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Amiloride Inhibition of γ-Aminobutyric Acid$_A$ Receptors Depends upon the α Subunit Subtype

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ABSTRACT

γ-Aminobutyric acid$_A$ (GABA$_A$) receptors (GABARs) are responsible for most fast inhibitory neurotransmission in the mammalian brain. The GABARs contain several allosteric modulatory sites, many of which are useful clinically. The activity of most of these modulators depends upon the subunit composition of the receptor. The diuretic amiloride was previously reported to inhibit GABARs in frog sensory neurons. We measured its effects on recombinant GABARs to determine its mechanism of action at mammalian receptors and to examine the effect of subunit composition. Amiloride acted primarily as a competitive antagonist, reducing the sensitivity of the receptor to GABA without affecting the maximal current amplitude. Receptors containing an α6 subunit were about 10-fold more sensitive to amiloride than those containing other α subunits. In contrast, the identity of the β or γ subtype had little effect on amiloride sensitivity. Although several other modulators have specific effects at α6-containing receptors, amiloride is the first inhibitor to be reported with no additional dependence on the identity of the β or γ subunit. Therefore, it probably represents a unique modulatory site on the GABAR, which could be useful for developing drugs targeting these receptors. The selective activity of amiloride could also be helpful for isolating the contribution of receptors composed of α6 subtypes in heterogeneous native GABAR populations.

The γ-aminobutyric acid$_A$ (GABA$_A$) receptor (GABAR) is a target for many clinically and experimentally important drugs. The relatively large number of allosteric regulatory sites on the receptor has been successfully exploited for the development of drugs used as sedatives, anxiolytics, and antiepileptics (Sieghart, 1995; Mehta and Ticku, 1999). The structure of the GABAR is complex, with seven different subunit families and 16 different subunit subtypes in mammalian species [α(1–6), β(1–3), γ(1–3), δ(1), ϵ(1), π(1), and θ(1)] (Whiting et al., 1999). Interestingly, the subunit composition of the receptor has a profound impact on the pharmacological properties of the receptor (Mehta and Ticku, 1999; Whiting et al., 1999). This variation has been useful experimentally to identify the structurally heterogeneous populations of receptors produced in neurons and has raised the possibility that selective drugs could be developed to target specific GABAR isoforms.

The diuretics amiloride and furosemide were first reported to inhibit the activity of GABARs in frog sensory neurons (Inomata et al., 1988). The activity of furosemide was subsequently found to depend upon the α and β subtype composition of receptor, with α6β2/3-containing receptors being 20- to 100-fold more sensitive to furosemide than receptors containing other α subtypes (Korpi et al., 1995; Thompson et al., 1999). Furosemide was found to act via a unique modulatory site, with its high-affinity effect localized to an isoleucine residue in the first transmembrane domain of the α6 subunit (Thompson et al., 1999). The action of amiloride on mammalian GABARs has not received further attention. Amiloride is used clinically as a K^+-sparing diuretic through its inhibitory action on renal Na^+ channels. Amiloride also inhibits several Na^+ transporters, including the Na^+/H^+ antiporter and Na^+/Ca^{2+} exchanger (Frelin et al., 1988; Kleyman and Cragoe, 1988).

We examined the effect of amiloride on the activity of recombinant GABARs expressed transiently in L929 fibroblasts to determine its mechanism of action on mammalian GABARs and to determine whether the subunit composition of the receptor influenced its sensitivity to amiloride.

Materials and Methods

Transfection of L929 Cells. Full-length cDNAs for the rat GABARs α1, α3–α6, β1–β3, γ1–γ3, δ, and human α2 subunits in pCMV, pCDNA1.1Amp, or pCDM8 expression vectors were transfected into the mouse fibroblast cell line L929 (American Type Culture Collection, Manassas, VA). For selection of transfected cells, the plasmid pHook-1, which encodes a single-chain surface antibody (sFv), was

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ABBREVIATIONS: GABA$_A$, γ-aminobutyric acid$_A$; GABAR, γ-aminobutyric acid$_A$ receptor; BES, *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid.

also transfected into the cells (Invitrogen, Carlsbad, CA). L929 cells were maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged by a 5-min incubation with 0.5% trypsin/0.2% EDTA solution in phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.3).

The cells were transfected using a calcium phosphate method optimized for the L929 cells (Chen and Okayama, 1987; Angelotti et al., 1993). Plasmids encoding GABAR subunit cDNAs were added to the cells in a 1:1 ratio of 4 μg each plus 2 μg of the plasmid encoding sFv (pHook-1). After a 4- to 6-h incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 sec. The selection procedure for pHook expression was performed 20 to 28 h later as described in Greenfield et al. (1997). The cells were passaged and mixed with 4 μl of magnetic beads coated with hapten specific for the pHook antibody (approximately 6×10^5 beads) (Invitrogen). After a 30- to 60-min incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into Dulbecco's modified Eagle's medium, plated onto poly-lysine–coated glass coverslips, and used for recordings 18 to 28 h later.

