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Mutations of the GABA-A Receptor α1 Subunit M1 Domain Reveal Unexpected Complexity for Modulation by Neuroactive Steroids

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ABSTRACT

Neuroactive steroids are among the most efficacious modulators of the mammalian GABA-A receptor. Previous work has proposed that receptor potentiation is mediated by steroid interactions with a site defined by the residues α1Asn407/Tyr410 in the M4 transmembrane domain and residue α1Gln241 in the M1 domain. We examined the role of residues in the α1 subunit M1 domain in the modulation of the rat $\alpha1\beta2\gamma2$L GABA-A receptor by neuroactive steroids. The data demonstrate that the region is critical to the actions of potentiating neuroactive steroids. Receptors containing the α1Q241W or α1Q241L mutations were insensitive to (3α,5α)-3-hydroxypregnan-20-one (3α5αP), albeit with different underlying mechanisms. The α1Q241S mutant was potentiated by 3α5αP, but the kinetic mode of potentiation was altered by the mutation. It is noteworthy that the α1Q241L mutation had no effect on channel potentiation by (3α,5α)-3-hydroxymethylpregnan-20-one, but mutation of the neighboring residue, α1Ser240, prevented channel modulation. A steroid lacking an H-bonding group on C3 (5α-pregnan-20-one) potentiated the wild-type receptor but not the α1Q241L mutant. The findings are consistent with a model in which the α1Ser240 and α1Gln241 residues shape the surface to which steroid molecules bind.

Potentiating neurosteroids are among the most efficacious modulators of the mammalian GABA-A receptor having potential applications as anxiolytics, anticonvulsants, sedatives, and anesthetics. Recent work has given significant insights into the functional and structural mechanisms of steroid actions. Potentiating steroids [e.g., (3α,5α)-3-hydroxypregnan-20-one (3α5αP) and (3α,5β)-3-hydroxypregnan-20-one (3α5βP)] act on the GABA-A receptor by modifying the channel open and closed times, leading to an increase in the open probability of the channel, enhanced macroscopic peak current, and a slower current decay when exposure to agonist is terminated. The putative steroid binding site is located in the membrane-spanning regions of the α subunit of the receptor, extending from the α1Gln241 residue in the M1 membrane-spanning region to the residues α1Asn407 and α1Tyr410 in the M4 domain (Hosie et al., 2006). Mutations that reduce the H-bonding ability of these residues reduce receptor potentiation by both 5α- and 5β-reduced steroids. It was proposed that a common interaction site mediates the effects of the two classes of steroids, the α1Gln241 residue acting as an H-bond acceptor to the 3α-hydroxyl group of the steroid molecule and the α1Asn407/Tyr410 residues interacting with the ketone group in the side chain on the D ring of steroids (Hosie et al., 2006). Subsequent studies showed that mutations that disrupt channel potentiation by steroids also affect modulation by a tricyclic benz[*e*]indene neurosteroid analog (Li et al., 2006), enantiomers of natural steroids (Li et al., 2007a), and the marine cembranoid eupalmerin acetate (Li et al., 2008), suggesting that the site may function as a common interaction site for a number of GABA-A receptor modulators.

To fully understand the role of the α1Gln241, α1Asn407, and α1Tyr410 residues in steroid actions, it is essential to establish, first, the role of these residues in normal receptor activity. Many channel modulators act in a state-specific manner. Therefore, a lack of responsiveness to a drug appli-

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ABBREVIATIONS: 3α5αP, (3α,5α)-3-hydroxypregnan-20-one; 3α5βP, (3α,5β)-3-hydroxypregnan-20-one; 3αCH$_2$OH5βP, (3α,5α)-3-hydroxymethylpregnan-20-one; DMSO, dimethyl sulfoxide; PDB, Protein Data Bank; DOPE, discrete optimized protein energy; 3deoxy5αP, 5α-pregnan-20-one; P4S, piperidine-4-sulfonic acid; 17-PA, (3α,5α)-17-phenylandrost-16-en-3-ol; PS, pregnenolone sulfate.

cation may result from changes in channel baseline kinetic properties rather than reflect the inability of the drug to interact with the receptor. In addition, previous single-channel work has demonstrated that steroids modify several kinetic parameters, effects that may be mediated by steroid interactions with two or more distinct binding sites (Akk et al., 2004; Li et al., 2007a). Consequently, it is of interest to assess the effect of mutations to the putative steroid site residues on the full spectrum of kinetic variables that are modified in the presence of neurosteroids.

In this study, we have used a combination of whole-cell and single-channel recordings to characterize the effects of mutations to residues in the α1 subunit M1 membrane-spanning domain on channel modulation by neuroactive steroids. A structural model showing the locations of the residues studied is shown in Fig. 1. We show that mutations to the α1Gln241 site strongly affect channel modulation by 3α5αP, albeit through different kinetic mechanisms. Channel potentiation by (3α,5α)-3-hydroxymethylpregnan-20-one (3αCH$_2$OH5βP) was disrupted in the α1S240L but not α1Q241L mutant receptor. We also show that the steroid 5α-pregnan-20-one (3deoxy5αP) potentiates the wild-type receptor but not a receptor containing the α1Q241L mutation. The findings are most compatible with a model in which the steroids bind to a hydrophobic surface on the receptor α1 subunit, the α1Ser240 and α1Gln241 residues acting to shape the surface to accommodate a variety of structurally distinct steroids.

Materials and Methods

All experiments were carried out on human embryonic kidney 293 cells expressing rat wild-type and mutant α1β2γ2L GABA-A receptors. The details of receptor expression and electrophysiology have been described in detail previously (Akk et al., 2001, 2004; Li et al., 2006). The mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA), and the mutated subunits were fully sequenced to confirm that only the desired mutation had been produced. The α1 subunit is epitope (FLAG)-tagged (Ueno et al., 1996) in the amino-terminal end of the subunit. Cells expressing high levels of receptors were determined using a bead-binding technique in which the presence of the FLAG peptide was detected with a mouse monoclonal antibody to the FLAG epitope (M2; Sigma-Aldrich, St. Louis, MO), which had been adsorbed to beads with a covalently attached goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA).

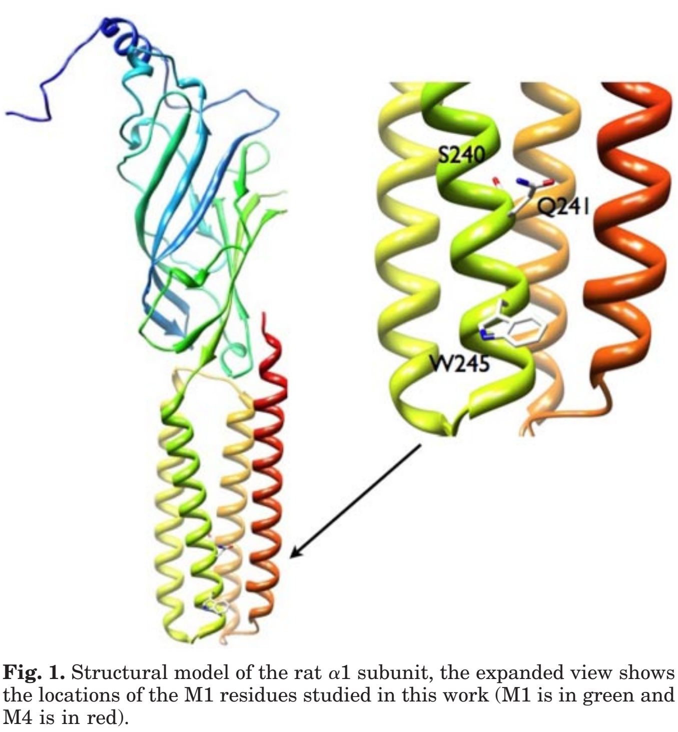

Fig. 1. Structural model of the rat α1 subunit, the expanded view shows the locations of the M1 residues studied in this work (M1 is in green and M4 is in red).

The electrophysiological experiments were carried out using standard single-channel patch clamp and whole-cell voltage clamp methods. The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. In single-channel recordings, the pipet solution contained 120 mM NaCl, 5 mM KCl, 10 mM MgCl$_2$, 0.1 mM CaCl$_2$, 20 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, and 10 mM HEPES, pH 7.4. In whole-cell recordings, the pipet solution contained 140 mM CsCl, 4 mM NaCl, 4 mM MgCl$_2$, 0.5 mM CaCl$_2$, 5 mM EGTA, and 10 mM HEPES, pH 7.4.

The agonist (GABA or piperidine-4-sulfonic acid) and steroid modulators were added to the pipet solution in single-channel recordings, or applied through the bath using a fast perfusion stepper system (SF-77B; Warner Instruments, Hamden, CT) in whole-cell experiments. The steroids were initially dissolved in DMSO at 5 to 10 mM concentration and diluted immediately before the experiment. The maximal DMSO concentration in diluted steroid solutions was 0.1%. We have found previously that channel activation by GABA is not affected by the presence of up to 0.3% DMSO (Li et al., 2007a). All experiments were carried out at room temperature (19–22°C).

The recording and analysis of single-channel currents have been described in detail previously (Akk et al., 2001, 2004). The pipet potential was held at +60 to +80 mV, which translates to an approximately −120 to −100 mV potential difference across the patch membrane. The channel activity was recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass–filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software (Molecular Devices). The key features of the analysis of single-channel currents are as follows. When possible, the analysis was limited to clusters (i.e., episodes of intense activity originating from the activation of a single ion channel or fragments of clusters containing no overlapping currents). The currents were low-pass–filtered at 2 to 3 kHz, and the data were idealized using the segmented-k-means algorithm (Qin et al., 1996). The open and closed times were estimated from the idealized currents using a maximum likelihood method that incorporates a correction for missed events (QuB Suite; http://www.qub.buffalo.edu). Under certain conditions (e.g., in the presence of P4S or low concentrations of GABA), no clear-cut clusters were observed. In these cases, episodes of activity containing no overlapping currents were used for analysis, and the analysis was limited to estimation of channel open time durations.

Throughout the manuscript, the open- and closed-time components, as determined from the respective histograms, are referrred to as OT1–3 and CT1–3, respectively. The numerical designation applies to the lifetime of the component (i.e., OT1 and CT1 are the briefest components in the respective histograms) but does not necessarily connote a specific state in the activation scheme. For example, the CT3 component at one GABA concentration does not have to match up with the CT3 component at another GABA concentration, and the components may involve dwells in different activation states. Thus, no mechanism is implied. In some cases, when we believe that a particular closed-time component can be associated with sojourns in a particular activation state, we have used additional nomenclature to illustrate the mechanistic implications. For example, a closed-time component whose duration inversely correlates with agonist concentration probably results from sojourns in the un-, mono- and diliganded closed states. We have designated the corresponding closed-time component CT$_\beta$.

The recording and analysis of whole-cell currents was carried out as described previously (Li et al., 2006). The cells were clamped at −60 mV. The cells were exposed to GABA and steroids for 4 s with 30-s washouts separating successive applications. The current traces were low-pass–filtered at 2 kHz and digitized at 10 kHz. The analysis of whole-cell currents was carried out using the pClamp 9.0 software package and was aimed at determining the peak amplitude. Each cell was, before testing the effects of steroids, examined using two GABA concentrations to determine the approximate GABA EC_{50} for the cell to verify the expression of γ subunit in the receptor complexes (Boileau et al., 2003).

