BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **253,** 10–15 (1998)
ARTICLE NO. RC989706

Cloning and Tissue Distribution of Novel Splice Variants of the Rat $GABA_B$ Receptor

Shojiro Isomoto,*,† Muneshige Kaibara,* Yasuko Sakurai-Yamashita,* Yuji Nagayama,* Yasuhito Uezono,‡ Katsusuke Yano,† and Kohtaro Taniyama*,[1]

**Department of Pharmacology and †Third Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan; and ‡Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807-8555, Japan*

Received October 8, 1998

We have identified two novel splice variants of the metabotropic γ-aminobutyric acid receptor ($GABA_BR1$), designated $GABA_BR1c$ and $GABA_BR1d$, when screening a rat cerebellum cDNA library. $GABA_BR1c$ has an amino acid sequence identical to $GABA_BR1b$, a member of $GABA_BR1$ isoforms, and an additional 93-bp insertion that generates an additional 31-amino-acid sequence in the fifth transmembrane region of $GABA_BR1b$. Thus, $GABA_BR1c$ may have a structural variation in the second extracellular loop and fifth transmembrane region. $GABA_BR1d$ also has an amino acid sequence identical to $GABA_BR1b$ and an additional insertion of 566 bp that generates a divergent amino acid sequence in the carboxyl-terminal end. Reverse-transcription polymerase chain reaction analysis showed that in various rat tissues $GABA_BR1c$ mRNA was ubiquitously expressed and $GABA_BR1d$ mRNA in forebrain, cerebellum, eye, kidney, and urinary bladder. $GABA_BR1$ isoforms may function not only in the central nervous system but also in various peripheral tissues.

Key Words: **molecular cloning; splice variants; $GABA_B$ receptor; reverse-transcription polymerase chain reaction.**

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system and is also present in several peripheral tissues, where it could have a functional role in the regulation of muscle contraction or hormonal secretion. The receptors for GABA are pharmacologically classified into two major subtypes, ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. The $GABA_A$ receptor is the target of several centrally active drugs, including barbiturates and benzodiazepines, and responds to the antagonist bicuculline (1). Activation of the $GABA_A$ receptor results in opening of a chloride channel and hyperpolarization of postsynaptic cells. The $GABA_B$ receptor is operationally defined by selective activation by baclofen and the lack of sensitivity towards bicuculline, and functions through G proteins to inhibit adenylyl cyclase activity, stimulate phospholipase A_2, activate K^+ channels, inactivate voltage-dependent Ca^{2+} channels, and modulate inositol phospholipid hydrolysis (2, 3). Recently, we have suggested that $GABA_B$ receptors activate Kir3.1 and Kir3.2 heterologously expressed in *Xenopus* oocytes with poly(A)$^+$ RNA derived from rat cerebellum (4).

The cDNA encoding the $GABA_B$ receptor was recently isolated from a rat brain cDNA library by expression cloning using a high-affinity $GABA_B$ receptor antagonist, [^{125}I]CGP64213 (5). This clone, designated $GABA_BR1a$, encodes a protein of 960 amino acids. Further screening of the cDNA library by low-stringency hybridization using the $GABA_BR1a$ cDNA as a probe revealed a shorter form, designated $GABA_BR1b$, which encodes an 844-amino-acid protein. The membrane topology of $GABA_BR1a$ and -b is similar to that of G-protein-coupled receptors, such as muscarinic, serotonergic, opioid, somatostatin, and metabotropic glutamate receptors: i.e., seven putative transmembrane regions with the amino (N)- and carboxyl (C)-terminal regions located extracellularly and intracellularly, respectively. The sequences of $GABA_BR1a$ and -b differ at the N-terminus and are otherwise identical, hence they may be formed by alternative splicing of a single rat gene. The availability of cDNAs for $GABA_B$ receptors led us to clone their variants on the basis of homology. We screened a rat cerebellum cDNA library and identified two novel isoforms of the receptor, designated $GABA_BR1c$ and -d. Tissue distributions of their mRNAs were examined using reverse tran-

[1] To whom correspondence should be addressed at Department of Pharmacology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Fax: +81-95-849-7048. E-mail: taniyama@net.nagasaki-u.ac.jp.

