1521-0111/98/4/280–291$35.00
MOLECULAR PHARMACOLOGY

https://doi.org/10.1124/mol.120.119842
Mol Pharmacol 98:280–291, October 2020

Analysis of Modulation of the ρ1 $GABA_A$ Receptor by Combinations of Inhibitory and Potentiating Neurosteroids Reveals Shared and Distinct Binding Sites[S]

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Received February 17, 2020; accepted July 7, 2020

ABSTRACT

The ρ1 $GABA_A$ receptor is prominently expressed in the retina and is present at lower levels in several brain regions and other tissues. Although the ρ1 receptor is insensitive to many anesthetic drugs that modulate the heteromeric $GABA_A$ receptor, it maintains a rich and multifaceted steroid pharmacology. The receptor is negatively modulated by 5β-reduced steroids, sulfated or carboxylated steroids, and β-estradiol, whereas many 5α-reduced steroids potentiate the receptor. In this study, we analyzed modulation of the human ρ1 $GABA_A$ receptor by several neurosteroids, individually and in combination, in the framework of the coagonist concerted transition model. Experiments involving coapplication of two or more steroids revealed that the receptor contains at least three classes of distinct, nonoverlapping sites for steroids, one each for the inhibitory steroids pregnanolone (3α5βP), 3α5βP sulfate, and β-estradiol. The site for 3α5βP can accommodate the potentiating steroid 5αTHDOC. The findings are discussed with respect to receptor modulation by combinations of endogenous neurosteroids.

SIGNIFICANCE STATEMENT

The study describes modulation of the ρ1 $GABA_A$ receptor by neurosteroids. The coagonist concerted transition model was used to determine overlap of binding sites for several inhibitory and potentiating steroids.

Introduction

The ρ1 $GABA_A$ receptor is a member of the Cys-loop family of transmitter-gated ion channels. It is expressed at high levels in the retina where it modulates the processing of visual signaling (Lukasiewicz et al., 2004). Additionally, the ρ1 receptor has been detected in several brain regions, such as the hippocampus, superior colliculus, visual cortex, and cerebellum, and in the anterior pituitary gland, dorsal root ganglia, and the pancreatic islets (Wegelius et al., 1998; Rozzo et al., 2002; Maddox et al., 2004; Alakuijala et al., 2005; Nakayama et al., 2006; Jin et al., 2013).

The physiologic role of the ρ1 receptor in the brain is not fully understood, but the receptor is highly sensitive to GABA and shows little desensitization in the presence of ambient concentrations of GABA, making it well-suited to contribute to tonic inhibition (Amin and Weiss, 1994; Alakuijala et al., 2006). In rat pancreatic islets, locally released GABA can activate $GABA_A$ receptors, including those comprising ρ1 subunits, on the glucagon-releasing α-cells, thereby affecting glucose homeostasis (Jin et al., 2013). Activation of ρ receptors in the rat anterior pituitary cells has been shown to enhance the secretion of the luteotropic hormone prolactin associated with milk production (Nakayama et al., 2006). The ρ1 receptor has also been implicated in the behavioral effects of ethanol; single nucleotide polymorphisms in the *GABRR1* gene encoding for the ρ1 subunit are significantly associated with early onset alcohol dependence (Xuei et al., 2010; Blednov et al., 2014). Modulation of ρ receptor function may have clinical significance.

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This work was supported by National Institutes of Health National Institute of General Medical Sciences [Grant R01 GM108580] and [Grant R01 GM108799] and funds from the Taylor Family Institute for Innovative Psychiatric Research.

https://doi.org/10.1124/mol.120.119842.

[S] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: *c*, ratio of the equilibrium dissociation constant of the open receptor to that of the closed receptor; β-estradiol, (8*R*,9S,13S,14S,17*S*)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthrene-3,17-diol; GLIC, *Gloeobacter violaceus* ligand-gated ion channel; 3α5βP, 1-[(3*R*,5*R*,8*R*,9*S*,10S,13S,14S,17*S*)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl]ethanone (pregnanolone); PDB ID, Protein Data Bank Identification; P_{open}, open probability of the receptor; $P_{open,\ const}$, open probability of the constitutively active receptor; 3α5βPS, [(3*R*,5*R*,8*R*,9*S*,10S,13S,14S,17*S*)-17-acetyl-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl]ethanone hydrogen sulfate (pregnanolone sulfate); 5αTHDOC, (3α,5α)-3,21-dihydroxypregnan-20-one (allotetrahydrodeoxycorticosterone).

