Identification of Channel-Lining Residues in the M2 Membrane-spanning Segment of the GABA$_A$ Receptor α_1 Subunit

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ABSTRACT The γ-aminobutyric acid type A (GABA$_A$) receptors are the major inhibitory, postsynaptic, neurotransmitter receptors in the central nervous system. The binding of γ-aminobutyric acid (GABA) to the GABA$_A$ receptors induces the opening of an anion-selective channel that remains open for tens of milliseconds before it closes. To understand how the structure of the GABA$_A$ receptor determines the functional properties such as ion conduction, ion selectivity and gating we sought to identify the amino acid residues that line the ion conducting channel. To accomplish this we mutated 26 consecutive residues (250–275), one at a time, in and flanking the M2 membrane-spanning segment of the rat α_1 subunit to cysteine. We expressed the mutant α_1 subunit with wild-type β_1 and γ_2 subunits in *Xenopus* oocytes. We probed the accessibility of the engineered cysteine to covalent modification by charged, sulfhydryl-specific reagents added extracellularly. We assume that among residues in membrane-spanning segments, only those lining the channel would be susceptible to modification by polar reagents and that such modification would irreversibly alter conduction through the channel. We infer that nine of the residues, α_1Val257, α_1Thr261, α_1Thr262, α_1Leu264, α_1Thr265, α_1Thr268, α_1Ile271, α_1Ser272 and α_1Asn275 are exposed in the channel. On a helical wheel plot, the exposed residues, except α_1Thr262, lie on one side of the helix in an arc of 120°. We infer that the M2 segment forms an α helix that is interrupted in the region of α_1Thr262. The modification of residues as cytoplasmic as α_1Val257 in the closed state of the channel suggests that the gate is at least as cytoplasmic as α_1Val257. The ability of the positively charged reagent methanethiosulfonate ethylammonium to reach the level of α_1Thr261 suggests that the charge-selectivity filter is at least as cytoplasmic as this residue.

INTRODUCTION

The γ-aminobutyric acid type A (GABA$_A$) receptors form anion-selective channels at inhibitory synapses in the mammalian central nervous system. The GABA$_A$ receptors are the targets for several classes of clinically useful drugs which potentiate GABA-induced currents including benzodiazepines, barbiturates, and several general anesthetics (Franks and Lieb, 1994; Macdonald and Olsen, 1994). In addition, epileptogenic drugs such as picrotoxin, penicillin and TBPS bind to the GABA$_A$ receptors and inhibit GABA-induced currents. In insects, the GABA$_A$ receptors are inhibited by the commonly used cyclodiene insecticides (ffrench-Constant et al., 1993). Multiple GABA$_A$ receptor subunits have been cloned (Burt and Kamatchi, 1991; Wisden and Seeburg, 1992; Macdonald and Olsen, 1994). The

subunits are members of a ligand-gated ion channel gene superfamily which includes the subunits of the nicotinic acetylcholine (Numa, 1989), serotonin (Maricq et al., 1991), and glycine (Betz, 1992) receptors and the avermectin-sensitive, glutamate-gated, chloride channel (Cully et al., 1994). Studies of heterologously expressed GABA$_A$ receptors of defined subunit composition have provided insights into the pharmacological diversity of GABA$_A$ receptors in situ (Burt and Kamatchi, 1991; Wisden and Seeburg, 1992; Macdonald and Olsen, 1994), however, there have been few studies of the structure of the ion conduction pathway.

Based on studies of the GABA$_A$ receptor and the homologous nicotinic acetylcholine receptor, the GABA$_A$ receptors are presumably composed of five subunits arranged pseudo-symmetrically around a central channel (Unwin, 1993; Nayeem et al., 1994); the subunit stoichiometry, however, is uncertain (Backus et al., 1993; Macdonald and Olsen, 1994). The subunits have a $\sim$200 amino acid NH$_2$-terminal extracellular domain, three closely spaced membrane-spanning segments

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(M1–M3), a cytoplasmic domain of variable length, a fourth membrane-spanning segment (M4) and a short, extracellular COOH terminus. The results of numerous experiments on the acetylcholine receptor indicate that residues in the M2 membrane-spanning segment line the ion conducting channel (Sakmann, 1992; Lester, 1992; Karlin, 1993), however, there is little information identifying the channel-lining residues in the $GABA_A$ receptors. We previously applied the scanning-cysteine-accessibility method to four residues, α_1Thr268 to α_1Ile271, in the M2 membrane-spanning segment of the rat α_1 subunit (Xu and Akabas, 1993). We showed that two residues near the extracellular end of the M2 segment, α_1Ile271 and α_1Thr268, are exposed in the channel lumen (Xu and Akabas, 1993). In addition, using a variation of this method on the residues α_1Val257 to α_1Thr261 we demonstrated that picrotoxin, a noncompetitive inhibitor, appears to bind in the channel at the level of α_1Val257 (Xu et al., 1995). We now present the results of a scanning-cysteine-accessibility analysis of all of the residues in and flanking the M2 membrane-spanning segment of the rat α_1 subunit.

The scanning-cysteine-accessibility method provides a systematic approach to the identification of the residues lining an ion channel. We mutate individual residues in largely hydrophobic, membrane-spanning segments to cysteine. We express the cysteine-substitution mutants in Xenopus oocytes and examine their functional properties in situ. If mutant channels are near-normal in their responses, we proceed to test whether the new cysteine is on the water-accessible surface of the protein. We test the ability of small, charged, hydrophilic, sulfhydryl-specific reagents to react covalently with the new cysteine. We assume that of the residues in membrane-spanning segments only those exposed in the channel lumen will be accessible to react with these sulfhydryl reagents. If the reagents react with a cysteine in the channel lining we assume that they will irreversibly alter ion conduction. We infer that for a mutant channel whose conduction is irreversibly altered by the sulfhydryl-specific reagents, the side chain of the corresponding wild-type residue lines the ion channel. The reagents we have used in this study include the organic mercurial derivatives p-chloromercuribenzenesulfonate (pCMBS) and p-chloromercuribenzoate (pCMB), the methanethiosulfonate (MTS) derivatives MTS-ethylsulfonate (MTSES) and MTS-ethylammonium (MTSEA), and iodoacetate. The structures of these reagents and their reactions with free sulfhydryls are shown in Fig. 1.