0006-291X/98 $25.00

scription-polymerase chain reaction (RT-PCR) analysis. The functional expression of these clones in mammalian cells and in *Xenopus* oocytes heterologously expressing Kir3.1 and Kir3.2 was attempted.

EXPERIMENTAL PROCEDURES

Screening of a rat cerebellum cDNA library. A rat cerebellum cDNA library (Clontech, Palo Alto, CA) was screened under conditions of mild stringency, using a ^{32}P-labeled DNA fragment encoding rat $GABA_BR1$ (nucleotide positions 653–1789 in $GABA_BR1b$), obtained by RT-PCR from rat cerebellum RNA. Hybridization was conducted in 30% formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, at 37°C for 18 h. Filters were washed with 2× SSC, 0.1% SDS at room temperature for 20 min and then exposed to X-ray film overnight at −80°C with an intensifying screen. Positive clones were subcloned into the pCR2.1 vector (Invitrogen, San Diego, CA) for sequencing. DNA sequencing was performed on both strands using an ABI 377 automated DNA sequencer (Perkin–Elmer, Foster, CA).

Electrophysiological studies using the Xenopus oocyte expression system. Preparation of oocytes, cRNA injection and electrophysiological measurements were as described (4, 6). Cloned rat $GABA_B$ receptor cRNAs, as well as the human m2-muscarinic receptor cRNA, were coinjected with cRNAs for rat Kir3.1 and mouse Kir3.2, cloned G protein-gated K^+ channel subunits, into *Xenopus* oocytes. Rat Kir3.1 and mouse Kir3.2 cDNAs were kindly provided by Dr. H. A. Lester (Caltech, CA), and human m2-receptor cDNA by Dr. E. G. Peralta (Harvard Univ.). Electrophysiological studies were done 3-7 days after the cRNA injection. To induce the K^+ currents, either baclofen, a $GABA_B$ receptor agonist, or acetylcholine, a m2-receptor agonist, were used.

Cell culture and Radioligand binding assays. CHO cells and HEK-293 cells (ATCC: CRL 1573) were maintained under standard culturing conditions for these cell lines. Cells were transfected with pCR3 vector (Invitrogen, San Diego, CA) ligated each of $GABA_BR1b$, $GABA_BR1c$ and $GABA_BR1d$ in the sense orientation. Cells were transfected with these plasmids by the Lipofectin method (Life Technologies, Gaithersburg, MD) and stable cell lines were established by G418 (geneticin) selection (500 μg ml^{-1} for CHO, 800 μg ml^{-1} for HEK293). The cell lines were subsequently grown in the presence of G418 (500 μg ml^{-1}). The transfected CHO cells were cultured to semiconfluence in 10-cm dishes, and were then passed into 24-well culture plate. After two days, the cells were washed twice with Krebs'–Tris buffer (20 mM Tris–HCl pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$, pH 7.4), and incubated for 90 min with ^{3}H-baclofen (20 nM and 50 nM) (New England Nuclear, Boston, MA) in room temperature in the presence or absence of (±)-baclofen (100 μM) (Sigma, St. Louis, MO). The cells were then cooled on ice and washed twice with ice-cold Krebs'–Tris buffer, and then solubilized with 1% SDS. Subsequently radioactivity was measured by liquid scintillation counting (7). Cell membranes of the transfected HEK293 cells were prepared according to the protocol described in Morrow *et al.* (8). Membranes were suspended in Krebs'–Tris buffer (50 mM Tris–HCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated with 20 nM ^{3}H-baclofen for 30 min at room temperature in the presence or absence of (±)-baclofen (100 μM). The incubation was terminated by filtration through GF/B Whatman glass fiber filters, and radioactivity was measured by liquid scintillation counting. The specific activity of ^{3}H-baclofen was 31.5 Ci mmol^{-1}.

