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The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β_2 and β_3 subunit

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ABSTRACT **Type A γ-aminobutyric acid ($GABA_A$) receptors of the mammalian nervous system are a family of ligand-gated ion channels probably formed from the coassembly of different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ) in the arrangement $\alpha\beta\gamma$ or $\alpha\beta\delta$. The activation of these receptors by GABA can be modulated by a range of compounds acting at distinct allosteric sites. One such compound is the broad-spectrum anticonvulsant loreclezole, which we have recently shown to act via a specific modulatory site on the β subunit of the $GABA_A$ receptor. The action of loreclezole depends on the type of β subunit present in the receptor complex; receptors containing β_2 or β_3 subunits have >300-fold higher affinity for loreclezole than receptors containing a β_1 subunit. We have used this property to identify the amino acid residue in the β subunit that determines the subunit selectivity of loreclezole. Chimeric β_1/β_2 human $GABA_A$ receptor subunits were constructed and coexpressed in *Xenopus* oocytes with human α_1 and γ_{2s} subunits. The chimera β_1/β_2Lys237–Gly334 conferred sensitivity to 1 μM loreclezole. Within this region there are four amino acids that are conserved in β_2 and β_3 but differ in β_1. By mutating single amino acids of the β_1 subunit to the β_2/β_3 equivalent, only the β_1 mutation of Ser-290 → Asn conferred potentiation by loreclezole. Similarly, mutation of the homologous residue in the β_2 and β_3 subunits to the β_1 equivalent (Asn → Ser) resulted in loss of sensitivity to loreclezole. The affinity for GABA and the potentiation by flunitrazepam were unchanged in receptors containing the mutated β subunits. Thus, a single amino acid, β_2 Asn-289 (β_3 Asn-290), located at the carboxyl-terminal end of the putative channel-lining domain TM2, confers sensitivity to the modulatory effects of loreclezole.**

The mammalian type A γ-aminobutyric acid ($GABA_A$) receptor gene family is now known to consist of a number of subunit polypeptides (α_1–α_6, β_1–β_3, γ_1–γ_3, δ, ρ_1–ρ_2) (1, 2). There is an increased body of evidence that suggests that in neurons these subunits coassemble in the arrangement $\alpha\beta\gamma$ or $\alpha\beta\delta$ (3, 4), probably as pentamers (by analogy with the related nicotinic receptor), to give a family of receptor subtypes that have different spatial and temporal patterns of expression (5).

The activity of $GABA_A$ receptors can be allosterically modulated by a number of agents, including ethanol and neurosteroids, and the clinically important barbiturates and benzodiazepines (BZs) (6, 7). The BZ pharmacology of $GABA_A$ receptor subtypes has been studied in some detail. Only receptors made up of an α, β, and γ subunit exhibit high-affinity BZ binding (8), and the pharmacology is determined by the type of α (9–11) and γ (12), but not the β (13), subunit present, suggesting that this modulatory site is constituted by determinants on both the α and γ subunits. The structural determinants that contribute to the other modulatory sites of the $GABA_A$ receptor are currently unclear.

Loreclezole, {(*Z*)-1-[2-chloro-2-(2,4-dichlorophenyl) ethenyl]-1,2,4-triazole}, is a broad-spectrum anticonvulsant compound that was suggested to act via the $GABA_A$ receptor (14, 15). Until recently the precise mode of action of loreclezole was unclear. Using recombinant human $GABA_A$ receptors expressed in *Xenopus* oocytes and transfected cells, we have recently shown that loreclezole potentiates the action of GABA by acting at a specific modulatory site on the β subunit (16). Further, it was found that loreclezole is subunit selective, having a >300-fold higher affinity for receptors containing β_2 or β_3 subunits over those containing β_1 subunits.

In this report we describe the results of experiments done to identify the structural determinants responsible for the subunit-selective action of loreclezole. By determining the modulation by loreclezole of recombinant $GABA_A$ receptors containing a series of β-subunit chimeras and point mutants, we have been able to demonstrate that a single amino acid, Asn-289 of the β_2 subunit (Asn-290 of the β_3 subunit) confers sensitivity to this modulatory compound.

