GABA and Pentobarbital Induce Different Conformational Rearrangements in the GABA_A Receptor α_1 and β_2 Pre-M1 Regions*

Jose Mercado¹ and Cynthia Czajkowski¹,²
From Department of Physiology¹ and the Neuroscience Training Program¹,²
University of Wisconsin-Madison, Madison, Wisconsin 53706

Running head: Pentobarbital-induced structural changes
Address correspondence to: Dr. Cynthia Czajkowski, University of Wisconsin-Madison, 601 Science Drive, Madison, WI 53711. Tel: (608) 265-5863; e-mail: czajkowski@physiology.wisc.edu

GABA binding to GABA_A receptors (GABA_ARs) triggers conformational movements in the α_1 and β_2 pre-M1 regions that are associated with channel gating. At high concentrations, the barbiturate pentobarbital opens GABA_AR channels with similar conductances as GABA suggesting that their open state structures are alike. Little, however, is known about the structural rearrangements induced by barbiturates. Here, we examined if pentobarbital activation triggers movements in the GABA_AR pre-M1 regions. α_1β_2 GABA_ARs containing cysteine substitutions in the pre-M1 α_1 (K219C, K221C) and β_2 (K213C, K215C) subunits were expressed in Xenopus oocytes and analyzed using two-electrode voltage clamp. The cysteine substitutions had little to no effect on GABA and pentobarbital EC_50 values. Tethering chemically diverse thiol-reactive methanethiosulfonate reagents onto α_1K219C and α_1K221C affected GABA- and pentobarbital-activated currents differently, suggesting that the physicochemical pre-M1 structural elements important for GABA and pentobarbital current activation are distinct. Moreover, pentobarbital altered the rates of cysteine modification by methanethiosulfonate reagents differently than GABA. For α_1K221Cβ_2 receptors, pentobarbital decreased the rate of cysteine modification while GABA had no effect. For α_1β_2K215C receptors, pentobarbital had no effect while GABA increased the modification rate. The competitive GABA antagonist, SR-95531 and a low, non-activating concentration of pentobarbital did not alter their modification rates suggesting that the GABA- and pentobarbital-mediated changes in rates reflect gating movements. Overall, the data indicate that the pre-M1 region is involved in both GABA- and pentobarbital-mediated gating transitions. PB, however, triggers different movements in this region than GABA suggesting their activation mechanisms differ.

Ligand gated ion channels (LGICs) are integral membrane proteins that mediate fast synaptic transmission between cells in the brain and at the neuromuscular junction. The type A γ-aminobutyric acid receptor (GABA_AR) is the main inhibitory LGIC in the brain and is the target for a wide range of therapeutic agents such as benzodiazepines, barbiturates, and anesthetics. Barbiturates, such as pentobarbital (PB), have three distinct effects on GABA_AR activity. At low concentrations, PB modulates GABA-mediated Cl^- current (I_GABA). At higher concentrations, PB directly activates the GABA_AR in the absence of GABA, and at still higher concentrations, PB blocks channel activity (1). Little is known, however, about the structural rearrangements underlying these functional effects.

Single channel studies from mouse spinal neurons (2-4) and from rat hippocampal neurons (5) have shown that currents evoked by PB are similar in conductance from those evoked by GABA, suggesting that the open state structures stabilized by PB binding are similar to those stabilized by GABA. However, GABA and PB bind to distinct sites on the GABA_AR (Fig. 1). The GABA binding site is located at the interfaces of the α_1 and β_2 subunits in the extracellular domain, while the PB/general anesthetics binding site(s) are believed to be located ~ 50 Å below the GABA binding site in a water-accessible pocket located between the four transmembrane helices (M1-4) of the receptor (Fig. 1). Mutational analyses as well as photolabeling studies have identified positions in the GABA_AR transmembrane helices that are important for mediating the effects of PB/anesthetics, with a
proposed binding pocket involving residues in M1 (α1M236), M2 (β2N265) and M3 (β2M286) (6-8).

The structural machinery associated with coupling agonist binding to channel gating in the cys-Loop family of LGIC’s likely involves distributed movements of, and interactions between, several discrete domains. Recent evidence suggests that binding of neurotransmitter in the extracellular domain triggers a series of molecular motions (conformational wave) that initiates in the ligand-binding pocket, followed by movements in Loop 2, Loop 7 (Cys-loop), the pre-M1 region, the M2-M3 linker and finally the transmembrane domains to gate the channel (9-11). Since PB- and GABA-activated channel open state structures are alike (3,12) but PB and GABA bind to different sites, we were interested in determining whether gating motions induced by PB are similar to those induced by GABA.

We previously demonstrated that the α1 and β2 pre-M1 regions of the GABAAR, which connect the extracellular domain of each subunit with the transmembrane domain, undergo structural rearrangements during GABA activation (13). In this study, we measured PB mediated changes in the accessibility of cysteines engineered into the pre-M1 region to monitor structural movements induced by activating concentrations of PB and compared these changes to those induced by GABA.

EXPERIMENTAL PROCEDURES

**Mutagenesis-Rat** cDNAs encoding α1 and β2 GABAAR subunits were used for all molecular cloning and functional studies. Cysteine mutants were made as previously described (13).

