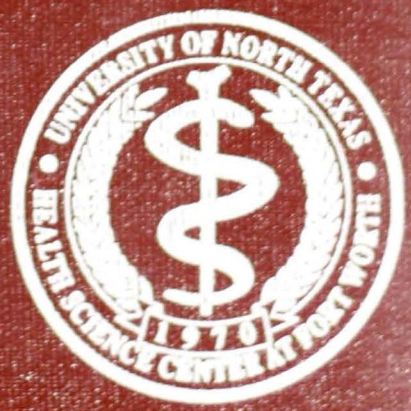
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Picrotoxin, a CNS convulsant inhibits all anionic ligand gated ion channels. The mechanism and the binding site for picrotoxin and its related ligands are still undefined. The second transmembrane (TMII) domain of these ligand gated ion channels is found to play a key role in the mechanism of block by picrotoxin. It has been shown that the incorporation of a phenylalanine residue in place of threonine at position 6' within the TMII domain of β2 subunit conferred high resistance toward picrotoxin in $GABA_A$ α3β2(T6'F)γ2 receptors. Mediating their blocking effect through the PTX-site, PTZ, TBPS, and U-93631 lost their inhibitory effects due to the same mutation β2(T6'F). Interestingly, this mutation uncovered a low affinity, highly efficacious stimulatory site for PTZ. PTZ seems to mediate its stimulatory effect through a novel distinct site different from that for benzodiazepine.

The effect of varying subunit configuration of $GABA_A$ receptors dramatically affected the ability of the mutation β2(T6'F) to abolish the inhibitory effect of picrotoxin. While picrotoxin failed to block the current induced by GABA in α3β2(T6'F)γ2 receptors, picrotoxin partially blocked the current in α3β2(T6'F) receptors. In β2(T6'F)γ2 receptors, picrotoxin restores its full efficacy. When phenylalanine was incorporated at position 6' in the α1 subunit, picrotoxin completely blocked the current induced by GABA in α1(T6'F)β2γ2 receptors. The combined results showed that the

ability of (T6'F) mutation to regulate the inhibitory mechanism of picrotoxin was dependent on the subunit configurations and at which subunit is mutated.

In addition, picrotoxin is known to inhibit $GABA_A$ receptors in use-facilitated mechanism, while it inhibits the glycine receptor in a non-use facilitated fashion. The molecular determinant behind the use-facilitated mechanism was modulated by the nature of the amino acid at position 15' within the second transmembrane domain. The mutation of serine 15' to either glutamine or asparagine in the glycine $\alpha 1$ receptors converted picrotoxin from a non-use facilitated blocker to a use-facilitated one. The latter finding suggested that this residue might reside within the PTX binding site or play a key role in the transduction pathway for picrotoxin mechanism. The overall results further support the fact that TMII domain plays a key role in the picrotoxin mechanism.

ANIONIC LIGAND-GATED ION CHANNELS:

THE CONVULSIVE SITE AND MECHANISM OF ACTION

MOHAMMED I. DIBAS, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

Committee Member

Chair, Department of Pharmacology and Neuroscience

Dean, Graduate School of Biomedical Sciences

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Finally, I really dedicate this thesis to my parents and my wife. Both are my life and hope.

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LIST OF ABBREVIATIONS

GABA	γ-aminobutyric acid
GABAA	Type A GABA receptor
DMSO	Dimethylsulfoxide
HEK	Human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
PTX	Picrotoxin
PTZ	Pentylenetetrazole
TBPS	t-butylbicyclophosporothionate
U-93631	[4-Dimethyl-3-t-butylcarboxy-4,5-dihydro(1,5-a) quinoxaline]
Gly	Glycine

CHAPTER I. BACKGROUND AND SIGNIFICANCE

THE STRUCTURE OF LIGAND-GATED ION CHANNEL

Ligand-gated ion channels (LGICs) belong to a superfamily that includes excitatory nicotinic acetylcholine and serotonin (5HT3) receptors, which are cation-selective, and the inhibitory γ- aminobutyric acid ($GABA_AR$ and $GABA_CR$) and glycine (GlyR) receptors, which are anion-selective [8, 13, 24]. There is a wide range of variability in the subunit subtypes comprising these receptors, which confers extreme diversity in the pharmacological behavior of LGICS. However, they are all transmembrane proteins that are made up of five subunits; each subunit, regardless of which receptor it belongs, contains the same structural domains (as shown in Fig 1) [13]:

1) Putative signal peptide.
2) N-terminal that contains the agonist binding site as well consensus glycosylation sites.
3) Four hydrophobic transmembrane domains (TM1-TM4).
4) Two-conserved cysteine residues that are possibly linked by a disulfide bond.
5) A large cytoplasmic domain that contains multiple consensus phosphorylation sites.

All LGICs adopt a pentameric structure that is conferred by a pseudosymmetric arrangement of five subunits around a central pore that is presumably lined by the second transmembrane domain [96]. The second transmembrane domain (TMII) has been

implicated as the major determinant for ion conduction for all LGICs (Fig2) [49, 62]. Noticeably, the TMII domain shows the most amino acid sequence homology among all LGICS. It has been proposed that the agonist binding to its recognition site, which is located in the N- terminal domain, induces a conformational change that leads to opening of the ion channel [8]. These channels usually exist in three main states, open state (when the agonist binds), closed state (when the agonist is not bound), and desensitized state (when the agonist is still bound, but the channel is nonconducting). A number of studies have demonstrated that these channels exist in equilibrium of all three states, such that the agonist binding favors one state over the other [8, 26, 27, 101]. Although there are lots of biochemical, electrophyiological, and biophysical studies performed on LGICS, there is no clear idea about how the gating and opening of these ion channels are being processed.

The anionic ligand gated ion channels

The anionic ligand gated ion channels play crucial roles in the central nervous system; for example, GABA receptors form the major inhibitory circuit in the CNS while the glycine receptors form the major inhibitory circuit in the spinal cord [13, 17, 84]. As mentioned above, when the agonists GABA or glycine bind to their respective ion channels, a conformational change is induced that leads to opening of the channel gate; thus the channel opening allows chloride ions to pass through the pore toward the inside of the neurons. Accumulation of chloride ions on the intracellular side of the neurons makes the inside more negative and thus more hyperpolarizing (i.e., depressing the neuron activity) [13]. Consequently, any drug (anti-convulsant) that stimulates these ion channels

will depress neuronal activity; conversely, any drug (convulsant) that inhibits these channels will lead to excitability of the neurons.

ANIONIC CHANNELS DIVERSITY

Anionic LGICs are made of many diverse subunits [39, 40, 84]. For example, there are seven major subunits (α, β, γ, π, ε, δ, and θ) in the $GABA_A$ family; each subunit has many isoforms. The alpha subunit has six isoforms; α1 form is the predominant subunit in the CNS. The β subunit has three isoforms; β2 is the predominant form. The γ subunit contains three isoforms; γ2 has two-splice variants (γ2-S, L; S denotes Short form while L denotes for Long form); γ2 is the predominant form [84]. The ability of these different subunits to co-assemble demonstrates not only the great complexity of these ion channels receptors, but also the crucial role these channels play in the fine modulation of the CNS. Through immunohistochemistry and pharmacological studies, it has been shown that the αβγ combination is the predominant form of $GABA_A$ receptors in the CNS. Nevertheless, other combinations might play some crucial roles in CNS modulation **(reviewed in 84)**.

ANIONIC LIGAND GATED ION CHANNELS ARE TARGETS OF THERPEUTICS AND ENVIRONMENTAL TOXINS

For many years these ion channels have been the subjects of extensive research due to the crucial roles they play in the CNS. These channels have been linked directly or indirectly to many neurological diseases, such as hyperplexia and epilepsy, leading

many researchers to explore these channels at the molecular, neurological, physiological, and pharmacological levels [13, 84]. In fact, these channels possess many important allosteric sites, besides the agonist site, where many therapeutics as well as toxins exert their effects.

Drugs that stimulate the function of $GABA_A$ receptors

A) Benzodiazepines (BZ)

BZ are very widely used drugs because of their multi-therapeutic uses such as sedative-hypnotic, anxiolytic, anti spastic and anti-epileptic [13, 84]. Their main therapeutic actions are mediated through $GABA_A$ receptors. Binding studies have shown that BZ do not compete with the GABA recognition site, but rather interact with a distinct allosteric site that is presumably located at the interface between the α and γ subunits. Previous electrophysiological studies have demonstrated that BZ need both α and γ subunits to have their full efficacy [77, 78, 100]. The mechanism by which BZ stimulate GABA induced current is still unknown; however, single channel studies have indicated that BZ increase the frequency of channel opening [82]. BZ do not open the channel in the absence of GABA, unlike barbiturate, which has a direct action. Depending on their effect, there are three main classes of BZ drugs: 1) BZ agonists like diazepam (Valium), 2) BZ antagonists (flumazenil) and 3) BZ inverse agonists (β-carboline) (reviewed in (104)). Although these BZ are widely used in the market, they have multiple unwanted side effects like sedation, dependence and withdrawal [58, 64]. Thus, there is ongoing research directed toward developing better BZs or other better alternatives.

B) BARBITURATE

Barbiturates are strong depressants that have dual action on $GABA_A$ receptors. Whereas at low concentration, barbiturates facilitate the GABA induced current, barbiturates directly gate the $GABA_A$ coupled chloride channels at high concentration [60, 90]. The latter feature highlights the dangerous side effect of barbiturate overdose. Based on electrophysiological and binding studies, the barbiturate site is distinct from the GABA and BZ binding sites [16, 19, 20, 72, 87, 89].

C) α-ALKYLATED γ-BUTYROLACTONE

Alkylated γ-butyrolactone (GBL) and alkylated γ-thiobutyrolactone (TBL) have both convulsant and anti-convulsant effects at the $GABA_A$ receptors depending on the location of the alkyl group [55, 56]. The existence of an alkyl group at the β position (β-alkylated GBL or β-alkylated TBL) leads to inhibition of $GABA_A$ receptors. By contrast, α-alkylated GBLs or TBLs are anticonvulsant drugs that stimulate the current induced by GABA. It has been postulated that all these lactone agents work through the convulsive picrotoxin-site where picrotoxin and β-alkylated GBL/TBL are the agonist and the α-alkylated GBL and α-alkylated TBL are the antagonists [55, 56]. However, binding studies have demonstrated that α-GBL and α-TBL accelerate the dissociation of [S^{35}] *t*-butylbicyclophosphorothionate (TBPS), a radioligand for the PTX binding site. Furthermore, a mutation, which knocked out the inhibitory effect of β-GBL and PTX, had no effect on the α-GBL or α-TBL [102]. Further studies also have suggested that

these α-lactones do not interact with the GABA, BZ, or the barbiturate binding sites [56, 57]. Thus, it has been suggested that these lactones interact with a distinct site (the lactone site) that is yet to be defined. The α-lactones are the most promising alternatives for benzodiazepines.

DRUGS THAT INHIBITS ANIONIC LIGAND GATED ION CHANNELS

PICROTOXIN

General history

The history of picrotoxin began almost three centuries ago [74]. It was first isolated as a bitter substance from the poisonous plant from the family *Menispermaceae*, a plant that is found in India [reviewed in 74]. Practical uses of PTX included stunning fish and killing body lice. Picrotoxin did not receive the attention of researchers until late 1920s when picrotoxin was found to antagonize chloral hydrate poisoning. It was later found that picrotoxin can be used as an analeptic in barbiturate overdose poisoning [51, 74]. Other studies have also demonstrated that picrotoxin could produce convulsions, muscle excitation, respiratory center irritability, induction of vomiting center in cats, sensitizing pain centers, hyperglycemic induction, and has a temperature lowering effect [reviewed in 74]. However, due to the extreme convulsions induced by picrotoxin, therapeutic usage was only restricted to its use as an antidote in barbiturate overdose [74].

Many researchers then used picrotoxin as a research tool to study the function of the CNS. Even with the advent of molecular biology techniques including cloning and cDNA library screening, picrotoxin is still used extensively trying to elucidate the mechanism of GABAergic neurons.

In the late 1980s, the first three GABA clones were isolated from rat tissue; in the following years a large number of these ion channel subunits have been discovered and characterized [reviewed in 84]. Upon deciphering the molecular structure of these ion channels, picrotoxin mechanism and site action started to become clearer and more interesting. The TMII domain, which lines the conduction pathway has received enormous attention from research groups [48, 62] because TMII domain has been implicated in ion conduction, ion selectivity, desensitization, activation- deactivation, and many other aspects of channel functions [48, 59, 62]. TMII is also implicated as the target of the convulsive acting drug picrotoxin and insecticides [12, 25, 30, 31, 35- 37, 65, 67-71, 83, 88, 91, 95, 105]. Thus, defining the mechanism and site of action of picrotoxin help us design safer insecticides, which selectively target the insects system rather than the mammalian counterparts.

Picrotoxin (PTX) is a convulsant drug that acts non-selectively on all anionic LGICs including γ-amino butyric acid-gated (GABA) channels, glycine-gated channels, *drosophila* GABA receptors and invertebrate glutamate-gated Cl^- channels [5, 7, 11, 14, 33-38,, 43, 45, 47, 50]. The location of the inhibitory site of PTX and its related compounds like pentylenetetrazole (PTZ), U93631, *t*-butylbicyclophosphorothionate (TBPS), and insecticides is still controversial [34, 50, 71]. The fact that PTX acts nonselectively on all anionic ligand gated ion channels supports the contention that PTX interacts with a conserved site among all targeted anionic LGICs. The second transmembrane (TMII) region that lines the channel pore is the most conserved region among all ligand gated ion

channels. Thus, most researchers have focused on the TMII domain to define the PTX action domain.

TMII is the probable domain where picrotoxin binds

Since a fine structural resolution of these ligand gated ion channels has not yet been defined, the exact site of picrotoxin action is still unknown. However, binding studies have shown that picrotoxin does not compete for GABA and glycine binding sites in their respective receptors [63, 75]. In addition, PTX and its related compounds do not compete for BZ or barbiturate binding sites [29, 46, 53, 88]; thus these studies have suggested that PTX interacts with a unique recognition site that is distinct but allosterically coupled to the agonist, BZ or barbiturate recognition sites.

Several lines of evidence indicate that the TMII domain is an essential region for picrotoxin inhibitory action. First, glycine receptors composed of only α subunits were found sensitive to picrotoxin; the co-expression of glycine β subunit along with any glycine α subunit produces picrotoxin-insensitive αβ glycine receptors [76]. Using site-directed mutagenesis, the TMII domain of the β subunit was identified as a major determinant of picrotoxin resistance [76]. Based on these results, authors have suggested that picrotoxin acts as a channel blocker of the glycine receptor [76]. The strongest evidence supporting the importance of TMII for action of picrotoxin came from Reddy et al. [81]. A synthetic peptide corresponding only to the TM2 of the glycine receptor formed anion-selective channels in artificial lipid membrane that were blocked by PTX [81]. Nevertheless, the exact location of picrotoxin within the TMII is still unknown.

There is a need to explore the structure –activity relationship of picrotoxin and its related compounds, which helps us identify potential amino acid residues that might play a role in the mechanism of interaction of picrotoxin and its related compounds.

Structure-activity relationship

Many structure-activity studies have suggested that the picrotoxin and its related compounds' convulsive activity is dependent on the presence of the following domains: a hydrophobic domain and the two electronegative domains. Relating to the picrotoxin structure (Fig. 3), the lipophilic group is provided by the isopropenyl moiety, while the lactone and epoxy groups provide the electronegative domain [51]. The convulsive activity of picrotoxin will be abolished if the isopropenyl moiety is hydrated like in picrotin (the inactive component of picrotoxin) or the lactone group is cleaved [51]. Klunk and Covey [55, 56] have demonstrated that β-alkylated γ butyrolactone compounds act through the same picrotoxin site. Their structure-activity studies have indicated that the convulsive activity of the β-alkylated γ-butyrolactone compounds is dependent on the presence of a hydrophobic group at the β site along with an electronegative group that is provided by the butyrolactone ring [55, 56]. Other studies of the other convulsive agents like TBPS, PTZ, TBOB, EBOB and the insecticides have supported the previous findings [21-23]. A recent study [86] has indicated that the common convulsive site could consist of a flat hydrophobic structure element, interacting with the hydrophobic region of the picrotoxin, and the hydrogen bond donors interacting with the lactone ring.

Such structural studies give us an insight about which amino acids located within the TMII that might be involved in the interaction with the two essential domains of picrotoxin.

MUTATIONAL STUDIES OF PICROTOXIN SITE

Site-directed mutagenesis has been a valuable tool to explore the convulsive site of picrotoxin. Many site-directed mutagenesis studies were aimed to find where PTX binds. All studies so far have targeted the TMII. Referring to Fig. 4, the residues in TMII are numbered by Miller numbering method to avoid any confusion with the different numbering of the different subunit [66].

Residue 2'

Numerous studies have shown that changing the amino acid residue at position 2' has a dramatic effect on the sensitivity of PTX. LGICs show a wide variability at position 2'. The most striking evidence supporting the importance of the residue at position 2' for picrotoxin convulsive action stemmed from a field-isolated *Drosophila* mutant Rdl (resistant to dieldren) [37]. These Rdl mutant insects show resistance toward dieldren and picrotoxin. The fact that the *Drosophila* insect has acquired a natural mutation at position 2' in drosophila GABA receptor (Alanine → Serine, Alanine →glycine) to resist the insecticides, supports that position 2' residue in TMII is an important residue for picrotoxin as well as insecticides. The author pointed out that A2'S mutation affected PTX action in two ways: it first disrupted the binding site of PTX and second, it affected the mechanism by which PTX stabilizes the closed state conformation

of the receptor [109]. The author also suggested that residue at position 2' might be part of picrotoxin binding site [109].

Wang et al. [99] studied the effect of PTX on GABAc receptors, which are predominant in the retina. In their studies, they also identified position 2' in TMII to be an important position determining picrotoxin sensitivity. GABAc ρl subunit, which can form homomeric receptors, has a proline at position 2'. When proline is mutated to the corresponding amino acids that exist in other family subunits in the $GABA_AR$ and glycine receptors, the biggest increase in sensitivity toward PTX was observed when alanine was present. Interestingly, mutation (Proline2 ' → Serine mutation in $GABA_C$) showed conflicting results, with PTX sensitivity being either increased or reduced [33, 94]. In addition, mutations at position 2' also affected the inhibitory behavior of picrotoxin in $GABA_C$ receptors [73]. Picrotoxin acted as a competitive inhibitor in recombinant rat GABAc ρ1. Cutting's group also found that altering the proline residue at position 2' to other amino acids, which exist in $GABA_A$ and glycine receptors, would change the picrotoxin from being competitive to noncompetitive antagonist [99].

Furthermore, glutamate-gated chloride channel cloned from insects, nematodes and crustacean are blocked with variable potency by picrotoxin [34]. The first two cloned subunits (α and β) of glutamate-gated chloride channels in nematodes can form homomeric channels that are blocked by picrotoxin. The sensitivity to picrotoxin block of homomeric GluClβ was much lower than that in GluClα. The large difference in sensitivity toward picrotoxin was attributed to the nature of amino acid at position 2' in TMII. A point mutation in GluClβ (Ala2'→threonine, threonine exists in the (α subunit)

dramatically reduced the picrotoxin sensitivity. Thus, 2' position in TMII has an essential role in the mechanism by which picrotoxin inhibits the ligand gated anionic channel ($GABA_AR$, $GABA_CR$, Glu gated channel, and glycine receptor).

In 1992, Akabas and Karlin [4] introduced a new mutational technique called substituted-cysteine accessibility method (SCAM). SCAM has been extensively utilized to identify water accessible residues in nicotinic receptors, $GABA_A$ receptors, dopamine receptors, NMDA receptors, ATP receptors, bradykinin receptors, and the dopmaine transporter [1-3, 9, 10, 15, 32, 52, 54, 79, 85, 103, 104, 107]. This method basically utilizes the ability of a sulfhydryl regent to react irreversibly with an engineered cysteine in place of the residue in study to explore the accessibility of the studied residue.

