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Structural determinants of the pharmacological properties of the $GABA_A$ receptor α6 subunit

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ABSTRACT

$GABA_A$ receptors (GABARs) are responsible for fast inhibitory neurotransmission in the mammalian brain and are the targets for many clinical drugs that act as antiepileptics, anxiolytics, and sedatives. The pharmacological characteristics of the receptor are largely determined by its subunit composition. Compared to all other α subtypes, the α6 subtype confers unique pharmacological properties. In particular, α6-containing receptors are more sensitive to both the agonist GABA and the antagonist amiloride. Results from chimeric constructs of the α1 and α6 subunits suggested that structural differences within the extracellular N-terminal domain were responsible for both these characteristics. Within this domain we examined fifteen amino acid residues unique to the α6 subtype. Each of these sites was individually mutated in the α6 subunit to the corresponding residue of the α1 subunit. The mutated subunits were expressed in HEK-293T cells along with wild-type β3 and γ2L subunits and sensitivity to GABA and amiloride determined with whole-cell electrophysiological recordings. Serine83 in the α6 subunit influenced sensitivity to both GABA and amiloride. Leucine174 and tyrosine175 were also found to contribute to inhibition by amiloride, but did not affect GABA sensitivity. These structural differences are at least partly responsible for the unique pharmacological properties associated with the α6 subunit.

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The $GABA_A$ receptor (GABAR) is responsible for most fast inhibitory neurotransmission in the mammalian central nervous system through the activation of an intrinsic, chloride-permeable ion channel. Agents that potentiate GABAR activity are widely used clinically as antiepileptics, anxiolytics, and sedatives. The structure of the receptor influences its sensitivity to many of these modulators. GABARs are pentameric, and can be constructed from a diverse family of GABAR subunits (Nayeem et al., 1994; Karlin and Akabas, 1995). To date, seven subunit families and sixteen subunit subtypes (α(1-6), β(1-3), γ(1-3), δ(1), ε(1), π(1), and θ(1)) have been found in mammalian species (Whiting et al., 1999). Most native GABARs are believed to contain 2 α, 2 β, and 1 γ subunit (Chang et al., 1996; Tretter et al., 1997).

The α subtype composition of the receptor is a major determinant of its functional properties. In particular, GABARs containing the α6 subtype exhibit distinctive pharmacological properties. Differences associated with the α6 subtype compared to the α1 subtype include a higher sensitivity to GABA, insensitivity to benzodiazepines, higher sensitivity to inhibition by zinc, furosemide, and amiloride, inhibition rather than potentiation by lanthanum, and greater direct activation by pentobarbital (Draguhn et al., 1990; Wieland et al., 1992; Knoflach et al., 1996; Thompson et al., 1996; Korpi and Luddens, 1997; Saxena et al., 1997; Fisher, 2002). Although some of these properties are shared with the structurally homologous α4 subunit, higher sensitivity to GABA and amiloride are unique to the α6 subtype (Ducic et al., 1995; Knoflach et al., 1996; Fisher, 2002). The diuretic amiloride is best known for its inhibitory actions on epithelial sodium channels (Kleyman and Cragoe, 1988). However, it has also been shown to inhibit the activity of GABARs through both competitive and non-competitive mechanisms (Inomata et al, 1988; Fisher, 2002).

The structural determinants of these pharmacological differences between the α1 and α6

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subtypes are known for only a few of these modulators. The insensitivity of the α6 subunit to benzodiazepines is due, at least in part, to an arginine residue in its extracellular N-terminal domain. Replacing this arginine with the histidine found in this location in other α subunits confers sensitivity to these positive modulators (Wieland et al., 1992). The ability of furosemide to inhibit α6-containing receptors is linked to an isoleucine residue in the first transmembrane (TM1) domain (Thompson et al., 1999). Higher sensitivity to zinc is associated with a histidine residue in the extracellular TM2-TM3 domain of the α6 subunit, which is replaced by an asparagine residue in the α1 subunit (Fisher and Macdonald, 1998). These studies have provided important information regarding the structural differences that underlie the distinct pharmacological properties associated with the α6 subunit. However, the structures responsible for its higher sensitivity to GABA or amiloride have yet to be described.

To determine the role of the extracellular N-terminal domain in determining subtype-specific pharmacological properties, we used chimeric subunits that exchange this region between the α1 and α6 subtypes (Fisher et al., 1997). We focused on this region as it represents the largest extracellular domain of the subunit, and is believed to contributes to the binding sites of many GABAR agonists and modulators. Previous studies suggested this domain plays a significant role in regulating GABA sensitivity (Fisher et al., 1997). We then used point mutations to determine the role of single amino acid differences within this domain in regulating sensitivity to GABA and amiloride. Excluding the initial highly variable segment of the N-terminus, there are fifteen amino acid residues in the extracellular N-terminal domain that are unique to the α6 subtype compared to other α subunits (Figure 1). Each of these residues was individually mutated to the corresponding amino acid of the α1 subunit. The wild-type, chimeric, or mutated subunits were transiently transfected into the HEK-293T cell line along

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with wild-type β3 and γ2L subunits and the sensitivity of the receptor to GABA and amiloride determined using whole-cell patch clamp recordings.

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MATERIALS AND METHODS

Construction of chimeric and mutated Subunits

The α1/α6 chimeric subunits were constructed with a splice site in the 1st transmembrane domain as described by Fisher et al. (1997). The commercially available QuickChange Site-Directed Mutagenesis and StrataPrep PCR Purification Kits (Stratagene, La Jolla, CA) were used to generate the desired point mutation from the wild-type plasmid and purify the product. Mutations were verified by sequencing (University of South Carolina DNA core).

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Transfection of HEK-293T cells

Full-length cDNAs for the wild-type GABAR subunits in the pCMVNeo expression vector were obtained from Dr. Robert Macdonald (Vanderbilt University). Recombinant receptors were transiently expressed in the human embryonic kidney cell line HEK-293T (GenHunter, Nashville, Tennessee). HEK-293T cells express the SV-40 T antigen, which allows the plasmid to replicate within the cell, leading to increased protein expression. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged with 0.05% trypsin/0.02% EDTA solution in phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH = 7.3).

