Distinct Structural Changes in the $GABA_A$ Receptor Elicited by Pentobarbital and GABA

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ABSTRACT The barbiturate pentobarbital binds to γ-aminobutyric acid type A ($GABA_A$) receptors, and this interaction plays an important role in the anesthetic action of this drug. Depending on its concentration, pentobarbital can potentiate (~10–100 μM), activate (~100–800 μM), or block (~1–10 mM) the channel, but the mechanisms underlying these three distinct actions are poorly understood. To investigate the drug-induced structural rearrangements in the $GABA_A$ receptor, we labeled cysteine mutant receptors expressed in *Xenopus* oocytes with the sulfhydryl-reactive, environmentally sensitive fluorescent probe tetramethylrhodamine-6-maleimide (TMRM). We then used combined voltage clamp and fluorometry to monitor pentobarbital-induced channel activity and local protein movements simultaneously in real time. High concentrations of pentobarbital induced a decrease in TMRM fluorescence (F_{TMRM}) of labels tethered to two residues in the extracellular domain (α_1L127C and β_2L125C) that have been shown previously to produce an increase in F_{TMRM} in response to GABA. Label at β_2K274C in the extracellular end of the M2 transmembrane helix reported a small but significant F_{TMRM} increase during application of low modulating pentobarbital concentrations, and it showed a much greater F_{TMRM} increase at higher concentrations. In contrast, GABA decreased F_{TMRM} at this site. These results indicate that GABA and pentobarbital induce different structural rearrangements in the receptor, and thus activate the receptor by different mechanisms. Labels at α_1L127C and β_2K274C change their fluorescence by substantial amounts during channel blockade by pentobarbital. In contrast, picrotoxin blockade produces no change in F_{TMRM} at these sites, and the pattern of F_{TMRM} signals elicited by the antagonist SR95531 differs from that produced by other antagonists. Thus, with either channel block by antagonists or activation by agonists, the structural changes in the $GABA_A$ receptor protein differ during transitions that are functionally equivalent.

INTRODUCTION

Many drugs with anesthetic, hypnotic, sedative, convulsant, and anticonvulsant actions act upon γ-aminobutyric acid type A ($GABA_A$) receptors. These drugs belong to a number of distinct chemical classes that include barbiturates, neurosteroids, and benzodiazepines (1,2). In addition to the principal agonist binding site to which GABA binds, the $GABA_A$ receptor has structurally distinct binding sites for many of its other ligands. The structural basis for the diverse actions of different classes of drugs on the same molecular target represents a major challenge in the investigation of structure-function relationships in the $GABA_A$ receptor.

The barbiturate pentobarbital binds to $GABA_A$ receptors and, depending on its concentration, modifies the function of this protein in three distinct ways. Below ~100 μM, pentobarbital has little or no effect on its own, but it potentiates channel activity induced by subsaturating concentrations of GABA (3–5). Intermediate concentrations of pentobarbital (~100 μM–1 mM) act as a receptor agonist to gate the channel in the absence of GABA (6). Above ~1 mM, pentobarbital becomes inhibitory and blocks current through the channel (6–8). The structural mechanisms underlying these different actions are not well understood.

$GABA_A$ receptors are members of the Cys-loop family of ligand-gated ion channels. These heteropentamers are formed from various combinations of structurally homologous subunits (9,10). Each subunit contains an extracellular N-terminus of ~200 amino acids, 4 transmembrane α-helices (M1–M4), and a short extracellular C-terminus. GABA binds to sites within pockets formed by the interfaces of the N-termini of adjacent α- and β-subunits (11). Barbiturates such as pentobarbital are thought to bind to a completely different site, possibly formed by parts of M1, M2, and M3 of the β-subunits (12–14). Indeed, barbiturates can directly gate channels formed by β-subunit homomeric receptors, but GABA cannot (15,16). Thus, β-subunits harbor structural elements sufficient for barbiturate binding, but activation by GABA requires additional elements. When pentobarbital acts as an agonist, the structural changes in the $GABA_A$ receptor resemble those induced by GABA in an important respect; the channels have the same unitary conductance (8,17). This finding raises an interesting question about how GABA and pentobarbital induce similar activated states by binding to different sites.

Simultaneous electrophysiology and site-specific fluorometry have shown that labels tethered to residues in the N-terminal domains of $GABA_A$ receptors undergo changes in their environment in response to GABA and other ligands (18,19). Similar experiments in the related nicotinic acetylcholine (20) and glycine receptors (21) showed that two different agonists induce distinct protein movements during activation. In this study, we used this approach to investigate the molecular rearrangements of the $GABA_A$ receptor

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induced by pentobarbital. We found sites in the N-terminus and upper M2 that undergo distinct structural changes depending on whether the receptor binds GABA or pentobarbital; these changes also reveal differences in the molecular mechanisms of action of these two ligands.

MATERIALS AND METHODS

Molecular biology and oocyte expression

Individual cysteine substitutions were made by recombinant polymerase chain reaction-driven site-directed mutagenesis (as described in the works by Muroi et al. (19) and Boileau et al. (22)) in DNA encoding rat $GABA_A$ α_1-subunit (E122C, L127C, K278C), β_2-subunit (P120C, L125C, K274C), and γ_2-subunit (N135C, L140C, K289C) in the pGH19 vector (23). In this work, the key sites studied in depth are highlighted in Fig. 1 *B*. The mutations were confirmed by DNA sequencing. Capped mRNA was prepared using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Oocytes were surgically isolated from anesthetized adult female *Xenopus* laevis and defolliculated after agitation in 1 mg/mL collagenase (Roche, Basel, Switzerland) in zero Ca^{2+}-ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.7). Before and after injection, the oocytes were stored in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) supplemented with 100 μg/mL gentamicin and 100 μg/mL bovine serum albumin (Fisher, Pittsburgh, PA). Oocytes were injected 24–48 h after surgery with ~30 nL of an injection cocktail containing α/β-subunit encoding mRNA in a 1:1 ratio (6.7–18 ng/subunit) or $\alpha/\beta/\gamma$-subunit encoding mRNA in a 1:1:10 ratio (4.5–27 ng/subunit). Injected oocytes were incubated at 18°C for 3–10 days before the experiments.

Fluorophore labeling

Oocytes were incubated in 10 μM tetramethylrhodamine-6-maleimide (TMRM; Invitrogen, Carlsbad, CA) in ND96 on ice for 30 min in the dark. They were then washed with ND96 and used for recording immediately or stored on ice for use within 3 h. A 10 mM stock of TMRM was prepared in dimethyl sulfoxide and stored at −80°C.