Using SCAM, Xu and Akabas have identified nine (2', 6', 7', 9', 10', 13', 16', 17', and 20') residues within the TMII of the GABAA α1 subunit that are water accessible to the lumen of the channel [103]. Interestingly, based on the fact that picrotoxin might bind within the channel lumen, Xu and Akabas used SCAM to screen which mutated exposed residues in TMII of rat $GABA_A$ α1 subunit would be protected by picrotoxin from sulfhydryl reagent modification [104]. They found that picrotoxin protected an engineered cyteine at position 2' from irreversible modification by the reagent [104]. Thus they speculated that picrotoxin might bind within the TMII as far as the level of valine 2' to either block the channel pore or stabilize the desensitized state of ion channel [104].

Residue 6'

The expression of rat glycine β along with glycine α subunit produces picrotoxin insensitive αβ glycine receptors [76]. Pribilla et al. [76] found that TMII region of glycine β subunit conferred picrotoxin insensitivity. Based on sequence homology between $GABA_A$Rs and glycine receptors, Gurley et al., [42] has shown that the mutation of the conserved threonine 6' in rat $GABA_A$ β2 to phenylalanine residue that exists in glycine β subunit, abolishes PTX sensitivity in rat αlβ2(T6'F)γ2 receptor. They inferred that mutating this conserved threonine in other $GABA_A$ subunit alpha or gamma would also abolish the picrotoxin activity. A possible mechanism of resistance is controversial. But, phenylalanine could block the access of the PTX toward its binding site within the channel lumen [42].

Zhang et al. [99] have shown that rat $GABA_C$ ρ1 alone could form an ion channel that was sensitive to picrotoxin. But, co-expression of rat ρ2 along ρ1 would form channels that were insensitive to picrotoxin. Using site directed mutagenesis, they found that 6' residue in TMII (a methonine in ρ2 in place of threonine in rat ρl) is the determinant of picrotoxin resistance in rat ρ1ρ2 receptor. So it is temping to infer from the previous two reports that methionine or phenylalanine presence at position 6' could block the access of picrotoxin to its site probably at position 2'. But, Xu and Akabas found that the irreversible reaction of the sulfhydryl reagent ($MTSEA^+$) with an engineered cysteine at position 6' in GABA α1 subunit generated a moiety (Cys-MTSEA) that is larger than the methionine and phenylalanine, however, rat GABA

α1(C-MTSEA)β2γ2 was still sensitive to picrotoxin [104]. In addition, PTX could not protect an engineered cysteine at position 6' from irreversible modification by the sulfhydryl reagent [104]. The residue at position 6' plays a crucial role in modulating either the PTX-site or the PTX-mechanism.

Zhorov et al., based on molecular modeling of the convulsive site, have proposed that threonine at position 6' is important for hydrogen bonding with the hydrophilic domain of picrotoxin; the picrotoxin hydrophilic domain is provided by the lactone ring. In addition, the same studies have proposed that the hydrophobic nature of position 2' is important for interaction with picrotoxin hydrophobic domain [110]. The same studies have suggested that the incorporation of phenylalanine at position 6' abolishes the hydrogen bonding interaction for PTX molecule to dock. If their proposal is true, the incorporation of alanine at position 6', which abolishes the hydrogen bonding interaction , is needed for picrotoxin docking, should abolish the inhibitory activity of picrotoxin. This is not the case in $GABA_C$ study, where Pan et al. [73] substituted the threonine at position 6' for alanine. The mutant ρ1(T6'A) receptors formed spontaneous open channels that were blocked by picrotoxin with slight effect on picrotoxin sensitivity [73]. However, mutational studies at position 6' were done in different receptor systems; thus, it is better to do this analysis in each system and then compare the results.

In summary, the mechanism by which the incorporation of phenylalanine at position 6' instead of the conserved threonine abolishes the picrotoxin activity is still yet to be defined.

Other TMII residues affecting the PTX activity

Residues 3', and 19'

Recent studies have shown the mutation of leucine at position 3' in rat GABA β2 to the corresponding F in α1 subunit produced spontaneously open mutant α1β2(L3'F) channels that are sensitive to picrotoxin but with a dramatic reduction in affinity. They inferred that the residue at 3' might be located near PTX binding site [18].

R19'L and R19'Q uncovers a stimulatory site for picrotoxin

Most studies mentioned above were focused at defining the picrotoxin site at the intracellular side of TMII (2'-6'). However, Lynch et al. found that mutation at 19' in human glycine α1 receptors, which exist at the extracellular domain of TMII, uncovered a stimulatory site for picrotoxin at low concentration; this stimulation is masked by the inhibitory effect at high concentration [63]. They concluded that picrotoxin might have two binding sites 1) stimulatory site which exists in the mutant receptors and 2) inhibitory site. Indeed, other studies have suggested the existence of two binding sites for picrotoxin in ligand gated anion channels [105]. However, these mutational studies underscore the complexity of picrotoxin interaction with these ligand gated ion channels.

Notably, TMII domain plays a key role in the mechanism of picrotoxin. However, our knowledge of where and how picrotoxin interacts with the TMII domain of the ligand gated anion channels remains limited.

The mechanism of Block:

Based on $GABA_AR$ studies, Inoue and Akaike [50] proposed that picrotoxin might act within the channel pore to directly block the current flow. This proposal stemmed from the use-dependent feature of picrotoxin action at the $GABA_A$ receptors [5, 6, 50]. This phenomenon suggests that PTX binding is dependent on the existence of an agonist, which opens the channel. Thus, PTX needs an open channel to have better access to its binding site. This suggests that PTX might bind within the channel pore and block the ion conduction pathway. It has been shown that picrotoxin inhibits $GABA_A$ and glutamate gated Cl- channels in a use-dependent fashion [5, 34, 50]. However, Lynch et al., found that PTX lacked the use dependent feature in the recombinant human α1glycine receptor [63]. Using a PTX analogue, Yoon et al., have suggested the existence of two sites, 1) a non-use dependent site and 2) a use dependent site [105].

Based on single channel studies involving $GABA_A$ receptors, it has been shown that picrotoxin lacks the flickery effect [71]; the flickery effect is a characteristic of a classical ion channel blocker. Thus, they proposed that PTX inhibited GABA-mediated current allosterically through a site within the channel pore rather than by blocking the pore of the channel. These results [71] also have lead the authors to propose that PTX might induce a conformational change that would stabilize a closed state or a desensitized state through a site that might exist in the TMII domain.

Competitive Vs noncompetitive:

Since picrotoxin acts on a wide spectrum of ligand gated chloride channels such as $GABA_{A/C}R$, GlyR, and glutamate-gated Cl- receptor, the binding site of picrotoxin seems to be almost conserved among all these receptors. Consequently, we might predict that the mechanism of inhibition by picrotoxin is similar among all these receptors. The pharmacology of the antagonistic action of picrotoxin is rather confusing. Picrotoxin antagonized $GABA_AR$s competitively [41, 93] or by a mixed competitive plus non-competitive action [28]. In rat hippocampal glycinergic neurons, picrotoxin acted as competitive inhibitor on glycine receptors at low concentration, and noncompetitive at high concentration [61, 106]. In recombinant $GABA_A$ receptors, Krishek et al., have shown that picrotoxin inhibits GABA-mediated current with a mixed/non-competitive inhibitory mechanism [57]. However, picrotoxin inhibited glycine-induced current in a pure competitive fashion in recombinant human glycine receptor $\alpha 1$ [63]. The basis for the difference in the pharmacological behavior of picrotoxin between native and recombinant studies is not known since the exact subunit composition is not defined in the native tissue. However, some recent mutational studies [99] suggested that there is a molecular basis within TMII behind the conflicting behavior of picrotoxin (see mutational studies).

General discussion of other PTX related compounds used in the present study

1) t-butylbicyclophosphoro-thionate (TBPS)

TBPS was first introduced in the 1970s as a potent convulsant that inhibits GABA receptors [21, 22, 45, 80]. Many binding studies have used [S^{35}] TBPS to characterize the PTX-binding site; it has been found that PTX and its related compound inhibit TBPS binding competitively suggesting that all these agents interact with a common or overlapping sites [80]. Strengthening the contention that TBPS interacts with PTX-binding site, TBPS inhibits GABA mediated current in noncompetitive and use-dependent fashion similar to PTX [98].

2) *Pentylenetetrazole (PTZ)*

Pentylenetetrazole (PTZ) is a central nervous system convulsant that inhibits GABA-activated Cl^- channels [47]. Although initial radioligand binding studies have suggested that the site of action of PTZ is the benzodiazepine site of the $GABA_A$ receptor, subsequent work from the same group has indicated the action of PTZ is mediated at the picrotoxin site [89]. Consequently, PTZ likely mediates its inhibitory effect through interaction at the picrotoxin site within TM2 domain. Haung et al., have shown that that PTZ inhibits the current induced by GABA in a competitive and dose-dependent manner [47]. In addition, the same study has shown that PTZ inhibitory effect is independent of the GABA configurations and it lacks the use dependent feature [47].

3) *[4-Dimethyl-3-t-butylcarboxyl-4,5-dihydro (1,5-a) quinoxaline] (U93631)*

U-93631 is a novel convulsant compound that is found to interact with PTX binding site [31]. Dillon et al., have demonstrated that U-93631 competitively inhibits [35S] TBPS, a radio-ligand for PTX-binding site; U93631 inhibits the current induced by GABA in a non-competitive fashion [31]. In addition, U93631 sensitivity shows some subunit dependence; GABA $\beta\gamma$ shows more sensitivity to U93631 than that to $\alpha\beta\gamma$ or $\alpha\beta$ [11].

Most of our knowledge about the action of these PTX-related compounds has come from electrophyiological and binding studies. No previous studies have attempted to study the effect of mutation (T6'F), which was shown to block the inhibitory effect of PTX, on the other convulsive agents that are presumably interacting with the same PTX-site. In chapter II, the effect of (T6'F) on the inhibitory effect of these PTX-related compounds will be evaluated.

OBJECTIVES:

It is clear that TMII domain plays a key role in mechanism of inhibition by PTX and its related ligands in the ligand-gated anion channels. However, many questions concerning the PTX mechanism of inhibition have been unanswered and still need further exploration. The PTX blockade of $GABA_A$ receptors is abolished by the mutation (T6'F). As mentioned above, many binding and electrophysiological studies have shown that PTZ, TBPS, and U93631 inhibit GABA-mediated current in a similar

fashion to PTX. However, no mutations studies have attempted to evaluate the possibility that mutations like (T6'F) affecting the PTX mechanism might also affect the inhibition by others that are proposed to act through the same site. In addition, subunit dependence of PTX blockade of $GABA_A$ receptors is still controversial. Whereas studies have demonstrated the lack of subunit dependence of PTX block, others have shown that PTX block is enhanced in $GABA_A$ receptors composed of $\beta\gamma$ in comparison to $\alpha\beta\gamma$ and $\alpha\beta$ configurations. No studies have attempted to elucidate the effect of varying $GABA_A$ subunit configurations on the PTX inhibitory abolishment due to mutation of (T6'F). In chapter III, the effect of varying the receptors configuration involving β2(T6'F) on PTX pharmacology is evaluated.

The mechanism by which PTX inhibit the anionic ligand gated ion channels is still equivocal. Whereas some studies have demonstrated the use dependence of PTX in $GABA_A$ receptors, others have shown that PTX lacks the use dependent block in the glycine channels. Many studies have attempted to elucidate the mechanism of block by PTX; no studies have been successful in doing so.

Thus, the overall of this study was to further explore the PTX binding site and its mechanism of inhibition. The first objective was to test the hypothesis that the mutation (T6'F) that previously abolished PTX inhibition blocked the inhibitory effect of PTZ, U93631, and TBPS (see chapter II). The second objective was to test the effect of varying the subunit configurations on PTX blockade (see chapter III). Finally, the third objective of this thesis was to test the molecular determinant of picrotoxin use dependent blockade (see chapter IV).

Fig. I-1 A description of the general topology of LGICs. Each subunit is a long polypeptide that presumably spans the membrane four times (TM1-TM4).

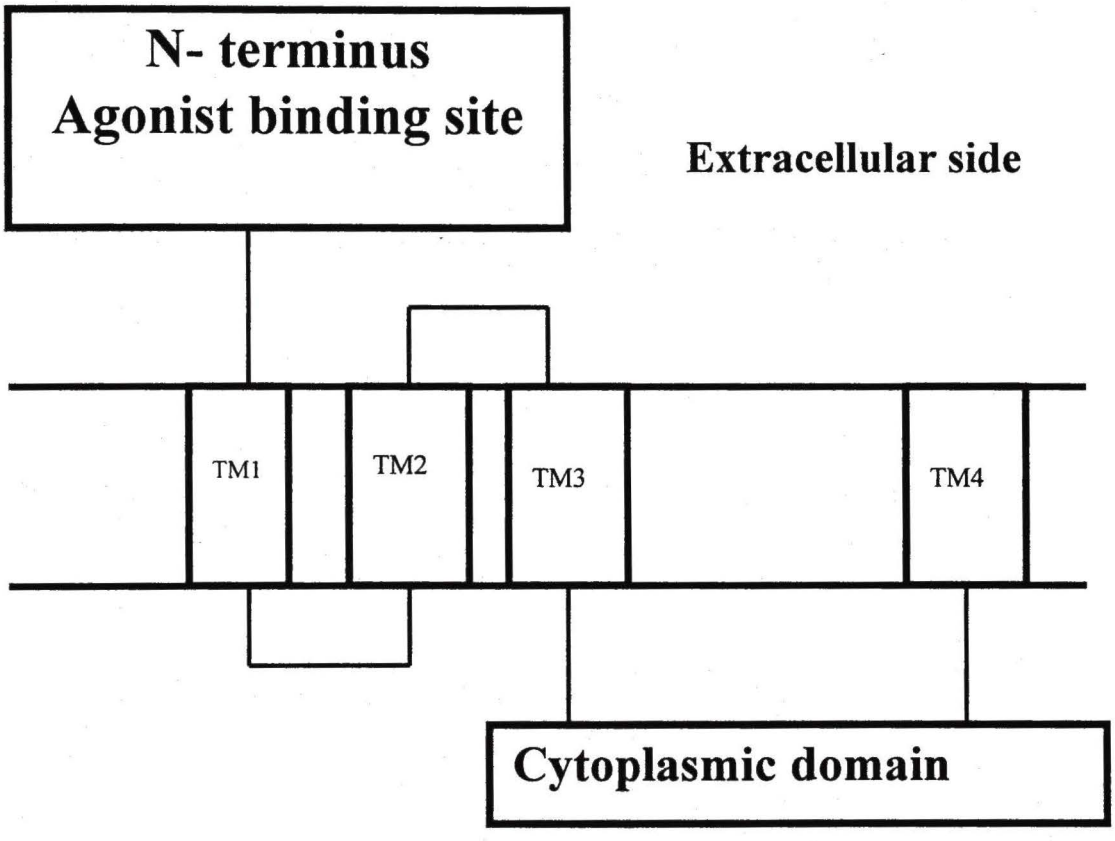

N- terminus
Agonist binding site
Extracellular side
TM1
TM2
TM3
TM4
Cytoplasmic domain
Intracellular side

Fig. I-2 Upper view of the ligand gated ion channels. LGIC adopt a pentameric structure where the second transmembrane region spans the lumen of the channel.

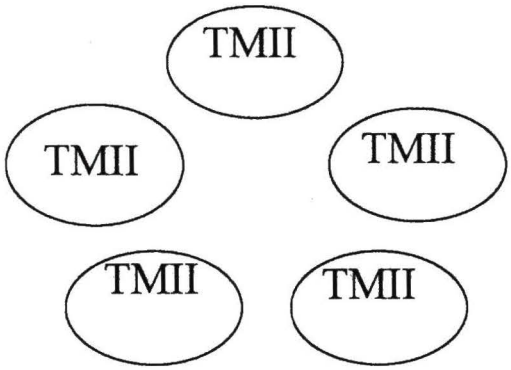
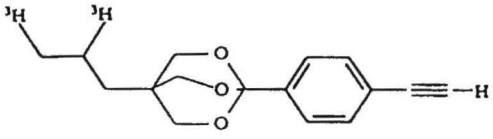
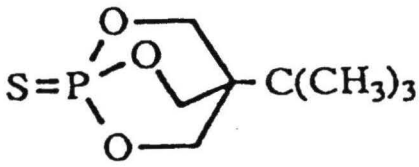

TMII
TMII
TMII
TMII
TMII

Fig. I-3 The structure of convulsant compounds acting through PTX-site. All convulsant compounds presumably acting through the picrotoxin site share two homologous domain, a hydrophilic domain and a hydrophobic domain. Picrotoxin is the prototype convulsant compound acting on ligand gated chloride channel.

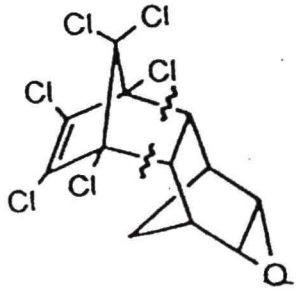
Structures of the Convulsant Compounds



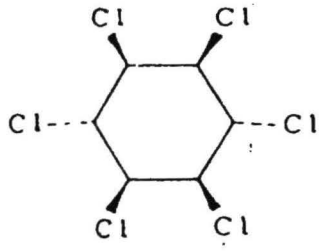
3H EBOB



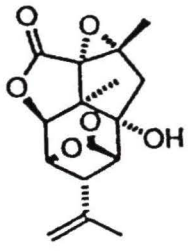
TBPS



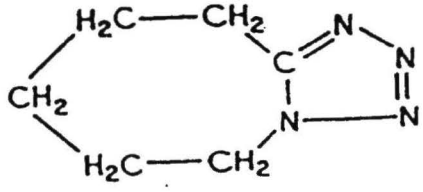
Dieldrin



Lindane



PTX



PTZ

Fig. I-4 A map of TMII which is mostly conserved among ligand gated ion channels. H denotes for human, M denotes for muscle, and R denotes rat. Glycine receptors have two subunits α and β. Glycine α subunit exists in multiple forms (α1-α4). $GABA_AR$ are heteromeric receptors. $GABA_AR$ has multiple subtypes α (1-6), β (1-4), γ (1-3), δ, θ, ε, π and finally $GABA_CR$ which consists of ρ subunit; ρ exists in multiple forms (1-3). Neuronal acetylcholine receptor α7 forms homomeric channels. **Miller (63) numbering system for TMII will be followed to avoid any confusion with the different numbering for amino acids among LGICs.**

Receptor	0'	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'
Hglyα2	R	**V**	**A**	**L**	**G**	**I**	**T**	**T**	**V**	**L**	**T**	**M**	**T**	**T**	**Q**	**S**
Hglyβ	**R**	**V**	**P**	**L**	**G**	**I**	**F**	**S**	**V**	***L***	**S**	**L**	**A**	**S**	**E**	**C**
RGABAα1	**R**	**T**	**V**	**F**	**G**	**V**	**T**	**T**	**V**	***L***	**T**	**M**	**T**	**T**	**L**	**S**
RGABAβ2	**R**	**V**	**A**	**L**	**G**	**I**	**T**	**T**	**V**	***L***	**T**	**M**	**T**	**T**	**I**	**N**
R5HT3	**R**	**V**	**S**	**F**	**K**	**I**	**T**	**L**	**L**	***L***	**G**	**Y**	**S**	**V**	**F**	**L**
RAchRα7	**K**	**I**	**S**	**L**	**G**	**I**	**T**	**V**	**L**	***L***	**S**	**L**	**T**	**V**	**F**	**L**
MachRα1	**K**	**M**	**T**	**L**	**S**	**I**	**S**	**V**	**L**	***L***	**S**	**L**	**T**	**V**	**F**	**M**

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THE CNS CONVULSANT PENTYLENETETRAZOLE STIMULATES GABA-ACTIVATED CURRENT IN PICROTOXIN-RESISTANT $GABA_A$ RECEPTORS

Mohammed I. Dibas and Glenn H. Dillon

Department of Pharmacology
University of North Texas Health Science Center at Fort Worth
3500 Camp Bowie Blvd., Fort Worth, TX 76107

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Corresponding author:
Glenn H. Dillon, Ph.D.
Dept. of Pharmacology
University of North Texas Health Science Center at Fort Worth
3500 Camp Bowie Blvd.
Fort Worth, TX 76107
Phone: (817)735-2055
Fax: (817)735-2091
Email: gdillon@hsc.unt.edu

Chapter II. THE CNS CONVULSANT PENTYLENETETRAZOLE STIMULATES GABA-ACTIVATED CURRENT IN PICROTOXIN-RESISTANT $GABA_A$ RECEPTORS

ABSTRACT

Picrotoxin (PTX) and related drugs like ^{t}-butylbicyclophosphoro-thionate (TBPS), pentylenetetrazole (PTZ) and U93631 are convulsant drugs that inhibit GABA-mediated Cl^- current. The binding site where these convulsive drugs interact is still unknown. As previously shown, the mutation of the conserved threonine (6') in β2 to phenylalanine abolished PTX sensitivity in rat α1β2γ2 receptors. This mutation within the second transmembrane domain (TMII) supported the contention that picrotoxin and its related compounds act deep within the channel pore that is lined by TM2. We have tested the effect of this mutation on the ability of other presumed PTX-site ligands to block $GABA_A$ receptors. The inhibitory effect of PTZ and TBPS on rat α3β2(T6'F)γ2 was abolished, as was observed for picrotoxin. In addition, U93631 inhibited GABA-mediated chloride current with decreased affinity in the mutant receptors. Sensitivity to these convulsant agents was drastically reduced or abolished in the mutant receptor, confirming the hypothesis that PTZ, TBPS, and U93631 act at the picrotoxin site. Quite unexpected, however, was our finding that PTZ elicited marked stimulation (up to 400% of control) in the mutated receptors. This stimulatory effect was not mediated via an interaction with the benzodiazepine site, as preincubation with the benzodiazepine antagonist flumazenil did not block the PTZ-induced stimulation. Our results reveal the existence of a novel stimulatory domain of PTZ in $GABA_A$ receptors.

