Structural Determinants of the Pharmacological Properties of the GABA\textsubscript{A} Receptor \(\alpha 6\) Subunit

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ABSTRACT

GABA\textsubscript{A} receptors 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 with all other \(\alpha\) subtypes, the \(\alpha 6\) subtype confers unique pharmacological properties. In particular, \(\alpha 6\)-containing receptors are more sensitive to both the agonist GABA and the antagonist amiloride. Results from chimeric constructs of the \(\alpha 1\) and \(\alpha 6\) subunits suggested that structural differences within the extracellular N-terminal domain were responsible for both these characteristics. Within this domain, we examined 15 amino acid residues unique to the \(\alpha 6\) subtype. Each of these sites was individually mutated to the corresponding residue of the \(\alpha 1\) subtype. The mutated subunits were expressed in human embryonic kidney-293T cells along with wild-type \(\beta 3\) and \(\gamma 2L\) subunits and sensitivity to GABA and amiloride determined with whole-cell electrophysiological recordings. Serine83 in the \(\alpha 6\) 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 \(\alpha 6\) subunit.

The GABA\textsubscript{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 (Nayef et al., 1994; Karlin and Akabas, 1995). To date, seven subunit families and 16 subunit subtypes ([\(\alpha 1\)–6], \(\beta 1\)–3, \(\gamma 1\)–3, \(\delta 1\), \(\epsilon 1\), \(\pi 1\), and \(\theta 1\)) have been found in mammalian species (Whiting et al., 1999). Most native GABARs are believed to contain two \(\alpha\), two \(\beta\), and one \(\gamma\) subunit (Chang et al., 1996; Tretter et al., 1997).

The \(\alpha\) subtype composition of the receptor is a major determinant of its functional properties. In particular, GABARs containing the \(\alpha 6\) subtype exhibit distinctive pharmacological properties. Differences associated with the \(\alpha 6\) subtype compared with the \(\alpha 1\) 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., 1999; Wieland et al., 1992; Knoflach et al., 1996; Thompson et al., 1996; Korpi and Lüddens, 1997; Saxena et al., 1997; Fisher, 2002). Although some of these properties are shared with the structurally homologous \(\alpha 4\) subunit, higher sensitivity to GABA and amiloride are unique to the \(\alpha 6\) subtype (Ducet 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 noncompetitive mechanisms (Inomata et al., 1988; Fisher, 2002).

The structural determinants of these pharmacological differences between the \(\alpha 1\) and \(\alpha 6\) subtypes are known for only a few of these modulators. The insensitivity of the \(\alpha 6\) 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 \(\alpha\) subunits confers sensitivity to these positive modulators (Wieland et al., 1992). The ability of furosemide to inhibit \(\alpha 6\)-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

### ABBREVIATIONS

GABAR, \(\gamma\)-aminobutyric acid receptor; TM, transmembrane; HEK, human embryonic kidney.
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 because it represents the largest extracellular domain of the subunit and is believed to contribute 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 15 amino acid residues in the extracellular N-terminal domain that are unique to the α6 subtype compared with other α subunits (Fig. 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 with wild-type β3 and γ2L subunits and the sensitivity of the receptor to GABA and amiloride determined using whole-cell patch-clamp recordings.

Materials and Methods

Construction of Chimeric and Mutated Subunits. The α1/α6 chimeric subunits were constructed with a splice site in the first transmembrane domain as described by Fisher et al. (1997). The commercially available QuikChange 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 to purify the product. Mutations were verified by sequencing (University of South Carolina DNA core).

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, Nashville, TN). Recombinant receptors were transiently expressed in the human embryonic kidney cell line HEK-293T (GenHunter, Nashville, TN). HEK-293T cells express the simian virus 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's medium 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 Na2HPO4, 150 mM NaCl, pH 7.3). HEK-293T cells were transfected using calcium phosphate precip-
ituation (Angelotti et al., 1993). Plasmids encoding the selected GA-
BAR 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, Carlsbad, CA) plasmid encoding a surface anti-
tbody, sFc, were also transfected.