For example, intravitreal injections of the ρ1 inhibitors *cis*- and *trans*-(3-aminocyclopentanyl)butylphosphinic acid prevent the development of experimental myopia in the chick (Chebib et al., 2009).

The ρ1 receptor is structurally homologous to heteromeric $\alpha\beta\gamma$ and $\alpha\beta\delta$ $GABA_A$ receptors but exhibits some notable pharmacological differences (Naffaa et al., 2017). It is insensitive to the competitive antagonist bicuculline and is not activated or modulated by pentobarbital (Shimada et al., 1992). The ρ1 receptor is also insensitive to volatile anesthetics and the intravenous anesthetic propofol (Mihic and Harris, 1996).

Many neurosteroids modulate the ρ1 receptor, in which case the configuration of the steroid at C5 determines the type of the effect. The ρ1 receptor is potentiated by 5α-reduced steroids, such as allopregnanolone and allotetrahydrodeoxycorticosterone (5αTHDOC), but, unlike heteromeric $GABA_A$ receptors, is inhibited by 5β-reduced steroids, such as pregnanolone (3α5βP) (Morris et al., 1999; Goutman and Calvo, 2004). The ρ1 receptor is also inhibited by sulfated neurosteroids and the neurosteroid/sex hormone (8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthrene-3,17-diol (β-estradiol) (Li et al., 2007; Eaton et al., 2014). The abundance of neurosteroids in the brain, coupled with the structural diversity of synthetic steroid analogs, raises the prospect for development of steroid-based clinical agents targeting the ρ receptor family. One weakness of this approach, however, has been the relatively low apparent affinity of the ρ1 receptor to many neurosteroids (Li et al., 2007). Hence, it would be beneficial to explore ways to lower the effective concentrations.

In recent work examining the actions of combinations of allosteric potentiators on the heteromeric $GABA_A$ receptor, we showed that the magnitude of effect strongly depends on whether the paired potentiators act through the same or distinct binding sites (Shin et al., 2019). This raises a possibility that combinations of neurosteroids or steroid analogs can be identified for employment at practical doses to modulate the ρ1 receptor. Here, we have examined the actions of several inhibitory and potentiating steroids on the human ρ1 receptor. The data, analyzed and interpreted in the framework of the coagonist concerted transition model (Forman, 2012; Ehlert, 2014; Steinbach and Akk, 2019), indicate that the inhibitory steroids 3α5βP, 3α5βP sulfate (3α5βPS), and β-estradiol (structures shown in Fig. 1) act by binding to distinct, nonoverlapping binding sites to independently modulate receptor function. Interestingly, the potentiating steroid 5αTHDOC (Fig. 1) is shown to share a binding site with the inhibitory steroid 3α5βP, thereby presenting a case of divergent action for two steroids acting at the same site i.e., binding at overlapping sites elicits functionally opposite effects.

Materials and Methods

Receptors and Expression. The human wild-type (GenBank Accession No. M62400) and mutant (I307Q) ρ1 $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes. Harvesting of oocytes was conducted under the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. The animal protocol was approved by the Animal Studies Committee of Washington University in St. Louis (Approval No. 20170071).