**Expression in Xenopus laevis Oocytes-** Oocytes were prepared as previously described (14). Capped cRNAs encoding the α1, β2, α1K219C, α1R220C, α1K221C, β2K213C, β2K215C and β2R216C subunits in the vector pGH19 (15,16) were transcribed *in vitro* using the mMessage mMachine T7 kit (Ambion, Austin, TX). Single oocytes were injected within 24 hr with 27 nL of cRNA (10 ng/μL per subunit) in a ratio 1:1. Oocytes were incubated at 18°C in ND96 (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.2) supplemented with 100 μg/mL gentamycin and 100 μg/mL BSA for 2-7 days before use.

**Two-electrode Voltage Clamp-** Oocytes were continuously perfused at a rate of ~5 mL/min with ND96 while being held under two-electrode voltage clamp at -80 mV. The bath volume was ~200 μL. Stock solutions of GABA (Sigma-Aldrich, St. Louis, MO) and PB (Research Biochemicals, Natick, MA) were prepared fresh daily in ND96. Borosilicate electrodes (Warner Instruments, Hamden, CT) were filled with 3 M KCl and had resistances between 0.7 to 2 MΩ. Electrophysiological data were acquired with a GeneClamp 500 (Axon Instruments, Foster City, CA) interfaced to a computer with an ITC16 analog-to-digital device (Instrutech, Great Neck, NY) and recorded using Whole Cell Program 3.2.9 (kindly provided by J. Dempster; University of Strathclyde, Glasgow, Scotland).

**Concentration-Response Analysis-** PB concentration-responses were measured and the resulting data were fit to the following equation: 

\[ I = I_{\text{max}} / \left(1 + (EC_{50}/[A])^n\right) \]

where \( I \) is the peak response to a given concentration of PB, \( I_{\text{max}} \) is the maximum amplitude of current, \( EC_{50} \) is the concentration of PB that evokes half-maximal response, \([A]\) is the agonist concentration and \( n \) is the Hill coefficient. At high PB concentrations, currents were partially blocked during PB application. Thus, peak PB currents were measured immediately after PB washout, when a prominent tail current appears (21). GraphPad Prism 4 software (San Diego, CA) was utilized for data analysis and fitting.

**Modification of Introduced Cysteine Residues by MTS reagents-** Three derivatives of methanethiosulfonate (CH₃SO₂X; MTS) were used to covalently modify the introduced cysteines: MTS-N-biotinylaminoethyl \([X=\text{SCH}_2\text{CH}_2\text{NH-biotin}]; \text{MTSEA-biotin}\), MTS-ethyltrimethylammonium \([X=\text{SCH}_2\text{CH}_2\text{N(CH}_3)_3^+];\)
MTSET\textsuperscript{+} and MTS-ethylsulfonate \([X=\text{SCH}_2\text{CH}_2\text{SO}_3^-; \text{MTSES}]\) (Biotium, Hayward, CA). MTSET\textsuperscript{+} is positively charged while MTSES\textsuperscript{-} is negatively charged at neutral pH. Stocks solutions (100 mM) were made in DMSO for all MTS reagents, aliquoted into microcentrifuge tubes and rapidly frozen on ice before storage at -20°C. For each application of MTS reagent, a new aliquot was thawed, diluted in ND96 to the working concentration and used immediately to avoid hydrolysis of the MTS compound. The final DMSO concentrations were \(\leq 2\%\), which had no effect on PB-mediated current responses.

MTS modifications of the engineered cysteines were assayed by measuring changes in PB evoked current \(I_{\text{PB}}\). The effects of MTSEA-biotin, MTSET\textsuperscript{+} and MTSES\textsuperscript{-} were studied using the following protocol: PB (EC\textsubscript{40-60}) current responses (10 sec) were measured from oocytes expressing wild type (\(\alpha_1\beta_2\)) or mutant receptors and stabilized. Stability was defined as < 10% variance of peak current responses to PB on two consecutive applications. After stabilization, the MTS reagent (2 mM) was bath-applied for 2 min, followed by a 5 min wash, and then \(I_{\text{PB}}\) was measured at the same concentration as before the MTS treatment. The effect of the MTS application was calculated as: \([(I_{\text{after}}/I_{\text{initial}}) -1] \times 100\)%, where \(I_{\text{after}}\) is the peak PB current elicited after the MTS application and \(I_{\text{initial}}\) is the peak current before MTS.