Two-electrode voltage clamp

All recordings were performed at room temperature in a darkened room. Oocytes were clamped at −80 mV with an OC-725C Oocyte Clamp amplifier (Warner Instruments, Hamden, CT). The oocyte was perfused continuously in a ~200 μL chamber with ND96 (~5 mL/min). Glass micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. Fluorescence and current signals were read into a personal computer running Clampex 8 software (Molecular Devices, Sunnyvale, CA).

Substituted cysteines were checked for reactivity with TMRM using a functional assay in which current responses to ~EC_{50} GABA were compared before and after 5-min treatments with 100–500 μM TMRM in ND96. A successful cysteine modification resulted in a change of $> 20\%$ in current amplitude. Cysteine mutant-expressing oocytes incubated with TMRM displaying significant changes in fluorescence (see below) compared to wild-type upon ligand addition were also assumed to have been successfully modified. Incorporation of the γ_2-subunit was confirmed by testing for potentiation of responses to low GABA concentrations by flurazepam (22).

Concentration-response curves were analyzed as described previously (19). Plots of fluorescence change versus concentration were fitted to the Hill equation as follows:

$$y = F_{\max}\left(x^n/\mathrm{EC}_{50}^n + x^n\right),$$

where $F_{\max}$ is the maximum fluorescence change, x is the ligand concentration, n is the Hill coefficient, and EC_{50} is the ligand concentration producing a half-maximum response. Fitting was performed with either Origin software (MicroCal, Northampton, MA) or Prism software (GraphPad, San Diego, CA).

For studies of the concentration dependence of block by pentobarbital, the extent of block was calculated from the peak ''tail'' current, determined by extrapolating the exponential decay (fitted with Clampfit 8; Molecular Devices) of the off-response (as the drug was removed) back to the start of drug removal (see Fig. 7, *insets*).

Simultaneous current and fluorescence recording

Parallel measurements of fluorescence and current were conducted as described previously (19). In brief, illumination from a 100 W halogen lamp was filtered through an excitation filter (535/50 nm excitation) and reflected with a 565 dichroic mirror onto the preparation through a Zeiss × 10 0.50 NA objective (Carl Zeiss, Thornwood, NY). Fluorescent light passed through a 610/75 nm emission filter and was measured with a Hamamatsu (Bridgewater, NJ) photomultiplier tube. An electronic shutter controlled illumination.

Structural modeling

A homology model based on the crystal structure of the *Lymnaea* acetylcholine-binding protein (24) for the extracellular domain and the 4 Å structure of the *Torpedo* nicotine acetylcholine receptor (25) for the transmembrane domain was constructed for the rat $GABA_A$ receptor as described previously (26). In brief, amino acid sequences of the $GABA_A$ receptor were aligned and threaded onto the parent structures and then energy minimized with SYBYL software (Tripos, St. Louis, MO). The two domains were then physically docked and again energy minimized in SYBYL.

The $GABA_A$ receptor model images in Fig. 1 were developed using the PyMOL Molecular Graphics System (DeLano Scientific, Palo Alto, CA).

Statistical analysis

A one-way analysis of variance (ANOVA) was performed using Prism software (GraphPad) to compare fluorescence changes induced by similar drug concentration (e.g., activating pentobarbital or $EC_{90–99}$ GABA) between mutants and controls (wild-type receptors). The post hoc Dunnett's test was performed after the ANOVA. The error bars in the graphs represent mean ± SE for $n \geq 3$ trials.

RESULTS

Previous studies of the extracellular domain of the $GABA_A$ receptor demonstrated that fluorescent labels attached to residues E122C and L127C of the α_1-subunit and to the homologous residues of the β_2-subunit (P120C and L125C, respectively) report changes in their chemical environments when either GABA or the competitive antagonist SR95531 bind to the receptor (19). The homologous residues of the γ_2-subunit (residues N135C and L140C, respectively) were also examined. Substituting a cysteine at γ_2L140 had a dominant negative effect and suppressed the formation of functional $GABA_A$ receptors. $\alpha_1\beta_2\gamma_2$N135C expressed well; when labeled with TMRM, however, this receptor showed no changes in fluorescence (F_{TMRM}) in response to GABA or SR95531. Labeling of this receptor was confirmed by showing a change in its current response, and so this site does not appear to be sensitive to the binding of these ligands. We used TMRM labeling to determine whether these same extracellular domain sites that reported changes

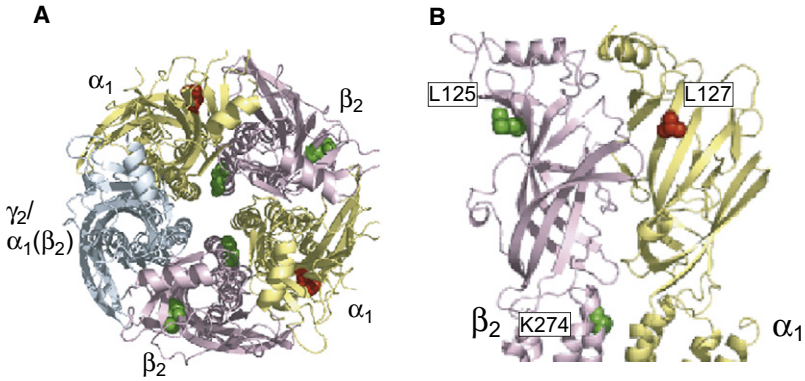

FIGURE 1 Three TMRM-labeled sites in the $GABA_A$ receptor showing fluorescence changes in response to pentobarbital. Structural model of the heteropentameric $GABA_A$ receptor from an extracellular, top-down view (*A*) and from a side view at the GABA binding interface (*B*). Labels indicate subunit subtypes. Residues at sites reporting changes in fluorescence in the β_2-subunit are shown as green and in the α_1-subunit are shown as red. Fluorescence changes are summarized in Table 1 and Fig. 6.

in response to GABA and SR95531 would also report changes in response to pentobarbital at modulating, activating, and blocking concentrations. We also investigated F_{TMRM} signals from labels at β_2K274 and aligned residues in other subunits. Key sites in the N-terminus and upper M2 are indicated in Fig. 1.