INTRODUCTION

$GABA_A$ receptors are the predominant inhibitory neurotransmitter receptors in the vertebrate central nervous system. The primary function of $GABA_AR$ is to increase the permeability of Cl^- ions in the neurons leading to hyperpolarization. $GABA_AR$ are hetero-oligomeric pentameric glycoproteins composed of assemblies of different subunits polypeptide. These subunits are characterized into seven different subunits families designated as $\alpha, \beta, \gamma, \delta, \varepsilon, \pi$, and recently discovered θ (17). Based on hydropathy studies, each subunit spans the membrane four times (TM1- TM4) with TM2 of each subunit spans the channel pore (14, 23).

$GABA_A$ receptors possess a variety of allosteric binding sites through which different drugs can modulate the GABA-mediated Cl^- current. Benzodiazepines are known to potentiate allosterically GABA-mediated current (3, also reviewed in 23). Conversely, convulsant drugs like picrotoxin (PTX), TBPS, and pentylenetetrazole (PTZ) are known to depress GABA-mediated current (1, 2, 21, 22).

Picrotoxin is a central nervous system convulsant that inhibits GABA- activated Cl-channel (13). PTX and other related PTX-site ligands including TBPS, PTZ, U-93631, and the cyclodiene insecticides like dieldren are proposed to bind within the channel pore that is formed by TM2 domain (1, 2, 7,10, 13, 15, 16, 19). However, the binding site of picrotoxin is not yet been located.

Moreover, the mechanism of block by PTX and other related compounds is still equivocal. Based on the use-dependent characteristic of PTX, PTX may act within the channel lumen to block the channel (13). However, single channel studies have proven

that PTX lacks the flickery effect which is characteristic of a channel block. In addition, picrotoxin does not affect the channel burst duration. These single channel studies are inconsistent with open channel blocking mechanism by PTX (19). The fact that PTX-induced inhibition is voltage independent also does not support the open channel blocking mechanism (19).

Studies in recent years have identified amino acid residues in the second transmembrane domain of the $GABA_A$ receptor that are involved in the actions of picrotoxin (8, 25). In particular, mutation to phenylalanine of a threonine residue at position 6' of the β2 subunit (β2T6'F) of the receptor has been shown to abolish sensitivity to picrotoxin in α1β2(T6'F)γ2 receptors (9). Based on the notion that PTZ, TBPS and U93631 presumably interacts at the same site in the receptor as does picrotoxin, it might be expected that this mutation would similarly abolish the effects of PTZ, TBPS, and U93631. Because of the often complex interactions of a ligand with its binding domain, it is of course also feasible that a mutation that affects the binding and/or functional characteristics of one ligand may not affect binding and/or functional characteristics of another ligand from the same general class. Recent examples of this have been reported for benzodiazepines (3, 12). Thus, our original aim was simply to test the hypothesis that the β2T6'F mutation would abolish the ability of PTZ, TBPS and U93631 to block the GABA-gated Cl^- channel. Our hypothesis was confirmed; i.e., PTZ and TBPS did not block GABA-gated current in receptors made resistant to picrotoxin. In addition, U93631 inhibitory effect was greatly reduced due to the mutation. Of

particular interest, however, was the unexpected finding that high concentrations of PTZ potentiated GABA-mediated current in picrotoxin-resistant receptors.

METHODS

Site-directed mutagenesis and transient transfection. The 6' threonine was mutated to phenylalanine in the second transmembrane domain of β2 subunits using the Gene Editor mutagenesis kit (Promega, Madison, WI). Any DNA isolation step was performed using GeniePrep (Ambion, Austin, TX). Mutations were confirmed by DNA sequencing. Untransfected HEK293 cells were plated onto 25mm coverslips. Rat α3, β2(T6'F), β2 and γ2S (S denotes short isoform) subunits were transiently transfected in a number of different combinations using the modified calcium phosphate precipitation method (6). 16-20 μg total DNA was used in the transfection step. Cells were analyzed electrophysiologically 48-72 hours after transfection.

Electrophysiology. Whole-cell patch recordings were made at room temperature (22-25 °C). Cells were voltage-clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1-2.5 MΩ for whole-cell recordings. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. Coverslips containing cultured cells were placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 $MgCl_2$, 3.0 $CaCl_2$, 20 HEPES, 25 D-glucose, pH 7.3. GABA-induced Cl^- currents were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. Currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and

stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data was not included in the analysis.

Experimental protocol. Once a whole-cell recording was established, GABA with or without the drug was prepared in the extracellular solution and then was applied from independent reservoirs by gravity flow to a cell using a Y-shaped tube positioned within 100 μm of the cell. With this system, the 10-90% rise time of the junction potential at the open tip averages less than 50 ms. Receptors were typically activated with roughly the EC_{50} GABA concentration. Once a control GABA response was determined, the effect of a drug on the response was examined. Recovery from drug-induced effect was usually obtained. GABA applications were separated by at least 2-min intervals to ensure both adequate washout of GABA from the bath and recovery of receptors from desensitization, if present.

Data analysis. GABA concentration-response profiles were fitted to the following equation: $I/I_{max}= 1/(1+(EC_{50}/[GABA])^n)$, where I and I_{max} represent the normalized GABA-induced current at a given concentration and the maximum current induced by a saturating [GABA], EC_{50} is the half-maximal effective GABA concentration, and n is the slope factor.

Materials:

GABA, PTZ, diazepam, flumazenil and picrotoxin were obtained from Sigma (St. Louis, MO); U-93631 was generously provided by Pharmacia-Upjohn (Kalamazoo, MI). TBPS was obtained from Tocris (Ballwin, MO).

Results

We first evaluated the effect of the β2 TMII mutation (T6'F) on the GABA dose response curve. The expression of the α3 along with β2(T6'F) and γ2 resulted in receptors with twofold right shift in the EC50 for GABA as shown in Fig. 1. The EC50 of the wild type α3β2γ2 and the mutant α3β2(T6'F)γ2 receptors were 21.3 ± 2.2 and 50.4 ± 3.3 μM and the hill coefficient were 1.4 ± 0.18 and 0.94 ± 0.04 respectively (N = 3-8). Thus, the sensitivity to GABA and allosteric modulation were only modestly affected by the mutation.

Figure 2 illustrates the picrotoxin effect on the response of $GABA_A$ α3β2γ2 and α3β2(T6'F) γ2 receptors. Consistent with the report from Gurley et al. (9), who found that expression of β2(T6'F) with α1 and γ2 subunits abolished picrotoxin sensitivity, we found that the expression of β2(T6'F) along with α3 and γ2 subunits resulted in a similar loss of sensitivity to picrotoxin. Exposure of the cells to 100 μM picrotoxin resulted in minimal inhibition; this compares to an IC_{50} for picrotoxin in wild type α3β2γ2 receptors of 5.1 ± 0.7 μM (1).

Previous studies showed that U93631 and TBPS inhibited GABA induced current in dose dependent fashion in the wild type α3β2γ2 receptors (7, 20). We evaluated the effect of mutation T6'F in β2 subunit on the inhibitory activity of U93631 and TBPS. As shown in Fig. 3, the inhibition by U93631 was greatly reduced by the mutation compared to the control, although a modest inhibition was present at 50 μM of U93631. In addition, 20 μM TBPS inhibited 87 ± 3.1 % of the GABA induced current in the wild

type α3β2γ2 receptors. However, the mutation completely abolished the TBPS ability to inhibit GABA induced current in the mutant receptors as shown in Fig. 4. Thus, our results suggest that the functional domains of U93631 and TBPS appear to be similar possibly overlapping to that of picrotoxin.

The pharmacological effect of PTZ on wild type α3β2γ2 and on α3β2(T6'F)γ2 receptors is illustrated in Fig. 5. In wild-type receptors, PTZ inhibited GABA-gated current in a concentration-dependent manner; the IC_{50} for PTZ in α3β2γ2 receptors is approximately 1 mM (Fig. 5A, also (1)). In contrast, up to 50 mM PTZ had no inhibitory effect on GABA-activated current in α3β2(T6'F)γ2 receptors. Indeed, at PTZ concentrations of greater than 50 mM, we observed a robust stimulation of GABA-gated current (Fig. 5B, C).

The fact that the PTZ-induced stimulatory effect occurred at high concentrations raised the possibility that the stimulation was due to an osmotic effect on the cell membrane. High concentrations of PTZ (100 mM) completely abolished the GABA-gated current in wild type α3β2γ2 receptors (Fig. 6Aa); no evidence of a stimulatory effect was observed. Moreover, PTZ applied by itself to either wild type or α3β2(T6'F)γ2 receptors, in the absence of GABA, had no effect (Fig. 6Aa, Ab). Taken together, these results confirm that the effect of PTZ was due to an allosteric potentiation of the GABA-activated current.

At least two possibilities exist to explain the stimulatory effect of PTZ in the mutant receptors. First, it is possible that PTZ is acting at the same receptor site, but the mutation has converted its effect from an antagonist to an allosteric potentiator (15).

Although this possibility is difficult to rule out definitively, the fact that neither picrotoxin, nor the picrotoxin-site ligand U-93631 (1) demonstrate similar behavior argues against this scenario. A second possibility is that PTZ-mediated stimulation is due to its binding at a second, distinct site on the receptor. Benzodiazepine site is a well-known stimulatory site on $GABA_AR$. To assess whether this stimulatory domain for PTZ overlaps with the benzodiazepine site of the receptor, we examined its effects on GABA current in the presence of a saturating concentration (15 μM) of the benzodiazepine antagonist flumazenil. As illustrated in Fig. 6B, flumazenil had no effect on the ability of PTZ to stimulate the GABA-gated current (PTZ enhanced GABA-gated current to 262 ± 24.3 % (n = 3) and to 268 ± 2.4 % (n = 3) in the absence and presence of flumazenil, respectively). Thus PTZ stimulation is not mediated through an interaction at the benzodiazepine site of the $GABA_A$ receptor.

To determine if the stimulatory effect of PTZ requires the presence of the α subunit, we tested its actions in cells expressing β2(T6'F)γ2 $GABA_A$ receptors. Interestingly, unlike in the α3β2(T6'F)γ2 configuration, receptors expressing only β2(T6'F) and γ2 subunits were sensitive to the inhibitory effects of PTZ. Sensitivity to picrotoxin inhibition was also present in β2(T6'F)γ2 receptors (see chapter 2). Subsequent experiments have demonstrated that sensitivity to both picrotoxin and PTZ also exists in α3β2(T6'F) receptors. Thus we were unable to determine if the stimulatory effect of PTZ is associated with a specific subunit of the $GABA_A$ receptor.

DISCUSSION

In the present report, we evaluated the effect of the TMII mutation of the conserved threonine 6' to phenylalanine in β2 subunit on the inhibitory pharmacology of the picrotoxin related compounds like TBPS, U93631 and PTZ.

Previous binding and electrophysiological studies suggested that the convulsants U93631, PTZ, and TBPS interact with a domain that might overlap with the picrotoxin site (10, 13, 16, 19-21). The aim of our studies is to further test the hypothesis that these ligands indeed interact with the picrotoxin site.

In the present report, mutation (T6'F) within the TMII that previously abolished the picrotoxin inhibitory effect on $GABA_A$ receptors, did also abolish the effect of TBPS, and PTZ. The same mutation also drastically decreased the effect of U93631; up to 50 μM U93631 showed a modest inhibition. Thus, combining our studies with others, we suggest that all the picrotoxin related ligands interact with a domain that might be homologous or overlapping with the picrotoxin action domain. In addition, our studies further support the contention that the TMII domain is important for the action of these ligands.

The mechanism by which the incorporation of phenylalanine at position 6' abolishes the inhibitory effect of the picrotoxin and picrotoxin related compounds, is still unknown. One possible mechanism is that the bulky phenyl group might block the access of the picrotoxin agents toward their site (9). Other studies suggested that the phenylalanine at position 6' abolished the hydrogen bonding conferred by the threonine

that is needed to interact with the hydrophilic head that is essential for the activity of these ligands (27).

It is noteworthy to suggest that the T6' residue might reside in the picrotoxin action domain or play a key role in the transduction pathway upon picrotoxin binding. Whether the molecular size or the loss of the hydrogen bonding or both are behind the abolishment of the inhibitory effect of the picrotoxin and its related drugs is yet to be determined. In addition, our studies support the contention that PTX and its related compounds interact with the TMII domain.

Interestingly, T6'F mutation uncovered a low affinity, but highly efficacious stimulatory site for the PTZ. It was found that other agents that act at domains that overlap the picrotoxin site have also been shown to have stimulatory effects at the $GABA_A$ receptor. The γ-butyrolactones (11, 15) and dieldrin (18) have dual effects on $GABA_A$ mediated current. The inhibitory effect of these compounds is mediated through the picrotoxin site; the site where potentiation is produced has not been determined. Whether or not the stimulatory actions of any of these picrotoxin-site ligands are mediated through a common site remains to be determined.

Although the presumed affinity of PTZ for its stimulatory site is low, its efficacy (greater than 3-fold increase in the current amplitude) is similar to that observed for many GABAergic therapeutics, including benzodiazepines and barbiturates. The present results suggest the PTZ molecule may serve as a template for development of novel therapeutics with agonist actions at the $GABA_A$ receptor. The α-substituted γ-butyrolactones are new and promising drugs that, like PTZ, interact with an undefined

site that stimulates GABA-activated current (11, 24). These compounds are the subject of ongoing structure-activity analysis to delineate the unique structural features that determine their interaction with the inhibitory picrotoxin site and the stimulatory lactone site (4, 5). The ultimate goal of these investigators is to develop compounds that have high affinity for the lactone site, while having minimal activity at the picrotoxin site (5). Whether the stimulatory effects of PTZ are mediated via interaction with the lactone site or some other undefined site has not been determined. Nevertheless, the current results indicate a similar structure-activity analysis of pentylenetetrazole might prove to be useful in development of new GABA-related compounds.

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Fig. II-1 GABA concentration-response profiles for transiently transfected $\alpha_3\beta_2\gamma_2$ and $\alpha_3\beta_2$ (T6'F) γ_2 $GABA_A$ receptor. EC_{50} for the mutant was right-shifted approximately twofold (to 50.4 ± 4.3 μM, from 21.3 ± 2.2 μM in the wild type). The hill coefficient were 1.4 ± 0.18 and 0.94 ± 0.04 in the wild type and the mutant receptors, respectively. N = 3-8 for each data point.

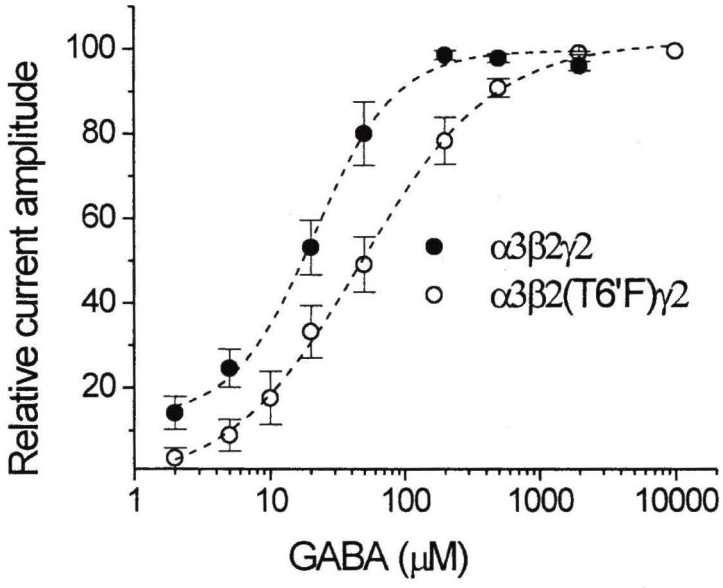

Relative current amplitude
100
80
60
40
20
1
10
100
1000
10000
GABA (μM)
α3β2γ2
α3β2(T6'F)γ2

Fig. II-2 Diminished sensitivity to picrotoxin in $\alpha 3\beta 2(T6'F)\gamma 2$ $GABA_A$ receptors. 100 μM picrotoxin had minimal effects on the response to 25 μM GABA in the mutant receptors. In contrast, the GABA response is markedly reduced by picrotoxin in $\alpha 3\beta 2\gamma 2$ $GABA_A$ receptors, as the IC_{50} is approximately 5 μM [1]. Drug application time (bars) was 10 s.

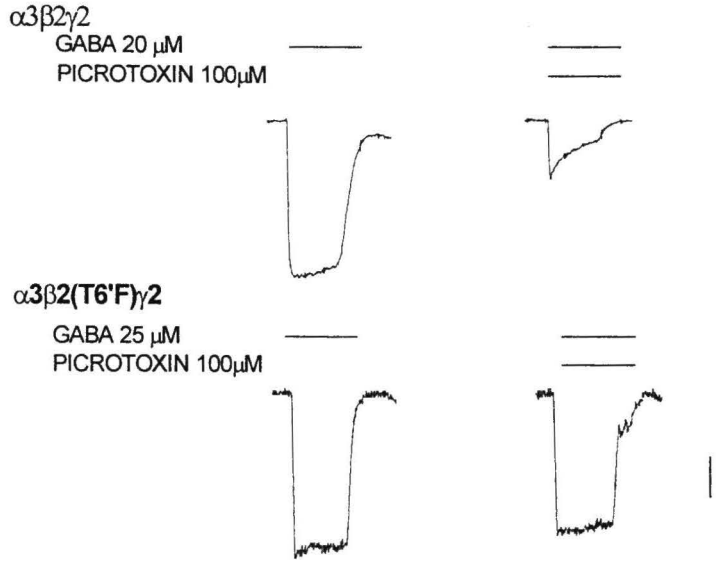

α3β2γ2
GABA 20 μM
PICROTOXIN 100μM
α3β2(T6'F)γ2
GABA 25 μM
PICROTOXIN 100μM

Fig. II-3 The effect of T6'F mutation on the U93631. The mutation (T6'F) drastically reduced the inhibitory action of U93631. N = 5-8 for each data point.