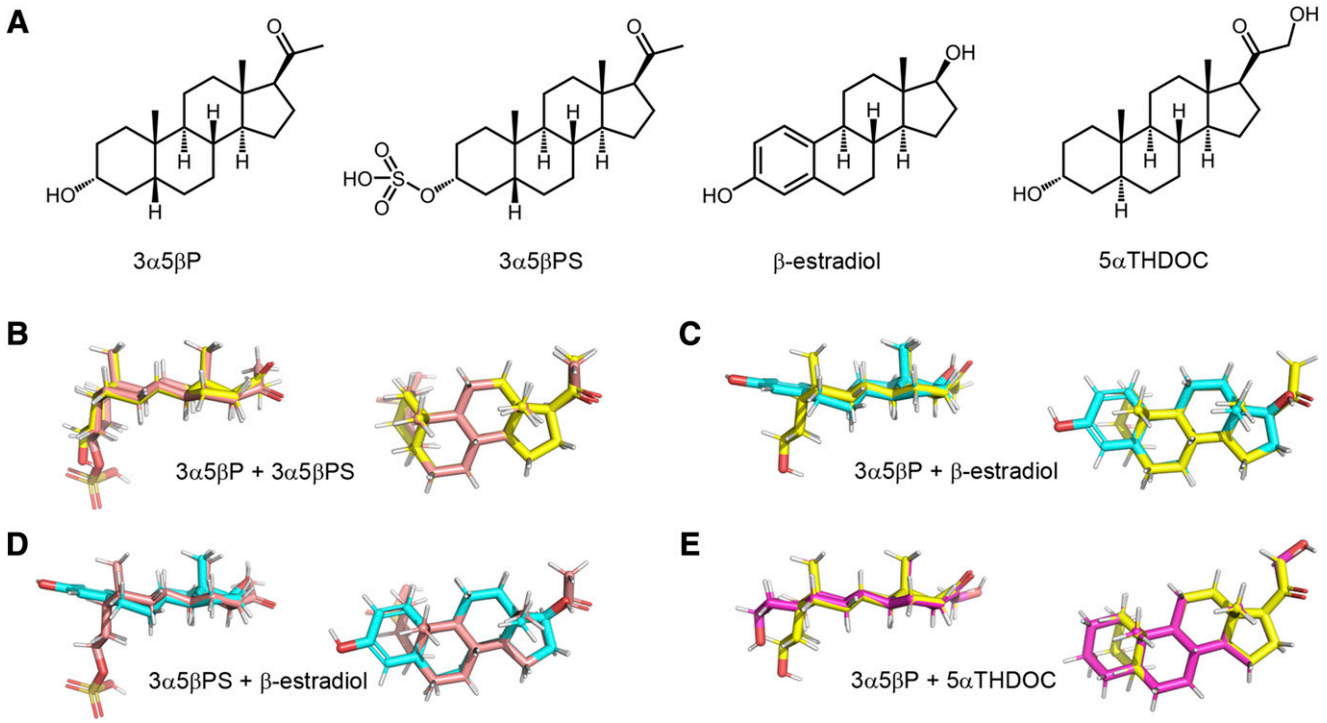

Fig. 1. Steroid structures. (A) Chemical structures of 3α5βP, 3α5βPS, β-estradiol, and 5αTHDOC. (B) Overlay of 3α5βP (yellow) and 3α5βPS (pink). (C) Overlay of 3α5βP (yellow) and β-estradiol (cyan). (D) Overlay of 3α5βPS (pink) and β-estradiol (cyan). (E) Overlay of 3α5βP (yellow) and 5αTHDOC (magenta). The structures show strong similarities, as expected since the B, C, and D rings are identical, and all the C17 substituents are in the β configuration. The major differences concern the orientation of the A ring (5α vs. 5β fusion vs. the flattened extension of the unsaturated A ring of β-estradiol) and the presence of a bulky charged substituent on the sulfated steroid.

The cDNA for the ρ1 subunit was subcloned into the pGEMHE expression vector in the T7 orientation and linearized with NheI (New England Biolabs, Ipswich, MA). The ρ1 (I307Q) mutation was generated using QuikChange (Agilent Technologies, Santa Clara, CA). The complementary RNA was synthesized using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with 5 ng of complementary RNA per oocyte and incubated at 15°C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) with supplements (2.5 mM Na pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin) for 1–3 days before conducting the electrophysiological recordings.