HEK-293T cells were transfected using calcium phosphate precipitation (Angelotti et al., 1993). Plasmids encoding the selected GABAR subunit cDNAs were added to the cells in 1:1:1 ratios of 4 μg each. To isolate the transfected cells, 2 μg of the Capture-Tec pHook-1 (Invitrogen) plasmid encoding a surface antibody, sFv, were also transfected.

The isolation procedure for the transfected cells was conducted 20-28 hours later. The cells were first passaged with trypsin and then mixed with magnetic beads (approximately 7.5 x

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10^5 beads) coated with antigen specific for the pHook antibody (Chesnut et al. 1996). Bead-bound cells were separated with a magnetic stand, resuspended into DMEM, and plated onto coverslips coated with poly-lysine and collagen. Cells were used for recording 20-28 hours later.

Electrophysiological recording techniques

Whole-cell recording configurations were used to record the activity of GABAR channels from transfected cells. The external and internal solutions created a chloride equilibrium potential near 0 mV. The external solution contained 142 mM NaCl, 8.1 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES buffer maintained at a pH of 7.4 with osmolarity adjusted to 295-305 mOsm. Recording electrodes were filled with internal solution composed of 153 mM KCl, 1 mM $MgCl_2$, 5 mM K-EGTA, 10 mM HEPES buffer, and 2 mM MgATP with pH = 7.4 and osmolarity adjusted to 295-305 mOsm. The MgATP was added on the day of recording to ensure effectiveness. Both external and internal solutions were sterile filtered. A Narishige PP-830 electrode puller (Narishige, Tokyo, Japan) was used to pull patch pipettes from thick-walled borosilicate glass with an internal filament (World Precision Instruments, Sarasota, Florida) to a resistance of 5-10 MΩ.

Drugs were applied to the cells using a computer-controlled stepper solution exchanger (SF-77B, Warner Instruments, Hamden, CT), allowing solution changes to the cell in <50 ms (open tip). There was a continuous flow of external solution through the chamber. Chloride currents were recorded at -50 mV with an Axon 200B patch clamp amplifier and the pClamp 8 suite of software programs (Axon Instruments Inc., Union City, CA). The recordings were stored on a computer hard drive for off-line analysis.

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Analysis of whole-cell currents

Whole-cell current recordings were analyzed using the pClamp8 suite (Axon Instruments Inc., Union City, CA) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). To determine GABA concentration response relationships, peak current amplitudes were normalized to the maximum current elicited by 1 mM GABA for each cell. The inhibitory effect of amiloride was expressed as a percentage of the response to GABA alone. Normalized concentration-response data were fit with a four-parameter logistic equation: current = [minimum current + (maximum current – minimum current)] / [1 + (10^(log EC_{50} – log [GABA]) x n), where n represents the Hill number. The EC_{50} or IC_{50} represents the concentration that produces 50% of the maximal effect. To examine the non-competitive site of amiloride action, the current was measured at the peak response and at the end of the 5 sec. drug application period. Statistical comparisons were performed using the Tukey-Kramer multiple comparisons test (Instat, GraphPad) with a significance level of $p < 0.05$.

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RESULTS

Effect of mutations in the α6 subunit on GABA sensitivity

HEK-293T cells were transfected with wild-type β3 and γ2L subunits, and either an α1 or α6 subunit. Cells were patch-clamped at -50 mV and GABA was applied for 5 sec at concentrations from 0.01 μM to 1 mM. The α1β3γ2L isoform (average EC_{50} = 17.9 ± 2.6 μM, N = 6) is ~12-fold less sensitive to GABA compared to the α6β3γ2L receptor (average EC_{50} = 1.3 ± 0.3 μM, N = 7). Of the 15 mutation sites examined in the α6 subunit, 14 had no effect on GABA EC_{50} compared to the α6β3γ2L wild-type (p>0.05) (Table 1, Figure 2). None of these mutations significantly altered the average maximal current amplitude or Hill slope (p>0.05) compared to α6β3γ2L or had any obvious effects on activation or desensitization kinetics at the whole-cell level.

Only one of these mutations in the α6 subunit significantly altered GABA sensitivity (p<0.05 compared to α6β3γ2L). The $\alpha6_{(S83R)}$β3γ2L decreased GABA sensitivity ~3-fold (average EC_{50} = 4.3 ± 0.5 μM, N = 7) (Table 1, Figure 2B). Exchanging this residue in the α1 subunit increased the sensitivity to GABA to a similar degree, with an average EC_{50} of 3.5 ± 0.9 μM ($\alpha1_{(R84S)}$β3γ2L, N = 5) (p<0.05 compared to α1β3γ2L). These findings suggest that this residue may be partly responsible for the uniquely low GABA EC_{50} associated with the α6 subtype.

Although most of the amino acid residues previously reported to alter GABA sensitivity in α subunits are conserved sites (see Korpi et al. 2002), one exception is isoleucine120 of the α1 subunit. Mutation of this residue to valine reduced GABA sensitivity nearly 10-fold (Westh-Hansen, 1997). The α6 subunit, along with the α3 and α5 subtypes, has a leucine residue in the

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homologous location. Because this site does not contain a residue unique to the α6 subtype, it was not included in our original screen. However, as it has been identified as potentially contributing to GABA sensitivity, we examined the effect of exchanging the α1 and α6 residues at this location. Exchanging the leucine and isoleucine residues had no effect on the GABA sensitivity for either the $\alpha1_{(I120L)}\beta3\gamma2L$ (average EC_{50} = 14.4 ± 3.0 µM, N=4) or the $\alpha6_{(L119I)}\beta3\gamma2L$ (EC_{50} = 1.4 ± 0.2 µM, N=3) receptors. Therefore, although changes at this site to some residues may influence receptor function, the structural heterogeneity at this location is not responsible for the functional differences between the α1 and α6 subtypes.