MATERIALS AND METHODS

cDNA Clones. cDNAs encoding human $GABA_A$ receptor α_1, β_1, β_2, β_3, and γ_{2s} subunits have been described (11–13, 17, 18).

Construction of Chimeric β Subunits. The human β subunit nucleotide sequences do not contain any conserved restriction enzyme sites that would be useful for constructing chimeras. As such, a PCR approach was used to introduce into the β_1 sequence restriction enzyme sites already present in the β_2 sequence, allowing the subsequent construction of chimeric β_1/β_2 subunits. Each chimera was constructed in the eukaryotic expression vector pCDM8 (Invitrogen), using strategies described below. PCR was performed with *Taq* polymerase (Perkin–Elmer/Cetus) as described (19), using human β_1 and β_2 subunit cDNAs as template. Oligonucleotide primers were prepared by using an Applied Biosystems 380B synthesizer and purified by using oligonucleotide purification cartridges, according to the manufacturer's instructions. PCR products were extracted with phenol/chloroform, ethanol-precipitated, digested with the appropriate restriction enzyme, purified by agarose-gel electrophoresis, and ligated as required. Constructs were verified by DNA sequencing. The construction of each β subunit chimera was as follows:

Chimera β2Δ1.1: A β_1 PCR product was generated by using the oligonucleotide primers 5′-CTAAGTTTTCGTCTTAAGAGAAACATTGG-3′ (sense, bp 734–762 of β_1 cDNA, introducing an *Afl* II site) and 5′-TTGATACCATGGCGGGATCCAGACAT-3′ (antisense primer located in the

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Abbreviations: $GABA_A$, γ-aminobutyric acid type A; BZ, benzodiazepine.

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pCDM8 vector, bp 3348–3371). This was digested with *Afl* II and *Not* I (site in polylinker of pCDM8) and ligated to similarly cut β_2 cDNA to generate β2Δ1.1.

Chimera β2Δ1.2: A β_1 PCR product was generated with primers 5′-CCAATGTTTCTCTTAAGACGAAAACT-TAG-3′ (antisense, bp 734–762 of β_1 cDNA introducing an *Afl* II site) and 5′-AGTCCGAAAGAATCTGCTCCCTGCTT-3′ (sense primer located in the pCDM8 vector, bp 1375–1400), digested with *Afl* II and *Hin*dIII (site in polylinker of pCDM8), and ligated into similarly cut β_2 cDNA to generate β2Δ1.2.

Chimera β2Δ1.3: The chimera β2Δ1.2 was digested with *Xho* I (bp 1330 of β_2 cDNA) and *Xba* I (site in polylinker), removing cDNA-encoding C-terminal amino acids 373–end. This was ligated with the corresponding β_1 sequence generated by PCR using primers 5′-CTCAGCACCCTCGAGATC-CGGAATGA-3′ (sense, bp 1036–1061 of β_1 cDNA, containing an *Xho* I site) and the antisense primer present in pCDM8 (bp 3348–3371) described above, also digested with *Xho* I and *Xba* I (site in polylinker of pCDM8) to create β2Δ1.3.

Chimera β2Δ1.4: A *Hin*dIII (bp 929) *Apa* I (bp 1221) β_2 cDNA fragment was subcloned into pBluescript (Stratagene). A β_1 PCR product was generated by using oligonucleotides 5′-CTTTGGGAAAGGGCCCCAGAAAAAGG-3′ (sense, bp 1024–1049, incorporating an *Apa* I site) and 5′-ACCAATTGGAGGGCCCAATCTCTTACC-3′ (bp 1548–1574, incorporating an *Apa* I site), digested with *Apa* I and ligated into the *Apa* I-cut β_2 pBluescript DNA. This construct was then digested with *Afl* II and *Xho* I and ligated to similarly cut β2Δ1.3 to generate β2Δ1.4.