Electrophysiology. The electrophysiological recordings were conducted at room temperature using the standard two-electrode voltage-clamp technique. The oocytes were clamped at −60 mV. The chamber (RC-1Z; Warner Instruments, Hamden, CT) was perfused with ND96 at the rate of 5–8 ml/min. Bath and drugs were gravity-applied from glass containers via Teflon tubing to the recording chamber (RC-1Z; Warner Instruments) at the rate of 5–8 ml/min.

The current responses were amplified with Axoclamp 900A (Molecular Devices, Sunnyvale, CA) or OC-725C amplifiers (Warner Instruments), digitized with Digidata 1320 or 1200 series digitizers (Molecular Devices), and stored using Clampex (Molecular Devices). Analysis of the current traces was done using Clampfit (Molecular Devices).

The GABA concentration-response relationship was determined by exposing the oocytes to 0.1–10 μM GABA (seven concentration points). Constitutive activity was measured by comparing the effect of 100 μM picrotoxin to the peak response to saturating GABA. The effects of steroids were determined by exposing an oocyte to a low concentration (0.2–0.8 μM) of GABA for 1.5–3 minutes, followed by GABA + steroid (another 2 to 3 minutes), and washout in ND96. No desensitization of the current was observed during the application of low GABA. Each cell was also exposed to a reference solution containing a saturating concentration (10 μM) of GABA. Because of slow washout of the steroids, the steroid concentration-response relationships were determined by exposing each oocyte to a single concentration of steroid. The maximal steroid concentration was 50 μM because of limitations imposed by solubility in aqueous solution.

All experiments were conducted in an exploratory manner. The minimum number of replicates (i.e., cells tested per experimental condition) was five. The sample size was not set before data collection. All experimental observations are included (i.e., no data were excluded).

Data Analysis. Descriptive analysis of current responses to GABA was aimed at determining the peak current amplitude. Initial characterization of the data was done by fitting the Hill equation to the GABA concentration-response data.

In the second step, the raw amplitudes of current traces were converted to units of open probability (P_{open}) by comparing a response amplitude to the response to saturating (10 μM) GABA in the same cell (Eaton et al., 2016). No adjustment for constitutive activity was done because of its negligible value ($P_{open,\,const} = 0.0011$; see below). No potentiation of the response to saturating GABA was observed during coapplication with the potentiating steroid 5αTHDOC; accordingly, the response to 10 μM GABA had a P_{open} experimentally indistinguishable from one. The GABA concentration-P_{open} data were fitted to eq. 1:

$$P_{open} = \frac{1}{1 + L \times \left[\frac{1+[GABA]/K_{C,GABA}}{1+[GABA]/(K_{C,GABA}c_{GABA})}\right]^{N_{GABA}}} \quad (1)$$

in which [GABA] is the concentration of GABA, $K_{C,GABA}$ is the equilibrium dissociation constant for the transmitter in the closed receptor, and c_{GABA} is the ratio of the equilibrium dissociation constant for GABA in the open receptor to $K_{C,GABA}$. N_{GABA}, the number of transmitter binding sites, was constrained to five based on the 5-fold symmetry of the homomeric ρ1 receptor. The parameter L is a measure of unliganded gating that was calculated from the experimentally determined constitutive open probability ($P_{open,\,const}$) as:

$$L = (1\text{-}P_{open,\,const})/P_{open,\,const} \quad (2)$$

The effects of steroids on GABA-activated receptors were analyzed in the framework of the coagonist concerted transition model to estimate the affinities of the closed and open receptors to the steroid (Forman, 2012; Akk et al., 2018). The experimental concentration-response relationships were fitted to eq. 3:

$$P_{open} = \frac{1}{1 + L_{+GABA} \times \left[\frac{1+[steroid]/K_{C,steroid}}{1+[steroid]/(K_{C,steroid}c_{steroid})}\right]^{N_{steroid}}} \quad (3)$$

in which [steroid] denotes the concentration of steroid, $K_{C,steroid}$ is the equilibrium dissociation constant for the steroid in the closed receptor, and $c_{steroid}$ is the ratio of the equilibrium dissociation constant for steroid in the open receptor to $K_{C,steroid}$. The number of steroid binding sites ($N_{steroid}$) was constrained to five for all steroids. The parameter L_{+GABA} is a measure of background activity in the presence of GABA calculated from experimental data as $(1-P_{open,+GABA})/P_{open,+GABA}$. The K and c values are given as best-fit parameter ± S.E. of the fit from the analysis of averaged data from at least five cells. Curve fitting was carried out using Origin v. 7.5 (OriginLab, Northhampton, MA).