Amiloride sensitivity is regulated by the extracellular N-terminal domain

Chimeric subunits exchanging the extracellular N-terminal domains of the α1 and α6 subunits were constructed previously to examine the role of this structure in the pharmacological characteristics associated with these subtypes (Fisher et al., 1997). Earlier work studied the response of the chimeric subunits to GABA, pentobarbital, diazepam, zinc, lanthanum, and furosemide, but not amiloride (Fisher et al., 1997). Therefore, we determined the sensitivity of receptors containing the chimeric subunits to inhibition by amiloride.

The α6β3γ2L (average IC_{50} = 44.0 ± 6.1 µM, N = 5) receptor was approximately 7-fold more sensitive to amiloride compared to the α1β3γ2L receptor (average IC_{50} = 304.4 ± 69 µM, N = 6) (Figure 3). Receptors containing the α1/α6 chimeric subunit, which includes the N-terminal domain of the α1 subunit, had low sensitivity to amiloride (average IC_{50} = 281.5 ± 37.4 µM, N = 5), comparable to the α1β3γ2L receptor. Likewise, receptors containing the α6/α1 chimeric subunit had high sensitivity to amiloride (average IC_{50} = 35.3 ± 2.9 µM, N = 6), similar to the α6β3γ2L receptor. This suggests that the higher sensitivity to inhibition by amiloride associated

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with the α6 subunit is conferred by structural differences in the extracellular N-terminal domain. Therefore, we examined the effect of mutations within this region on amiloride sensitivity.

Effect of single mutations in the α6 subunit on amiloride sensitivity

To determine which amino acid differences within the N-terminal domain conferred sensitivity, we examined the sensitivity of receptors containing each of the point mutants to 100 μM amiloride. This concentration of amiloride had little effect on the α1β3γ2L isoform (81.8 ± 2.6 % of the current observed in response to GABA alone, N = 6) while the α6β3γ2L isoform was substantially inhibited (27.2 ± 4.7 % of the current observed in response to GABA alone, N = 5) (Figures 3,4). Amiloride was applied with a submaximal GABA concentration (EC_{20-30}) for each isoform. Twelve of the fifteen mutation sites had no effect on the ability of 100 μM amiloride to inhibit the receptor (Figure 4)

Mutations at three sites in the N-terminal domain of the α6 subunit altered inhibition by amiloride (Figures 4,5). Incorporation of the $\alpha6_{(S83R)}$ mutation, earlier shown to reduce GABA sensitivity, also reduced sensitivity to amiloride to a level similar to the α1β3γ2L wild-type receptor (average IC_{50} = 265.7 ± 60.6 μM, N = 5) (Figure 5). A higher GABA concentration (1 μM), providing an equivalent effect (~EC_{20}), was used to examine effects of amiloride on this mutated subunit. Mutations at two adjacent sites in the α6 subunit also reduced amiloride sensitivity, but only to an intermediate level compared to the wild-type receptors (Figures 4, 5A). The average amiloride IC_{50}s for these point mutants were 102.8 ± 23.2 μM (N = 5) for $\alpha6_{(L174A)}\beta3\gamma2L$, and 102.1 ± 15.1 μM, (N = 6) for $\alpha6_{(Y175R)}\beta3\gamma2L$. Neither of these mutations altered GABA sensitivity of the receptor (Figure 2). Interestingly, none of the individual mutations at these sites in the α1 subunit had any effect on amiloride sensitivity (Figure 5A).

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This suggests that alteration of any single residue was unable to reproduce α6-like pharmacology, and that instead that multiple structural changes might be necessary.

Effects of combining mutations at multiple sites on GABA and amiloride sensitivity

To determine whether these three sites might combine to produce the increased amiloride sensitivity of the α6 subtype, we created α1 and α6 subunits with multiple mutations. The $\alpha6_{(L174A,Y175R)}\beta3\gamma2L$ receptor had reduced sensitivity to amiloride compared to the mutation of either of these sites alone (Figure 5A). The combination of mutations at these sites did not alter sensitivity to GABA, however (average EC_{50} = 1.8 ± 0.2 µM, N=3). Incorporating mutations at all three sites (S83R, L174A, and Y175R) produced a receptor with α1-like sensitivity to amiloride (average IC_{50}= 348.3 ± 100.9 µM, p>0.1 compared to α1) (Figure 5). The GABA EC_{50} for the triple mutant (average EC_{50} = 6.7 ± 0.9 µM, N=5) was not significantly different from that of the $\alpha6_{(S83R)}$ mutation alone, additional evidence that L174 and Y175 do not regulate GABA sensitivity.

The effect of combining the mutations in the α1 subunit on GABA and amiloride sensitivity was also examined. The $\alpha1_{(A175L,R176Y)}$ double mutant did not increase sensitivity to amiloride (Figure 5A) or alter GABA sensitivity of the receptor (average EC_{50} = 17.7 ± 1.7 µM, N=4, p>0.1 compared to α1). Mutation of all three sites in the α1 subunits was required to increase sensitivity to inhibition by amiloride (average IC_{50} = 64.5 ± 13.7 µM, N=5, p<0.001 compared to α1, p>0.05 compared to α6) (Figure 5B). As with the mutations in the α6 subunit, the GABA EC_{50} of the triple mutant (average EC_{50} = 4.5 ± 0.4 µM, N=5) was not significantly different from the $\alpha1_{(R84S)}$ mutation alone.