The sites tested with pentobarbital in simultaneous voltage clamp and fluorometry recording are indicated in Table 1. In all of these labeled receptors, GABA or pentobarbital generally elicited currents in the range of 10–25 μA, indicating these mutants expressed well. At most of these sites, pentobarbital elicited no significant F_{TMRM} signal. Residues at which labels showed a fluorescence change in response to 10 mM pentobarbital in either $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_2$ receptors are indicated in Table 1 with bold type.

The extracellular domain of the $GABA_A$ receptor

Modulating concentrations of pentobarbital (<100 μM) applied in the absence of GABA induced little or no current response and failed to elicit statistically significant changes in F_{TMRM} at any of the sites in the extracellular domain (see Fig. 6). We know that pentobarbital binds to the $GABA_A$ receptor at this concentration because the same low concentrations of pentobarbital elicited a fluorescence change in TMRM-β_2K274C (data presented below). Furthermore, previous studies have shown that potentiating pentobarbital concentrations alter the accessibilities of cysteines introduced at the GABA binding pocket to thiol-reactive reagents (27). Thus, in the absence of GABA, occupation of a high-affinity modulating site on the $GABA_A$ receptor by pentobarbital produces no detectable structural changes in the vicinity of residues α_1E122, α_1L127, β_2P120, or β_2L125, whereas these locations undergo clear changes in response to GABA and, in some cases, to SR95531 as well (19). We tested pentobarbital concentrations of 300 μM, 800 μM, and 10 mM and observed no change in fluorescence in TMRM-γ_2N135C.

To investigate $GABA_A$ receptor activation by pentobarbital, we tested concentrations in the range of 300–800 μM. These intermediate concentrations elicited small fluorescence decreases in some of these extracellular domain labels (Fig. 2). Activating concentrations of pentobarbital elicit $GABA_A$ receptor current while showing little or no evidence of channel block (as judged by tail current; see below). In receptors either lacking ($\alpha_1\beta_2$, Fig. 2 *A*) or containing ($\alpha_1\beta_2\gamma_2$, Fig. 2 *B*) a γ_2-subunit, TMRM-α_1L127C showed a fluorescence increase in response to GABA and a fluorescence decrease in response to pentobarbital. The opposite sign of the F_{TMRM} changes produced by these two agonists suggest that GABA and pentobarbital induce different molecular rearrangements in the extracellular domain. However, a more thorough analysis of the concentration dependence of pentobarbital-induced fluorescence changes presented below indicates that the fluorescence changes induced by intermediate, activating concentrations of pentobarbital reflect a small amount of blocking action. We will return to this issue after presenting additional data.

TMRM-β_2L125C reported no change in fluorescence in response to pentobarbital, regardless of whether a γ_2-subunit was present (Fig. 2, *C* and *D*). TMRM-β_2L125C did show a fluorescence change in response to GABA when the

TABLE 1 TMRM fluorescence changes

	$\alpha_1\beta_2\gamma_2$		$\alpha_1\beta_2$	
Label site	PB	GABA	PB	GABA
α_1E122	–	↓	–	↓
α_1L127	–	↑	↓	↑
α_1K278	–	–	nt	nt
β_2P120	–	–	–	–*
β_2L125	–	–	↓	–*
β_2K274	↑	↓	↑	–
γ_2N135	–	–	–	–
γ_2K289	–	–	nt	nt

Fluorescence increases (*upward arrow*) and decreases (*downward arrow*) elicited by 10 mM pentobarbital (*PB*) and $EC_{90–99}$ GABA. "–" denotes no significant change in fluorescence. "nt" denotes not tested. Label sites in bold type denote sites at which PB elicited fluorescence changes in either $\alpha_1\beta_2\gamma_2$ or $\alpha_1\beta_2$ receptors.

*In a previous report (19), a label at β_2P120 showed a fluorescence decrease, and a label at β_2L125 showed a fluorescence increase. These changes were statistically significant compared to control recordings in wild-type receptors ($p < 0.05$) using the *t*-test. However, when the ANOVA technique was used to analyze the data set in this study, these changes were no longer significant.

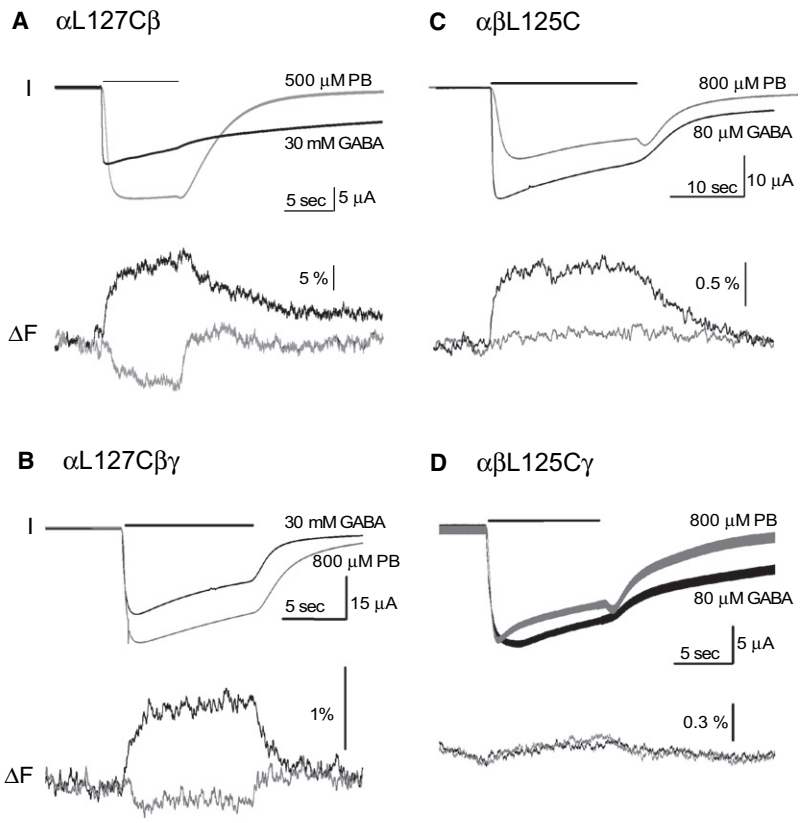

FIGURE 2 Current and fluorescence responses of N-terminus labels to GABA and activating concentrations of pentobarbital. Simultaneous current (*top*) and fluorescence (*bottom*) changes induced by $EC_{90–99}$ GABA (*black traces*: 30 mM in *A* and *B*; 80 μM in *C* and *D*) and an activating pentobarbital concentration (*gray traces*: 500–800 μM). (*A*) TMRM-α_1L127Cβ_2 receptors, (*B*) TMRM-α_1L127C$\beta_2\gamma_2$ receptors, (*C*) TMRM-$\alpha_1\beta_2$L125C receptors, and (*D*) TMRM-$\alpha_1\beta_2$L125Cγ_2 receptors.

