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Residues in Transmembrane Domains I and II Determine γ-Aminobutyric Acid Type A_A Receptor Subtype-Selective Antagonism by Furosemide

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ABSTRACT

$GABA_A$ receptors in cerebellar granule cells are unique in expressing a subtype containing the α6 subunit. This receptor subtype has high affinity for GABA and produces a degree of tonic inhibition on cerebellar granule cells, modulating the firing of these cells via spillover of GABA from GABAergic synapses. This receptor subtype also has selective affinity for the diuretic furosemide over receptors containing other α-subunits. Furosemide exhibits approximately 100-fold selectivity for α6-containing receptors over α1-containing receptors. By making α1/α6 chimeras we have identified a transmembrane region (209–279) responsible for the high furosemide sensitivity of α6β3γ2s receptors. Within the α1 transmembrane region, a single amino acid was identified that when mutated from threonine to isoleucine, increased furosemide sensitivity by 20-fold. We demonstrate the β-subunit selectivity of furosemide to be due to asparagine 265 in the β2 and β3 transmembrane-domain II similar to that observed with potentiation by the anticonvulsant loreclezole. We also show that Ile in transmembrane-domain I accounts for the increased GABA sensitivity observed at α6β3γ2s compared with α1β3γ2s receptors, but did not affect direct activation by pentobarbital or potentiation by the benzodiazepine flunitrazepam. Location of these residues within transmembrane domains leads to speculation that they may be involved in the channel-gating mechanism conferring increased receptor activation by GABA, in addition to conferring furosemide sensitivity.

In the mammalian brain, inhibitory neurotransmission is mainly mediated via activation of $GABA_A$ receptors, which belong to a superfamily of ligand-gated ion channels. The mammalian $GABA_A$ receptor gene family consists of a number of subunit polypeptides (α_{1-6}, β_{1-3}, γ_{1-2}, δ, and ϵ) that are thought to coassemble as pentamers (Whiting et al., 1995; Sieghart, 1995). Native $GABA_A$ receptor subtypes most likely consist of α and β subunits together with a γ, δ, or ϵ subunit. The binding of GABA to the receptor complex results in the opening of an anion channel through which chloride ions flow. In addition to the GABA binding site, a number of allosteric sites have been identified on the receptor, which can modulate GABAergic activity. These include the benzodiazepines and anesthetics, which potentiate GABAergic responses, and antagonists such as picrotoxin and zinc, which act in a noncompetitive manner (Macdonald and Olsen, 1994).

Another compound identified as a noncompetitive antagonist at $GABA_A$ receptors is the diuretic compound furosemide. This blocker of the $Na^+/2Cl^-/K^+$ cotransporter, has also been shown to be receptor subtype-selective, eliciting approximately 100-fold greater sensitivity for α6β2γ2s receptors than for α1β2γ2s receptors (Korpi et al., 1995), as well as selectivity for α6β3γ2s over α6β1γ2s. The aim of this study was to identify the amino acids within the α6 subunit and β3 subunit that are responsible for conferring high affinity for this antagonist, using chimeric receptors and point mutations.

Materials and Methods

Cloning of human $GABA_A$ receptor subunit cDNAs (α1-α6, β3, and γ2s) has been described previously (Hadingham et al., 1993a,b, 1996; Wafford et al., 1996). Chimeric and point-mutated cDNAs were generated by standard techniques as described previously (Wingrove et al., 1994). Mutations were confirmed by DNA sequencing.

Expression of Human $GABA_A$ Receptors in *Xenopus* Oocytes. Adult female *Xenopus laevis* were anesthetized by immersion in a 0.4% solution of 3-aminobenzoic acid ethylester for 30 to 45 min (or until unresponsive). Ovary tissue was removed via a small abdominal incision and stage V and VI oocytes were isolated with fine forceps. After mild collagenase treatment to remove follicle cells (Type IA, 0.5 mg ml^{-1} for 6 min), the oocyte nuclei were directly injected with 10 to 20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7, filtered through nitro-cellulose) containing different combinations of human $GABA_A$ subunit cDNAs (20 ng μl^{-1}) engineered into the expression vector pCDM8 or pcDNAI/Amp. After incubation for 24 to 72 h, oocytes were placed in a 50 μl bath and perfused at 4 to 6 ml/min^{-1} with modified Barth's medium consisting

ABBREVIATIONS: TM; transmembrane-domain; HEK, human embryonic kidney.

of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, at pH 7.5. Cells were impaled with two 1 to 3 MΩ electrodes containing 2 M KCl and voltage-clamped at −70 mV.

In all experiments drugs were applied in the perfusate until the peak of the response was observed. Noncumulative concentration-response curves to GABA and pentobarbital were constructed with an interval of 3 min between each agonist application.

Inhibition curves to furosemide (0.3–3000 μM) were constructed using a GABA EC_{50} concentration. Furosemide was preapplied for 30 s before addition of the GABA EC_{50} concentration and furosemide. The effects of flunitrazepam were examined on control GABA EC_{20} responses with a preapplication time of 30 s. A minimum of two *Xenopus* toads were used for each data set.