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Separate structures regulate the competitive and non-competitive actions of amiloride

In addition to amiloride's action as a competitive antagonist, inhibition consistent with a noncompetitive channel block is observed at high concentrations of GABA (Fisher, 2002). Compared to the $\alpha1$ subunit, the $\alpha6$ subunit also confers higher sensitivity to this non-competitive action of amiloride. To determine whether distinct structures are responsible for these two mechanisms of action of amiloride, the sensitivity of the chimeric subunits to channel block by amiloride was examined using 1 mM GABA and 1 mM amiloride. At high GABA concentrations, amiloride has no effect on the peak current of $\alpha6\beta3\gamma2L$ receptors, but instead causes a rapid decay in current amplitude followed by a rebound current at the end of drug application (Figure 6A). This is consistent with amiloride both binding to and unbinding from the open channel.

In contrast to the competitive inhibition by amiloride, both the $\alpha1/\alpha6$ and the $\alpha6/\alpha1$ chimeric subunits conferred some sensitivity to block by amiloride at high concentrations of GABA (Figure 6). To enable construction of the chimeric subunits, a single amino acid change was generated within the first transmembrane domain of the $\alpha1$ subtype (Fisher et al., 1997). As a result, both of the chimeras as well as the $\alpha6$ subunit share a threonine residue at this site, while the $\alpha1$ wild-type subunit contains a leucine. Since both chimeras and the $\alpha6$ subunit also share sensitivity to non-competitive block by amiloride, point mutations were generated to determine whether this site was responsible. Neither the $\alpha6_{(T230L)}\beta3\gamma2L$ nor the $\alpha1_{(L231T)}\beta3\gamma2L$ receptors differed significantly from their wild-type counterparts (Figure 6B). This suggests that structural differences within several different domains of the subunit regulate sensitivity to this action of amiloride.

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Since the N-terminal domain did appear to contribute to channel block by amiloride, we examined whether any of the point mutations within the α6 subunit that reduced sensitivity to competitive inhibition by amiloride also contributed to its non-competitive action. None of these mutations alone or combined altered the sensitivity to block by amiloride at high GABA concentrations however (Figure 6C), suggesting that the two actions of amiloride have distinct structural determinants.

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DISCUSSION

We examined the role of amino acids within the extracellular N-terminal domain of the GABAR α6 subunit in determining its unique high sensitivity to activation by GABA and inhibition by amiloride. Mutations were made exchanging these residues for those found at the homologous sites in the α1 subunit, which has lower sensitivity to both GABA and amiloride compared to the α6 subtype. Three residues in the N-terminal domain (serine83, leucine174, and tyrosine175) were found to influence GABA and/or amiloride sensitivity. Our results also suggest that separate structures determine the subtype-specificity of the competitive and non-competitive actions of amiloride.

Mutation of α6 serine83 to arginine or α1 arginine84 to serine produced GABARs with intermediate GABA sensitivity, different from either wild-type receptor, and comparable to that of α4βxγ2 containing receptors (Knoflach et al., 1996; Whittemore et al., 1996). Residues shared between α4 and α6 in the extracellular domain may therefore also contribute to regulation of GABA sensitivity, and mutations at these sites may be necessary in combination with Ser83 to completely exchange the properties of the α1 and α6 subunits.

Identification of the structures in the α6 subunit responsible for amiloride sensitivity was more complex, with multiple residues (S83, L174 and Y175) contributing to this property. Each one of the individual mutations alone decreased the sensitivity to amiloride, but the effects of L174 and Y175 appeared to be additive. This suggested that the combination of all these sites is required for produce high sensitivity to inhibition. Consistent with this, individual mutations at these sites within the α1 subunit did not increase inhibition. Only the triply mutated α1 subunit showed enhanced sensitivity to amiloride.

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According to a structural model of the N-terminal domain of ligand-gated channels, position 83 of the α6 subunit lies at the beginning of the 3rd beta sheet (β3) (Brejc et al., 2001; Ernst et al., 2003). This region has not been suggested as a participant in the GABA binding pocket. However, this region does connect GABA binding loops D and E, and may influence the positioning of these loops or their movement during signal transduction. Isoleucine121 in the α1 subunit lies at the end of the 5th beta sheet, predicted to be in close proximity to serine83. Mutations at this location altered GABA sensitivity (Westh-Hansen et al., 1997), although we found that this site was not responsible for the differences between the α1 and α6 subunits. This supports the possibility that structures in this region affect responsiveness to agonists. The exchange of serine and arginine at this location replaces a smaller, uncharged sidechain with a bulkier, positively charged residue. Whether the volume or the charge of the residue, or a combination of these characteristics, is the more important factor in determining GABA sensitivity may provide insight into the role that this structure plays in regulating receptor function.

Serine 83, leucine174, and tyrosine175 combined to regulate sensitivity to amiloride. Based on the structural models, leu174 and tyr175 lie in loop 9, between the 8th and 9th beta sheets (Brejc et al., 2001; Ernst et al., 2003). This region is adjacent to loop F, which is believed to form part of the GABA binding pocket, and connects loop B and C, implicated in benzodiazepine binding. This region is also a determinant of the unique benzodiazepine pharmacology associated with the α6 subunit (Im et al., 1997). These domains may play an important general role in transmitting conformational changes from one area of the receptor structure to another (Ernst et al., 2003). However, this region has little homology with the acetylcholine binding protein from which the crystal structure was derived, and therefore may

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have distinct structural characteristics (Ernst et al., 2003). It is notable that the mutation at serine83 influenced sensitivity to both GABA and amiloride. This is consistent with amiloride acting as a competitive antagonist and inhibiting the ability of GABA to bind to the receptor.

These mutations generally exchanged smaller uncharged residues for bulkier, positively charged residues. Amiloride carries a positive charge in our solutions (pH= 7.4) (Kleyman and Cragoe, 1988) and it is therefore possible that electrostatic interactions with the charged arginine side chains reduced the binding of amiloride.