γ_2-subunit was absent (as evidenced in a comparison between panels *C* and *D* in Fig. 2). The significance of this change cannot be assessed unambiguously. In a previous study (19), we found this change to be significant according to the *t*-test; in this work, however, ANOVA with a larger data set (additional mutants) indicated that this change was not statistically significant. In the same manner, the TMRM-α_1E122C and TMRM-β_2P120C fluorescence also did not change in response to activating concentrations of pentobarbital (data not shown), but we previously reported that these sites change their fluorescence in response to GABA (19). Furthermore, GABA and SR95531 produced fluorescence changes opposite in sign in both TMRM-α_1E122C and TMRM-α_1L127C (19). Thus, depending on the ligand and the site, labels in this region reported increases, decreases, or no change in fluorescence, indicating that this part of the $GABA_A$ receptor undergoes different conformational rearrangements in response to the binding of different ligands.

Above a concentration of 1 mM, pentobarbital blocks the $GABA_A$ receptor channel by an unknown mechanism (7,28). The peak current elicited by a concentration of 10 mM pentobarbital was lower than that elicited by 300–800 μM. Upon drug removal, the current increased transiently before returning to baseline (Fig. 3). This ''tail'' current indicates that pentobarbital dissociates from a blocking site on the receptor more rapidly than from an activation site. At a concentration of 10 mM, pentobarbital produced prominent fluorescence changes in TMRM-α_1L127C and TMRM-β_2L125C, which were considerably greater than those elicited by activating concentrations (as evidenced by a comparison of Fig. 3 with Fig. 2). Upon pentobarbital removal, F_{TMRM} decayed rapidly, but the current increased as the tail appeared. This divergence of the current and fluorescence upon drug removal contrasts sharply with the more similar time courses of changes in these two signals as the drug was applied. The tail current reached a peak close to the time when the fluorescence returned to baseline. This finding suggests that the F_{TMRM} change induced by pentobarbital reflects blockade rather than receptor activation and channel opening. As with activating concentrations, blocking concentrations of pentobarbital only produced fluorescence changes in labels at the two aligned residues α_1L127C and β_2L125C. Labels at the other sites in the extracellular domain tested in this study failed to show fluorescence changes with any pentobarbital concentration (Table 1).

The upper end of M2

The extracellular end of M2 (the pore-lining helix) harbors a conserved lysine residue (position 24′) implicated in the transduction of binding to gating in the $GABA_A$ receptor (29–31). We therefore used TMRM labeling to investigate structural changes induced by pentobarbital and other

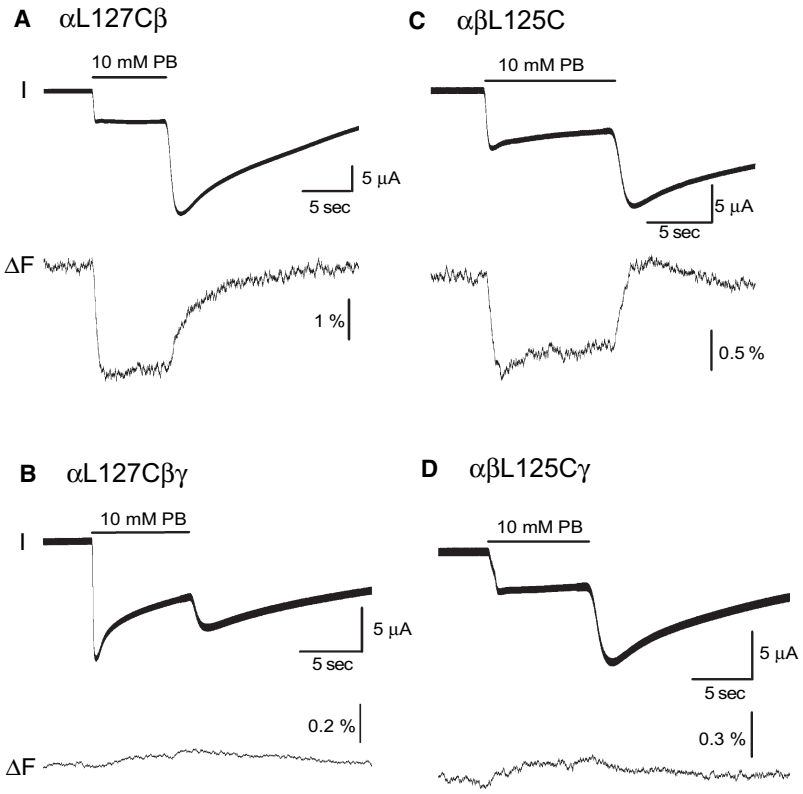

FIGURE 3 Current (*top*) and fluorescence (*bottom*) responses of N-terminal labels to blocking concentrations of pentobarbital (10 mM). (*A*) TMRM-α_1L127Cβ_2 receptors, (*B*) TMRM-α_1L127C$\beta_2\gamma_2$ receptors, (*C*) TMRM-$\alpha_1\beta_2$L125C receptors, and (*D*) TMRM-$\alpha_1\beta_2$L125C γ_2 receptors. Note prominent tail currents upon pentobarbital removal.

ligands. The mutant β_2K274C was incorporated into functional receptors either with or without a γ_2-subunit. Activating concentrations of pentobarbital (300–800 μM) elicited fluorescence changes from a label at this site (Fig. 4), but labels at the aligned positions in the other subunits (α_1K278 and γ_2K289) showed no significant changes (Table 1). As with the label at α_1L127C in the extracellular domain, the fluorescence change elicited by GABA in the TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors was opposite in sign to that elicited by pentobarbital (Fig. 4 *A*).

In contrast to labels in the extracellular domain, TMRM-β_2K274C (in $\alpha_1\beta_2$ receptors) produced a change in fluorescence even in response to a low, potentiating concentration (60 μM) of pentobarbital (Fig. 4 *B*). Because this concentration failed to elicit detectable current, these fluorescence signals reflect an electrically silent event in the $GABA_A$ receptor protein. This result was specific for $\alpha_1\beta_2$ receptors; modulating concentrations of pentobarbital failed to elicit a statistically significant fluorescence change in TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors.