The scanning-cysteine-accessibility method has been used to study the acetylcholine receptor (Akabas et al., 1992, 1994a) the $GABA_A$ receptor (Xu and Akabas, 1993; Xu et al., 1995), the cystic fibrosis transmembrane conductance regulator (Akabas et al., 1994b), potassium channels (Kurz et al., 1995; Lu and Miller,
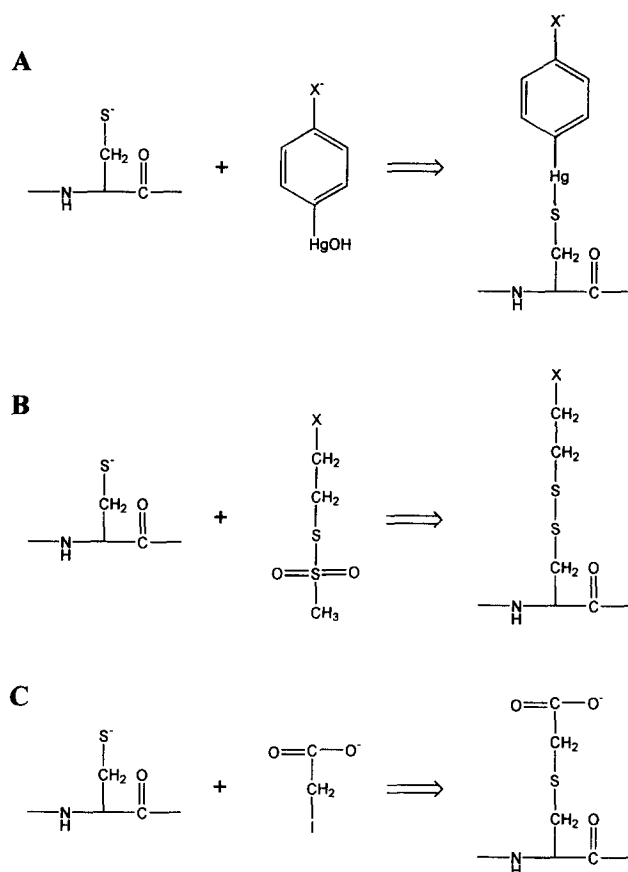

FIGURE 1. Chemical structures of the sulfhydryl reagents and their reactions with cysteine. The structure of the reactive ionized thiolate form of cysteine is shown on the left of each panel; the structure of the reagents follows the + sign and the structure of the products are to the left of the arrow. Note that all of these reagents transfer the charged portion of the reagent onto the sulfhydryl of cysteine resulting in a charged product. (A) Organic mercurial derivatives. For pCMBS, $X^- = SO_3^-$; for pCMB, $X^- = COO^-$. (B) Methanethiosulfonate derivatives. For MTSES, $X = SO_3^-$; for MTSEA, $X = NH_3^+$. (C) Iodoacetate.

1995; Pascual et al., 1995), bacterial toxin channels (Mindell et al., 1994; Slatin et al., 1994), the lactose permease (Jung et al., 1993), and the dopamine D2 receptor (Javitch et al., 1995).

We now report on the analysis of 26 residues in and flanking the M2 segment from α_1Glu250 to α_1Asn275, using $GABA_A$ receptors formed by the expression of α_1, β_1 and γ_2 subunits. We show that nine of these residues are exposed in the channel lumen. We found that the positively charged reagent, MTSEA, can penetrate from the extracellular end of the channel to the level of α_1Thr261 suggesting that the charge selectivity filter is at a more cytoplasmic position. Furthermore, we show that both negatively and positively charged reagents can enter the channel in the closed state implying that the gate is near the cytoplasmic end of the M2 segment.

The results for the residues 257 to 261 using the reagents pCMBS and MTSEA were published previously (Xu et al., 1995) and are included in Figs. 5 and 9 for completeness.

METHODS

Oligonucleotide-mediated Mutagenesis

The cDNA's encoding the rat α_1 and γ_2 subunits in the pBluescript SK(-) plasmid (Stratagene Corp., La Jolla, CA) were obtained from Dr. P. Seeburg (Shivers et al., 1989; Ymer et al., 1989), and the β_1 subunit in the pBluescript SK vector from Dr. A. Tobin (Khrestchatisky et al., 1989). The Altered-sites mutagenesis procedure (Promega Corp., Madison, WI) was used to substitute cysteine residues, one at a time, in the α_1 subunit as previously described (Xu et al., 1995). Mutations were confirmed by DNA sequencing.

Preparation of mRNA and Oocytes

The in vitro mRNA transcription and the preparation and injection of *Xenopus* oocytes was performed as described previously (Xu et al., 1995). Oocytes were injected with 10 ng of mRNA encoding the α_1, β_1, and γ_2 subunits in a 1:1:1 ratio.

Reagents

MTSEA$^+$ and MTSES$^-$ were synthesized as described previously (Stauffer and Karlin, 1994). pCMBS$^-$, pCMB$^-$, and iodoacetate were obtained from Sigma Chemical Co. (St. Louis, MO).

Electrophysiology

GABA-induced currents were recorded from individual oocytes under two-electrode voltage clamp, at a holding potential of -80 mV. Electrodes were filled with 3 M KCl and had a resistance of <2 MΩ. The ground electrode was connected to the bath via a 3 M KCl/Agar bridge. Data was acquired and analyzed on a 486/33 MHz computer using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN), a TL1-125 DMA data interface (Axon Instruments, Foster City, CA) and software written using the Axobasic language (Axon Instruments). The oocyte was perfused at 5 ml/min with Ca^{2+}-free Frog Ringer solution (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl$_2$, 10 mM HEPES pH 7.5 with NaOH) at room temperature. The recording chamber had a volume of $\sim$0.25 ml.

Experimental Protocol

We tested the susceptibility of wild-type and mutant GABA$_A$ receptors to a 1-min application of the sulfhydryl reagents applied in the presence or in the absence of 100 μM GABA. The following sequence of perfusing solutions was used: 100 μM GABA (10 s), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min), sulfhydryl reagent $\pm$ 100 μM GABA (1 min), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min) and 100 μM GABA (10 s). The average of the two peak currents before the sulfhydryl reagent was applied was compared with the average of the two peak currents after. The fractional effect was taken as $[1 - (I_{GABA, after}/I_{GABA, before})]$.