The isolation procedure for the transfected cells was conducted 20
to 28 h later. The cells were first passaged with trypsin and then
mixed with magnetic beads (approximately 7.5 × 10^6 beads) coated
with antigen specific for the pHook antibody (Chesnut et al., 1996).
Bead-bound cells were separated with a magnetic stand, resus-
pended into Dulbecco’s modified Eagle’s medium, and plated onto
coverslips coated with poly-lysine and collagen. Cells were used for
recording 20 to 28 h later.

**Electrophysiological Recording Techniques.** Whole-cell re-
cording 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 m M
CaCl_2, 10 mM glucose, and 10 mM HEPES buffer maintained at a pH
of 7.4 with osmolarity adjusted to 295 to 305 mM osM. 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 to 305 mM osM.
The MgATP was added on the day of recording to ensure effective-
ness. 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 (WPI, Sarasota, FL) to a resistance of 5 to 10
MΩ.

Drugs were applied to the cells using a computer-controlled step-
er solution exchanger (SP-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 a 200B patch-clamp ampli-
fier and the pCLAMP 8 suite of software programs (Axon Instruments,
Union City, CA). The recordings were stored on a computer hard
drive for off-line analysis.

**Analysis of Whole-Cell Currents.** Whole-cell current recordings
were analyzed using the pClamp 8 suite (Axon Instruments) and
GraphPad Prism (GraphPad Software Inc., San Diego, CA). To de-
termine 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. Normal-
ized concentration-response data were fit with a four-parameter
logistic equation: current = [minimum current + (maximum cur-
rent − minimum current)/[1 + (10^α (log EC_{50} − log [GABA]) × 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 noncompetitive site of amiloride action, the current was mea-
sured at the peak response and at the end of the 5-s drug application
period. Statistical comparisons were performed using the Tukey-
Kramer multiple comparisons test (Instat; GraphPad Software Inc.)
with a significance level of p < 0.05.

**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 s 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 with 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}

<table>
<thead>
<tr>
<th>Isomorf</th>
<th>GABA EC_{50} (μM)</th>
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<tbody>
<tr>
<td>α1β3γ2L</td>
<td>17.9 ± 2.6 (6)</td>
</tr>
<tr>
<td>α6β3γ2L</td>
<td>1.3 ± 0.3 (7)</td>
</tr>
<tr>
<td>α6β2223β3γ2L</td>
<td>0.9 ± 0.3 (5)</td>
</tr>
<tr>
<td>α6γ438β3γ2L</td>
<td>1.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>α6A274Rβ3γ2L</td>
<td>1.0 ± 0.2 (5)</td>
</tr>
<tr>
<td>α6γ536Rβ3γ2L</td>
<td>4.3 ± 0.5 (7)^*</td>
</tr>
<tr>
<td>α6γ1124Rβ3γ2L</td>
<td>1.0 ± 0.2 (5)</td>
</tr>
<tr>
<td>α6γ1124Rβ3γ2L</td>
<td>1.5 ± 0.5 (5)</td>
</tr>
<tr>
<td>α6γ2134Rβ3γ2L</td>
<td>1.0 ± 0.1 (5)</td>
</tr>
<tr>
<td>α6γ5145Dβ3γ2L</td>
<td>1.6 ± 0.4 (5)</td>
</tr>
<tr>
<td>α6γ5165γ2L</td>
<td>1.2 ± 0.3 (5)</td>
</tr>
<tr>
<td>α6γ1407γ2L</td>
<td>0.9 ± 0.1 (5)</td>
</tr>
<tr>
<td>α6γ2477γ2L</td>
<td>1.1 ± 0.3 (5)</td>
</tr>
<tr>
<td>α6γ2717γ2L</td>
<td>0.7 ± 0.2 (5)</td>
</tr>
<tr>
<td>α6γ1568γ2L</td>
<td>1.2 ± 0.3 (5)</td>
</tr>
<tr>
<td>α6γ2408γ2L</td>
<td>1.6 ± 0.1 (5)</td>
</tr>
<tr>
<td>α6γ2411γ2L</td>
<td>1.0 ± 0.1 (5)</td>
</tr>
</tbody>
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\(^*p < 0.05\)

Fig. 2). None of these mutations significantly altered the aver-
age maximal current amplitude or Hill slope (p > 0.05) compared with 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 with α6β3γ2L). The α6β3γ2L or had any obvious effects on the α6 subunit increased the sensitivity to GABA to a similar degree, with an average EC_{50} of 3.5 ± 0.9 μM [α1β3γ2L; n = 5] (p < 0.05 compared with α6β3γ2L). These findings suggest that this residue may be partly responsible for the uniquely low GABA EC_{50} value associated with the α6 subtype.