**Rate of MTS Modification**—The rates at which the various MTS reagents modified the engineered cysteines were determined by measuring the effect of sequential applications of low concentrations of MTS reagents on \(I_{\text{GABA}}\) as described previously (17). The protocol is described as follows: EC\textsubscript{40-60} GABA was applied for 10 sec every 3- to 5-min until \(I_{\text{GABA}}\) stabilized (< 3% variance). After a 40 s ND96 washout, MTS reagents were applied for 5- to 20-s and the cell was then washed for an additional 2.5 to 4.5 min. The procedure was repeated until \(I_{\text{GABA}}\) no longer changed indicating that the reaction had proceeded to apparent completion. Concentration of MTS reagent and time of application varied as follows: \(\alpha_1\text{K219C: MTSEA-biotin, 10 }\mu\text{M, 20 s; }\alpha_1\text{K221C: MTSEA-biotin, 10 }\mu\text{M, 20 s; }\beta_2\text{K213C: MTSET}^+, 30 \mu\text{M, 20 s; }\beta_2\text{K215C: MTSET}^+, 30 \mu\text{M, 20 s. The effects of co-applying GABA, SR-95531 (GABA antagonist), or PB (modulator) on reaction rates were assayed by co-applying GABA (EC\textsubscript{80-90}), 10 \mu\text{M SR-95531, or PB (50 or 500 }\mu\text{M) with the MTS reagent. For these experiments, }I_{\text{GABA}}\text{ was stabilized as follows: EC\textsubscript{40-60} GABA was applied for 10 s, washed for 40 s, high concentrations of GABA, SR-95531 or PB were applied for 5-20 s, and the oocyte washed for 2.5 to 5 min. The procedure was repeated until }I_{\text{GABA}}\text{ from EC\textsubscript{40-60} GABA was < 3% of the previous }I_{\text{GABA}}\text{ peak. This allowed complete wash-out of the different drugs and ensured that any alteration in the current amplitudes following MTS treatment in the presence of drug was the result of MTS modification and not a result of inadequate wash-out of drug. Concentrations of MTS reagents and times of applications in the presence of GABA (EC\textsubscript{80-90}) were as follows: }\alpha_1\text{K219C: MTSEA-biotin, 30 }\mu\text{M, 20 s; }\alpha_1\text{K221C: MTSEA-biotin, 10 }\mu\text{M, 20 s; }\beta_2\text{K213C: MTSET}^+, 30 \text{ or } 60 \mu\text{M, 10 s; }\beta_2\text{K215C: MTSET}^+, 30 \mu\text{M, 10 s. In the presence of 500 }\mu\text{M PB: }\alpha_1\text{K219C: MTSEA-biotin, 30 }\mu\text{M, 20 s; }\alpha_1\text{K221C: MTSEA-biotin, 10 }\mu\text{M, 20 s; }\beta_2\text{K213C: MTSET}^+, 30 \mu\text{M, 20 s; }\beta_2\text{K215C: MTSET}^+, 30 \mu\text{M, 10 s. In the presence of 50 }\mu\text{M PB: }\alpha_1\text{K219C: MTSEA-biotin, 30 }\mu\text{M, 20 s; }\alpha_1\text{K221C: MTSEA-biotin, 10 }\mu\text{M, 20 s; }\beta_2\text{K213C: MTSET}^+, 30 \mu\text{M, 20 s; }\beta_2\text{K215C: MTSET}^+, 50 \mu\text{M, 10 s. In the presence of 10 }\mu\text{M SR-95531: }\alpha_1\text{K219C: MTSEA-biotin, 100 }\mu\text{M, 10 s; }\alpha_1\text{K221C: MTSEA-biotin, 100 }\mu\text{M, 10 s; }\beta_2\text{K213C: MTSET}^+, 50 \mu\text{M, 10 s; }\beta_2\text{K215C: MTSET}^+, 75 \mu\text{M, 20 s.}

For all rate experiments, the decrease or increase in GABA-induced current was plotted versus cumulative time of MTS exposure. Peak current at each time point was normalized to the initial peak current \((t=0)\), and fit to a single exponential function using GraphPad Prism software to obtain a pseudo-first order rate constant \((k_1)\). The second order rate constant \((k_2)\) was calculated by dividing \(k_1\) by the concentration of the MTS reagent used (18).

**Statistical analysis**—Log (EC\textsubscript{50}) values, changes in PB EC\textsubscript{50} after MTS modification and second order \((k_2)\) rates were analyzed using a one-way analysis of variance, followed by a post-hoc Dunnett’s test
to determine the level of significance between wild-type and mutant receptors.

Structural Modeling- A model of the entire GABA<sub>A</sub> receptor was built as previously described (13).

RESULTS

Functional characterization of pre-M1 mutant receptors. We previously showed (13) that cysteine substitutions in pre-M1 region of the α<sub>1</sub> (K219C, K221C) and β<sub>2</sub> (K213C, K215C) subunits had no effects on GABA EC<sub>50</sub> (α<sub>1</sub>β<sub>2</sub> receptors, EC<sub>50</sub> = 6.9 ± 0.7 μM). To determine whether the same cysteine substitutions altered PB activation, we measured PB concentration-responses using two-electrode voltage clamp (Fig. 2). Cysteine substitutions of α<sub>1</sub>K219, α<sub>1</sub>K221 and β<sub>2</sub>K215 had little (2-fold) to no effect (β<sub>2</sub>K213) on PB EC<sub>50</sub> values relative to α<sub>1</sub>β<sub>2</sub> receptors (EC<sub>50</sub> = 271 ± 18 μM; Fig. 2; Table 1). Mutant receptors had Hill coefficients for PB activation that were not significantly different from wild type α<sub>1</sub>β<sub>2</sub> receptors (Table 1). PB maximal macroscopic currents elicited from mutant receptors were similar to α<sub>1</sub>β<sub>2</sub> receptors ranging from 600 nA to 18 μA. The fact that these mutations had little effect on GABA and PB EC<sub>50</sub> values, Hill coefficients or surface expression suggests that the side-chains of the introduced cysteines are in similar positions as the side chains of the native residues, making the introduced cysteines at positions α<sub>1</sub>K219, α<sub>1</sub>K221, β<sub>2</sub>K213 and β<sub>2</sub>K215 ideal candidates to probe the dynamics of the pre-M1 region induced by PB binding/gating.