As in oocytes expressing receptors with labels in the N-terminus (Fig. 3), blocking concentrations of pentobarbital (>1 mM) elicited current responses in TMRM-$\alpha_1\beta_2$K274Cγ_2 and TMRM-$\alpha_1\beta_2$K274C receptors with tail

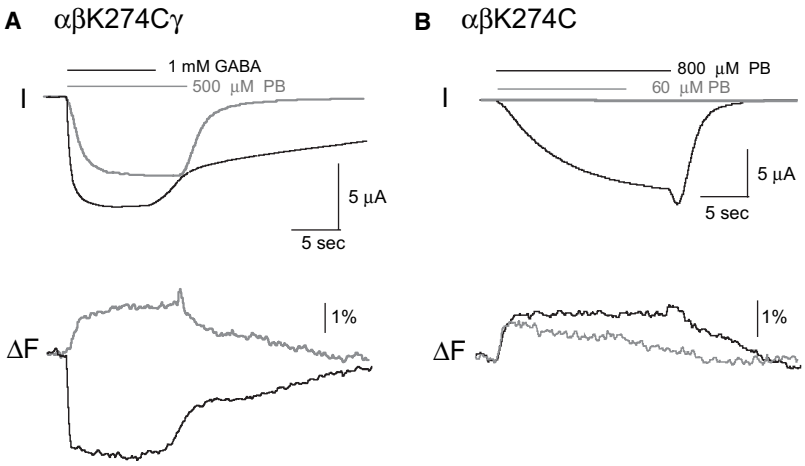

FIGURE 4 Current (*top*) and fluorescence (*bottom*) responses of labels in M2. (*A*) Activating concentrations of pentobarbital (*gray traces*) and GABA (*black traces*) on TMRM-$\alpha_1\beta_2$K274Cγ_2. (*B*) Activating (*black traces*) and modulating (*gray traces*) concentrations of pentobarbital on TMRM-$\alpha_1\beta_2$K274C receptors.

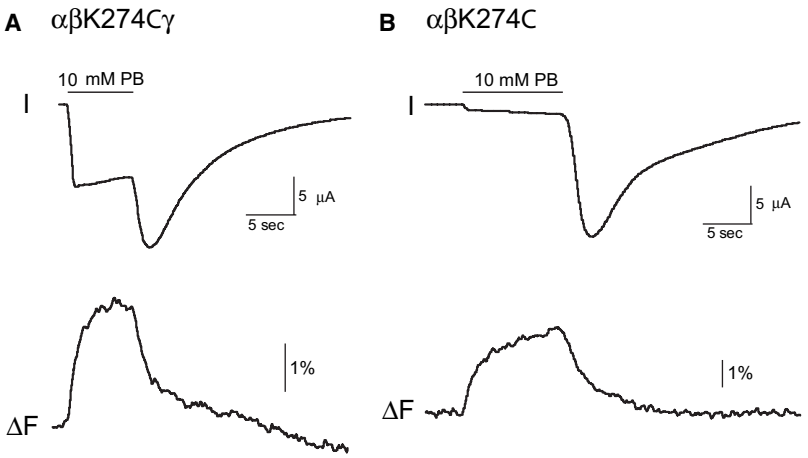

FIGURE 5 Current and fluorescence traces in response to a blocking concentration of pentobarbital (10 mM) on (*A*) TMRM-$\alpha_1\beta_2$K274Cγ_2 and (*B*) TMRM-$\alpha_1\beta_2$K274C receptors. Note prominent tail currents upon pentobarbital removal.

currents characteristic of receptor blockade (Fig. 5). With this high concentration of pentobarbital, the onset of the fluorescence and current responses moved roughly in parallel upon drug application, but the fluorescence decayed more rapidly upon drug removal. In summary, TMRM-$\alpha_1\beta_2$K274C receptors reported a fluorescence increase with all pentobarbital concentrations tested, but the magnitude varied depending on the concentration used.

Concentration dependence of pentobarbital actions

Fig. 6 summarizes the F_{TMRM} signals elicited by different pentobarbital concentrations from labels on residues α_1L127C, β_2L125C, and β_2K274C in $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors. Fluorescence changes elicited by GABA are also presented; these changes include results for GABA on α_1L127C and β_2L125C that were found in our previous work (19). Fig. 6 illustrates the contrasts in structural changes induced by potentiating, activating, and blocking pentobarbital concentrations; the relationship between these fluorescence signals and the distinct effects of pentobarbital on receptor function, however, were difficult to establish from the data as presented in Fig. 6. To address this issue, we plotted fluorescence and current responses versus pentobarbital concentration using TMRM-α_1L127Cβ_2 and TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors; we selected these receptors because they produced particularly clear F_{TMRM} signals.