The sulfhydryl reagents were applied in CFFR solution, except for iodoacetate which was applied in NaCl-free CFFR. Solutions of the MTS reagents were prepared immediately before each ex-

periment. Solutions of the mercurials were prepared each day and stored on ice until use. Solutions of iodoacetate were prepared daily and kept on ice in the dark until use.

Statistics

Data in Figs. 4 to 9 are presented as the means $\pm$ SE. Significance was determined by one-way analysis of variance using Duncan's post-hoc test ($p < 0.05$) (SPSS-PC).

RESULTS

Using site-directed mutagenesis we substituted cysteine, one at a time, for 26 consecutive residues (α_1Glu250 to α_1Asn275) in and flanking the M2 membrane-spanning segment. The mutant α_1 subunits were expressed in *Xenopus* oocytes with wild type β_1 and γ_2 subunits. One to two days after mRNA injection GABA- induced currents were elicited for all of the mutants. The average magnitude of the peak, inward currents induced by 100 μM GABA ($V = -80$ mV) was -1.43 ± 0.4 μA for wild type and for the mutants ranged between -0.16 ± 0.03 μA for P253C and -1.8 ± 0.2 μA for S272C (Fig. 2). In the absence of sulfhydryl reagents, the GABA-induced currents of wild-type and all of the mutants were stable for the duration of a typical experiment, $\sim$30 min.

For oocytes expressing wild-type GABA$_A$ receptor (defined as receptor formed by coexpression of the α_1, β_1, and γ_2 subunits), a 1-min application of either 0.5 mM pCMBS, 0.1 mM pCMB, 10 mM MTSES, 100 mM iodoacetate or 2.5 mM MTSEA either in the presence
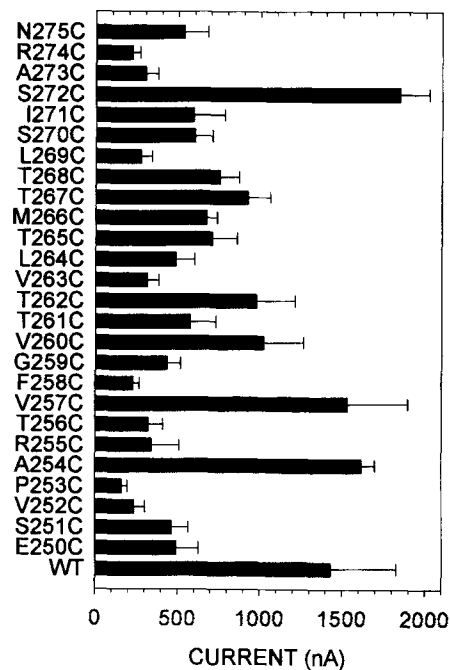
FIGURE 2. The average peak current induced by 100 μM GABA for oocytes expressing wild type or cysteine mutant GABA$_A$ receptors. Each bar represents the average current ($V = -80$ mV) and SEM for six oocytes.

of 100 μM GABA or in its absence caused a <10% change in the subsequent GABA-induced currents (Figs. 3 and 4). Changes of similar magnitude were observed after 1-min applications of GABA alone (data not shown). 100 mM iodoacetate reversibly reduced the peak current during coapplication with GABA (Fig. 3 C); coapplication of the other sulfhydryl reagents had no obvious reversible effects on the GABA-induced currents (Fig. 3).

Reactions with pCMBS

A 1-min application of 0.5 mM pCMBS, added in the presence of GABA, irreversibly inhibited the subsequent GABA-induced currents of the mutants V257C, T261C, L264C, T265C, T268C, I271C, and S272C significantly more than wild type[1] (Fig. 5 A). It is important to note that in the presence of GABA the channels undergo transitions between the open, desensitized and closed states; we cannot distinguish in which state(s) reaction is occurring in the presence of GABA.

A 1-min application of 0.5 mM pCMBS, added in the absence of GABA, irreversibly inhibited the subsequent GABA-induced currents of the mutants V257C, L264C, T265C, T268C, and S272C significantly more than wild type and significantly potentiated the subsequent GABA-induced currents of the mutant N275C (Fig. 5 B). GABA altered the accessibility of three of the mutants for reaction with pCMBS: the accessibility of N275C was reduced in the presence of GABA and the accessibilities of T261C and I271C were increased in the presence of GABA.

Reactions with pCMB

A 1-min application of 0.1 mM pCMB, added in the presence of GABA, irreversibly inhibited the subse-
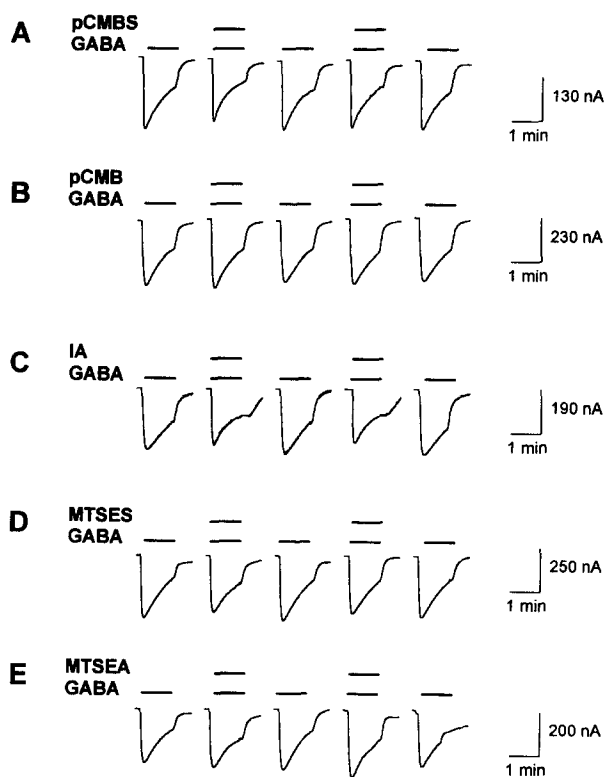

FIGURE 3. Two-electrode voltage clamp recordings demonstrating that 1-min applications of the sulfhydryl reagents have no irreversible effects on the wild-type GABA_A receptors. The panels show the application of (A) 0.5 mM pCMBS, (B) 0.1 mM pCMB, (C) 100 mM iodoacetate, (D) 10 mM MTSES, and (E) 2.5 mM MTSEA+. A 1-min application of 100 μM GABA precedes and follows the applications of the sulfhydryl reagents. Holding potential −80 mV.

quent GABA-induced currents of the mutants V257C, T261C, L264C, T265C, T268C, I271C, and S272C significantly more than wild type (Fig. 6 A).

A 1-min application of 0.1 mM pCMB, added in the absence of GABA, irreversibly inhibited the subsequent GABA-induced currents of the mutants L264C, T265C, T268C, and S272C significantly more than wild type (Fig. 6 B). As with pCMBS, a 1-min application of 0.1 mM pCMB, added in the absence of GABA, significantly potentiated the subsequent GABA-induced currents of the mutant N275C (Fig. 6 B). Coapplication of pCMB with GABA altered the accessibility of four of the residues: the accessibility of N275C was decreased in the presence of GABA and the accessibilities of V257C, T261C, and I271C, were increased in the presence of GABA.

Reactions with Iodoacetate

A 1-min application of 100 mM iodoacetate, added in the presence of GABA, irreversibly altered the subsequent GABA-induced currents of four of the mutants: the currents of the mutant T262C were potentiated
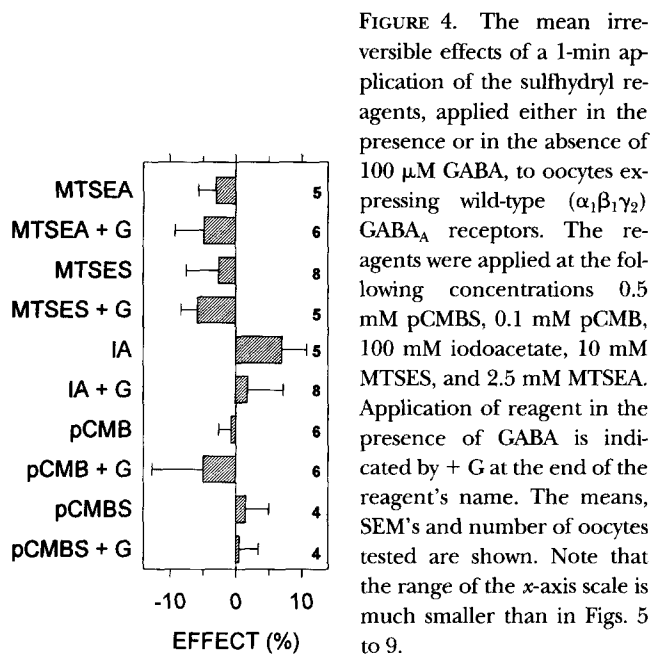
1. We identify engineered cysteine residues that reacted with the sulfhydryl reagents if exposure to the sulfhydryl reagents irreversibly altered the subsequent GABA-induced currents of a mutant significantly more than the subsequent currents of wild type. For mutants where the effects of modification are large it is easy to identify residues that have reacted, however, where the effects of modification are small we depend on statistical analysis to identify residues that have reacted with the sulfhydryl reagents. We determine the statistical significance by the oneway analysis of variance using Duncan's post-hoc test to identify the mutants that are significantly different from wild type. There are, however, a variety of post-hoc tests that differ in stringency and, therefore, in which mutants they identify as being different from wild type. For example, analysis of the effect of a 1-min application of pCMBS in the presence of GABA by the least significant difference test, a less stringent test than Duncan's, indicates that in addition to the seven mutants identified by Duncan's test, N275C is also significantly different; analysis of the same data by the Student-Newman-Keuls test, a more stringent post-hoc test, identifies the same mutants as Duncan's test. Therefore, for those mutants where the effects of modification are small, the stringency of the post-hoc test selected determines which mutants we consider to be significantly altered by the sulfhydryl reagents.

FIGURE 4. The mean irreversible effects of a 1-min application of the sulfhydryl reagents, applied either in the presence or in the absence of 100 μM GABA, to oocytes expressing wild-type $(\alpha_1\beta_1\gamma_2)$ GABA$_A$ receptors. The reagents were applied at the following concentrations 0.5 mM pCMBS, 0.1 mM pCMB, 100 mM iodoacetate, 10 mM MTSES, and 2.5 mM MTSEA. Application of reagent in the presence of GABA is indicated by + G at the end of the reagent's name. The means, SEM's and number of oocytes tested are shown. Note that the range of the x-axis scale is much smaller than in Figs. 5 to 9.

FIGURE 6. The irreversible effect of a 1-min application of 0.1 mM pCMB, applied in the presence (A) or in the absence (B) of 100 μM GABA, on the subsequent GABA-induced currents of wild type and mutant GABA$_A$ receptors. Solid bars indicate mutants for which the effect was statistically significantly different than the effect on wild type. Negative change indicates inhibition and positive change indicates potentiation of the subsequent GABA-induced currents. The means, SEM's and number of oocytes tested are shown.

and the currents of the mutants L264C, T268C, and I271C were inhibited (Fig. 7 A).

A 1-min application of 100 mM iodoacetate, added in the absence of GABA, irreversibly altered the subse-
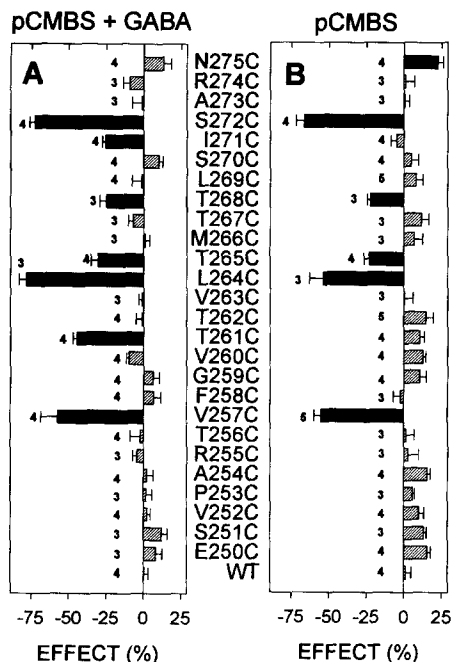

FIGURE 5. The irreversible effect of a 1-min application of 0.5 mM pCMBS, applied in the presence (A) or in the absence (B) of 100 μM GABA, on the subsequent GABA-induced currents of wild-type and mutant GABA$_A$ receptors. Solid bars indicate mutants for which the effect was statistically significantly different than the effect on wild type by a one way analysis of variance by Duncan's criteria. Negative change indicates inhibition and positive change indicates potentiation of the subsequent GABA-induced currents. The means, SEM's and number of oocytes tested are shown.
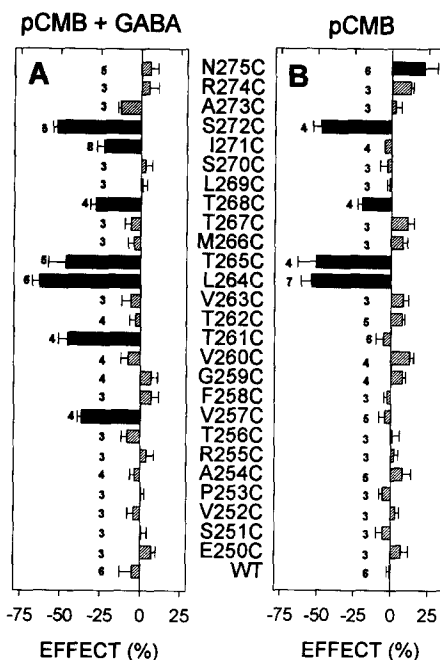
quent GABA-induced currents of five of the mutants significantly more than wild type. The subsequent currents of the mutants T261C, T265C, and N275C were potentiated and the currents of the mutants T268C and I271C were inhibited (Fig. 7 B).[2] Coapplication of GABA altered the accessibility of five of the mutants to reaction with iodoacetate: the accessibility of T261C, T265C, and N275C was decreased by coapplication of GABA and the accessibility of L264C was increased by coapplication of GABA.

Reactions with MTSES

A 1-min application of 10 mM MTSES, applied in the presence of GABA, inhibited the subsequent GABA-induced currents of the mutant V257C and potentiated

2. For the mutant T262C, iodoacetate applied either in the presence or absence of GABA potentiated the subsequent GABA-induced current by 20%. Using Duncan's post-hoc test, only the effect in the presence of GABA was significant, however, by the less stringent least significant difference test the effects in the presence and in the absence of GABA are significant (see footnote 1). Despite the difference in the significance by Duncan's test we do not believe that the accessibility of T262C is reduced in the absence of GABA. Similar considerations apply to the effects of MTSEA on this mutant.
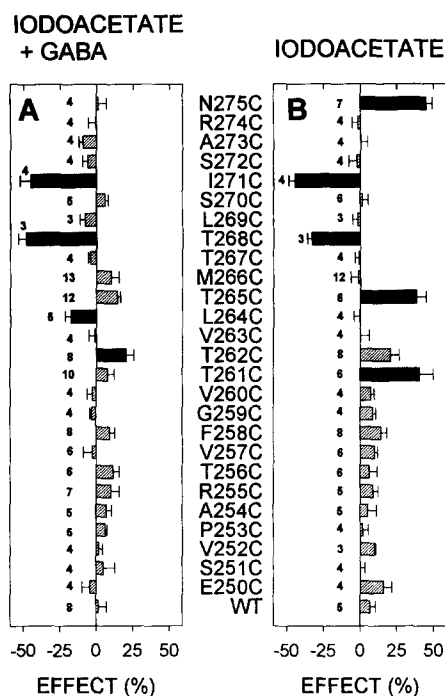
IODOACETATE + GABA   **IODOACETATE**

EFFECT (%)   EFFECT (%)

Figure 7. The irreversible effect of a 1-min application of 100 mM iodoacetate, applied in the presence (A) or in the absence (B) of 100 μM GABA, on the subsequent GABA-induced currents of wild type and mutant $GABA_A$ receptors. Solid bars indicate mutants for which the effect was statistically significantly different than the effect on wild type. Negative change indicates inhibition and positive change indicates potentiation of the subsequent GABA-induced currents. The means, SEM's and number of oocytes tested are shown.

MTSES⁻ + GABA   **MTSES⁻**

EFFECT (%)   EFFECT (%)

Figure 8. The irreversible effect of a 1-min application of 10 mM MTSES, applied in the presence (A) or in the absence (B) of 100 μM GABA, on the subsequent GABA-induced currents of wild type and mutant $GABA_A$ receptors. Solid bars indicate mutants for which the effect was statistically significantly different than the effect on wild type. Negative change indicates inhibition and positive change indicates potentiation of the subsequent GABA-induced currents. The means, SEM's and number of oocytes tested are shown.

the currents of the mutant N275C (Fig. 8 A). A 1-min application of MTSES, applied in the absence of GABA, only altered the subsequent currents of the mutant N275C; resulting in potentiation (Fig. 8 B). MTSES applied either in the presence or in the absence of GABA had no effect on the other mutants.

Reactions with the Cationic Reagent MTSEA

A 1-min application of the positively charged MTSEA (2.5 mM), applied in the presence of GABA, irreversibly altered the currents of six of the mutants: the currents of T261C, T262C, T265C, T268C, and N275C were potentiated and the current of the mutant I271C was inhibited (Fig. 9 A).

A 1-min application of MTSEA, applied in the absence of GABA, irreversibly altered the subsequent GABA-induced currents of three of the mutants. The currents of the mutants T261C and N275C were potentiated and the current of the mutant I271C was inhibited significantly more than wild type (Fig. 9 B). Thus, coapplication with GABA increased the accessibility of T265C and T268C for reaction with MTSEA.[2]
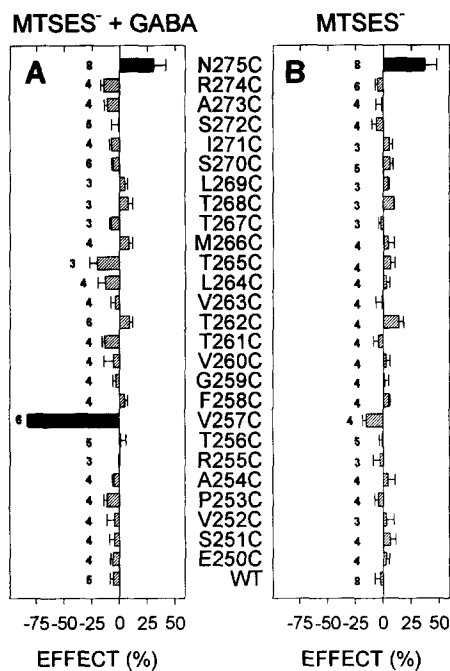
DISCUSSION