Effects of cysteine modification on PB and GABA evoked currents. We measured current responses elicited with PB EC<sub>50</sub> (I<sub>PB</sub>) concentrations before and after methanethiosulfonate (MTS) reagent application (Fig. 3) to examine how covalently modifying the introduced cysteines would affect PB currents. The MTS reagents used were 1) MTSEA-biotin, which covalently adds a neutral biotinylaminoethyl group (12 Å long), 2) MTSET<sup>+</sup>, which adds a positively charged ethyltrimethylammonium group (4.5 Å long), and 3) MTSES<sup>-</sup>, which adds a negatively charged ethylsulfonate group (4.8 Å long). Application of 2 mM MTSEA-biotin, MTSET<sup>+</sup> or MTSES<sup>-</sup> for 2 min to wild type receptors had no effect on I<sub>PB</sub> (≤ 5 ± 3% for all reagents) indicating that any effects observed in the mutant receptors are due to modification of the introduced cysteines (Fig. 3B). MTSEA-biotin modification of β<sub>2</sub>K213C and β<sub>2</sub>K215C increased I<sub>PB</sub> by 86 ± 12% and 31 ± 4% (n ≥ 3), respectively, and decreased I<sub>PB</sub> by 72 ± 3% (n = 6) in α<sub>1</sub>K221C containing receptors (Fig. 3B).

Similar functional effects on GABA EC<sub>50</sub> current responses (I<sub>GABA</sub>) were observed after MTSEA-biotin modification of β<sub>2</sub>K213C, β<sub>2</sub>K215C and α<sub>1</sub>K221C (Fig. 3B and (13)). In contrast, MTSET-biotin modification of α<sub>1</sub>K219C did not affect I<sub>PB</sub>, whereas I<sub>GABA</sub> was increased by 34 ± 2% (Fig. 3B). This indicates that MTSEA-biotin covalently modified α<sub>1</sub>K219C but modification had no functional effect (i.e. a “silent” reaction) on PB induced currents.

Derivatization of β<sub>2</sub>K213C and β<sub>2</sub>K215C with MTSES<sup>-</sup> increased I<sub>PB</sub> by 50 ± 3% and 53 ± 16%, respectively while MTSET<sup>+</sup> increased I<sub>PB</sub> 133 ± 2% and 56 ± 5%, respectively (n ≥ 3) (Fig. 3B). Similar functional effects on I<sub>GABA</sub> were observed after derivatization of β<sub>2</sub>K213C and β<sub>2</sub>K215C with MTSES<sup>-</sup> or MTSET<sup>+</sup> (13) (Fig. 3B). As we previously reported, modification of α<sub>1</sub>K219Cβ<sub>2</sub> and α<sub>1</sub>K221Cβ<sub>2</sub> by MTSES<sup>-</sup> and MTSET<sup>+</sup> differentially affected I<sub>GABA</sub> (Fig. 3B). Tethering a negative charge (MTSES<sup>-</sup>) onto α<sub>1</sub>K221C enhanced I<sub>GABA</sub> (98 ± 14%; n = 4) whereas treatment with the positively charged MTSET<sup>+</sup> had no functional effect on I<sub>GABA</sub> (Fig. 3B). In contrast, tethering a positive charge (MTSET<sup>+</sup>) onto α<sub>1</sub>K219C decreased I<sub>GABA</sub> (31 ± 7%; n = 3) whereas modification with MTSES<sup>-</sup> had no effect. These results are likely due to differences in the local electrostatic environments near α<sub>1</sub>K219C and α<sub>1</sub>K221C rather than steric effects since MTSET<sup>+</sup> and MTSES<sup>-</sup> are similar in size (Fig. 3B insets) and have a common reaction mechanism. Surprisingly, modification of α<sub>1</sub>K219Cβ<sub>2</sub> and α<sub>1</sub>K221Cβ<sub>2</sub> by MTSES<sup>-</sup> or MTSET<sup>+</sup> had no functional effects on I<sub>PB</sub> (Fig. 3A) indicating that introducing a positive or negative charge at these positions has different effects on PB and GABA current responses, suggesting that the physicochemical structural elements in the pre-M1 region important for GABA and PB current activation are distinct.
Effect of PB on MTS reaction rates. To determine if PB binding/gating induces different structural rearrangements in the pre-M1 regions than GABA, we measured the rates of MTS modification of α1K219C, α1K221C, β2K213C and β2K215C in the presence of a directly activating concentration of PB (500 μM) and compared these to rates measured in the presence of GABA (Figs. 4 and 5). The rate of modification of a cysteine by an MTS reagent mainly depends on the ionization of the thiol group and the access pathway of the reagent. Thus, changes in rates measured in the presence of PB or GABA provide a measure of structural changes in the receptor that are triggered by their binding. PB had no effect on the MTSET rate of modification of α1β2K215C while GABA increased the rate by 4-fold. For α1K221Cβ2 receptors, PB decreased the MTS-biotin rate by 2-fold while GABA had no significant effect. For α1β2K213C receptors and α1K219Cβ2 receptors, GABA increased and decreased their rates of modification by 3 and 4-fold, respectively. In contrast, when PB was present during the MTS reaction, the MTS treatment no longer altered subsequent GABA current responses for α1β2K213C and α1K219Cβ2 receptors (Fig. 4C). A subsequent application of MTS reagent in the absence of PB had little effect on GABA current indicating that the thiol was modified in the presence of PB but modification now resulted in no detectable effect on IGABA (no functional effect, NFE). While the mechanism underlying this loss of functional effect is unknown, one can conclude that cysteine modification in the presence of 500 μM PB is different than modification in the presence of GABA. A low concentration of PB that does not activate the receptor but potentiates GABA responses (50 μM) decreased the rate of modification of α1K219C by ~3 fold and had no effects on the rates of modification of α1K221C, β2K213C and β2K215C (Fig. 4, Table 2) indicating that the effects of PB (500μM) on α1K221Cβ2 and α1β2K213C receptors likely reflect gating-associated motions. Overall, these data indicate that GABA and PB induce different structural rearrangements in the pre-M1 regions of the α1 and β2 subunits.