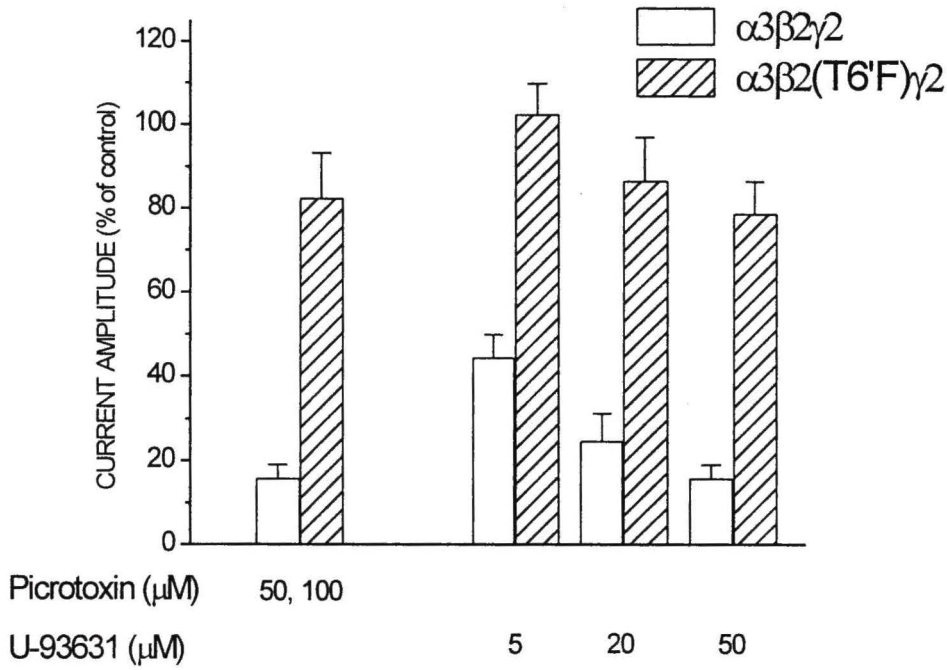

α3β2γ2
α3β2(T6'F)γ2
CURRENT AMPLITUDE (% of control)
120
100
80
60
40
20
0
Picrotoxin (μM)
50, 100
U-93631 (μM)
5
20
50

Fig. II-4 The T6'F mutation abolished the inhibitory effect of TBPS in $\alpha 3\beta 2$(T6'F)$\gamma 2$ $GABA_A$ receptors. In contrast, 20 μM TBPS greatly reduces the GABA-induced response in the wild type. Calibration bar equal to 300 pA in A and 50 pA in B. A minimum of 3 cells.

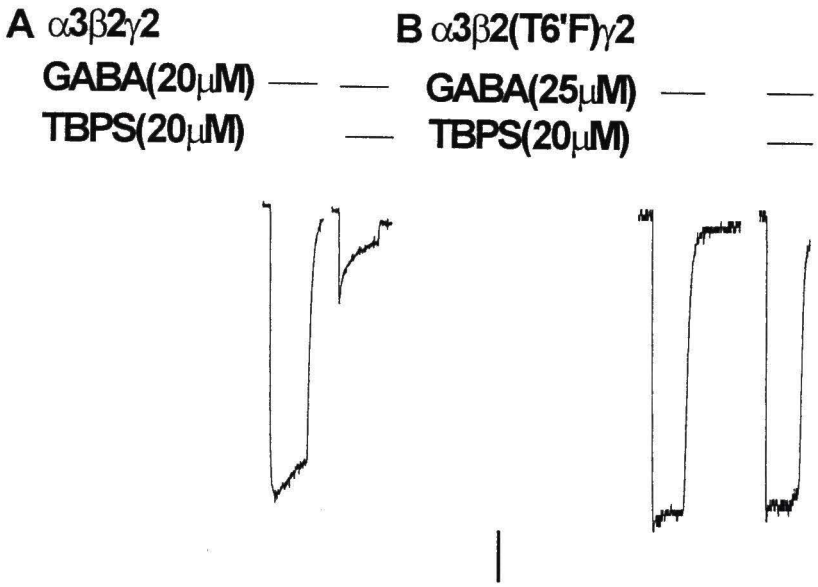

A α3β2γ2
GABA(20μM)
TBPS(20μM)
B α3β2(T6'F)γ2
GABA(25μM)
TBPS(20μM)

Fig. II-5. The effect of PTZ in wild type and picrotoxin-resistant $GABA_A$ receptors. A) Inhibition by PTZ in $\alpha3\beta2\gamma2$ receptors; the PTZ IC50 in these cells is approximately 1 mM. B) In $\alpha3\beta2$(T6'F)$\gamma2$ receptors, the inhibitory effect of PTZ was completely abolished. At higher concentrations of PTZ, GABA-gated Cl- current was markedly enhanced. C) Mean responses to PTZ in $\alpha3\beta2$(T6'F)$\gamma2$ receptors. The stimulatory effect was significant at 50, 100 and 200 mM PTZ. N = A minimum of three cells tested. Drug application time was 10 s; calibration bar = 300 pA for A, 200 pA for B.

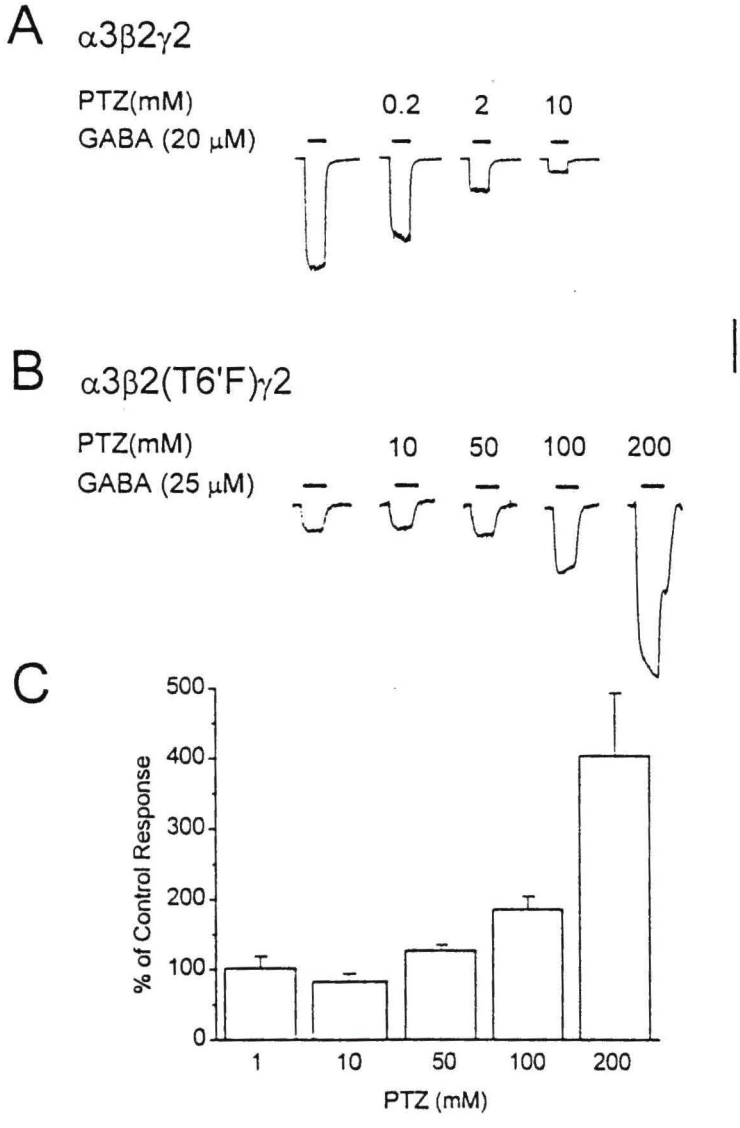
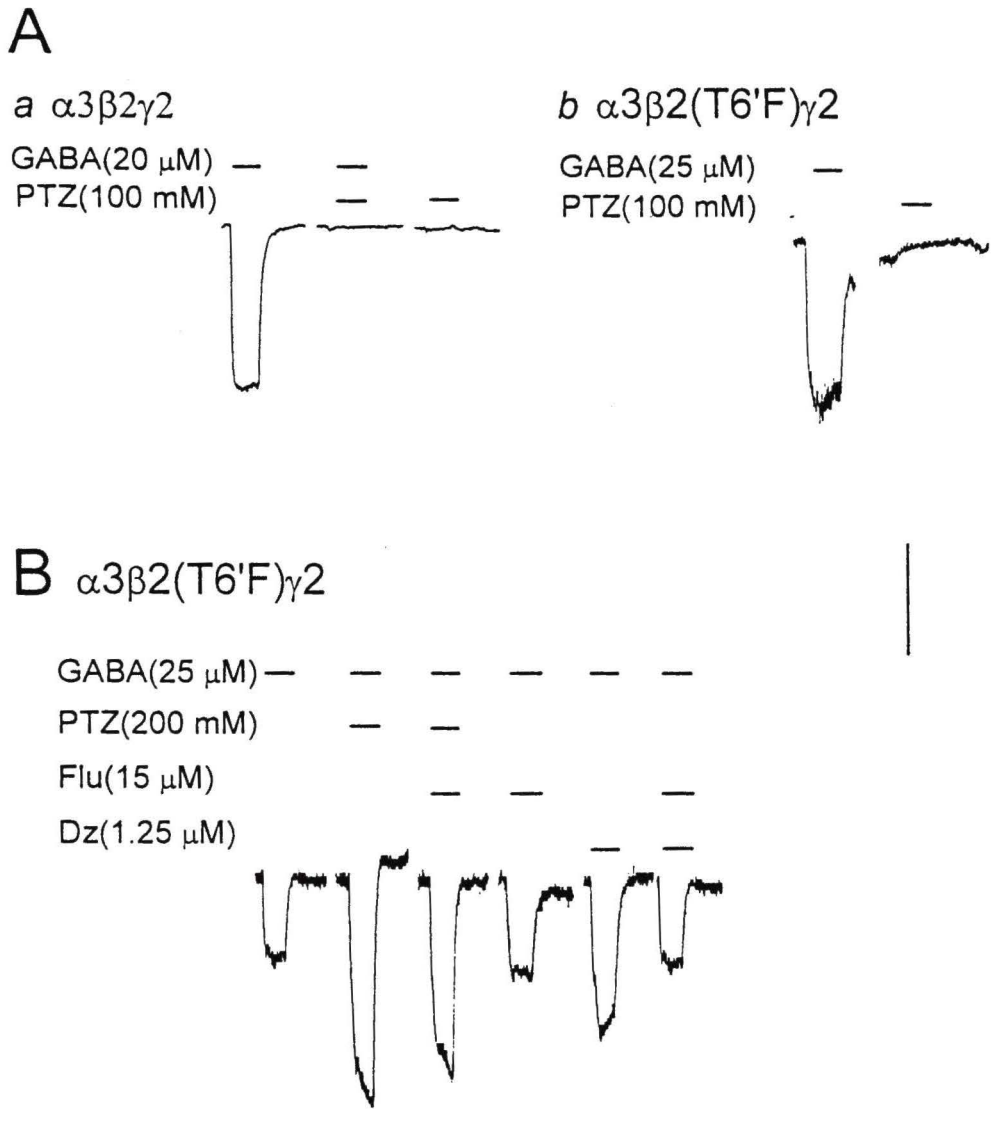

A
α3β2γ2
PTZ(mM)
GABA (20 μM)
0.2
2
10
B
α3β2(T6'F)γ2
PTZ(mM)
GABA (25 μM)
10
50
100
200
C
% of Control Response
500
400
300
200
100
0
1
10
50
100
200
PTZ (mM)

Fig. II-6. PTZ-mediated stimulation of $\alpha 3\beta 2(T6'F)\gamma 2$ receptors is not mediated via an interaction with the benzodiazepine site. (Aa) High concentration of PTZ had only inhibitory effects in $\alpha 3\beta 2\gamma 2$ $GABA_A$ receptors (middle trace). In addition, application of a similarly high concentration of PTZ by itself had no significant effect on the cell (right trace of (Aa)). A similar lack of effect of PTZ by itself was observed in $\alpha 3\beta 2(T6'F)\gamma 2$ receptors (Ab). B) Exposure of the cells to a saturating concentration of the benzodiazepine antagonist flumazenil did not inhibit the ability of PTZ to enhance GABA-gated current in the picrotoxin-resistant $\alpha 3\beta 2(T6'F)\gamma 2$ receptors (n = 3, third trace). Cells retained sensitivity to the Benzodiazepine diazepam (fifth trace), and this effect could be blocked by the flumazenil (last trace). Drug application time was 10 s; calibration bar = 1000 pA for (Aa), 300 pA for (Ab), and 150 pA for (B).


A
a α3β2γ2
GABA(20 μM)
PTZ(100 mM)
b α3β2(T6'F)γ2
GABA(25 μM)
PTZ(100 mM)
B α3β2(T6'F)γ2
GABA(25 μM)
PTZ(200 mM)
Flu(15 μM)
Dz(1.25 μM)

In the previous chapter, it has been shown that the mutation (T6'F) abolishes the inhibitory effect of PTX and its related compounds like PTZ, U-93631, and TBPS in $GABA_A$ $\alpha3\beta2$(T6'F)$\gamma2$ receptors. Thus, the incorporation of phenylalanine at position 6' in $\beta2$ affected the convulsive site pharmacology as a whole rather than the picrotoxin pharmacology alone. However, subunit dependence of picrotoxin ability to block $GABA_A$ receptors is still in debate. Some studies have shown that picrotoxin lacks the subunit dependence while other studies have shown otherwise. Thus, the ability of varying the subunit configurations involving $\beta2$(T6'F) to modulate picrotoxin pharmacology has been investigated in the next chapter.

PICROTOXIN RESISTANCE CONFERRED BY THE 6' PHENYLALANINE OF THE SECOND TRANSMEMBRANE DOMAIN OF $GABA_A$ RECEPTOR SUBUNITS IS DEPENDENT ON RECEPTOR CONFIGURATION

Mohammed Dibas[1], and Glenn H. Dillon[1]

Department of Pharmacology and Neuroscience

University of North Texas Health Science Center at Fort Worth

3500 Camp Bowie Blvd., Fort Worth, TX 76107[1].

Running title:

Picrotoxin inhibition of $GABA_A$ receptors

Corresponding author:

Glenn H. Dillon, Ph.D.
Dept. of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3500 Camp Bowie Blvd.
Fort Worth, TX 76107
Phone: (817) 735-2055
Fax: (817) 735-2091
Email: **gdillon@hsc.unt.edu**

CHAPTER III. PICROTOXIN RESISTANCE CONFERRED BY THE 6' PHENYLALANINE OF THE SECOND TRANSMEMBRANE DOMAIN OF $GABA_A$ RECEPTOR SUBUNITS IS DEPENDENT ON RECEPTOR CONFIGURATION

ABSTRACT

Picrotoxin (PTX) is a CNS convulsant that inhibits GABA-mediated Cl^- current. Although its site of action is unknown, it has been shown that introduction of a phenylalanine (F) residue at the 6' position of the β2 subunit second transmembrane domain (TM2) in α1β2γ2 receptors abolishes PTX sensitivity (Gurley et al., 1995). The mechanism by which this TM2 6' F confers resistance to PTX is still unclear. To gain further insight into the influence of this mutation on PTX inhibition, we have assessed its effects on PTX blockade of $GABA_A$ receptors composed of varying subunits. As expected, the T6'F mutation in the β2 subunit abolished inhibition induced by PTX in α3β2(T6'F)γ2 receptors. However, in α3β2(T6'F) receptors, picrotoxin acted as a partial antagonist, with a maximal efficacy approximately 40% of that observed in wild type receptors. Moreover, the expression of β2(T6'F) along with only the γ2 subunit formed channels with only modestly impaired picrotoxin sensitivity (PTX IC_{50} was 0.5 ± 0.05 and 1.7 ± 0.18 μM in β2γ2 and β2(T6'F)γ2 receptors, respectively). When the analogous 6' TM2 mutation was introduced in the α1 subunit and coexpressed with wild type β2 and γ2 subunits (α1(T6'F)β2γ2 receptors), the ability of PTX to block the GABA-gated current was not significantly affected. The present data demonstrate that varying the

subunits expressed with β2(T6'F) dramatically influences the picrotoxin blocking mechanism. In addition, the spatial arrangement of subunits within the lumen of the channel plays a crucial role in the mechanism of PTX inhibition.

INTRODUCTION

The plant alkaloid picrotoxin is a CNS convulsant that inhibits many ligand-gated Cl^- channels, including heteromeric $GABA_A$ receptors, glutamate-gated Cl^- channels, homomeric glycine receptors, *Drosophila* GABA receptors, and $GABA_C$ receptors (ffrench-Constant et al., 1993; Wang et al., 1995; Etter et al. 1997). Despite extensive study, its mechanism and site of action are still undefined. Some evidence indicates picrotoxin may act as a classical open channel blocker (Inoue and Akaike, 1988), while other reports suggest an allosteric mechanism is more likely (Newland and Cull-Candy, 1992; Yoon et al., 1993; Dillon et al., 1995). Although its precise site of action is unknown, results from a number of studies suggest the channel-forming second transmembrane domain (TM2) is the probable site of picrotoxin action. For instance, whereas glycine receptors composed of only α subunits are sensitive to picrotoxin, coexpression of the β subunit along with any glycine α subunit produces picrotoxin-insensitive receptors; this resistance was shown to be conferred by TM2 of the β subunit (Pribilla et al., 1992). Subsequent work demonstrated that spontaneously active channels formed from synthetic peptides equivalent to TM2 of the glycine α subunit were blocked by picrotoxin (Reddy et al., 1993), further supporting the role of TM2 in picrotoxin inhibition.

Within the second transmembrane domain, amino acid residues at the 2' and 6' positions have been shown to block PTX-induced antagonism of the GABA-gated Cl^- channel. The importance of the 2' position was demonstrated with the discovery that strains of *Drosophila* that are resistant to the insecticide dieldrin have a natural mutation

(A→S or A→G) at this position in TM2 of the insect GABA receptor; receptors encoding this mutation are also resistant to picrotoxin (ffrench-Constant et al., 1993). In both homomeric $GABA_C$ receptors (Wang et al., 1995) and glutamate-gated Cl^- channels of *C. elegans* (Etter et al., 1999), varying the nature of the amino acid residue at the 2' position has a dramatic effect on picrotoxin sensitivity. Evidence for involvement of the 2' position in picrotoxin inhibition of heteromeric vertebrate $GABA_A$ receptors has also been reported (Bell-Horner et al., 2000).

Several reports have demonstrated a role of the 6' TM2 residue in PTX inhibition. Gurley et al. (1995) demonstrated that substitution of a phenylalanine (F) residue (wild type is threonine) at the 6' position in TM2 of the β2 subunit of α1β2γ2 $GABA_A$ receptors produces resistance to PTX. Zhang et al. (1995) also demonstrated a role of the 6' position in PTX sensitivity of $GABA_C$ receptors. Homomeric $GABA_C$ receptors (formed from ρ1 subunits) are PTX sensitive. However, coexpression of ρ2 subunits along with ρ1 resulted in PTX-resistant receptors. The resistance to PTX was shown to be due to a methionine residue at the 6' position in ρ2 (ρ1 expresses threonine at the same position) (Zhang et al., 1995). While both findings demonstrated the importance of the 6' position for PTX-mediated inhibition, the mechanism for the effect was not determined. Recent molecular modeling results have led to the suggestion that the phenylalanine substitution at position 6' abolishes the hydrogen bonding necessary for picrotoxin binding (Zhorov and Bregestovski, 2000). However, mutation of the conserved 6' threonine to alanine (which also abolishes hydrogen bonding capability) in homomeric ρ1-expressing $GABA_C$ receptors formed constitutively open channels that

were blocked by picrotoxin (Zhang et al., 1995). Thus, the mechanism by which the 6' phenylalanine eliminates picrotoxin sensitivity is unknown.