Electrophysiological Recording Solutions and Techniques. For whole-cell recording, the external solution consisted of 142 mM NaCl, 8.1 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, and osmolarity was adjusted to 295 to 305 mOsM. Recording electrodes were filled with an internal solution of 153 mM KCl, 1 mM $MgCl_2$, 5 mM K-EGTA, and 10 mM HEPES, pH 7.4, and osmolarity was adjusted to 295 to 305 mOsM. These solutions provided a chloride equilibrium potential near 0 mV. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota, FL) on a two-stage puller (Narishige, Tokyo, Japan) to a resistance of 5 to 10 MΩ. Drugs were applied to cells using a stepper solution exchanger with a complete exchange time at an open tip of 20 to 30 ms (SF-77B; Warner Instruments, Hamden, CT). There was continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B patch-clamp amplifier (Axon Instruments, Union City, CA) and stored on digital audiotape (Dagan, Minneapolis, MN). All experiments were performed at room temperature (near 25°C).

Analysis of Whole-Cell Currents. Whole-cell currents were analyzed off-line using the programs Clampfit (pCLAMP8 suite; Axon Instruments) and Prism (GraphPad Software, San Diego, CA). Normalized concentration-response data for the different isoforms were fit with a four-parameter logistic equation: current = [minimum current + (maximum current − minimum current)] / [1 + ($10^{\log EC_{50} - \log [drug]}$] × n_H, where n_H represents the Hill number. All fits were made to normalized data with the current expressed as a percentage of the maximum current elicited by saturating GABA concentrations for each cell or, in the case of amiloride, to the response to GABA alone. Statistical tests were performed using the Instat program (GraphPad). Differences among log EC_{50} or IC_{50} values were determined with the Student's unpaired t test or Tukey-Kramer multiple comparisons test using a significance level of 0.05.

Construction of Mutated $\alpha 6_{(I228T)}$ Subunit. Complementary oligonucleotide primers encoding the mutation site were synthesized by the University of South Carolina DNA synthesis core facility (Columbia, SC). Point mutations were generated with the commercially available QuikChange kit (Stratagene, La Jolla, CA) and were verified by sequencing (University of South Carolina sequencing core facility). A mutation of the α6 nucleotide sequence from ATT to ACT was used to change the amino acid sequence from isoleucine to threonine. Including the signal sequence, this triplet represents base pairs 239 to 241 of the rat mRNA coding sequence.

Results

Amiloride Inhibits the Activity of $\alpha 6\beta 3\gamma 2L$ Recombinant Receptors. Fibroblasts were transiently transfected with α6, β3, and γ2L subunits and voltage-clamped at −50 mV. Whole-cell currents were recorded in response to applications of 1 μM GABA and 1 μM GABA + amiloride. Peak currents decreased, in a concentration-dependent manner,

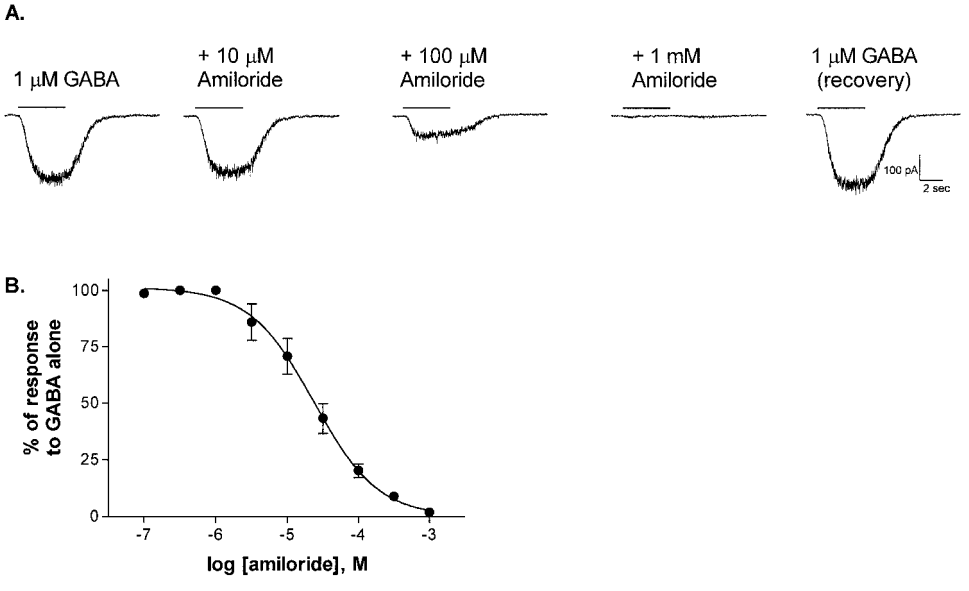

Fig. 1. Amiloride inhibits the activity of $\alpha 6\beta 3\gamma 2L$ recombinant receptors. Fibroblasts were transfected with α6, β3, and γ2L subunits, and the peak current response to 1 μM GABA alone or with amiloride was measured in cells voltage-clamped at −50 mV. A, representative traces in response to GABA alone and GABA with increasing concentrations of amiloride. The amplitude of the current was reduced by amiloride in a concentration-dependent manner. Amiloride was coapplied with GABA, and the onset of the inhibitory effect was immediate. The last trace (recovery) shown in response to 1 μM GABA alone was obtained 2 min after the 1 mM amiloride application, indicating that the inhibition was readily reversible. All traces shown were obtained from the same cell. B, the concentration-response relationship was constructed by determining the inhibition of the peak current by amiloride as a percentage of the response to 1 μM GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation (solid line). The IC_{50} for amiloride inhibition of $\alpha 6\beta 3\gamma 2L$ receptors from the fit of the combined data was 23.5 μM with a Hill slope of −0.96 (N = 4).

with increasing concentrations of amiloride to a nearly complete inhibition with 1 mM amiloride (Fig. 1A). Inhibition by amiloride showed rapid onset, with no change in the amount of inhibition after repeated applications of GABA + amiloride. The inhibition was readily reversible by a 1- to 2-min wash with external solution. The average IC_{50} for amiloride inhibition of $\alpha6\beta3\gamma2L$ receptors from the fits of data from individual cells was 19.0 ± 5.3 μM ($N = 4$) (Fig. 1B).