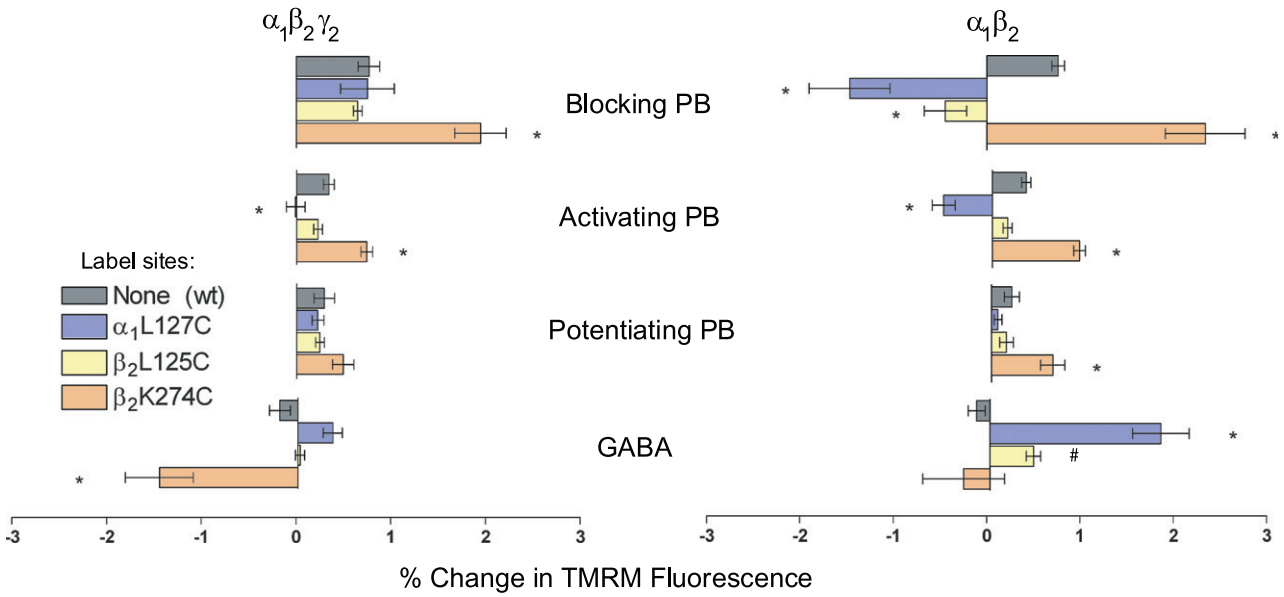

FIGURE 6 Summary of fluorescence changes elicited by pentobarbital (*PB*) and GABA. Each bar represents mean percent change in fluorescence in response to indicated ligands; error bars represent mean ± SE of $n = 3$–31 trials. Pentobarbital concentrations are 10 mM for blocking (tail current prominent), 300–800 μM for activating (current response with little or no tail current), and 50–100 μM for potentiating/modulating (little to no current). GABA concentrations are approximately EC_{90-99}. *$p < 0.05$ for one-way ANOVA compared to wild-type (*wt*) and post hoc Dunnett's test. #This change was significant by a *t*-test within a smaller data set (19).

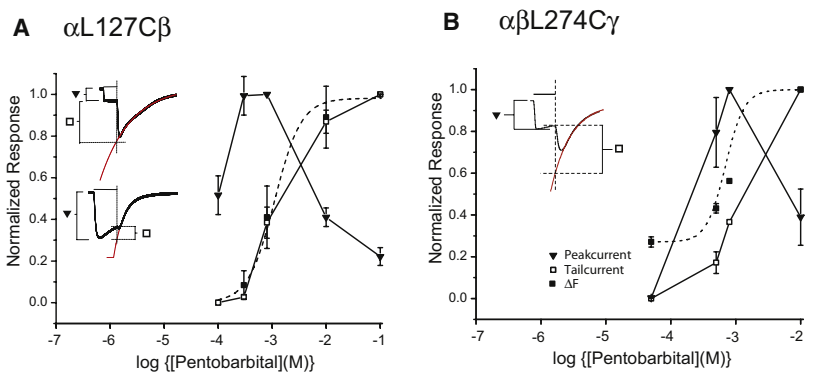

FIGURE 7 Changes in fluorescence parallel current blockade by pentobarbital. Plot of fluorescence change (ΔF, *solid squares*, *broken line*), current in the presence of pentobarbital (*downward solid triangles*), and current block (*open squares*). (*A*) TMRM-α_1L127Cβ_2. (*B*) TMRM-$\alpha_1\beta_2$K274Cγ_2. Current response points were connected by straight lines, whereas ΔF points were fitted using least-squares regression to the Hill equation (see Materials and Methods). All values were normalized to the maximum for that measurement. Error bars represent mean ± SE of experiments in ~10 oocytes. The insets show representative pentobarbital-induced current traces including guides for our measurements of current in the presence of pentobarbital (*downward solid triangles*) and extrapolated tail current amplitude (*open squares*). The decay of tail current was fitted to a single exponential (*red*), which was extrapolated to the time of buffer wash to estimate maximum current amplitude at the time of buffer wash and block relief (see Materials and Methods).

In this analysis, we attempted to establish parallels between the concentration dependence of channel activation, channel blockade, and F_{TMRM}.

Fig. 7 compares plots of the concentration dependence of pentobarbital-induced F_{TMRM}, current (during pentobarbital application), and block. Block was determined form the tail current, as illustrated in the insets in each panel of Fig. 7. The amplitude of the tail current (*open squares*) was determined by fitting the current decay to an exponential and extrapolating back in time to the start of drug removal. Taking this extrapolated current as an estimate of the full channel current in the absence of blockade (I_f) and the current immediately before pentobarbital removal as the current observed in the presence of blockade (I_b) allowed us to calculate the extent of block. We also determined the amplitude of the initial peak current before drug removal (*solid downward triangles*). Current, extent of block, and F_{TMRM} were each normalized to their maxima and plotted versus pentobarbital concentration (Fig. 7).

These plots revealed a close parallel between the concentration dependence of the F_{TMRM} change and receptor blockade. By contrast, there was a clear disparity between the concentration dependence of the F_{TMRM} signal versus the pentobarbital-induced receptor activation. These comparisons strongly support an association of the major component of the pentobarbital-induced fluorescence change with its blocking action. Because the concentration at which the pentobarbital-induced current peaks falls well below the concentration at which fluorescence saturates, the activated state of the receptor induced by pentobarbital appears to be associated with little, if any, change in fluorescence. The parallel is not as clear for TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors (Fig. 7 *B*), but this finding can be attributed to the fact that low, potentiating pentobarbital concentrations induced a small but significant fluorescence change (Fig. 4 *B*). The nonzero fluorescence signal at the point < 100 μM, where the current is zero, together with the increase in fluorescence in parallel with the blockade indicate that this site undergoes two distinct structural changes—one associated with potentiation and the other with blockade.

Picrotoxin action on the upper part of M2

Because the label at K274C shows a strong fluorescence change during block by pentobarbital, we examined the structural changes induced by other important $GABA_A$ receptor antagonists at this site. Picrotoxin is a $GABA_A$ receptor antagonist that interacts with residues in M2 that are deep within the ion channel, and it probably inhibits current by both steric channel obstruction and allosteric modulation (32–35). As expected, picrotoxin (100 μM ~IC_{99}) strongly inhibited the current elicited by 1 mM GABA in TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors, but the GABA-induced fluorescence decrease remained unchanged (Fig. 8). This experiment also showed that picrotoxin application on its own induced no change in F_{TMRM}. Thus,