Effect of SR-95531 on MTS reaction rates. To explore if the structural rearrangements in the pre-M1 regions induced by GABA reflect conformational movements associated with gating, we measured the rates of MTS modification of α1K219C, α1K221C, β2K213C and β2K215C in the presence of the GABA binding site competitive antagonist SR-95531 (10 μM) (Fig. 5). Since GABA and SR-95531 bind to the same site but GABA promotes channel opening/desensitization whereas SR-95531 does not, co-application of SR-95531 and MTS should capture motions associated with stabilization of a closed state whereas co-application of GABA and MTS should capture motions associated with open/desensitized states (i.e. gating). SR-95531 had no significant effect on the rate of modification of α1K221Cβ2, α1β2K213C and α1β2K215C receptors compared to control (Fig. 5 and Table 2) suggesting that occupancy of the GABA binding site alone does not induce structural rearrangements in or near these positions. SR-95531 slowed the MTSEA-biotin rate of modification of α1K219Cβ2 receptors threefold (Fig. 5 and Table 2) indicating that binding of a competitive antagonist can induce structural rearrangements in the pre-M1 region of the α1 subunit.

DISCUSSION

The Monod-Wyman-Changeux allosteric theory has been used with great success to model LGIC gating behavior (19). In this theory, channel gating is accomplished by a concerted quaternary movement of all the subunits switching from an inactive to an active conformation. GABA and PB activation both evoke the same single channel conductances (3), despite binding to different parts of the receptor (20). Thus, a key question is whether binding of an allosteric modulator such as PB triggers similar allosteric gating transitions as GABA.

For cys-loop LGICs, it has been suggested that the pre-M1 region acts as a central hub that couples neurotransmitter induced motions in the ligand-binding site to movements in Loop 2 and the M2-M3 linker, which then ultimately triggers movements in the M2 channel region that opens the channel (Fig. 1) (10,13). A key element in the
transduction pathway coupling neurotransmitter binding to gating of the channel is believed to be a salt bridge between two highly conserved residues present in all cys-loop LGIC subunits: an arginine in the pre-M1 region with a glutamic acid in Loop 2 (Fig. 1) (10). Previously, we showed that cysteine substitution of this highly conserved arginine (R216) in the β2 pre-M1 region of the GABA_{A}R abolished channel gating by GABA without altering binding of the GABA agonist [³H]muscimol (13) suggesting that this residue plays a key role in allosterically coupling GABA binding to gating. Interestingly, the β3R216C mutation also abolished channel gating by PB suggesting that this residue and the pre-M1 region may also play a role in PB activation (13).

Here, we provide evidence that the α1 and β2 pre-M1 regions move in response to PB activation of the GABA_{A}R and that PB triggers different movements in this region than GABA. Cysteine substitutions of the conserved pre-M1 lysine residues had little to no effects on PB EC_{50} (Fig. 2 and Table 1) and thus, the positions occupied by the cysteine side-chains in the mutant receptors are likely similar to the native lysine positions. The rates of modification of α1K221Cβ2, α1K219Cβ2, α1β2K213C and α1β2K215C receptors differ depending upon whether GABA or PB is present. For α1K221Cβ2 receptors, only 500 μM PB caused a significant change in the rate of MTS modification while for α1β2K215C receptors only GABA caused a change (Fig. 5; Table 2). For α1K219Cβ2 and α1β2K213C receptors, GABA altered their rates of modification, whereas in the presence of PB the mutant receptors were modified but MTS modification no longer altered subsequent GABA-induced current (Fig. 5; Table 2) demonstrating that PB stabilizes the receptor in a different conformation(s) than GABA. Moreover, structurally perturbing the pre-M1 regions, by tethering chemically diverse thiolreactive groups onto these mutant cysteines, had different effects on PB and GABA current responses (Figs. 2, 3). Based on these data, we infer that the structural transitions evoked and/or stabilized by PB channel activation differ from those triggered by GABA in this region of the receptor.