Comparative structural models of the wild-type and mutant α1 subunits were developed using the program Modeler (Sali and Blundell, 1993). The alignments used for the models were produced using the program MUSCLE (Edgar, 2004) by aligning the rat α1 sequence with the acetylcholine binding protein of *Lymanea stagnalis* (PDB code 1I9B) (Brejc et al., 2001) and the nicotinic acetylcholine receptor of *Torpedo marmorata* (PDB code 2BG9) (Unwin, 2005). To improve the quality of the models, the 72-residue cytoplasmic loop connecting the M3 and M4 domains was replaced with seven glycine residues. There are no conserved residues in the cytoplasmic loop, and only 30 amino acid residues could be aligned to positions on the templates. By replacing this region with seven glycines, optimal for stabilizing two α helices (Liu et al., 2003), a large region of conformational uncertainty was removed. For each protein, a total of 10 models was produced using molecular dynamics-based refinement for each model. The model quality was then assessed using the default penalty function, and further scored with "DOPE" (Discrete Optimized Protein Energy), and the best model was then used for further analysis (Shen and Sali, 2006). Although DOPE was developed from a nonredundant set of 1472 structures from the PDB, perhaps biasing toward soluble proteins rather than membrane-bound proteins, the idealized reference state is independent of the composition of the protein and the scoring function represents the current state of the art. The molecular graphics images shown in this work were produced with the UCSF Chimera package (Pettersen et al., 2004) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health grant P41-RR01081).

The synthesis of $3\alpha CH_2OH5\beta P$ will be described in detail in future publications. The compound had spectroscopic properties consistent with the assigned structure. It was chromatographically pure and gave the correct elemental analysis. Steroid 3deoxy5αP was obtained from Steraloids, Inc (Newport, RI). Other chemicals, including 3α5αP, 3α5βP, and pregnenolone sulfate, were purchased from Sigma-Aldrich (St. Louis, MO).

Results

The Effects of the α1Q241W Mutation on Channel Activation by GABA. We first examined the activation of the α1Q241W mutant receptor by GABA. The goal of the experiments was to establish the baseline kinetic properties of the mutant receptor for subsequent studies of steroid modulation.

In whole-cell recordings, the GABA dose-response relationship of the α1Q241W receptor was shifted to lower agonist concentrations compared with the wild-type receptor. The midpoint of the dose-response curve was at 2.5 ± 0.2 μM, and the Hill slope was 1.0 ± 0.1 (Fig. 2A).

The principal characteristics of single-channel activity were unchanged by the mutation. Similar to what we have observed previously for wild-type receptors (e.g., Steinbach and Akk, 2001; Li et al., 2007b), exposure to high concentrations of GABA led to channel activity in easily identifiable single-channel clusters. Figure 3 shows sample currents from the mutant receptor exposed to 2, 10, or 50 μM GABA. At 2 μM GABA, channel activity was characterized by isolated openings interspersed with brief bursts of activity, but no single-channel clusters were evident. When channel activity was elicited by 10 or 50 μM GABA, the openings were condensed into 1- to 10-s clusters of activity.

We examined the intracluster open- and closed-time distributions to gain mechanistic insight into the activation properties of the mutant receptor. The open-time histograms were best fit to sums of three exponentials. In the presence of

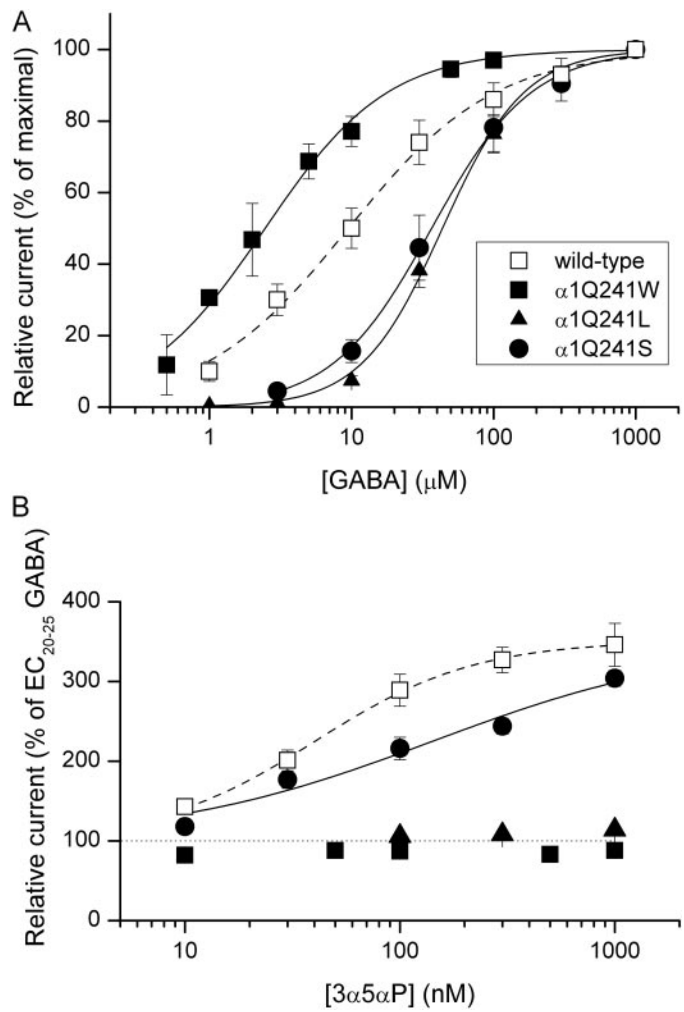

Fig. 2. Activation and modulation of the wild-type and α1Q241W, α1Q241L, and α1Q241S mutant receptors. A, wild-type and mutant receptors were activated by GABA, and the mean fractional peak responses (± S.E.M.) from 5 to 15 cells are plotted as a function of GABA concentration. Curve fitting to the Hill equation yields the following parameters. Wild-type: EC_{50} = 9.4 ± 1.1 μM, n_H = 0.9 ± 0.1; α1Q241W: EC_{50} = 2.5 ± 0.2 μM, n_H = 1.0 ± 0.1; α1Q241L: EC_{50} = 43.6 ± 1.7 μM, n_H = 1.5 ± 0.1; α1Q241S: EC_{50} = 36.6 ± 1.5 μM, n_H = 1.2 ± 0.1. The wild-type receptor data are from Li et al. (2006). B, wild-type and mutant receptors were activated by GABA in the presence of 10 to 1000 nM 3α5αP. The GABA concentration used corresponded to EC_{20-25} in the macroscopic dose-response curve and was 5 μM for the wild-type receptor, 1 μM for α1Q241W, 20 μM for α1Q241L, and 15 μM for the α1Q241S mutant receptor. The data points show mean ± S.E.M. from four to six cells. Fitting to the Hill equation was conducted for data from the wild-type and α1Q241S receptors. The best-fit parameters for the wild-type are: maximal potentiation = 351 ± 4%, EC_{50} = 41 ± 2 nM, and n_H = 1.2 ± 0.1. The best-fit parameters for the α1Q241S mutant receptor are: maximal potentiation = 350 ± 85%, EC_{50} = 142 ± 173 nM, and n_H = 0.7 ± 0.3. Offset was fixed at 100%. The test applications lasted 4 s and were separated from flanking control (GABA alone) applications by 30-s washouts.

100 μM GABA (a saturating concentration), the mean durations of the open-time components (averaged from four patches) were 0.36 ± 0.10 ms (33%), 3.4 ± 1.7 ms (34%), and 9.0 ± 2.1 ms (32%). Reduction of the GABA concentration to 10 μM had had little effect on the intracluster open-time distributions (see Table 1). However, the relative number of brief openings (OT1) in the record was increased to 48 ± 1% (n = 2 patches) when 1 μM GABA was used to activate the receptors. This indicates that a portion of the brief openings originates from un- or monoliganded receptors.

The α1Q241W mutation resulted in an increase in the relative frequency of the longest-lived open-time component, OT3. At GABA concentrations evoking equivalent responses, the prevalence of OT3 is 13 ± 4% in the wild-type receptor (Li et al., 2007b) and 52 ± 12% in the α1Q241W mutant receptor (Table 1). In addition, there was a trend toward an increase in the duration of OT3 in the mutant receptor. As a result, the mean open duration was enhanced from 2.1 ± 0.3 to 5.6 ± 0.6 ms in the presence of the α1Q241W mutation.

In the presence of 10 to 100 μM GABA, the intracluster closed-time distributions contained three components. At 10 μM GABA, the mean durations of the closed-time components (see also Table 2) were 0.13 ± 0.01 ms (58%; CT1), 1.9 ± 0.4 ms (27%; CT2), and 20.3 ± 2.0 ms (15%; CT3). In the presence of 100 μM GABA, the mean durations and fractions of the closed times (n = 4 patches) were 0.18 ± 0.04 ms (63%; CT1), 1.7 ± 0.2 ms (29%; CT2), and 8.8 ± 2.7 ms (8%; CT3).

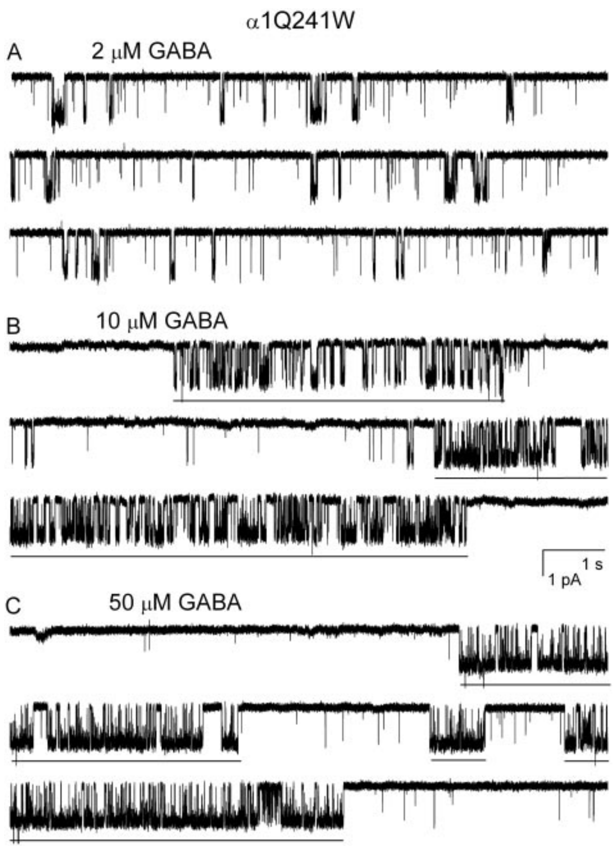

Fig. 3. Single-channel activity from the α1Q241W mutant receptor. Each section shows three consecutive 10-s segments at a specific concentration of GABA. Channel openings are shown as downward deflections. A, channel activation by 2 μM GABA resulted in isolated openings and brief (<0.5 s) bursts of openings. No clusters were evident. B, channel activation by 10 μM GABA resulted in high-open-probability clusters of activity (shown with lines underneath the current traces). In addition to the clusters, the record contained brief bursts of activity and isolated openings of unknown origin, which were not included in the kinetic analysis. C, channel activation by 50 μM GABA resulted in clusters of activity and isolated openings. The clusters were defined as long-lived (>0.5 s) groups of activity separated from each other by periods of inactivity, ignoring isolated openings greater than 1 s.