In the present study, we assessed the effect of varying the configuration of the subunit coexpressed with β2(T6'F) on picrotoxin's inhibitory action. We found that expressing β2(T6'F) along with both α3 and γ2 produced channels that were highly resistant to picrotoxin. Interestingly, β2(T6'F)γ2 channels were highly sensitive to picrotoxin, and α3β2(T6'F) were partially sensitive to picrotoxin. More surprisingly, we found that the equivalent T6'F mutation introduced into the α1 subunit did not abolish picrotoxin inhibition of α1(T6'F)β2γ2 receptors. Our results further underscore the complexity of picrotoxin blockade of $GABA_A$ receptors. Furthermore, our data are consistent with the suggestion that orientation of channel-lining residues may vary subtly depending on receptor subunit expression.

MATERIALS AND METHODS

Site-directed mutagenesis and transient transfection. The 6' threonine was mutated to phenylalanine in the second transmembrane domain of α1 and β2 subunits using the Gene Editor mutagenesis kit (Promega, Madison, WI). Any DNA isolation step was performed using GeniePrep (Ambion, Austin, TX). Mutations were confirmed by DNA sequencing. Untransfected HEK293 cells were plated onto 25mm coverslips. Rat α1(T6'F), α3, β2(T6'F), β2 and γ2S (S denotes short isoform) subunits were transiently transfected in a number of different combinations using the modified calcium phosphate precipitation method (Chen and Okayama, 1987). 16-20 μg total DNA was used in the transfection step. Cells were analyzed electrophysiologically 48-72 hours after transfection.

Electrophysiology. Whole-cell patch recordings were made at room temperature (22-25 °C). Cells were voltage-clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1-2.5 MΩ for whole-cell recordings. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. Coverslips containing cultured cells were placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 $MgCl_2$, 3.0 $CaCl_2$, 20 HEPES, 25 D-glucose, pH 7.3. GABA-induced Cl^- currents were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. Currents were low-pass

filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data was not included in the analysis.

Experimental protocol. Once a whole-cell recording was established, GABA with or without the drug was prepared in the extracellular solution and then was applied from independent reservoirs by gravity flow to a cell using a Y-shaped tube positioned within 100 μm of the cell. With this system, the 10-90% rise time of the junction potential at the open tip averages less than 50 ms (Huang and Dillon, 1999). Receptors were typically activated with roughly the EC_{50} GABA concentration. Once a control GABA response was determined, the effect of a drug on the response was examined. Recovery from drug-induced effect was usually obtained, and application of high [GABA] was used to facilitate recovery from picrotoxin. β2(T6'F)γ2 receptors generated relatively small currents in response to GABA. This was not due to the mutation alone, as wild type β2γ2 also generate relatively small currents compared to other receptor configurations (Sigel et al., 1990; Bell-Horner et al., 2000). Thus, in some cases roughly 2X the GABA EC_{50} was used to activate these receptors; the effect of PTX was similar in both cases. GABA

applications were separated by at least 2-min intervals to ensure both adequate washout of GABA from the bath and recovery of receptors from desensitization, if present.

Data analysis. GABA concentration-response profiles were fitted to the following equation: $I/I_{max}= 1/(1+(EC_{50}/[GABA])^n)$, where I and I_{max} represent the normalized GABA-induced current at a given concentration and the maximum current induced by a saturating [GABA], EC_{50} is the half-maximal effective GABA concentration, and n is the slope factor. The antagonism profile of the non-competitive antagonist picrotoxin was analyzed by constructing concentration-inhibition relationships. The data were fitted to the equation: $I/I_{max}= 1/(1+(IC_{50}/[picrotoxin])^n)$, where I is the steady state current at a given concentration of picrotoxin, I_{max} is the maximum current induced by GABA, IC_{50} is the picrotoxin concentration that is half maximally effective, and n is the slope factor.

Materials:

GABA, PTZ, diazepam and picrotoxin were obtained from Sigma (St. Louis, MO); U-93631 was generously provided by Pharmacia-Upjohn (Kalamazoo, MI).

RESULTS

We first evaluated the effect of the mutation of threonine at 6' to phenylalanine in the β2 subunit on the GABA EC_{50} in all configurations tested: α3β2(T6'F)γ2, α3β2(T6'F), and β2(T6'F)γ2. As previously shown, there was a two-fold shift in the GABA EC_{50} in α3β2(T6'F)γ2 receptors (Bell-Horner et al., 2000). This was similar to the shift observed in the α1β2(T6'F)γ2 receptors (Gurley et al., 1995). When the β2(T6'F) was coexpressed with the α3, there was a roughly five-fold rightward shift in the GABA EC_{50}. The GABA EC_{50} for α3β2 and α3β2(T6'F) receptors was 3.5 ± 0.5 and 19.4 ± 1.4 μM, respectively (Fig. 2A). Interestingly, the concentration response curve for the β2(T6'F)γ2 was shifted to the left, indicating an increase in GABA sensitivity. The GABA EC_{50} for wild type β2γ2 and β2(T6'F)γ2 receptors was 85 ± 13 and 44 ± 7 μM, respectively (Fig. 2B). The decrease in GABA EC_{50} in β2(T6'F)γ2 receptors is consistent with that found in human glycine α3(T6'F) receptors (Steinbach et al., 2000).

Effect of the β2 subunit TM2 T6'F mutation on PTX sensitivity is dependent upon coexpressed subunits

Previous studies have shown that the single mutation T6'F, when present in only the β2 subunit, was sufficient to block picrotoxin action in α1β2(T6'F)γ2 receptors (Gurley et al., 1995). Recent studies have shown that picrotoxin inhibition, and the ability to influence its antagonism, displays some subunit dependence (Bell-Horner et al., 2000, Chang et al., 1999). We thus attempted to assess the effects on picrotoxin inhibition of changing the subunit(s) coexpressed with β2(T6'F). Based on the

possibility that the phenylalanine existence at position 6' was able to block the picrotoxin access to its site, we expected that picrotoxin inhibition would also be abolished in other receptors configurations containing the β2(T6'F). We initially assessed the effects of PTX in α3β2(T6'F)γ2 receptors. In wild type α3β2γ2 receptors, 100 μM PTX nearly completely abolished the response to GABA (Fig. 3A). In contrast, up to 1 mM PTX had no significant effect on GABA-gated current in α3β2(T6'F)γ2 receptors (Fig. 3B). These data are consistent with findings in α1β2(T6'F)γ2 receptors (Gurley et al., 1995).

We next tested the effect of PTX in mutant receptors lacking the γ2 subunit. In wild type α3β2 receptors, PTX inhibited the GABA induced current with an IC_{50} of 7.1 ± 1.1 μM (not shown). In α3β2(T6'F) receptors, PTX acted as a partial antagonist. 100 μM PTX was able to inhibit 43 ± 7.1% of GABA-gated current (n=3, Fig. 3C). Thus, in spite of the presence of at least 2 (Im et al., 1995; Gorrie et al., 1997) and possibly 3 phenylalanines at the 6' position (Tretter et al., 1997), picrotoxin retained the ability to inhibit GABA-mediated current in α3β2(T6'F) receptors.

Expression of the β2(T6'F) along with only the γ2 subunit had even more notable effects on picrotoxin action. Picrotoxin completely blocked GABA-mediated current in β2(T6'F)γ2 receptors, with an IC_{50} of 1.7 ± 0.2 μM (Fig. 3D, 3E). The IC_{50} of PTX in these β2(T6'F)γ2 receptors is only modestly higher than that observed in wild type β2γ2 receptors (0.5 ± 0.05 μM, Bell-Horner et al., 2000). These findings prompted us to test the effects of other picrotoxin-site ligands on GABA-gated in β2(T6'F)γ2 receptors.

Sensitivity to other picrotoxin-site ligands is present in β2(T6'F)γ2 receptors

Pentylenetetrazole (PTZ) and U-93631 block GABA-gated current at a domain comparable to that of PTX (Bell-Horner et al., 2000; Dibas and Dillon, 2000). Because PTX was effective in blocking β2(T6'F)γ2 receptors, we tested the hypothesis that these compounds may also retain the ability to block GABA-gated current in these receptors. In wild type α3β2γ2 and β2γ2 receptors, the PTZ IC_{50} is approximately 1 mM, and GABA current is fully blocked at 20 mM PTZ (unpublished observations, manuscript submitted). Up to 100 mM PTZ failed to inhibit GABA-gated current in α3β2(T6'F)γ2 receptors (Dibas and Dillon, 2000), confirming the involvement of the 6' phenylalanine in PTZ-mediated block. However, as seen with picrotoxin, PTZ retained the ability to block GABA-mediated current in β2(T6'F)γ2 receptors (Fig. 4, n = 3). Recent studies from our lab have shown that PTZ inhibits GABA-gated current in a competitive fashion (unpublished observation, manuscript submitted). Thus, because a relatively high concentration of GABA was used to gate the β2(T6'F)γ2 receptors, a complete inhibition of the GABA current was not observed. Nevertheless, the data confirm the fact PTZ, like picrotoxin, is efficacious in blocking GABA-mediated current in β2(T6'F)γ2 receptors.

We found a similar effect for the PTX-site ligand U-93631. In wild type α3β3γ2 and β2γ2 receptors, U-93631 inhibits GABA-gated current with an IC_{50} of 5.2 ± 0.3 μM and 0.3 ± 0.06 μM, respectively (Bell-Horner et al., 2000). Up to 50 μM U-93631 failed to inhibit GABA-mediated current in α3β2(T6'F)γ2 receptors (Bell-Horner et al., 2000). In contrast, in the present report 5 μM U-93631 was able to inhibit 64 ± 8.1 % of the

current in β2(T6'F)γ2 receptors (Fig. 4, n = 3). Thus, whereas the three picrotoxin-site ligands are all ineffective in α3β2(T6'F)γ2 receptors, all could inhibit GABA-gated current in β2(T6'F)γ2 receptors.

The equivalent mutation in the α1 subunit (α1T6'F) does not block picrotoxin sensitivity

As noted, previous work has shown that the β2(T6'F) mutation was sufficient to confer picrotoxin resistance in α1β2γ2 receptors (Gurley et al., 1995). Most current stoichiometric models of the $GABA_A$ receptor suggest receptors incorporating α, β, and γ subunits express 2α, 2β, and 1 γ subunit (Im et al., 1995; Chang and Weiss, 1996; Tretter et al., 1997). Thus, it might be expected that the mutation equivalent to β2(T6'F), when placed in the α1 subunit, would similarly block picrotoxin inhibition. We tested this hypothesis in the present investigation. α1(T6'F)β2γ2 receptors responded to GABA with a roughly 2-fold decrease in sensitivity (to 24.4 ± 3.0 μM) and a Hill coefficient (1.2 ± 0.15) unchanged from control (Fig. 5A). Interestingly, whereas 100 μM picrotoxin had no effect on α1β2(T6'F)γ2 (Gurley et al., 1995) or α3β2(T6'F)γ2 receptors (above), it completely (n = 10) blocked GABA-gated current in α1(T6'F)β2γ2 receptors (Fig. 5B). Thus, the effect on picrotoxin sensitivity of the 6' phenylalanine also depends on which subunit expresses the mutated phenylalanine.

DISCUSSION

Previous reports have shown that the nature of the amino acid residues at the 6' position of TM2 has dramatic effects on picrotoxin blockade of $GABA_A$ receptors (Gurley et al., 1995; Xu et al., 1995). Consistent with these findings, mutation of the 6' threonine to phenylalanine of the β2 subunit almost completely abolished the inhibitory effect of picrotoxin in α3β2(T6'F)γ2 receptors (Bell-Horner et al., 2000). In addition, the same mutation abolished the inhibitory effect of the convulsants pentylenetetrazole and U-93631, which presumably act at a domain that overlaps the picrotoxin site (Bell-Horner et al., 2000; Dibas and Dillon, 2000). Whereas a lack of subunit-dependent inhibition by picrotoxin has been reported (Sigel et al., 1990; Krishek et al., 1996), recent work has demonstrated that picrotoxin inhibition is significantly enhanced in β2γ2 receptors, in comparison to receptors also expressing an α subunit (Bell-Horner et al., 2000). We thus assessed the effects of the β2(T6'F) mutation in receptors expressing the mutant subunit with either the α3 or the γ2 subunit alone. Interestingly, picrotoxin was partially efficacious in α3β2(T6'F) receptors, and PTX and related ligands (U-93631 and PTZ) displayed near normal inhibitory activity in β2(T6'F)γ2 receptors. We further assessed stoichiometric influences of the 6' phenylalanine on PTX inhibition by evaluating PTX sensitivity of α1(T6'F)β2γ2 receptors. We unexpectedly observed that these receptors also retain sensitivity to PTX.

One might conclude from the results of Gurley et al. (1995) that the presence of a phenylalanine residue at the 6' position in any of the $GABA_A$ receptor subunits should confer PTX resistance. Our demonstration that is not the case is consistent with an earlier

report from Pribilla et al. (1992), which in fact formed the basis for the studies of Gurley et al. Homomeric glycine αx receptors (x = 1-3) displayed sensitivity to picrotoxin comparable to that of $GABA_A$ receptors. However, coexpression of the glycine β subunit with any of the glycine α subunits abolished the picrotoxin sensitivity (Pribilla et al., 1992). Subsequent β/α3 chimeric studies demonstrated that the TM2 domain of the β subunit conferred PTX resistance (Pribilla et al., 1992). One of the chimeric constructs (β-A, Fig. 1) retained the 6' F in the β subunit. PTX was fully efficacious in this chimera, and had only about a 10-fold reduction in potency (Pribilla et al., 1992). This is consistent with our findings, and indicates the presence of multiple phenylalanine residues at the 6' position is not necessarily adequate to confer PTX resistance. Additional factors must be considered to account for the mechanism of PTX blockade in the current and previous studies (Pribilla et al., 1992; Xu et al., 1995; Zhang et al., 1995).

The nature of the amino acid at the 2' position of TM2 is also clearly important for picrotoxin-mediated inhibition. A natural mutation at this position (alanine → serine or glycine) in the GABA receptor of *Drosophila* confers resistance to the insecticide dieldrin and picrotoxin (ffrench-Constant et al., 1993). Subsequent work has demonstrated that the 2' position in $GABA_C$ receptors and glutamate-gated Cl^- channels, both of which share considerable TM2 homology with $GABA_A$ receptors, dramatically affects picrotoxin sensitivity. The presence of alanine at the 2' position results in the greatest sensitivity to PTX (Wang et al., 1995; Etter et al., 1999). In addition, the increased picrotoxin sensitivity of $GABA_A$ β2γ2 receptors compared to α1β2γ2 or α1β2 receptors may be due to an increased number of alanines at the 2' position in β2γ2

receptors (Bell-Horner et al., 2000). Thus, in the present report, the PTX-inhibiting effects of the 6' phenylalanines could be partially offset by the PTX-facilitating effects of the 2' alanine. However, if the 6'F effect on PTX action is due to steric hindrance, this mechanism is unlikely, especially when one considers that the β2(T6'F)γ2 receptors likely have 3 phenylalanine residues at the 6' position. Moreover, potential changes in the number of alanine residues at the 2' position could not account for the partial antagonist actions of picrotoxin in α3β2(T6'F) receptors.

A more likely explanation for the current data is that the spatial arrangement of the 6' phenylalanine varies in the different receptor configurations analyzed. Observations from a number of previous studies support this contention. Horenstein and Akabas (1998) have demonstrated that inhibition by Zn^{++} of the $GABA_A$ receptor is enhanced by the presence of histidine (H) at the 17' position of either α1 or β1 subunits. However, the presence of H in the γ2 subunit produced Zn^{++}-insensitive receptors, even when all other subunits also expressed H at the 17' position. Although several possibilities exist, a potential explanation for this effect is that the presence of the γ2 subunit may alter the conformation of the adjacent subunits such that they can no longer coordinate the Zn^{++} ion in the channel. Additionally, Dalziel et al. (2000) have shown that mutation of the 9' leucine to phenylalanine or tyrosine of the β1 subunit in α1β1 $GABA_A$ receptors greatly affected GABA EC_{50} but had no effect on peak current. When the same mutations were introduced into the equivalent position of the α1 subunit of the α1β1 receptor, minimal effects on GABA EC_{50} were observed, but peak currents were greatly reduced. This indicates the equivalent positions in the different subunits perform

distinct roles in channel function. Additional subunit-specific effects of equivalent TM2 mutations have also been described (Chang and Weiss, 1999; Dalziel et al., 1999). Subunit-dependent differences in orientation of equivalent residues toward the ion channel could account for these effects. We suggest that subunit-dependent differences in channel-oriented residues account for the data in the present report.

The strongest evidence for this contention comes from work analyzing channel-accessible residues of nicotinic receptor subunits. Through use of the substituted cysteine accessibility method (SCAM), Zhang and Karlin (1998) have shown that there are substantial differences in channel access in equivalent TM2 residues of the α and β subunits of the nicotinic receptor. For instance, 9 residues are exposed in the α subunit in the closed state, and 8 in the open channel state (Akabas et al., 1994; Zhang and Karlin, 1998, considering residues from the 0' to 19' position). In the β subunit, 5 residues are exposed in the closed state, and 4 in the open state. More importantly, residues at equivalent positions in the two subunits are not consistently exposed, and the channel access of these residues is differentially affected by channel gating (Zhang and Karlin, 1998). This demonstrates clearly that, in spite of conserved physical traits of equivalent amino acids in different subunits, they are not similarly oriented in the TM2 domain. Thus, in the present report, the differential effects of the presence of phenylalanine at the 6' position in α1 compared to β2 subunits, as well as in double α3β2(T6'F) or β2(T6'F)γ2 vs. triple α3β2(T6'F)γ2 receptors, may be due to differences in their orientation toward the ion channel. At present, this contention cannot be confirmed unequivocally due to the lack of high structural resolution of these ligand

gated ion channels. Xu and Akabas have identified channel-lining residues of TM2 of the α1 subunit $GABA_A$ receptor. However, channel-lining residues for β and γ subunits of the $GABA_A$ receptor have not yet been reported.

In those receptors that the presence of phenylalanine at the 6' position abolishes picrotoxin-mediated inhibition, by what mechanism does this occur? Because the mechanism of picrotoxin blockade is not known, the question cannot be answered definitively. Recent molecular modeling suggests that the hydrogen bonding needed for PTX binding is lost upon phenylalanine incorporation (Zhorov and Bregestovski, 2000). However, substitution of alanine (A, unable to hydrogen bond) at the 6' position in $GABA_C$ receptors produces spontaneously open channels that are effectively blocked by picrotoxin (Pan et al., 1997, see Fig. 6). Thus, the mechanism by which the 6' phenylalanine blocks picrotoxin-mediated inhibition remains unresolved. The present data demonstrate clearly, however, that its effects are receptor subunit- and stoichiometry-dependent.

In summary, many residues (like 2', 3', 6', 9', 19') within TMII domain have been implicated to affect PTX mechanism, however, picrotoxin's mechanism and site of action are still elusive (Buhr et al., 2000, Chang and Wiess 1999, Etter et al., 1999, ffrench-Constant et al., 1993, Gurley et al., 1995, Lynch et al., 1995). Our results demonstrate that the ability of the 6' phenylalanine to block picrotoxin-mediated inhibition is subunit- and receptor configuration-dependent. This likely results from differences in the orientation of the 6' residues toward the ion channel in the different subunits and receptor configurations. An important aspect of our study is that future

studies involving the effect of TMII mutation on the pharmacological action of a drug on $GABA_A$ receptor should be conducted in different receptor configurations to draw any conclusion. Additional studies are ongoing to more precisely define the molecular mechanism by which the TM2 6' position influences picrotoxin action of other LGICs.

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Fig. III-1 Alignment of TM2 residues of $GABA_A$ and glycine receptor subunits. $GABA_A$ receptor subunits are α3 (Malherbe et al., 1990), and β2(Ymer et al., 1989). Glycine receptor subunits shown are α3 (Kuhse et al., 1990) and β (Grenningloh et al., 1990). Gly β-A and Gly β-B are chimeric subunits from Pribilla et al. (1992). Numbering system follows that of Miller (1989), where 0' and 19' residues are at the cytoplasmic and extracellular ends, respectively, of the ion channel. All sequences are from rat.