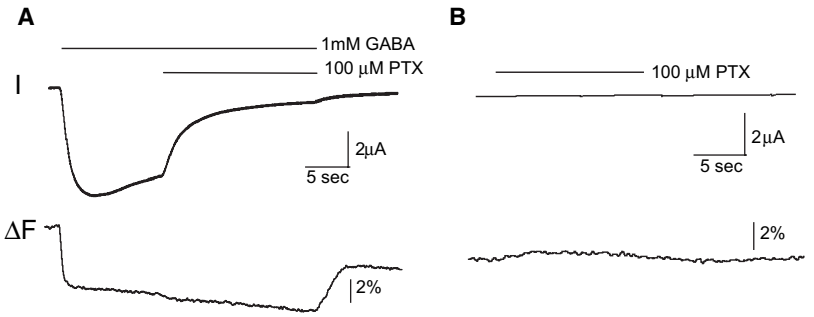

FIGURE 8 Picrotoxin blocks $GABA_A$ receptor channels without altering fluorescence. Current (*top*) and fluorescence (*bottom*) change in TMRM-$\alpha_1\beta_2$K274Xγ_2 receptors challenged with $EC_{90–99}$ GABA and 100 μM picrotoxin (*A*) or picrotoxin alone (*B*).

picrotoxin antagonism entails no detectable changes in the environment near β_2K274C. Another $GABA_A$ receptor antagonist SR95531 is a competitive inhibitor of GABA binding and induces fluorescence changes in labels attached to agonist-binding pocket residues in the α-subunit (19). This compound at a concentration of 10 μM induced no change in the fluorescence of TMRM-$\alpha_1\beta_2$K274Cγ_2 receptors ($n = 5$; data not shown). Thus, residue 274 of the β_2-subunit responds differently when challenged with different antagonists.

DISCUSSION

As better structural pictures of Cys-loop ligand-gated ion channels emerge, the question of how allosteric modulators and activators induce structural transitions between distinct functional states comes into sharper focus. In this study, we used site-specific fluorescent labels to probe transitions in the $GABA_A$ receptor induced by pentobarbital; our results demonstrate that functionally equivalent states induced by the binding of pentobarbital and GABA have nonequivalent structures. Furthermore, pentobarbital-induced channel block triggers structural changes that are distinct from those seen during activation and modulation. These structural changes are also distinct from those elicited by other antagonists.

Distinctions between GABA- and pentobarbital-induced conformational changes

GABA and pentobarbital both induce the opening of the channel associated with the $GABA_A$ receptor, but these two ligands bind to different sites (12). The action of pentobarbital depends on residues in β-subunits toward the extracellular end of M2 (13,14). Our study found that, among homologous locations in M2 (24′), the β_2-subunit label—but not the α_1- or γ_2-subunit labels—demonstrated a fluorescence change in response to pentobarbital. Although we could not distinguish between a direct interaction involving pentobarbital and the β_2K274C label and ligand-induced protein movement at this site, our results provide further support for the hypothesis that the extracellular end of M2 of a β-subunit has an important role in pentobarbital action. GABA and pentobarbital appear to stabilize an open-state channel structure with a similar single-channel conductance (8,17) and similar chemical reactivity of residues at the 6′ position in the channel-lining M2 helix (36).

In the extracellular N-terminus, GABA induced F_{TMRM} signals at positions α_1L127C, α_1E122C, β_2L125C, and β_2P120C (19). We found that activation by pentobarbital did not change the fluorescence of labels at these sites. The F_{TMRM} signals induced by pentobarbital at α_1L127C and β_2L125C reflected blockade rather than activation, and were opposite in sign to those elicited by GABA. When GABA activates the $GABA_A$ receptor, the N-terminal domains undergo a global, quasisymmetrical change in structure in which the homologous sites in the α- and β-subunits undergo similar but unequal movements (19). Activation by pentobarbital occurs without a fluorescence change at these same sites in the N-terminus. On this basis, we conclude that pentobarbital and GABA both gate $GABA_A$ receptor channels without inducing the same global structural changes in the N-terminus.

A residue near the extracellular end of M2 (β_2K274C, which is at the 24′ position) also reports different movements in response to GABA and pentobarbital. Potentiating concentrations of pentobarbital elicited a fluorescence change at this site in $\alpha_1\beta_2$ receptors but not in $\alpha_1\beta_2\gamma_2$ receptors. GABA elicited a fluorescence change of the opposite sign, but it did so in $\alpha_1\beta_2\gamma_2$ receptors rather than in $\alpha_1\beta_2$ receptors. Although the dependence of these changes on subunit composition complicates the interpretation, the results in $\alpha_1\beta_2$K274Cγ_2 receptors offer an example where GABA activation induces a movement near the top of M2, whereas pentobarbital activation does not. Site-specific fluorescent labeling studies in other Cys-loop ligand-gated channels (namely, the nicotinic receptor (20) and the glycine receptor (21)) offer more examples of ligand-specific structural changes during activation. These studies (20,21) also demonstrate that the sites exhibiting this behavior reside near the extracellular end of M2. In the pre-M1 region, which is quite close to the extracellular end of M2, GABA and pentobarbital have different effects on the rates of modification of introduced cysteines (37). A number of residues in the N-terminus also respond to activation by pentobarbital by changes in cysteine accessibility, and many of these changes differ from those induced by GABA (27,38–41). Taken together, these findings suggest that the structural mechanisms underlying channel opening in Cys-loop ligand-gated ion channels can differ substantially depending on the choice of ligand used to trigger a response.

The present fluorescent labeling results together with the above-cited cysteine accessibility study (37) identify a fairly extensive part of the $GABA_A$ receptor, including the N-terminus, pre-M1 region, and extracellular end of M2 of the β_2-subunit, where GABA and pentobarbital induce different movements. However, deeper in the membrane, the structural changes induced by these two ligands appear to be more similar, as indicated by chemical reactivity (36) and single-channel conductance (8,17). Rate-equilibrium free energy analysis in the related nicotinic acetylcholine receptor has indicated that the transduction of binding to gating progresses through a sequence of coupled movements of domains with roughly nanometer-sized dimensions. The extracellular ligand-binding domain moves first, followed by loops 2 and 7, which are located at the interface between the extracellular domain and the transmembrane domain (pre-M1); then the extracellular M2-M3 linker moves. Finally, the transmembrane domains move, with the extracellular and intracellular ends of M2 moving before the middle (42,43). General anesthetics including pentobarbital

likely bind in a water-filled pocket in the upper transmembrane region of the $GABA_A$ receptor (13,14,44–47); this region lies along the transduction pathway elucidated by the rate-equilibrium free energy analysis in the nicotinic receptor. Occupation of a site in this region could thus enable pentobarbital to initiate channel opening at a deeper location, triggering movements at the upper ends of the transmembrane domains that propagate toward the gate of the channel. In this way, the transduction of pentobarbital binding to channel gating could exhibit altered coupling to movements in N-terminal domains.