Studies of the effects of two simultaneously applied steroids were aimed at comparing the experimental observations with simulations based on two models. In the first model, in which the paired steroids interact with distinct, nonoverlapping sites, the effect of one steroid was considered to modify the value of L_{+GABA} as follows:

$$L_{+GABA+steroid\ 1} = (1\text{-}P_{open,\ +GABA+steroid\ 1})/P_{open,\ +GABA+steroid\ 1} \quad (4)$$

in which $L_{+GABA+steroid\ 1}$ is the modified L_{+GABA} in the presence of GABA and steroid 1, and $P_{open,\ +GABA\ +\ steroid\ 1}$ is the open probability in the presence of GABA and that steroid. The predicted P_{open} for the steroid combination was then calculated with eq. 3 using the $K_{C,steroid\ 2}$ and $c_{steroid\ 2}$ values determined in the absence of steroid 1 and $L_{+GABA+steroid\ 1}$ substituting for L_{+GABA}. In this simulation, it is not critical which of the two paired steroids is considered steroid 1 that modifies L_{+GABA} and which is considered steroid 2 whose effect at modified L_{+GABA} (i.e., $L_{+GABA+steroid}$) is examined. Switching the designation of modulators produces identical results (Shin et al., 2019).

In the second model, we assumed that the two steroids compete for binding to the same site. In this case, the predicted P_{open} was calculated using eq. 5:

$$P_{open} = \frac{1}{1 + L_{+GABA} \times \left[\frac{1+[steroid\ 1]/K_{C,steroid\ 1}+[steroid\ 2]/K_{C,steroid2}}{1+[steroid\ 1]/(K_{C,steroid\ 1}c_{steroid\ 1})+[steroid\ 2]/(K_{C,steroid\ 2}c_{steroid\ 2})}\right]^{N}} \quad (5)$$

in which steroid 1 and steroid 2 are the paired steroids, N is the number of shared sites (constrained to five), $K_{C,steroid\ 1}$ and $K_{C,steroid\ 2}$ are the equilibrium dissociation constants for steroid 1 and steroid 2 in the closed receptor, and $c_{steroid\ 1}$ and $c_{steroid\ 2}$ are the ratios of the equilibrium dissociation constants for the two steroids in the open receptor to $K_{C,steroid\ 1}$ and $K_{C,steroid\ 2}$, respectively. The equation can be expanded by adding more terms to the denominator to analyze the combined effect of more than two steroids. Note that in both of these models, the functional parameters for a given steroid were determined in the absence of other steroids.

The results of modeling were compared by first calculating the difference in second-order Akaike information criterion scores of the two models:

$$\Delta = \mathrm{n}\ln\left(\frac{\mathrm{RSS}_{\mathrm{Model\ 1}}}{\mathrm{n}}\right) - \mathrm{n}\ln\left(\frac{\mathrm{RSS}_{\mathrm{Model\ 2}}}{\mathrm{n}}\right) \quad (6)$$

in which $\mathrm{RSS}_{\mathrm{Model\ 1}}$ and $\mathrm{RSS}_{\mathrm{Model\ 2}}$ are the residual sums of squares for the two models showing larger and smaller deviations from experimental data, respectively, and n is the number of cells. This was followed by determining Akaike weights for each model (w), which indicate the probability that a given model is the better model (Wagenmakers and Farrell, 2004; Burnham et al., 2011):

$$w_{\mathrm{Model\ 1}} = \frac{\exp\left[-\frac{1}{2}\Delta\right]}{\exp\left[-\frac{1}{2}\Delta\right] + 1} \quad (7)$$

in which $w_{\mathrm{Model\ 1}}$ is the probability that model 1 is the best model describing data. The probability of model 2 was then calculated as $1-w_{\mathrm{Model\ 1}}$. The calculated w values rank the two chosen models without providing specific insight into an absolute best model.

