Two Novel Residues in M2 of the γ-Aminobutyric Acid Type A Receptor Affecting Gating by GABA and Picrotoxin Affinity*

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An amino acid residue was found in M2 of γ-aminobutyric acid (GABA) type A receptors that has profound effects on the binding of picrotoxin to the receptor and therefore may form part of its binding pocket. In addition, it strongly affects channel gating. The residue is located N-terminally to residues suggested so far to be important for channel gating. Point mutated α1β3 receptors were expressed in Xenopus oocytes and analyzed using the electrophysiological techniques. Coexpression of the α1 subunit with the mutated β3 subunit β2L253F led to spontaneous picrotoxin-sensitive currents in the absence of GABA. Nanomolar concentrations of GABA further promoted channel opening. Upon washout of picrotoxin, a huge transient inward current was observed. The reversal potential of the inward current was indicative of a chloride ion selectivity. The amplitude of the inward current was strongly dependent on the picrotoxin affinity. A kinetic model is presented that mimics the gating behavior of the mutant receptor. The point mutation in the neighboring residue β2A252V resulted in receptors that displayed an about 6-fold increased apparent affinity to GABA and an about 10-fold reduced sensitivity to picrotoxin.

The GABAA \( \gamma \) receptors are the major inhibitory neuronal ion channels in the mammalian central nervous system. Two subunits termed \( \alpha \) and \( \beta \) have initially been purified from bovine brain (1) and the corresponding DNAs have been cloned (2). Many mammalian subunits have been cloned since (3–8). These subunits show a high degree of homology to subunits of nicotinic acetylcholine receptors, the glycine receptor and the GABA \( \gamma \) receptors confers cyclodiene resistance (15). A leucine residue is strictly conserved in the middle of the M2 region of all subunit isoforms and is located at position 263 of the \( \alpha_1 \) subunit. Substitution to a serine in \( \alpha_1, \beta_1, \beta_2, \) or \( \gamma_2 \) resulted in an abnormally high apparent GABA affinity for channel opening (16). Some point mutations of this leucine on \( \alpha_1, \beta_1, \beta_2, \) or \( \rho_1 \) subunits resulted in spontaneous open channels (17–21).

During work aimed at the understanding of the site in M2 involved in the recognition of tert-butylbicyclophosphorothionate (22), we investigated the properties of chimeric \( \alpha_1, \beta_3, \) receptor subunits coexpressed with \( \alpha_1 \) subunits. The results indicated an importance of Ala252 and Leu253 for the tert-butylbicyclopentylphosphorothionate binding affinity. Here, we show that substitutions of \( \beta_2A252 \) and \( \beta_2L253 \) result in reduced picrotoxin affinity. For \( \beta_2L253F \) affinity is more than 100-fold reduced, and a huge transient opening of the channel upon removal of picrotoxin was evident. Such a transient opening has not been observed for any other mutation before, and our mathematical model suggests that this is a consequence of the strongly decreased affinity for picrotoxin. Residue Val256, neighboring Phe257, the homologue on the \( \alpha_1 \) subunit, has been shown to covalently interact with other noncompetitive blockers acting at the picrotoxin binding site (14). Leucine 253 of the \( \beta_2 \) subunit (\( \beta_2L253 \)) may therefore be part of the contact site for picrotoxin together with \( \alpha_1V256 \). In addition, we report that single point mutation \( \beta_2L253F \) confers abnormal gating properties to \( \alpha_1, \beta_2 \) receptors. These include spontaneous opening of the channels and a very high GABA sensitivity for channel gating. Thus, our work points to the involvement in GABAA \( \gamma \) receptor channel gating of more N-terminally located amino acid residues than previously suggested.

EXPERIMENTAL PROCEDURES

Amino Acid Residue Numbering—Residues are numbered according to the mature rat sequences.

Construction of Receptor Subunits—The cDNAs coding for the \( \alpha_1, \beta_3, \) and chimeric subunits of the rat GABAA \( \gamma \) receptor channel have been described elsewhere (22, 23). Site-directed mutagenesis was done using the QuikChange mutagenesis kit (Strategene). In vitro synthesized sequences have been verified by DNA sequencing.