Residues Exposed in the Channel Lumen

The GABA-induced currents of 9 out of 26 cysteine-substitution mutants in and flanking the M2 membrane-spanning segment were irreversibly altered by extracellular application of charged, sulfhydryl-specific reagents (Table I). We infer from the irreversible alteration of the GABA-induced current that the sulfhydryl reagents reacted with an engineered cysteine. For several reasons we assume that these reagents will only react with sulfhydryls that are on the water accessible surface of the protein. First, pCMBS was essentially impermeable through the erythrocyte membrane during a 90-min incubation, although pCMB was permeable (VanSteveninck, Weed, and Rothstein, 1965). Second, the rate of reaction of the MTS reagents with an ionized thiolate anion is 5×10^9 times faster than the rate of reaction with the unionized thiol (Roberts et al., 1986) and the rate of reaction of the mercurials with an ionized thiolate anion is 2×10^3 times faster than the rate with the unionized thiol (Hasinoff et al., 1971); only sulfhydryls exposed to water will ionize to a significant extent. Thus, we infer that engineered cysteine

MTSEA⁺ + GABA   **MTSEA⁺**

A

N275C
R274C
A273C
S272C
I271C
S270C
L269C
T268C
T267C
M266C
T265C
L264C
V263C
T262C
T261C
V260C
G259C
F258C
V257C
T256C
R255C
A254C
P253C
V252C
S251C
E250C
WT

B

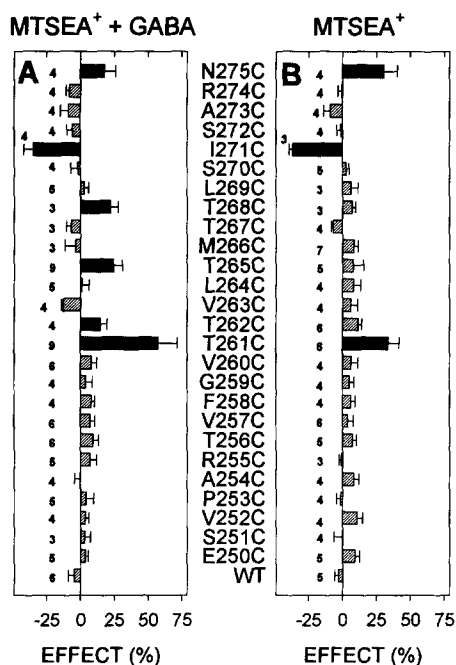-25 0 25 50 75   -25 0 25 50 75

EFFECT (%)   EFFECT (%)

FIGURE 9. The irreversible effect of a 1-min application of the positively charged MTSEA (2.5 mM), applied in the presence (A) or in the absence (B) of 100 μM GABA, on the subsequent GABA-induced currents of wild type and mutant GABA_A receptors. Solid bars indicate mutants for which the effect was statistically significantly different than the effect on wild type. Negative change indicates inhibition and positive change indicates potentiation of the subsequent GABA-induced currents. The means, SEM's and number of oocytes tested are shown.

residues that react with these sulfhydryl reagents are on the water accessible surface of the protein. Furthermore, we assume that the only water-accessible surface for residues in membrane-spanning segments is the surface exposed in the ion channel lumen. We infer that the structure of each mutant is similar to the structure of wild type because we observed GABA-induced

currents in oocytes expressing each of the 26 cysteine-substitution mutants. Therefore, for those mutants that reacted with the sulfhydryl reagents, we infer that the corresponding wild-type residues α_1Val257, α_1Thr261, α_1Thr262, α_1Leu264, α_1Thr265, α_1Thr268, α_1Ile271, α_1Ser272, and α_1Asn275 are exposed in the channel lumen forming the lining of the ion conducting pathway.

Our inference that a residue is exposed in the channel if it reacts with the sulfhydryl reagents is independent of whether the reaction results in potentiation or inhibition of the subsequent GABA-induced currents. Elucidation of the detailed mechanisms for these effects will require the determination of the effect of modification on single channel conductance and open probability. Nevertheless, the observation that reaction of an individual reagent gave inhibition for some mutants and potentiation for others (Table I) implies that the effects of modification are not solely due to local electrostatic or steric effects.

It is tempting to assume that the residues for which the sulfhydryl reagents had no effect are not exposed in the channel lumen. Although this is the likely explanation it may not be correct in all cases. Residues that are exposed in the channel may not react due to local steric factors such as those that limit the ability of MTSES to react with most of the channel-lining residues. Alternatively, because we have measured the effects of modification using macroscopic currents it is possible that, for example, modification might increase open probability but decease single-channel conductance or vice versa resulting in an unaltered macroscopic current.

α_1Val257 was the most cytoplasmic mutant accessible to the reagents applied extracellularly; we do not know whether the reagents can penetrate beyond this point in the channel. MTSES is impermeable through the channel; we were unable to measure outward currents when the 115 mM NaCl in the extracellular bath was re-

TABLE I

Summary of the Cysteine-substitution Mutants and the Sulfhydryl Reagents with Which They Reacted*

	pCMBS	pCMBS/G	pCMB	pCMB/G	IA	IA/G	MTSES	MESES/G	MTSEA	MTSEA/G
N275	+				+		+	+	+	+
S272	−	−	−	−						
I271		−		−	−	−			−	−
T268	−	−	−	−	−	−				+
T265	−	−	−	−	+					+
L264	−	−	−	−		−				
T262						+				+
T261		−		−	+				+	+
V257	−	−		−					−	

*These results are abstracted from Figs. 3 to 7. Only the mutants that reacted with the sulfhydryl reagents are shown. Abbreviations: +, covalent modification caused potentiation of the subsequent GABA-induced current; −, covalent modification caused inhibition of the subsequent GABA-induced currents; blank indicates no effect; /G indicates the sulfhydryl reagent was applied in the presence of GABA; IA is iodoacetate.

placed by 115 mM NaMTSES (data not shown). Consistent with this, isethionate, which also contains a sulfonate, was not measurably permeant (Bormann et al., 1987). The narrow region of the channel is likely to be at or below α_1Val257 because picrotoxin, a channel blocker that is roughly spherical with a diameter of 9 Å appears to bind in the channel at the level of α_1Val257 (Xu et al., 1995). Furthermore, the residues aligned with α_1Val257 in the acetylcholine receptor are thought to be at the narrow point in the channel (Villarroel et al., 1991; Imoto et al., 1991; Cohen et al., 1992). Thus, the extracellularly applied reagents may not be able to reach the mutants, E250C to T256C, that are more cytoplasmic than α_1Val257 (Fig. 10). Alternatively, reduced glutathione, an anion, may be able to enter the cytoplasmic end of this anion-selective channel and protect or regenerate the free sulfhydryl of exposed residues in the region from 250 to 256. Experiments utilizing patch clamp recording will be necessary to resolve these issues.