Amiloride Inhibition Decreases with Increasing GABA Concentration. The results of Inomata et al. (1988) suggested that amiloride was a competitive antagonist at GABARs in frog sensory neurons. Therefore, we examined the effect of GABA concentration on inhibition of the $\alpha6\beta3\gamma2L$ receptor by amiloride. The amount of inhibition of the peak current by 100 μM amiloride decreased with increasing GABA concentration, and amiloride had no effect on the peak current in response to 1 mM GABA, suggesting a competitive mechanism of action (Fig. 2, A and B). In the presence of 100 μM amiloride, the concentration-response relationship for GABA was shifted to the right, consistent with competitive inhibition (Fig. 2C). Without amiloride, the GABA EC_{50} averaged 4.1 ± 0.9 μM ($N = 3$). In the presence of 100 μM amiloride, the GABA EC_{50} increased to 12.5 ± 3.9 μM ($N = 4$, $p \leq 0.05$). The Hill slope was unaffected by the presence of amiloride, averaging 1.5 ± 0.3 without amiloride and 1.4 ± 0.2 with amiloride ($p > 0.5$). Higher concentrations of amiloride produced a similar effect. A 1 mM concentration of amiloride did not affect the peak amplitude response to maximal concentrations of GABA (3–10 mM) but shifted the GABA concentration-response curve to the right (GABA EC_{50} with 1 mM amiloride = 13.5 ± 2.1 μM, $N = 3$).

Although these results are consistent with a competitive inhibition, at higher GABA concentrations another effect of amiloride appeared. This effect is most apparent with 1 mM GABA (Fig. 2A). Coapplication of 100 μM amiloride with GABA did not affect the rate of rise or the peak of the current. However, the current rapidly declined after reaching the peak, compared with the response with GABA alone. At the end of the drug application, this current rebounded to a level similar to the control current, and the deactivation rate was similar to that with GABA alone. These characteristics are consistent with an open-channel block mechanism in which amiloride cannot bind to its site until the channel is opened. Therefore, at high GABA concentrations, it would affect neither activation rate nor peak current. The rebound current suggests that the channel cannot close until amiloride has unbound. These results indicate that amiloride may have at least two sites of action at the GABAR.

The rapid decrease in current caused by amiloride may have produced some error in measuring the peak current for the GABA concentration-response relationships. However, as the peak current in response to 1 mM GABA was unaffected, it seems that the drug application was rapid enough to allow accurate measurement of the initial current response. If the suggested open-channel block site affected measurement of the response to lower GABA concentrations, a change in Hill slope should have been observed.

Effect of Voltage on Amiloride Inhibition. Many modulators of ion channels exhibit voltage dependence in their activity. Inhibition of the response to 1 μM GABA by 100 μM amiloride was similar at holding potentials of −50 ($N = 6$) and +50 mV ($N = 4$) for the $\alpha6\beta3\gamma2L$ isoform ($p > 0.5$) (Fig. 3). However, the rapid reduction in current after the peak observed with 1 mM GABA + 100 μM amiloride was reduced at +50 mV ($N = 4$) compared with −50 mV ($N = 6$) ($p \leq 0.05$), although some inhibition was still apparent (Fig. 3). These dual effects of amiloride may therefore occur at distinct sites, only one of which is influenced by membrane voltage.

Higher Sensitivity to Amiloride Is Associated with the $\alpha6$ Subtype. The α subunit family is the most diverse of