Whole Cell Patch-Clamp of Human Embryonic Kidney (HEK) 293 Cells Transiently Transfected with Human $GABA_A$ Receptors. Experiments were performed on HEK 293 cells transiently transfected with human cDNA combinations α1β3γ2s, α6β3γ2s, α1T230Iβ3γ2s, and α6I228Tβ3γ2s (6 μg of cDNA total per coverslip) using calcium phosphate precipitation (Chen and Okayama, 1988) as described previously (Hadingham et al., 1993a). Glass coverslips containing the cells in a monolayer culture were transferred to a perspex chamber on the stage of Nikon Diaphot inverted microscope. Cells were continuously perfused with a solution containing 124 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM D-glucose, at pH 7.2, and observed using phase-contrast optics. Patch-pipettes were pulled with an approximate tip diameter of 2 μm and a resistance of 4 MΩ with borosilicate glass and filled with 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, 3 mM Mg^+-ATP, pH adjusted to 7.3 with CsOH. Cells were patch-clamped in whole-cell mode using an Axopatch-200B patch-clamp amplifier (Axon Inst., Foster City, CA). Drug solutions were applied by a double-barreled pipette assembly, controlled by a stepping motor attached to a Prior manipulator, enabling rapid equilibration around the cell. Increasing GABA concentrations were applied for 5-s pulses with a 30-s interval between applications.

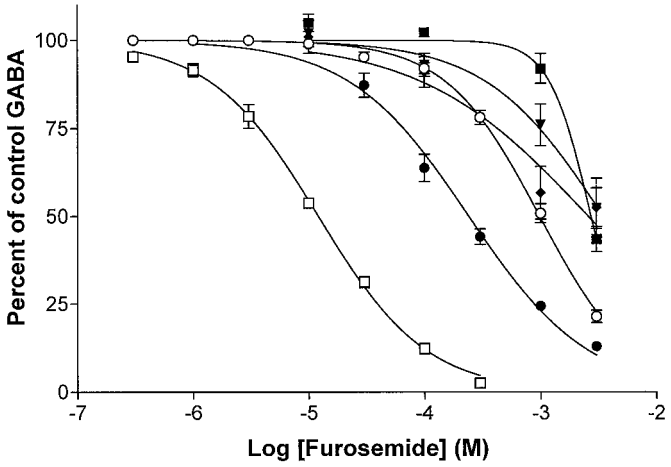

Fig. 1. Concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes expressing human $GABA_A$ receptors composed of α1β3γ2s (○), α2β3γ2s (▼), α3β3γ2s (♦), α4β3γ2s (●), α5β3γ2s (■), and α6β3γ2s (□) Data represents the mean ± S.E.M. of at least three individual cells.

Analysis. Curves were fitted 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. EC_{50} and IC_{50} values are shown as mean (95% CL), $n = 3$ or more, and differences between means were evaluated by Student's t test and considered significant if $P < .05$.

Drugs Used. γ-Aminobutyric acid (Sigma Chemical Co., St. Louis, MO) was prepared as a 1 M stock solution in modified Barth's medium. Concentrated stock solutions of furosemide (1 M) and flunitrazepam (10 mM) (both obtained from Sigma) were freshly prepared in 100% dimethyl sulfoxide. Pentobarbital was obtained from Rhône Mérieux (Harlow, UK) as a concentrate in alcohol (Sagatal for injection containing 60 mg ml^{-1} pentobarbitone sodium). The concentrates were diluted into buffer and the maximal final vehicle concentration was 0.3% v/v for dimethyl sulfoxide and 0.4% v/v for the alcohol. No effects on GABA currents were observed with either vehicle.

Results and Discussion

As has previously been reported (Korpi et al., 1995; Wafford et al., 1996) furosemide displays a greater sensitivity for