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed essentially as has been described (20), with the modification that mutagenic primers were designed so as to also contain a silent base change that would result in the introduction or removal of a restriction site. This result allowed rapid screening of mutants by restriction digest. All mutations were confirmed by DNA sequencing. Mutagenesis of β_1 subunit cDNA was performed with the subunit cDNA in pBluescript (Stratagene); mutants were subcloned into pCDM8 for expression studies. Mutagenesis of β_2 and β_3 subunit cDNAs was performed by using the vector pCDNA I Amp (Invitrogen). The oligonucleotides used to generate each β subunit mutant were as follows: for β_1 with Thr-255 → Ile, 5′-TGTAATCAGTATCGATGGCATGTAGG-3′ (incorporating a *Cla* I site); for β_1 with Ser-290 → Asn, 5′-CTCCCTGAGATGGGTGTTGATGGTTG-3′ (removes *Sau* I site); for β_1 with Ile-308 → Met, 5′-CCATCAGATACAT-GTCAATCGCTTTG-3′ (incorporating an *Afl* III site); for β_2 with Asn-289 → Ser, 5′-ACAATCAGCACCCACCTCAGG-GAAAC-3′ (incorporating a *Sau* I site); for β_3 with Asn-290 → Ser, 5′-ACCATCAGCACCCACCTTAGGGAGAC-3′ (incorporating a *Sau* I site).

***Xenopus* Oocyte Expression.** Oocytes were removed from anesthetized toads and manually defolliculated; the follicular cells were removed by mild collagenase treatment (type IA, 0.5 mg/ml for 10 min). The oocyte nuclei were directly injected by using the "blind" method with 10–20 nl of injection buffer (88 mM NaCl/1 mM KCl/15 mM Hepes, pH 7.0; filtered through nitrocellulose) containing $GABA_A$ receptor subunit cDNAs (6 ng/μl) and used 24–96 hr later. Approximately 80% of injected oocytes had responses to applied GABA. For electrophysiological recording, oocytes were placed in a 50-μl bath and perfused at 4–6 ml/min with modified Barth's medium [88 mM NaCl/1 mM KCl/10 mM Hepes/0.82 mM $MgSO_4$/0.33 mM $Ca(NO_3)_2$/0.19 mM $CaCl_2$/2.4 mM $NaHCO_3$, pH 7.5]. Cells were impaled with two 1- to 3-MΩ electrodes containing 2 M KCl and voltage-clamped between −40 and −70 mV. Drugs were applied in the perfusate. GABA modulators were preapplied for 30 sec before GABA addition, which was applied until the peak of the response was observed, normally 30 sec or less. At least 3 min was allowed between each GABA application to prevent desensitization. Concentration–response curves were calculated by using a nonlinear square-fitting program to the equation $f(x) = B_{MAX}/[1+(EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. Where the effect of a modulatory compound (loreclezole or flunitrazepam) was being determined, an EC_{20} concentration of GABA was used (between 3 and 30 μM), predetermined for each individual oocyte.

Loreclezole was a gift from Janssen, and all other compounds were obtained from Sigma or Research Biochemicals (Natick, MA).