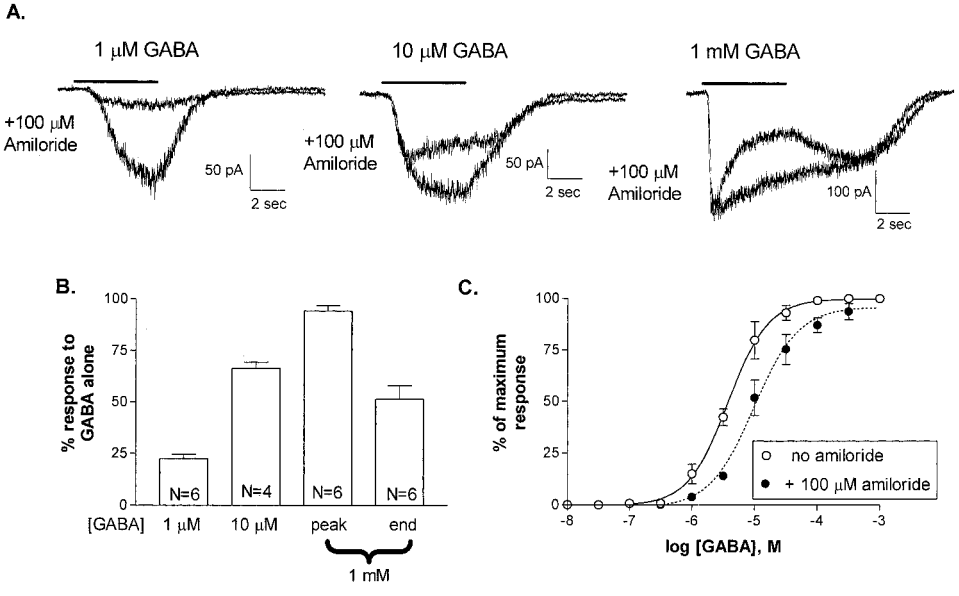

Fig. 2. Effect of GABA concentration on inhibition by amiloride. A, Representative traces from $\alpha6\beta3\gamma2L$ receptors in response to 1 μM, 10 μM, or 1 mM GABA, alone and with 100 μM amiloride. Responses with and without amiloride are superimposed to clarify the amount of inhibition. The traces shown with different GABA concentrations are not all from the same cell. B, comparison of the amount of inhibition produced by 100 μM amiloride for different GABA concentrations. The amplitudes of the end currents in response to 1 mM GABA were measured at the end of the 5-s drug application period. Bars represent mean ± S.E.M. C, 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 for each cell. Points shown are mean ± S.E.M. Data were fit with a four-parameter logistic equation. EC_{50} values from these fits were 3.8 μM without amiloride ($N = 3$) and 9.9 μM in the presence of 100 μM amiloride ($N = 4$).

the GABAR subunits, with six different subtypes (α1–α6). The α subtype composition of the receptor influences many functional properties of the receptor, and the activity of virtually all GABAR modulators shows some dependence upon the α-subtype composition of the receptor (Mehta and Ticku, 1999). To determine the effect of α subtype on sensitivity to amiloride, concentration-response relationships were determined for receptors, composed of each of the different α subtypes. All α subunits were coexpressed with the same β and γ subunits (β3 and γ2L) to provide a common background for comparison, and a submaximal GABA concentration (EC_{20-40}) was used for each isoform.

A high sensitivity to inhibition by amiloride depended upon the presence of an α6 subtype (Fig. 4). Receptors containing any of the other α subtypes were significantly less sensitive to amiloride ($p \leq 0.001$, compared with $\alpha6\beta3\gamma2L$). The IC_{50} values for amiloride were about 10-fold greater than those for the $\alpha6\beta3\gamma2L$ receptor, with averages of 250.0 ± 25.0 μM ($\alpha1\beta3\gamma2L$, $N = 4$), 261.6 ± 30.4 μM ($\alpha2\beta3\gamma2L$, $N = 4$), 250.8 ± 73.9 μM ($\alpha3\beta3\gamma2L$, $N = 3$) 334.2 ± 45.9 μM ($\alpha4\beta3\gamma2L$, $N = 3$), and 274.3 ± 17.3 μM ($\alpha5\beta3\gamma2L$, $N = 5$).

Because the rapid decrease in current observed with high GABA concentrations suggested another site of action for amiloride at $\alpha6\beta3\gamma2L$ receptors, we also examined whether inhibition of the maximal response to GABA showed α subtype dependence. With the $\alpha1\beta3\gamma2L$ receptor, 100 μM amiloride had little effect on either the peak or the late current (measured at the end of the 5-s application) in response to 1 mM GABA at −50 mV. The average amount of current compared with GABA alone was 98.1 ± 2.0 of the peak current and 98.8 ± 1.5 of the late current ($N = 5$, data not shown).

The β Subunit Subtype Does Not Affect Sensitivity to Amiloride. Three β subtypes have been cloned from mammalian species. The nature of the β subtype influences some pharmacological properties of the GABAR (Mehta and Ticku, 1999), including sensitivity to the positive modulator loreclezole (Wafford et al., 1994) and the inhibitor furosemide (Korpi et al., 1995). In both of those cases, receptors containing a β2 or β3 subtype were sensitive to modulation, whereas those containing a β1 subtype were insensitive. To determine whether the β subtype affected sensitivity to amiloride, the β1, β2, and β3 subtypes were coexpressed with α6 and γ2L subunits. Amiloride was coapplied with either 1 μM GABA