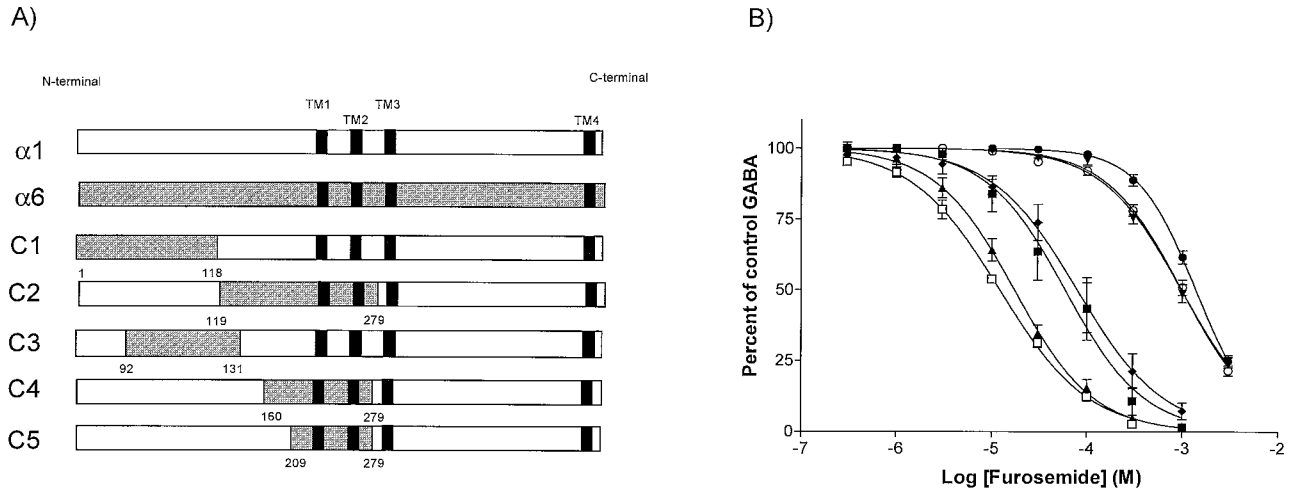

Fig. 2. A, schematic diagram of the five α1/α6 chimeras generated for this study. Numbering is according to mature α1 polypeptide and for each chimera the first and last amino acid of the α6 sequence are numbered. The transmembrane domains are illustrated. Shaded area represents α6 sequence and clear areas represent α1 sequence. B, concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes expressing human $GABA_A$ receptors composed of α1β3γ2s (○), α6β3γ2s (□), and the chimeras C1 (●), C2 (▲), C3 (▼), C4 (♦), and C5 (■) expressed with β3γ2s. Data represents the mean ± S.E.M. of at least four individual cells.

$\alpha6\beta3\gamma2s$ receptors [IC_{50} = 12.1 (11.4, 12.9) μM] compared with $\alpha1$–$5\beta3\gamma2s$ receptors [IC_{50} values ranging from 234.9 (212.3, 260) μM for $\alpha4\beta3\gamma2s$ to ≥3 mM for $\alpha2\beta3\gamma2s$] (Fig. 1). In addition, the γ-subunit appears not to be required for furosemide antagonism, as $\alpha6\beta3$ receptors are also highly sensitive to block by furosemide, with an IC_{50} of 14.4 (9.5, 21.9) μM (data not shown).

A number of chimeras (C1-C5) were made encompassing different regions of the $\alpha1$ and $\alpha6$ subunits (Fig. 2A) and expressed with human $\beta3$ and $\gamma2$ subunits in *Xenopus* oocytes. Chimeras 1 and 3 both displayed $\alpha1$-like furosemide sensitivity

TABLE 1

Summary of furosemide IC_{50} values and GABA EC_{50} values determined for the different chimeras and point mutants in this study, expressed using (a) *Xenopus* oocytes or (b) HEK cells. Data represents mean (±95% confidence limits) of $n \geq 3$, n.d. (not determined)

Receptor	GABA EC_{50}	Furosemide IC_{50}
	μM	μM
(a) *Xenopus* oocytes		
$\alpha1\beta3\gamma2s$	11.3 (8.8, 14.5)	980 (889, 1080)
$\alpha4\beta3\gamma2s$	n.d.	235 (212, 260)
$\alpha6\beta3\gamma2s$	3.85 (3.45, 4.3)	12.1 (11.4, 12.9)
Chimera 1	17.4 (13.1, 23.1)	1380 (1320, 1450)
Chimera 2	13.2 (10.8, 16.1)	17.1 (14.9, 19.7)
Chimera 3	9.29 (7.03, 12.28)	980 (870, 1100)
Chimera 4	7.53 (7.00, 8.11)	78.6 (55.8, 110.6)
Chimera 5	6.41 (3.79, 10.86)	56.3 (37.6, 84.3)
$\alpha1T230I\beta3\gamma2s$	5.38 (3.69, 7.85)	40.9 (34.6, 48.3)
$\alpha6I228T\beta3\gamma2s$	n.d.	127.6 (86.3, 188.6)
$\alpha4T234I\beta3\gamma2s$	n.d.	22.3 (18.5, 26.9)
Chimera 6	n.d.	1520 (1350, 1700)
(b) HEK cells		
$\alpha1\beta3\gamma2s$	3.29 (2.50, 5.37)	2240 (1710, 2940)
$\alpha6\beta3\gamma2s$	0.89 (0.74, 1.08)	69 (55, 87)
$\alpha1T230I\beta3\gamma2s$	0.84 (0.77, 0.91)	211 (147, 305)
$\alpha6I228T\beta3\gamma2s$	0.71 (0.59, 0.86)	311 (244, 397)