RESULTS

Structural Determinants Necessary for the Modulation of $GABA_A$ Receptors by Loreclezole Are Located Between Lys-237 and Gly-334 of the β_2 Subunit. We have previously demonstrated that the modulatory action of loreclezole is at least 300-fold selective for $GABA_A$ receptors containing a β_2 or β_3 subunit over those containing a β_1 subunit (ref. 16 and Fig. 1). This property was used to delineate the region of the β_2 subunit that confers sensitivity to loreclezole. A series of β_1/β_2 chimeric subunits were constructed and coexpressed in *Xenopus* oocytes with α_1 and γ_{2s} cDNAs. Large GABA-gated currents (0.5–2 μA) were observed with all receptors containing either wild-type or mutated β subunits, with approximately the same affinities for GABA. The ability of 1 μM loreclezole (the EC_{50} concentration for modulation of $\alpha_1\beta_2\gamma_{2s}$ receptors; see ref. 16) to potentiate submaximal GABA responses ($\approx EC_{20}$, between 3 and 30 μM GABA, determined for each individual oocyte) at $GABA_A$ receptors containing chimeric β subunits was then determined (Fig. 1). The determinants required for potentiation by loreclezole were delineated to the sequence encompassed by Lys-237 and Gly-334 of the β_2 subunit (chimera β2Δ1.4). As a control, all receptor subunit combinations were examined for their potentiation by the BZ flunitrazepam (Fig. 1). Application of 1 μM flunitrazepam (which has an EC_{50} of 29 nM; ref. 21) potentiated the GABA responses of all subunit combinations, confirming their expression and coassembly, although interestingly the potentiation of receptors containing the chimeras β2Δ1.3 and β2Δ1.4 was somewhat reduced.

Asn-289 of the β_2 Subunit (Asn-290 of the β_3 Subunit) Is Required for Potentiation by Loreclezole. Site-directed mutagenesis was used to precisely identify the determinants within the sequence Lys-237–Gly-334 of the β_2 subunit required for potentiation by loreclezole. In Fig. 2 partial amino acid sequences of the human β_1, β_2, and β_3 subunits have been aligned. Because receptors containing β_2 and β_3 subunits, but not those containing β_1, are potentiated by 1 μM loreclezole, we identified as targets for site-directed mutagenesis amino acid residues that were conserved in β_2 and β_3 subunits but are not conserved in β_1. There are four such residues: β_1 Thr-255, β_1 Ser-290, β_1 Ile-308, and β_1 Lys-334 (Fig. 2). The latter was disregarded, as it is located within the putative large cytoplasmic domain between TM3 and TM4. Site-directed mutagenesis was performed on the β_1 subunit, individually changing each of the other three residues to their β_2/β_3 equivalent. When coexpressed with α_1 and γ_{2s} subunits, only one mutant, β_1 Ser-290 → Asn, conferred loreclezole sensitivity (Fig. 3). To confirm the critical role of this residue, two other mutants were constructed: β_2 Asn-289 → Ser and β_3 Asn-290 → Ser. In both these mutants the asparagine residue of the β_2 and β_3 subunits, thought to be critical for loreclezole potentiation, were replaced by serine found in the homologous position of the β_1 subunit. When coexpressed with α_1 and γ_{2s}, in both cases the mutation