We have previously postulated that the intracluster closed-time histograms from the wild-type receptor contain a component whose duration (but not prevalence) depends on agonist concentration, as well as several components whose durations are not modulated by changes in agonist concentration (Steinbach and Akk, 2001). The agonist concentration-dependent component (CT_β) results from dwells in unliganded, monoliganded, and diliganded closed states. The lifetime of the CT_β component inversely correlates with agonist concentration because the movement from states with lower ligation status to the diliganded open state involves the binding of agonist. The agonist concentration-independent closed states probably result from dwells in various blocked or short-lived desensitized states. The relative frequencies of the closed states are not affected by GABA concentration (Steinbach and Akk, 2001).

It is typically not feasible to resolve all states at a given GABA concentration; i.e., the CT_β state may overlap in duration with dwells in the blocked or short-lived desensitized states, but, by altering the agonist concentration, it is possible to manipulate the duration of the CT_β state and separate it from other closed-time components. For example, we previously showed that the single-channel clusters from the wild-type receptor contain a long-lived (10–20 ms) but infrequent (2% of all intracluster closed events) closed-time component (Steinbach and Akk, 2001). We associated this closed event with a short-lived desensitized state (CT_{SD}), because it seemed to be related to the long-lived closed times observed among channel openings after a brief pulse of agonist (Jones and Westbrook, 1995). In the presence of 50 μM GABA, where the activation-related closed-time component is prolonged, the CT_β and CT_{SD} states have indistinguishable lifetimes, and both states contribute to the longest-lived intracluster closed-time component (CT3). In contrast, at saturating GABA concentrations, where the activation-related closed times are brief, the infrequent CT_{SD} state is the sole contributor to the long-lived CT3 closed-time component.

Single-channel currents from the α1Q241W mutant receptor indicated the presence of a similar, long-lived closed state. At 100 μM GABA (a saturating concentration), the longest-lived closed component (CT3) had a relative frequency of 8%, but when the receptors were activated by 10 μM GABA, the prevalence of CT3 was 15%. We suggest that the CT3 component at saturating GABA concentrations consists of dwells in the short-lived desensitized state (CT_{SD}), whereas at 10 μM GABA, the CT3 component contains dwells in the CT_{SD} state as well as sojourns in the mono- and unliganded closed states (i.e., CT_β). We can therefore estimate the prevalence of CT_β by subtracting the relative frequency of CT_{SD}, determined at 100 μM GABA (8 ± 3%), from the relative frequency of CT3 measured at 10 μM GABA (15 ± 4%). Assuming that the relative frequencies of the two states are unaffected by changes in the GABA concentration, we get a prevalence of approximately 7% for the activation-related closed-time component. This value is lower than that in the wild-type receptor (27 ± 6%; Li et al., 2007b).

In sum, kinetic analysis of the single-channel data from the α1Q241W mutant receptor shows both similarities to and differences from the wild-type data. In the presence of elevated GABA concentrations, the mutant receptor single-channel activity consists of high-open-probability clusters that contain three open-time and three closed-time components. However, the properties of the open and closed states are dissimilar to those in the wild-type receptor. The prevalence and duration of the longest-lived open-time component (OT3) are increased, and the prevalence of the activation-related closed-time component (CT_β) is decreased compared with the wild-type receptor. These are the same kinetic parameters that are affected when the wild-type receptor is activated by GABA in the presence of neurosteroid 3α5αP (Akk et al., 2005).

The α1Q241W Mutant Receptor Is Not Potentiated by 3α5αP. Previous work (Hosie et al., 2006), confirmed by us (Fig. 2B), showed that 3α5αP is ineffective at potentiating macroscopic currents from the α1Q241W mutant receptor. To determine the effect of the steroid on single-channel currents, we compared single-channel currents elicited by 10 μM GABA in the absence and presence of 1 μM 3α5αP. This steroid concentration elicits maximal potentiation in the wild-type receptor. As predicted by macroscopic recordings, the presence of steroid did not affect the kinetic properties of single-channel activity. Sample currents are shown in Figs. 4, A and B, and the data are summarized in Tables 1 and 2.

In the wild-type α1β2γ2L GABA-A receptor, 3α5αP potentiates the currents by increasing the duration and prevalence of OT3 and by decreasing the prevalence of CT3 (Akk et al., 2005). As a result, the mean open time is enhanced and the mean intracluster closed time is reduced in the presence of steroid. Our findings indicate that none of these changes occur when the glutamine residue in the α241 position is replaced with tryptophan. But the baseline values for the duration and prevalence of OT3 and the prevalence of CT3 in the α1Q241W receptor are similar to those observed with the wild-type receptor in the presence of steroid, suggesting that the amino acid substitution mimics the presence of steroid.

The α1Q241W Mutation Modifies Channel Activation by Piperidine-4-sulfonic Acid. Piperidine-4-sulfonic acid (P4S) is a high-affinity, low-efficacy agonist of the GABA-A receptor. In macroscopic recordings, the concentration producing half-maximal response from wild-type α1β2γ2L receptors is 16.5 ± 0.3 μM (i.e., similar to that for GABA), but the peak current elicited by saturating concentrations of P4S is only 63 ± 13% of that for GABA (data not shown). Unlike GABA, P4S, even at saturating concentrations, elicits single-

TABLE 1

Summary of the effects of 1 μM 3α5αP on the intracluster open times from the α1β2γ2L wild-type receptor and α1Q241W, α1Q241L, and α1Q241S mutant receptors

The intracluster open-time histograms were fitted to sums of three exponentials (wild-type and α1Q241W) or two exponentials (α1Q241L and α1Q241S). In the wild-type receptor, 3α5αP potentiates the currents by enhancing the mean duration and prevalence of OT3. In addition, the presence of steroid influences the mean duration and prevalence of OT1, and the prevalence of OT2. The presence of steroid was without effect on the properties (mean durations and fractions) of channel openings from the α1Q241W and α1Q241L receptors but prolonged the mean duration of OT2 in the α1Q241S receptor. Wild-type data are from Li et al. (2007b). The statistical analysis applies to the effect of the steroid for a given receptor (wild type or mutant).

Mutation	GABA	3α5αP	OT1	Fraction OT1	OT2	Fraction OT2	OT3	Fraction OT3	*n*
	μM	*μM*	*ms*		*ms*		*ms*		
None	50		0.28 ± 0.05	0.22 ± 0.02	3.0 ± 0.7	0.65 ± 0.06	7.3 ± 3.2	0.13 ± 0.07	4
None	50	1	0.41 ± 0.04*	0.39 ± 0.07**	2.4 ± 0.9	0.23 ± 0.03***	14.1 ± 2.1*	0.38 ± 0.04*	3
α1Q241W	10		0.33 ± 0.08	0.26 ± 0.09	2.2 ± 1.5	0.22 ± 0.07	9.6 ± 4.1	0.52 ± 0.12	4
α1Q241W	10	1	0.30 ± 0.04	0.33 ± 0.06	3.1 ± 2.3	0.27 ± 0.19	8.5 ± 3.2	0.40 ± 0.17	3
α1Q241L	50		0.60 ± 0.25	0.47 ± 0.32	1.9 ± 0.7	0.53 ± 0.32			4
α1Q241L	50	1	0.54 ± 0.14	0.23 ± 0.03	2.2 ± 0.1	0.77 ± 0.03			3
α1Q241S	50		0.69 ± 0.21	0.60 ± 0.21	1.8 ± 0.6	0.40 ± 0.21			7
α1Q241S	50	1	0.66 ± 0.16	0.40 ± 0.13	5.7 ± 1.5***	0.60 ± 0.13			6

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

TABLE 2

Summary of the effects of 1 μM 3α5αP on intracluster closed times from the α1β2γ2L wild-type receptor and α1Q241W, α1Q241L, and α1Q241S mutant receptors

The intracluster closed-time histograms were fitted to sums of three exponentials. In the wild-type receptor, 3α5αP potentiates the currents by decreasing the prevalence of the longest-lived closed-time component, CT3. In addition, the presence of steroid influences the mean duration of CT1 and the prevalence of CT2. The presence of steroid was without effect on the properties (mean durations and fractions) of intracluster closed times from the α1Q241W receptor. In receptors containing the α1Q241L mutation, 3α5αP affected the fraction of CT1. In receptors containing the α1Q241S mutation, 3α5αP affected the mean duration and fraction of CT1, and the fraction of CT2. Wild-type data are from Li et al. (2007b). The statistical analysis applies to the effect of the steroid for a given receptor (wild-type or mutant).

Mutation	GABA	3α5αP	CT1	Fraction CT1	CT2	Fraction CT2	CT3	Fraction CT3	*n*
	μM	*μM*	*ms*		*ms*		*ms*		
None	50		0.15 ± 0.01	0.60 ± 0.10	1.5 ± 0.2	0.13 ± 0.05	14.4 ± 4.2	0.27 ± 0.06	4
None	50	1	0.22 ± 0.04*	0.64 ± 0.12	1.4 ± 0.2	0.30 ± 0.10*	14.3 ± 1.2	0.05 ± 0.01***	4
α1Q241W	10		0.13 ± 0.01	0.58 ± 0.07	1.9 ± 0.4	0.27 ± 0.04	20.3 ± 2.0	0.15 ± 0.04	4
α1Q241W	10	1	0.16 ± 0.03	0.55 ± 0.15	1.8 ± 0.3	0.24 ± 0.04	19.7 ± 9.6	0.21 ± 0.11	3
α1Q241L	50		0.22 ± 0.05	0.54 ± 0.02	2.0 ± 1.1	0.17 ± 0.05	11.3 ± 2.7	0.29 ± 0.05	4
α1Q241L	50	1	0.17 ± 0.03	0.61 ± 0.05*	1.2 ± 0.3	0.13 ± 0.03	13.2 ± 5.6	0.25 ± 0.04	3
α1Q241S	50		0.21 ± 0.03	0.70 ± 0.07	1.5 ± 0.5	0.10 ± 0.02	14.4 ± 2.7	0.20 ± 0.05	7
α1Q241S	50	1	0.14 ± 0.03**	0.63 ± 0.04*	1.4 ± 0.3	0.18 ± 0.05**	14.6 ± 6.9	0.19 ± 0.03	6

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

channel currents best-characterized as monotonous, low open probability episodes of activity (Fig. 5A) (also Steinbach and Akk, 2001). The channel open-time histograms contain two components, with mean durations resembling those of OT1 and OT2 for receptors activated by GABA. The studies of closed times are typically inconclusive because the number of active receptors in the patch producing the single-channel activity is unknown.

Coapplication of 3α5αP with P4S cardinally changes the mode of activity. Instead of isolated openings, channel activity in the presence of the steroid takes place in easily identifiable clusters. Sample recordings from the wild-type receptor activated by 1 mM P4S + 1 μM 3α5αP are shown in Fig. 5B. The analysis of intracluster open time histograms revealed the emergence of the third, long-lived open state. Averaged from 5 patches, the mean open times were 0.21 ± 0.04 ms (33%; OT1), 1.4 ± 0.5 ms (28%; OT2), and 8.4 ± 3.1 ms (40%; OT3).