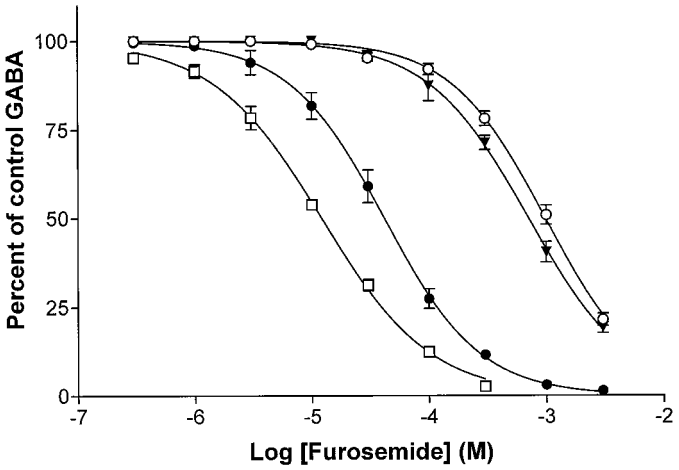

Fig. 4. Concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes expressing human $GABA_A$ receptors composed of $\alpha1\beta3\gamma2s$ (○), $\alpha6\beta3\gamma2s$ (□), and the individual point mutants $\alpha1V227M\beta3\gamma2s$ (▼) and $\alpha1T230I\beta3\gamma2s$ (●). Data represents the mean ± S.E.M. of four individual cells.

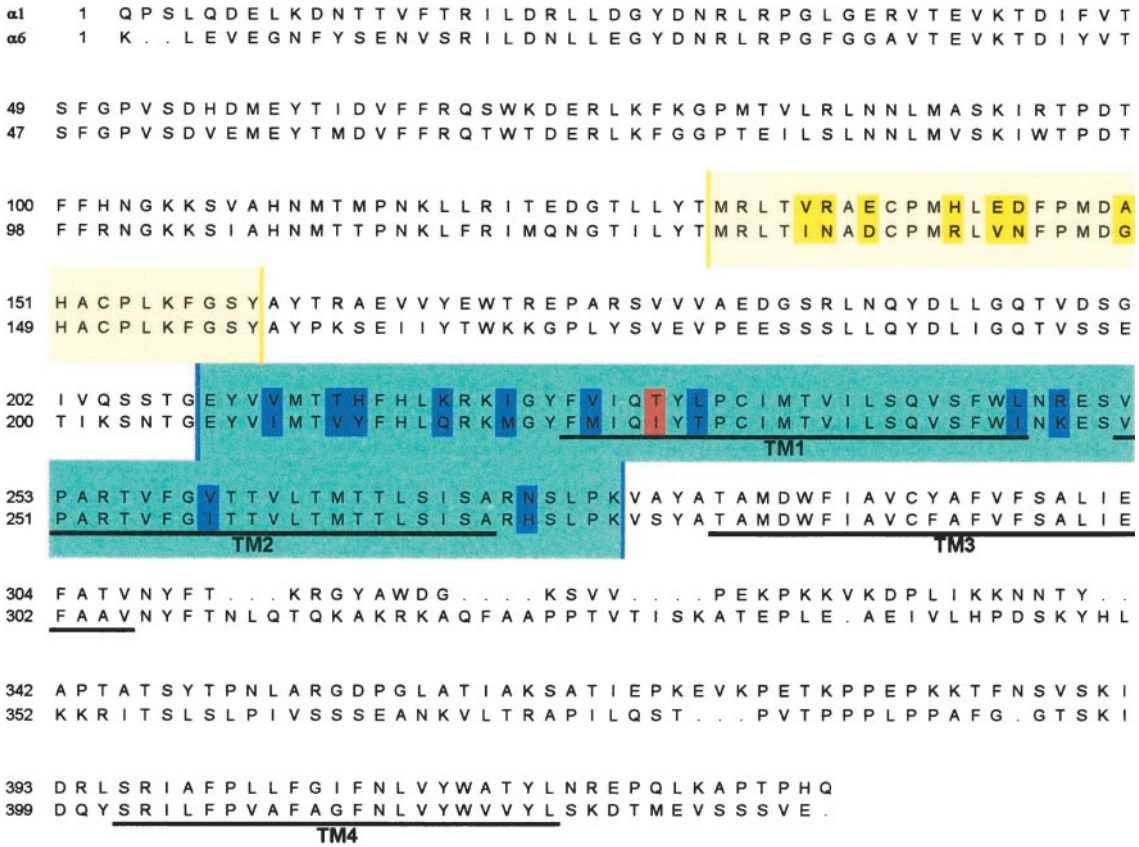

Fig. 3. Alignment of human $GABA_A$ $\alpha1$ and $\alpha6$ subunits. Numbering is according to mature $\alpha1$ polypeptide. The figure shows the four putative transmembrane domains and the two regions containing the amino acids responsible for the high sensitivity at $\alpha6$-containing receptors identified from the $\alpha1/6$ chimeras. Within the first region shaded in yellow (131–160) there are seven amino acid differences between $\alpha1$ and $\alpha6$ whereas the second region shaded in blue (209–279) has 12.