RT-PCR assay for $GABA_B$ receptors. The cDNAs synthesized from total RNAs extracted from various rat organs with oligo-(dT) primers were used as templates for PCR amplification. Primers for the amplification of the $GABA_BR1$ isoforms were as follows: $GABA_BR1c$, 5′-AGTGGAGGAAGACCCTAGAG-3′ (forward), and 5′-ATCATGGTCACAGGAGCAGT-3′ (reverse), corresponding to nucleotide positions 1747–1766 and 2216–2197 of the $GABA_BR1c$ cDNA sequence; $GABA_BR1d$, 5′-TGAAACGCAGGACACCATGA-3′ (forward), and 5′-TCACTTGTAAAGCAAATGTACT-3′ (reverse), corresponding to nucleotide positions 2253–2272 and 3102–3081 of the $GABA_BR1d$ cDNA sequence. The PCR condition was as follows: an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 8 min. Amplified DNA fragments for $GABA_BR1c$ and -d were electrophoretically fractionated on 2 and 0.8% agarose gels, respectively. The RT-PCR products from rat cerebellum and kidney were subcloned into pCR2.1 vector, and were sequenced as described above to confirm the sequences of $GABA_BR1$ isoforms.

RESULTS

We obtained 71 positive clones after screening approximately 6 x 10^5 plaques of the rat cerebellum cDNA library. A full-length $GABA_BR1b$ cDNA encoding 844 amino acids was obtained by restriction enzyme digestion and religation of two overlapping clones. The nucleotide at position 2457 of this clone was A, while that of rat $GABA_BR1b$ reported by Kaupmann *et al.* (4) was G. This substitution did not alter the deduced amino acid sequence, and might be due to polymorphism in rat $GABA_BR1$ gene.

Two of positive clones obtained, named RGB33 and RGB69, were sequenced. The nucleotide sequence of RGB33 revealed a single open reading frame encoding a protein of 875 amino acid residues. RGB33 had a sequence identical to $GABA_BR1b$, and possessed an additional 93-bp insertion between nucleotide positions 1961 and 1962 of $GABA_BR1b$. The insertion of 93 bp generated an additional 31-amino-acid sequence between Gly^{654} and Ile^{655} in $GABA_BR1b$. Thus, RGB33 may have a structural variation in the fifth transmembrane region and the second extracellular loop (Fig. 1). RGB69, that also had a sequence identical to $GABA_BR1b$, lacked 5′-untranslated region and 1438 bp of coding region in the N-terminus of $GABA_BR1b$ and had an additional 566-bp insertion in the C-terminus between nucleotide positions 2361 and 2362 of $GABA_BR1b$. RT-PCR with a specific primer for $GABA_BR1b$ and that for RGB69 using the cDNAs synthesized from total RNAs extracted from rat cerebellum as a template yielded transcripts for RGB69, while RT-PCR with specific primers for $GABA_BR1a$ and RGB69 could not yield them (data not shown). Therefore, a full-length cDNA for RGB69 was obtained by combining with the 5′-end of $GABA_BR1b$, and encoded a protein of 812 amino acids. Insertion of the 566 bp generated divergent amino acid sequences in the C-termini between RGB69 and $GABA_BR1b$. Thus, RGB69 had an amino acid sequence identical to $GABA_BR1b$ through Glu^{787} and then diverged in the C-terminal ends (Fig. 2). These findings indicated that RGB33, RGB69 and $GABA_BR1a$/-b may be formed by alternative splicing