Alternatively, one could argue that some of the differences measured in the effects of modifying these cysteines on GABA and PB current responses result from the pre-M1 residues being located near the PB binding site pocket. Several lines of evidence indicate that that α1K219, α1K221, β2K213 and β2K215 do not form part of the PB binding site. First, mutations of these residues to cysteine had little to no effect on PB EC_{50}. If these residues directly formed part of the PB binding site, one would expect larger shifts in PB EC_{50} especially due to the non-conservative cysteine for lysine substitution. Second, modification of β2K213C and β2K215C with a variety of MTS reagents all increased PB-induced current and modification of α1K219C had no effects on PB-induced currents. If these residues were lining the PB binding site, one would expect that tethering bulky/charged groups at these positions would sterically inhibit the ability of PB to bind and would decrease PB-mediated current. While modification of α1K221C with MTSEA-biotin caused a decrease in PB-mediated current, modification with MTSET and MTSES had no effect on PB-mediated currents again suggesting that this residue is not part of the PB binding site. Third, PB caused an increase in the rate of MTS modification of β2K215C. If β2K215C were part of the PB binding site, one would expect that PB would decrease the rate of modification. Moreover, based on our homology model of the GABA_{A}R and given the size of PB (~7 Å), it seems unlikely that α1 and β2 pre-M1 region residues are forming part of the general anesthetic binding site. Residues in the α1 and β2 pre-M1 regions are separated by 20 Å or more from the residues that have been previously identified as forming the potential PB/general anesthetic binding site (Fig. 1) (8,21-24). Mutations in M2 (N265) and M3 (M286) in the beta subunit eliminate PB activation of the receptor (8,25) and the actions of the related general anesthetics etomidate and propofol (26-28). More recently, α1M236 (in M1) and βM286 (in M3) have been directly identified as being part of a general anesthetic binding site by photolabeling with an etomidate analog (6). Propofol blocks covalent modification of BM286C by sulfhydryl specific reagents indicating that this residue forms part of a general anesthetic binding site (29). Furthermore, a knock-in mouse for βN265M removes the immobilizing and hypnotic actions of PB as well
as the actions of etomidate and propofol (30). Taken together, the data indicate that general anesthetics including PB likely share a similar binding site, which is located in a water-filled pocket approximately 50 Å below the GABA binding site between M1, M2 and M3.

In the presence of GABA, the receptor undergoes transitions between an ensemble of open and desensitized states (31,32). If PB was stabilizing similar states as GABA, one might expect similar changes in the rate of modification in the presence of PB and GABA. Since this was not the case, we infer that PB binding/gating induces structural rearrangements near the pre-M1 region that are structurally distinct from movements induced by GABA. Interestingly, a recent paper using disulfide-trapping experiments demonstrated that GABA and PB induce a similar open state structure at the 6’ position in M2 (12). This is consistent with functional studies that have shown similar single-channel conductances regardless of whether GABA,A_R channels are opened by GABA or by PB. We speculate that the unique movements induced by PB and GABA in the pre-M1 regions are the result of their binding to different sites and triggering different activation pathways that lead to their functional effects.

We envision that PB binding between transmembrane helices initiates a conformational change in the M2-M3 linker that propagates to the pre-M1 region via Loop 2 (Fig. 1). Mutational studies have implicated the \( \alpha_1 \) and \( \beta_2 \) M2-M3 linker as involved in PB activation (21,33). The movements in the pre-M1 region triggered by PB are then likely to be transmitted to various regions of the GABA,A_R extracellular ligand binding domain as well as channel membrane domain. The pre-M1 region may be the conduit by which the actions of PB are propagated to the GABA binding site. Binding studies have shown that PB enhances GABA apparent affinity (34) suggesting that the structure of the GABA binding site changes in the presence of PB. Moreover, we have identified 13 positions in the GABA binding site interface that change accessibility during pentobarbital binding/gating (\( \beta_2 \)T160C, \( \beta_2 \)D163C, \( \beta_2 \)G203C, \( \beta_2 \)S204C, \( \beta_2 \)R207C, \( \beta_2 \)S209C, \( \beta_2 \)D62C, \( \alpha_1 \)S68C, \( \alpha_1 \)E122C, \( \alpha_1 \)R131C, \( \alpha_1 \)V180C, \( \alpha_1 \)A181C, and \( \alpha_1 \)R186C; (17,35-38) indicating that the extracellular domain undergoes conformational rearrangements during PB binding/gating.

In summary, we have shown that the \( \alpha_1 \) and \( \beta_2 \) pre-M1 regions of the GABA,A_Rs are structural elements involved in both GABA and PB-mediated channel activation. PB binding, however, induces different structural movements in this region than when the receptor binds GABA suggesting that PB stabilizes a different state or ensembles of states than GABA. These differences reveal distinct molecular mechanisms of action of these two ligands.