RECEPTOR	0'	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'	16'	17'	18'	19'
GABA α3	R	T	**V**	F	G	I	**T**	T	V	L	T	M	T	T	L	S	I	S	A	R
GABA β2	R	V	**A**	L	G	I	**T**	T	V	L	T	M	T	T	I	N	T	H	L	R
Gly α3	R	V	**A**	L	G	I	**T**	T	V	L	T	M	T	T	Q	S	S	G	S	R
Gly β	R	V	**P**	L	G	I	**F**	S	V	L	S	L	A	S	E	C	T	T	L	A
Gly β-A	R	V	**P**	L	G	I	**F**	T	V	L	T	M	T	T	Q	S	S	G	S	R
Gly β-B	R	V	**A**	L	G	I	**T**	T	V	L	T	M	T	T	Q	S	S	G	S	R

Fig. III-2 Concentration-response curves for $GABA_A$ α3β2 and α3β2(T6'F) (A) and β2γ2 and β2(T6'F)γ2 (B) receptors. GABA EC_{50}s are 3.5 ± 0.5, 19.4 ± 1.4, 85 ± 13, and 44 ± 7 μM for α3β2, α3β2(T6'F), β2γ2 and β2(T6'F)γ2 receptors, respectively. Slope factors are 1.0 ± 0.1, 0.96 ± 0.06, 0.8 ± 0.05, and 0.8 ± 0.05 for α3β2, α3β2(T6'F), β2γ2 and β2(T6'F)γ2 receptors, respectively. Values are mean ± sem from a minimum of four to five cells.

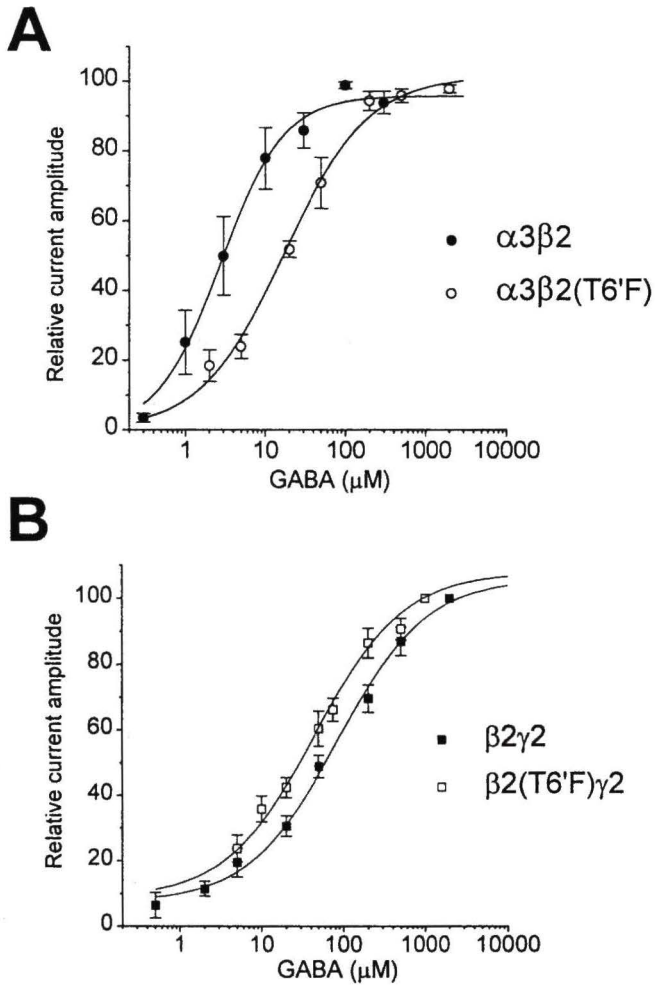

A
Relative current amplitude
100
80
60
40
20
0
1
10
100
1000
10000
GABA (μM)
α3β2
α3β2(T6'F)
B
Relative current amplitude
100
80
60
40
20
0
1
10
100
1000
10000
GABA (μM)
β2γ2
β2(T6'F)γ2

Fig. III-3 Effect of β2(T6'F) mutation on picrotoxin sensitivity depends on coexpressed subunits. 100 μM PTX fully inhibits wild-type α3β2γ2 receptors (A), while 1 mM PTX had insignificant effects on α3β2(T6'F)γ2 receptors (B). PTX is a partial antagonist in α3β2(T6'F), with maximal inhibition occurring at 100 μM PTX (C). Receptors expressing β2(T6'F) receptors, however, retained full sensitivity to PTX (D). (E) Concentration-response profiles for the four receptor configurations illustrated. PTX inhibited GABA-mediated current of β2(T6'F)γ2 receptors with an IC_{50} of 1.7 ± 0.2 and a slope factor unchanged from the wild type (1.2 ± 0.1, Bell-Horner et al., 2000). N= 3-5 for each data point. GABA was applied (10 s) to all receptors at roughly its EC_{50} concentration (EC_{70} for β2(T6'F)). Calibration bar = 480 pA for A and 150 pA for B-D.

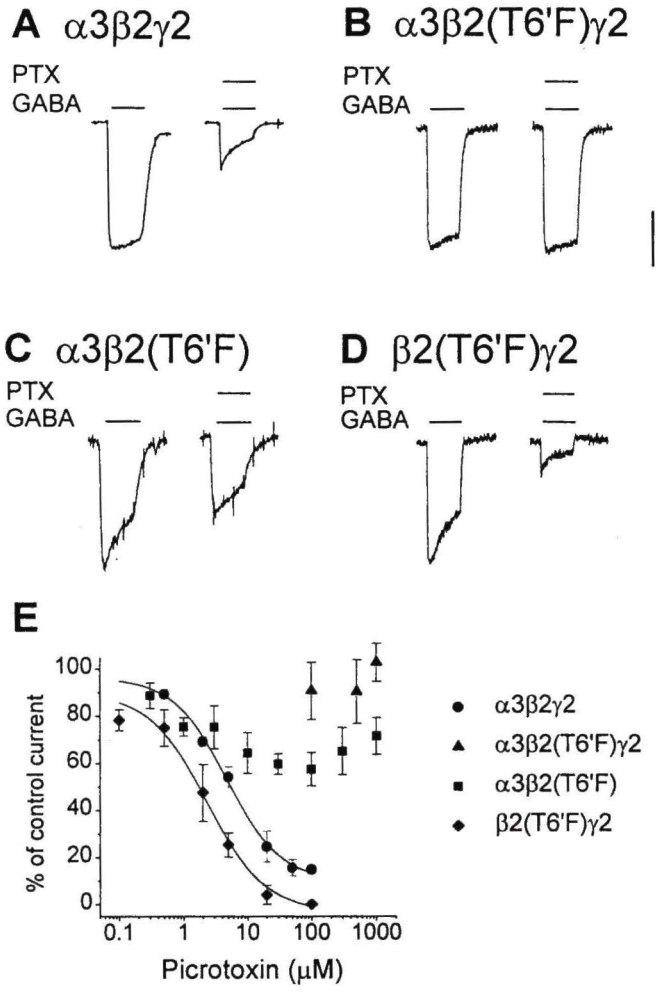

A α3β2γ2
PTX
GABA
B α3β2(T6'F)γ2
PTX
GABA
C α3β2(T6'F)
PTX
GABA
D β2(T6'F)γ2
PTX
GABA
E
% of control current
100
80
60
40
20
0
0.1
1
10
100
1000
Picrotoxin (μM)
α3β2γ2
α3β2(T6'F)γ2
α3β2(T6'F)
β2(T6'F)γ2

Fig. III-4 Effect of other picrotoxin-site ligands in β2(T6'F)γ2 receptors. Both pentylenetetrazole and U-93631, the actions of which are blocked in α3β2(T6'F)γ2 receptors (Bell-Horner et al., 2000; Dibas and Dillon, 2000), also retained efficacy in β2(T6'F)γ2 receptors. 100 mM PTZ inhibited 64 ± 3.6 % and 5 μM U93631 inhibited 64 ± 8.1 % of the GABA-mediated current. N= 3 for each data point. Calibration = 125 pA; ligand application time was 10 s.

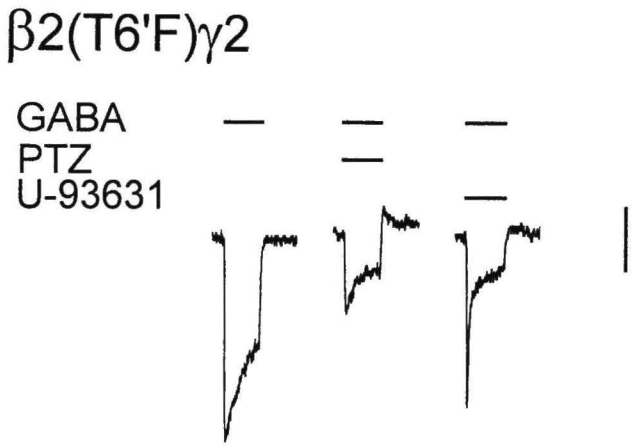

β2(T6'F)γ2
GABA
PTZ
U-93631

Fig. III-5 Subunit-dependent effect of T6'F mutation on picrotoxin sensitivity. (A) Concentration-response relationship for $\alpha1\beta2\gamma2$ and $\alpha1$(T6'F)$\beta2\gamma2$ receptors; GABA sensitivity was shifted approximately 2-fold by the mutation. Note that picrotoxin (100 μM) could completely block GABA-gated current in these receptors. Diazepam (DZ, 1 μM) significantly enhanced the GABA-mediated current, indicating the presence of all three subunits. N = 6 cells for the effect of PTX; n = 3 for diazepam.

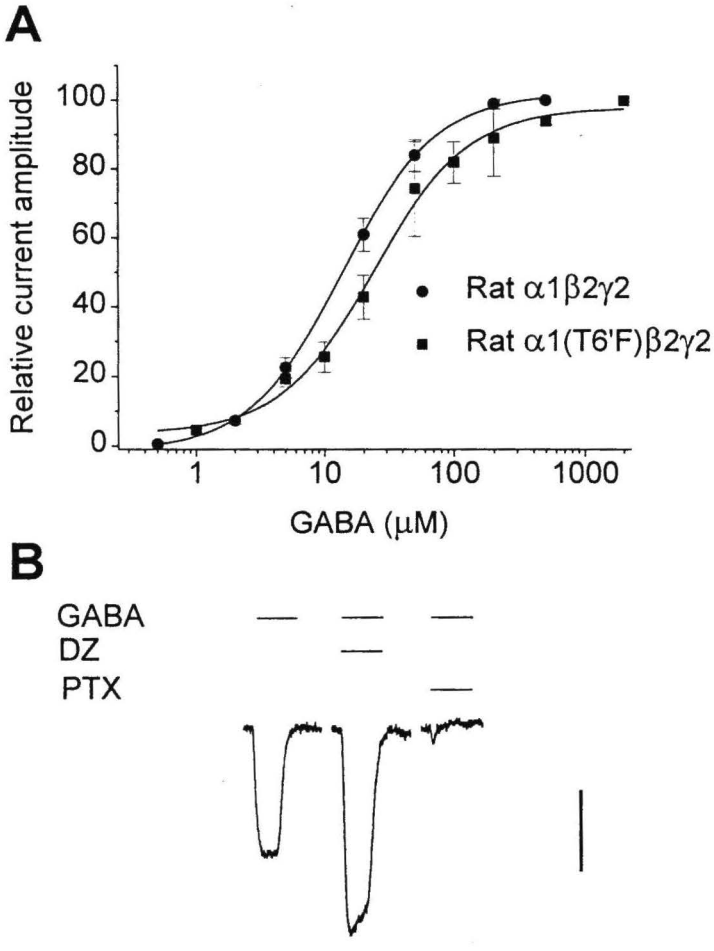
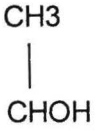
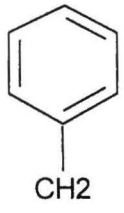

A
Relative current amplitude
100
80
60
40
20
0
1
10
100
1000
GABA (μM)
Rat α1β2γ2
Rat α1(T6'F)β2γ2
B
GABA
DZ
PTX

Fig. III-6 Schematic representation of the side chains present or mutated into the 6' position (including studies conducted by others). Threonine (T), with a molecular volume (MV) of the side chain equal to 121.5 A^3, exists in wild type of all $GABA_A$ subunits and glycine α subunits; phenylalanine (F), with a side chain MV of 257.9 A^3, exists in the glycine β subunit. PTX sensitivity is retained with incorporation of C-$MTSEA^+$ (MV = 296.4 A^3) at the 6' position of $\alpha1\beta1\gamma2$ receptors (Xu et al., 1995). In addition, PTX sensitivity is retained when alanine (MV = 51.8 A^3), which lacks hydrogen bonding capability, is substituted for the native threonine at this position in $GABA_C$ $\rho1$ receptors (Pan et al., 1997). The presence of methionine (MV = 212 A^3) at the 6' position of $GABA_C$ $\rho2$ receptors confers PTX resistance (Zhang et al., 1995). Based on these values, neither molecular volume nor lack of hydrogen bonding can account for the abolishment of PTX antagonism. The molecular volume was computed by Hyperchem 6.0 software (Hypercube Inc., Gainesville, Florida), and was computed by subtracting the molecular volume of the glycine side chain, which has only a hydrogen atom as the side chain. The MTSEA-cys was built using the cysteine as a backbone. The ranking of the molecular volume as computed by the Hyperchem software follows the molecular volume ranking reported in Tsai et al. (1999).



Threonine



Phenylalanine

Modified cysteine by MTSEA+

Alanine

Methionine

Fig. III-7 Summary figure of PTX pharmacology in $GABA_A$ and glycine receptors. Companion table illustrating changes at these positions in various configurations and mutations of $GABA_A$ and glycine receptors, and the resulting PTX sensitivity. See text for further discussion.

a, Pribilla et al., 1992
b, Gurley et al., 1995
c, Bell-Horner t al., 2000
d, present study

Receptor	2'	6'	PTX	REF.
Gly α3	A	T	sensitive	a
Gly α3β	3A/2P	3T/2**F**	**resistant**	a
Gly α3β–A	3A/2P	3T/2**F**	**sensitive**	a
Gly α3β–B	A	T	Sensitive	a
GABAα1/3β2γ2	2V/2A/S	T	Sensitive	b, c
GABAα1β2(T6'F)γ2	2V/2A/S	3T/2**F**	**resistant**	b
GABAα3β2(T6'F)γ2	2V/2A/S	3T/2**F**	**Resistant**	b
GABAα3β2(T6'F)	3V/2A	3T/2**F**	Partial resist.	d
GABA β2(T6'F)γ2	3A/2S	3**F**/2T	Sensitive	d
GABAα1(T6'F)β2γ2	2V/2A/S	2**F**/3T	Sensitive	d

Picrotoxin site and mechanism of inhibition of the anionic ligand gated ion channels are still controversial. The residue at position 6' plays a key role in the picrotoxin inhibition of these channels. However, it is still not known if picrotoxin binds near residue 6'. In addition, lots of studies aimed to decipher the complex mechanism of inhibition of picrotoxin. Picrotoxin inhibits $GABA_A$ and Glutamate gated chloride channels in a use-facilitated manner while it inhibits glycine channels in non use-facilitated fashion. The molecular determinant for such oddly behavior is still undefined. Since all ligand gated ion channels have conserved threonine at position 6', it is unlikely that residue 6' might explain the use-facilitated feature of picrotoxin. In the next chapter, the molecular determinant of use- facilitated feature of picrotoxin is resolved.

IDENTIFICATION OF A KEY RESIDUE WITHIN THE SECOND TRANSMEMBRANE DOMAIN THAT CONFERS USE-FACILITATED BLOCK BY PICROTOXIN IN GLYCINE α1 RECEPTORS

Mohammed Dibas[1], Cathy Bell-Horner[1], and Glenn H. Dillon[1]

[1] From the Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth3500 Camp Bowie Blvd., Fort Worth, TX 76107

Corresponding author:

Glenn H. Dillon, Ph.D.
Dept. of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3500 Camp Bowie Blvd.
Fort Worth, TX 76107
Phone: (817) 735-2055
Fax: (817) 735-2091
Email: **gdillon@hsc.unt.edu**

Abbreviation:
GABA, γ-aminobutyric acid; GABAA, type A GABA receptor; DMSO, dimethylsulfoxide; HeK, human embryonic kidney; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PTX, picrotoxin.

CHAPTER IV. IDENTIFICATION OF A NOVEL RESIDUE WITHIN THE SECOND TRANSMEMBRANE DOMAIN THAT CONFERS USE-FACILITATED BLOCK BY PICROTOXIN IN GLYCINE α1 RECEPTORS

SUMMARY

The CNS convulsant picrotoxin (PTX) inhibits $GABA_A$ and glutamate-gated Cl^- channels in a use-facilitated fashion, while PTX inhibition of glycine and $GABA_C$ receptors displays little or no use- facilitated block. In the present study, we identified a key residue in the extracellular aspect of the second transmembrane domain (TMII) that converted the mechanism of picrotoxin block of glycine α1 receptors from non use-facilitated to use-facilitated. In wild type α1 receptors, PTX inhibited glycine-gated Cl^- current in a concentration-dependent, competitive manner. The degree of inhibition by PTX was similar on peak and steady-state currents, confirming a lack of use-facilitated blockade. Mutation of the TMII 15' serine to glutamine (α1S15'Q receptors) dramatically altered PTX blockade. The mutation converted the mechanism of PTX blockade from competitive to non-competitive. More notable, however, was the fact that in the α1S15'Q receptors, PTX had insignificant effects on peak current amplitude, and dramatically enhanced current decay kinetics. Similar results were found in α1(S15'N) receptors. Our results implicate a specific amino acid at the extracellular aspect of the ion channel in determining use-facilitated characteristics of picrotoxin blockade of glycine α1 receptors. Moreover, the data are consistent with the suggestion that picrotoxin may interact with two domains in ligand-gated anion channels.

INTRODUCTION

Glycine receptors belong to a superfamily of ligand-gated chloride channels that include $GABA_A$ receptors, $GABA_C$ receptors, and glutamate-gated chloride channels (1). In native tissue, glycine receptors exist as either α homomers or αβ heteromers (1). They are made of five subunits (usually 3α and 2β subunits) arranged asymmetrically around the ion pore. Each subunit is made up of a large N-terminal, four transmembrane domains (TM), and a large cytoplasmic domain; TMII forms the channel lumen (2). Glycine receptors are targets of therapeutics such as anesthetics as well as toxins like the CNS convulsant picrotoxin (1).

Picrotoxin inhibits all known anionic ligand-gated Cl^- channels (3-5). The mechanism of action and the exact location of picrotoxin binding are still unknown (6-12). However, a number of studies have indicated that TMII is the probable site for picrotoxin action (6, 13-21). For example, TMII of the glycine β subunit was found to be responsible for conferring resistance to picrotoxin in heteromeric glycine αxβ receptors (x = 1-3) (6). Subsequent work defined the existence of a phenylalanine residue at the 6' position of the TMII glycine β subunit in conferring insensitivity to picrotoxin (16). In addition, other TMII residues (2' and 19') have also been implicated directly or indirectly in the mechanism by which picrotoxin inhibits these channels (13, 15, 16). Mutations at positions 2' and 19' have been shown to affect the type of the inhibition (competitive vs. non-competitive) by picrotoxin in $GABA_C$ and glycine α1 receptors, respectively (13, 20).

The ability of some antagonists to block channel activity is enhanced when the channel is open. This trait is generally referred to as use-dependent or use-facilitated block, and suggests the site of action of the antagonist may be in the channel lumen. Whereas picrotoxin block of $GABA_C$ and glycine $\alpha 1$ receptors displays weak or no use-facilitation (13, 21), picrotoxin inhibition of $GABA_A$ receptors and glutamate-gated Cl^- channels is strong use-facilitated (3, 5, 11, 12). The molecular basis underlying this trait is unknown. Thus, we sought to determine the mechanism that confers use-facilitated blockade by picrotoxin. Because of its prominent role in picrotoxin action, we focused on the TMII domain as the probable region that would underlie the differing mechanisms of picrotoxin blockade. In our analysis of the TMII domain of Cl^- channels of the LGIC superfamily, we observed that $GABA_A$ and glutamate-gated channels have neutral acidic polar residues (asparagine (N) and glutamine (Q)) at the 15' position; these receptors both demonstrate use-facilitated block by picrotoxin. Glycine $\alpha 1$ receptors, which do not display use-facilitated picrotoxin blockade, have a dissimilar residue (S) at the 15'position.

We demonstrate here that (S15'Q) and (S15'N) mutations in TMII confer use-facilitated block by picrotoxin. In addition, the S15'Q mutation converted picrotoxin block from competitive to non-competitive. Our data demonstrate the involvement of the 15' position in the mechanism of picrotoxin block, and suggest that PTX may be acting at two distinct sites in ligand-gated anion channels.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis and transient transfection The wild glycine receptor α1 was a generous gift from Dr. Betz. Mutant α1 cDNAs were kindly provided by Qing Ye, N. L. Harrison, and R.A. Harris. All cDNAs had been subcloned into the mammalian expression vector pCIS. Untransfected TSA-201 cells (transformed HEK293 cells) were plated onto 25mm coverslips. These cells were cultured as described previously (24). Glycine WT α1, α1(S15'Q) and α1(S15'N) subunits were transiently transfected using the modified calcium phosphate precipitation method (25). 15-20 μg total of DNA was used in the transfection step. Cells were analyzed electrophysiologically 48-72 hours after transfection.

Electrophysiology Whole-cell patch recordings were made at room temperature (22-25 °C). Cells were voltage-clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1-2.5 MΩ for whole-cell recordings. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. Coverslips containing cultured cells were placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 $MgCl_2$, 3.0 $CaCl_2$, 20 HEPES, 25 D-glucose, pH 7.3. Glycine-induced Cl^- currents from the whole-cell configuration of the patch clamp technique were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. Currents were low-pass filtered at 5 kHz,

monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data were not included in the analysis.

Experimental protocol Glycine with or without PTX was prepared in the extracellular solution and then was applied from independent reservoirs by gravity flow to cells using a Y-shaped tube positioned within 100 μm of the cell. With this system, the 10-90% rise time of the junction potential at the open tip is 12 - 51 ms (26). Receptors were typically activated with roughly the EC_{50} glycine concentration. Once a control glycine response was determined, the effect of PTX on the response was examined. Glycine applications were separated by at least 2-min intervals to ensure both adequate washout of glycine from the bath and recovery of receptors from desensitization, if present.

Data analysis Glycine concentration-response profiles were fitted to the following equation: $I/I_{max} = 1/(1+(EC_{50}/[glycine])^n)$, where I and I_{max} represent the normalized glycine-induced current at a given concentration and the maximum current induced by a saturating [glycine], EC_{50} is the half-maximal effective glycine concentration, and n is the Hill coefficient. The antagonism profile of the non-

competitive antagonist picrotoxin was analyzed by constructing concentration-inhibition relationships. The data were fitted to the equation: $I/I_{max}= 1/(1+(IC_{50}/[picrotoxin])^n)$, where I is the steady state current at a given concentration of picrotoxin, I_{max} is the maximum current induced by glycine, IC_{50} is the picrotoxin concentration that is half maximally effective, and n is the Hill coefficient.

Materials- Glycine and picrotoxin were obtained from Sigma (St. Louis, MO).

RESULTS

Previous work has shown that mutations at the 15' position affect glycine sensitivity to varying degrees (23). Thus, we first examined the glycine concentration-response curve for the wild type, the mutant α1(S15'Q), and mutant α1(S15N) receptors (Fig. 2). The EC_{50} and Hill coefficient for glycine in the wild type receptors were 44 ± 5.3 μM and 2.1 ± 0.5, respectively. The substitution of the 15' serine (S) by glutamine (Q) caused a roughly two-fold right shift in the glycine dose response curve. The incorporation of N at position 15' caused a dramatic rightward shift (roughly 15-fold) in the concentration-response curve for glycine. The EC_{50} and Hill coefficient values for all receptors are summarized in Table 1.

S15'Q mutation confers use-facilitated block by picrotoxin In wild type α1 receptors, picrotoxin lacks the use-facilitated feature that exists in $GABA_A$ and glutamate-gated Cl^- channels (13). We observed a similar lack of use-facilitated block in the present studies. As shown in Fig. 3A, picrotoxin inhibited the peak as well as the steady state current induced by 200 μM glycine with equal potency in the wild type receptors. Picrotoxin did not increase the rate of current decay, even at a concentration of 3 mM. Introduction of the 15'Q mutation had striking effects on picrotoxin blockade of the glycine receptor. Co-application of variable concentrations of picrotoxin (1-1000 μM) with 1 mM glycine in α1(S15'Q) receptors had little effect on initial peak current, but subsequently induced a time-dependent current decay to steady state (Fig. 3B). In addition, increasing the picrotoxin concentration enhanced the exponential decay rate of the glycine-induced current (Fig. 3B, 4). The application of increasing picrotoxin

concentration caused a concentration-dependent decrease in the time constant of the current decay τ (from 11.5 ± 1.0 sec at 3 μM PTX, to 0.23 ± 0.02 sec with 1 mM PTX, Fig. 4B). Thus, the time constant for the decay was inversely related to the concentration of picrotoxin. We also evaluated the effect of picrotoxin on the current induced by glycine in α1(S15'N) receptors. The conversion to use-facilitated block was evident in α1(S15'N) receptors as well (Fig. 3C). Thus, the incorporation of N or Q changed the mechanism by which picrotoxin inhibits glycine receptors. The α1(S15'Q) mutation had a more marked effect on picrotoxin inhibition than the α1(S15'N) mutation, and had a lesser effect on glycine sensitivity (Fig. 2). Hence, remaining experiments were conducted using α1(S15'Q) receptors.

We next tested whether increasing the glycine concentration would increase the rate of the block by picrotoxin in α1(S15'Q) receptors. When the channel was gated by 100 μM glycine, 100 μM picrotoxin enhanced the glycine current decay rate to a time constant (τ) of 4.7 ± 0.3 sec (Fig. 4A). When the α1(S15'Q) receptors were gated with a saturating glycine concentration (1 mM), 100 μM picrotoxin caused a much greater enhancement of glycine-activated current (to a τ of 0.87 ± 0.5 sec). The enhancement of current decay with an increase in glycine concentration further demonstrates the use-facilitated nature of picrotoxin blockade in the mutant receptor.

It might be argued that the mutation has affected the glycine activation kinetics, by enhancing glycine binding and/or the gating transition. In this scenario, the data would not necessarily reflect a use- facilitated block, but could instead be explained via

Picrotoxin can access its site in the S15'Q mutant in the absence of channel opening- The ability of picrotoxin to access its site is poor in the absence of agonist in $GABA_A$ and glutamate-gated Cl^- channels (3, 5). However, picrotoxin can efficiently access its site without channel opening in glycine α1 receptors (13); we confirmed this finding in the present investigation (data not shown). We subsequently tested whether the S15'Q mutation affected the channel state-dependence of picrotoxin access. Receptors were preincubated in 100 μM picrotoxin for 3 min. At the 3 min. timepoint, glycine (1 mM) and picrotoxin (100 μM) were co-applied to the cell, while still equilibrated in bath picrotoxin. As is evident from Fig. 6, pretreatment with picrotoxin abolished the use-facilitated aspect of block (Fig. 6, n = 4). A similar effect was found when using 10 μM picrotoxin. Thus, whereas the S15'Q mutation clearly induced a use-facilitated picrotoxin effect, it did not prevent picrotoxin from accessing its site in the closed channel state.

DISCUSSION

The mechanism of block by picrotoxin The inhibitory mechanism of picrotoxin in ligand-gated anion channels is a complex phenomenon. Based on the fact that the onset of picrotoxin block is facilitated in the presence of agonist in $GABA_A$- and glutamate-gated Cl^- channels, it has been suggested that picrotoxin acts as an ion channel blocker (3, 5). However, single channel analysis has shown that picrotoxin lacks the flickery effect that is typical of a classical channel blocker (12). In addition, picrotoxin inhibition is voltage-independent in these channels (3, 12, 13). Furthermore, other reports have demonstrated little or no use-facilitated block by picrotoxin in glycine and $GABA_C$ receptors, respectively (13, 21). Thus, picrotoxin might bind at an allosteric site to stabilize a closed or desensitized state of these channels (11, 12, 32). To resolve the complexity of the picrotoxin interaction with these channels, the existence of multiple binding sites for picrotoxin has been suggested (7, 10). Upon analyzing actions of picrotoxin and related compounds, Yoon et al. (7) suggested that picrotoxin might bind to both a use-dependent and a use-independent sites in $GABA_A$ receptors (7). Regardless of whether picrotoxin inhibition results from an interaction at one or two sites (additional discussion below), a molecular basis for the use-facilitated block has not been described.

In the present study, mutation of the wild type glycine α1 TMII 15' serine to glutamine (Q) or asparagine (N), which exist in glutamate- and $GABA_A$-gated Cl^- channels, respectively (3, 15), conferred use-facilitated block by picrotoxin. Use-facilitated block was concluded based on the following criteria: 1) minimal effect on initial peak current, and prominent effect on current decay kinetics; 2) glycine current

decay rate was picrotoxin concentration-dependent, and 3) current decay rate in the presence of picrotoxin was dependent on glycine concentration. None of these traits are present in wild type glycine α1 receptors, and all are observed in $GABA_A$- and glutamate-gated Cl^- channels (3, 7, 11). Thus, the introduction of N or Q at the 15' position of TMII appears to be sufficient to confer use-facilitated block by picrotoxin. At first inspection, one aspect of our data appears to be inconsistent with this conclusion. Pretreatment studies showed that picrotoxin can still access its site in the absence of channel opening. In the strictest sense, "use-dependent" implies the channel must be gated for the drug to access its site. There are a number of possibilities that could account for this somewhat unexpected finding. First, although picrotoxin is polar, it is not charged at physiological pH, and thus could access its site through both hydrophilic (the channel lumen) and hydrophobic (the lipid bilayer) pathways (5, 27). Second, even in $GABA_A$ receptors, picrotoxin can gain access to its site in the closed channel state (11, 12). The picrotoxin association rate is slowed approximately 100-fold, however, when the channel is closed (11). Nevertheless, the inference is that channel opening is not an absolute requirement for picrotoxin to access its site. For this reason, we have chosen to refer to the phenomenon as use-facilitation rather than use-dependence. Third, additional, as yet unidentified residues may exist in the vicinity of the 15' position in both $GABA_A$ and glutamate-gated Cl^- channels, but not in glycine receptors, that further interfere with picrotoxin access to its binding site in the closed state. Agonist binding in $GABA_A$ and glutamate-gated Cl^- channels could induce a conformational change that relieves the hindrance of picrotoxin binding. This is speculative, and no structural data

exists to support or refute this suggestion. Finally, we cannot definitively rule out the possibility that during our picrotoxin incubation experiments, some spontaneous channel openings may have occurred that allowed picrotoxin to access its site. Indeed, in a number of experiments we did note a modest (30-50 pA) decrease in holding current when cells were incubated in picrotoxin. This may account for some of the reduction of glycine current amplitude following picrotoxin incubation. It is worth noting that the picrotoxin-site agonist βEMGBL (β-ethyl-β-methyl-γ-butyrolactone) shows prominent use-dependent block in $GABA_A$ receptors, but can also readily access its site in the absence of channel opening (7). Indeed, in many respects the block of $GABA_A$ receptors by βEMGBL closely resembles the block of α1(S15'Q) receptors by picrotoxin we describe here. The authors of that paper advanced similar thoughts pertaining to βEMGBL access in the absence of channel opening (7).

Location of the picrotoxin site(s)- Precisely where picrotoxin binds has not been determined conclusively. A number of studies have implicated residues in the cytoplasmic aspect of TMII as the picrotoxin binding site (28-32). Mutations introduced at both the 2' and 6' positions of TMII confer PTX resistance (15, 16, 28). Picrotoxin can protect a cysteine engineered into the 2' position from irreversible modification by reactive sulfhydryl reagents (28). When cysteine is mutated into the 6' position, picrotoxin cannot protect it from suflhydryl modification. Based on these studies, it has been suggested that the picrotoxin binding site lies between the 2' and 6' positions of TMII (28, 31). However, more recent work has shown that picrotoxin can also protect a cysteine engineered into the extracellular aspect of TMII (17' position) from irreversible

modification by chemically reactive picrotoxin-related compounds (33). While not definitive, these data are consistent with earlier suggestions that picrotoxin and related ligands may interact with multiple sites (7, 10, 34, 35). The present data give additional credence to this possibility, and indicate the second site of picrotoxin interaction may exist in the 15' to 17' vicinity. In the $GABA_A$ receptor $\alpha 1$ subunit, the 15' residue appears to be directed away from the lumen of the channel (36). However, a cysteine residue at this position can be modified irreversibly by sulfhydryls, and it may form a water-filled crevice along with residues in the TMIII domain (36). Taken together, these studies indicated that this putative extracellular site and the more intracellular site, near the 2' position, could account for the use-dependent and use-independent picrotoxin sites described by Yoon et al. (7). If two picrotoxin sites do exist, they must be of comparable affinity or of significantly different efficacy, as Hill coefficients of near unity are routinely recorded in functional assays (3, 9, 11, 13,16, 28, but see 10 and 20).

Other interpretations of the data must be considered, however. A number of recent studies have demonstrated the important role of the 15' residue in modulating the effects of other ligands, including anesthetics, alcohol, barbiturates, and diazepam (37-41). The fact that the 15' position strongly influences such a structurally and functionally diverse class of drugs makes it difficult to argue that this residue might form a common binding site for all of these compounds. An alternative explanation is that it may be part of a pivotal signal transduction point through which the effects of many of these drugs converge (13). Indeed, conformational changes corresponding to different functional states are greatly influenced by mutations within TMII (13, 30, 42-43). In the present

experiments, substitution of glutamine or asparagine for the native serine may have influenced a state transition that altered picrotoxin inhibition of the channel, resulting in the use-facilitated blockade. Several studies have suggested that picrotoxin may inhibit $GABA_A$ receptors by stabilization of a desensitized state (11, 12, 30). We observed no evidence of this at the whole-cell level, but alterations in kinetic transitions can only be definitively observed with single-channel recordings.

In conclusion, we have successfully identified a residue at the extracellular aspect of TMII that confers use-facilitated (i.e., use-dependent) block by picrotoxin. It is possible that this 15' residue plays a role in a transduction step necessary for the expression of picrotoxin's inhibitory effect. Alternatively, it may form part of a second binding site for picrotoxin, the existence of which has been alluded to previously. Further studies should be conducted to explore the importance of this residue for picrotoxin action in other ligand-gated ion channels, as well as the action of other convulsants that might interact with the picrotoxin site.

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Fig. IV-1 TMII map of ligand-gated ion channels. Glycine α1 receptors, which express serine at the 15' position, display no use-facilitated block by picrotoxin. $GABA_A$ receptors incorporating the β2 subunit and glutamate-gated chloride channels (homomeric β channels, Glu-gated β) show use-facilitated inhibition by picrotoxin. These receptors express asparagine and glutamine, respectively, at the 15' position, the focus of the present study. Residues at the 2', 6' and 19' positions are also known to affect picrotoxin sensitivity.

RECEPTOR	0'	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'	16'	17'	18'	1
Gly α1	R	V	**G**	L	G	I	**T**	T	V	L	T	M	T	T	Q	**S**	S	G	S	I
GABA β2	R	V	**A**	L	G	I	**T**	T	V	L	T	M	T	T	I	**N**	T	H	L	I
Glu-gated β	R	V	**A**	L	G	V	**T**	T	L	L	T	M	T	T	M	**Q**	S	A	I	I

Fig. IV-2 Glycine sensitivity in wild type and mutant glycine α1 receptors. Both mutations induced a rightward shift in the glycine concentration-response curve. EC_{50} values were 44 ± 5.3, 102 ± 7.6, and 670 ± 38 μM in wild type, α1(S15'Q), and α1(S15'N) receptors, respectively. See Table 1 for Hill coefficients. All data points are from a minimum of 3-4 cells.

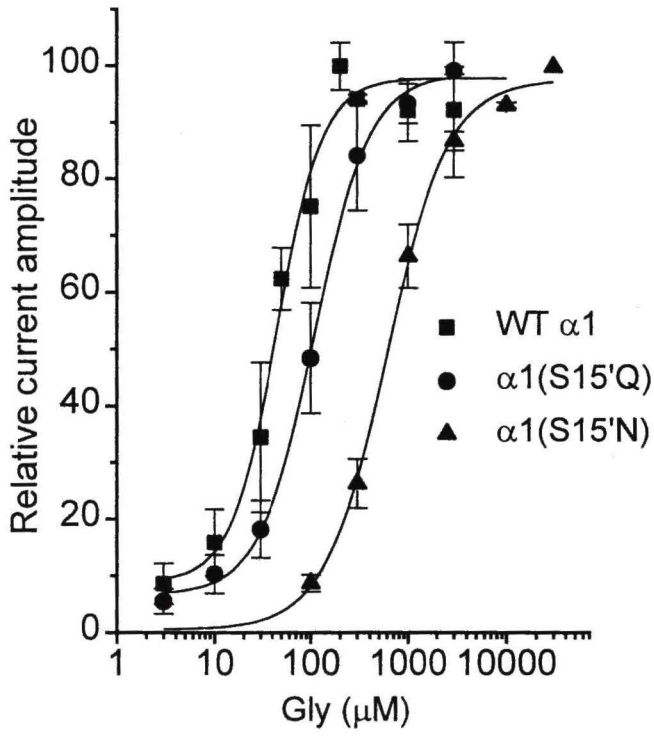
Relative current amplitude
100
80
60
40
20
0
1
10
100
1000
10000
Gly (μM)
WT α1
α1(S15'Q)
α1(S15'N)

Fig. IV-3 Use-facilitated picrotoxin block conferred by the S15'Q and S15'N mutations. A) *a,* An example of response to picrotoxin in wild type α1glycine receptors. *b,* Mean concentration-response profile for picrotoxin in the wild type receptors. Note that picrotoxin equally inhibited peak and steady-state currents. B) *a,* Response to picrotoxin in α1(S15'Q) receptors. Note insignificant effects on peak current, and marked enhancement of current decay in this receptor, indicative of use-facilitated blockade. *b,* Mean concentration-response profile for effect of picrotoxin on initial peak and steady-state currents. Picrotoxin up to 1 mM had minimal effect on peak current. C) *a,* Similar use-facilitated blockade was observed in α1(S15'N) receptors, although the degree of use-facilitation was not as great as that induced with the S15'Q mutation (*b*). Calibration bar equal to 360 pA in A, 285 pA in B and 885 pA in C. See Table 1 for IC_{50} and Hill coefficient values. Ligand application time in this and all other figures is 10 sec., unless otherwise noted.

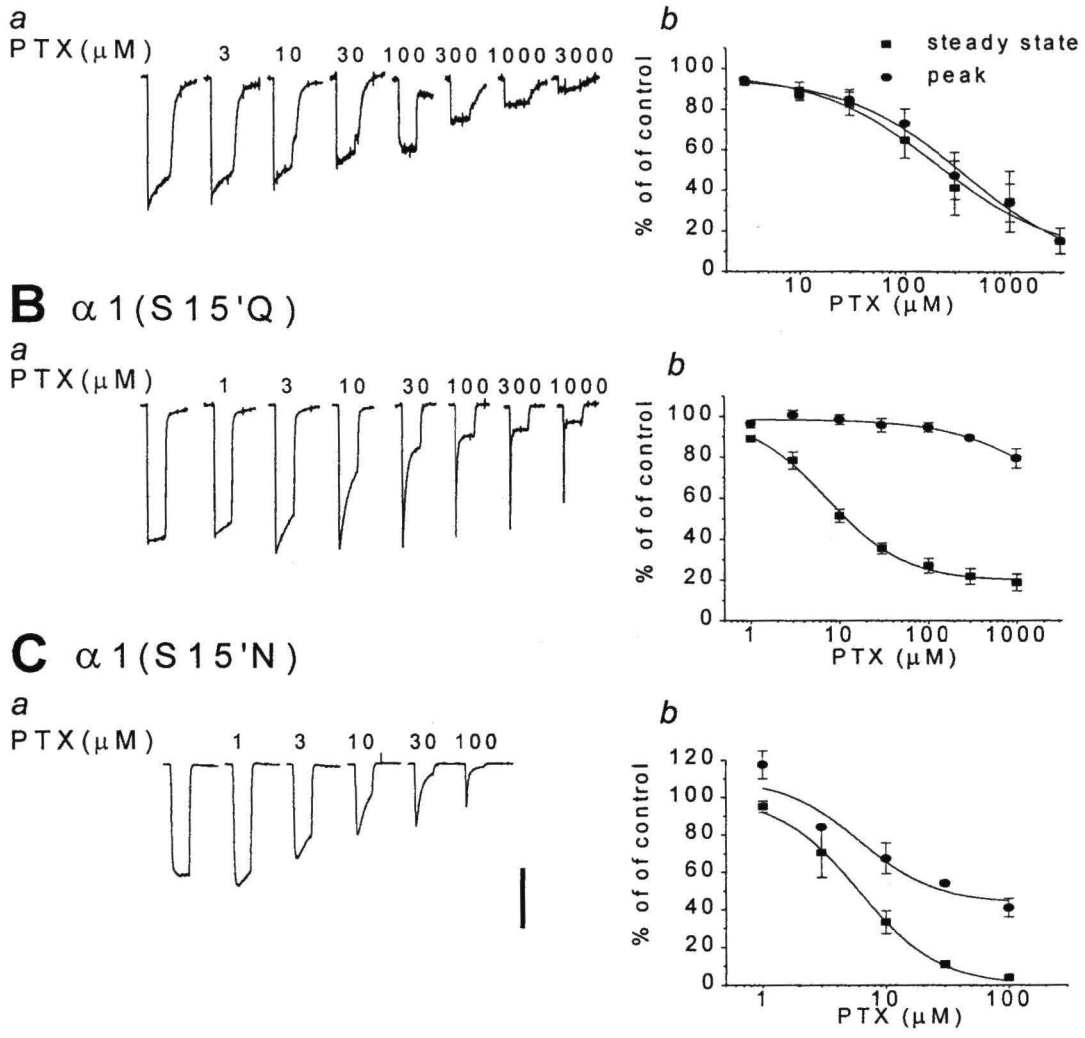
A WT α1
a
PTX(μM)
3 10 30 100 300 1000 3000
b
steady state
peak
% of of control
PTX (μM)
B α1(S15'Q)
a
PTX(μM)
1 3 10 30 100 300 1000
b
% of of control
PTX (μM)
C α1(S15'N)
a
PTX(μM)
1 3 10 30 100
b
% of of control
PTX (μM)

Fig. IV-4 Rate of block in α1(S15'Q) receptors is dependent on both glycine and picrotoxin concentration. A) Rate of picrotoxin-induced current decay is significantly slower when the channel is gated by 100 μM glycine (^{a}) than when gated by 1000 μM glycine (^{b}), a trait consistent with use-facilitated blockade. B) The time constant (τ) for use-facilitated block picrotoxin is inversely proportional to picrotoxin concentration. Decaying currents were fitted with a single exponential function. Note also the summary data for the experiment in A), illustrating a 5-fold enhancement in current decay kinetics by 100 μM picrotoxin when glycine is increased from 100 to 1000 μM. All data points are from a minimum of three cells; calibration bar equal to 175 pA in A^{a} and 375 pA in A^{b}.

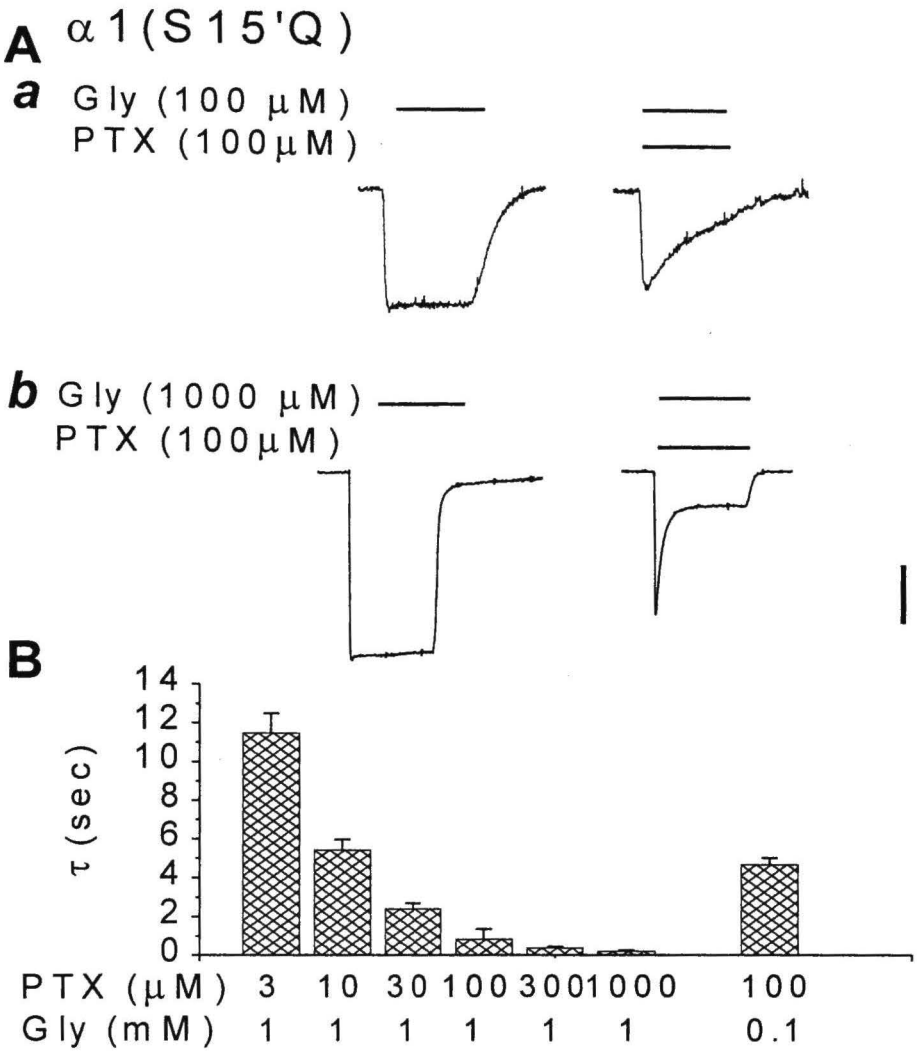
A
α1(S15'Q)
a
Gly (100 μM)
PTX (100μM)
b
Gly (1000 μM)
PTX (100μM)
B
τ (sec)
14
12
10
8
6
4
2
0
PTX (μM) 3 10 30 100 300 1000 100
Gly (mM) 1 1 1 1 1 1 0.1

Fig. IV-5 S15'Q mutation converts picrotoxin blockade from competitive to non-competitive. A) Consistent with previous findings (13), picrotoxin inhibition of wild type glycine α1 receptors is competitive. B) In contrast, picrotoxin-induced blockade in α1(S15'Q) receptors was converted to non-competitive. See Table 1 for summary information.

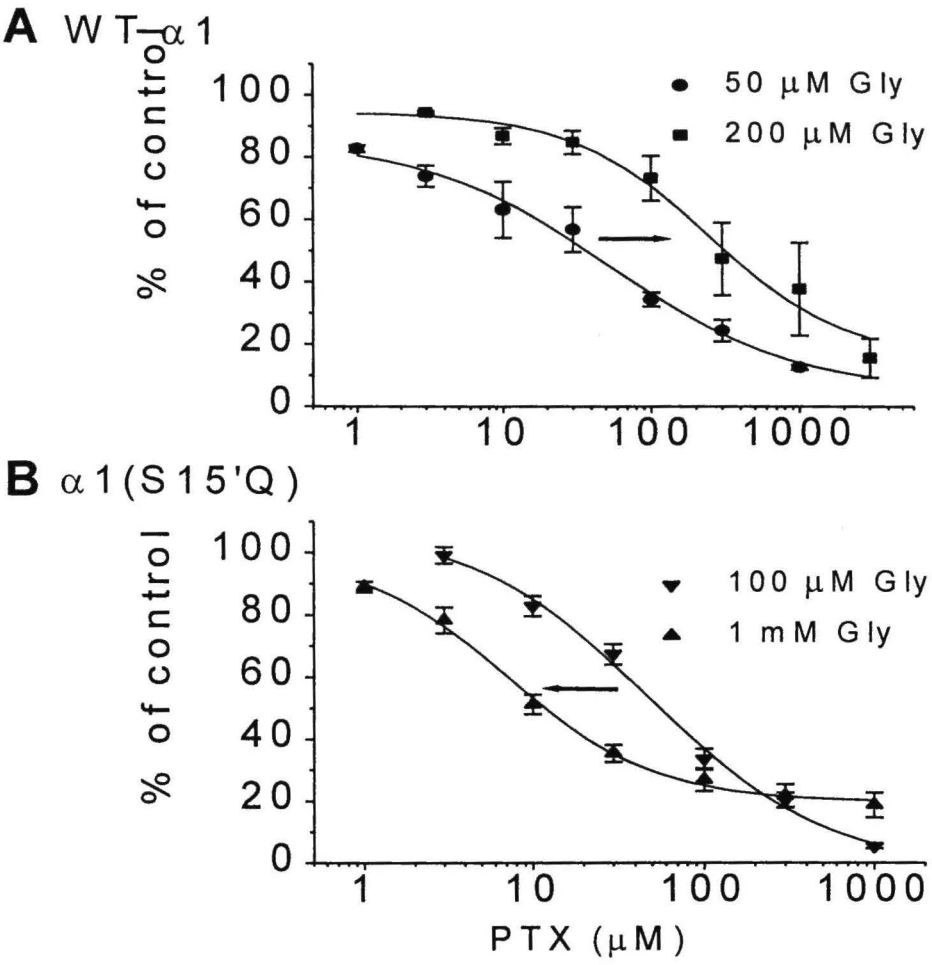
A WT α1
% of control
100
80
60
40
20
0
1
10
100
1000
50 μM Gly
200 μM Gly
B α1(S15'Q)
% of control
100
80
60
40
20
0
1
10
100
1000
PTX (μM)
100 μM Gly
1 mM Gly

Fig. IV-6 Picrotoxin can accesses its site in the absence of the glycine application in α1(S15'Q) receptors. Following establishment of the control glycine current (initial trace, 1 mM), receptors were incubated in 100 μM picrotoxin for 3 minutes. Picrotoxin and glycine were subsequently co-applied to the cells. The dotted line represents the presence of picrotoxin in the bath solution. Dashes represent 5 sec. co-application of picrotoxin (100 μM) and glycine (1 mM), separated by 3 second intervals. Similar results were obtained in 4 cells expressing the S15'Q mutation, as well as wild type glycine α1 receptors (not shown). Calibration bar equal to 230 pA.

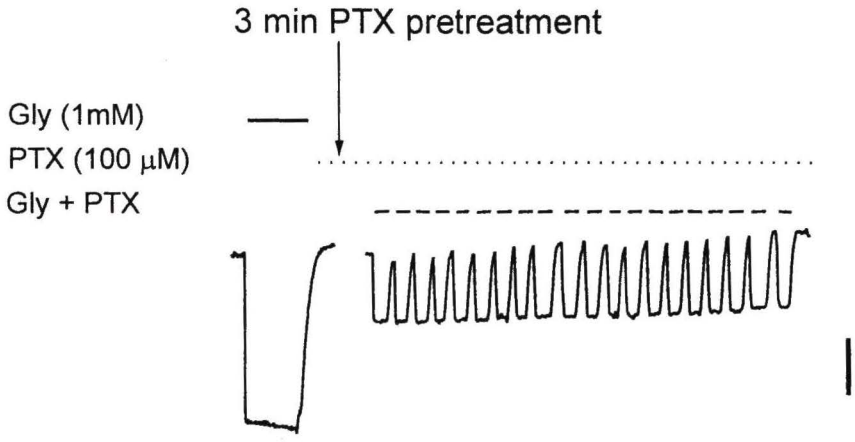
3 min PTX pretreatment
Gly (1mM)
PTX (100 μM)
Gly + PTX

Table IV-1 Glycine sensitivity and picrotoxin inhibition in wild type and mutant glycine receptors. Values for glycine EC_{50} and picrotoxin IC_{50} are in μM. PTX IC50 is the value obtained when gated by the glycine EC_{50} (PTX IC_{50}-A) or when gated by saturating glycine (PTX IC_{50}-B). n_H, Hill coefficient; n.d., not determined.

Receptor	Gly EC_{50}	Gly n_H	PTX IC_{50}-A	PTX n_H-A	PTX IC_{50}-B	PTX n_H-B
WT α1	44 ± 5.3	2.1 ± 0.5	39 ± 6	0.7 ± 0.05	339 ± 40	1.0 ± 0.13
α1 (S15'Q)	102 ± 7.6	1.5 ± 0.2	54 ± 14	0.85 ± 0.25	9.3 ± 1.5	1.0 ± 0.17
α1 (S15'N)	670 ± 38	1.5 ± 0.1	5.0 ± 0.26	1.2 ± 0.07	n.d.	n.d.

CHAPTER V. SUMMARY AND DISCUSSION

The main goal of the present study was to further understand the molecular interaction of picrotoxin and its related ligands with the anionic ligand-gated ion channels, mainly $GABA_A$ and glycine receptors. The results of these experiments further illustrate the complex mechanism by which picrotoxin inhibits these channels.

As shown earlier, the mutation (T6'F) within the second transmembrane domain of β2 subunit that abolished picrotoxin sensitivity in rat $GABA_A$ α3β2(T6'F)γ2 also abolished the inhibitory effect of other convulsive drugs like PTZ, TBPS, and U93631. Consistent with other electrophysiological and binding studies, the mutation studies further support the contention that PTX, PTZ, TBPS, and U-93631 interact with a common and probably overlapping site that is presumably within the second transmembrane domain that lines the lumen of the channel. Surprisingly, the same mutation has uncovered a stimulatory site for PTZ that is distinct from the well-known BZ site. This raises the possibility of existence of another site for picrotoxin and its related ligands to interact; this site is possibly the lactone site that is yet to be defined. However, PTZ induced stimulation might be mediated through another site that is distinct from the proposed lactone site. Unfortunately, this stimulation, which was evident in α3β2(T6'F)γ2 receptor, disappeared in the combinations α3β2(T6'F) and β2(T6'F)γ2 receptors. Thus, it was not possible to determine the if PTZ stimulation is dependent on the $GABA_A$ subunit configurations.

We further tried to determine the effect of varying the $GABA_A$ subunits on the picrotoxin pharmacology. It is logical to conclude that the incorporation of a large

moiety like phenylalanine residue at position 6' instead of the small hydrophilic threonine within TMII of β2 subunit would block the picrotoxin access to its site regardless the subunit configurations. Our data indicated this is not the case. PTX pharmacology was dramatically modulated by the subunit configurations. Whereas PTX was not able to block GABA induced current in α3β2(T6'F)γ2, it partially or completely blocked the current in α3β2(T6'F) and β2(T6'F)γ2 configurations, respectively. Moreover, the incorporation of the same mutation within the TMII of the α1 subunit renders picrotoxin sensitivity in α1(T6'F)β2γ2 receptors. The latter result was also not expected based on the published literature about the effect of the phenylalanine incorporation at position 6'. It seems that the spatial arrangement of phenylalanine is different from one combination to another and from one subunit to another. A number of studies have supported the latter finding [1, 2]. However, the mechanism by which phenylalanine existence at the 6' position affects the pharmacology of PTX is still complex and undefined. A recent study has suggested that the residue at position 6' might not reside within the picrotoxin binding site [3].

Whether the 6' residue resides in PTX recognition site or it is an important residue for PTX transduction pathway to inhibit these channels is yet to be defined. Since we do not have a fine crystal of these transmembrane channels, this hypothesis is yet to be further resolved in the future.

Much of this study dealt with the most puzzling feature of picrotoxin, (i.e. use-facilitated or use-dependent phenomenon). As noted, while PTX inhibits $GABA_A$ and glutamate channels in a use-facilitated manner, PTX inhibits glycine and GABAC

channels in non-use facilitated fashion. Using sequence homology, site directed mutagenesis, and whole cell patch clamp technique, we identified a key residue at position 15' in the second transmembrane (TMII) that controls the use-facilitated block by picrotoxin in these ligand gated anion channels. Substitution of serine at position 15' by glutamine (Q) or asparagine (N) converted the picrotoxin from non-use facilitated blocker to a use- facilitated blocker. However, the mechanism by which this residue affects the mechanism of block by picrotoxin needs to be further explored.

This latter finding underscores the complexity of picrotoxin interaction with these ion channels. The results presented in the present work along with other studies suggest the existence of two binding sites: the first one is the vicinity of 2' or 6'residues and the second site may be located near the 15' residue (as shown in Fig. 1).

One inherent difficulty in defining the mechanism of block by these convulsant drugs is the lack of structural design for these ion channels. Any mutation studies concerning the mechanism or the site of action of any drug should be interpreted carefully; mutations at the 6' or the 15' may affect directly or indirectly the site or/and the mechanism of block by picrotoxin.

In conclusion, residue 6' and 15' play key roles in the picrotoxin inhibitory effect. Future studies should be conducted to further elucidate the mechanism of picrotoxin on these ion channels.

Future directions

In the present study, the nature of amino acids at positions 6' and 15' were shown to have a dramatic effect on picrotoxin pharmacology. Our knowledge of mechanism of block by picrotoxin is enhanced but is still limited. Using single-channel recordings will help us understand the effect of these mutations on the picrotoxin kinetic effect. In addition, the effect of these mutations can be explored through knock-in techniques to evaluate the effect of the mutations on picrotoxin pharmacology in vivo. However, the most powerful tool to explore the mechanism of picrotoxin is X-ray crystallography. Recently, a bacterial potassium channel (KcsA channel) was the first ion channel to have its structure resolved at the crystal level [4]. Knowing the crystal structure of $GABA_A$ receptors in the unbound and PTX-bound states will help determine the specific domain for the convulsive drugs.

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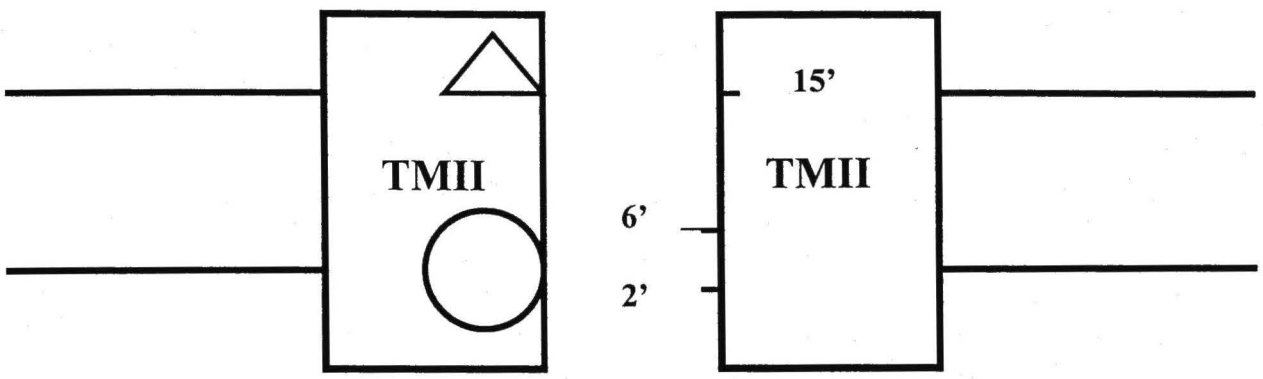
3) Shan, Q., Haddrill, J. L., and Lynch, J. W. J. Neurochem. 76: 1109-1120, 2001.

4) Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. Science 280: 69-77, 1998.

Fig. V-1 **Picrotoxin might have two binding sites;** one is near the 2' and 6' residues (circle). The second site might be located near the 15' position (rectangle).


TMII
15'
TMII
6'
2'