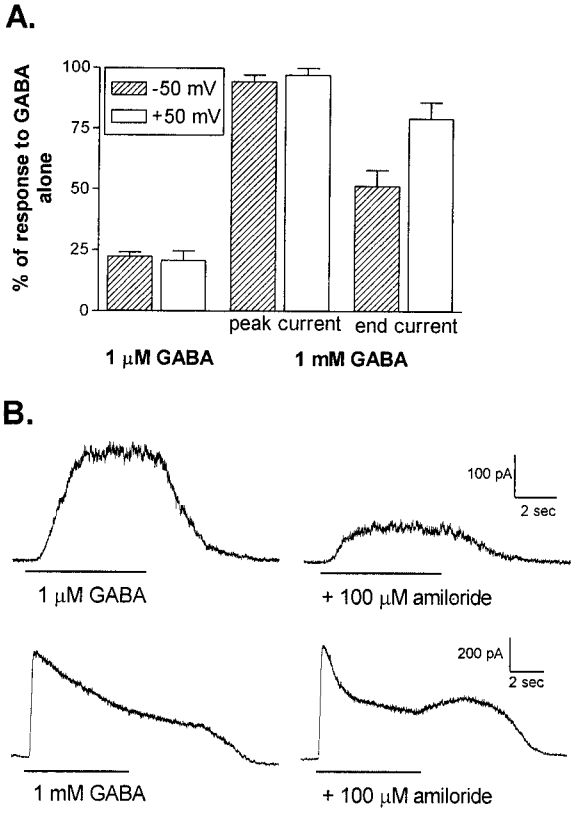

Fig. 3. Effect of membrane voltage on inhibition by amiloride. A, the response of $\alpha6\beta3\gamma2L$ receptors to 1 μM or 1 mM GABA, alone or with 100 μM amiloride, was measured at a holding potential of −50 or +50 mV. End current for 1 mM GABA applications was measured at the end of the 5-s drug application. Bars represent mean ± S.E.M of the percentage of the response to GABA alone. B, representative current traces from $\alpha6\beta3\gamma2L$ receptors in response to 1 μM or 1 mM GABA, alone and with 100 μM amiloride at +50 mV.

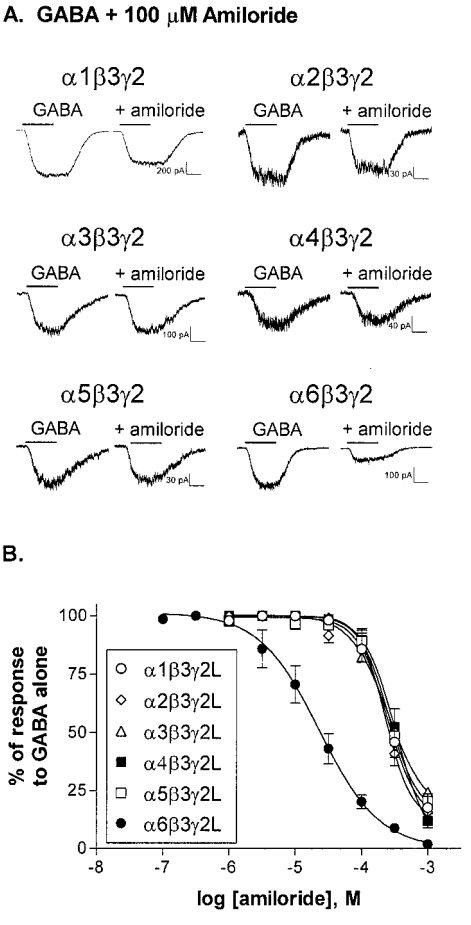

Fig. 4. Amiloride sensitivity depends upon the α-subtype composition of the receptor. A, representative traces from receptors composed of β3, γ2L, and one of the α subtypes, as indicated, showing the current response to GABA alone or GABA + 100 μM amiloride from transfected cells voltage-clamped at −50 mV. Standard horizontal bars indicate 2 s for all traces. GABA concentration was 1 μM (α6), 3 μM (α4, α5) or 10 μM (α1, α2, α3). B, concentration-response relationships were constructed by measuring the peak current with coapplication of amiloride as a percentage of the response to GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation. IC_{50} values from the fits of combined data shown were 250.0 μM ($\alpha1\beta3\gamma2L$, $N = 4$), 231.3 μM ($\alpha2\beta3\gamma2L$, $N = 4$), 259.8 μM ($\alpha3\beta3\gamma2L$, $N = 3$), 333.2 μM ($\alpha4\beta3\gamma2L$, $N = 3$), 257.3 μM ($\alpha5\beta3\gamma2L$, $N = 5$), and 23.5 μM ($\alpha6\beta3\gamma2L$, $N = 4$). Hill numbers from these fits were −1.89 ($\alpha1\beta3\gamma2L$), −2.22 ($\alpha2\beta3\gamma2L$), −1.49 ($\alpha3\beta3\gamma2L$), −1.80 ($\alpha4\beta3\gamma2L$), −1.97 ($\alpha5\beta3\gamma2L$), and −0.96 ($\alpha6\beta3\gamma2L$).

(β2, β3) or 3 μM GABA (β1), which represented approximately EC_{20-30} GABA concentrations for these isoforms. All these receptors were equally sensitive to inhibition by amiloride (Fig. 5, A and B). Average amiloride IC_{50} concentrations from the fits of the individual data were 18.8 ± 2.3 μM ($\alpha6\beta1\gamma2L$, $N = 4$) and 24.6 ± 7.5 μM ($\alpha6\beta2\gamma2L$, $N = 3$) ($p > 0.5$ among β1-, β2-, and β3-containing isoforms).