(C1 IC_{50} = 1.38 (1.32, 1.45) mM and C3 IC_{50} = 0.98 (0.87, 1.10) mM). The furosemide sensitivity for chimera 2 was not significantly different from $\alpha6\beta3\gamma2s$ receptors [17.1 (14.9, 19.7) μM compared with 12.1 (11.4, 12.9) μM] whereas chimeras 4 and 5 displayed intermediate sensitivity [78.6 (55.8, 110.6) μM and 56.3 (37.6, 84.3) μM respectively; Fig. 2B] (Table 1a). These results suggest that there are at least two amino acids responsible for the high furosemide sensitivity of $\alpha6$-containing receptors, the first being located within a region between amino acids 131 and 160 and the second between 209 and 279 (Fig. 3).

Identification of Isoleucine228 in Transmembrane-Domain (TM) 1. In the region between positions 209 and

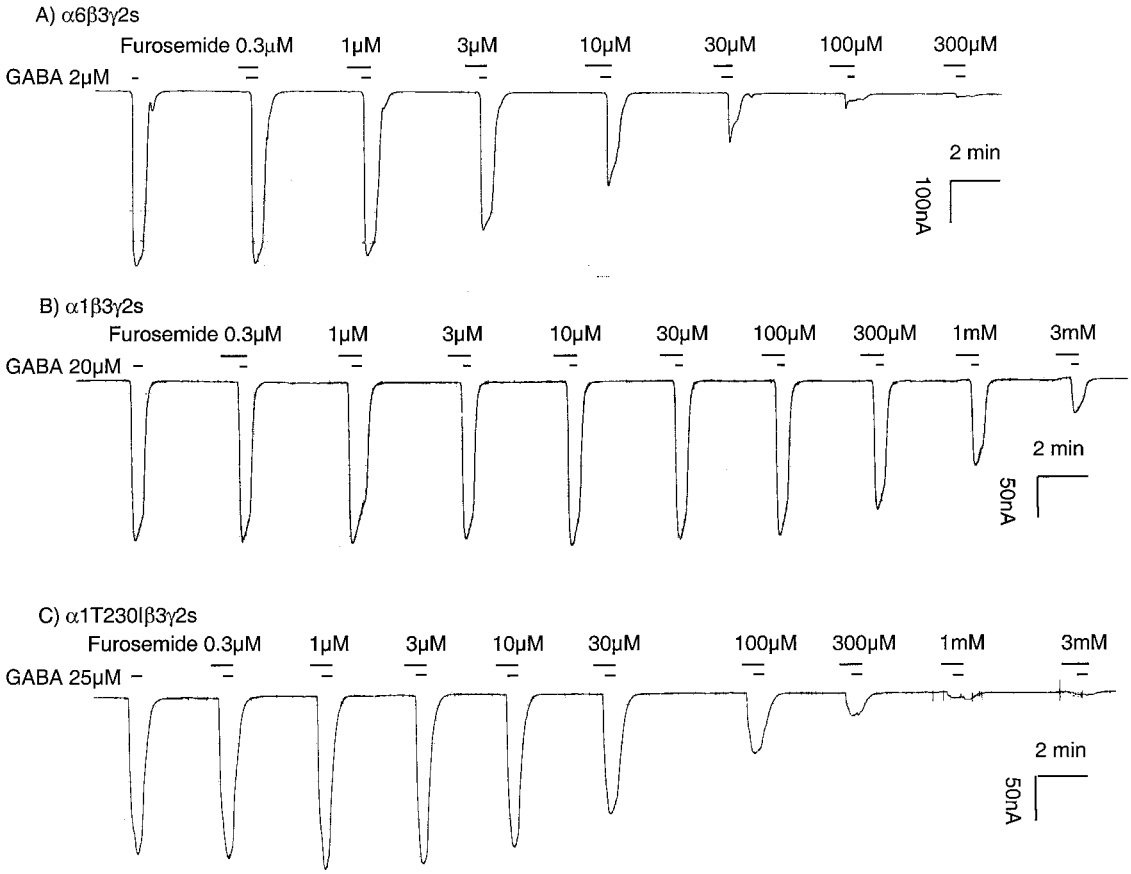

Fig. 5. The sensitivity of furosemide is determined in part by the presence of isoleucine at position 228 within the $\alpha6$ subunit. Representative current recordings illustrate the concentration-dependent inhibition by furosemide (0.3 μM –3 mM) of the inward current evoked by an EC_{50} concentration of GABA on *Xenopus* oocytes expressing $\alpha6\beta3\gamma2s$ (A), $\alpha1\beta3\gamma2s$ (B), and $\alpha1T230I\beta3\gamma2s$ (C) receptors. Drug application is illustrated by the horizontal bars above the current records.

