

# The Location of a Closed Channel Gate in the GABA<sub>A</sub> Receptor Channel

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Considerable controversy surrounds the location of the closed channel gate in members of the Cys-loop receptor family of neurotransmitter-gated ion channels that includes the GABA<sub>A</sub>, glycine, acetylcholine, and 5-HT<sub>3</sub> receptors. Cysteine-accessibility studies concluded that the gate is near the cytoplasmic end of the channel in acetylcholine and GABA<sub>A</sub> receptors but in the middle of the 5-HT<sub>3A</sub> receptor channel. Zn<sup>2+</sup> accessibility studies in a chimeric 5-HT<sub>3</sub>-ACh receptor suggested the gate is near the channel's cytoplasmic end. In the 4-Å resolution structure of the acetylcholine receptor closed state determined by cryoelectron microscopy, the narrowest region, inferred to be the gate, is in the channel's midsection from 9' to 14' but the M1–M2 loop residues at the channel's cytoplasmic end were not resolved in that structure. We used blocker trapping experiments with picrotoxin, a GABA<sub>A</sub> receptor open channel blocker, to determine whether a gate exists at a position more extracellular than the picrotoxin binding site, which is in the vicinity of α<sub>1</sub>Val257 (2') near the channel's cytoplasmic end. We show that picrotoxin can be trapped in the channel after removal of GABA. By using the state-dependent accessibility of engineered cysteines as reporters for the channel's structural state we infer that after GABA washout, with picrotoxin trapped in the channel, the channel appears to be in the closed state. We infer that a gate exists between the picrotoxin binding site and the channel's extracellular end, consistent with a closed channel gate in the middle of the channel. Given the homology with acetylcholine and 5-HT<sub>3</sub> receptors there is probably a similar gate in those channels as well. This does not preclude the existence of an additional gate at a more cytoplasmic location.

## INTRODUCTION

In the closed, resting state, ion conduction through the Cys-loop receptor neurotransmitter-gated ion channels is blocked by a gate (or gates) in the transmembrane, channel-forming domain. Agonist binding to the extracellular domain induces a propagated conformational change that opens the closed state gate, allowing ion conduction through the channel (Karlin, 2002; Auerbach, 2003; Absalom et al., 2004; Akabas, 2004; Lester et al., 2004). In the continued presence of agonist, the channels enter nonconducting desensitized state(s). The conformations of the channels are different in the closed and desensitized states, and the gate(s) that block ion conduction in these kinetically distinct states may be different (Auerbach and Akk, 1998; Williams and Akabas, 1999; Wilson and Karlin, 2001; Purohit and Grosman, 2006). To understand the conformational changes that occur as the Cys-loop receptor channels open it is important to know the location of the closed channel gate(s). Evidence from a variety of experiments has led to differing conclusions regarding the location of the closed channel gate.

The ion channel in the Cys-loop receptors is largely lined by residues from the five M2 membrane-spanning segments that form an inner ring of α helices (Imoto et al., 1988; Leonard et al., 1988; Akabas et al., 1994;

Xu and Akabas, 1996; Reeves et al., 2001; Miyazawa et al., 2003; Goren et al., 2004). The other three transmembrane segments, M1, M3, and M4, form an outer ring of helices that separate the M2 helices from the lipid bilayer (Blanton and Cohen, 1992; Miyazawa et al., 2003; Unwin, 2005). To facilitate comparisons between superfamily members, residues in the channel-lining M2 segment are referred to by an index numbering system (Miller, 1989); 0' is the absolutely conserved positively charged residue at the cytoplasmic end of M2, aligned with GABA<sub>A</sub> receptor α<sub>1</sub>Arg255, and 20' is the extracellular ring of charge, aligned with α<sub>1</sub>Asn275 (Imoto et al., 1988). The M2 segment appears to be α helical from –1' to 27' (Bera et al., 2002; Miyazawa et al., 2003).

Determining the location of the closed channel gate has been a subject of considerable controversy. Using the substituted cysteine accessibility method (SCAM), we and others showed that when applied extracellularly in the absence of agonist, charged sulfhydryl-reactive reagents reacted with cysteines substituted at the 2' level in the acetylcholine (ACh) and GABA<sub>A</sub> receptors.

Abbreviations used in this paper: ACh, acetylcholine; MTSEA<sup>+</sup>, methanethiosulfonate ethylammonium; MTSET<sup>+</sup>, methanethiosulfonate ethyltrimethylammonium; pCMBS<sup>–</sup>, p-chloromercuribenzenesulfonate; SCAM, substituted cysteine accessibility method.

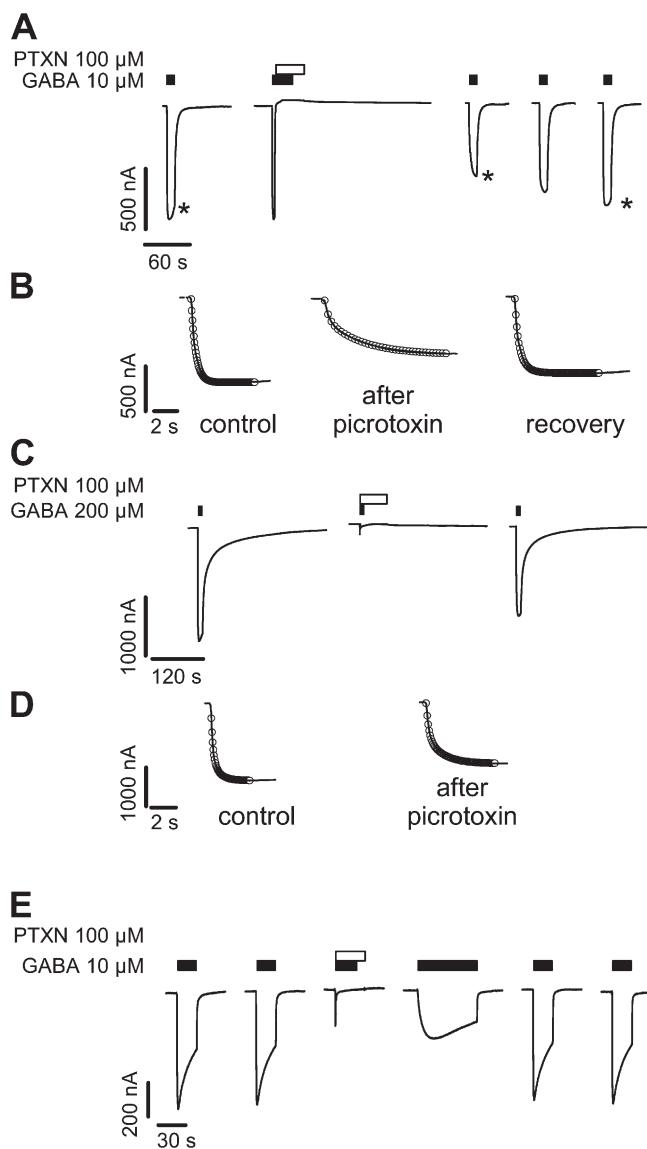
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The gate was inferred to be below the 2' level, at the channel's cytoplasmic end (Akabas et al., 1994; Xu and Akabas, 1996). The conclusions of these studies regarding the location of the channel gate were limited by the fact that the sulfhydryl reagent reaction rates were not measured. To address this shortcoming in the ACh receptor, the reaction rates of methanethiosulfonate (MTS) reagents with channel-lining, M2 segment-substituted cysteines were measured in the absence and presence of agonist (Pascual and Karlin, 1998; Wilson and Karlin, 1998). At some positions and for some reagents, rates differed by 1,000-fold, whereas at certain adjacent positions they only differed by a few fold. Based on these results and on differences in the reaction rates of sulfhydryl reagents applied from the extracellular and cytoplasmic sides in the absence of ACh, it was concluded that the gate was located near the cytoplasmic end of M2 (Wilson and Karlin, 1998). Two potential caveats to the interpretation of these results are the possibility that the cysteine mutations may increase the spontaneous open probability and/or that some of the sulfhydryl reagents might act as weak, partial agonists, thereby allowing reaction to occur in the open rather than the closed state (Akabas, 2004). A subsequent SCAM study in the 5HT<sub>3A</sub> receptor attempted to address this issue by applying the sulfhydryl reagents in the presence of a high affinity competitive antagonist. They concluded that the gate was in the middle of the channel, below the 14' level (Panicker et al., 2002). In contrast, in a chimeric ACh-5HT<sub>3</sub> receptor using a related approach, substituting histidines for M2 residues and measuring Zn<sup>2+</sup> block, it was concluded that the gate was near the cytoplasmic end of the channel (Paas et al., 2005). The problem of the mutations altering the spontaneous open probability was not addressed in this work and some of the records show evidence of significant increases in spontaneous open probability with inhibition of baseline current by Zn<sup>2+</sup> before the addition of agonist. Biochemical studies in the ACh receptor showed photoincorporation of a noncompetitive antagonist, 3-(trifluoromethyl)-3-(m-[(125)I]iodophenyl)diazirine, in the 9' and 13' region in the absence of agonist but not in the presence of agonist. This was inferred to be a hydrophobic environment in the closed state, perhaps consistent with a channel gate (White and Cohen, 1992). Finally, in the 4-Å resolution structure of the ACh receptor closed state that was solved using cryoelectron microscopic images of two-dimensional helical crystals, the narrowest region in the channel was a constriction, ~6 Å in diameter, in the midsection from 9' to 14' that was inferred to be the channel gate, "a hydrophobic girdle" (Miyazawa et al., 2003). It should be noted that the 4-Å resolution structure failed to resolve the residues in the M1-M2 loop, the gate location inferred by other studies. Molecular dynamics simulations based on the ACh receptor structure

have suggested that the 9' to 14' region may form a hydrophobic gate (Amiri et al., 2005; Corry, 2006). Thus, disparate methods have led to divergent conclusions regarding the position of the closed channel gate in Cys-loop receptors.