Our results do not preclude residues in other domains from contributing to sensitivity to GABA or amiloride. The proposed open channel block observed with amiloride is likely to be mediated in part by residues within the transmembrane domains. Interactions between charged residues in the extracellular N-terminal domain and the region linking TM2 and TM3 have been proposed as a mechanism for signal transduction of GABA binding to channel activity (Kash et al., 2003). Previous reports show that structural differences within different domains regulate other pharmacological properties of the α6 subunit, such as sensitivity to inhibition by zinc and furosemide (Fisher and Macdonald, 1998; Thompson et al, 1999). We focused on the extracellular N-terminal domain as our results with α6/α1 subunit chimeras indicated that structural differences within this region were responsible for the higher sensitivity of the α6 subunit to GABA and amiloride.

Since the GABAR is responsible for most fast inhibitory neurotransmission in the CNS, it is a target for many drugs used clinically. The structural diversity of GABAR subunits and their different expression patterns creates the possibility for subunit-selective treatments targeting distinct GABAR populations (Laurie et al., 1992a, 1992b; Wisden et al, 1992). The use of many non-selective GABAR modulators is limited due to development of side effects including

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sedation, amnesia, muscle weakness and depression (Korpi et al., 2002). Continued research on the structural differences among the GABAR subunits that are responsible for their unique properties is imperative for the development of subunit-selective drugs. Novel treatments that target specific populations of GABARs have the potential to provide selective modulation while limiting harmful side effects.

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FIGURE LEGENDS

Figure 1. Location of the 15 mutation sites in the rat α6 subunit.

The extracellular amino-terminal domain amino acid sequence of the α subunit family (α1-α6) consists of approximately 220 residues. Rat sequence from Tyndale et al. (1995). The sequence shown excludes the initial variable region for each subtype, which consists of around fourteen residues. Residue numbering is given for the mature sequence. The highlighted regions represent the amino acid sites where the α6 residue is unique. Each of these sites was individually mutated to the corresponding residue in the α1 subunit.

Figure 2. Effect of single point mutations in the α1 and α6 subunits on GABA sensitivity.

A. Concentration-response relationships were constructed by expressing the peak response to each concentration of GABA as a percentage of the current response to 1 mM GABA of each cell. The points shown are mean ± SEM. Data were fit with a four-parameter logistic equation (solid line for wild-type, dashed lines for point mutants). For average EC_{50} values, see Table 1.

B. Representative whole-cell traces from transfected HEK-293T cells. GABA was applied at the concentrations indicated for 5 sec (bar) to cells voltage-clamped at -50 mV.

Figure 3. Amiloride sensitivity is conferred by the extracellular N-terminal domain of the α6 subunit

A. Representative whole-cell traces from transfected HEK-293T cells. GABA or GABA plus 100 μM amiloride was applied for 5 sec (bar) to cells voltage-clamped at -50 mV. The GABA concentration used was near the $EC_{20\text{-}30}$ value for each isoform. Traces shown for each isoform are from the same cell and are superimposed to clarify the amount of inhibition.

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B. Concentration-response relationships were constructed by expressing the inhibition of the peak current by amiloride as a percentage of response to GABA alone for each cell. The points shown are mean ± SEM. Data were fit with a four-parameter logistic equation (solid line for wild-type and dashed line for chimeric constructs). IC_{50} values of these fits to averaged data were 41.6 μM for α6β3γ2L (N=5), 35.7 μM for α6/α1β3γ2L (N=6), 263.4 μM for α1β3γ2L (N=6), and 285.9 μM for α1/α6β3γ2L (N=5).

Figure 4. Effect of single point mutations in the α6 subunit on amiloride sensitivity.

The effect of 100 μM amiloride on GABAR activity is presented as a percentage of the response to GABA alone. Bars indicate the mean ± SEM (N=4-6). The dashed lines represent the responses of the wild-type receptors. *** ($p < 0.001$) or * ($p < 0.05$) indicates a significant difference from the response of α6β3γ2L.

Figure 5. Effect of multiple mutations on amiloride sensitivity.

A. Average inhibition by 100 μM amiloride of receptors containing subunits with single or multiple mutations. Bars indicate the mean ± SEM (N=5-7). The dotted lines represent the responses of the wild-type receptors.

B. Concentration-response relationships were constructed by expressing the inhibition of the peak current by amiloride as a percentage of response to GABA alone for each cell. The points shown are mean ± SEM. Data were fit with a four-parameter logistic equation (solid line for wild-type, dashed lines for mutated receptors). IC_{50} values of these fits were 41.6 μM for α6β3γ2L (N=5), 370.3 μM for $\alpha 6_{(S83R,\ L174A,Y175R)}\beta 3\gamma 2L$ (N=5), 263.4 μM for α1β3γ2L (N=6), and 62.3 μM for $\alpha 1_{(R84S,\ A175L,R176Y)}\beta 3\gamma 2L$ (N=5). Representative whole-cell traces show

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responses to GABA or GABA plus 100 μM amiloride applied for 5 sec (bar) to cells voltage-clamped at -50 mV. The GABA concentration used was near the EC_{20-30} value for each isoform.

Figure 6. Competitive and non-competitive effects of amiloride have distinct structural requirements.

A. Representative whole-cell traces from transfected HEK-293T cells. 1 mM GABA or GABA plus 1 mM amiloride was applied for 5 sec (bar) to cells voltage-clamped at -50 mV. The α1β3γ2L +amiloride trace is reduced by 10% to clarify the lack of effect on decay kinetics. Amiloride had no effect on the peak amplitude of the current.

B. Average inhibition of the current at the end of the drug application by 1 mM amiloride. Bars show the mean ± SEM. ** (compared to α1β32L) and # (compared to α6β3γ2L) indicate significant difference from wild-type ($p<0.01$).

Table 1. Average GABA EC_{50} values for single point mutation of the α6 subunit.

Only the mutation of serine83 to arginine caused a significant change in GABA EC_{50} compared to the α6β3γ2L wild-type receptor (* indicates $p<0.05$). Average Hill slopes were 1.3 ± 0.2 (α1) and 1.4 ± 0.3 (α6) for the wild-type receptors and were not affected by any of the single point mutations ($p>0.05$), which had average values ranging from 1.0 to 1.5. N = number of cells.

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Table 1

Isoform	**GABA EC_{50}, μM (N)**
α1β3γ2L	17.9 ± 2.6 (6)
α6β3γ2L	1.3 ± 0.3 (7)
$\alpha6_{(E22D)}\beta3\gamma2L$	0.9 ± 0.3 (5)
$\alpha6_{(T69K)}\beta3\gamma2L$	1.1 ± 0.2 (5)
$\alpha6_{(A79M)}\beta3\gamma2L$	1.0 ± 0.2 (5)
$\alpha6_{(S83R)}\beta3\gamma2L$	**4.3 ± 0.5 (7)***
$\alpha6_{(H121E)}\beta3\gamma2L$	1.0 ± 0.2 (5)
$\alpha6_{(N134R)}\beta3\gamma2L$	1.5 ± 0.3 (5)
$\alpha6_{(D136E)}\beta3\gamma2L$	1.0 ± 0.1 (5)
$\alpha6_{(N143D)}\beta3\gamma2L$	1.6 ± 0.4 (5)
$\alpha6_{(I165V)}\beta3\gamma2L$	1.2 ± 0.3 (5)
$\alpha6_{(K170T)}\beta3\gamma2L$	0.9 ± 0.1 (5)
$\alpha6_{(L174A)}\beta3\gamma2L$	1.1 ± 0.3 (5)
$\alpha6_{(Y175R)}\beta3\gamma2L$	0.7 ± 0.2 (5)
$\alpha6_{(L187N)}\beta3\gamma2L$	1.2 ± 0.3 (5)
$\alpha6_{(N204S)}\beta3\gamma2L$	1.6 ± 0.1 (5)
$\alpha6_{(Q218K)}\beta3\gamma2L$	1.0 ± 0.1 (5)

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α1  21LLDGYDNRLRPGLGERVTEVKTDIFVTSFGPVSDHDMEYTIDVFFRQSWKDERL
α2  21LLDGYDNRLRPGLGDSITEVFTNIYVTSFGPVSDTDMEYTIDVFFRQKWKDERL
α3  46LLDGYDNRLRPGLGDAVTEVKTDIYVTSFGPVSDTDMEYTIDVFFRQTWHDERL
α4  20LLDGYDNRLRPGFGGPVTEVKTDIYYTSFGPVSDVEMEYTMDVFFRQTWIDKRL
α5  25LLDGYDNRLRPGLGERITQVRTDIYVTSFGPVSDTEMEYTIDVFFRQSWKDERL
α6  20LLEGYDNRLRPGFGGAVTEVKTDIYVTSFGPVSDVEMEYTMDVFFRQTWTDERL
       22                                            69

α1    KFKGPMTVLRLNNLMASKIWTPDTFFHNGKKSVAHNMTMPNKLLRITEDGTLLY
α2    KFKGPMNILRLNNSMASKIWTPDTFFHNGKKSVAHNMTMPNKLLRIQDDGTLLY
α3    KFDGPMKILPLNNLLASKIWTPDTFFHNGKKSVAHNMTTPNKLLRLVDNGTLLY
α4    KYDGPIEILRLNNMMVTKVWTPDTFFRNGKKSVSHNMTAPNKLFRIMRNGTILY
α5    RFKGPMQRLPLNNLLASKIWTPDTFFHNGKKSIAHNMTTPNKLLRLEDDGTLLY
α6    KFKGPAEILSLNNLMVSKIWTPDTFFRNGKKSIAHNMTTPNKLFRLMHNGTILY
           79  83                                    121

α1    TMRLTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTREPARSVVVAE
α2    TMRLTVQAECPMHLEDFPMDAHSCPLKFGSYAYTTSEVTYIWTYNPSDSVQVAP
α3    TMRLTIHAECPMHLEDFPMDVHACPLKFGSYAYTKAEVIYSWTLGKNKSVEVAQ
α4    TMRLTISAECPMRLVDFPMDGHACPLKFGSYSYPKSEMIYTWTKGPEKSVEVPK
α5    TMRLTIDAECPMQLEDFPMDAHACPLKFGSYAYPNSEVVYVWTNGSTKSVVVAE
α6    TMRLTINADCPMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKKGPLYSVEVPE
           134 136    143                  165  170 174,175

α1    DGSRLNQYDLLGQTVDSGEVQSSTGEYVVMTTHFHLKRK
α2    DGSRLNQYDLLGQSIGKETIKSSTGEYTVMTAHFHLKRK
α3    KGSRLNQYDLLGHVVGTEIIRSSTGEYVVMTTHFHLKRK
α4    ESSSLVQYDLIGQTVSSETIKSITGEYIVMTVYFHLRRK
α5    DGSRLNQYHLMGQTVGTENISTSTGEYTIMTAHFHLKRK
α6    ESSSLLQYDLIGQTVSSETIKSNTGEYVIMTVYFHLQRK
           187              204           218
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Start of 1st Transmembrane Domain