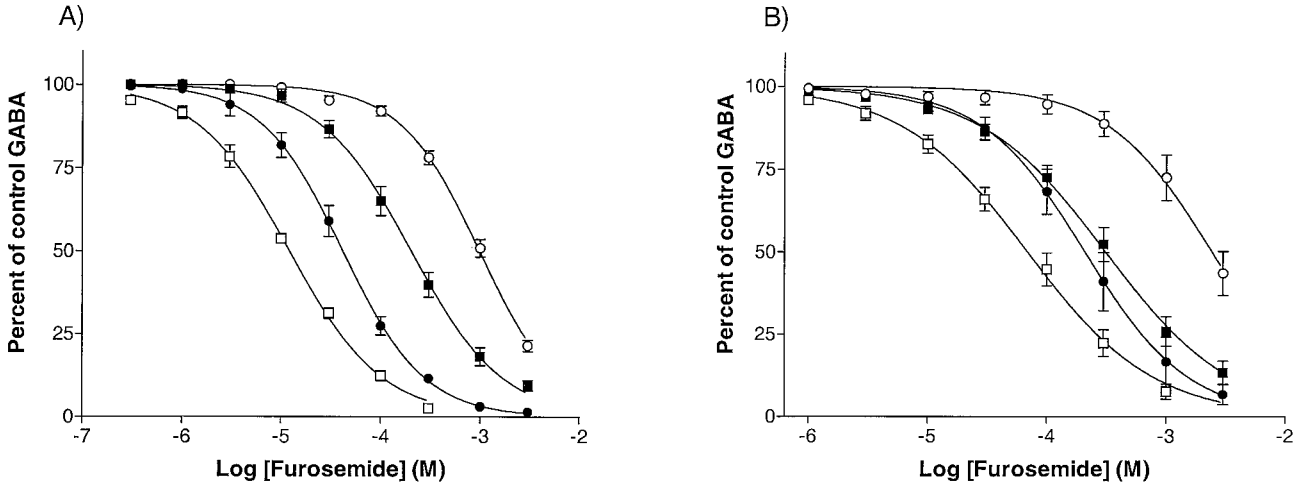

Fig. 6. Concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes (A) and transiently transfected HEK cells (B) expressing $\alpha1\beta3\gamma2s$ (○), $\alpha1T230I\beta3\gamma2s$ (●), $\alpha6\beta3\gamma2s$ (□), and $\alpha6I228T\beta3\gamma2s$ (■ $GABA_A$ receptors. Data represents the mean ± S.E.M. of at least four individual cells.

279 there are 12 amino acid differences between α1 and α6. Fisher et al. (1997) described a rat α6/α1 chimera with a splice site within TM1 that conferred high furosemide sensitivity and a α1 point mutation (α1L258T) where furosemide sensitivity was unchanged. These results eliminated 5 of the 12 amino acids identified within this region. The remaining seven amino acids were mutated (in groups of 2 or 3) in α1 to the α6 equivalent and the furosemide IC_{50} determined.

α1V212I,T215V,H216Yβ3γ2s and α1K220Q,I223Mβ3γ2s receptors both displayed α1-like furosemide sensitivity [IC_{50} = 2.12 (1.78, 2.51) mM and 1.15 (0.94, 1.40) mM respectively]. α1V227M,T230Iβ3γ2s receptors, however, revealed an intermediate sensitivity [IC_{50} = 51.4 (44.6, 59.2) μM] similar to that of chimeras 4 and 5. Individual point mutations produced IC_{50} values of 0.7 (0.63, 0.78) mM for α1V227Mβ3γ2s and 40.9 (34.6, 48.3) μM for α1T230Iβ3γ2s (Fig. 4), demonstrating a critical role for isoleucine 228 within the α6 subunit in conferring furosemide selectivity.

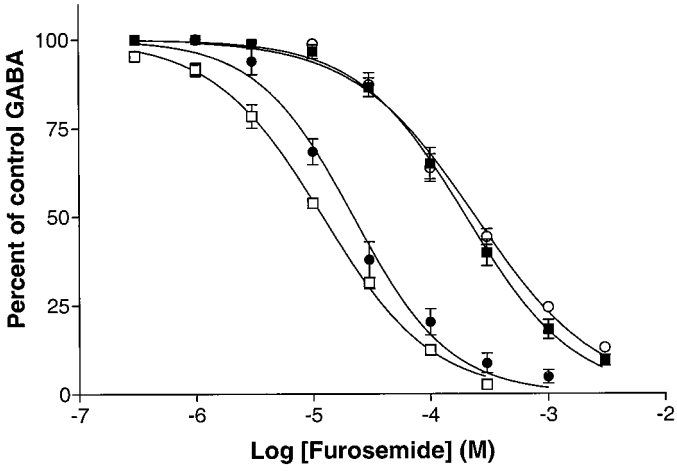

Fig. 7. Concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes expressing human $GABA_A$ receptors composed of α4β3γ2s (○), α4T234Iβ3γ2s (●), α6β3γ2s (□), and α6I228Tβ3γ2s (■). Data represents the mean ± S.E.M. of four individual cells.