In the present work we sought to use an alternative approach to define the position of the channel gate in GABA<sub>A</sub> receptors. We have used open channel blocker trapping experiments to define the gate location relative to a known channel blocker binding site. Blocker trapping was used previously to determine the location of the channel gate in potassium channels (Armstrong, 1971; Holmgren et al., 1997). We used picrotoxin, a GABA<sub>A</sub> receptor open channel blocker that binds near the 2' position near the cytoplasmic end of the channel. We hypothesized that if picrotoxin was trapped in the channel after the removal of GABA by closing of a more extracellular channel gate, then the currents elicited by a subsequent GABA application should appear to activate more slowly than before picrotoxin application because picrotoxin's off-rate is slower than the GABA-induced channel opening rate (Newland and Cull-Candy, 1992; Maconochie et al., 1994). It would then be necessary to demonstrate that the picrotoxin-trapped state of the channel in the absence of GABA is the closed state and not an open-blocked or desensitized state. We used the state-dependent reactivity of various engineered cysteine residues as reporters for the channel's conformational state to show that the picrotoxin-trapped state is likely to be a closed state.

Picrotoxin inhibition of GABA<sub>A</sub> receptors is complex but most consistent with a noncompetitive, use-dependent, open channel block mechanism (Newland and Cull-Candy, 1992; Yoon et al., 1993; Chang and Weiss, 2002; Muroi et al., 2006; Olsen, 2006). Picrotoxin is an uncharged, rigid, roughly spherical molecule that is ~9 Å in diameter (Xu et al., 1995). Picrotoxin binds at a site that is only accessible from the extracellular side in the open state (Newland and Cull-Candy, 1992). Several lines of experimental evidence support the location of a picrotoxin binding site in the GABA<sub>A</sub> receptor channel at the 2' level. First, 2' mutations alter picrotoxin block (Ffrench-Constant et al., 1993; Zhang et al., 1994). Second, picrotoxin protects a cysteine substituted for α<sub>1</sub>Val257 (2') from modification by charged sulfhydryl reagents applied extracellularly without protecting cysteines substituted at more extracellular channel lining positions (Xu et al., 1995). Third, the β<sub>3</sub> 2' mutation A252V increases GABA affinity but decreases picrotoxin affinity (Buhr et al., 2001). Finally, phenylalanine substitutions at the 6' level in any subunit eliminate picrotoxin block, presumably by sterically blocking access to the more cytoplasmic 2' binding site (Gurley et al., 1995). Thus, picrotoxin appears to be a GABA<sub>A</sub> receptor open channel blocker that binds in the channel lumen at the 2' level. We



**Figure 1.** PicROTOXIN bound in the open state was trapped in the channel after channel closure and slows subsequent GABA-induced channel opening. (A) Two electrode voltage clamp recordings from wild-type  $\alpha_1\beta_1\gamma_{2S}$  expressing *Xenopus* oocyte. Black bars above traces indicate 10  $\mu\text{M}$  GABA application; open bar, 100  $\mu\text{M}$  picROTOXIN (PTXN) application. In the second trace, the GABA application was begun before the coapplication of GABA and picROTOXIN. The stars (\*) in A indicate traces shown at expanded time scale in B. Currents during 5-min washes between traces are not shown. (B) Expanded time scale of traces from A, before (control) and after (picROTOXIN) application of picROTOXIN and after recovery. Exponential fit (open circles) overlays current traces (black line). In some places the black line cannot be seen because the fitted line overlaps the data completely. Note the slowed current rise time after picROTOXIN application (picROTOXIN) compared with before picROTOXIN and after recovery. See text for average time constants. (C) Similar experiment as in A except that a saturating GABA concentration (200  $\mu\text{M}$ ) was used. The application of picROTOXIN (open bar) is concomitant with the application of GABA. (D) Extended time scale view of the first and third traces in C. Exponential fit (open circles), currents (black lines). (E) After picROTOXIN trapping, recovery from picROTOXIN-induced inhibition and return of the current to its previous amplitude and

present additional experimental support for picROTOXIN binding in the channel at the 2' level. It may also bind at a second allosteric site outside the channel (Yoon et al., 1993) and we will present evidence consistent with this and demonstrate that this interaction only decreases GABA-induced currents by 20–30%, discriminating it from the complete block caused by binding within the channel.

## MATERIALS AND METHODS

### In Vitro Transcription and Oocyte Expression

The rat GABA<sub>A</sub> receptor subunit cDNAs in the pGEMHE vector were linearized with NheI for in vitro transcription using T7 RNA polymerase (Promega) (Goren et al., 2004). mRNA was stored at  $-80^\circ\text{C}$  in diethylpyrocarbamate-treated water at a concentration of 200  $\mu\text{g}/\text{ml}$ . Stage V–VI *Xenopus laevis* oocytes were defolliculated by treatment with 2 mg/ml collagenase Type IA (Sigma-Aldrich) for 65 min. They were washed in OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5 with NaOH) and kept in OR3 (70% Leibovitz L-15 medium [Invitrogen] supplemented with 10 mM HEPES, 50  $\mu\text{g}/\text{ml}$  tetracycline, and 50  $\mu\text{g}/\text{ml}$  gentamicin). Oocytes were injected 24 h after isolation with 50 nl of a 1:1:1 mixture of  $\alpha_1:\beta_2:\gamma_{2S}$  subunit mRNA and were kept in OR3 medium for 2–4 d at  $17^\circ\text{C}$ . Mutant subunit mRNA was substituted for wild-type subunits where necessary.

### Electrophysiological Recordings

The electrophysiological recordings were conducted at room temperature in an  $\sim 250\text{-}\mu\text{l}$  chamber continuously perfused at a rate of 5–6 ml/min with nominally calcium-free frog ringer (CFRR) composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.5 with NaOH. Currents were recorded from *Xenopus* oocytes using two electrode voltage clamp recording at a holding potential of  $-60$  mV. The ground electrode was connected to the bath via a 3 M KCl/agar bridge. Glass microelectrodes filled with 3 M KCl had a resistance of  $<2$  M $\Omega$ . Data were acquired and analyzed using a TEV-200 amplifier (Dagan Instruments), a Digidata 1322A data interface (Axon Instruments), and pClamp 8 software (Axon Instruments). Currents were elicited by 10–20-s applications of GABA separated by at least 4 min of CFRR wash to allow complete recovery from desensitization. Currents were judged to be stable if the variation between consecutive GABA pulses was  $<10\%$ . GABA, picROTOXIN, and penicillin were obtained from Sigma-Aldrich.

### Sulfhydryl Reactions

Application of sulfhydryl reagents was performed as described previously (Williams and Akabas, 1999; Bera et al., 2002). Methanethiosulfonate-ethyltrimethylammonium (MTSET<sup>+</sup>) was obtained from Biotium, Inc. p-chloromercuribenzenesulfonate (pCMBS<sup>-</sup>) was obtained from Toronto Research Chemicals, Inc.

### Curve Fitting and Data Analysis

The time course of currents elicited by GABA was fitted using a mono- or biexponential function using the Chebyshev algorithm implemented in Clampfit (Axon Instruments). Data are presented as mean  $\pm$  SEM.

opening rate can be accelerated by increasing the duration of GABA application (fourth trace) rather than requiring multiple short GABA applications as in A.

## RESULTS

### Picrotoxin Application Slows the Subsequent Channel Opening Rate

We expressed wild-type rat  $\alpha_1\beta_2\gamma_{2S}$  GABA<sub>A</sub> receptors in *Xenopus* oocytes. Prior to picrotoxin application, the time course of the current increase due to application of 10  $\mu$ M GABA (EC<sub>50</sub>) could be fit with a single exponential function. The average rise time was  $340 \pm 75$  ms ( $n = 6$ ) (Fig. 1, A and B, control). Coapplication of 100  $\mu$ M picrotoxin and 10  $\mu$ M GABA almost completely inhibited the GABA-induced current (Fig. 1 A, trace 2). After inhibition by picrotoxin, GABA was washed out, presumably allowing the channels to close, and then picrotoxin was washed out for 5 min. Upon reapplication of GABA, a double exponential function was required to fit the current increase with rise times of  $400 \pm 85$  ms and  $4200 \pm 700$  ms ( $n = 6$ ). The amplitude of slow component was  $76 \pm 4\%$  and of the fast component  $24 \pm 4\%$  ( $n = 12$ ). The longer rise time was significantly slower than the initial rise time (two tailed Student's *t* test,  $P < 0.05$ ) (Fig. 1 B, compare control and picrotoxin traces). We infer that the slower opening component represented the opening of channels in which picrotoxin had remained trapped during the washout period. The faster opening component activates with a time course similar to the activation before picrotoxin application. We assume that picrotoxin exited from some of the channels either as GABA was washed out or during the 5-min period after the picrotoxin washout. The presence of this component does not affect our results. The trapped picrotoxin could be washed out of the channels either by repeated 10–20-s EC<sub>50</sub> GABA applications (Fig. 1 A, traces 3–5) or by a single prolonged application of saturating GABA (Fig. 1 E). After these GABA applications, the current rise could once again be fit with a single exponential function with a rise time of  $370 \pm 70$  ms ( $n = 6$ ) (Fig. 1 B, compare control and recovery traces). This demonstrated the expected reversibility of the picrotoxin block. Picrotoxin trapping can be repeated several times on the same oocyte (unpublished data).

Similar results were obtained using 200  $\mu$ M GABA, a saturating concentration, for the test responses before and after picrotoxin trapping. The average rise time of the GABA-induced current before picrotoxin was faster,  $150 \pm 50$  ms ( $n = 6$ ). The fast rise time was limited by the solution exchange rate at the oocyte surface, a physical constraint imposed by the size of the cell. This does not affect our results as the rise time after picrotoxin application is significantly slower than the solution exchange time. After picrotoxin washout, the current increase required a bi-exponential fit with rise times of  $150 \pm 30$  and  $1200 \pm 200$  ms ( $n = 6$ ) (Fig. 1, C and D).

Application of 100  $\mu$ M picrotoxin in GABA's absence had no effect on the current rise time of subsequent

GABA applications, consistent with picrotoxin being an open channel blocker, that is, the binding site was not accessible in the closed state (unpublished data). This is consistent with the results of Muroi et al. (2006) who showed that picrotoxin application in the absence of GABA did not alter the fluorescence intensity of site-specific fluorescent-labeled GABA receptors, whereas GABA activation did.