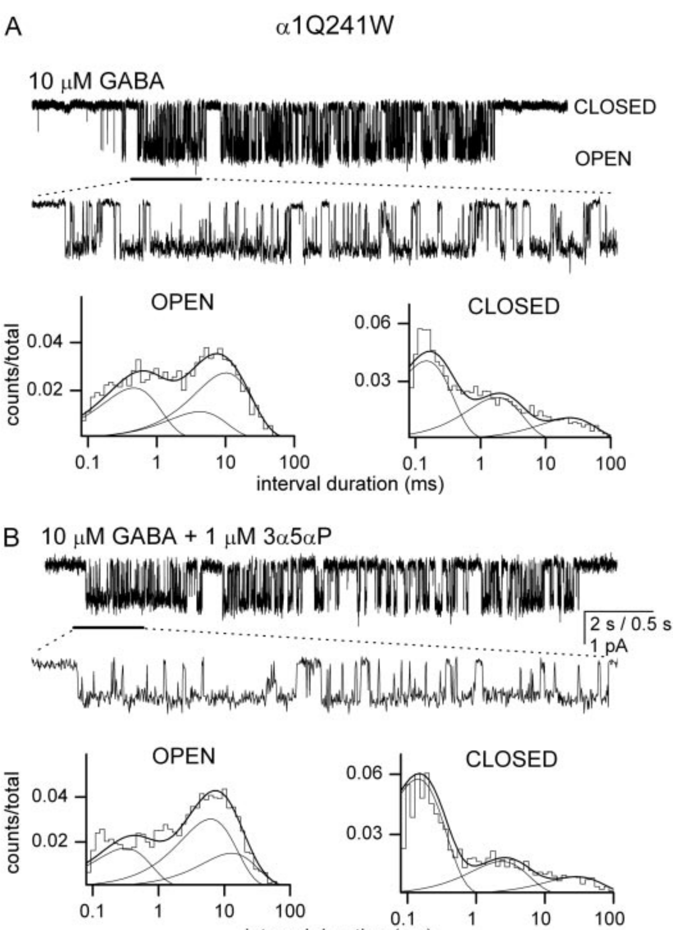

Fig. 4. The single-channel currents from the α1Q241W mutant receptor are not modulated by 3α5αP. A, sample single-channel currents elicited by 10 μM GABA, and the open and closed time histograms. The lower trace gives a portion of the current trace (shown with a thick line underneath the data trace) at higher resolution. The histograms were fitted to sums of three exponentials. The open times were 0.42 ms (19%), 3.9 ms (19%), and 9.3 ms (45%). The closed times were 0.14 ms (56%), 1.6 ms (29%), and 21.7 ms (15%). B, sample single-channel currents elicited by 10 μM GABA + 1 μM 3α5αP, and the open- and closed-time histograms. The lower trace gives a portion of the current trace at higher resolution. The histograms were fitted to sums of three exponentials. The open times were 0.29 ms (28%), 5.7 ms (49%), and 11.9 ms (23%). The closed times were 0.13 ms (70%), 2.2 ms (19%), and 27.0 ms (11%). The open- and closed-time parameters apply to the specific patch. Averaged values from multiple patches are given in the text.

The data described above suggested that in the presence of GABA, the α1Q241W mutation acts by mimicking the effects of steroid. We were interested in testing whether channel activation by P4S is similarly modified by the mutation. Accordingly, we next examined the activation of the α1Q241W mutant receptor by P4S. The major finding was that mutant receptors activated by P4S exhibited clear-cut clusters that contained three classes of open events. Sample single-channel activity is shown in Fig. 5C. At 1 mM P4S, the intracluster open- and closed-time histograms contained three components. The mean open times (averaged from four patches) were 0.41 ± 0.21 ms (23%; OT1), 3.1 ± 1.0 ms (66%; OT2), and 8.3 ± 3.5 ms (11%; OT3).

The presence of the long-lived (~8 ms) OT3 component in the α1Q241W receptor activated by P4S is qualitatively similar to the emergence of OT3 when the wild-type receptor is activated by P4S + 3α5αP. We conclude that the α1Q241W mutation and the presence of steroid 3α5αP have qualitatively similar effects on GABA-A receptor activation by P4S.

The α1Q241W Mutation Does Not Affect GABA-A Receptor Inhibition by Pregnenolone Sulfate. Pregnenolone sulfate is an endogenous neurosteroid that inhibits GABA-A receptor activation (Majewska et al., 1988). In macroscopic recordings, the effect manifests as an increase in the apparent rate of desensitization (Shen et al., 2000). Previous studies have suggested that distinct, nonoverlapping sites mediate the inhibitory effect of pregnenolone sulfate and the effects of potentiating neuroactive steroids (Park-Chung et al., 1999; Akk et al., 2001). Here, we sought to confirm this by probing the effect of the α1Q241W mutation on channel modulation by pregnenolone sulfate. We hypothesized that if the site mediating the effect of pregnenolone sulfate were dis-

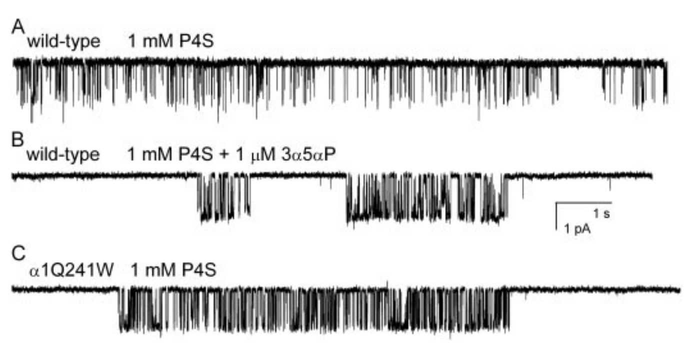

Fig. 5. The α1Q241W mutation affects channel activation by a low-efficacy agonist piperidine-4-sulfonic acid. A, sample single-channel activity from the wild-type receptor elicited by 1 mM P4S. No single-channel clusters were evident. Occasional overlaps seen in the data segment indicate that two or more channels contributed to the single-channel activity shown. The open times, measured from portions of the record without overlaps, were 0.18 ms (32%) and 1.6 ms (68%). No long duration openings (OT3) were apparent in the record. The closed times were 0.27 ms (24%), 8.8 ms (30%), and 30.3 ms (46%). B, exposure of wild-type receptors to 1 mM P4S + 1 μM 3α5αP results in increased open-time durations and the appearance of grouped openings (i.e., single-channel clusters). The intracluster open times were 0.15 ms (22%), 1.3 ms (40%), and 13.3 ms (38%). The intracluster closed times were 0.22 ms (47%), 1.8 ms (33%), and 34.5 ms (20%). C, exposure of the α1Q241W mutant receptor to 1 mM P4S elicits currents qualitatively similar to those from the wild-type receptor activated by P4S + 3α5αP. The intracluster open times were 0.55 ms (30%), 4.3 ms (62%), and 12.7 ms (8%). The intracluster closed times were 0.17 ms (53%), 2.0 ms (21%), and 13.7 ms (26%). The open- and closed-time parameters apply to the specific patch. Averaged values from multiple patches are given in the text.

tinct from the 3α5αP binding site, then the α1Q241W mutation would be likely to have no effect on channel modulation by the inhibitory steroid.

Whole-cell recordings were conducted on cells expressing wild-type or α1Q241W mutant receptors. The receptors were activated by a saturating concentration of GABA (1 mM for wild type, 250 μM for the mutant) in the absence and presence of 2 to 50 μM pregnenolone sulfate. In the wild-type receptor, the major effect of pregnenolone sulfate was a dose-dependent enhancement in the apparent rate of desensitization. In control recordings from cells exposed to 1 mM GABA, the desensitization time constant was 6.3 ± 2.8 s (n = 5 cells). When 50 μM pregnenolone sulfate was coapplied with GABA, the decay time constant was 656 ± 172 ms (n = 5 cells). In cells expressing α1Q241W mutant receptors, the mode of action of pregnenolone sulfate was analogous. The decay time constant was 4.1 ± 1.8 s (n = 4 cells) in the presence of GABA and 413 ± 78 ms (n = 4 cells) in the presence of GABA + 50 μM pregnenolone sulfate. In addition, there was a slight (<10%) decrease in peak response in some cells exposed to pregnenolone sulfate. Sample current traces are shown in Fig. 6, A and B.

The rate of development of block in the presence of 50 μM pregnenolone sulfate can be estimated from the relationship $k_{+PS} = \{1/\tau_{decay}(PS) - 1/\tau_{des}\}/50\ \mu M$, where τ_{des} is the macroscopic current desensitization time constant in the presence of GABA, and $\tau_{decay}(PS)$ is the decay time constant in the presence of GABA + pregnenolone sulfate. We estimate that the k_{+PS} was 0.03 ± 0.01 $\mu M^{-1}s^{-1}$ in the wild-type receptor and 0.04 ± 0.01 $\mu M^{-1}s^{-1}$ in the α1Q241W mutant receptor.

We examined the concentration-dependence of the effect of pregnenolone sulfate by comparing the area of the response (total charge transfer) during a 4-s application of GABA and 2 to 50 μM steroid (Fig. 6C). The data show that pregnenolone sulfate had a half-maximal effect at 7.4 ± 0.4 and 7.7 ± 2.2 μM on wild-type and mutant receptors, respectively. In sum, we infer that the α1Q241W mutation is without effect on channel modulation by the inhibitory steroid pregnenolone sulfate, indicating that distinct sites underlie channel modulation by potentiating and inhibitory steroids.

Properties of Activation, and Lack of Modulation by 3α5αP of the α1Q241L Mutant Receptor. We next examined the activation properties of the α1Q241L mutant receptor. This mutation also blocks potentiation of macroscopic currents by 3α5αP (Hosie et al., 2006; Fig. 2B), and we were curious to see whether the leucine substitution, similar to the more bulky tryptophan substitution, mimicked the presence of the steroid.

At GABA concentrations of 50 to 1000 μM, clear-cut clusters were observed (Fig. 7, A and B). The intracluster open-time distributions contained two components. In the presence of 1 mM GABA (a saturating concentration), the open times were 0.79 ± 0.03 ms (47%) and 1.9 ± 0.3 ms (53%) (averaged from three patches). The open-time distributions were unchanged when the GABA concentration was reduced to 50 μM (Table 1). The intracluster closed-time histograms were best fit to three exponentials. When the receptors were exposed to 1 mM GABA, the closed-time components had mean durations and prevalence of 0.23 ± 0.07 (57%), 1.0 ± 0.2 ms (35%), and 13.3 ± 5.1 ms (8%). In the presence of 50 μM GABA, the prevalence of the CT3 component was 29% (Table 2). By applying the reasoning used above in the analysis of single-channel activity from the α1Q241W mutant receptor, we estimate that the prevalence of CT_β is approximately 21% in the α1Q241L receptor. This value is comparable with the prevalence of CT_β in the wild-type receptor (27 ± 6%; Li et al., 2007b), and we conclude that the leucine substitution, in contrast to the tryptophan substitution, does not decrease the fraction of CT_β. The intracluster open-time histograms from the α1Q241L mutant lacked the long-lived OT3 component, and it is thus not possible to directly compare the open-time data from the wild-type and α1Q241L receptors. Nonetheless, the α1Q241L mutation, unlike the α1Q241W, did not result in a frequent, long-lived open-time component.