REFERENCES

1. Belelli, D., Pistis, M., Peters, J. A., and Lambert, J. J. (1999) Trends Pharmacol Sci 20, 496-502
2. Mathers, D. A., and Barker, J. L. (1980) Science 209, 507-509
3. Jackson, M. B., Lecar, H., Mathers, D. A., and Barker, J. L. (1982) J Neurosci 2, 889-894
4. MacDonald, R. L., Rogers, C. J., and Twyman, R. E. (1989) J Physiol 417, 483-500
5. Rho, J. M., Donevan, S. D., and Rogawski, M. A. (1996) J Physiol 497 (Pt 2), 509-522
6. Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) J Neurosci 26, 11599-11605
7. Pistis, M., Belelli, D., McGurk, K., Peters, J. A., and Lambert, J. J. (1999) J Physiol 515 (Pt 1), 3-18
8. Amin, J. (1999) Mol Pharmacol 55, 411-423
9. Grosman, C., Zhou, M., and Auerbach, A. (2000) Nature 403, 773-776
10. Lee, W. Y., and Sine, S. M. (2005) Nature 438, 243-247
11. Chakrapani, S., Bailey, T. D., and Auerbach, A. (2004) J Gen Physiol 123, 341-356
12. Rosen, A., Bali, M., Horenstein, J., and Akabas, M. H. (2007) Biophys J 92, 3130-3139
13. Mercado, J., and Czajkowski, C. (2006) J Neurosci 26, 2031-2040
14. Boileau, A. J., Kucken, A. M., Evers, A. R., and Czajkowski, C. (1998) Mol Pharmacol 53, 295-303.
15. Liman, E. R., Tytgat, J., and Hess, P. (1992) Neuron 9, 861-871
16. Robertson, G. A., Warmke, J. M., and Ganetzky, B. (1996) Neuropharmacology 35, 841-850
17. Holden, J. H., and Czajkowski, C. (2002) J Biol Chem 277, 18785-18792
18. Pascual, J. M., and Karlin, A. (1998) J Gen Physiol 111, 717-739
19. Monod, J., Wyman, J., and Changeux, J. P. (1965) J Mol Biol 12, 88-118
20. Amin, J., and Weiss, D. S. (1993) Nature 366, 565-569
21. Siegwart, R., Jurd, R., and Rudolph, U. (2002) J Neurochem 80, 140-148
22. Sigel, E., Buhr, A., and Baur, R. (1999) J Neurochem 73, 1758-1764.
23. Olsen, R. W. (1982) Annu Rev Pharmacol Toxicol 22, 245-277
24. Wagner, D. A., and Czajkowski, C. (2001) J Neurosci 21, 67-74.
25. Newell, J. G., and Czajkowski, C. (2003) J Biol Chem 278, 13166-13172
26. Kloda, J. H., and Czajkowski, C. (2007) Mol Pharmacol 71, 483-493

FOOTNOTES

*This work was supported in part by the Diversity Program in Neuroscience of the American Psychological Association (to J.M.) and by NINDS, National Institutes of Health Grant NS34727 (to C.C). We thank Dr. Ken Satyshur for assistance in construction of the structural model.

The abbreviations used are: GABA<sub>R</sub>, γ-aminobutyric type A receptor; AChBP, acetylcholine binding protein; SCAM, substituted cysteine accessibility method; EM, electron microscopy; PB, pentobarbital.

FIGURE LEGENDS

**Figure 1. Structural model of the GABA<sub>R</sub> α<sub>1</sub> and β<sub>2</sub> subunit.** A, The extracellular binding domain is colored in white. Domains believed to contribute to the GABA transduction mechanism (loop 2, loop 7, M2-M3 linker, and pre-M1) are highlighted in yellow. The Loop C region of the GABA binding site is highlighted in green. The transmembrane domains (M1, M2 and M3) are colored in red. Residues in the pre-M1 region (R216) as well as residues forming the potential PB binding site (N265 and M286) in the
β₂ subunit and (M236) in the α₁ subunit are shown in a space-filled format. The M4 transmembrane helix has been omitted for illustration purposes. B, Detailed view of the interface between the ligand binding domain and the transmembrane domain.

Figure 2. PB concentration-response curves of wild type α₁β₂ and mutant GABAₐR. A, Representative current responses from an oocyte expressing α₁K219Cβ₂ receptors elicited by increasing concentrations of PB (mM). B, PB concentration-response curves from oocytes expressing α₁β₂ (▲; dashed line), α₁K219Cβ₂ (▼), α₁K221Cβ₂ (■), α₁β₂K213C (◊) and α₁β₂K215C (○) receptors. Peak PB-activated currents were measured after PB washout (tail current) and used for concentration-response fitting. Data points represent the mean ± SEM from four to six independent experiments. Data were fit by nonlinear regression analysis as described in Materials and Methods. PB EC₅₀ and n_H values are reported in Table 1.

Figure 3. Effects of MTS reagents on α₁β₂ and mutant GABAₐR. A, Representative current traces elicited by GABA (top traces) or PB (bottom traces) of EC₅₀ concentrations from oocytes expressing α₁K219Cβ₂ and α₁K221Cβ₂ receptors before and after treatment with MTSET⁺ and MTSES⁻ (2 min, 2 mM). MTSET⁺ and MTSES⁻ treatment altered the GABA current responses but had no effects on PB current responses. B, Summary of the effects of a 2 min application of 2 mM MTSEA-biotin (top), MTSET⁺ (middle) or MTSES⁻ (bottom) on GABA (EC₅₀) activated currents (I_GBₐ) (previously reported in: Mercado and Czajkowski, 2006) and PB (EC₅₀) activated currents (I_PB) from α₁β₂ and mutant receptors. The absolute percent change in I_PB and I_GBₐ after MTS treatment is defined as: [(I_after/I_initial) -1] x 100]. Bars represent the mean from at least 3 independent experiments. Values >20% are significantly different from α₁β₂ values (p<0.01).

Figure 4. Rates of MTSET⁺ modification of α₁β₂K215C receptors in the presence and absence of GABA or PB. A, Representative GABA current traces recorded while applying MTSET⁺ (30 μM) in the presence of PB (500 μM). GABA EC₄₀-₆₀ current responses were recorded before and after successive application (10 s) of 30 μM MTSET⁺ co-applied with PB (arrows). B, Normalized GABA current responses were plotted versus cumulative time of MTSET⁺ (▲), MTSET⁺ co-applied with EC₈₀-₉₀ GABA (■) and MTSET⁺ co-applied with 500 μM PB (●) and fit with single-exponential functions. Data were normalized to the maximal amount of potentiation of I_GBₐ for each experiment and represent mean ± SEM from at least three independent experiments. C, Representative GABA mediated current traces from oocytes expressing α₁β₂K213C receptors. Currents elicited by an EC₅₀ concentration of GABA were recorded before and after MTSET⁺ (2 mM, 2 min) in the absence or presence of 500 μM PB. MTSET⁺ treatment alone potentiated the subsequent current response (95%) but when MTSET⁺ was co-applied with 500 μM PB the treatment had no functional effect. On the subsequent GABA current and following washout, MTSET treatment alone no longer resulted in a significant potentiation of current.