Amiloride Sensitivity Does Not Depend on the γ Subtype. In mammalian species, there are three different γ GABAR subtypes. The γ2 subtype also has splice variants (γ2L and γ2S). The γ subtype influences some pharmacological properties of recombinant GABARs, in particular, the benzodiazepine sensitivity (Benke et al., 1996). The γ subtypes were coexpressed with the same α (α6) and β (β3) subunits. Amiloride was coapplied with 1 μM GABA. The nature of the γ subunit had no effect on the sensitivity of the receptor to amiloride (Fig. 5, A and C). The average IC_{50} values were 19.7 ± 5.1 μM ($N = 4$) for the $\alpha6\beta3\gamma1$ receptor and 25.6 ± 10.2 ($N = 3$) for the $\alpha6\beta3\gamma3$ receptor ($p > 0.5$ compared with $\alpha6\beta3\gamma2L$).

To determine whether the presence of a γ subunit affected amiloride sensitivity, we also examined the properties of $\alpha6\beta3$ δ receptors. Incorporation of a δ subunit has many effects on the functional and pharmacological properties of the GABAR (Saxena and Macdonald, 1994). However, the δ subunit did not affect inhibition by amiloride (Fig. 5, A and C). The average IC_{50} of the $\alpha6\beta3$ δ receptor was 22.5 ± 7.9 μM ($N = 2$), not significantly different from the $\alpha6\beta3\gamma2L$ isoform ($p > 0.5$).

Separate Structures Regulate Amiloride and Furosemide Sensitivity. The diuretic furosemide shares a similar profile with amiloride in that the α6 subtype confers higher sensitivity to inhibition compared with other α subtypes. An isoleucine residue (I228) located in the first transmembrane domain of the subunit was found to be important for high-affinity inhibition by furosemide (Thompson et al., 1999). When this residue was mutated in the α6 subunit to the threonine residue found at the homologous location in the α1 subunit, sensitivity to furosemide was reduced. To determine whether this site was also important for amiloride activity, $\alpha6_{(I228T)}$ subunits were coexpressed with wild-type β3 and γ2L subunits. The amiloride sensitivity of the $\alpha6_{(I228T)}\beta3\gamma2L$ receptors (average IC_{50}, 15.4 ± 3.7 μM; $N = 4$) was not significantly different from the wild-type $\alpha6\beta3\gamma2L$ isoform ($p > 0.4$) (Fig. 6).

Pentobarbital-Activated Currents Were Less Sensitive to Amiloride Inhibition. In addition to acting as a positive allosteric modulator of the GABAR, pentobarbital also directly activates the channel. Its agonist site is distinct from the GABA binding site, as direct activation is not blocked by the competitive antagonist bicuculline (Thompson et al., 1996). Interestingly, the α6 subunit confers higher affinity and efficacy for the agonist action of pentobarbital (Thompson et al., 1996). Therefore, the effect of amiloride on pentobarbital-activated currents was examined. Amiloride was much less effective in inhibiting currents activated by 10 μM pentobarbital (EC_{20-30} concentration) compared with 1 μM GABA (Fig. 7). The average IC_{50} was 169.9 ± 29.7 μM ($N = 3$), significantly different from the IC_{50} with GABA-activated currents ($p \leq 0.01$). This suggests that, like bicuculline, amiloride competitively antagonizes only the GABA binding site, and not that of pentobarbital. Unlike bicuculline, however, amiloride does have some inhibitory action on these currents. This may represent the proposed second site for amiloride, responsible for the appearance of open-channel block at high GABA concentrations.