Functional Expression and Characterization—Xenopus laevis oocytes were prepared, injected, and defolliculated, and currents were recorded as described (24, 25). Briefly, oocytes were injected with 50 nl of capped polyadenylated cRNA dissolved in 5 mM K-HEPES, pH 6.8. This solution contained the transcripts coding for the different subunits at concentrations of 75 nM. RNA transcripts were synthesized from linearized plasmids encoding the desired protein using the mMessage mMACHINE kit (Ambion) according to the recommendations of the manufacturer. A
chimeras were coexpressed together with the a subunit of picrotoxin alone (Fig. 2). At 1 mM picrotoxin, the amplitude of the transient inward current was 11 times (average of 3 repeats) the amplitude of the inward current upon application of 1 mM picrotoxin in the absence of GABA (Fig. 2A). Interestingly, a strong transient inward current was detected during washout of picrotoxin in the absence of GABA (Fig. 2A). Wild type a1b2 receptors were activated by GABA and showed no response to the application of picrotoxin alone (Fig. 2B). Injection of cRNA coding for CH7 alone did not result in ion currents induced by picrotoxin or GABA (data not shown), indicating that the channel with these unusual properties was formed from a1CH7. Three additional chimeras were coexpressed together with the a1 subunit to localize residues important for the unusual gating behavior. Only a1CH74 also displayed the picrotoxin washout current found in a1CH7 receptors (Table I). An amino acid comparison between the chimera revealed that a threonine-valine-phenylalanine motif was common to CH7 and CH74 and absent in the other two constructs. Three mutant subunits were constructed by individually introducing these three residues of a1 into the homologous positions of b2, with the aim to identify the amino acid residue responsible for the abnormal properties.

Expression of Point Mutated Receptors—Wild type and mutant b2 subunits were coexpressed together with the a1 subunit in Xenopus oocytes. All three mutant receptors expressed GABA-activated chloride currents. Receptors with a leucine to phenylalanine substitution in the channel pore-forming region at position 253 (a1b2L253F) could be activated by very low concentrations of GABA (30 nM). Repeated applications elicited increasingly smaller current amplitudes even when a washout period of up to 15 min was used (data not shown). For this reason it was technically not possible to measure the apparent affinity of GABA for channel opening in a1b2L253F. Dose response curves for wild type a1b2, a1b2V251T, and a1b2A252V revealed 3.5- and 6.0-fold increases in the apparent affinities to GABA for these mutated receptors (Table II), respectively.

Response to Picrotoxin—Application of 1 mM picrotoxin in the absence of GABA did not induce any apparent outward or inward currents in a1b2V251T and a1b2A252V receptors. In contrast, in oocytes expressing a1b2L253F receptors, picrotoxin application resulted in apparent outward currents (Fig. 2C) similar to oocytes expressing a1CH7. The mutated channel a1b2L253F also showed a huge transient inward current upon washout of picrotoxin (Fig. 2C). At 1 mM picrotoxin, the amplitude of the transient inward current was 11 times (average of three determinations) the amplitude of the apparent outward current.

To exclude that the current properties upon injection of cRNAs coding for a1 and b2L253F resulted from homomorphic b2L253F, we also expressed this mutated subunit alone. No
detectable signal could be obtained with 300 μM picrotoxin (two independent batches of oocytes).

A voltage ramp protocol was used to measure ion currents in mutant α1β2L253F channels before and during and the application of picrotoxin and about 5 s after its removal (Fig. 3A). The voltage ramp had a duration of 0.13 s, such that the amplitude of the inward current showed little change during the application of the ramp. Picrotoxin application resulted in a reduction of the membrane conductance, indicating that part of the receptors are spontaneously in an open conformation. All three curves obtained had the same intersection at −38 ± 1 mV (three experiments). Therefore, it can be concluded that the spontaneous current and the transient inward current have the same ion permeability. Replacing the 95 mM Cl− with 9.5 mM Cl− and 84.5 mM acetate− in the outside medium resulted in an about 60 mV shift to the right in the reversal potential of the transient inward current (not shown), in line with a chloride selective conductance. From the reversal potential of −38 mV determined at an extracellular chloride concentration of 95 mM, an intracellular chloride concentration of −23 mM may be estimated. The same voltage ramp protocol was used before and during the application of a subsaturating concentration of GABA to oocytes expressing the wild type α1β2 receptor (Fig. 3B). The intersection of the two curves was found at −25 ± 2 mV (three experiments), indicating an intracellular chloride ion concentration of about 37 mM. The difference in the intracellular chloride concentration in oocytes expressing wild type and the point mutated receptor can be explained by a more negative membrane potential of the oocyte than the chloride reversal potential. Under conditions where the permeability for this ion is increased, the membrane potential drives chloride ions out of the cell.