Homology Modeling and Molecular Docking. Because there are no known structures of the ρ1 $\mathrm{GABA_A}$ receptor, it was necessary to develop a homology-based model of the receptor. We used the $\mathrm{GABA_A}$ β3 homopentamer structure [PDB ID: 4COF; (Miller and Aricescu, 2014)] and the structure of the chimeric GLIC-α1 receptor bound with 5αTHDOC [PDB ID: 5OSB; (Laverty et al., 2017)] as templates. The sequence of the ρ1 subunit was modified by truncating the N terminus by removing residues 1–59 because none of the chosen templates had structural information for this region. The next modification was the replacement of the cytoplasmic loop (residues 371–453) with the sequence SQPARA (Jansen et al., 2008). Both of the template structures used had also replaced the cytoplasmic loop with this sequence. The ρ1 sequence was then aligned to the β3 and GLIC-α1 sequences using MUSCLE (Edgar, 2004). The sequence alignment of a single subunit is given in Supplemental Fig. 1. This alignment was used as the structural input for Modeler version 9 (Sali and Blundell, 1993). A total of 100 models were built and ranked by discrete optimized protein energy score (Shen and Sali, 2006). The top-ranked model (Supplemental Fig. 2) was deemed acceptable using the MolProbity server (Chen et al., 2010) and used for subsequent docking studies.

The model of the ρ1 $\mathrm{GABA_A}$ receptor was read into the program Chimera (Meng et al., 2006) in which the structure was aligned with the 5αTHDOC-bound (PDB ID: 5OSB) and pregnenolone sulfate–bound structures [PDB ID: 5OSC; (Laverty et al., 2017)]. The bound neurosteroids in these structures were used to define the center of a docking box on each subunit (Supplemental Table 1). The docking boxes were set to be 20 × 20 × 20 Å allowing a large search volume. The intersubunit site (Hosie et al., 2006) was defined by the centroid of the bound 5αTHDOC molecules. The intrasubunit site near the top of the third and fourth membrane-spanning domains (Hosie et al., 2006; Chen et al., 2019) was defined as a centroid defined by residues A322, V329 on TM3 and F442, and I449 on TM4 (numbering in mature peptide). Lastly, the intrasubunit site for pregnenolone sulfate (Laverty et al., 2017) was defined as the centroid of the bound pregnenolone sulfates. The four neurosteroids were individually docked into the various sites using AutoDock Vina (Trott and Olson, 2010). Docking scores are provided in the text. The best-scored docking poses of the four steroids in each of the three sites are given in Supplemental Figs. 3–5.

Superimposition of Neurosteroids. Each of the four neurosteroids was aligned to a model of β-estradiol using the "pair alignment" function in the program PyMOL version 2.2.3 (Schrodinger, LLC). As all four neurosteroids share a common steroid backbone, 3α5βP, 3α5βPS, and 5αTHDOC were initially aligned to β-estradiol. By aligning the six ring fusion carbons of β-estradiol to the corresponding carbons in the other steroids, the alignment function superimposes the steroid backbone as closely as possible given the different conformations of the A rings. The use of β-estradiol as the common reference automatically aligns the other steroids into a common orientation, as seen in Fig. 1.

Materials, Drugs, and Solutions. The inorganic salts used in ND96, GABA, and picrotoxin were purchased from Sigma-Aldrich (St. Louis, MO). The steroids (3α5βP, 3α5βPS, β-estradiol, and 5α-THDOC) were bought from Sigma-Aldrich or Steraloids (Newport, RI). The stock solution of GABA was made in ND96 at 500 mM, stored in aliquots at −20°C, and diluted as needed on the day of experiments. The steroids were dissolved in DMSO at 10–50 mM and stored at room temperature (3α5βP, β-estradiol, 5αTHDOC) or at 5°C (3α5βPS).