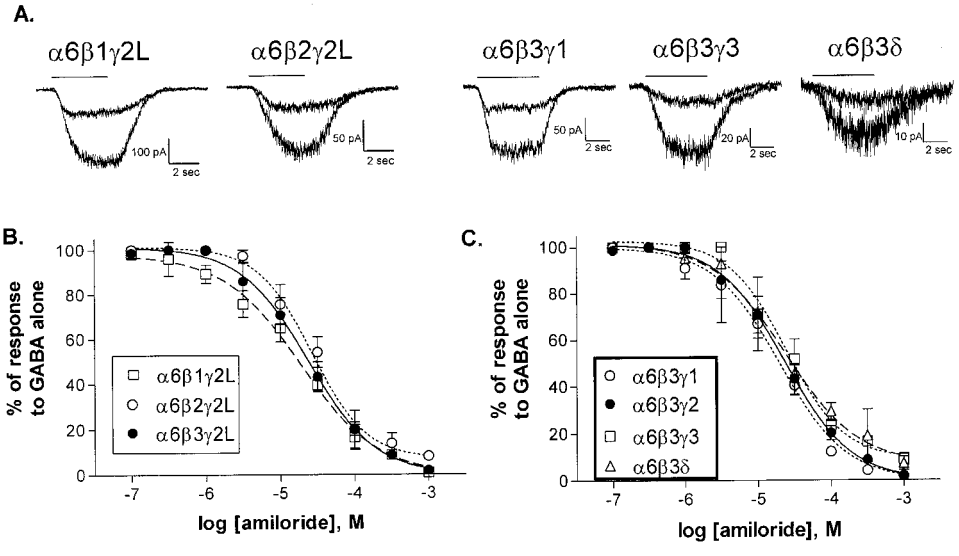

Fig. 5. Amiloride sensitivity does not depend upon the β- or γ-subtype composition of the receptor. A, representative traces from receptors composed of α6 and various β-, γ-, and δ-subunit combinations showing the current response to GABA alone or GABA + 100 μM amiloride. Traces with and without amiloride are superimposed. GABA concentration was EC_{20-30} for each isoform. B, concentration-response relationships were constructed by measuring the peak current with coapplication of amiloride as a percentage of the response to GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation. IC_{50} values from the fits of the combined data were 18.6 μM ($\alpha6\beta1\gamma2L$, $N = 4$), 26.5 μM ($\alpha6\beta2\gamma2L$, $N = 3$), and 23.5 μM ($\alpha6\beta3\gamma2L$, $N = 4$). Hill numbers from these fits were −0.84 ($\alpha6\beta1\gamma2L$), −1.19 ($\alpha6\beta2\gamma2L$), and −0.96 ($\alpha6\beta3\gamma2L$). C, concentration-response relationships were constructed by measuring the peak current with coapplication of amiloride as a percentage of the response to 1 μM GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation. IC_{50} values from the fits of the combined data were 19.9 μM ($\alpha6\beta3\gamma1$, $N = 4$), 23.5 μM ($\alpha6\beta3\gamma2L$, $N = 4$), 24.4 μM ($\alpha6\beta3\gamma3$, $N = 3$), and 23.9 μM ($\alpha6\beta3\delta$, $N = 2$). Hill numbers of these fits were −0.96 ($\alpha6\beta3\gamma1$), −0.96 ($\alpha6\beta3\gamma2L$), −1.1 ($\alpha6\beta3\gamma3$), and −0.88 ($\alpha6\beta3$ δ).

Discussion

The diuretic amiloride is best known for its inhibitory action on Na^+ channels and transporters, but it has been reported to have activity at several diverse targets. We found that amiloride inhibited the activity of recombinant GABARs of mammalian origin, confirming an earlier report with GABARs in frog sensory neurons (Inomata et al., 1988). Amiloride has also been shown to modulate the activity of neurotransmitter receptors, including the adenosine (Garritsen et al., 1990) and adrenergic receptors (Howard et al., 1987; Nunnari et al., 1987). Therefore, it may not be surprising that amiloride also modulates the activity of GABARs. Amiloride seemed to act directly on the GABAR, with an immediate and reversible inhibition of the response of the receptor. Because of the time course and subunit specificity of activity by amiloride, it is unlikely that indirect effects such as alterations in intracellular pH were responsible for the decrease in GABAR activity.

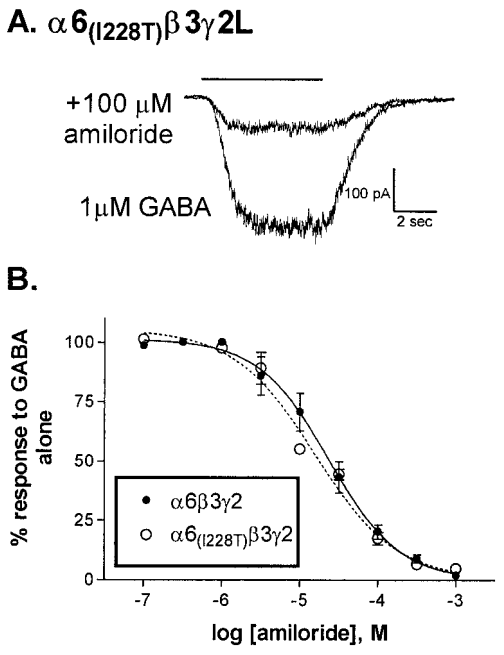

Fig. 6. The M1-domain isoleucine residue that regulates furosemide sensitivity of the $\alpha 6$ subunit does not affect amiloride sensitivity. A, representative traces from the $\alpha 6_{(I228T)}\beta 3\gamma 2L$ receptor showing the current response to 1 μM GABA alone or GABA + 100 μM amiloride. Traces with and without amiloride are superimposed. B, concentration-response relationships were constructed by measuring the peak current with coapplication of amiloride as a percentage of the response to 1 μM GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation. IC_{50} values from the fits of the combined data were 23.5 μM ($\alpha 6\beta 3\gamma 2L$, $N = 4$) and 15.6 μM ($\alpha 6_{(I228T)}\beta 3\gamma 2L$, $N = 4$). Hill numbers of these fits were −0.96 ($\alpha 6\beta 3\gamma 2L$) and −0.84 ($\alpha 6_{(I228T)}\beta 3\gamma 2L$).

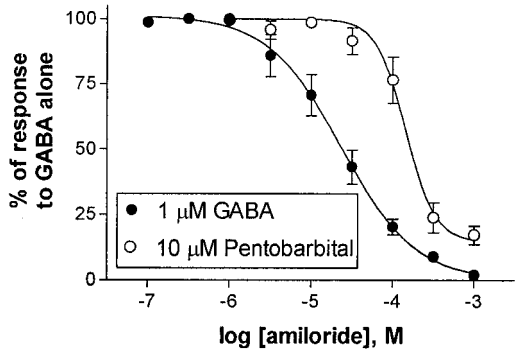

Fig. 7. $\alpha 6\beta 3\gamma 2L$ receptors directly activated by pentobarbital are less sensitive to inhibition by amiloride. Concentration-response relationships were constructed by measuring the peak current with coapplication of amiloride as a percentage of the response to 10 μM pentobarbital or 1 μM GABA alone for each cell. Symbols and bars represent the mean ± S.E.M. Data were fit with a four-parameter logistic equation. The IC_{50} and Hill number (n) from the fit of the combined data for pentobarbital-activated currents was 144.3 μM, $n = -2.4$ ($N = 3$) compared with 23.5 μM, $n = -0.96$ for GABA-activated currents ($N = 4$).