In the next set of experiments, we tested the effect of 1 μM 3α5αP on channel activation elicited by 50 μM GABA. Sample currents are shown in Fig. 7C, and the data are summarized in Tables 1 and 2. The data indicate that the presence of steroid is without effect on the intracluster kinetic param-

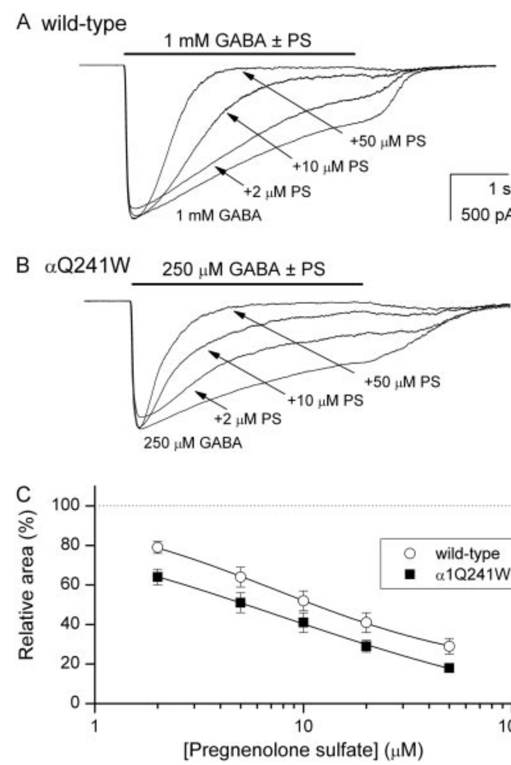

Fig. 6. The α1Q241W mutation does not affect channel inhibition by pregnenolone sulfate. A, sample macroscopic recordings from a cell expressing wild-type α1β2γ2L receptors. The receptors were activated by 1 mM GABA (a saturating concentration) in the absence and presence of 2, 10, or 50 μM pregnenolone sulfate (PS). The current decay phases were fitted by a single exponential to a constant level yielding 6794 ms (GABA), 4214 ms (GABA + 2 μM PS), 840 ms (GABA + 10 μM PS), and 464 ms (GABA + 50 μM PS). B, sample macroscopic recordings from a cell expressing α1Q241W mutant receptors. The receptors were activated by 250 μM GABA (a saturating concentration) in the absence and presence of 2, 10, or 50 μM PS. The current decay phases were fitted by a single exponential to a constant level yielding 3345 ms (GABA), 1268 ms (GABA + 2 μM PS), 864 ms (GABA + 10 μM PS), and 465 ms (GABA + 50 μM PS). C, the relative area (total charge carried) of the macroscopic response as a function of PS concentration. The data points show mean ± S.E.M. from four or five cells. The curves were fitted to: $Y([steroid]) = Y_0 + (Y_{max} - Y_0)\,[steroid]^{n_H}/([steroid] + EC_{50})^{n_H}$. The best-fit parameters for the wild-type receptor were Y_0 = 100% (constrained), Y_{max} = 15 ± 2%, EC_{50} = 7.4 ± 0.4 μM, n_H = 0.8 ± 0.1. The best-fit parameters for the α1Q241W mutant receptor were Y_0 = 100% (constrained), Y_{max} = −12 ± 8%, EC_{50} = 7.7 ± 2.2 μM, n_H = 0.6 ± 0.1.

eters. These findings are consistent with a model where the leucine substitution disrupts the interaction of 3α5αP with the receptor.

The α1Q241S Mutation Modifies Channel Potentiation by 3α5αP. In macroscopic recordings, the α1Q241S mutation shifts the steroid potentiation curve to higher steroid concentrations but has no effect on maximal potentiation (Hosie et al., 2006; Fig. 2B). The previous results (see above) had indicated that mutations to α1Gln241 produced changes in potentiation by neurosteroids and in the kinetic properties of single-channel currents elicited by GABA alone. Accordingly, it was of interest to examine the consequences of a mutation of this residue, which had relatively small effects on potentiation.

Single-channel clusters were recorded at 50 and 1000 μM GABA (Fig. 8, A and B). At 1 mM GABA, the open-time distributions of currents from the α1Q241S receptor contained two components with mean durations and fractions of 0.76 ± 0.23 ms (75%) and 2.0 ± 1.0 ms (25%) (n = 3 patches). Lowering the GABA concentration to 50 μM did not alter the open time distributions (Table 1).

We next tested the effect of 1 μM 3α5αP on channel activation elicited by 50 μM GABA. Sample currents are shown in Fig. 8C, and the summary of the effects of the presence of steroid is given in Tables 1 and 2. The data indicate that 3α5αP has a complex effect on the intracluster open- and closed-time distributions. The mean duration of the longer-lived open-time component was increased in the presence of 3α5αP. This effect is in qualitative agreement with the findings obtained from the wild-type receptor. However, we note that, in contrast to its effect on wild-type receptors, the application of 3α5αP did not lead to a statistically significant increase in the prevalence of long-lived openings. The presence of 3α5αP also did not affect the prevalence of CT3 in the α1Q241S mutant receptor. These findings indicate that the mutation modifies the mode of action of neurosteroid 3α5αP on the GABA-A receptor.

The α1S240L but Not the α1Q241L Mutation Disrupts Channel Potentiation by a 3α-Hydroxymethyl Steroid. Lack of potentiation by 3α5αP in the α1Q241L mutant has been proposed to stem from the inability of the leucine residue to act as an H-bond acceptor to the C3-OH group of the steroid (Hosie et al., 2006). Previous studies have shown that steroid analogs with substitutions other than the hydroxyl group in the C3 position can be efficacious potentiators of the GABA-A receptor. For example, pregnane steroids with a carboxylic acid, or its amide derivative, at C3 are positive modulators of GABA-A receptor activity (Mennerick et al., 2001). The greater distance between the backbone of the steroid's A ring and the H-bonding group on C3 in such steroids as well as the rotational freedom of the C3 substituents suggests that the steroid may be interacting with a locus other than the one used by 3α5αP, and so we hypothesized that such steroids may remain capable of potentiating receptors containing the α1Q241L mutation.

To test this hypothesis, we compared wild-type and α1Q241L mutant channel potentiation by the steroid 3αCH_2OH5βP (Fig. 9A) that has a hydroxymethyl group at C3. Sample currents and steroid dose-response curves are shown in Fig. 9, B to D. The data demonstrate that the α1Q241L mutation has minimal effect on channel potentiation by 3αCH_2OH5βP. The steroid, at 10 μM, potentiated macroscopic responses elicited by an $EC_{20–25}$ concentration of GABA from the wild-type and mutant receptors by 2.7 ± 0.4-fold (n = 5 cells) and 2.2 ± 0.2-fold (n = 4 cells), respectively.

We next examined the effects of mutations to the residues in the vicinity of α1Gln241 on channel potentiation by 3α5αP and 3αCH_2OH5βP. First, we tested the effect of substituting leucine for serine in position 240.

The GABA dose-response curve from the α1S240L mutant receptor was shifted to higher agonist concentrations, having an EC_{50} of 38 ± 4 μM (Fig. 10A). The steroid effects were examined in the presence of GABA concentrations producing peak responses equal to ~10 to 20% of maximal current. We found that coapplication of 3αCH_2OH5βP with GABA did not lead to potentiation of peak response in the α1S240L receptor (Fig. 10, B–D). The peak response was 109 ± 39% of control

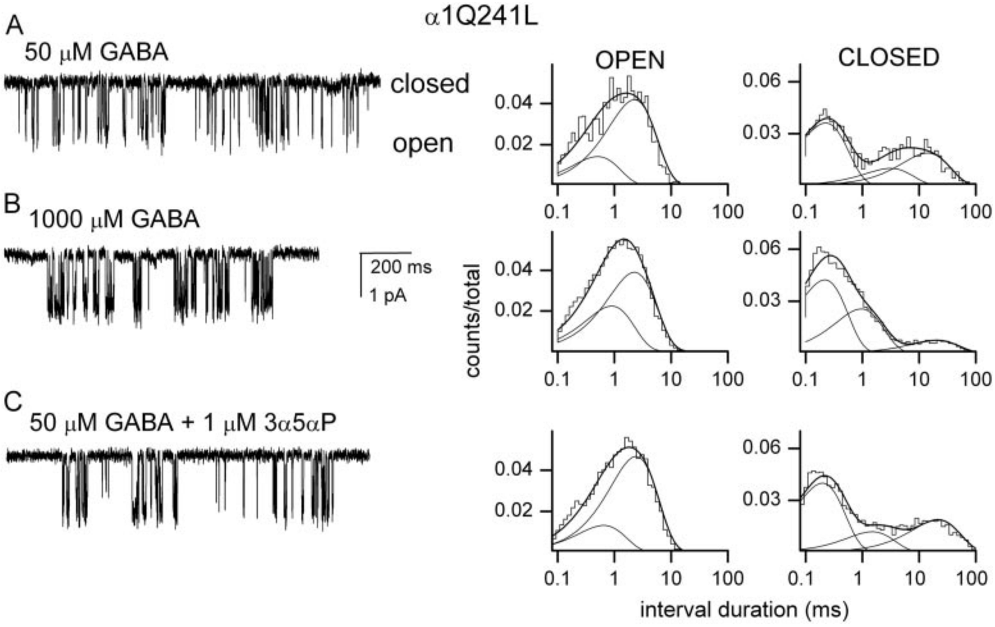

Fig. 7. 3α5αP does not modulate single-channel activity from the α1Q241L mutant receptor. A, a sample single-channel cluster elicited by 50 μM GABA and the open- and closed-time histograms. The open times were 0.47 ms (26%) and 2.1 ms (74%). The closed times were 0.20 ms (58%), 2.9 ms (15%), and 13.0 ms (27%). B, a sample single-channel cluster elicited by 1000 μM GABA, and the open- and closed-time histograms. The open times were 0.82 ms (38%) and 2.0 ms (62%). The closed times were 0.19 ms (59%), 0.9 ms (32%), and 18.9 ms (9%). C, a sample single-channel cluster elicited by 50 μM GABA in the presence of 1 μM 3α5αP, and the open- and closed-time histograms. The open times were 0.60 ms (22%) and 2.2 ms (78%). The closed times were 0.18 ms (58%), 1.4 ms (16%), and 19.6 ms (26%). The open- and closed-time parameters apply to the specific patch. Averaged values from multiple patches are given in the text.

(n = 8 cells) when 10 μM 3αCH$_2$OH5βP was coapplied with 10 or 25 μM GABA.

In contrast, the mutation was essentially without effect on potentiation by 3α5αP. Application of 3 μM 3α5αP potentiated currents from the α1S240L mutant to 284 ± 103% of control (n = 7 cells) indicating that the α1Ser240 residue is critical in channel modulation by 3αCH$_2$OH5βP but not 3α5αP.

Effects of Mutations to α1Trp245 and α1Ser243 Residues on Channel Potentiation by 3αCH$_2$OH5βP and 3α5αP. We next examined the effects of leucine mutations to residues α1Trp245 and α1Ser240 on channel activation by GABA, and modulation by 3αCH$_2$OH5βP and 3α5αP. In the α1W245L mutant receptor, the EC$_{50}$ for GABA-elicited currents was 24 ± 2 μM (Fig. 10A). The effects of steroids were studied in the presence of 5 or 10 μM GABA. The data show that the steroids were ineffective at producing channel modulation (Fig. 10D). Coapplication of 10 μM 3αCH$_2$OH5βP with GABA resulted in peak current of 101 ± 15% of control (n = 9 cells). When 3 μM 3α5αP was coapplied with GABA, the peak response was 108 ± 32% of control (n = 11 cells).