Results

Analysis of the ρ1 Receptor Activation by GABA. We recorded the GABA concentration-response relationship by exposing oocytes expressing human ρ1 receptors to 0.1–10 μM GABA. Fitting the Hill equation to the concentration-response data yielded an $\mathrm{EC_{50}}$ of 0.82 ± 0.09 μM (mean ± S.D.; n = 5 cells) and a Hill coefficent of 2.85 ± 0.45. These are similar to the values reported previously (Amin and Weiss, 1996; Chang and Weiss, 1998; Li et al., 2006). Sample currents and the GABA concentration-response curve are given in Fig. 2.

Next, we converted the current amplitudes to units of open probability ($\mathrm{P_{open}}$). To that end, the current amplitudes were matched with estimated $\mathrm{P_{open}}$ of constitutive activity and that of the peak response to saturating GABA (Eaton et al., 2016). To evaluate the level of constitutive open probability, we compared the effects of 100 μM picrotoxin, which inhibits constitutively active receptors, and the saturating concentration (10 μM) of GABA. In 14 cells, picrotoxin elicited apparent outward current with the mean amplitude of 0.106% ± 0.037% of the peak response to saturating GABA. To estimate the open probability elicited by saturating GABA, we probed the ability of the potentiating steroid 5αTHDOC (Morris et al., 1999; Li et al., 2007) to potentiate the response to the transmitter. In this approach, it is assumed that coapplication of a potentiator with saturating GABA elicits a response with $\mathrm{P_{open}}$ near one that enables, by comparison of the peak responses, determination of the $\mathrm{P_{open}}$ of response to GABA in the absence of potentiator (Forman, 2012; Eaton et al., 2016). In five cells, no enhancement of the current response was observed when 25–50 μM 5αTHDOC was coapplied with saturating GABA. Furthermore, coapplication of 1 mM amiloride hydrochloride hydrate, a known potentiator of the ρ1 receptor (Snell and Gonzales, 2015), also did not enhance the peak or steady-state responses to saturating GABA. These observations suggest that the $\mathrm{P_{open}}$ of the ρ1 receptor in the presence of saturating GABA is high and experimentally indistinguishable from one. We thus estimated that the $\mathrm{P_{open}}$ of constitutive activity was 0.0011 and, using eq. 2, calculated a value for L of 1018 ± 244 (n = 14).

To gain further insight into the activation properties of the ρ1 receptor, we normalized the current responses to the peak response to 10 μM GABA. The $\mathrm{P_{open}}$ in the presence of 10 μM GABA was constrained to values between 0.92 and 0.999, and the concentration-$\mathrm{P_{open}}$ relationships were fitted with eq. 1. Essentially, we reasoned that a small (less than 8%) potentiating effect of 5αTHDOC might be masked by experimental imprecision and that the true $\mathrm{P_{open}}$ of the ρ1 receptor in the presence of 10 μM GABA falls within the range of 0.92–0.999.

The fitting results, summarized in Table 1, provide a plausible range of $\mathrm{K_{GABA}}$ (affinity of the resting receptor to GABA) and c_{GABA} values (a measure of GABA efficacy) for the human ρ1 receptor. The estimates for $\mathrm{K_{GABA}}$ ranged from 1.1 μM