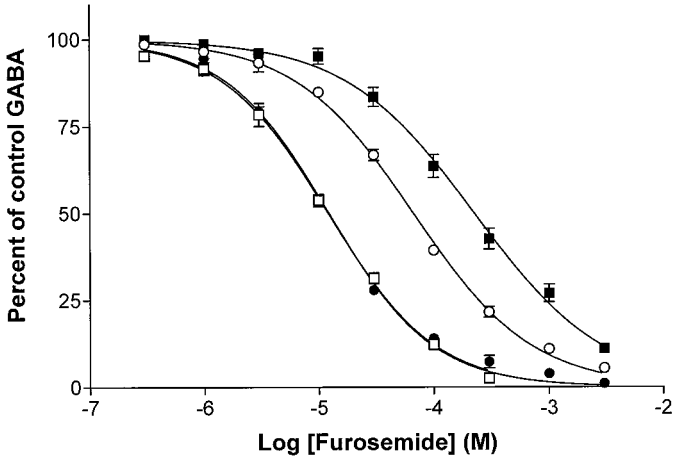

Fig. 8. Concentration-inhibition curves for furosemide versus a GABA EC_{50} response on *Xenopus* oocytes expressing human $GABA_A$ receptors composed of α6β1γ2s (○), α6β1S265Nγ2s (●), α6β3γ2s (□), and α6β3N265Sγ2s (■). Data represents the mean ± S.E.M. of at least four individual cells.

Figure 5 illustrates the effects of furosemide on oocytes expressing wild-type α1β3γ2s, α6β3γ2s, and α1T230Iβ3γ2s receptors. The effects of furosemide were shown to be significantly reduced on the equivalent α6 receptor mutant (α6I228T), producing a 10-fold reduction in furosemide sensitivity with an IC_{50} of 127.6 (86.3, 188.6) μM (Fig. 6A). In addition, when the same wild-type and equivalent mutants were expressed in HEK cells and studied using whole cell-patch-clamp techniques, similar differences were observed in furosemide sensitivity (Fig. 6B, Table 1b).

Interestingly, the threonine residue is conserved in all other α-subunits, including the α4 subunit, that has intermediate furosemide sensitivity (Wafford et al., 1996), and so cannot account for the higher affinity of α4-containing receptors for furosemide. Mutation of this conserved threonine to isoleucine in α4 produced a 10-fold increase in furosemide sensitivity with an IC_{50} of 22.3 (18.5, 26.9) μM, similar to α6β3γ2s receptors. (Fig. 7).

The location of this residue in TM1 suggests that TM1 may form part of the ion channel with the residue providing a binding site for furosemide. The residue may also have a role in ion-channel gating, possibly making the receptor more amenable to block by furosemide. Thus mutation of a single amino acid within the α1 subunit from threonine to the α6 equivalent isoleucine at position 230 produced a 20-fold increase in furosemide sensitivity. This single amino acid change, however, did not shift the IC_{50} completely to that observed on α6β3γ2 receptors, suggesting that other residues are also involved.

Additional Determinants Affecting Furosemide Sensitivity. Our results from the chimera studies identified a possible second domain (131–160) within the α6 subunit, which may contribute to the high furosemide sensitivity. Within the transmembrane domain of α1, a single amino acid changed to the α6 equivalent, α1T230I, increased the furosemide sensitivity of α1 by 20-fold. A further 5-fold increase in sensitivity is required to bring the furosemide IC_{50} to that seen on α6β3γ2s or chimera 2. Single amino acid mutations or insertion of the region 131 to 160 into α1 however, did not affect the furosemide IC_{50} (see Table 1a, chimera 6), so it is currently unclear how this small additional component is

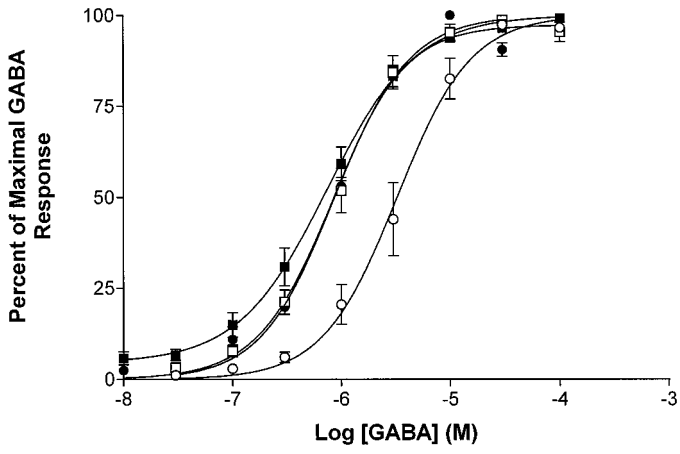

Fig. 9. GABA concentration-response curves on transiently transfected HEK cells expressing α1β3γ2s (○), α1T230Iβ3γ2s (●), α6β3γ2s (□), and α6I228Tβ3γ2s (■) $GABA_A$ receptors. Data represents the mean ± S.E.M. of at least eight individual cells.