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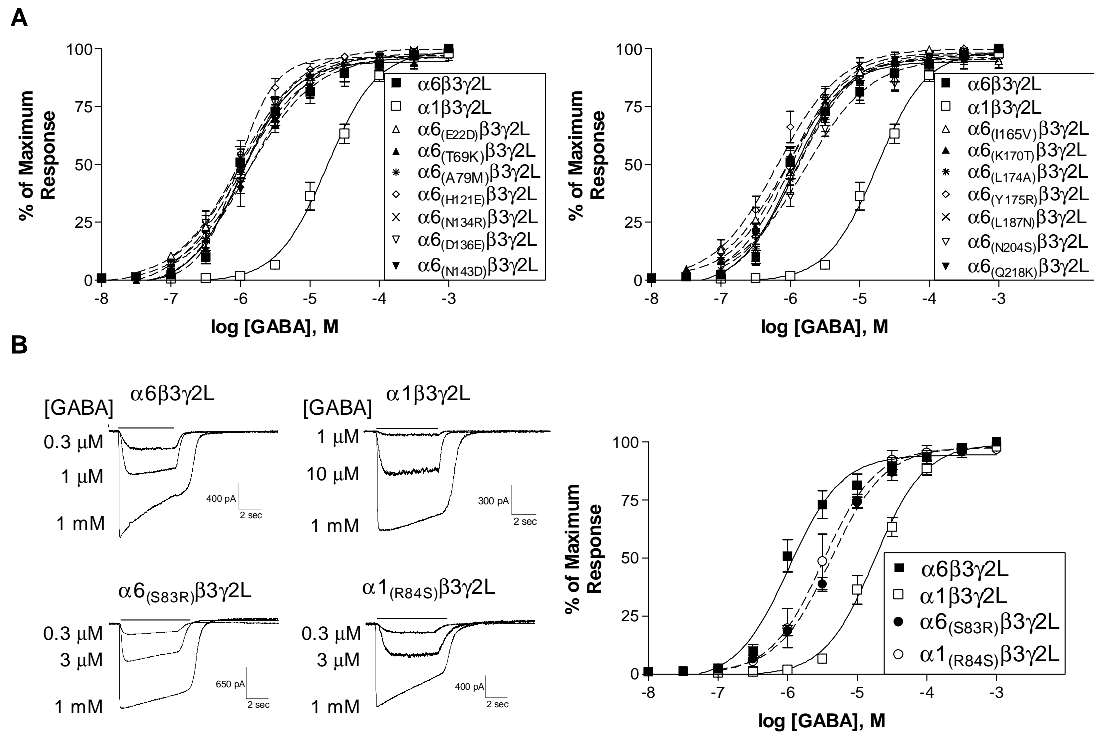

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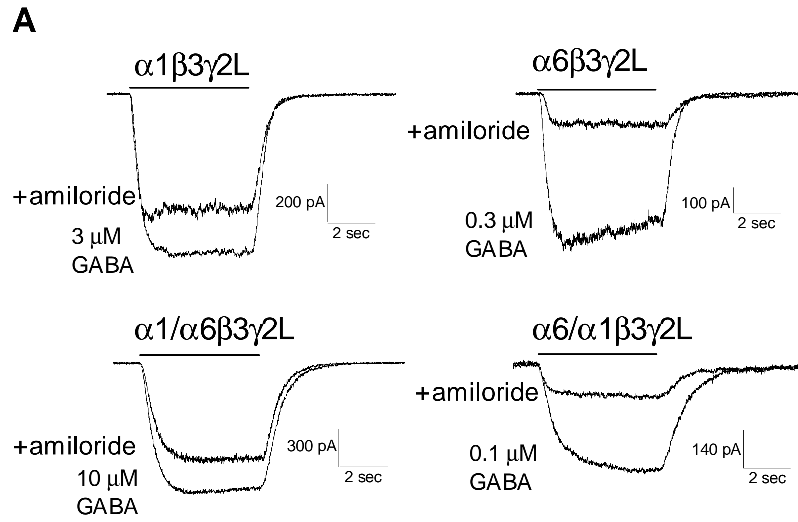

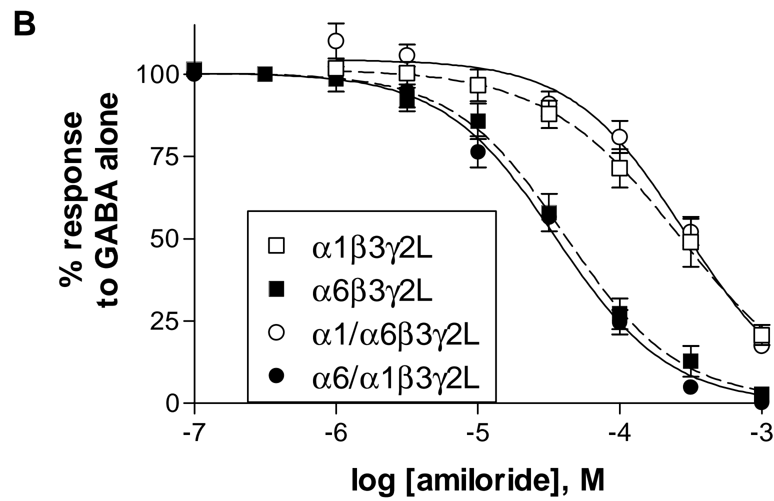

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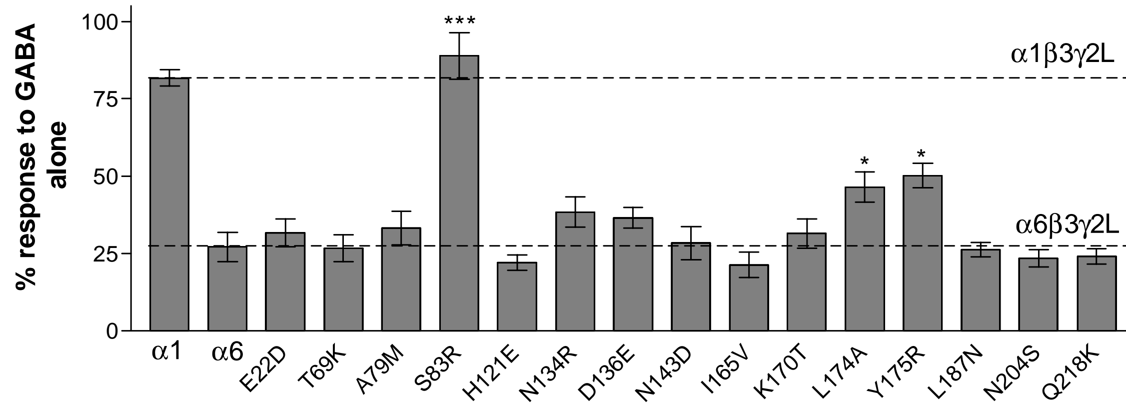