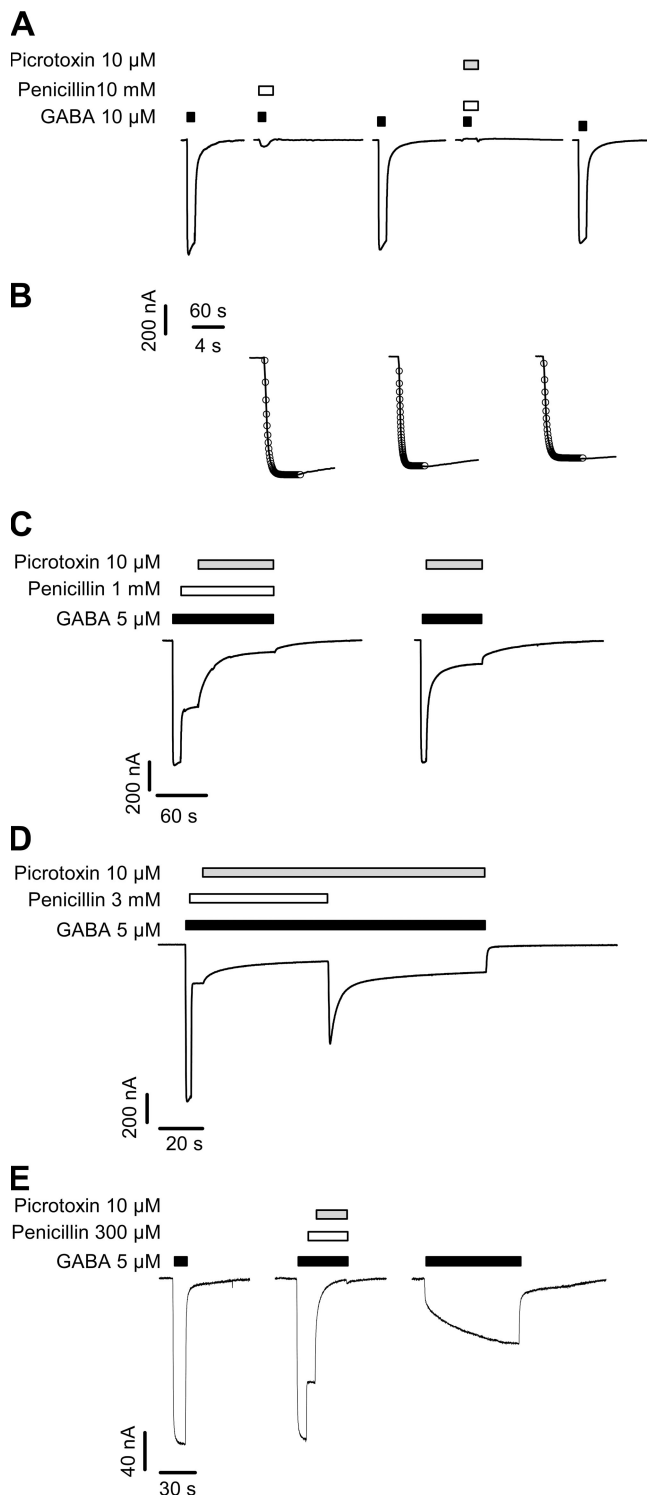
### Penicillin, a Voltage-dependent, Open Channel Blocker, Prevents Picrotoxin Block

To use picrotoxin to determine the position of the gate we must establish that picrotoxin binds in the channel. While most evidence supports the hypothesis that picrotoxin binds in the channel, some experiments have suggested the existence of a second site. Therefore, we sought to provide additional evidence that the major component of picrotoxin block is mediated via binding in the channel.

Because picrotoxin is uncharged, picrotoxin block is not voltage dependent. In contrast, penicillin is an anion at physiological pH 7.5 and blocks GABA-induced currents in a noncompetitive, voltage-dependent manner (Chow and Mathers, 1986; Twyman et al., 1992; Fujimoto et al., 1995; Sugimoto et al., 2002). The amount of block by 1 mM penicillin was  $67 \pm 3\%$  when measured at  $-60$  mV and  $79 \pm 3\%$  at 0 mV ( $n = 4$ ) (unpublished data). The voltage dependence is consistent with the penicillin binding/blocking site being in the ion channel where it can be influenced by the transmembrane voltage.

Penicillin (10 mM) blocked  $\sim 95\%$  of the GABA-induced current but did not alter the subsequent rate of channel reopening (Fig. 2, A and B). We could not detect penicillin trapping in the closed channel. This may be due to the low affinity of penicillin for the channel and its high on and off rates (Chow and Mathers, 1986; Twyman et al., 1992). Alternatively, penicillin may bind at a position that prevents channel closing while penicillin is in the channel, as was seen with QX-222 in the ACh receptor channel (Neher and Steinbach, 1978).

When coapplied, 10 mM penicillin and 10  $\mu$ M picrotoxin blocked the GABA-induced current (Fig. 2 A, trace 4) but after a 5-min wash, the subsequent channel opening rate was not slowed (Fig. 2, A and B, right trace). We infer that penicillin, with a higher on-rate, blocked the majority of the channels, preventing picrotoxin from binding. Thus, after washout of picrotoxin and penicillin from the bath, the subsequent channel opening rate was not affected by the prior presence of picrotoxin because in the presence of both penicillin and picrotoxin most of the channels had been blocked by penicillin and not by picrotoxin. This is consistent with a competitive interaction between penicillin and picrotoxin for inhibition of GABA<sub>A</sub> receptors. This



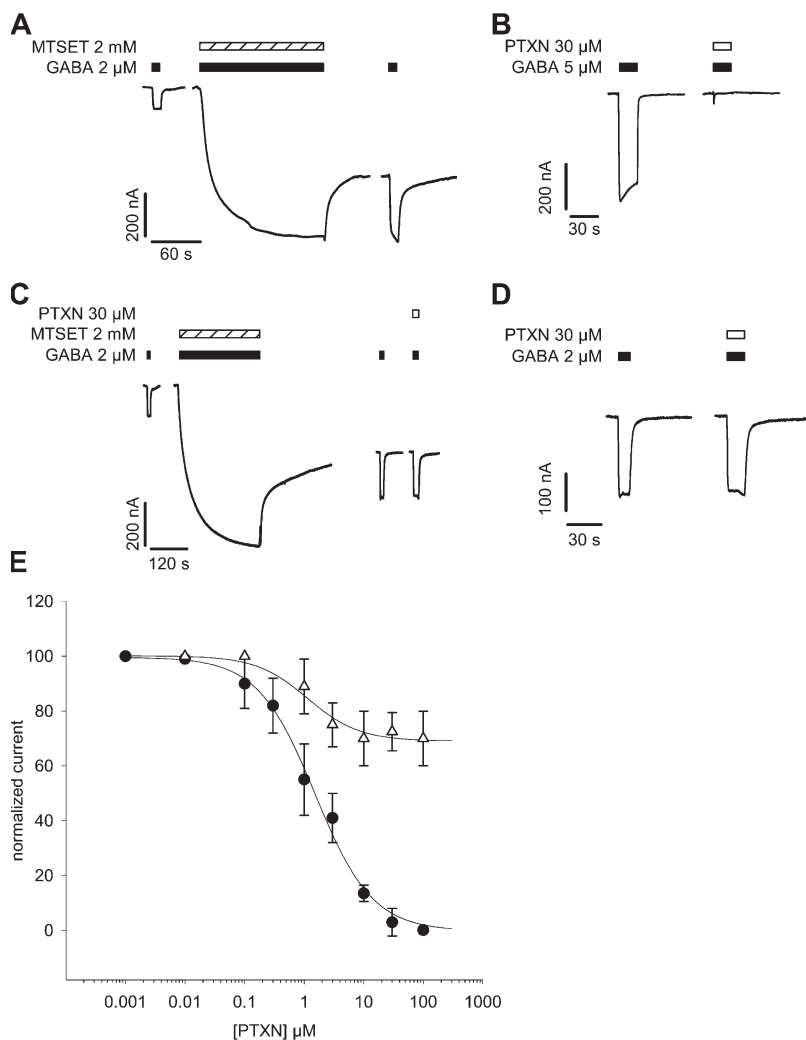
**Figure 2.** Mutually exclusive inhibition of GABA<sub>A</sub> receptors by penicillin and picrotoxin. (A) Application of 10 mM penicillin in the presence of EC<sub>50</sub> GABA (10  $\mu$ M) followed by coapplication of 10 mM penicillin and 10  $\mu$ M picrotoxin in the presence of the same GABA concentration. (B) Expanded time scale of the GABA-induced currents from A before (left), after penicillin alone (center), and after coapplication of penicillin and picrotoxin (right). Experimental values of the opening time constants were not significantly different and all were well fit by a monoexponential function. This implies that coapplication of penicillin and picro-

toxin prevented picrotoxin trapping. (C) Comparison of the extent of inhibition of GABA-induced current by 10  $\mu$ M picrotoxin after inhibition by a submaximal concentration of penicillin (left) and without penicillin (right). The amount of current inhibited by picrotoxin does not depend on the presence of penicillin, and is, in this illustrative example, equal to 80% of the remaining current. (D) During a continuous 5  $\mu$ M GABA (dark bar) application, channels were first blocked by a submaximal 3 mM penicillin application (white bar) and then by 10  $\mu$ M picrotoxin application (gray bar) in the ongoing presence of penicillin and GABA. Washout of the penicillin led to a rapid rise in the current and a subsequent fall as picrotoxin blocked the channels. This demonstrates that even in the presence of picrotoxin, the penicillin-blocked channels were not inhibited by picrotoxin until the penicillin was washed out. (E) Picrotoxin trapping can occur in the presence of a submaximal penicillin concentration. After application of GABA, application of 300  $\mu$ M penicillin led to a 35% block. After steady state is reached, 10  $\mu$ M picrotoxin completely inhibited the residual current. Note that when picrotoxin, penicillin, and GABA are removed, the residual current is close to zero. The third trace shows the current recorded after activation by 5  $\mu$ M GABA. A biexponential function is needed to fit the current increase and the rapid component represents 35% of the total, a fraction equal to the amount of current blocked by penicillin initially.

interaction could arise because penicillin and picrotoxin bind at the same or overlapping sites. Alternatively, penicillin may bind at a more extracellular position in the channel and prevent picrotoxin from gaining access to a more cytoplasmically located binding site. We cannot distinguish between these possibilities but it does not alter the interpretation that this result provides support for the presence of a picrotoxin binding site in the channel lumen that mediates the picrotoxin trapping shown in Fig. 1.

Additional evidence to support a competitive interaction between penicillin and picrotoxin comes from sequential application and washout experiments. In the presence of GABA, 3 mM penicillin blocks  $\sim$ 80% of the GABA current (Fig. 2 D). In the presence of GABA and penicillin, application of 10  $\mu$ M picrotoxin, a concentration that by itself blocks  $\sim$ 80% of the GABA-induced current (Fig. 2 C, right trace), blocked most of the remaining current (Fig. 2 D). Washout of penicillin, in the continuing presence of GABA and picrotoxin, resulted in a transient current increase that was blocked, presumably by picrotoxin still present in the bath (Fig. 2 D). The current appearing upon penicillin washout arose because the off-rate of penicillin is much faster than the on-rate for picrotoxin. This implies that picrotoxin could not bind to penicillin-blocked channels because if both picrotoxin and penicillin were simultaneously bound and inhibiting the GABA<sub>A</sub> receptors, then penicillin washout would not have resulted in a transient rise in the current. In contrast, once penicillin is removed, the channels that were blocked by penicillin are opened and can be blocked by picrotoxin, which is once again consistent with a competitive interaction most likely in the channel lumen.

toxin prevented picrotoxin trapping. (C) Comparison of the extent of inhibition of GABA-induced current by 10  $\mu$ M picrotoxin after inhibition by a submaximal concentration of penicillin (left) and without penicillin (right). The amount of current inhibited by picrotoxin does not depend on the presence of penicillin, and is, in this illustrative example, equal to 80% of the remaining current. (D) During a continuous 5  $\mu$ M GABA (dark bar) application, channels were first blocked by a submaximal 3 mM penicillin application (white bar) and then by 10  $\mu$ M picrotoxin application (gray bar) in the ongoing presence of penicillin and GABA. Washout of the penicillin led to a rapid rise in the current and a subsequent fall as picrotoxin blocked the channels. This demonstrates that even in the presence of picrotoxin, the penicillin-blocked channels were not inhibited by picrotoxin until the penicillin was washed out. (E) Picrotoxin trapping can occur in the presence of a submaximal penicillin concentration. After application of GABA, application of 300  $\mu$ M penicillin led to a 35% block. After steady state is reached, 10  $\mu$ M picrotoxin completely inhibited the residual current. Note that when picrotoxin, penicillin, and GABA are removed, the residual current is close to zero. The third trace shows the current recorded after activation by 5  $\mu$ M GABA. A biexponential function is needed to fit the current increase and the rapid component represents 35% of the total, a fraction equal to the amount of current blocked by penicillin initially.



**Figure 3.** In  $\alpha_1A254E/V257C\beta_1\gamma_{2s}$  receptors, modification of the 2' substituted-cysteine residue by covalent reaction with the positively charged MTSET<sup>+</sup> increased the GABA current amplitude but reduced sensitivity to picrotoxin. (A) Currents from an oocyte expressing  $\alpha_1A254E/V257C\beta_1\gamma_{2s}$  receptors. Application of 2 mM MTSET<sup>+</sup> for 150 s in the presence of 2 μM GABA results in a significant increase in the GABA-induced current magnitude both during and after the application of MTSET<sup>+</sup> and causes an increase in the holding current in the absence of GABA after MTSET<sup>+</sup> washout. (B) Prior to MTSET<sup>+</sup> application, 30 μM picrotoxin inhibits the currents induced by 5 μM GABA in an oocyte expressing  $\alpha_1A254E/V257C\beta_1\gamma_{2s}$  receptors. (C) After reaction with 2 mM MTSET<sup>+</sup> in the presence of GABA, inhibition by picrotoxin is markedly reduced. Note that the two GABA test currents after MTSET<sup>+</sup> application are similar in size although one is with 30 μM picrotoxin and the other has no picrotoxin. Note also that 30 μM picrotoxin has no effect on the large holding current. These two traces are shown enlarged in D. (D) Enlargement of the final two traces in C showing the limited effect of 30 μM picrotoxin on the GABA-induced currents after MTSET<sup>+</sup> modification of  $\alpha_1A254E/V257C\beta_1\gamma_{2s}$  channels. (E) Normalized picrotoxin concentration-inhibition relationship in the double mutant  $\alpha_1A254E/V257C$  before (dark circle) and after (open triangle) treatment with 2 mM MTSET<sup>+</sup> for 1 min. Each point represents mean  $\pm$  SEM of at least three experimental determinations. Note that the reaction with the substituted cysteine in  $\alpha_1A254E/V257C$  channels goes to completion during a 1-min application of 2 mM MTSET<sup>+</sup>.

Finally, we first applied a submaximal concentration of penicillin (300 μM) to block about one third of the GABA current and then applied 10 μM picrotoxin to block the remainder of the GABA current (Fig. 2 E, trace 2). 5 min after simultaneous washout of GABA, penicillin, and picrotoxin, the best fit to the rise of the GABA current was a biexponential function where the fast component accounted for about one third of the total current (Fig. 2 E, trace 3). This result is also consistent with a mutually exclusive interaction of penicillin and picrotoxin with GABA<sub>A</sub> receptors. Thus, in light of the voltage dependence of penicillin block, which implies that penicillin binds in the channel, and the competition between penicillin and picrotoxin that we have demonstrated, we infer that picrotoxin also binds in the channel lumen.

#### Picrotoxin Binds in the Channel Near the 2' Position

We previously showed that anionic sulfhydryl-reactive reagents reacted with an engineered cysteine at the 2' position in  $\alpha_1V257C\beta_1\gamma_2$  receptors, inhibiting subsequent GABA-induced currents, whereas cationic reagents did

not react (Xu and Akabas, 1996). The inhibition after modification of  $\alpha_1V257C$  by anionic sulfhydryl reagents precluded the possibility of determining whether the modification prevented picrotoxin binding. To circumvent this problem we used a double mutant,  $\alpha_1A254E/V257C$  with a cysteine at the 2' position and a glutamate at the adjacent -1' position, the amino acid found at the aligned position in the ACh receptor intermediate ring of charge (Imoto et al., 1988). In oocytes expressing  $\alpha_1A254E/V257C\beta_2\gamma_2$ , a 150-s coapplication of GABA and 2 mM MTSET<sup>+</sup> caused a significant increase in the GABA-induced current during the application and resulted in a significant increase in the holding current after GABA washout (Fig. 3 A). We infer that in the presence of the engineered -1' glutamate, the cationic MTSET<sup>+</sup> was able to penetrate sufficiently deep into the channel to allow reaction with the engineered 2' cysteine. The increased holding current after MTSET<sup>+</sup> modification implies that the spontaneous open probability was increased in the MTSET<sup>+</sup>-modified channels. As can be seen in the figure (Fig. 3 A), the reaction went to completion during the 150-s application.

Prior to reaction with MTSET<sup>+</sup>, the  $\alpha_1A254E/V257C\beta_2\gamma_2$  receptors were almost completely blocked by picrotoxin (Fig. 3 B) in a concentration-dependent manner (Fig. 3 E, filled circles). After reaction with MTSET<sup>+</sup>, the extent of inhibition by picrotoxin was significantly reduced but not eliminated (Fig. 3 D). The increased holding current was not inhibited but the remaining GABA-induced current was inhibited by ~20% in a concentration-dependent manner (Fig. 3 E, open triangles). We infer that covalently modifying the 2' cysteine in one or both  $\alpha_1$  subunits with the positively charged thioethyltrimethylammonium portion of MTSET<sup>+</sup> significantly increases the Cl<sup>-</sup> conductance but prevents picrotoxin from binding in the channel at its channel-blocking, binding site. These data provide evidence, in addition to previously published results (Ffrench-Constant et al., 1993; Zhang et al., 1994; Gurley et al., 1995; Wang et al., 1995; Xu et al., 1995), to support the contention that picrotoxin binds in the GABA<sub>A</sub> receptor channel near the 2' level.

The 20% residual inhibition by picrotoxin after MTSET<sup>+</sup> reaction could be due to incomplete reaction of MTSET<sup>+</sup> with the channels but as we noted above, the MTSET<sup>+</sup> reaction appeared to go to completion (Fig. 3 A). Some of the Cys could be modified by endogenous sulfhydryls or oxidized to higher order oxidation states that preclude reaction with MTSET<sup>+</sup>. Alternatively, the residual inhibition may be due to binding at another site on the GABA<sub>A</sub> receptor, outside of the channel, where inhibition is mediated by allosteric effects rather than by direct channel block. The existence of a second allosteric site outside the channel has been suggested by other investigators (Yoon et al., 1993).

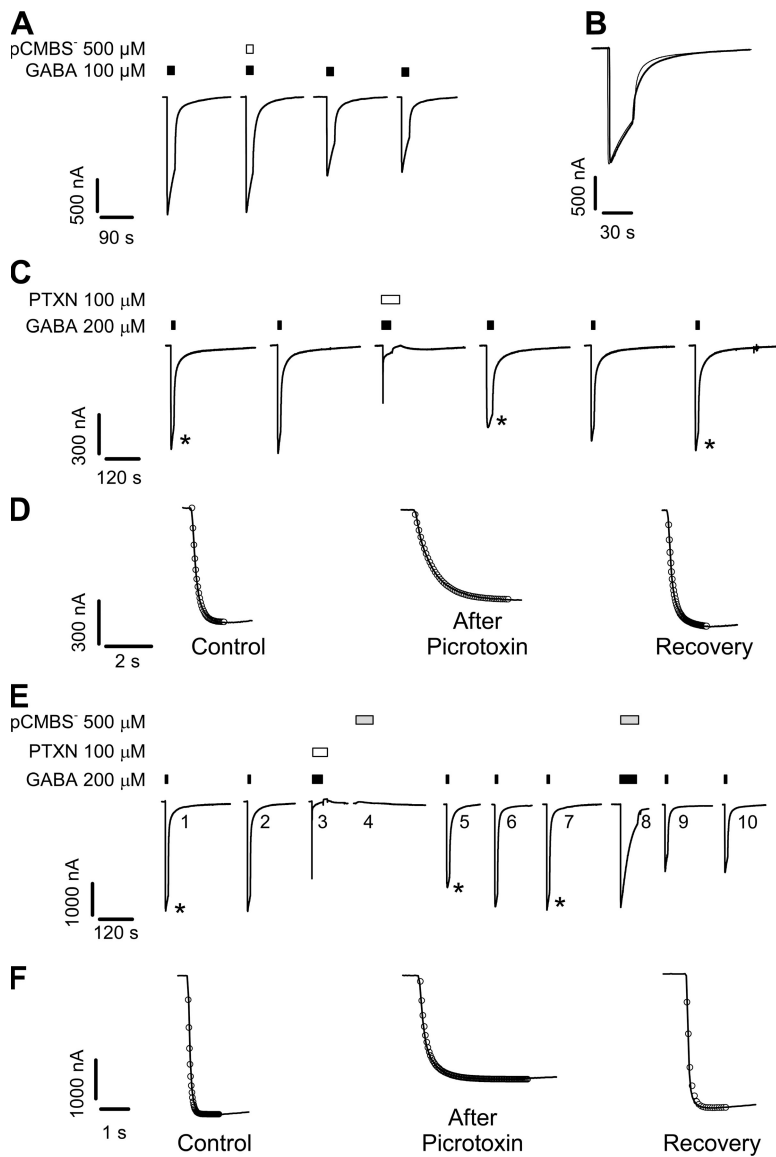
#### In the Absence of GABA, the Picrotoxin-blocked Channel Appears To Be in the Close State and Not in the Desensitized State

Thus far we have provided evidence to support the hypothesis that picrotoxin binds within the GABA<sub>A</sub> receptor channel. Furthermore, we have shown the picrotoxin can be trapped in channels after the removal of GABA. To use picrotoxin trapping to locate the position of a closed channel gate we need to determine the conformational state of the picrotoxin-trapped (presumably agonist-free) channels. To accomplish this we used the state-dependent reactivity of two engineered cysteines,  $\alpha_1E303C$  and  $\alpha_1V280C$ , as reporters for the channel's conformational state. Previously, we showed that reaction rates at these positions were state dependent (Williams and Akabas, 1999, 2000, 2001; Bera et al., 2002). We chose these positions because in the structure of the homologous ACh receptor the aligned residues are separated by 20–30 Å and both are a similar distance from the picrotoxin binding site (Miyazawa et al., 2003). This separation is greater than the 9-Å diameter of picro-

toxin so that picrotoxin could not simultaneously protect both positions.

The mutant  $\alpha_1E303C$  (in M3) fulfills two conditions that are important for this particular experiment. (1) pCMBS<sup>-</sup> does not react with  $\alpha_1E303C$  in the absence of GABA where the channels are presumably in the closed state (Williams and Akabas, 1999). (2) As we showed previously (Williams and Akabas, 1999), reaction with pCMBS<sup>-</sup> occurs in the presence of GABA because the subsequent GABA test currents are reduced (Fig. 4 A, compare the magnitude of trace 1 with traces 3 and 4). However, the reaction in the presence of GABA does not appear to occur in the open state because the current during the application of GABA + pCMBS is not altered compared with the current induced by GABA alone (Fig. 4 B). Note that the initial GABA-induced current and the current during the GABA + pCMBS<sup>-</sup> application can be superimposed (Fig. 4 B). This leads us to conclude that reaction of pCMBS<sup>-</sup> with  $\alpha_1E303C$  in the presence of GABA most likely occurs in a desensitized state but it cannot be the fast desensitized state from which channels frequently return to the open state. We infer that reaction occurs in a deeper desensitized state from which channels rarely return to the open state. Therefore, if pCMBS<sup>-</sup> reacts with  $\alpha_1E303C$  in the picrotoxin-trapped state it would suggest that this is a desensitized state.

Picrotoxin applied in the presence of GABA can be trapped in the  $\alpha_1E303C\beta_1\gamma_{2S}$  channels just as in wild-type channels. This trapping resulted in slowed channel opening after picrotoxin trapping and the need for a biexponential fit to the opening rate after picrotoxin was trapped (Fig. 4, C and D). We then examined the reactivity of pCMBS<sup>-</sup> with  $\alpha_1E303C$  in the picrotoxin-trapped state (Fig. 4 E). After two GABA test pulses (Fig. 4 E, traces 1 and 2), we coapplied picrotoxin and GABA and washed them out to create the picrotoxin-trapped state (Fig. 4 E, trace 3). Next we applied 500  $\mu$ M pCMBS<sup>-</sup> for 1 min in the absence of GABA (Fig. 4 E, trace 4). After pCMBS<sup>-</sup> washout, we applied 200  $\mu$ M GABA twice to allow complete escape of the trapped picrotoxin (Fig. 4 E, traces 5 and 6). After allowing time for recovery from desensitization, we applied a GABA test pulse (Fig. 4 E, trace 7), which had the same magnitude and time course as the initial GABA currents (trace 1). This implies that the cysteine at  $\alpha_1E303C$  was not modified during the pCMBS<sup>-</sup> application to the picrotoxin-trapped channels. To demonstrate that the cysteine was still available to react with pCMBS<sup>-</sup>, we next applied pCMBS<sup>-</sup> in the presence of GABA (Fig. 4 E, trace 8). The subsequent GABA test currents (Fig. 4 E, traces 9 and 10) were markedly reduced compared with the previous test current (Fig. 4 E, trace 7). This demonstrates that the cysteine did not react during the first pCMBS<sup>-</sup> application in the picrotoxin-trapped channels because it was available to react during the second pCMBS<sup>-</sup>



**Figure 4.** In the picROTOXIN-trapped (agonist-free) state, the lack of reactivity of the cysteine-substitution mutant  $\alpha_1E303C\beta_1\gamma_{2S}$  suggests that this state is not a desensitized state. (A) Current recordings from an oocyte expressing  $\alpha_1E303C\beta_1\gamma_{2S}$  illustrating the effect of pCMBS<sup>-</sup> application in the presence of GABA on the currents. The first trace shows the response to 100  $\mu$ M GABA. The second trace illustrates the response to coapplication of 100  $\mu$ M GABA and 500  $\mu$ M pCMBS<sup>-</sup>. The third and fourth traces show the response to 100  $\mu$ M GABA after the pCMBS<sup>-</sup> application. Note that the current magnitudes after the pCMBS<sup>-</sup> application are significantly smaller than the initial current, indicating that the pCMBS<sup>-</sup> has covalently modified the substituted cysteine. However, note that the current during the coapplication of pCMBS<sup>-</sup> and GABA is virtually identical to the initial current. This can be seen more clearly in B where the first and second traces are overlaid on an expanded time scale. (B) Overlay of the first two current traces in A on an expanded time scale illustrating that the currents in the presence of GABA and during coapplication of GABA and pCMBS<sup>-</sup> are similar. This implies that pCMBS<sup>-</sup> must react in a nonconducting state of the receptor that rarely returns to the open state, i.e., a desensitized state. (C) Current recordings demonstrating that picROTOXIN was trapped in the  $\alpha_1E303C\beta_1\gamma_{2S}$  receptors. Black bars above traces indicate application of saturating 200  $\mu$ M GABA; open bar, 100  $\mu$ M picROTOXIN. The stars (\*) in C indicate traces shown at expanded time scale in D. (D) Expanded time scale of traces from C with exponential fits (open circles) overlaid as described in Fig. 1 B legend. Average current rise time before picROTOXIN was 165  $\pm$  50 ms ( $n$  = 6). For the first GABA current after picROTOXIN washout, a biexponential function was required with rise times of 250  $\pm$  75 ms and 1200  $\pm$  350 ms ( $n$  = 6). After recovery, the current rise could be fit with a single exponential with a rise time of 240  $\pm$  70 ms ( $n$  = 6). (E) With picROTOXIN trapped, 500  $\mu$ M pCMBS<sup>-</sup> was applied (gray bar, trace 4). After the pCMBS<sup>-</sup> application, GABA was applied twice to washout the picROTOXIN (traces 5 and 6). The subsequent GABA

current (trace 7), after the picROTOXIN washout, is of similar magnitude to the initial GABA test currents (traces 1 and 2), indicating that pCMBS<sup>-</sup> has not modified the engineered Cys. Subsequent application of 500  $\mu$ M pCMBS<sup>-</sup> + GABA (trace 8) resulted in the irreversible inhibition of the subsequent GABA currents (traces 9 and 10) with no recovery over time, as we observed previously (Williams and Akabas, 1999). The individual current traces are numbered to correspond to the text. \* indicates traces shown on an expanded time scale in F. (F) Expanded time scale of traces 1, 5, and 7 (indicated with \*) from E showing a monoexponential fit to traces 1 and 7 and slowed reopening in trace 5 requiring a biexponential fit to the current rise. This demonstrates that picROTOXIN was trapped during the first pCMBS<sup>-</sup> application and removed after the picROTOXIN washout.

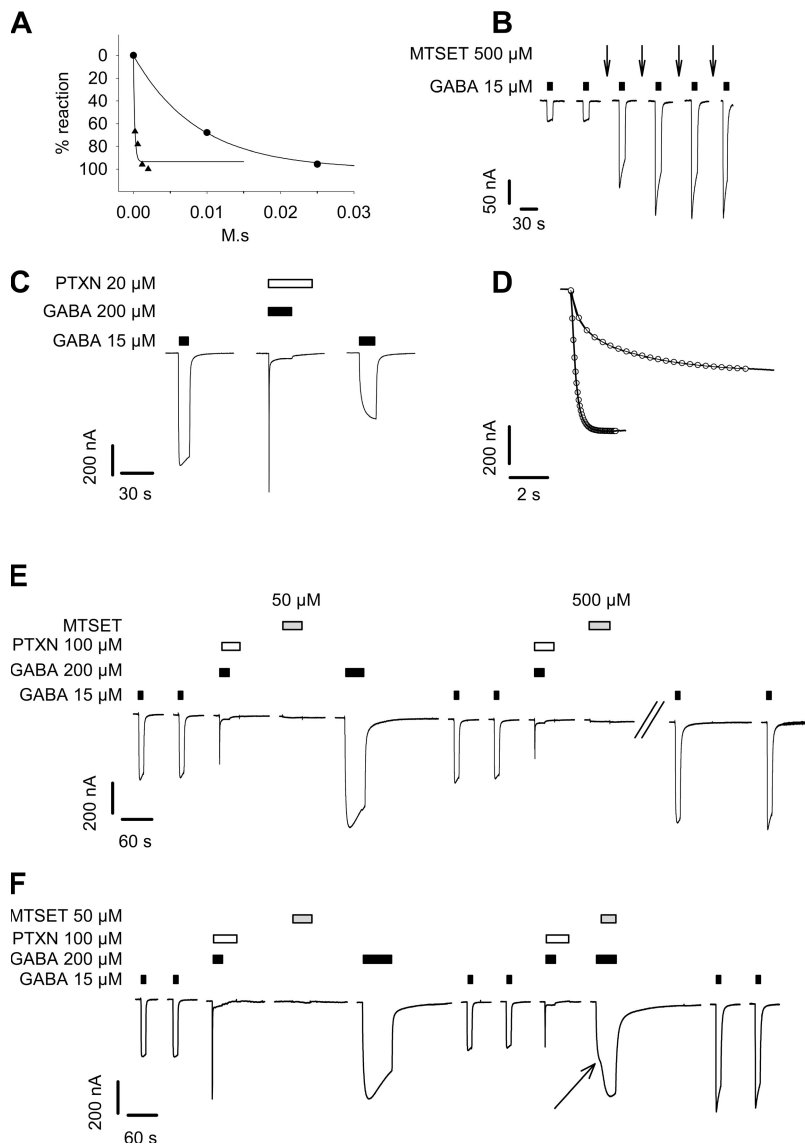
application in the presence of GABA. Based on the fact that  $\alpha_1E303C$  does not react in the closed state but does react in an activated/desensitized state (Williams and Akabas, 1999), we infer that the picROTOXIN-trapped state is not a desensitized state.

#### In the Absence of GABA, the PicROTOXIN-blocked Channel Is in the Closed State

To provide further support for the hypothesis that the picROTOXIN-trapped channels are in the closed state we used another cysteine mutant,  $\alpha_1V280C$  (M2 25'), that is located near the extracellular end of the channel-lining

M2 segment. Reaction with MTSET<sup>+</sup> significantly potentiated the subsequent GABA-induced currents (Fig. 5 B). The reaction rate of MTSET<sup>+</sup> with this mutant was approximately seven times faster in the presence of GABA than in its absence (closed state rate 185 M<sup>-1</sup>s<sup>-1</sup>; open/activated state rate 1353 M<sup>-1</sup>s<sup>-1</sup>) (Fig. 5, A and B) (Bera et al., 2002). We could therefore use the MTSET<sup>+</sup> reaction rate with  $\alpha_1V280C$  as a marker for the closed state. As with wild-type receptors, picROTOXIN was trapped inside  $\alpha_1V280C\beta_1\gamma_{2S}$  receptors, leading to a slower opening rate after picROTOXIN trapping that was best fit by a double exponential function (Fig. 5, C and D).





**Figure 5.** MTSET<sup>+</sup> reactivity with the cysteine substitution mutant  $\alpha_1$ V280C $\beta_1$  $\gamma_{25}$  supports the inference that the picrotoxin-trapped state is structurally similar to the closed channel. (A) The reaction rate of MTSET<sup>+</sup> with  $\alpha_1$ V280C $\beta_1$  $\gamma_{25}$  receptors is sevenfold faster in the presence of GABA than in its absence. Graph shows the cumulative effect of MTSET<sup>+</sup> applied in the absence (circles) and in the presence (triangles) of a submaximal GABA concentration. Note the units for the abscissa axis M-s represents the product of the concentration and the duration of MTSET<sup>+</sup> application. (B) Data used to construct the plot shown in A (dark triangles). GABA-induced test currents after repeated applications of a submaximal concentration of MTSET<sup>+</sup> in the presence of GABA (traces during MTSET<sup>+</sup> application are not shown). Note the significant increase in the GABA-induced currents after MTSET<sup>+</sup> application, indicating covalent modification of the substituted cysteine. (C) Picrotoxin is trapped in the  $\alpha_1$ V280C $\beta_1$  $\gamma_{25}$  channels as it was in wild-type channels. The rate of current rise after coapplication of GABA and picrotoxin is significantly slower than before picrotoxin trapping. (D) Overlay of the first and third traces from C on an expanded scale (dark line) along with the best mono- and biexponential fits (open circles) for the current traces before and after trapping, respectively. (E) Illustration that a 50  $\mu$ M MTSET<sup>+</sup> application that would lead to complete reaction in channels in the activated state has no effect on the picrotoxin-trapped channels, but a 500  $\mu$ M MTSET<sup>+</sup> application that would lead to complete reaction in channels in the closed state reacts completely. After two initial GABA test pulses, GABA and picrotoxin are coapplied to completely block the  $\alpha_1$ V280C $\beta_1$  $\gamma_{25}$  channels. In the picrotoxin-blocked state 50  $\mu$ M MTSET<sup>+</sup> was applied, and after washout, a prolonged, high concentration of GABA was applied to wash out the trapped picrotoxin. The magnitude of the subsequent GABA test currents is the same as the initial pair of currents, indicating that MTSET<sup>+</sup>

did not react with the substituted cysteines. GABA and picrotoxin were coapplied again to trap picrotoxin, but this time 500  $\mu$ M MTSET<sup>+</sup> was applied to the picrotoxin-trapped channels. After a prolonged, high concentration GABA application (not depicted) to wash out the trapped picrotoxin, the subsequent GABA test responses are significantly larger than the initial GABA test responses, indicating that the MTSET<sup>+</sup> covalently modified the substituted cysteines. (F) Similar experiment to that in E, except for the application of 50  $\mu$ M MTSET<sup>+</sup> in the presence of the second prolonged, high concentration GABA application to show that as the picrotoxin washes out of the blocked channels, 50  $\mu$ M MTSET<sup>+</sup> can react with the cysteines, while it could not in the picrotoxin-blocked state. The arrow indicates an inflexion in the current concomitant with MTSET<sup>+</sup> application.

To determine whether the channels with picrotoxin trapped in them were in the open/activated or closed states, we examined the reactivity of  $\alpha_1$ V280C in the picrotoxin-trapped channels. We chose an MTSET<sup>+</sup> concentration (50  $\mu$ M) and a duration of application (30 s) that should react with >80% of open/activated-state channels but with <5% of closed-state channels (0.0015 M-s). After picrotoxin trapping (Fig. 5 E, trace 3), 50  $\mu$ M MTSET<sup>+</sup> was applied for 30 s (Fig. 5 E, trace 4). After application of 200  $\mu$ M GABA to facilitate washout of the trapped picrotoxin (Fig. 5 E, trace 5), the subsequent GABA test currents were similar to the initial

GABA test currents (Fig. 5 E, compare traces 1, 2 and 6, 7). To prove that the engineered cysteine was reactive with a higher concentration of MTSET<sup>+</sup> we repeated the experiment in the same oocyte, but after picrotoxin trapping, we tested the effect of a 30-s application of 10 times higher concentration of MTSET<sup>+</sup> (500  $\mu$ M, 0.015 M-s) (Fig. 5 E, trace 9). After a 200  $\mu$ M GABA application to wash out the trapped picrotoxin (trace not shown in figure between the two slashes), the subsequent GABA currents were significantly potentiated (Fig. 5 E, final two traces compared with initial two). These results imply that in the picrotoxin-trapped state

the reactivity of  $\alpha_1$ V280C was consistent with the closed channel reaction rate. Lending further support to this hypothesis, a 30-s coapplication of 50  $\mu$ M MTSET<sup>+</sup> during washout of the trapped picrotoxin with a prolonged GABA application allowed reaction to proceed, consistent with the open/activated channel rate (Fig. 5 F). This resulted in the potentiation of the subsequent GABA test currents (Fig. 5 F, final two traces compared with initial two traces).

## DISCUSSION

The goal of the present study was to use picrotoxin blocker trapping to determine whether a closed channel gate exists in the GABA<sub>A</sub> receptor between the picrotoxin binding site and the extracellular end of the channel. We have provided evidence that picrotoxin can be trapped in the GABA<sub>A</sub> receptor after removal of agonist. The existence of the picrotoxin-trapped state was demonstrated by the slowed rate of opening on the next application of GABA (Fig. 1). The slowing of GABA<sub>A</sub> receptor channel opening during the acquisition of a current–voltage relationship in the continuous presence of picrotoxin was previously noted (Newland and Cull-Candy, 1992). Considerable evidence in the literature supports the contention that picrotoxin binds in the GABA<sub>A</sub> receptor channel at the 2' level near  $\alpha_1$ V257 (Ffrench-Constant et al., 1993; Zhang et al., 1994; Gurley et al., 1995; Xu et al., 1995). We provided additional evidence to support this hypothesis through the penicillin competition experiments (Fig. 2) and by the inhibition of picrotoxin binding by MTSET<sup>+</sup> modification of  $\alpha_1$ A254E/V257C channels (Fig. 3). Thus, in order to keep picrotoxin trapped in its binding site after the removal of agonist we infer that a gate must exist between the picrotoxin binding site at the 2' level and the extracellular solution because picrotoxin can only access its binding site from the extracellular side (Newland and Cull-Candy, 1992). We hypothesize that in the absence of agonist the picrotoxin-trapped state is likely to be either a closed state or a desensitized state, although we cannot exclude the possibility that it is a unique conformation of the receptor. We used the state-dependent reactivity of two cysteine substitution mutants, one in the M2 segment ( $\alpha_1$ V280C) and one in the M3 segment ( $\alpha_1$ E303C), in an attempt to distinguish whether the picrotoxin-trapped state resembles a closed or desensitized conformation. The  $\alpha_1$ V280C mutant reacted with MTSET<sup>+</sup> at a rate consistent with reaction in the closed state and not in a GABA-activated state (Fig. 5). The  $\alpha_1$ E303C mutant did not react with pCMBS<sup>-</sup>, consistent with the picrotoxin-trapped channels being in the closed and not a desensitized state (Fig. 4). Thus, we infer that in the absence of agonist the picrotoxin-trapped state resembles the closed state. Furthermore, we infer that the gate that lies at a position

extracellular to the picrotoxin binding site and that traps picrotoxin in its binding site is likely to be the closed state gate. This would be consistent with the narrow region between 9' and 14' observed in the 4-Å resolution ACh receptor structure forming a closed channel gate (Miyazawa et al., 2003; Unwin, 2005) and with the inference from SCAM experiments on the 5-HT<sub>3</sub> receptor that the extracellular side of a closed channel gate was at the 14' level (Panicker et al., 2002) and with the inference from photolabeling experiments that the ACh receptor might have a gate at the 13' level (White and Cohen, 1992).

Further support for the existence of a closed channel gate between the extracellular end of the channel and the 2' level comes from the fact that picrotoxin is an open channel blocker. Picrotoxin blocks GABA currents only when it is applied from the extracellular side in the presence of GABA (Newland and Cull-Candy, 1992). It is unable to access its binding site in the closed state (Newland and Cull-Candy, 1992). Application of picrotoxin in the absence of GABA to GABA<sub>A</sub> receptors labeled with site-specific fluorophores caused no change in fluorescence, whereas activation by GABA in the absence of picrotoxin produced complex changes in fluorescence intensity (Muroi et al., 2006). Similar results were observed with GABA<sub>C</sub> receptors (Chang and Weiss, 2002). The inability of picrotoxin to access its binding site in the closed channel state is consistent with the presence of a closed channel gate between the extracellular end of the channel and the 2' level. An alternative explanation that we cannot exclude at present for picrotoxin not acting on the closed channel is that the binding site is only formed by a conformational change in the 2' region that occurs during channel activation. Our current results demonstrating picrotoxin trapping would argue against this alternative explanation.

Our current results do not preclude the possibility that there are additional gates at positions more cytoplasmic than the picrotoxin binding site. Picrotoxin is a roughly spherical molecule  $\sim$ 9 Å in diameter. A constriction that would limit picrotoxin movement through the channel in the closed state might not be sufficient to limit the flux of small ions through the channel. In fact, the channel lumen presumably narrows below the 2' level to prevent picrotoxin from moving into the cytoplasmic channel vestibule. We think that picrotoxin does not move to a position below 2' because it would not have been able to protect  $\alpha_1$ V257C (2') from modification by extracellularly applied pCMBS<sup>-</sup> (Xu et al., 1995). Furthermore, we think that picrotoxin binding in the presence of GABA does not cause closure of a channel gate more extracellular than 6' because a cysteine substituted at 6',  $\alpha_1$ T261C, could react with extracellularly applied MTS reagents when the GABA-activated channels were blocked by picrotoxin (Xu et al., 1995).

Establishing the location of the picrotoxin binding site in the channel is critical for the interpretation of these experiments. As reviewed in the Introduction, considerable evidence indicates that a picrotoxin binding site is located near the cytoplasmic end of the GABA<sub>A</sub> receptor channel at the 2' level (Ffrench-Constant et al., 1993; Zhang et al., 1994; Gurley et al., 1995; Xu et al., 1995). We have provided additional evidence that picrotoxin binds in the channel lumen by showing the ability of penicillin to prevent picrotoxin binding (Fig. 2). Picrotoxin is uncharged and its block of GABA-induced currents is not voltage dependent (Newland and Cull-Candy, 1992). In contrast, penicillin is an anion at neutral pH and blocks GABA<sub>A</sub> receptors in a voltage-dependent manner consistent with it blocking by binding in the ion channel where it can experience the transmembrane voltage (Chow and Mathers, 1986; Twyman et al., 1992; Fujimoto et al., 1995; Sugimoto et al., 2002). Preapplication of penicillin to GABA-activated channels prevented subsequent inhibition by picrotoxin (Fig. 2). This implies that penicillin binds in the channel at and/or above the picrotoxin binding site and prevents picrotoxin from reaching its binding site from the extracellular end of the channel. This experiment strongly supports the hypothesis that there is a picrotoxin binding site in the channel. However, because the location of the penicillin binding site within the channel is not known it does not place additional constraints on the location of a picrotoxin binding site other than that it is in the channel.

To further support the location of a picrotoxin binding site near the M2 segment 2' level we demonstrated that a mutant channel that binds picrotoxin no longer binds picrotoxin after modification of a 2' cysteine. We previously showed that picrotoxin protected a Cys substituted for  $\alpha_1$ Val257 (2') from modification by extracellularly applied pCMBS<sup>-</sup> (Xu et al., 1995). Reaction of negatively charged sulfhydryl reagents with  $\alpha_1$ V257C caused significant inhibition of subsequent GABA-induced currents (Xu and Akabas, 1996) and this precluded investigating whether picrotoxin could still block the residual currents after modification. Furthermore, positively charged reagents did not react at this position (Xu and Akabas, 1996). In the current work, we have taken advantage of an observation that the cationic reagent MTSET<sup>+</sup> can react with  $\alpha_1$ V257C (2') in the presence of a mutation at the adjacent -1' position,  $\alpha_1$ A254E, that inserts the amino acid present at the aligned position in the cation-selective Cys-loop family members. Picrotoxin blocked the unmodified double mutant  $\alpha_1$ A254E/V257C channels with similar affinity to wild-type channels (unpublished data). Thus, the mutations alone did not significantly alter the structure of the picrotoxin binding site. Reaction of MTSET<sup>+</sup> with  $\alpha_1$ A254E/V257C in the presence of GABA had two effects: it caused a significant increase in the holding

current and it increased the subsequent GABA-induced current. The increased holding current was most likely due to a large increase in the spontaneous open probability after MTSET<sup>+</sup> modification. The increased GABA-induced currents were most likely due to a shift in the GABA EC<sub>50</sub> of the modified channels. We cannot rule out the possibility that the increased currents also resulted from an increase in the single channel conductance but this would not affect the interpretation of the subsequent experiments with picrotoxin. In the MTSET<sup>+</sup>-modified channels, there is a major decrease in the extent of picrotoxin inhibition. We infer that modification of the 2' Cys by MTSET<sup>+</sup> prevented picrotoxin from binding to its binding site within the channel lumen. This is unlikely to be an electrostatic interaction with picrotoxin because picrotoxin is uncharged. The inhibition of picrotoxin block could be due to direct steric interactions between the modified Cys and picrotoxin or it could be an indirect allosteric effect or electrostatic effect on the protein structure whereby modification of the 2' Cys alters a site at which picrotoxin normally binds. We believe that in light of the previously published result from our lab that picrotoxin protects the 2' Cys from modification by sulfhydryl reagents (Xu et al., 1995) and the mutagenesis results of others that show that 2' mutations prevent picrotoxin block (Ffrench-Constant et al., 1993; Zhang et al., 1995), the most likely interpretation of our result is that the MTSET<sup>+</sup>-modified 2' Cys prevents picrotoxin binding by a direct steric effect. Thus, we infer that the majority of the inhibition due to picrotoxin is due to binding at a site within the channel lumen.

There may be a second picrotoxin binding site on the GABA<sub>A</sub> receptor that accounts for the residual inhibition by picrotoxin after MTSET<sup>+</sup> modification of the  $\alpha_1$ A254E/V257C channels. We cannot exclude the possibility that the residual inhibition by picrotoxin occurs in channels that did not react with MTSET<sup>+</sup> although we think this is unlikely because reaction seems to have gone to completion. The residual inhibition is rapidly reversible (unpublished data). Work by others has suggested the existence of a second picrotoxin binding site besides the site within the channel (Yoon et al., 1993). Thus, based on the previously published results from our lab and from several others and on our current results, we conclude that picrotoxin binds at the 2' level in the GABA<sub>A</sub> receptor channel.

We have presented evidence that picrotoxin can be trapped for minutes in the GABA<sub>A</sub> receptor channel after washout of GABA and picrotoxin (Fig. 1). This implies that a gate or constriction exists in the channel between the picrotoxin binding site at the 2' level and the extracellular end of the channel. To infer that this is a closed state gate we need to establish the state of the GABA<sub>A</sub> receptor in the absence of GABA with picrotoxin trapped in the channel. We do not think that the

channel can be in an open state because in the open state picrotoxin can leave its binding site. Thus, the channels are either in a desensitized state or a closed state, both of which are nonconducting states in which a gate or gates block ion conduction. There is extensive evidence using a variety of techniques including photoaffinity labeling, spectroscopy, cysteine accessibility, and kinetic analysis to indicate that in Cys-loop receptors the closed and desensitized states are structurally distinct (Galzi et al., 1991; Revah et al., 1991; White and Cohen, 1992; Jones and Westbrook, 1995; Baenziger and Chew, 1997; Auerbach and Akk, 1998; Williams and Akabas, 1999; Krampfl et al., 2000; Bianchi et al., 2001; Wilson and Karlin, 2001; Chang et al., 2002; Arevalo et al., 2005; Muroi et al., 2006; Purohit and Grosman, 2006). Several lines of evidence suggest that different gates occlude ion flow in the closed and desensitized states (Auerbach and Akk, 1998; Wilson and Karlin, 2001; Purohit and Grosman, 2006).

We used the accessibility of engineered cysteines in order to distinguish whether the GABA<sub>A</sub> receptors with picrotoxin trapped were in a closed or desensitized conformation. Previously, we showed that pCMBS<sup>-</sup> does not react with the M3 segment Cys mutant  $\alpha_1$ E303C in the closed state (Williams and Akabas, 1999). Furthermore, when pCMBS<sup>-</sup> is applied in the presence of GABA, the GABA currents during the application of pCMBS<sup>-</sup> are identical with GABA currents in the absence of pCMBS<sup>-</sup> (Fig. 4 B). However, the subsequent GABA-induced currents are markedly reduced compared with the GABA currents before pCMBS<sup>-</sup> application. Based on the absence of effect on the currents during the pCMBS<sup>-</sup> application, we inferred that pCMBS<sup>-</sup> must react in a desensitized state from which the channels rarely return to the open state. Thus, we infer that  $\alpha_1$ E303C is accessible to react in a desensitized state but not in the closed state. We showed in Fig. 4 E that in the picrotoxin-trapped state that pCMBS<sup>-</sup> does not react with  $\alpha_1$ E303C receptors. The reactivity, or lack thereof, of  $\alpha_1$ E303C in the picrotoxin-trapped state is consistent with it being a closed state where the cysteine is inaccessible to react with pCMBS<sup>-</sup> as opposed to an activated/desensitized state where the cysteine would have been reactive.

To further characterize the picrotoxin-trapped state we used another cysteine mutant,  $\alpha_1$ V280C (25'), in the M2  $\alpha$  helix that extends above the level of the membrane. We had previously shown that the rate of reaction of MTSET<sup>+</sup> with this residue was seven times faster in the presence of GABA than in the absence of GABA (Bera et al., 2002). We exposed picrotoxin-trapped  $\alpha_1$ V280C receptors to an MTSET<sup>+</sup> concentration for a duration of time that would have resulted in >80% reaction if the channels were in the activated state. After removal of MTSET<sup>+</sup> and GABA-induced washout of the trapped picrotoxin, we found that the

cysteine had not been modified (Fig. 5 E). In contrast, when we applied a higher concentration of MTSET<sup>+</sup> that would react completely with picrotoxin-trapped  $\alpha_1$ V280C channels if they were in the closed state, we found that the cysteine was modified. Thus, the cysteine was accessible for modification, but in the picrotoxin-trapped channels it reacted at a rate similar to the rate at which it reacted with  $\alpha_1$ V280C channels in the closed state. It is important to recognize that during the time course of the sulfhydryl accessibility experiments the channels are undergoing transitions between multiple states. Thus, the reaction rates reflect the ensemble average of states present during the period of application. We cannot determine precisely in which state reaction is occurring. For  $\alpha_1$ V280C, the sevenfold difference in MTSET<sup>+</sup> reaction rates in the absence and presence of GABA indicates that the ensemble average of states is different in the two conditions. The MTSET<sup>+</sup> reactivity of  $\alpha_1$ V280C in the picrotoxin-trapped state is consistent with the inference that the picrotoxin-trapped channels are in a state resembling the closed state. Thus, we conclude that the gate that traps picrotoxin in the channel after the removal of GABA is a closed state gate. This gate must lie between the 2' level where picrotoxin binds and the extracellular end of the channel.

As outlined in the Introduction, inferences as to the location of the closed channel gate in the members of the Cys-loop receptors have varied depending on the channel studied and the approach used. Our current results for the GABA<sub>A</sub> receptor imply that a closed state gate exists between the 2' level and the extracellular end of the channel. Based on cysteine accessibility experiments, we had previously concluded that the ability of the sulfhydryl reagents to react with the 2' Cys mutant in the absence of GABA implied that the closed state gate was at a more cytoplasmic position than the 2' level (Xu and Akabas, 1996). There are several aspects of our previous work that limited the strength of the conclusion regarding the position of the gate. First, we had not measured the reaction rates of the sulfhydryl reagents and therefore did not know the extent to which the rates in the presence and in the absence of GABA differed. Second, we did not take the measures necessary to preclude the possibility that the reaction in the absence of GABA was truly in the closed state and not during either spontaneous openings or during brief "agonist-induced openings" where the sulfhydryl reagents could have acted as weak partial agonists. This would have entailed measuring the spontaneous open probability of the Cys mutant channels and applying the sulfhydryl reagents with a strong competitive antagonist to prevent the sulfhydryl reagents from activating the channels by binding in the agonist binding sites. In retrospect, the fact that pCMBS<sup>-</sup> reacted with  $\alpha_1$ V257C (2') in the absence of GABA but pCMB<sup>-</sup> did

not, may have been a clue that pCMBS<sup>-</sup> could act as a weak agonist at least for the  $\alpha_1V257C$  channels (Xu and Akabas, 1996).

In the ACh receptor based on cysteine accessibility experiments similar conclusions were reached that the location of the gate was at a position more cytoplasmic than 2' (Akabas et al., 1994; Pascual and Karlin, 1998; Wilson and Karlin, 1998; Zhang and Karlin, 1998). Although rates were measured in some of these experiments they did not measure the spontaneous open probability of the Cys mutants nor did they perform the experiments in the presence of a competitive antagonist that would have prevented the reagents from acting as weak agonists. Based on the methanethiosulfonate ethylammonium (MTSEA<sup>+</sup>) reaction rate with the ACh receptor 2' position,  $\alpha T244C$ , being significantly greater than  $10 \text{ M}^{-1}\text{s}^{-1}$  in the absence of ACh and on differences in reaction rates due to application from the extracellular and cytoplasmic ends of the channel in the absence and presence of ACh, Wilson and Karlin (1998) concluded that the closed channel gate was more cytoplasmic than the 2' position. A potential weakness in these studies was that the inference that the extracellular side of the gate is below the 2' M2 position was largely based on the reactivity of the ACh receptor 2' cysteine substitution mutant,  $\alpha T244C$ , with MTSEA<sup>+</sup> in the absence of ACh (Wilson and Karlin, 1998). The rates of other MTS derivatives tested with this cysteine substitution mutant were significantly slower in the absence of ACh than in its presence. A doubly charged thio-sulfonate, 2-aminoethyl-2-aminoethanethiosulfonate (AEAETS<sup>2+</sup>), reacted with  $\alpha T244C$  at  $1.25 \text{ M}^{-1}\text{s}^{-1}$  in the absence of ACh and  $63,000 \text{ M}^{-1}\text{s}^{-1}$  in the presence of ACh (Pascual and Karlin, 1998). This might be consistent with the presence of a gate between the extracellular end of the channel and the 2' position. It is possible that MTSEA<sup>+</sup>, like other organic ammonium compounds, may act as a weak partial agonist of the ACh receptor. We previously observed that the quaternary ammonium MTS reagent MSTET<sup>+</sup> displayed agonist activity with many of the ACh receptor M2 cysteine substitution mutants (Akabas et al., 1994). Perhaps little or no current was seen during the MTSEA<sup>+</sup> applications to  $\alpha T244C$  in the absence of ACh because the very fast open state reaction rate,  $16800 \text{ M}^{-1}\text{s}^{-1}$ , led to rapid reaction and complete inhibition of the channels as soon as they opened (Pascual and Karlin, 1998). These potential problems may limit the strength of the conclusion that in the ACh receptor there is no closed channel gate more extracellular than 2'. It should be noted that our present results do not rule out the existence of an additional, more cytoplasmic gate as was inferred from the ACh receptor SCAM experiments (Wilson and Karlin, 1998).

Using engineered histidines to create Zn<sup>2+</sup> binding sites in ACh-5-HT<sub>3</sub> chimeric channels it was concluded

that the closed channel gate was more cytoplasmic than the 2' level (Paas et al., 2005). It is, however, clear from the experimental records shown in the published work that there is a significant level of spontaneous opening that can be seen when Zn<sup>2+</sup> is applied and the holding currents decrease. Thus, it is likely that Zn<sup>2+</sup> was reaching the engineered histidines during spontaneous openings, rather than in the closed state as was inferred in the paper. This undermines their conclusion that the gate is more cytoplasmic than 2' in the chimeric channel.

In the 5-HT<sub>3</sub> receptor, in order to avoid the possibility of the MTS reagents acting as weak partial agonists and to reduce the potential for spontaneous channel opening, the closed state cysteine accessibility experiments were done in the presence of a high affinity, competitive antagonist (Panicker et al., 2002). These experiments were consistent with the extracellular side of the gate being at the 14' level, i.e., near the middle of the channel (Panicker et al., 2002).

Finally, structural evidence from the 4-Å resolution electron density maps indicates that the narrowest region in the closed ACh receptor is in the middle of the channel between the 9' and 14' residues (Miyazawa et al., 2003; Unwin, 2005). In this region the pore narrows to  $\sim 6 \text{ Å}$  in diameter. Several theoretical molecular dynamics studies have suggested that hydrated monovalent cations could not pass through a region this narrow at a significant rate, suggesting that it may form a hydrophobic gate (Amiri et al., 2005; Corry, 2006). Our present results, using a different functional approach, open channel blocker trapping, are consistent with a gate located in the middle of the channel as was inferred from the ACh receptor cryoelectron microscopic studies and from the 5-HT<sub>3A</sub> receptor SCAM experiments (Panicker et al., 2002; Miyazawa et al., 2003).

If picrotoxin can be trapped in the closed channel near the 2' level, it implies that in the closed state there must be a vestibule near 2' that can accommodate an  $\sim 9\text{-Å}$  diameter picrotoxin molecule. This vestibule may not change size significantly during channel gating, consistent with the tight packing and low mobility that we previously inferred for this region (Goren et al., 2004) and that was suggested for the 5HT<sub>3</sub> receptor (Panicker et al., 2004). Channel gating may involve movements of the M2 segments in a region more extracellular than the 6' residue.

In summary, we conclude that picrotoxin can be trapped in the closed GABA<sub>A</sub> receptor channel. To trap picrotoxin in its binding site near the cytoplasmic end of the channel, at the 2' level, there must be a gate located at a more extracellular position. Given that the reactivity of substituted cysteines is consistent with the picrotoxin-blocked channel being in the closed state, we suggest that this gate is a closed state gate. This is consistent with the 5-HT<sub>3A</sub> receptor SCAM experiments

and with the 4-Å ACh receptor structure that inferred the existence of a gate in the middle of the channel in the 9'-14' region (Panicker et al., 2002; Miyazawa et al., 2003). It is likely that the gate location is similar in all members of the Cys-loop receptor superfamily. Establishing the location of the channel gate now focuses attention on where conformational changes must occur in order to transduce agonist binding in the extracellular domain into opening of the channel gate.

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