We looked for conformational movements produced by pentobarbital at modulating, activating, and blocking concentrations, thereby probing three distinct modes of action on the $GABA_A$ receptor. The sites to which pentobarbital binds to induce these various changes in receptor function are still unknown. With regard to the two positive effects on receptor function, potentiation and activation, examining the mechanisms by which other drugs exert similar actions on the $GABA_A$ receptor provides a useful guide. Benzodiazepines bind to a single site at the interface between the extracellular domains of the α- and γ-subunits. It is likely that benzodiazepines both potentiate and activate the receptor by an allosteric enhancement of channel opening by binding to this one site (48–50). In contrast, neurosteroids likely activate the receptor and potentiate GABA-induced activity by binding to two distinct sites among the transmembrane domains (51). Thus, there are precedents for binding at both a single site and multiple sites in the mediation of the related actions of potentiation and activation. Ascertaining whether barbiturates like pentobarbital target one or two sites on the $GABA_A$ receptor for the two positive actions will require further experiments.

The influence of a γ-subunit

Labeled α_1L127C and β_2L125C mutants produced significant fluorescence changes in response to pentobarbital in $\alpha_1\beta_2$ receptors but not in $\alpha_1\beta_2\gamma_2$ receptors. Likewise, the presence of the γ_2-subunit eliminated the fluorescence change of TMRM-β_2K274C that was induced by potentiating concentrations of pentobarbital, but this subunit was necessary for GABA-induced fluorescence changes in this mutant. Previous work (19) has shown that the presence of the γ_2-subunit alters the fluorescence responses of labels on other subunits induced by both GABA and SR95531. Thus, we have a number of examples demonstrating that the γ_2-subunit influences ligand-induced structural changes in other subunits. If the fluorescence changes were the result of direct quenching by a ligand rather than a conformational change in the protein, we would not expect the incorporation of another subunit that has no direct role in ligand binding to have this kind of an impact. $GABA_A$ receptors usually contain either a γ- or δ- subunit, but recent work showing $GABA_A$ receptors composed from α- and β-subunits in neurons (52) makes the question of how a γ-subunit alters ligand-induced structural transitions especially interesting. The presence of a γ-subunit thus alters the structural changes that drugs induce in other subunits. In our structural models (Fig. 1), all of our labeled sites are located at or near interfaces with other subunits, and so subunit-subunit interactions could influence the structural changes within each subunit.

Distinctions between conformational changes during block by pentobarbital and picrotoxin

Millimolar concentrations of pentobarbital inhibit the $GABA_A$ receptor noncompetitively. Our fluorescence measurements in combination with tail currents indicate that the blocking action is actually detectable even before the current starts to decline with increasing pentobarbital concentration (Fig. 7). Single-channel recording has revealed shorter mean open times and rapid flickering at increased pentobarbital concentrations; this result is expected if pentobarbital binds to the open state to induce channel block (8). However, a kinetic model put forward to describe pentobarbital actions proposed that pentobarbital can bind to its blocking site when the channel is closed (14); this model supports the hypothesis that block by pentobarbital involves more than a steric plugging of the pore. The pentobarbital-binding site responsible for this antagonism has not been identified, and the stoichiometry remains controversial. One single-channel study (8) suggested that the $GABA_A$ receptor contains a single low-affinity pentobarbital channel-blocking site, whereas another study (7) suggested that receptors contain two or more blocking sites. Interestingly, our study found large fluorescence changes in TMRM-α_1L127C and TMRM-β_2K274C that closely tracked the time course and concentration dependence of the blocking action of pentobarbital. The changes detected with N-terminal labels at α_1L127C and β_2L125C indicate that this block induces an allosteric transition that can extend a considerable distance from the channel to the extracellular domain of the receptor. These results thus provide strong evidence that pentobarbital does more than simply occlude an open channel.

Picrotoxin, another well-established channel blocker of the $GABA_A$ receptor, has been shown to interact with residues deep within the channel pore (32,35,53,54); studies have also demonstrated that picrotoxin antagonism has an allosteric component (35,55,56). Surprisingly, picrotoxin had no discernible effect on the GABA-induced change in fluorescence of TMRM-β_2K274C in our study, even though current was completely blocked. Thus, picrotoxin can block the channel without inducing a structural change at this location. Picrotoxin also failed to alter fluorescence signals in labels at α_1E122C and α_1L127C (19), and a recent study in the glycine receptor (21) reported that picrotoxin had no effect on the glycine-induced fluorescence changes in a label at the outer end of M2. In a fluorescent label study of the N-terminus of the homooligomeric ρ1 $GABA_A$ receptor

(18), picrotoxin block of GABA-induced current failed to block the GABA-induced fluorescence change at one site, slightly blocked the fluorescence change at another, and completely blocked the fluorescence change at a third site. Thus, picrotoxin can cause allosteric movements in this particular $GABA_A$ receptor subtype, but the extent of these movements appears to be limited.

Picrotoxin block of the channel did not change the fluorescence of TMRM-α_1L127C and TMRM-β_2K274C, whereas pentobarbital block did. Inhibition by both SR95531 and pentobarbital produced similar fluorescence changes in labels at α_1L127C (19). In TMRM-β_2K274C, however, pentobarbital induced a fluorescence change, whereas SR95531 did not (data not shown). Furthermore, SR95531 did not induce fluorescence changes in any of the β_2-subunit labels tested here (the present results and (19)) but pentobarbital did (Fig. 3 *C*). Thus, these three different drugs can block the $GABA_A$ receptor with different profiles of structural changes in the receptor protein. These patterns may hold clues as to how diverse classes of drugs can antagonize the same receptor by different mechanisms.

CONCLUSIONS

Fluorescent labels in the N-terminal agonist-binding domain and in the extracellular end of the M2 membrane-spanning segment have revealed that GABA, pentobarbital, SR95531, and picrotoxin each induce different structural rearrangements in the $GABA_A$ receptor. The generation of an open channel with the same conductance, however, does not necessarily mean that the entire protein undergoes the same structural change. Different forms of pharmacological activation can elicit transitions to similar final open-channel states in which other parts of the protein assume different structures. Moreover, the extracellular end of the M2 segment and the extracellular binding domain can undergo a variety of conformational movements in response to channel activation and channel block. By mapping out the conformational movements that different drugs induce in different parts of a protein, site-specific fluorescent labeling promises to contribute a great deal to the elucidation of detailed molecular mechanisms of drug action at $GABA_A$ receptors.

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