conferred. The action of furosemide has also been shown to depend on the β-subunit variant, being weaker on β1-containing receptors than on β2- and β3-containing receptors (Korpi et al., 1995). Potentiation of $GABA_A$ receptors by the anticonvulsant loreclezole has been shown to be dependent on the β-subunit (Wafford et al., 1994) and is dependent on the presence of asparagine 265 in the β2 or β3 subunit (Wingrove et al., 1994). We have compared the effects of furosemide on α6β1γ2 and α6β3γ2 receptors, confirming selectivity for α6β3γ2. We have also used β point mutants, β1S265N and β3N265S, coexpressed with α6 and γ2s to demonstrate that the β-subunit selectivity is determined by the same asparagine residue as loreclezole (Fig. 8). Mutation of the serine within β1 to asparagine (the β3 counterpart) increased furosemide sensitivity [from an IC_{50} of 66.5 (63.3, 70.0) μM to 12.3 (11.8, 12.9) μM]. Conversely, mutation of the asparagine within β3 to serine decreased furosemide sensitivity [from an IC_{50} of 12.4 (11.4, 12.9) μM to 224 (190, 263) μM]. It is interesting to note that mutation within the β1 subunit revealed an identical IC_{50} as α6β3γ2s whereas mutation within the β3 subunit produced a significantly higher IC_{50} than α6β1γ2s. Like the threonine/isoleucine we have identified in TM1, the asparagine/serine is located on the extracellular end of TM2 and it is possible that these two amino acids are located close to each other at the extracellular face of the channel.

Isoleucine 228 in α6 Also Confers Higher GABA Affinity. Interestingly, concentration-response curves for GABA expressing the wild-type α1β3γ2, α6β3γ2, and the corresponding Thr/Ile mutants revealed significant differences in GABA affinity. The GABA EC_{50} for α1T230Iβ3γ2s receptors [0.84 (0.77, 0.91) μM] in HEK cells was significantly lower than α1β3γ2s receptors [3.29 (2.50,5.37) μM] but not different from α6β3γ2s receptors [0.89 (0.74, 1.08) μM (Fig. 9; Table 1b]. However, the equivalent mutation in α6 (I228T) did not affect GABA EC_{50} [0.71 (0.59, 0.86) μM]. Hence, this mutation could also account for the higher GABA affinity of α6-containing receptors. GABA concentration-response curves in *Xenopus* oocytes were also carried out on the mutant α1 and α6 receptors, as well as all the α1/α6 chimeras, however, the greater intrinsic variability in the oocyte expression system precluded the significant detection of such a 5-fold difference. The location in TM1 makes it unlikely that this residue forms part of the GABA binding site, which has been shown to be formed by residues in the α and β-subunit N-terminal regions (Sigel et al., 1992; Amin and Weiss, 1993). The EC_{50} value is a function of both the GABA binding affinity and the isomerization rate constants for transitions between the various closed, open, and desensitized states. Channel gating involves conformational changes in the membrane-spanning domains and we hypothesize that mutation from threonine 230 to isoleucine within TM1 alters the transduction process, resulting in a lower EC_{50} value. The high GABA affinity of α6-containing receptors has recently been shown to be critical to their function in granule cells, as mediating a tonic inhibition via spillover of GABA from Golgi to granule cell synapses (Brickley et al., 1996; Rossi and Hamann, 1998).

Similar to GABA and furosemide, direct activation by pentobarbital displays α6 selectivity (Thompson et al., 1996) and was therefore examined on *Xenopus* oocytes expressing α1β3γ2s and α1T230Iβ3γ2s receptors. No differences were observed in either the EC_{50} or maximum response as a percentage of the maximum GABA response (189 μM and 75% for α1β3γ2 compared with 191 μM and 66% for α1T230Iβ3γ2s). Additionally, potentiation of a GABA EC_{20} by the benzodiazepine flunitrazepam (1 μM) was unaffected by the threonine to isoleucine mutation (104 ± 13% for α1β3γ2s and 90 ± 8% for α1T230Iβ3γ2s). Although mutation of Thr230 to Ile within the α1 subunit significantly increased furosemide and GABA affinity, it did not alter the direct activation of pentobarbital or the potentiation elicited by flunitrazepam.

The role of the putative membrane spanning TM1 has also been investigated in the muscle nicotinic receptor (Akabas and Karlin, 1995) using cysteine substitution experiments. They suggest that the top third (N terminal) of TM1 contributes to the lining of the ion channel and hypothesize that in the closed state, TM1 segments intercalate between TM2 at the extracellular end. On receptor activation, movements of TM1 and TM2 could flip a gate, possibly formed by the cytoplasmic loop between them. If the same is true in the homologous $GABA_A$ receptor, by interacting directly with TM1, furosemide could be stabilizing this closed state of the ion channel gate. The position of the asparagine in β2 and β3, however, is hypothesized to be facing away from the lumen of the channel (Xu and Akabas, 1996); if this is the case, it may interact with the residues identified within TM1 in this study. Further study of the effects of this mutation at the single channel level will enhance our understanding of how this residue affects channel function and the mechanism of furosemide antagonism.

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