Although most of the amino acid residues previously reported
to alter GABA sensitivity in α subunits are conserved sites (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 et al., 1997). The α6 subunit, along with the α3 and α5 subtypes, has a leucine residue in the homologous location. Because this site does not contain a residue unique to the α6 subtype, it was not in-
cluded in our original screen. However, because it has been iden-
tified as potentially contributing to GABA sensitivity, we exam-
ned 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 α1γ2Lβ3β2L (average EC_{50} = 14.4 ± 3.0 μM; n = 4) or the α6γ2408γ2L (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
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. Therefore, we determined the sensitivity of receptors containing the chimeric subunits to inhibition by amiloride.

The α6β3γ2L (average IC50 = 44.0 ± 6.1 μM; n = 5) receptor was approximately 7-fold more sensitive to amiloride compared with the α1β3γ2L receptor (average IC50 = 304.4 ± 69 μM; n = 6) (Fig. 3). Receptors containing the α1/α6 chimeric subunit, which includes the N-terminal domain of the α1 subunit, had low sensitivity to amiloride (average IC50 = 281.5 ± 37.4 μM; n = 5), comparable with the α1β3γ2L receptor. Likewise, receptors containing the α6/α1 chimeric subunit had high sensitivity to amiloride (average IC50 = 35.3 ± 2.9 μM; n = 6), similar to the α6β3γ2L receptor. This suggests that the higher sensitivity to inhibition by amiloride associated 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), whereas the α6β3γ2L isoform was substantially inhibited (27.2 ± 4.7% of the current observed in response to GABA alone; n = 5) (Figs. 3 and 4). Amiloride was applied with a submaximal GABA concentration (EC20) for each isoform. Twelve of the 15 mutation sites had no effect on the ability of 100 μM amiloride to inhibit the receptor (Fig. 4).

Mutations at three sites in the N-terminal domain of the α6 subunit altered inhibition by amiloride (Figs. 4 and 5). Incorporation of the α6β3γ2L, mutation, earlier shown to reduce GABA sensitivity, also reduced sensitivity to amiloride to a level similar to the α1β3γ2L wild-type receptor (average IC50 = 265.7 ± 60.6 μM; n = 5) (Fig. 5). A higher GABA concentration (1 μM), providing an equivalent effect (~EC50), was used to examine the effects of amiloride on this mutated subunit. Mutations at two adjacent sites (L174 and Y175) in the α6 subunit also reduced amiloride sensitivity, but only to an intermediate level compared with the wild-type receptors (Figs. 4 and 5A). The average amiloride IC50 values for these point mutants were 102.8 ± 23.2 μM (n = 5) for α6(L174A)β3γ2L, and 102.1 ± 15.1 μM (n = 6) for α6(Y175R)β3γ2L. Neither of these mutations altered GABA sensitivity of the receptor (Fig. 2). Interestingly, none of the individual mutations at these sites in the α1 subunit had any effect on amiloride sensitivity (Fig. 5A). 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 $\alpha_6$ subtype, we created $\alpha_1$ and $\alpha_6$ subunits with multiple mutations. The $\alpha_6^{L174A,Y175R}$ receptor had reduced sensitivity to amiloride compared with the mutation of either of these sites alone (Fig. 5A). The combination of mutations at these sites, however, did not alter sensitivity to GABA (average EC$_{50}$ = 1.8 ± 0.2 μM; n = 3). Incorporating mutations at all three sites (S83R, L174A, and Y175R) produced a receptor with $\alpha_1$-like sensitivity to amiloride (average IC$_{50}$ = 348.3 ± 100.9 μM; p > 0.1 compared with $\alpha_1$) (Fig. 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 $\alpha_6^{S83R}$ mutation alone, additional evidence that L174 and Y175 do not regulate GABA sensitivity.