As mentioned above, the channel opens to a certain degree spontaneously producing an inward current. The initial response to the application of picrotoxin is an apparent outward current reflecting channel closure. The picrotoxin concentration dependence of the peak current amplitude is illustrated in Fig. 4A. It increases between 10 and 1000 μM picrotoxin and shows no saturation up to 1 mM picrotoxin (Fig. 4C). The maximum apparent outward current may, however, be estimated, assuming an infinite membrane resistance for the case where all channels are in the closed state. Assuming that most channels are in the open state upon removal of picrotoxin, it can be estimated that less than 9% of the channels are spontaneously open prior to the application of picrotoxin. The shape of the late apparent outward current response during perfusion with picrotoxin depends on the concentration of picrotoxin used (Fig. 4A). In the presence of low concentrations, only a transient inhibition of the current could be detected, followed by reopening of the channels. The time course of this reopening seemed picrotoxin concentration independent in the range of 10–300 μM. Because of the small amplitudes of this component, it could only be estimated. Assuming a mono-exponential time course reopening was characterized by a τ in the range of −6–13 s (not shown).

A large transient inward current was observed during washout of picrotoxin. The concentration dependence on picrotoxin

### Table I

| Subunit combination | GABA-induced current | Picrotoxin wash-out current |
|---------------------|----------------------|-----------------------------|
| α1β2                | +                    | −                           |
| α1CH3               | +                    | −                           |
| α1CH4               | +                    | −                           |
| α1CH7               | −                    | +                           |
| α3L253F             | −                    | +                           |

### Table II

| Subunit combination | GABA EC50 (μM) | Hill coefficient |
|---------------------|----------------|------------------|
| α1β2                | 8.4 ± 3.7 (n = 8) | 1.2 ± 0.2 |
| α1β2V251T           | 2.4 ± 1.4 (n = 5) | 1.2 ± 0.1 |
| α1β2A252V           | 1.4 ± 0.5 (n = 4) | 1.1 ± 0.2 |
| α1β2L253F           | amplitude not reproducible | |

**Fig. 2. Electrophysiological properties of α1CH7 and of α1β2 receptors.** Currents were recorded under voltage clamp conditions at −80 mV from oocytes injected with α1 and CH7 (A), with α1 and β2 (B), or with α1 and β2L253F (C) subunits. 1 mM GABA and 1 mM picrotoxin (Ptx) were applied for 20 s each (A and B, bars) or 1 min (C). Two additional identical experiments in each case gave similar results. The same behavior of the channels was observed by using lower concentrations of picrotoxin in three additional experiments and shorter application times in 17 additional experiments.

The Picrotoxin-binding Site of GABA<sub>A</sub> Receptors
of this current is illustrated in Fig. 4C. Its amplitude increases using increasing concentrations of picrotoxin and does not saturate up to 1 mM picrotoxin. Half of the amplitude observed at 1 mM picrotoxin was observed at about 300 μM (Fig. 4C). Fig. 5A shows that the size of this current increases with the duration of picrotoxin application. For this experiment 300 μM picrotoxin was applied during different time intervals between ~1 and 60 s. The time dependence of the increase in inward current amplitude is well fitted with a mono-exponential function with \( \tau = 9.1 \pm 2.2 \text{s (n = 3)} \). Reclosure of the channel after transient opening was independent of the picrotoxin concentration and followed a bi-exponential time course with \( \tau_i = 5.5 \pm 2.8 \text{s and } \tau_a = 23.3 \pm 12.0 \text{s (means ± S.D., three experiments at five different concentrations each).}

The picrotoxin sensitivity of GABA-activated currents was also measured for wild type \( \alpha_3\beta_3 \) receptors and mutant \( \alpha_3\beta_3 V251T \) and \( \alpha_3\beta_3 A252V \) receptors. A GABA concentration eliciting 10–15% of the maximum current was used in these experiments. Because \( \alpha_3\beta_3 V251T \) and \( \alpha_3\beta_3 A252V \) receptors displayed an increased apparent affinity to GABA (Table II), a lower concentration of agonist had to be used for these two mutant receptors. \( \alpha_3\beta_3 A252V \) receptors displayed an about 10-fold reduced sensitivity to picrotoxin compared with wild type and \( \alpha_3\beta_3 V251T \) receptors (Fig. 6).

**Kinetic Modeling**—To explain the following two phenomena, namely the reopening of the channels during continuous application of picrotoxin and the transient inward current after its removal, we performed computer simulations based on the model shown in Fig. 7. Rate constants used are given in the figure legends of Figs. 4B. It is assumed that about 5% of the channels are spontaneously open, which determines the equilibrium state of the system and the initial conditions for the simulations. The channels switch between this open state and the closed states giving rise to the observed inward resting current. Washout of picrotoxin is assumed to lead to fast dissociation from the receptor without rebinding. This results in the immediate emptying of all bound states. The number of picrotoxin less closed states is then given by the relaxation process upon picrotoxin removal. The double exponential decay determined in the experiments requires a minimal model which consists of three states: an open state \( O \) and two closed states \( C \) and \( R \). Applying picrotoxin to the system leads to a pronounced reduction in the number of open channels. The resulting initial decrease of the inward current (apparent outward current) is followed by the relaxation into a new equilibrium state. We propose that only the state \( (O, \text{PTX}) \) (binding of picrotoxin to the open channels) is kinetically relevant because after withdrawal of picrotoxin these channels are able to pro-

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**FIG. 3.** Voltage dependence of ion currents of \( \alpha_3\beta_3 L253F \) (A) and \( \alpha_3\beta_3 \) (B) receptors. Continuous voltage ramps of 130 ms duration were generated from a holding potential of −80 to +50 mV, and ion currents were recorded. The experiment was first performed in the absence of picrotoxin (medium), then in the presence of 0.3 μM picrotoxin, and finally during the washout of the drug in A and first during perfusion with medium and then during perfusion with medium containing 0.3 μM GABA in B. Two additional experiments in each case gave similar results.

**FIG. 4.** The size of the apparent outward current and the transient inward current in \( \alpha_3\beta_3 L253F \) receptors both depend on the concentration of picrotoxin. A, current traces obtained by applying increasing concentrations of picrotoxin (bars) for 1 min. B, computer simulations describing the concentration dependence of the transient inward current evoked by picrotoxin withdrawal in the mutated \( \alpha_3\beta_3 L253F \) GABA\(_A\) receptor. The assumed concentrations of picrotoxin were 10, 30, 100, 300, and 1000 μM. The application time was 60 s. The rate constants are described in the legend to Fig. 7 and assume the following values: \( k_1 = 5.0 \times 10^{-2} \text{μM}^{-1}\text{s}^{-1}, k_2 = 1.5 \text{s}^{-1}, k_3 = 8.1 \times 10^{-2} \text{s}^{-1}, k_4 = 8.1 \times 10^{-5} \text{s}^{-1}, k_5 = 3.0 \times 10^{-4} \text{μM}^{-1}\text{s}^{-1}, k_6 = 1.0 \times 10^{-3} \text{s}^{-1}, k_7 = 5.0 \times 10^{-5} \text{μM}^{-1}\text{s}^{-1}, k_8 = 5.5 \times 10^{-2} \text{s}^{-1}, k_9 = 1.0 \times 10^{-5} \text{s}^{-1}, \) and \( k_{10} = 9.0 \times 10^{-3} \text{s}^{-1} \). C, size of the apparent outward current amplitudes and the inward current amplitudes from three different oocytes. Data are given as the means ± S.D.
duce a transient inward current. If in contrast picrotoxin binding to closed channels would be dominant, no transient inward current could be observed, because the channels would already be closed. Fig. 5B presents simulations of experiments where the duration of 300 μM picrotoxin application is varied. Because the equilibrium is on the side of the state (O.PTX) because of the excess of picrotoxin, the longer the transient filling of the state (O.PTX), the larger the transient inward current amplitude after removal of picrotoxin. Fig. 4B shows simulations of the current during perfusion with different concentrations of picrotoxin. The amplitude of the initial apparent outward current is limited at high picrotoxin concentration because of saturation of channel closure. The following relaxation process into the new equilibrium state depends only weakly on the concentration of picrotoxin (largest relaxation constant $t_5 = 13–16$ s), which is in agreement with the experiments. The simulations revealed that the closed state (R) serves as a reservoir for states C and O so that after binding of the channels to picrotoxin the two latter states become partly refilled by channels from state R. Obviously the higher the concentration of picrotoxin, the larger the population of the state (O.PTX), and as a consequence the amplitude of the rebound current augments with increasing picrotoxin concentration. The true $k_2$ that describes the transition rate from O.PTX to O is obscured by the rate of solution change (see “Experimental Procedures”). The model is constructed in such a way that the decay of the inward current after removal of picrotoxin is double exponential with time constants $\tau_1 = 8$ s and $\tau_2 = 12$ s. Both, the picrotoxin concentration dependence (Fig. 4B) and the time dependence (Fig. 5B) of the responses of the mutated channel to picrotoxin exposure and removal predicted by the model should be compared with the experimentally observed behavior (Figs. 4A and 5A). Except for the initial peak of the apparent outward current predicted from the model and almost absent in the experimental traces, the agreement between model and experiment is remarkable. The difference is probably mostly due to the limited rate of solution change (see “Experimental Procedures”), which has not been taken into account in the simulations.

**DISCUSSION**

We studied alterations in the channel lining part of the recombinant $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptor. A mutant ion channel was found that displayed a transient chloride current upon removal of the channel blocker picrotoxin. A single point mutation is able to confer this unusual property to the receptor, namely, of leucine 253 in the M2 region of the $\beta_3$ subunit to a phenylalanine, which is present in the homologous position of all $\alpha$ subunits. The point mutated $\beta_3$ was coexpressed together with the $\alpha_1$ subunit. These receptors can open spontaneously in the absence of GABA and are blocked by picrotoxin in a dose-dependent fashion. Washout of picrotoxin results in a strong transient inward current. The amplitude of this current is dependent on the concentration of picrotoxin and the time of its application. The point mutated receptors could be activated by very low concentrations of GABA. Obviously, this mutation...
strongly affects gating of the channel and its interaction with picrotoxin.

It is interesting to compare the newly identified position with other positions within M2 that are important for receptor function and modulation. Based on radioligand binding experiments to chimeric receptors, it has been suggested that $\beta_3L253$ is important for the binding of another channel blocker tert-butylbicyclophosphorothionate (22). This position is 12 amino acid residues N-terminal to the one on the $\beta_3$ subunit implicated in the action of loreclezole (27). It is located 6 residues C-terminal to the predicted cytoplasmic entry point into the membrane and is 6 residues N-terminal to the conserved leucine in the center of the M2 region. Point mutations of this leucine also resulted in spontaneous currents (17–20). The spontaneous currents were in some cases antagonized by GABA (18, 19) and were sensitive to picrotoxin (18–21). Spontaneous channel activity has also been reported with expression of the rat or mouse $\beta_3$ subunit alone (28, 29), the mouse $\beta_3$ subunit alone (30), and a combination of rat $\alpha$ and $\beta$ subunits (31). Using the mentioned amounts of cRNA, no spontaneous currents were observed for homomeric wild type $\beta_3$ and mutant $\beta_3L253F$ receptors. In all the mentioned cases an off-current upon picrotoxin washout was never reported. Our model presented below will predict why this is not the case.

The structure of the GABA_A receptor in M2 is presumably close to that of other members of the ligand-gated ion channel family. The above cited studies and studies of the homologous nicotinic acetylcholine receptor suggested that the centrally conserved leucine is structurally critical for gating and might even be a part of the channel gate (32). Other studies place the gate more N-terminal to the cytoplasmic end of the channel pore (13). Because $\alpha_1\beta_3L253F$ receptors display an altered channel gating our results support this proposal.