To test the possibility that the α1W245L mutation has a more global effect on receptor function preventing channel potentiation per se, we recorded whole-cell responses in the presence of pentobarbital. This GABA-A receptor modulator potentiates current responses at micromolar concentrations. The kinetic mechanism of action of pentobarbital resembles that of neuroactive steroids (e.g., Steinbach and Akk, 2001; Akk et al., 2004), which might imply commonality of the transduction elements involved in channel modulation by the two drugs, but the effects are considered to be mediated by drug interactions with distinct sites (Akk et al., 2004; Hosie et al., 2006). We reasoned that if the α1W245L mutation allows potentiation by pentobarbital, then this serves as indication of lack of global changes in receptor function. In five cells expressing the α1W245L mutant receptor, coapplication of 100 μM pentobarbital with 5 μM GABA (EC$_{10}$) increased the peak response to 906 ± 254% of control. We interpret the findings to indicate the selective involvement of the α1Trp245 residue in steroid actions.

As a negative control, we examined the effect of the α1S243L mutation on channel modulation by potentiating steroids. The α-helical configuration of the M1 membrane-spanning domain places the sidechain of this residue essentially in the opposite (compared with the α1Gln241 residue) surface of the domain, and we hypothesized that mutations to this residue would have minimal effect on channel modulation by either 3α5αP or 3αCH$_2$OH5βP. The α1S243L mutation shifted the GABA dose-response curve by almost 10-fold to higher concentrations (EC$_{50}$ = 80 ± 3 μM). Coapplication of 3 μM 3α5αP or 10 μM 3αCH$_2$OH5βP with 30 μM GABA enhanced the peak response to 374 ± 41% (n = 6 cells) or 183 ± 11% (n = 8 cells) of control, respectively. We conclude that the α1S243L mutation has minimal effect on channel potentiation by 3α5αP and 3αCH$_2$OH5βP.

Mutations to the M1 Domain Residues Affect Channel Modulation by 3α5βP. We next examined the effects of the mutations to the M1 membrane-spanning domain on channel modulation by 3α5βP. Single-channel experiments have shown that 3α5αP and 3α5βP are kinetically similar as modulators of the GABA-A receptor currents (Akk et al., 2005; Li et al., 2007a). Previous work employing mutations to

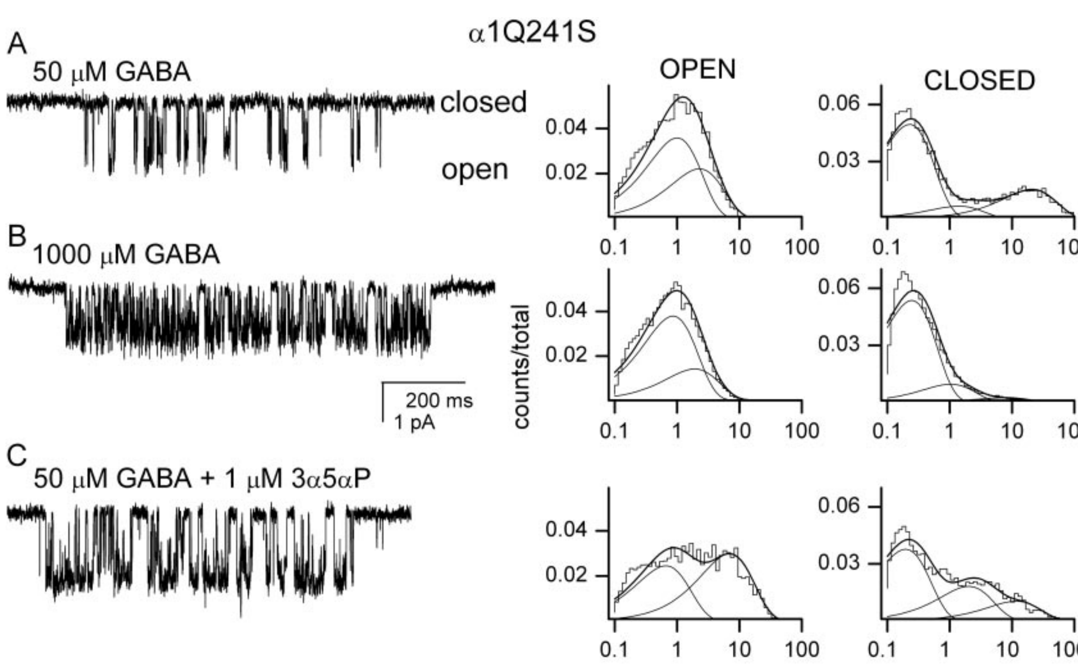

Fig. 8. Modulation of single-channel currents from the α1Q241S mutant receptor by 3α5αP. A, a sample single-channel cluster elicited by 50 μM GABA, and the open- and closed-time histograms. The open times were 0.92 ms (63%) and 2.1 ms (37%). The closed times were 0.20 ms (72%), 1.3 ms (9%), and 19.2 ms (20%). B, a sample single-channel cluster elicited by 1000 μM GABA and the open- and closed-time histograms. The open times were 0.72 ms (79%) and 1.7 ms (21%). The closed times were 0.21 ms (84%), 1.0 ms (12%), and 7.0 ms (3%). C, a sample single-channel cluster elicited by 50 μM GABA in the presence of 1 μM 3α5αP and the open- and closed-time histograms. The open times were 0.61 ms (46%) and 6.4 ms (54%). The closed times were 0.18 ms (58%), 1.9 ms (27%), and 10.9 ms (15%). The open- and closed-time parameters apply to the specific patch. Averaged values from multiple patches are given in the text.

the α1Gln241 site has indicated that 3α5αP and 3α5βP may interact with the same site to potentiate the GABA-A receptor (Hosie et al., 2006). In contrast, Mennerick et al. (2004) showed that a steroid analog (3α,5α)-17-phenylandrost-16-en-3-ol (17-PA) selectively antagonized channel potentiation and direct activation by 3α5αP but not 3α5βP, suggesting that different sites mediate the effects of these steroids. Here, we have evaluated the effects of mutations α1S240L, α1Q241L, α1S243L, and α1W245L on channel potentiation by 3α5βP to compare the differential effect that these mutations have on channel potentiation by 5α- and 5β-reduced steroids.

The summary of results is given in Fig. 11. Coapplication of 3 μM 3α5βP with GABA resulted in potentiation of peak current in α1β2γ2L wild-type receptors and α1S243L mutant receptors but not when the receptor contained the α1S240L, α1Q241L, or α1W245L mutation. Potentiation of receptors containing the α1S243L mutation is in agreement with our data suggesting that the orientation of the α1Ser243 residue is such that substitutions here have little effect on modulation by steroids. The lack of potentiation in the α1W245L mutant is similar to the effect of this mutation on modulation by 3α5αP, confirming our earlier conclusion that this residue is critical to channel potentiation by neuroactive steroids. The lack of potentiation by 3α5βP in the α1Q241L mutant is also similar to the effect of the mutation on modulation by 3α5αP. But the α1S240L mutation had different effects on potentiation by 3α5αP and 3α5βP. The mutation fully abolishes potentiation by 3α5βP (107 ± 19% of control, n = 5 cells) but is without effect on potentiation by 3α5αP (Fig. 10B). Thus, mutations to the α1 subunit M1 domain differentially affect channel modulation by 3α5αP and 3α5βP, suggesting that the two steroids are oriented differently in the binding pocket.

Potentiation of the GABA-A Receptor by 5α-Pregnan-20-one. The data presented above on the effects of the mutations to the α1Ser240 and α1Gln241 residues on channel potentiation by 3α5αP and 3αCH_2OH5βP could be interpreted as indicating that the hydroxyl group of 3α5αP interacts, possibly via H-bonding, with the α1Gln241 residue, and the hydroxymethyl group of 3αCH_2OH5βP interacts with the α1Ser240 residue. On the other hand, the finding that potentiation by 3α5βP is sensitive to the nature of the residue in both the α240 and α241 positions and the single-channel data on the α1Q241S mutation that retains but modifies the mechanism of potentiation by 3α5αP are not fully compatible with this simple model.

A possible explanation is that the α1Gln241 residue is not directly interacting with the C3-OH group of the steroid molecule but is rather a necessary component to appropriately shape the binding surface to accommodate the steroids 3α5αP and 3α5βP. To explore this hypothesis, we examined receptor modulation by 3deoxy5αP. This steroid (Fig. 12A) is devoid of H-bonding groups on C3 and is therefore predicted to not interact, at least via H-bonding, with the residues in the α1 subunit M1 domain. The interaction of 3deoxy5αP

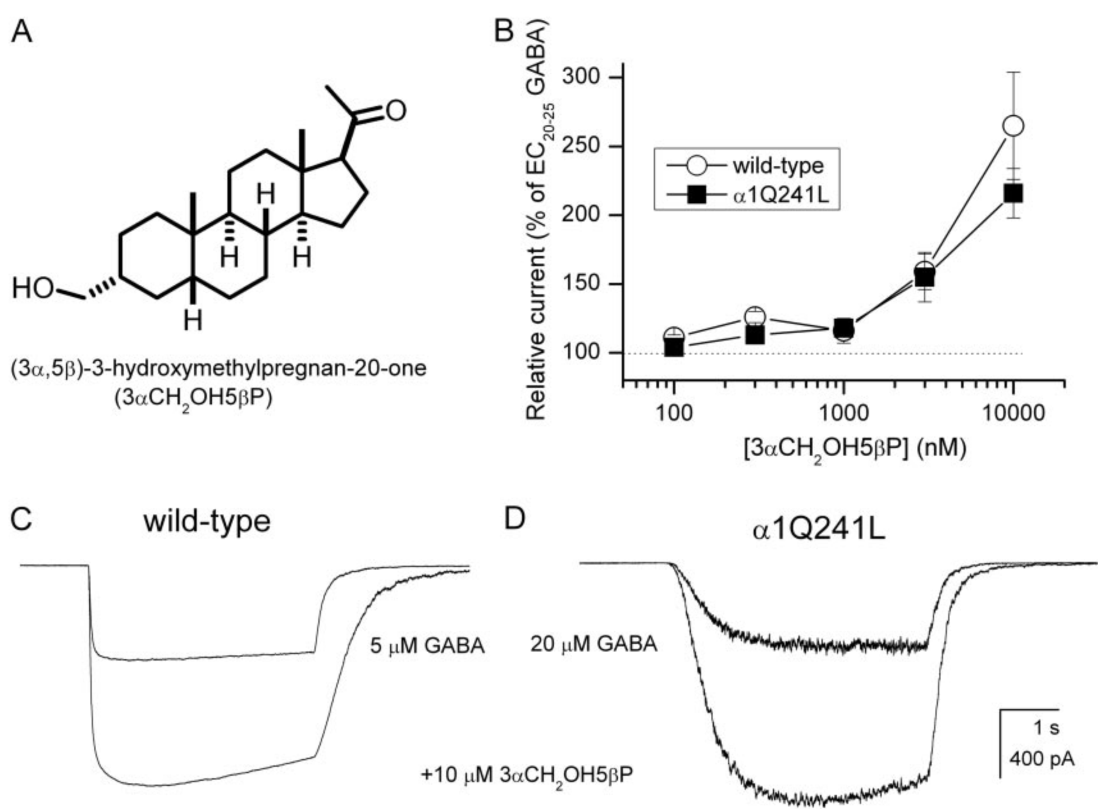

Fig. 9. The α1Q241L mutation does not affect potentiation by the steroid analog 3αCH_2OH5βP. A, structure of the steroid analog 3α$HOCH_2$5βP. B, potentiation dose-response curves for wild-type and α1Q241L mutant receptors. The data points show mean ± S.E.M. from four to five cells. Because of the absence of saturation, no curve fitting was attempted. C, sample macroscopic recordings from a cell expressing wild-type α1β2γ2L receptors. The receptors were activated by 5 μM GABA in the absence and presence of 10 μM 3αCH_2OH5βP. The peak responses in this cell were 581 pA (GABA), and 1346 pA (GABA + steroid). D, sample macroscopic recordings from a cell expressing α1Q241L mutant receptors. The receptors were activated by 20 μM GABA in the absence and presence of 10 μM 3αCH_2OH5βP. The peak responses in this cell were 559 pA (GABA) and 1480 pA (GABA + steroid).

with the wild-type receptor could be considered functionally analogous to 3α5αP interaction with the α1Q241L mutant receptor.