Figure 5. Summary of effects of GABA, PB and SR-95531 on MTS second order rate constants. Second order rate constants (k₂) for MTS modification of cysteine mutants in the absence and presence of EC₈₀-₉₀ GABA, 500 μM PB, 50 μM PB or 10 μM SR-95531. k₂ values are reported in Table 2. Data are the mean ± SEM from at least 3 independent experiments. *, ** indicate values significantly different from control at p<0.05 and p<0.001, respectively. NFE (no functional effect) MTS reagent reacts with the cysteine mutant in the presence of PB but has no functional effect on subsequent GABA responses.
TABLES

Table 1. PB concentration-response data for $\alpha_1\beta_2$ and mutant receptors. Concentration-response data for PB activation of wild-type and mutant receptors are tabulated. $EC_{50}$ and Hill coefficient ($n_H$) values are expressed as mean ± SEM for $n$ number of independent experiments from at least 2 batches of oocytes.

| Receptor            | $EC_{50}$ $\mu M$ | $n_H$   | $n$ |
|---------------------|-------------------|---------|-----|
| $\alpha_1\beta_2$   | 271 ± 18          | 2.7 ± 0.3| 6   |
| $\alpha_1$(K219C)$\beta_2$ | 495 ± 32**       | 2.4 ± 0.2| 4   |
| $\alpha_1$(K221C)$\beta_2$ | 471 ± 41*        | 2.8 ± 0.4| 5   |
| $\alpha_1\beta_2$(K213C) | 224 ± 5.1        | 2.5 ± 0.4| 5   |
| $\alpha_1\beta_2$(K215C) | 577 ± 44**       | 3.8 ± 0.5| 4   |

* $p<0.05$, ** $p<0.01$, significantly different from control.

Table 2. Second-order rate constants ($k_2$) for reaction of MTS reagents with mutant receptors in the absence (control) and presence of GABA, PB and SR95531.

| Receptor            | Control $^c$ $k_2$ (M$^{-1}$s$^{-1}$) | $n$ | GABA $^c$ $EC_{50-90}$ $k_2$ (M$^{-1}$s$^{-1}$) | $n$ | SR95531 (10 $\mu M$) $k_2$ (M$^{-1}$s$^{-1}$) | $n$ | Pentobarbital (500 $\mu M$) $k_2$ (M$^{-1}$s$^{-1}$) | $n$ | Pentobarbital (50 $\mu M$) $k_2$ (M$^{-1}$s$^{-1}$) | $n$ |
|---------------------|--------------------------------------|-----|-----------------------------------------------|-----|-----------------------------------------------|-----|-------------------------------------------------|-----|-------------------------------------------------|-----|
| $\alpha_1$K219C$\beta_2$ | 2070 ± 300                           | 4   | 580 ± 130**                                   | 3   | 700 ± 110**                                   | 4   | NFE                                             | 3   | 710 ± 190                                       | 4   |
| $\alpha_1$K221C$\beta_2$ | 2110 ± 290                           | 6   | 1600 ± 80                                     | 4   | 1570 ± 250*                                   | 4   | 970 ± 250*                                     | 4   | 1750 ± 260                                      | 3   |
| $\alpha_1\beta_2$K213C$^b$ | 1900 ± 340                           | 5   | 5540 ± 320**                                  | 7   | 1610 ± 200*                                   | 4   | NFE                                             | 3   | 1590 ± 340                                      | 3   |
| $\alpha_1\beta_2$K215C$^b$ | 1990 ± 220                           | 6   | 7560 ± 1750**                                 | 3   | 3050 ± 510*                                   | 3   | 2590 ± 250*                                    | 3   | 2850 ± 440                                      | 4   |

$^c$MTSEA-biotin reaction rates are reported.
$^a$MTSET$^+$ reaction rates are reported.
$^c$Reported in Mercado and Czajkowski, 2006 (13).
NFE, no functional effect, MTS reagents react but have no functional effect on subsequent GABA responses.
***Indicate values significantly different from control, with $p<0.05$ and $p<0.001$, respectively.
Values are the mean ± S.E.M.
Figure 1
Figure 2

A

$\alpha_1K219\beta_2$

PB mM 0.1 0.2 0.3 0.4 0.6 1 3

2 $\mu$A 6 s

B

Normalized $I_{PB}$

Log [Pentobarbital], M

1.0

0.6

0.2

0.0

-4.5 -4.0 -3.5 -3.0 -2.5

$\alpha_1\beta_2$

$\alpha_1K219\beta_2$

$\alpha_1K221\beta_2$

$\alpha_1\beta_2K213C$

$\alpha_1\beta_2K215C$
Figure 5
GABA and pentobarbital induce different conformational rearrangements in the GABAA receptor α1 and β2 Pre-M1 regions
Jose Mercado and Cynthia Czajkowski

J. Biol. Chem. published online April 3, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M708638200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts