors are coupled to K^+ and Ca^{2+} channels via G proteins, are selectively activated by baclofen, and do not respond to known $GABA_A$ receptor modulators. The molecular gene structure of these receptors has been just described (6). Recently, an emerging subclass of GABA receptors, called $GABA_C$ or GABAρ (7, 8), was clearly established by injecting bovine retina mRNA into *Xenopus* oocytes (9). These receptors display peculiar physiological and pharmacological properties that are clearly different from those of the A and B subclasses of GABA receptors. For example, both $GABA_A$ and $GABA_C$ receptors gate Cl^- channels, but the $GABA_C$ channels desensitize either very little, or not at all, after being activated by GABA. Furthermore, they are not blocked by bicuculline and are not activated by baclofen (9–12). Moreover, $GABA_C$ receptors are highly expressed in the vertebrate retina (9, 13–16).

Molecular cloning has revealed that the structure of $GABA_C$ receptors is related to the ligand-gated ion channel family of receptors (17, 18). Like other members of this family (i.e., the acetylcholine and glycine receptors), $GABA_C$ receptors have four highly hydrophobic regions, which potentially form transmembrane (TM) regions, a large cytoplasmic loop between the TM3 and TM4 segments, and a large hydrophilic extracellular N-terminal domain, which has a cysteine loop and is where the GABA binding site is presumed to be located. In contrast to the $GABA_A$ subclass of receptors, the members of the $GABA_C$ subclass form functional homooligomeric receptors. So far, three types of $GABA_C$ receptor cDNAs have been cloned (ρ1–ρ3), all of which show 30–38% amino acidic similarity with the members of the $GABA_A$ receptor subclass and 61–63% among themselves (17, 19, 20).

The coding potential of the $GABA_A$ receptor genes is increased by the mechanism of alternative splicing. For example, alternative splicing of the α6 and γ2 subunits leads to receptors with different properties. Furthermore, in the case of the α6 subunit, a small deletion in the N-terminal region inhibits the function of the receptor (21). On the other hand, splicing of the γ2 subunit introduces a series of eight residues in the second intracellular loop that forms a protein kinase C target site that, if differentially phosphorylated, changes the functional properties of the $GABA_A$ receptor (22).

After finding the $GABA_C$ receptor expressed by bovine retina mRNA (9) we proceeded to try to clone the receptor. While that work was going on, Cutting *et al.* (17) cloned the GABAρ1 subunit. We subsequently found that, in addition to that subunit, the human retina contained two shorter forms of ρ1. This paper describes these results presented earlier in brief form (23).

MATERIALS AND METHODS

PCR Amplification and Molecular Cloning of $GABA_{\rho 1}$ Receptor-Related Sequences from Human Retina cDNA Libraries. We used the published $GABA_{\rho 1}$ sequence (17) to design oligonucleotides that were used as primers for our PCR experiments. One set of oligonucleotides was primer Rho5′, 5′-ctcctcgagatgag(a/g)tt(t/c)gg(c/g)at(c/t)tt(c/t)-3′ and primer TM2a, 5′- gtg(c/g)(a/t)cat(t/g)gt(t/g/c)ag(a/c)ac(g/c)gt(t/g)gt-3′. The primer Rho5′ corresponds to a sequence at the start of the published GABAρ1 ORF, and TM2a corresponds to the reverse complement of part of the sequence encoding the TM2 domain.

A human adult retina cDNA library (kindly provided by J. Nathans, Johns Hopkins University) was amplified as 40

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Abbreviations: GABA, γ-aminobutyrate; TM, transmembrane.

*Present address: Department of Anatomy and Cell Biology, University of Miami School of Medicine, Miami, FL 33101.
†Present address: National Centre for Biological Sciences, TIFR, Banglore 560012, India.
‡To whom reprint requests should be addressed. e-mail: rmiledi@uci.edu.

independent pools, which were screened by PCR with the primers described above, and using the following conditions: one denaturation step of 95°C for 5 min, 10 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. A final step at 72°C was for 10 min. Three different-sized PCR products were obtained, purified by using Gene Clean (Bio 101), and cloned into the T-vector pCRII (Invitrogen). Their nucleotide sequences were determined by the Sanger method. Two more retina libraries, one adult and the other fetal (kindly provided by A. Swaroop, University of Michigan), were pooled and screened by PCR using the Rho5′ primer and a second antisense primer annealing to the 296–316 region of the GABA$_{\rho 1}$ cDNA: 5′-gccagggcctccgaagccagg-3′. The amplification conditions were: 95°C for 5 min, 35 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 50 sec with a final step of 72°C for 10 min. Two PCR products were obtained, cloned into pCRII, and sequenced.

A random primer ^{32}P-labeled probe was made by using one of the PCR products mentioned above, which eventually resulted in the GABA$_{\rho 1}\Delta$51 cDNA (see Fig. 1). Two plates containing 50,000 plaque-forming units (pfu) of the PCR-positive library pools (three pools from each of the fetal libraries and three more of the adult library donated by A. Swaroop) were plated and transferred to nitrocellulose filters. Phage DNA, bound to the membranes, was hybridized at 42°C for 16 h with 1×10^6 cpm/probe per ml of hybridization buffer (5× SSPE, 0.5% SDS, 1× Denhardt's solution). After hybridization, the filters were washed for 30 min at room temperature and 1 h at 60°C in 2× SSPE and 0.1% SDS. Five positive hybridization spots were picked up from each plate, replated at 10,000 pfu, transferred to nitrocellulose filters and hybridized and washed by using the same conditions as above. Two positive spots from each plate were analyzed by using PCR with the Rho5′ and the reverse primer 296–316 in one set of reactions and the Rho5′ and TM2 primers in another set. A cDNA containing the complete ORF and the other hybridizing cDNAs were partially restricted with *Eco*RI and cloned into the *Eco*RI site of pBluescript (Stratagene) and sequenced.

***In Vitro* Transcription and Voltage-Clamp Recording of Membrane Currents.** The resulting plasmids, containing the cDNAs, were restricted with *Sal*I and transcribed *in vitro* by using the T7 RNA polymerase (Promega). To increase the levels of expression, the cDNAs were transferred as an *Sma*I-*Eco*RV insert into the expression vector pSP64T (24) that was previously *Bgl*II restricted, filled in with the Klenow fragment, and treated with calf intestine alkaline phosphatase. pSP64T-derived plasmids were linearized with *Sal*I and *in vitro* transcribed with SP6 RNA polymerase (Promega).

Fifty nanograms of *in vitro* transcribed cRNA, dissolved in 50 nl of water, was injected into *Xenopus* oocytes. Donor frogs were from *Xenopus*1 and Nasco. The oocytes were handled as previously described, defolliculated with 0.5 mg/ml collagenase I (Sigma) in Ringer's solution for about 1 h (25), and stored in Barth's medium with 0.1 mg/ml gentamicin. The next day, if follicular cells remained attached, these were removed manually or by shaking the oocytes again. Voltage-clamp recordings were made at room temperature (20–22°C) from oocytes in a chamber (0.1 ml) continuously perfused with frog Ringer's solution (5–10 ml/min), with the membrane potential usually held at −60 mV (26).

Construction of a Full-Length-GABA$_{\rho 1}\Delta$51. The GABA$_{\rho 1}\Delta$51 clone was obtained from J. Nathan's human adult retina cDNA library as a 3′ end truncated form, missing part of the ORF. Therefore, to study the potential expression of a GABA$_{\rho 1}\Delta$51, a *Bgl*II-*Msc*I fragment of the GABA$_{\rho 1}$ cDNA, originally cloned from the same cDNA library, was exchanged by the same corresponding fragment of the 3′ end truncated form of GABA$_{\rho 1}\Delta$51. The derived plasmid contains a complete ORF and lacks the 51 nt, as in the GABA$_{\rho 1}\Delta$51, and its sequence is the same as the clones subsequently obtained from the other adult and fetal retina cDNAs.

RESULTS

Two Alternative Spliced Forms of the GABA$_{\rho 1}$ Receptor Are Found in Human Adult and Fetal cDNA Libraries. Fig. 1 shows that several pools of a human adult retina cDNA library produced three different amplicons in PCRs by using the primers Rho 5′ and TM2 described in *Materials and Methods*. Cloning and subsequent sequencing of these fragments indicated that besides the expected GABA$_{\rho 1}$, there seemed to be two additional forms of the GABA$_{\rho 1}$ cDNA that were thought to be the product of alternative splicing. Pools of the cDNA libraries that were identified to contain GABAρ1 were plated and hybridized by using GABA$_{\rho 1}$ cDNA and GABA$\rho 1\Delta$51 cDNA as probes, and cDNA clones that hybridized were isolated and characterized. The cDNA inserts from the phage were transferred to Bluescript and sequenced.

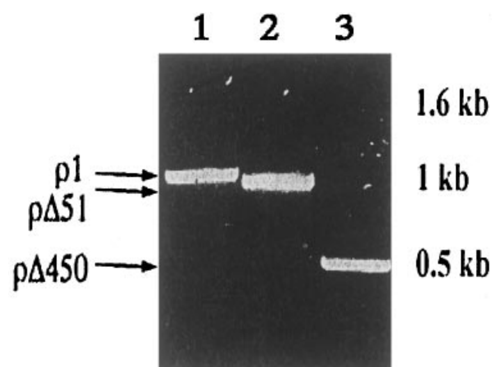

FIG. 1. Three forms of the GABA$_{\rho 1}$ receptor. A human retina cDNA library was pooled and screened by PCR. Two extra amplicons, besides the original form of GABA$_{\rho 1}$, were found in different pools. Lane 1 corresponds to the original human ρ1 cloned initially and used as control, lane 2 corresponds to a positive pool with GABA$_{\rho 1}\Delta$51, and lane 3 corresponds to GABA$_{\rho 1}\Delta$450.

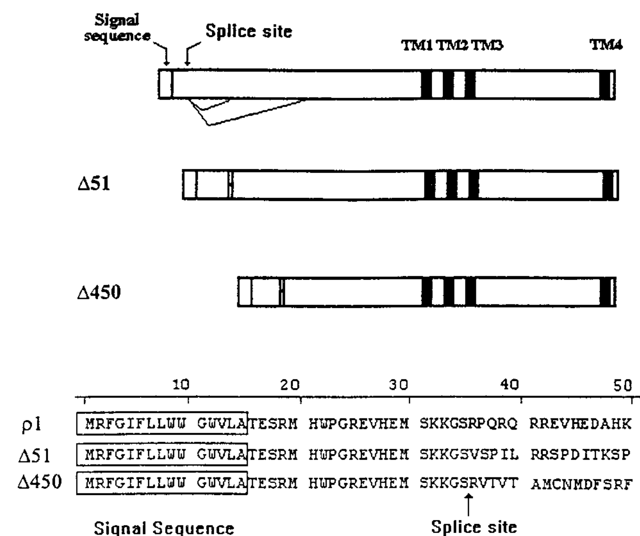

FIG. 2. Two alternative spliced variants of the GABA$_{\rho 1}$ receptor. The variants share a common 5′ end but extend to different 3′ ends. Cloning and sequencing analysis revealed that the deleted regions were in the extracellular N-terminal domain. An extra codon (AGT = Ser) not found in the first report of GABA$_{\rho 1}$ (17) was present in position 35, just upstream of the deleted regions.

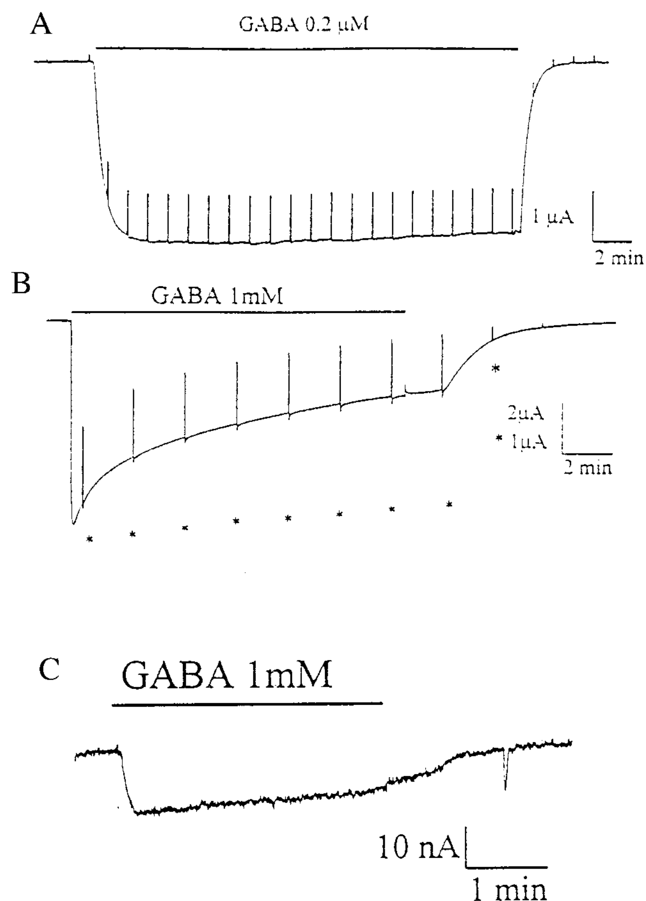

FIG. 3. Functional expression of GABA$_{\rho1}$Δ51 in *Xenopus* oocytes. cRNAs coding for GABA$_{\rho1}$Δ51 were injected into *Xenopus* oocytes, and 48–72 h after injection, the oocytes elicited a current when exposed to GABA. (*A*) Like the GABA$_{\rho1}$ receptor, the GABA$_{\rho1}$Δ51 receptor inactivates very low even after many minutes of exposure to GABA (holding potential, −60 mV). (*B*) Same oocyte as for *A*, but with the membrane potential held at −40 mV. With a higher GABA concentration the currents decayed, but, as indicated by (*), the conductance (reflected by the current of 1-sec pulse to −30 mV) was well maintained. (*C*) Currents elicited by 1 mM GABA in oocytes expressing low levels of receptors were also well maintained. Oocytes were from the same batch as in *A* and *B*.

The first clone was found to have a big deletion in the amino-terminal region, spanning 450 bases. However, this cDNA had a complete ORF that included a signal sequence and the four hydrophobic domains that have been suggested to form the transmembrane regions. The 5′ noncoding sequence of this cDNA extended up to 500 bp, larger than the other GABAρ1 cDNAs (Fig. 2). The second clone, GABA$_{\rho1}$Δ51, lacked 51 bases, coding for a segment of the N-terminal extracellular domain. The deletion starts at the same 5′ position as the GABA$_{\rho1}$Δ450 and extends 51 nt toward the C terminus. That clone did not have a complete ORF because it lacked the 3′ end of the coding region.

Because the GABA$_{\rho1}$Δ51 clone did not have a complete ORF, we presumed that it was probably the result of incomplete second-strand cDNA synthesis and that it was unlikely to express functional receptors. To obtain receptors without the 51 bp we screened three more cDNA libraries of adult and fetal retina cDNAs; these libraries yielded six clones carrying full-length cDNAs that expressed functional receptors in oocytes.

Expression of GABA$_{\rho1}$Δ51 and GABA$_{\rho1}$Δ450 in *Xenopus* Oocytes. Several preparations of cRNAs encoding the GABA$_{\rho1}$Δ450 injected into *Xenopus* oocytes did not induce them to acquire GABA responses. When this receptor mRNA was coinjected with that of the GABA$_{\rho1}$, the expression of functional GABA receptors was approximately half of that in oocytes injected with the full GABA$_{\rho1}$ alone. We tried to increase the expression of receptors by introducing the GABA$_{\rho1}$Δ450 into the expression plasmid pSP64T, which carries upstream and downstream noncoding sequences of the *Xenopus* β-globin gene. It is known that in some cases these sequences are useful to increase the stability of the cRNA. For example, 5HT1a receptor cRNA containing this sequence led to the expression of a large number of functional receptors that coupled quite efficiently to the phosphoinositide pathway (27). Again, no responses were elicited in oocytes expressing homooligomeric GABA$_{\rho1}$Δ450 receptors. On the other hand, GABA$_{\rho1}$Δ51 induced the oocytes to acquire GABA responses with similar characteristics to the GABA$_{\rho1}$ (4, 5). For example, GABA$_{\rho1}$Δ51 receptors were not blocked by bicuculline and they desensitized very little during prolonged applications of GABA. The currents elicited by low concentrations of GABA (0.1–0.2 μM) were very stable during applications of GABA, lasting many minutes (Fig. 3*A*). Even the currents elicited by GABA concentrations that were 10,000-fold higher were relatively well maintained. Sometimes, like in the oocyte used to illustrate Fig. 3*B*, the currents elicited by 1 mM GABA decayed appreciably in a few minutes. However, the conductance change was well maintained (Fig. 3*B*), and the equilibrium potential for GABA action changed during the exposure to the neurotransmitter. Moreover, the currents elicited by 1 mM GABA were well maintained in oocytes that were from the same batch but that expressed low levels of receptors (Fig. 3*C*). Therefore, in those cases, the decay of the GABA current was mostly due to changes in the Cl^- ion gradient across the membrane, brought about by the activation of large numbers of receptors. Finally, the GABA$_{\rho1}$Δ51 receptors are clearly inhibited by zinc in the same manner as the full-length GABA$_{\rho1}$ receptor (Fig. 4) (10).