We examined macroscopic currents from the wild-type receptor exposed to 5 μM GABA in the absence and presence of 0.2 to 5 μM 3deoxy5αP. Channel potentiation was observed at steroid concentrations 0.5 μM and higher, reaching maximal potentiation at 1 μM 3deoxy5αP. Exposure to 1 μM 3deoxy5αP increased the peak response to 202 ± 16% of control (n = 5 cells; Fig. 12, B and C). We suspect that poor solubility of the steroid in aqueous solutions prevented stronger potentiation when nominally higher steroid concentrations were used. In any case, channel potentiation by a steroid that is devoid of groups on C3 that can form an H-bond indicates that an H-bond between the A ring of the steroid and the receptor is not required to produce channel potentiation.

We also examined the effect of the α1Q241L mutation on channel potentiation by 3deoxy5αP. The summary of the data and sample currents are shown in Fig. 12, B and D. The data demonstrate that the steroid is unable to potentiate currents from the α1Q241L mutant receptor. Coapplication of 1 μM 3deoxy5αP with 20 μM GABA (~EC_{15}) led to peak response of 97 ± 1% of control (n = 7 cells). This finding is not consistent with a model where the lack of steroid potentiation in a receptor containing the α1Q241L mutation is due to the inability of the leucine residue to form an H-bond with the C3-OH group.

Discussion

We have examined the effects of mutations to residues in the GABA-A receptor α1 subunit M1 membrane-spanning region on channel modulation by neuroactive steroids. A previous report (Hosie et al., 2006) had implicated the α1Gln241 residue in the actions of neurosteroids by showing that when the glutamine residue was replaced with a tryptophan or leucine residue, potentiation by 3α5αP and 3α5βP was greatly diminished. It was concluded that the α1Gln241 interacts with the steroid 3α-hydroxyl group (Hosie et al., 2006). The goal of the present work was to provide a more detailed kinetic and pharmacological characterization of the effects of mutations on channel activation, and modulation by neurosteroids. Overall, our data are consistent with the

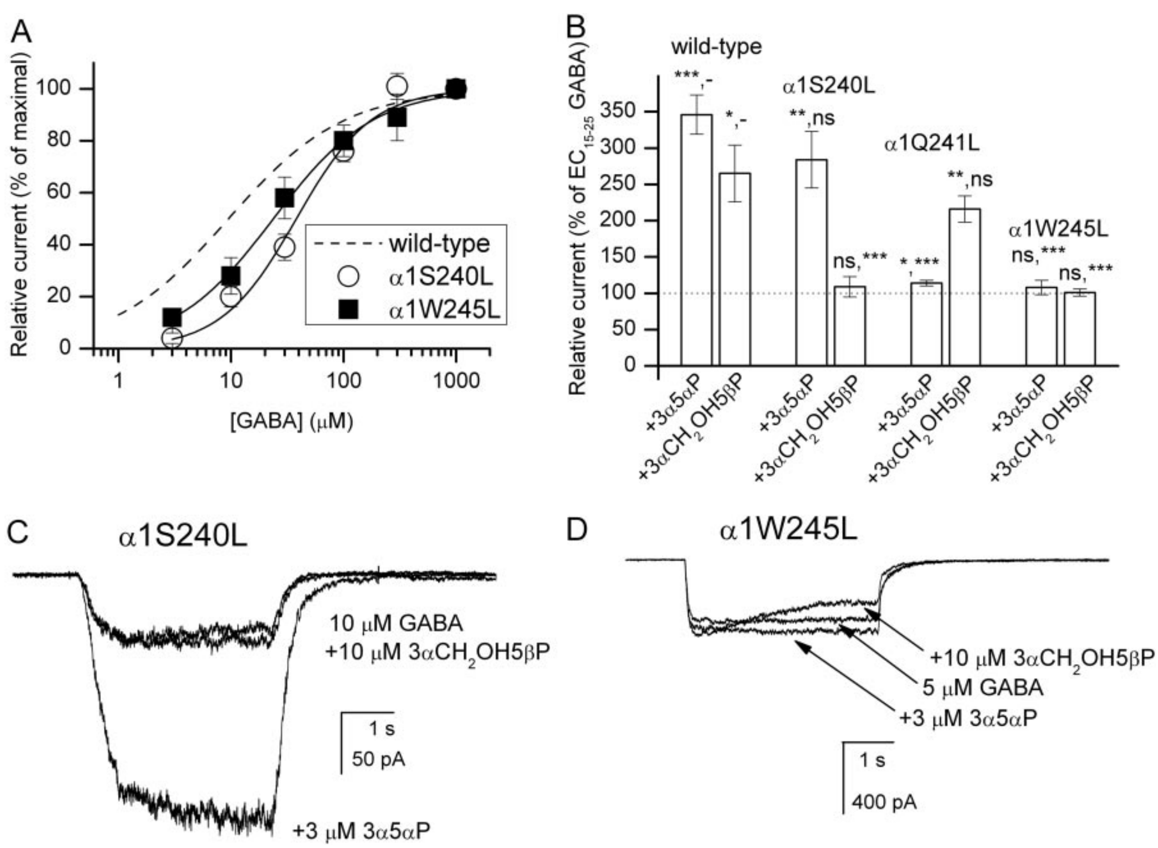

Fig. 10. Potentiation by $3\alpha CH_2OH5\beta P$ is abolished in the α1S240L and α1W245L mutant receptors. A, wild-type and mutant receptors were activated by GABA, and the mean fractional peak responses (± S.E.M.) from five to seven cells are plotted as a function of GABA concentration. Curve fitting to the Hill equation yields the following parameters. α1S240L: EC_{50} = 38.1 ± 4.3 μM, n_H = 1.3 ± 0.2; α1W245L: EC_{50} = 23.8 ± 1.6 μM, n_H = 1.0 ± 0.1. The wild-type receptor data (dashed line) are replotted from Fig. 1A. B, comparison of potentiation of wild-type, α1S240L, α1S241L, and α1W245L mutant receptors by 3α5αP or $3\alpha CH_2OH5\beta P$. The data (mean ± S.E.M.) show the levels of potentiation by 3 μM 3α5αP (1 μM for wild-type and α1Q241L) or 10 μM $3\alpha CH_2OH5\beta P$ of currents elicited by an EC_{15-25} concentration of GABA. Statistical tests (Student's t test) were carried out with respect to control (GABA alone) and to steroid-mediated potentiation of the wild-type receptor. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant; −, not applicable. C, sample macroscopic recordings from a cell expressing α1S240L mutant receptors. The receptors were activated by 10 μM GABA in the absence and presence of 10 μM $3\alpha CH_2OH5\beta P$ or 3 μM 3α5αP. The peak responses in this cell were 55 pA (10 μM GABA), 57 pA (10 μM GABA + 10 μM $3\alpha CH_2OH5\beta P$), and 210 pA (10 μM GABA + 3 μM 3α5αP). D, sample macroscopic recordings from a cell expressing α1W245L mutant receptors. The receptors were activated by 5 μM GABA in the absence and presence of 10 μM $3\alpha CH_2OH5\beta P$ or 3 μM 3α5αP. The peak responses in this cell were 352 pA (10 μM GABA), 311 pA (5 μM GABA + 10 μM $3\alpha CH_2OH5\beta P$), and 412 pA (5 μM GABA + 3 μM 3α5αP).

α1Gln241 residue acting as a critical site for channel modulation by 3α5αP. However, our data are most consistent with a model in which the α1Gln241 residue forms a crucial intraprotein contact rather than participates in direct interaction with the C3-OH group. We have identified an additional residue in the α1 subunit M1 domain (α1Ser240) that is required for modulation by the steroid analog 3αCH_2OH5βP but not 3α5αP and a residue (α1Trp245) that may participate as a transduction element in channel potentiation by steroids but not by pentobarbital.

The α1Q241W and α1Q241L mutations affected the kinetic properties of channels activated by GABA. The tryptophan substitution enhanced channel opening efficacy, whereas the leucine substitution had a slightly deleterious effect on channel function. Our data confirm that the α1Q241W and α1Q241L mutations drastically diminish receptor potentiation by 3α5αP, but we show that different mechanisms underlie the absence of potentiation in the mutant receptors. The α1Q241W mutation functionally mimics the presence of steroid, and receptors activated by GABA (or P4S) demonstrate single-channel kinetic properties found in wild-type receptors exposed to GABA (or P4S) in the presence of high concentrations of potentiating steroids. The coapplication of 3α5αP with GABA had no further effect on mutant receptor activation.

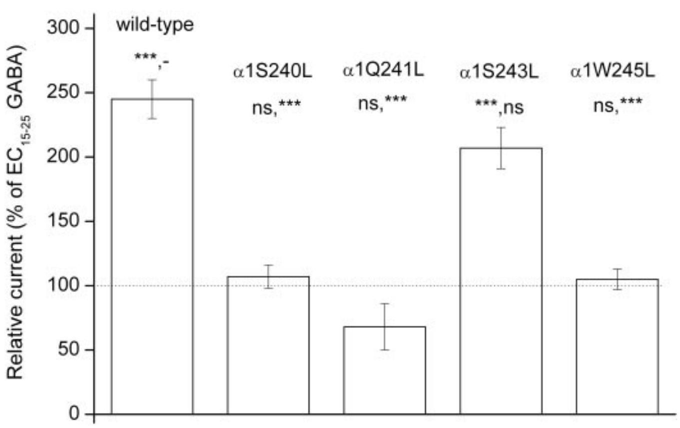

Fig. 11. Effects of mutations to the M1 domain on potentiation by 3α5βP. Comparison of potentiation of wild-type, and α1S240L, α1Q241L, α1S243L, and α1W245L mutant receptors by 3α5βP. The data (mean ± S.E.M.) from three to six cells show the levels of potentiation by 3 μM 3α5βP of currents elicited by an $EC_{15–25}$ concentration of GABA. Statistical tests (Student's *t* test) were carried out with respect to control (GABA alone) and to 3α5βP-mediated potentiation of the wild-type receptor. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant; −, not applicable.