The effect of combining the mutations in the $\alpha_1$ subunit on GABA and amiloride sensitivity was also examined. The $\alpha_1^{(A175L,R176Y)}$ double mutant did not increase sensitivity to amiloride (Fig. 5A) or alter GABA sensitivity of the receptor (average EC$_{50}$ = 17.7 ± 1.7 μM; n = 4; p > 0.1 compared with $\alpha_1$). Mutation of all three sites in the $\alpha_1$ subunits was required to increase sensitivity to inhibition by amiloride (average IC$_{50}$ = 64.5 ± 13.7 μM; n = 5; p < 0.001 compared with $\alpha_1$, p > 0.05 compared with $\alpha_6$) (Fig. 5B). As with the mutations in the $\alpha_6$ 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 $\alpha_1^{R84S}$ mutation alone.

Separate Structures Regulate the Competitive and Noncompetitive 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 with the $\alpha_1$ subunit, the $\alpha_6$ subunit also confers higher sensitivity to this noncompetitive 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 $\alpha_6$-γ2L receptors, but instead causes a rapid decay in current amplitude followed by a rebound current at the end of drug application (Fig. 6A). This is consistent with amiloride binding to and unbinding from the open channel.

In contrast to the competitive inhibition by amiloride, both the $\alpha_1$/$\alpha_6$ and the $\alpha_6$/$\alpha_1$ chimeric subunits conferred some sensitivity to block by amiloride at high concentrations of GABA (Fig. 6). To enable construction of the chimeric subunits, a single amino acid change was generated within the first transmembrane domain of the $\alpha_1$ subtype (Fisher et al., 1997). As a result, both of the chimeras as well as the $\alpha_6$ subunit share a threonine residue at this site, whereas the $\alpha_1$ wild-type subunit contains a leucine. Because both chimeras and the $\alpha_6$ subunit also share sensitivity to noncompetitive block by amiloride, point mutations were generated to determine whether this site was responsible. Neither the $\alpha_6^{T230L}$ nor the $\alpha_1^{L231T}$ receptors differed significantly from their wild-type counterparts (Fig. 6B). This suggests that structural differences within several different domains of the subunit regulate sensitivity to this action of amiloride.

Because the N-terminal domain did seem to contribute to channel block by amiloride, we examined whether any of the point mutations within the $\alpha_6$ subunit that reduced sensitivity to competitive inhibition by amiloride also contributed to its noncompetitive action. None of these mutations alone or combined, however, altered the sensitivity to block by amiloride at high GABA concentrations (Fig. 6C), suggesting that the two actions of amiloride have distinct structural determinants.

Discussion

We examined the role of amino acids within the extracellular N-terminal domain of the GABAR $\alpha_6$ 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 $\alpha_1$ subunit, which has lower sensitivity to both GABA and amiloride compared with the $\alpha_6$ 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 with that of α4β3γ2-containing receptors (Knöflach et al., 1996; Whittemore et al., 1996). Residues shared between 4 and 6 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 seemed to be additive. This suggested that the combination of all these sites is required for producing 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.

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 third 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 contribute to regulation of 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 side chain 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.

Serine83, leucine174, and tyrosine175 combined to regulate sensitivity to amiloride. Based on the structural models, leu174 and tyr175 lie in loop 9, between the eighth and ninth 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 δ6 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 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 δ6 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 δ6/α1 subunit chimeras indicated that structural differences within this region were responsible for the higher sensitivity of the δ6 subunit to GABA and amiloride.

Because the GABAR is responsible for most fast inhibitory neurotransmission in the central nervous system, 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,b; Wisden et al., 1992; Korpi et al., 1993; Brickley et al., 1996; Nusser et al., 1998). The use of many nonselective GABAR modulators is limited due to development of side effects including 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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