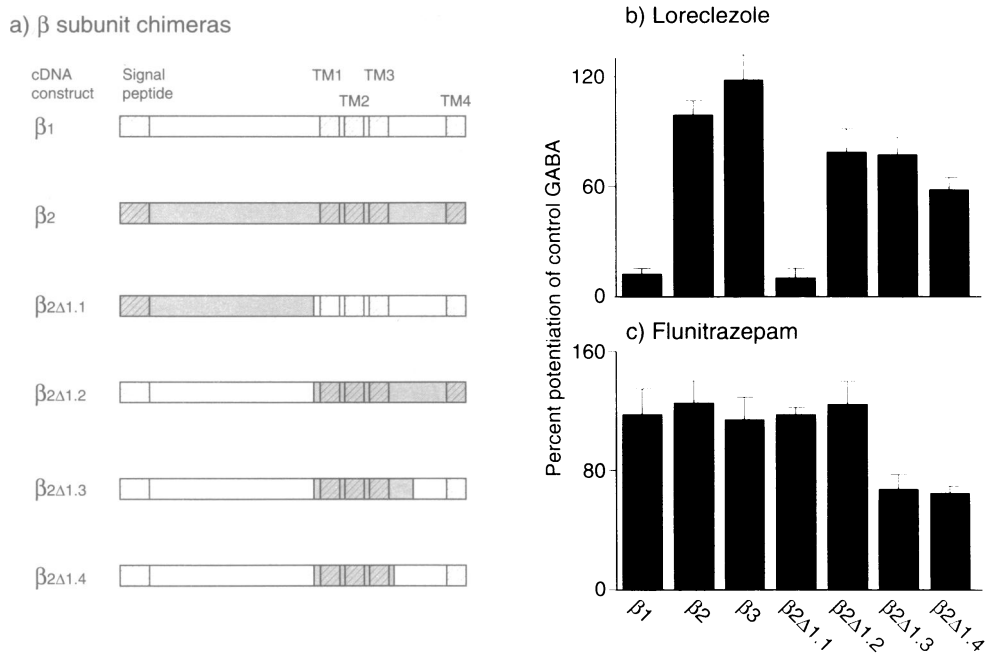

FIG. 1. Modulation by loreclezole and flunitrazepam of human $GABA_A$ receptors containing chimeric β_1/β_2 subunits. (*a*) Diagrammatic representation of β_1/β_2 chimeras. β_1 sequences are represented by clear boxes, and β_2 sequences are represented by shaded boxes. The hatched areas represent the putative signal peptide and transmembrane domains (TM1–4). Potentiation of human $\alpha_1\beta\gamma_{2S}$ subunit combinations by loreclezole (*b*) and by flunitrazepam (*c*). The β subunit present in each combination is indicated beneath each bar. Bars represent the percent potentiation of a submaximal GABA response (EC_{20}, predetermined for each individual oocyte). Data are the mean ± SEM of ≥ four oocytes.

resulted in loss of loreclezole sensitivity, confirming the importance of this asparagine residue. Typical responses demonstrating the effect of loreclezole on $GABA_A$ receptor subunit combinations containing wild-type or mutant β subunits are shown in Fig. 4. For all subunit combinations, the affinities for GABA were not affected, and flunitrazepam could potentiate the response to GABA (Fig. 3*b*), demonstrating that the receptor subunits had been expressed and correctly assembled.

Concentration–effect curves were generated to determine the EC_{50} for the potentiation by loreclezole of the response to GABA (Fig. 5). Receptors containing wild-type β_2 or mutant β_1 Ser-290 → Asn had essentially the same EC_{50} value (1.2 ± 0.4 μM and 2.0 ± 0.15 μM, respectively), Hill slope (0.7 ± 0.11 and 0.9 ± 0.05, respectively), and maximum potentiation (214 ± 9% and 181 ± 29%, respectively). These data demonstrate that Asn-289 of the β_2 subunit (Asn-290 of the β_3 subunit) is the critical determinant for potentiation of $GABA_A$ receptors by loreclezole.

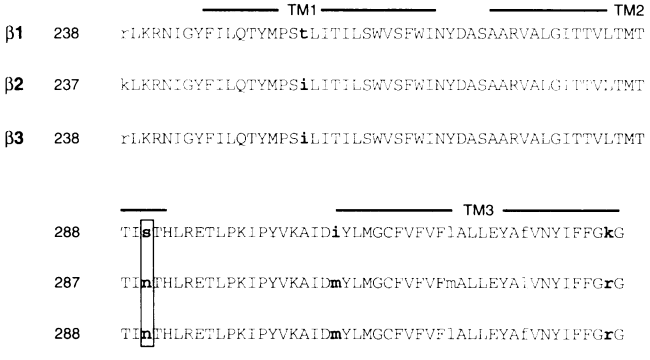

FIG. 2. Identification of the amino acid residue in the β subunit required for potentiation of $GABA_A$ receptor function by the anticonvulsant loreclezole; alignment of the predicted amino acid sequences of the human $GABA_A$ receptor β_1, β_2, and β_3 subunit sequences. Only the region of the subunit sequences corresponding to Lys-237–Gly-334 of β_2 are shown. Numbering indicates the amino acid number, assigning the initiating methionine as 1. The putative transmembrane domains are indicated by TM1–3. Residues in boldface type are those that are conserved only in β_2 and β_3. The boxed residue indicates the position of β_2 Asn-289 and β_3 Asn-290.

DISCUSSION

In this study we investigated the structural determinants that confer subunit-selective modulation of $GABA_A$ receptor function by the specific anticonvulsant compound loreclezole. A single amino acid residue, β_2 Asn-289 (β_3 Asn-290) was responsible for confering sensitivity to loreclezole: its removal from β_2 and β_3 resulted in loss of sensitivity (Fig. 3), whereas Ser → Asn substitution in the homologous position of β_1 resulted in the gain of loreclezole sensitivity (Figs. 3–5).

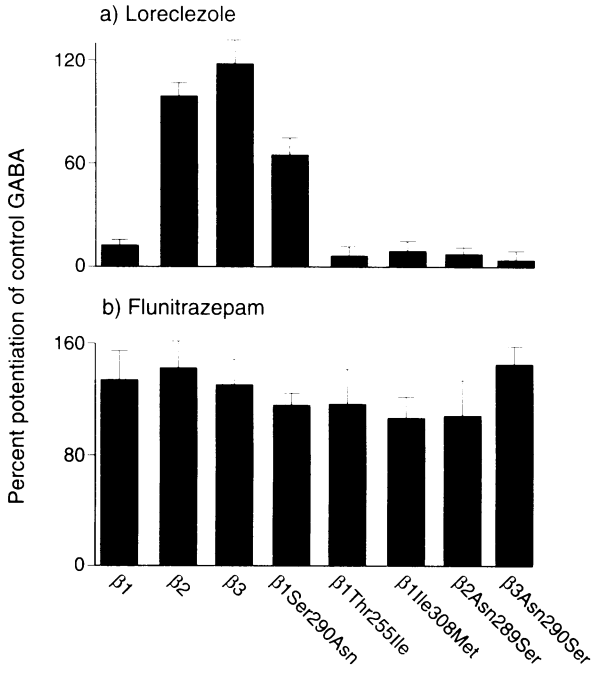

FIG. 3. Modulation by loreclezole and flunitrazepam of human $GABA_A$ receptor combinations containing mutated β subunits. Potentiation of human $GABA_A$ receptor subunit combinations by loreclezole (*a*) and flunitrazepam (*b*). The β subunit present in each subunit combination is indicated beneath each bar. Bars represent the percent potentiation of a submaximal GABA response (EC_{20}, predetermined for each individual oocyte). Data are the mean ± SEM of ≥ four oocytes.

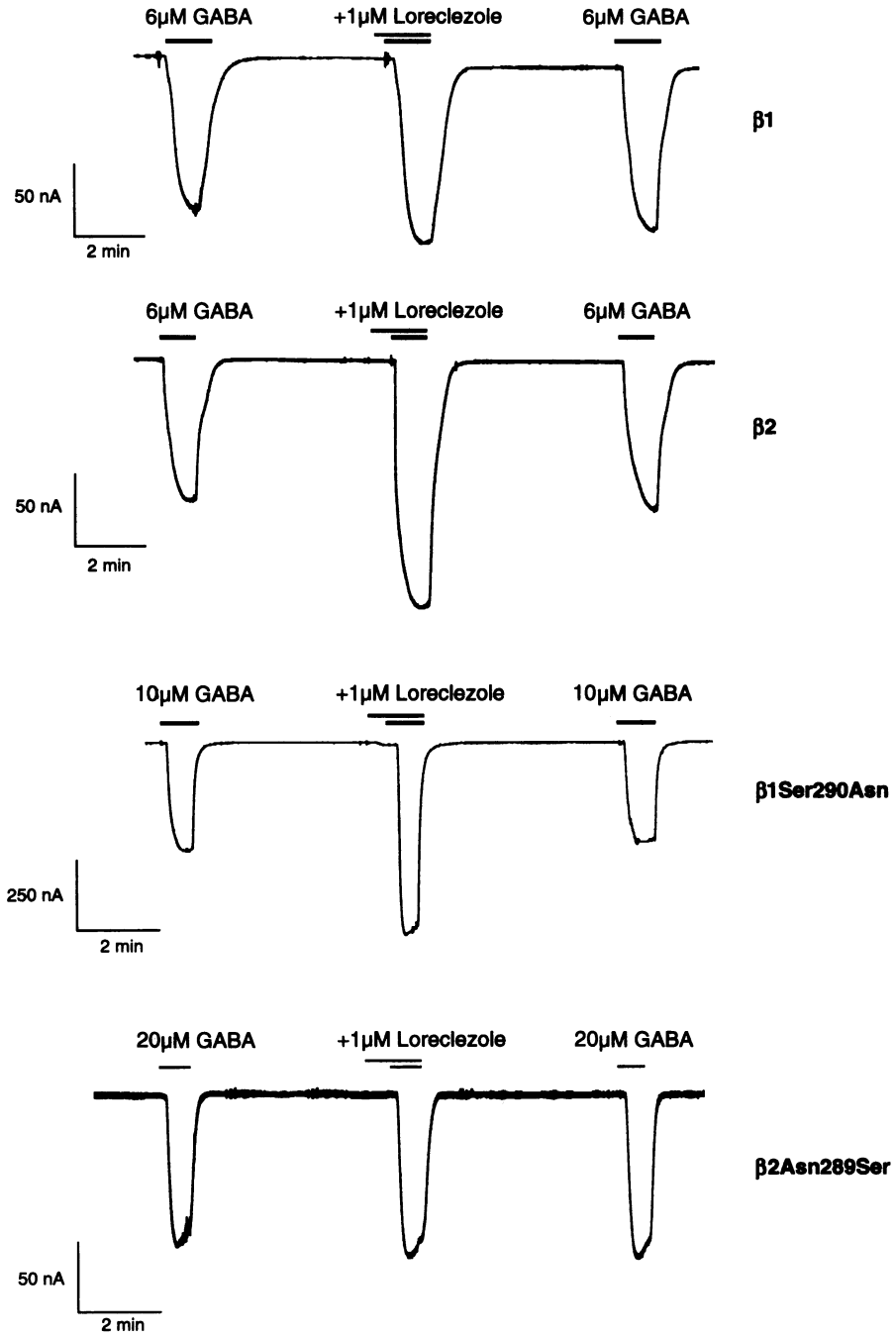

FIG. 4. Effect of 1 μM loreclezole on GABA-induced currents in *Xenopus* oocytes expressing human $GABA_A$ receptors composed of $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_1$(Ser290Asn)γ_2, and $\alpha_1\beta_2$(Asn289Ser)γ_2. Loreclezole was applied for 30 sec before coapplication of GABA (an EC_{20} concentration, predetermined for each oocyte) as indicated by the bars. Recovery was observed after a 3-min wash period.

Our data demonstrate that a single-amino acid residue determines the sensitivity of $GABA_A$ receptors to loreclezole. Further investigation is required to definitively determine whether this asparagine residue is required for binding of loreclezole (i.e., located at the binding site) or whether it is required for the transduction of the allosteric changes that result from the binding of loreclezole to the receptor. It is possible to speculate that the molecular interaction is via a hydrogen bond(s) between the triazole moiety of loreclezole and the amide group of asparagine. However, a single hydrogen bond contributes 0.5–1.8 kcal·mol^{-1} of binding energy, equivalent to perhaps a factor of 2–20 in the dissociation constant (22). Because the change in EC_{50} for loreclezole at $\alpha_1\beta_1\gamma_2$ versus $\alpha_1\beta_1$(Ser290Asn)γ_2 is at least 300-fold (Fig. 5), then other molecular interactions would be required. Unfortunately there is no radioligand for the loreclezole-binding site; such a radioligand would be a very useful tool for further characterization of the mechanism of interaction of loreclezole with the $GABA_A$ receptor.

It is only the β_2 and β_3 subunits that have an asparagine residue at position 289/290. The β_1 subunit (17) and all α (11, 23) and γ (24) subunits have a serine, and the δ subunit has a methionine (25). Considering other members of the ligand-gated ion channel gene superfamily, the glycine receptor α_1 subunit has a serine at the homologous position (26), whereas the β_1 subunit has a cysteine (27). The cation-gating 5-hydroxytryptamine-type 3 receptor (28) and *Torpedo* nicotinic acetylcholine receptor α, β, γ, δ subunits (see ref. 29) all have leucine at the homologous position. Thus, at least for the $GABA_A$ and glycine receptor subunits, there appears to be

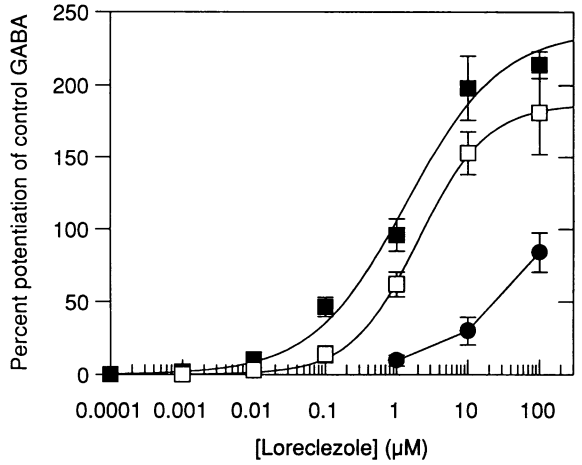

FIG. 5. Concentration–effect curves for the potentiation by loreclezole of human $GABA_A$ receptors expressed in *Xenopus* oocytes. ●, $\alpha_1\beta_1\gamma_2$; ■, $\alpha_1\beta_2\gamma_2$; □, $\alpha_1\beta_1$(Ser289Asn)γ_2. Data for the first two combinations are taken from Wafford *et al.* (16) and is shown for comparison. An EC_{20} concentration of GABA was used, predetermined for each individual oocyte. Data were fitted as described in the methods.

some flexibility regarding the amino acid found in this position.

Perhaps the most intriguing observation is the location of this asparagine residue is its location in the TM2 helical domain, now generally agreed to line the pore of the ion channel. The precise location of this residue is toward the carboxyl-terminal end of TM2, i.e., on the extracellular side, six residues away from the conserved leucine residue (β_2 Leu-283), which in *Torpedo* nicotinic acetylcholine receptor (Leu-251) is thought to be at the narrowest point of the channel (30). Although many residues in TM2 have been mutated and changes in channel function have been demonstrated (31), the residue at the position homologous to β_2 Asn-289 does not appear to have been subjected to site-directed mutagenesis.

Site-directed mutagenesis has also been used to begin to identify the structural determinants that contribute to the BZ-binding site. Studies have concentrated on the α subunit. A single amino acid substitution (α_1 Gly-228 → Glu) increases the affinity of several selective BZ compounds without affecting the affinity of nonselective compounds (32). Similarly, conversion of α_6 Arg-119 → His conferred high-affinity binding of diazepam to $\alpha_6\beta_2\gamma_2$ receptors (33). It is also clear that the γ subunit contributes to the BZ-binding site and influences the pharmacology (12, 34). Indeed, some BZ compounds will bind to recombinant $GABA_A$ receptors that lack an α subunit (ref. 35 and K.A.W., unpublished observations). It is thus likely that the BZ-binding site will be made up of contributions from both the α and γ subunits. Together, these data suggest that although a single amino acid residue can be a critical determinant for the binding of certain compounds to the BZ-binding site, ligand-binding sites are complex structures made up of numerous determinants that are not necessarily closely located in the primary sequence. This is certainly the case for the acetylcholine-binding site of the nicotinic acetylcholine receptor (31). In this report we have demonstrated that a single amino acid residue determines to a very large degree the modulatory effect of loreclezole. Further studies will be required to determine definitively if β_2 Asn-289 is, indeed, located at the loreclezole-binding site and what, if any, contributions are made by other amino acid residues.

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