The major effect of the α1Q241L mutation on channel activity was the loss of the longest-lived open-time component. The application of 3α5αP did not modify single-channel or whole-cell currents elicited by GABA, consistent with the previous hypothesis (Hosie et al., 2006) that the leucine substitution interferes with the ability of 3α5αP to interact with the steroid binding site.

As with all mutational studies, the possibility exists that the amino acid substitutions have a deleterious structural effect. Figure 13 shows a DOPE plot (Shen and Sali, 2006) of the M1 region, illustrating that there are no major energetic changes caused by the α1Q241W or α1Q241L mutations. DOPE provides a statistical potential for assessing the deviation of a particular structure from an idealized reference state, and the plots shown in Fig. 13 show the results of a sliding window of 13 residues through the models. Examining the M1 region, we see that the mutations studied produced small changes in this parameter, supporting the idea that no major structural consequences resulted from the mutations.

We also examined the effect of the α1Q241S mutation on channel activation and 3α5αP-induced potentiation. Hosie et al. (2006) had found that the α1Q241S mutation had little effect on steroid potentiation and concluded that the serine

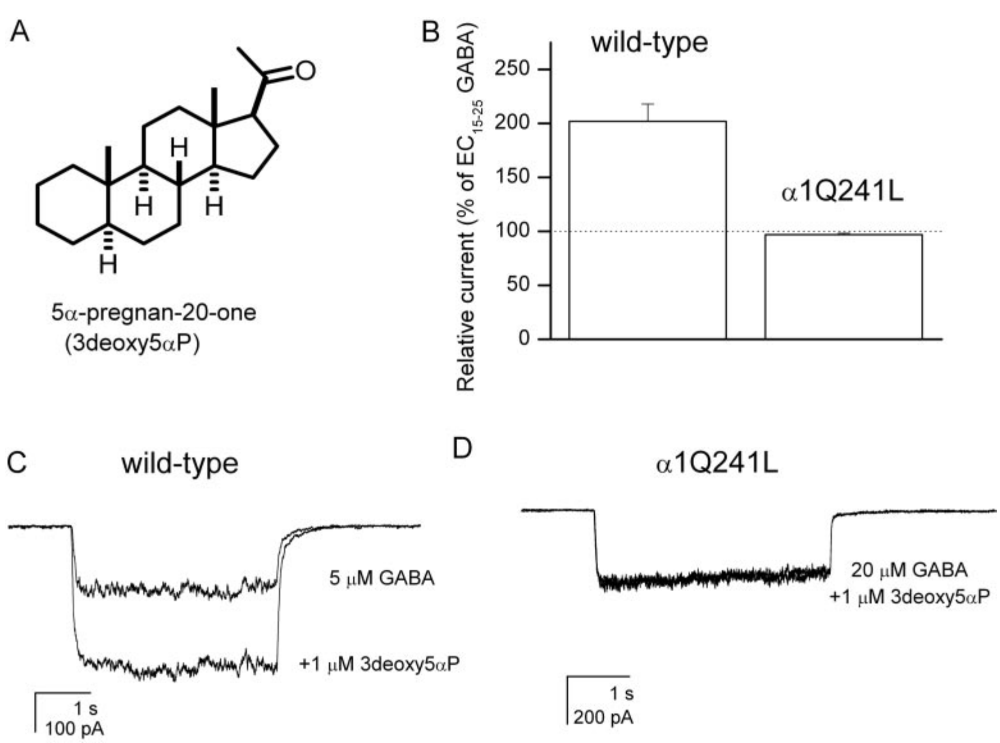

Fig. 12. Steroid 3deoxy5αP potentiates the wild-type but not the α1Q241L mutant receptor. A, structure of the steroid analog 3deoxy5αP. B, comparison of potentiation of the wild-type receptor and the α1Q241L mutant receptor by 1 μM 3deoxy5αP. The data show mean ± S.E.M. from five to seven cells. C, sample macroscopic recordings from a cell expressing wild-type receptors. The receptors were activated by 5 μM GABA in the absence and presence of 1 μM 3deoxy5αP. The peak responses in this cell were 114 pA (GABA) and 281 pA (GABA + 3deoxy5αP). D, sample macroscopic recordings from a cell expressing α1Q241L mutant receptors. The receptors were activated by 20 μM GABA in the absence and presence of 1 μM 3deoxy5αP. The peak responses in this cell were 317 pA (GABA) and 305 pA (GABA + 3deoxy5αP).

side-chain was able to replace glutamine as an H-bond acceptor and so retain potentiation. We confirm that 3α5αP elicits potentiation of macroscopic currents (Fig. 2B) but find that the single-channel potentiation profile is distinct in the mutant and wild-type receptors. In the wild-type receptor, the presence of 3α5αP results in increases in the duration and prevalence of the longest open-time component and a decrease in the prevalence of the activation-related closed-time component. In the α1Q241S mutant receptor, 3α5αP has a strong effect on the mean duration of the longest-lived open-time component but is largely without effect on the prevalence of long openings. The presence of steroid has a complex effect on the intracluster closed-time distributions. However, the signature feature of steroid modulation in the wild-type receptor—a decrease in the prevalence of CT3—is absent in the mutant receptor.

In addition, we have shown that the α1Q241W mutation does not interfere with the actions of the inhibitory neurosteroid pregnenolone sulfate. This is in agreement with previous studies that have concluded that the sites of action for potentiating and inhibitory neuroactive steroids are distinct (Park-Chung et al., 1999; Akk et al., 2001).

It is noteworthy that the α1Q241L mutation did not affect channel potentiation by the steroid analog 3αCH_2OH5βP. Site-directed mutagenesis of residues in the vicinity of α1Gln241 led to the identification of two additional residues adjacent to (or within) the steroid binding pocket: α1Ser240 and α1Trp245. Mutation of the α1Ser240 residue to leucine disrupted the potentiating effect of 3αCH_2OH5βP but not 3α5αP. The α1W245L mutation abolished potentiation by both steroids but left intact channel potentiation by pentobarbital and the marine cembranoid eupalmerin acetate (data not shown). In addition, the α1W245L mutation did not

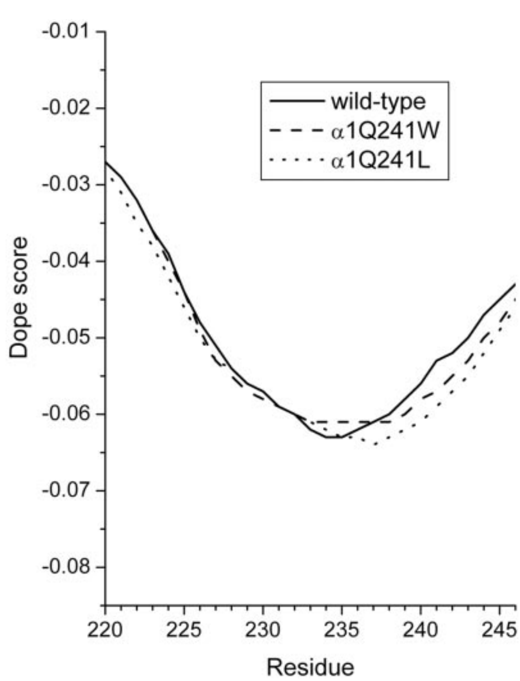

Fig. 13. The DOPE plots of the M1 domains of the wild-type (solid line), α1Q241W (dashed line), and α1Q241L mutant receptor (dotted line) over a 13-residue evaluation window. Negative scores show a favorable trend to the idealized reference state, whereas positive scores indicate unfavorable deviations from the reference state. There were only minimal changes in the scores arising from the mutations.

affect channel modulation by the inhibitory steroid pregnenolone sulfate (data not shown). We propose that the α1Trp245 residue participates as a transduction element in the actions of potentiating neuroactive steroids.

In single-channel recordings, some neuroactive steroids have three kinetically distinct effects on channel open- and closed-time distributions (Akk et al., 2004; Li et al., 2007a). Many of our previous findings could be interpreted as the effects being produced by steroid interactions with multiple nonoverlapping sites. The dose-response relationships for the effects on open and closed times can be different for a given steroid. For example, (3α,5β,17β)-3-hydroxy-18-norandrostance-17-carbonitrile elicits an increase in the prevalence of OT3 with an EC_{50} of <100 nM, whereas the EC_{50} for the increase of the duration of OT3 is >10 μM (Akk et al., 2004). We have also found that the steroid etiocholanolone, which has a single kinetic effect (to increase the prevalence of OT3), does not compete with a steroid having three kinetic effects (3α5βP) suggesting that etiocholanolone is unable to bind to the sites that mediate the increase in the duration of OT3 and the decrease in the prevalence of CT_β (Li et al., 2007a). Additional evidence for multiple binding sites for steroids comes from the finding that potentiation by 5α- but not 5β-reduced steroids is inhibited by a steroid analog 17-PA (Mennerick et al., 2004), and that the dose-response curves for steroid enhancement of muscimol-elicited $^{36}Cl^-$ uptake are biphasic (Morrow et al., 1990).

In contrast, the finding that the α1Q241L mutation abolishes all kinetically distinguishable effects that 3α5αP exerts in the wild-type receptor suggests that the steroid interacts with a single site on the receptor to produce potentiation. The presence of all three types of effects in the receptor containing the α1Q241W mutation, which we believe mimics the presence of steroid, is similarly in favor of a single binding site for steroid.

We hypothesize that the steroid binding pocket presents a hydrophobic surface capable of accommodating steroid molecules of different structure. Different steroids, through interactions with nonoverlapping loci, elicit a particular combination of kinetic effects that lead to channel potentiation. The lack of competition between etiocholanolone and 3α5βP could be explained if the α1Gln241 residue were not a docking site for the steroid per se, but rather one of the necessary components to maintain the structure of the surface to which steroids bind. Mutations to the M1 domain differentially affect the structure of the binding surface so that 3αCH_2OH5βP remains capable of interacting with its binding site after the α1Q241L but not the α1S240L mutation, whereas 3α5βP requires both α1Ser240 and α1Gln241 intact to modulate receptor activity. In this model, differential sensitivity of 3α5αP and 3α5βP to mutations in the M1 domain suggests that the two steroids bind to different loci, accounting for their different sensitivity to the steroid antagonist 17-PA.

Support for this hypothesis comes from the finding that the α1Q241S mutation alters the kinetic mode of action of 3α5αP. The data showing that the α1Q241L mutation disrupts potentiation by 3deoxy5αP is further indication that the glutamine-to-leucine mutation does not act by simply preventing H-bonding between the A ring of the steroid and the receptor. Instead, the mutation may alter the structure of the protein so that the binding site can no longer accommodate 3deoxy5αP. By extension, the data suggest that the α1Gln241 residue does not constitute the docking site for neurosteroids.

In sum, the present study confirms the crucial role of the amino acid residues in the α1 subunit M1 domain in GABA-A receptor modulation by potentiating neurosteroids. The data demonstrate a critical role of the α1Gln241 residue in channel potentiation by 3α-hydroxysteroids and 3deoxy5αP. We identified two additional residues in the α1 subunit M1 membrane-spanning domain, α1Ser240 and α1Trp245, whose mutation interferes with the ability of potentiating neuroactive steroids to modulate the GABA-A receptor. Our data are most consistent with a model in which mutations to residues in the M1 membrane-spanning domain shape the binding surface on the GABA-A receptor to which multiple steroid molecules can bind.

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