The functional effects of mutagenesis have been studied for several GABA$_A$ receptor M2 segment residues. In *Drosophila*, cyclodiene insecticides and picrotoxin bind to the GABA$_A$ receptor and inhibit GABA-induced currents. A point mutation of Ala to Ser in the residue which aligns with α_1Val257, a residue exposed in the channel, confers resistance to these agents (french-Constant et al., 1993). This mutation in the *Drosophila* GABA$_A$ receptor had a small effect on single channel conductance, a fivefold stabilization of the open state and a ninefold reduction in the rate of desensitization (Zhang et al., 1994). Mutation to Phe, in the rat GABA$_A$ receptor, of the residues at the positions aligned with α_1Thr261, an exposed residue, in either the α, β, or γ subunits also abolished picrotoxin sensitivity (Gurley et al., 1995). We have shown that coapplication of picrotoxin protected the mutant α_1V257C from modification by pCMBS but coapplication of picrotoxin did not protect the mutant α_1T261C from modification by

pCMBS or MTSEA (Xu et al., 1995). Picrotoxin also protected the mutant α_1V257C from modification by MTSES applied in the presence of GABA (data not shown). Based on these results we inferred that picrotoxin binds in the channel lumen at the level of α_1Val257 (Xu et al., 1995). The binding of the anticonvulsant drug loreclezole is dependent on the residue in the β subunit that aligns with α_1Ser270, a residue that is not exposed in the channel. Loreclezole binds when there is an Asn at this position in the β subunit, as there is in the β_2 and β_3 subunits but the affinity is 300-fold lower when there is a Ser at this position as occurs in the β_1 subunit (Wingrove et al., 1994). In the homologous glycine receptor, mutation of residues in the M2 segment altered single channel conductance (Bormann et al., 1993).

Secondary Structure of the M2 Membrane-spanning Segment

When the residues in and flanking the M2 segment are plotted on an α helical net or wheel representation the exposed residues, except α_1Thr262, lie on one side of the helix within an arc of 120° (Fig. 10 *B*) and form a stripe extending down the side of the helix (Fig. 10 *A*). Approximately 110° of arc would be exposed on an α helix if the channel were lined by five α helices arranged parallel to the channel axis; this is consistent with the presumed pentameric structure of the GABA$_A$ receptor (Unwin, 1993; Nayeem et al., 1994). The exposure of α_1Thr262 indicates that the helix may be distorted in this region, although we cannot exclude the possibility that the mutation distorts the structure of the T262C mutant. Interestingly, using the same approach a similar distortion of the α helical pattern was observed in the aligned region of the M2 segment of the acetylcholine receptor (Akabas et al., 1994a). In a 9-Å resolution structure of the acetylcholine receptor, Unwin (1993) noted that the channel-lining helix was kinked in the middle; assuming that homologous pro-
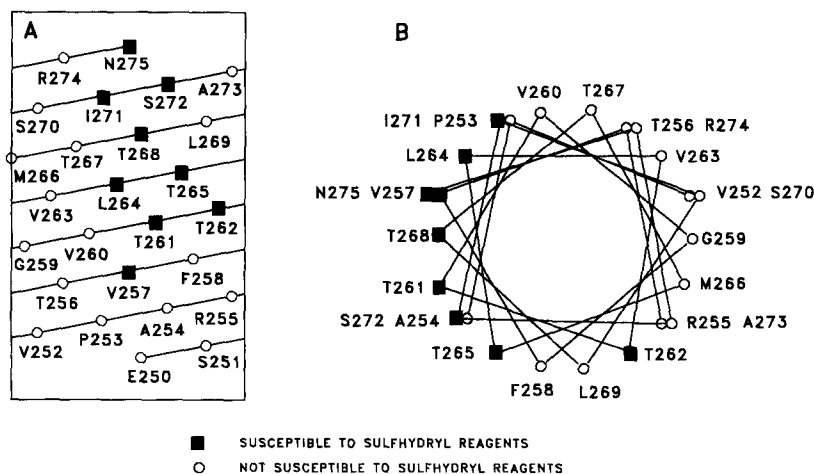

FIGURE 10. α helical projections of the residues in and flanking the M2 membrane spanning segment of the rat α_1 subunit. The mutants that reacted with the sulfhydryl reagents are indicated with solid black squares. The nonreactive mutants are indicated by open circles. (*A*) is a helical net projection. The *x*-axis represents the circumference of the helix. The *y*-axis represents distance along the helix, the extracellular end is at the top. (*B*) is a helical wheel representation.

■ SUSCEPTIBLE TO SULFHYDRYL REAGENTS
O NOT SUSCEPTIBLE TO SULFHYDRYL REAGENTS

teins have similar three-dimensional structures, the kink may correspond to the region of α_1Thr262.

Charge-selectivity Filter

The ability of the positively charged MTSEA to react with cysteines substituted for six of the channel lining residues (Fig. 9 and Table I) suggests that cations can enter the extracellular end of the GABA$_A$ receptor channel at least to the level of α_1Thr261.[3] This may imply that the charge selectivity filter that distinguishes between anions and cations is at least as cytoplasmic as α_1Thr261. Alternatively, because the channel may not be ideally anion selective (the upper limit of potassium permeability relative to chloride is 0.05 [Bormann et al., 1987]) the ability of MTSEA to react might be due to the small, but possibly finite cation permeability. Complementary to these results is our finding in the cation-selective acetylcholine receptor that the extracellular end of the channel is also accessible to anionic sulfhydryl reagents (Akabas et al., 1994a).