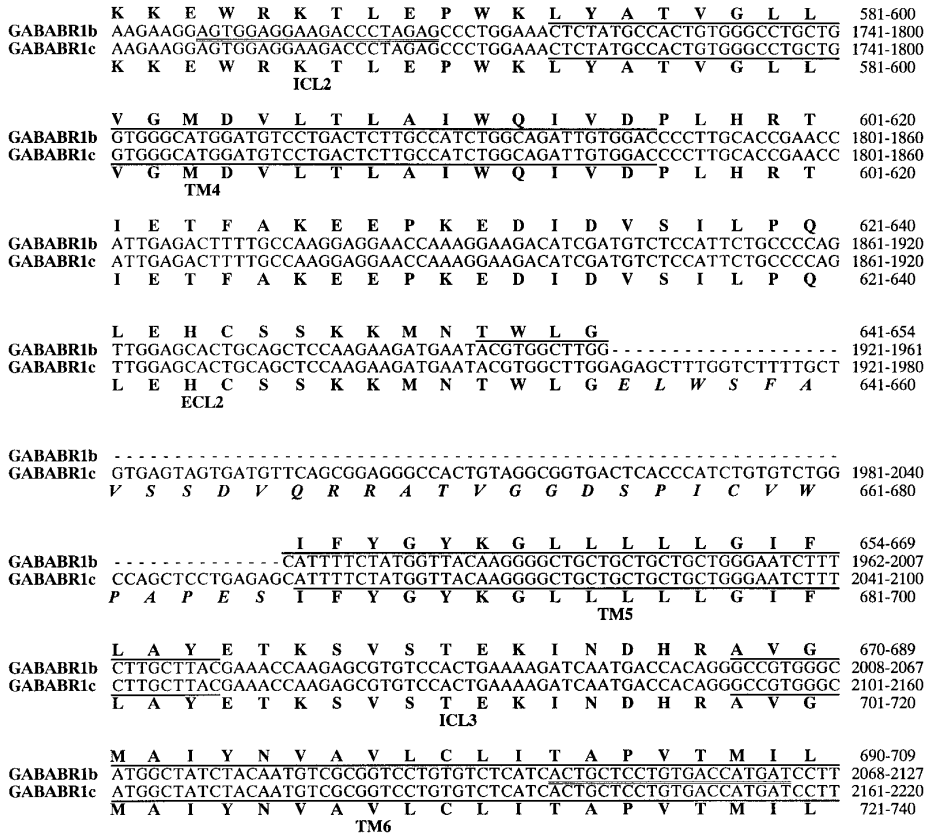

FIG. 1. Aligned nucleotide and deduced amino acid sequences in the transmembrane region of rat $GABA_BR1b$ and $GABA_BR1c$. The nucleotides and amino acids of $GABA_BR1b$ and -c are numbered to the right of each sequence. The putative transmembrane domains (TM4-6), that are lined, intracellular loops (ICL2 and -3) and an extracellular loop (ECL2) are indicated. $GABA_BR1c$ has an additional 93-bp insertion that generated an additional 31-amino-acid sequence. Additional amino acids are indicated in italic. Nucleotide sequences of sense and antisense primers for RT-PCR to determine tissue distributions of $GABA_BR1b$ and -c mRNAs are indicated by the double bars.

of a single rat $GABA_BR1$ gene. None of $GABA_BR1$ isoforms reported to date contained the additional segments of RGB33 and RGB69. Based on these results, we designated RGB33 and RGB69 as $GABA_BR1c$ and $GABA_BR1d$, respectively.

For functional expression of $GABA_BR1$ isoforms, the *Xenopus* oocyte expression system and mammalian cells were used. When m2-receptor cRNA was coinjected with Kir3.1 and Kir3.2 cRNAs into *Xenopus* oocytes, acetylcholine-induction of the inwardly rectifying K^+ current was enhanced dramatically, as noted in previous studies (6, 9). In contrast, baclofen up to 400 μM induced no significant current alterations in the oocytes where any one of cRNAs for $GABA_BR1b$, -c, and -d was coinjected with Kir3.1 and Kir3.2 cRNAs (data not shown). We could not detect a specific binding for ^{3}H-baclofen in either CHO or HEK293 cells transfected these $GABA_BR1$ clones (data not shown).

Northern blot analysis hybridized with a probe containing sequences common to all of rat $GABA_BR1$ isoforms revealed that their mRNAs were expressed at high levels in brain and at low levels in testis, but not in tissues from heart, spleen, lung, liver, skeletal muscle and kidney (5). To determine the distribution of mRNAs for $GABA_BR1c$ and -d in various rat tissues, RT-PCR analysis was done. Specific primers for amplification of $GABA_BR1c$ yielded transcripts of 470 bp for $GABA_BR1c$ and those of 377 bp for isoforms without 93-bp insertion of $GABA_BR1c$. In tissues where mRNAs for both $GABA_BR1c$ and any one of other isoforms are expressed, transcripts of 470 bp and 377 bp can be detected. The RT-PCR yielded two visible amplified products of 470 and 377 bp in all rat tissues examined: forebrain, cerebellum, eye, atrium, ventricle, lung, stomach, small intestine, colon, liver, spleen, kidney, urinary bladder and skeletal muscle (Fig. 3A). Thus, $GABA_BR1c$ mRNA is ubiquitously expressed in various rat tissues. Specific primers for amplification of $GABA_BR1d$ yielded transcripts of 850 bp for $GABA_BR1d$ and those of 284 bp for isoforms without 566-bp insertion of $GABA_BR1d$. $GABA_BR1d$ mRNA was expressed in forebrain, cerebellum, eye, kidney and urinary bladder, while the transcript of 284 bp was detected in all tissues examined (Fig. 3B).

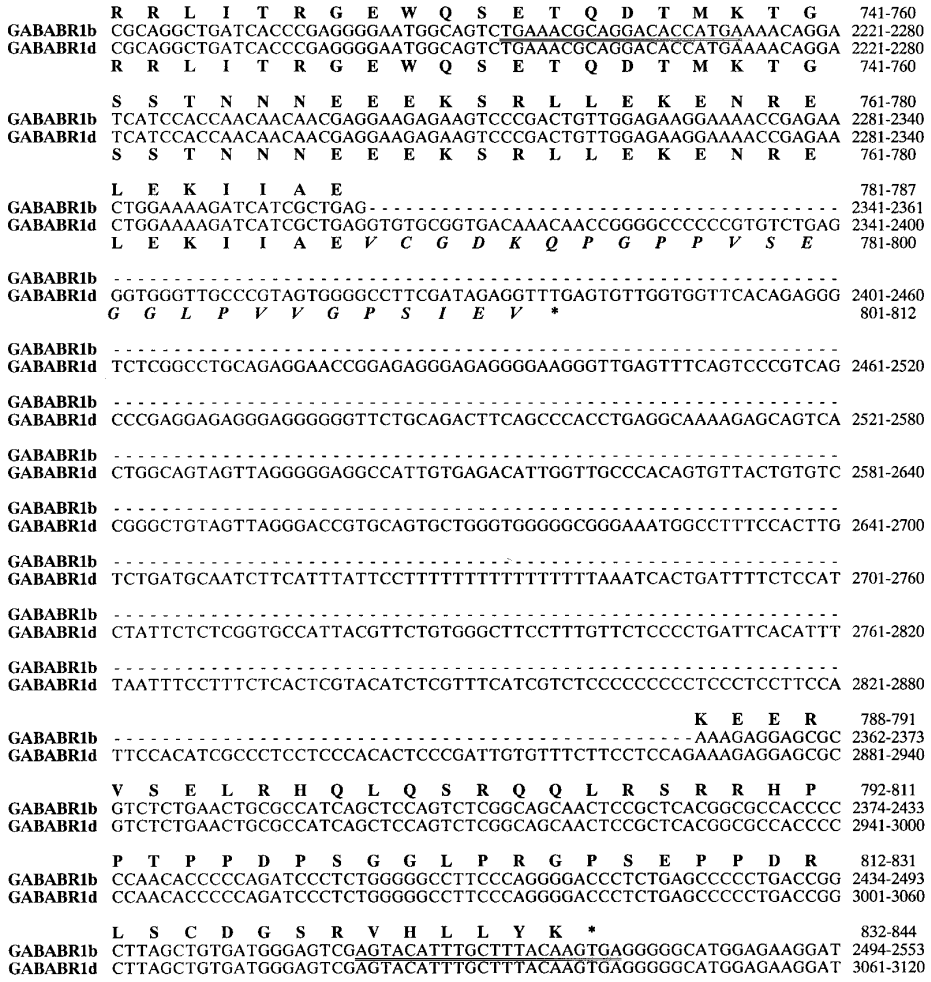

FIG. 2. Aligned nucleotide and deduced amino acid sequences in the transmembrane region of rat $GABA_BR1b$ and $GABA_BR1d$. The nucleotides and amino acids of $GABA_BR1b$ and -d are numbered to the right of each sequence. Asterisk indicates the terminal codon. $GABA_BR1d$ has an additional 566-bp insertion and diverged amino acids, indicated in italic. Nucleotide sequences of sense and antisense primers for RT-PCR to determine tissue distributions of $GABA_BR1b$ and -d mRNAs are indicated by the double bars.

DISCUSSION

We obtained evidence that $GABA_BR1c$ and -d have an additional splice in the transmembrane and 3′-translated regions of $GABA_BR1b$, respectively, while $GABA_BR1a$ differed from $GABA_BR1b$ in the 5′-translated nucleotide and amino acid sequences (5). Alternatively spliced variants have also been identified in G protein-coupled receptors such as glutamate, dopamine, β_1-adrenergic, and μ opioid receptors, and are implicated in altered receptor functions, including response to agonists, ligand binding and G protein coupling (10). For example, mGluR1a, a member of the metabotropic glutamate receptor gene family, generates faster responses in *Xenopus* oocytes (11), and is slightly activated even in the absence of an agonist in certain cell lines (12), compared with its splice variants lacking the long C-terminal domain. Thus, the divergent amino acid sequences resulted from use of the alternate exon or insertion of additional exons of $GABA_BR1$ isoforms might arise any functional differences. Kaupmann *et al.* (5) found $GABA_BR1a$ and -b to be pharmacologically identical and to have similar ligand-binding affinities. Biological functions of the $GABA_BR1$ isoforms remain to be determined.

When Kir3.0 channels are coexpressed in *Xenopus* oocytes with a G protein-coupled receptor, such as m2-muscarinic (6, 13), serotonin 1A (14) and δ-opioid receptors (15), each agonist for these receptors activated K^+ currents. We have reported that, in oocytes coinjected with the rat cerebellar poly(A)$^+$ RNA and cRNAs for Kir3.1 and Kir3.2, K^+ currents were elicited by $GABA_B$ receptor agonists such as baclofen and 3-aminopropylphosphinic acid, and that the baclofen-induced currents were inhibited by selective $GABA_B$ receptor antagonists such as 2-OH saclofen and CGP 35348 (4). These findings indicate that $GABA_B$ recep-

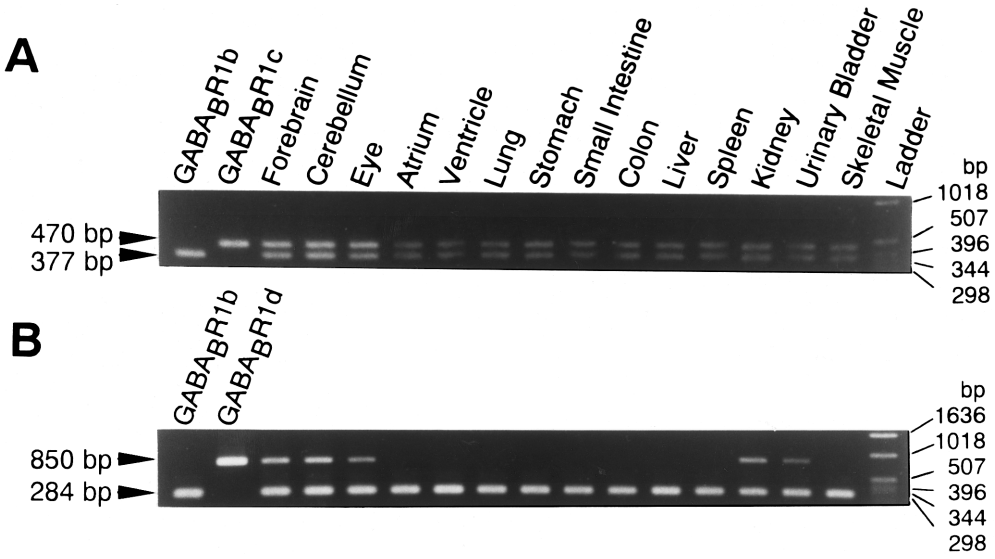

FIG. 3. RT-PCR detection of mRNAs for rat GABA$_B$R1 isoforms. (A) The specific primers for amplification of GABA$_B$R1c yielded transcripts of 470 bp for GABA$_B$R1c and those of 377 bp for isoforms not containing the 93-bp insertion of GABA$_B$R1c. GABA$_B$R1b and -c cDNAs were used as controls. The RT-PCR yielded two amplified products of 470 bp and 377 bp in all tissues examined. (B) The specific primers for amplification of GABA$_B$R1d yielded transcripts of 850 bp for GABA$_B$R1d and those of 284 bp for isoforms not containing the 566-bp insertion of GABA$_B$R1d. GABA$_B$R1b and -d cDNAs were used as controls. The RT-PCR products of 850 bp were detected in forebrain, cerebellum, eye, kidney and urinary bladder, and those of 284 bp in all tissues examined.

tors in rat cerebellum are functionally expressed in oocytes, and activate the Kir3.0 channels. Thus, for functional expression of cloned GABA$_B$ receptors, the *Xenopus* oocyte expression system could be used. Although it was demonstrated that GABA$_B$R1a and -b negatively couple to adenylyl cyclase, as described for native GABA$_B$ receptors when expressed in HEK293 cells (5), we found that baclofen did not induce any significant current alterations in oocytes where any one of GABA$_B$R1b, -c and -d was coexpressed with Kir3.1 and Kir3.2. These results suggest the possible existence of other classes for the GABA$_B$ receptor which can activate K^+ channels. Another possibility would be that accessory proteins may be needed to function as the receptor in *Xenopus* oocytes. A family of proteins that modify the activity of a member of G protein-coupled receptors has been identified and was shown to be expressed endogenously in certain cell lines, including HEK293T cells (16). Therefore, GABA$_B$R1 isoforms may require an accessory protein, which may possibly be expressed endogenously in HEK293 cells but not in oocytes, to functionally reconstitute the GABA$_B$ receptors. In our present study, we could not detect a specific binding for ^{3}H-baclofen in CHO or HEK293 cells transfected GABA$_B$R1 isoforms clones. The difference in a radioligand might explain the discrepancy between the previous results (5) and our present results. Although Kaupmann *et al.* (5) detected recombinant GABA$_B$R1 isoforms in COS cells and HEK293 cells using relatively high-affinity radioligands, very recently Couve *et al.* (17) reported that recombinant GABA$_B$R1a receptors fail to reach the cell surface and are retained in the endoplasmic reticulum when introduced into COS cells and HEK293 cells.

RT-PCR analysis showed that mRNAs for GABA$_B$R1 isoforms are expressed in various tissues, while Northern blot analysis revealed this expression only in brain and testis. Thus, GABA$_B$R1 isoforms are expressed at low levels in the organs other than brain and testis. Ubiquitous expression at low levels of the GABA$_B$R1 isoforms, including GABA$_B$R1c, suggests that the receptors may be expressed in subsets of cells which distribute widely in various tissues, e.g., vascular smooth muscle, endothelial and peripheral nerve cells, rather than in parenchymal organs. Actually, GABA$_B$ receptors were demonstrated in peripheral autonomic nerve terminals (18), where they mediate a decrease in evoked neurotransmitter release, resulting in inhibition of gastric motility, relaxation of urinary bladder and bronchus, reduction in vascular tone, stimulation of insulin, growth hormone and glucagon release, and hyperpolarization of sympathetic ganglia. On the other hand, GABA$_B$R1d was expressed in limited tissues, indicating that distribution of this isoform differs from that of other GABA$_B$R1 isoforms. Distribution of each isoform of GABA$_B$R1 in various tissues can be determined by *in situ* hybridization using the probes specific for each isoform, plus immunohistochemical techniques. Cloning of the GABA$_B$ receptors expressed in various tissues should prove to be an effective approach to elucidate molecular mechanisms of these receptors in peripheral organs.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan and The Ueda Memorial Trust Fund for Research of Heart Diseases. The $GABA_BR1c$ and -d cDNA sequences have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with Accession Nos. AB016160 and AB016161, respectively.

REFERENCES

1. Burt, D. B., and Kamatchi, G. L. (1991) *FASEB J.* **5,** 2916–2923.
2. Bowery, N. G., Hill, D. R., Hudson, A. L., Doble, A., Middlemiss, D. N., Shaw, J., and Turnbull, M. (1980) *Nature* **283,** 92–94.
3. Bowery, N. G. (1990) *Annu. Rev. Pharmacol. Toxicol.* **33,** 109–147.
4. Uezono, Y., Akihara, M., Kaibara, M., Kawano, C., Shibuya, I., Yanagihara, N., Toyohira, Y., Yamashita, H., Taniyama, K., and Izumi, F. (1998) *Neuroreport* **9,** 583–587.
5. Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., and Bettler, B. (1997) *Nature* **386,** 239–246.
6. Isomoto, S., Kondo, C., Takahashi, N., Matsumoto, S., Yamada, M., Takumi, T., Horio, Y., and Kurachi, Y. (1996) *Biochem. Biophys. Res. Commun.* **218,** 286–291.
7. Chazenbalk, G. D., Nagayama, Y., Kaufman, K. D., and Rapoport, B. (1990) *Endocrinology* **127,** 1240–1244.
8. Morrow, B. A., Lee, E. J. K., Taylor, J. R., Elsworth, J. D., Nye, H. E., and Roth, R. H. (1997) *JPET* **283,** 712–721.
9. Kofuji, P., Davidson, N., and Lester, H. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6542–6546.
10. Gudermann, T., Kalkbrenner, F., and Schultz, G. (1996) *Annu. Rev. Pharmacol. Toxicol.* **36,** 429–459.
11. Pin, J. P., Waeber, C., Prezeau, L., Bockaert, J., and Heinemann, S. F. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10331–10335.
12. Prézeau, L., Gomeza, J., Ahern, S., Mary, S., Galvez, T., Bockaert, J., and Pin, J. P. (1996) *Mol. Pharmacol.* **49,** 422–429.
13. Krapivinski, G., Gordon, E. A., Wickman, K., Velimirovic, B., Krapivinski, L., and Clapham, D. E. (1995) *Nature* **374,** 135–141.
14. Spauschus, A., Lentes, K.-U., Wischmeyer, E., Dißmann, E., Karschin, C., and Karschin, A. (1996) *J. Neurosci.* **16,** 930–938.
15. Lesage, F., Duprat, F., Fink, M., Guillemare, E., Coppola, T., Lazdunski, M., and Hugnot, J.-P. (1994) *FEBS Lett.* **353,** 37–42.
16. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) *Nature* **393,** 333–339.
17. Couve, A., Filippov, A. K., Connolly, C. N., Bettler, B., Brown, D. A., and Moss, S. J. (1998) *J. Biol. Chem.* **273,** 26361–26367.
18. Bowery, N. G., Dobel, A., Hill, D. R., Hudson, A. L., Shaw, J. S., Turnbull, M. J., and Warrington, R. (1981) *Eur. J. Pharmacol.* **71,** 53–70.