Valine 256 on the $\alpha_1$ subunit has been proposed to be exposed to the channel lumen (13) and to be in direct contact with picrotoxin (13, 14). A homologous residue has also been implicated in the action of the cyclodiene insecticide resistance in invertebrate GABA receptor subunit $Rdl$ (15). Because of the 5-fold symmetry of the channel pore, it is likely that the homologous residue on the $\beta_3$ subunit, alanine 252, is also facing the channel pore and might be close to the bound picrotoxin entity. Our results indeed show that replacement of alanine by valine in this position ($\beta_3A252V$) results in an about 10-fold reduced affinity for picrotoxin. Mutation of the adjacent leucine 253 to phenylalanine even more drastically reduces the picrotoxin affinity. It cannot completely be ruled out that the substitution might have some indirect effects on the picrotoxin binding pocket. However, it is tempting to speculate that picrotoxin is in direct contact with $\beta_3L253$. In apparent contrast to this speculation, Xu and Akabas (12) found that the homologous phenylalanine 257 on the $\alpha_1$ subunit after cysteine substitution is not accessible to sulfhydryl reagents. However, this residue is on the same side of the $\alpha$-helix as reactive positions and is also adjacent to them. Side chains other than cysteine might be at least partially accessible to the channel lumen and could be in contact with the picrotoxin molecule.

We propose a kinetic model for $\alpha_1\beta_3L253F$ describing its interaction with picrotoxin. It makes a number of predictions that are discussed in the following. Mutant $\alpha_1\beta_3L253F$ receptors assume an open and two closed states in the absence of GABA. The open state is inferred from the presence of spontaneously open channels, and the closed states are required because of the double exponential decay of the transient inward current upon picrotoxin removal. Picrotoxin is mostly bound to open channels that become nonconducting because of the bound channel blocker resulting in an apparent outward current. Reopening of channels in the presence of picrotoxin finds a simple explanation with the help of our model. The binding of picrotoxin to the open (O) and closed channel (C) is faster than the transition between the two closed states (R and C). After emptying states O and C by adding picrotoxin, the channels in the desensitized state R serve as a reservoir that slowly releases channels to the open state via state C. The equilibrium of this process is reached in about 1 min (Figs. 4 and 5). The regain of open channels is responsible for the increase of the inward current in the presence of picrotoxin.

The huge transient inward current after picrotoxin washout is the result of the decreased affinity (fast transition of O.PTX to O), and it depends on both the duration of picrotoxin exposure and on its concentration (Figs. 4 and 5). Because of the almost instantaneous transition of O.PTX to O upon picrotoxin removal, the amplitude of the initial washout current is approximately proportional to the population of state O.PTX. Therefore, we can use this amplitude to estimate the overall equilibrium affinity constant to picrotoxin. It is higher than 300 $\mu M$ in the present case for $\alpha_1\beta_3L253F$ receptors, although the local affinity constant of the reaction between O and O.PTX is 20 $\mu M$ in our model. The former value should be compared with 3 $\mu M$ picrotoxin that reduces GABA induced currents in $\alpha_1\beta_3$ to about 50% (Fig. 4C).

As mentioned above, substitution of the centrally conserved leucine in GABA receptors in some cases also resulted in the formation of spontaneously open channels. Our model shows that the large amplitude of the fast transient inward current requires a faster transition from O.PTX to O as compared with all other transition rates of the model. Increasing the time constant for this process reduces the amplitude and slows down the time course for the transient population of the open state. Both effects are observed for other GABA_A receptors with substitution of the centrally conserved leucine (18–21). Therefore, the model suggests that for these mutated GABA_A receptors the dissociation of picrotoxin from the blocked channel ($k_9$) is slowed down. The relative amplitude of this fast transient inward current is also limited by the fraction of channels that are in the open state in the absence of picrotoxin. The larger this fraction of spontaneously open channels is, the smaller the predicted inward current. Both factors may contribute to a different degree to the lack of an transient inward current in the initially cited cases of spontaneous currents.

In summary, we report two important observations. The first is that we describe two point mutations in the M2 region of the $\beta_3$ subunit close to the putative cytoplasmic end that both result in an increase of apparent GABA affinity. One of these, $\beta_3L253F$ results additionally in spontaneous channel opening and is thus structurally critical for channel gating. Our results therefore strongly suggest that the region important for channel gating has to be extended at least four amino acid positions.
toward the N terminus as compared with previous conclusions (18). The second observation is that these point mutations result in a reduced affinity for picrotoxin. The residue $\alpha_1V256$ has been shown to covalently interact with other noncompetitive blockers acting at the picrotoxin binding site (14). Mutation of the homologous residue $\beta_2A252$ shows a 10-fold effect. However, mutation of the neighboring residue $\beta_3L253$ has drastic effects on picrotoxin affinity and may therefore together with $\alpha_1V256$ form part of the contact site for picrotoxin.

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