Of the nine residues exposed in the channel, none are charged, six are polar and three are hydrophobic. This suggests that the electrostatic interactions between permeating anions and the channel wall are likely to be charge-dipole interactions rather than charge–charge interactions. The anion permeability sequence reported for the GABA$_A$ receptor is SCN > I > Br > Cl, with the reverse conductance sequence (Bormann et al., 1987). This is consistent with a weak interaction between anion binding sites in the channel and the permeating anions (Wright and Diamond, 1977).

Position of the Channel Gate

The ability of the charged sulfhydryl reagents to penetrate from the extracellular end of the channel to the level of α_1Val257 in the absence of GABA, i.e., in the closed state of the channel, suggests that the channel lumen is patent in the closed state and that the position of the gate is at least as cytoplasmic as α_1Val257.

To infer the position of the gate we must assume that in the closed state the sulfhydryl reagents reach the channel-lining cysteines by entering the extracellular end of the channel and moving down the channel. Is it possible that the reagents reach some of the more cytoplasmic residues in the closed state by an alternative pathway, such as through the lipid bilayer to the cytoplasm and then in the cytoplasmic end of the channel? Although these reagents are largely ionized at pH 7.5,

all will have some finite concentration of their unionized form which might be permeable through the lipid bilayer. The membrane permeability of pCMBS is very low (VanSteveninck et al., 1965) and it has been used to distinguish the sidedness of exposure of endogenous cysteine residues in a bacterial anion transporter (Yan and Maloney, 1993). Furthermore, if the reagents did reach the cytoplasm they would most likely be scavenged by the relatively high concentration of free sulfhydryls in the cytoplasm before they could enter the channel. Because the spontaneous open probabilities of our mutants are unknown, we cannot preclude the possibility that in the absence of GABA the sulfhydryl reagents can reach the channel lining cysteines during brief spontaneous openings of the channel. By measuring the rates of reaction of the sulfhydryl reagents with channel-lining cysteines in the presence and absence of GABA we can set a limit on the spontaneous open probability that would be necessary to explain our results.

Our inference that the gate is near the cytoplasmic end of the channel is consistent with the results of similar experiments on the homologous acetylcholine receptor (Akabas et al., 1994a).

GABA-induced Changes in the Reaction of Channel-lining Residues with the Sulfhydryl Reagents

All of the channel-lining residues reacted with at least one of the sulfhydryl reagents in both the presence and in the absence of GABA (see footnote 2 regarding T262C) (Table I). Thus, the same residues are exposed in the channel lumen in the presence and in the absence of GABA. In the absence of GABA the channel is mainly in the closed state, however, as noted above, in the presence of GABA the channel fluctuates between the open, desensitized and closed states; we cannot at present distinguish in which of these state(s) reaction with the sulfhydryl reagents is occurring. Nevertheless, GABA-induced conformational changes do not appear to involve a significant rotation of the channel-lining M2 segments.

Coapplication of GABA altered the ability of at least one of the sulfhydryl reagents to react with eight of the nine channel-lining residues (Table I). GABA-induced changes in the channel might effect the reaction of the sulfhydryl reagents with channel-lining cysteine residues in several ways. First, GABA-induced structural changes might alter local steric factors that influence the ability of a particular reagent to orient itself in order to react. Second, opening the channel gate may alter the electrostatic profile within the channel and this may change the residence time of ions at different positions along the channel; this might also alter the extent of ionization of the cysteine which would effect the reaction.

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3. To rule out the possibility that MTSEA might be entering the channel in its uncharged form we examined the ability of the permanently positively charged MTS-ethyltrimethylammonium (MTSET) to react with T261C. MTSET reacts with T261C in the presence of GABA, indicating that positively charged ions can enter the anion-selective channel of the GABA$_A$ receptor (Horenstein and Akabas, unpublished observation).

Differences between the Sulfhydryl Reagents

An interesting aspect of these experiments is that no single reagent reacted with all of the exposed residues; pCMBS and pCMB reacted with eight of nine, iodoacetate reacted with seven out of nine, MTSES reacted with two out of nine, and the cationic reagent, MTSEA, reacted with six out of nine (Table I). pCMBS, the largest of the reagents, reacted with all of the exposed residues except T262C; given that pCMBS is fairly rigid it is possible that steric factors limit its access to T262C. The smaller anionic reagent, iodoacetate, reacted with a subset of the residues accessible to the mercurials, but, in addition, it also reacted with T262C. The lack of reaction of iodoacetate with V257C and S272C might be due to the slower rate of reaction of iodoacetate with sulfhydryls compared to the mercurials and perhaps the residence time of iodoacetate in the region of these residues is insufficient to allow reaction.

Given that the chemistry of the MTS derivatives is similar, the lack of MTSES reaction with five mutants, T261C, T262C, T265C, T268C, and I271C, that reacted with MTSEA is surprising. The MTS derivatives were used at concentrations that give similar rates of reaction with small sulfhydryls in free solution (Stauffer and Karlin, 1994). MTSES is larger than MTSEA by 1 to 2 Å, but is similar in size to pCMBS, therefore size does not seem to be the crucial issue. MTSES is considerably more flexible than pCMBS; the benzene ring in pCMBS and pCMB is rigid, thereby keeping the anionic group away from the reactive mercurial group, however, the sulfonate group of MTSES can rotate to a position adjacent to the reactive thiosulfonate end of the molecule. In this conformation the reactivity with the negatively charged thiolate of the engineered cysteine may be reduced; why this conformation of MTSES should predominate in the channel is unclear and other factors may be important.

Comparison with the Acetylcholine Receptor

The scanning cysteine accessibility method has also been applied to the M2 segment of the α subunit of the acetylcholine (ACh) receptor (Akabas et al., 1994a). Ten residues between the ACh receptor residues αGlu241 and αGlu262 are exposed in the channel. Seven of the nine residues that we have shown are exposed in the GABA$_A$ receptor channel align with residues exposed in the ACh receptor channel. The ACh receptor residues that align with the GABA$_A$ residues α_1Thr262 and α_1Ser272 are not exposed in the ACh receptor channel. Thus, although the structures of these homologous proteins are similar there are differences. Further study of these differences may help us to understand the mechanisms of charge selectivity and ion conduction in the ligand-gated ion channels.

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