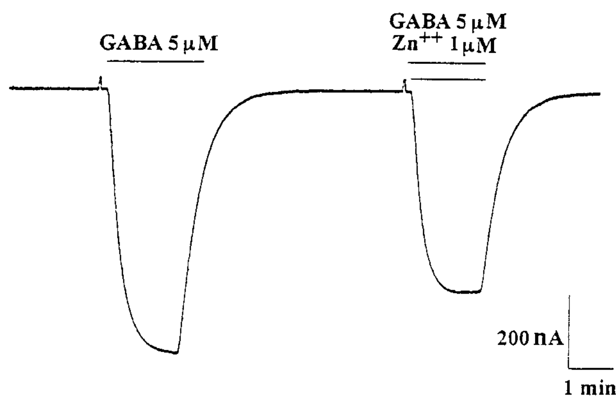

FIG. 4. Zinc blocks GABA$_{\rho1}$Δ51 receptor currents. Like the GABA$_{\rho1}$ receptor, the currents elicited by GABA in oocytes injected with GABA$_{\rho1}$Δ51 were blocked by zinc ions in a reversible manner.

DISCUSSION

The GABA$_C$ receptors show a clear relationship with other members of the ligand-gated ion channel receptor family (18). Some members of this family, including GABA$_A$ receptors (21–22), are known to increase their coding potential by differentially splicing coding regions, so it is not surprising that the GABA$_C$ receptors also show increased diversity through alternative splicing. As far as we have examined them, the properties of the receptors expressed by GABA$_{\rho1}$Δ51 were similar to those originally described for GABA$_{\rho1}$ receptors. Furthermore, in our analysis of the coding sequence, we did not find absent any obvious critical motif (PKC, CaMKi II, or N-glycosylation). The GABA$_{\rho1}$Δ51 receptor was able to re-

spond to glycine and alanine in the same way as the full $GABA_{\rho 1}$ receptor (ref. 10; results not shown). It would be interesting, however, to analyze in more detail the effect of other agonists and of antagonists of this receptor whose binding sites are yet to be well defined. In fact, it is surprising that with such a long deletion, no functional changes have yet been found for $GABA_{\rho 1}\Delta 51$ receptors.

The much larger deletion of $GABA_{\rho 1}\Delta 450$ appears to have lost the putative GABA-binding site motif and also potential N-glycosylation sites. The lack of activation by GABA of the receptors expressed by $GABA_{\rho 1}\Delta 450$ indicated that this cRNA is not able to form functional homomeric receptors, or that the receptors were not assembled in the plasma membrane. However, this cDNA still codes for a signal sequence and for all the four hydrophobic TM regions, so it is likely that the protein produced still gets anchored to the plasma membrane. It is also possible that the $GABA_{\rho 1}\Delta 450$ protein may be able to form complexes with the other varieties of the $GABA_C$ receptor, or with the different $GABA_A$ subunits, modifying in an as yet to be characterized way the properties of a heteromeric receptor. Because the four transmembrane segments are present, this polypeptide may well be able to contribute to the lining of the chloride channel in such receptors. Interestingly, when coinjected with $GABA_{\rho 1}$, the $GABA_{\rho 1}\Delta 450$ variant seems to cause a reduction in GABA-activated currents. This would suggest that $GABA_{\rho 1}\Delta 450$ is able to associate with $GABA_{\rho 1}$ subunits and form heteromeric receptors that are functionally different and altered in GABA binding.

This report of a functional alternative spliced form of a member of the $GABA_C$ subclass of receptors raises many questions. For example, are the alternative spliced forms expressed in a tissue-specific manner? Are they exclusively expressed in the human retina or are they present in other tissues? It is interesting that so far only $GABA_{\rho 1}$, but none of the other spliced forms, was detected in the rat pituitary by means of reverse transcription–PCR (28). All these results suggest interesting possibilities and new directions to be explored in trying to understand how the brain and the retina work.

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