The subunit dependence of action by amiloride is unlike that of any other GABAR inhibitor reported to date, suggesting that it acts through novel regulatory sites. The $\alpha 6$ subunit conferred higher sensitivity to amiloride in both its competitive and noncompetitive effects. The $\alpha 6$ subunit is the most functionally diverse of the α subunit family and has a unique pharmacological profile. Other differences associated with the $\alpha 6$ subtype compared with the $\alpha 1$ subtype include an insensitivity to benzodiazepines, higher sensitivity to inhibition by zinc and furosemide, inhibition rather than potentiation by lanthanum, and direct activation by pentobarbital (Lüddens et al., 1990; Korpi et al., 1995; Knoflach et al., 1996; Saxena and Macdonald, 1996; Thompson et al., 1996; Saxena et al., 1997). Unlike amiloride, however, the sensitivity to these other modulators is generally also affected by the β or γ subtype of the receptor and/or by the presence of a δ subunit. The lack of subtype dependence for the β and γ subunits does not necessarily mean that these subunits do not contribute at all to the action of amiloride, only that the subtypes do not differ in their contribution. It is interesting that the $\alpha 4$ subunit was unlike the $\alpha 6$ subunit in amiloride sensitivity, because these subunits are structurally very similar and often share pharmacological characteristics (Wafford et al., 1996). The amiloride site was clearly distinct from that for furosemide, as the mutation in the $\alpha 6$ subunit, found to reduce furosemide sensitivity (I228T), did not affect amiloride sensitivity (Thompson et al., 1999). Although both these agents are used clinically as diuretics, they are not structurally related, and they have different sites of action within the kidney. Therefore, despite their similar effects on GABAR function, it is not unexpected that they act at different sites on the receptor.

The results suggested that amiloride acted at two separate sites on the GABAR and that the $\alpha 6$ subunit conferred higher sensitivity at both sites. At the first proposed site, amiloride, acted as a competitive antagonist. This site had higher affinity than the second, was not voltage-dependent, and did not inhibit pentobarbital-activated currents. The other proposed amiloride site showed some voltage dependence, showed increased inhibition with increasing GABA concentration, and caused a rapid decrease in current after activation and a rebound current after removal of GABA and amiloride. All these characteristics are consistent with open-channel block, with amiloride unbinding more rapidly than GABA, allowing observation of the rebound current. Although the subunit dependence of these two sites is apparently similar, these sites are likely to have different structural components. Mutagenesis studies may allow identification of the amino acid residues responsible for the higher sensitivity conferred by the $\alpha 6$ subtype and isolate the structures responsible for each of the effects of amiloride. The extracellular N terminus probably determines the site for competitive antagonism, because many residues within this domain contribute to formation of the GABA binding site (Mehta and Ticku, 1999).

The site for open-channel block is probably located within or near the external vestibule of the channel pore. Many structural derivatives of amiloride are available, and their relative activity varies with the target protein (Frelin et al., 1988; Kleyman and Cragoe, 1988). Examining the effectiveness of these analogs at the GABAR could clarify the structural requirements of each of these sites.

Alterations or abnormalities in α6 subunit expression may underlie the development of several neurological and behavioral disorders. Chronic treatment with benzodiazepines increases α6 subunit expression and decreases α1 expression (O'Donovan et al., 1992). Because α6-containing receptors are insensitive to benzodiazepines, this change may lead to tolerance, which is a commonly observed clinical problem. A similar pattern of change is seen with alcohol dependence, as chronic ethanol administration increases α6 mRNA and decreases α1 mRNA (Mhatre and Ticku, 1992). The cerebellar localization of the α6 subunit suggests that it may be involved in motor control, particularly in the motor effects of ethanol and other sedatives (Korpi et al., 1993). Interestingly, recent work has shown that transgenic mice lacking either the α6 or α1 subunit exhibit no major phenotypic disturbances (Homanics et al., 1997; Jones et al., 1997; Nusser et al., 1999; Sur et al., 2001). This may indicate that the wide variety of GABAR subunits and subtypes could allow considerable flexibility to overcome deficits in the expression of a single subunit subtype.

In general, amiloride and its derivatives do not readily cross the blood-brain barrier (Sipos and Brem, 2000), suggesting that effects on GABAR function would be observed only in cases of high levels of amiloride or when this barrier is compromised. Therefore, it is likely that under most conditions, clinically relevant concentrations of amiloride would not affect $GABA_A$ receptors. However, amiloride is currently under investigation as a treatment for brain tumors, primarily because of its inhibition of a serine protease (Bubien et al., 1999; Sipos and Brem 2000). If amiloride is delivered directly to the cerebrospinal fluid as a part of that therapy, inhibition of GABAR activity could cause unexpected side effects. The α6 subunit-specific effect of amiloride will make it a useful tool for research purposes, serving to further differentiate native GABAR isoforms pharmacologically. Additionally, concentrations of amiloride above the micromolar range should be used with care in experimental protocols where direct effects on the GABAR activity could confound the results.

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