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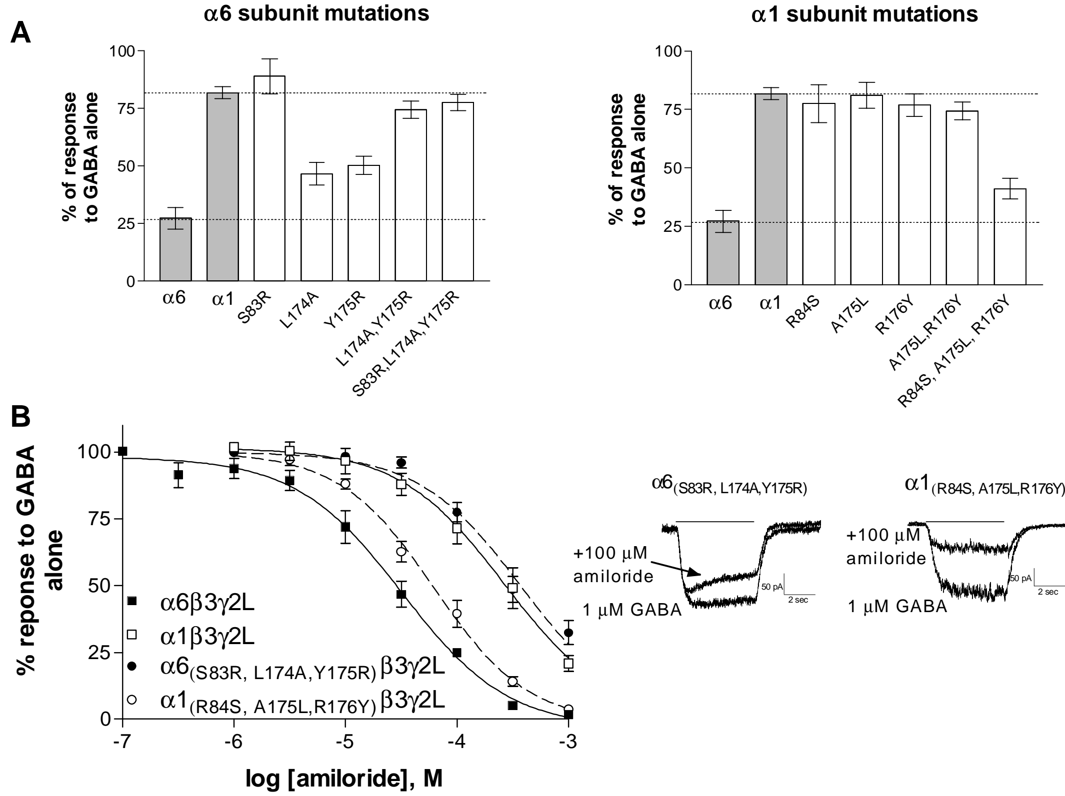

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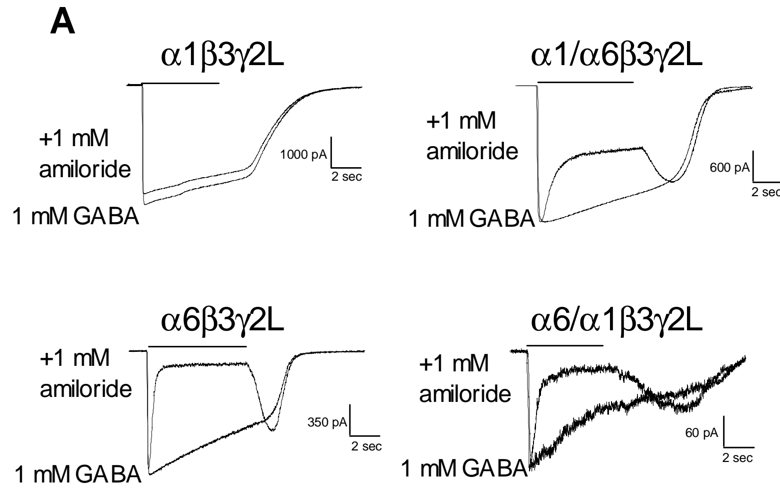

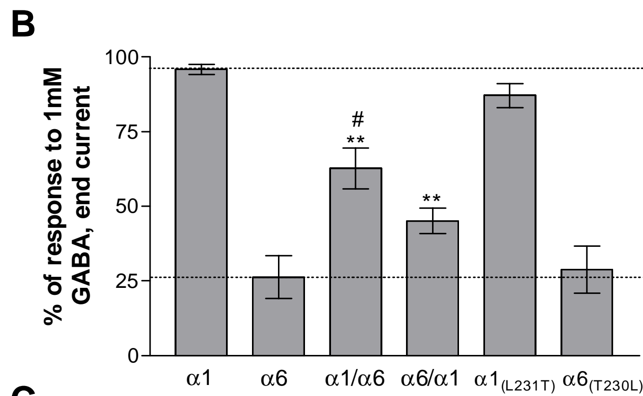

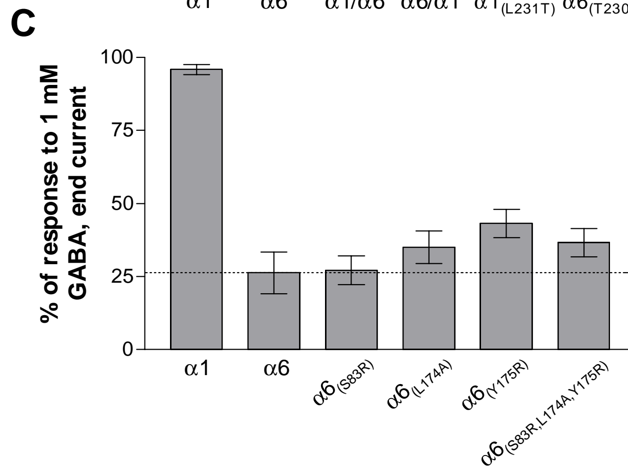

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