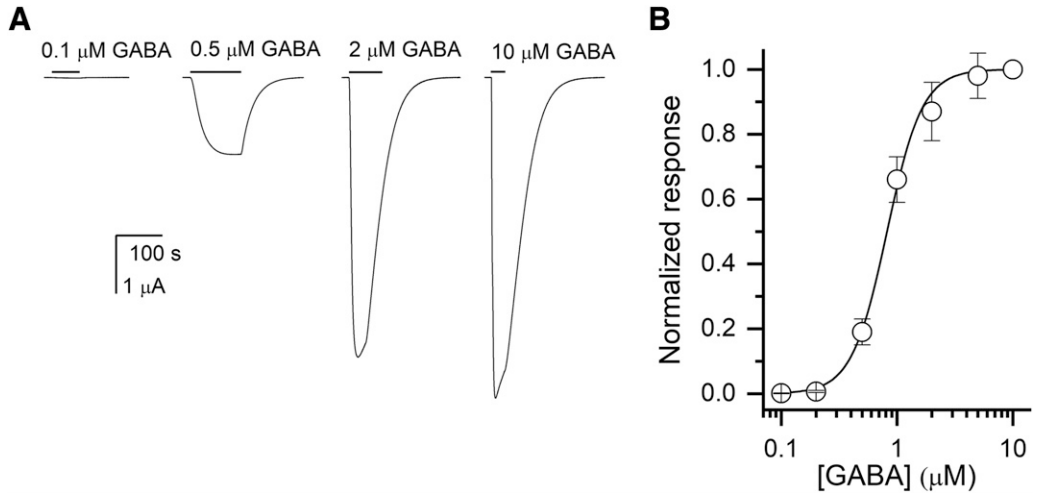

Fig. 2. Activation of the ρ1 wild-type receptor by GABA. (A) Sample current responses to applications of 0.1, 0.5, 2, or 10 μM GABA. The bars indicate the durations of applications of GABA. The applications of GABA were followed by 2–4 minutes washes in ND96. (B) The GABA concentration-response relationship from oocytes exposed to 0.1–10 μM GABA. The current amplitudes were normalized to the response to 10 μM in the same cell. The data points show mean ± S.D. from five cells. The concentration-response data from each cell were fitted separately yielding an EC_{50} of 0.82 ± 0.09 μM (mean ± S.D.) and an n_{Hill} of 2.85 ± 0.45. The curve shows a calculated concentration-response relationship based on the mean EC_{50} and n_{Hill}.

(with the P_{open} of the peak response to 10 μM GABA constrained to 0.92) to 2.1 μM (with P_{open} fixed at 0.999). The estimated c_{GABA} ranged from 0.085 to 0.128, corresponding to the binding of five GABA molecules providing 6.1–7.3 kcal/mol toward stabilization of the open state. In each case, the value of L was adjusted to take into consideration the altered ratio of the response to picrotoxin to the hypothetical response with P_{open} of 1.

In subsequent experiments examining the effects of steroids on responses to a low concentration of GABA, the peak response to 10 μM GABA was used as the "reference" response, which was assumed to have an open probability of one. This introduced a potential error in the estimates of P_{open} in these experiments. The extent of the error was established by assigning different values of P_{open} to the peak response to saturating GABA (see below).

Receptor Inhibition by the Steroids 3α5βP, 3α5βPS, and β-Estradiol. Inhibition of the ρ1 $GABA_A$ receptor by neurosteroids has been reported previously (Morris et al., 1999; Li et al., 2007; Eaton et al., 2014). Here, we analyzed the inhibitory effects of the steroids 3α5βP, 3α5βPS, and β-estradiol on the ρ1 receptor in the framework of the coagonist concerted transition model. Current responses were recorded in the presence of 0.4–0.8 μM GABA (P_{open} ~0.3) in the absence and presence of 0.1–50 μM steroid. Additionally, each cell was probed by application of a saturating concentration (10 μM) of GABA, initially assumed to generate a peak response with P_{open} of 0.999 (see *Materials and Methods* for more details). Analysis of the currents using eq. 3 yielded a $K_{3\alpha5\beta P}$ of 2.85 ± 0.62 μM and a $c_{3\alpha5\beta P}$ of 1.25 ± 0.02, a $K_{3\alpha5\beta PS}$ of 51.1 ± 25.3 μM and a $c_{3\alpha5\beta PS}$ of 2.34 ± 0.89, and a $K_{\beta\text{-Estradiol}}$ of 16.4 ± 4.8 μM and a $c_{\beta\text{-Estradiol}}$ of 1.47 ± 0.07. The number of binding sites was held at five for each steroid. The data and the fitted curves are shown in Fig. 3. We note that incomplete inhibition, as predicted by eq. 3, has been observed previously for the ρ1 receptor exposed to 3α5βP (Morris et al., 1999; Li et al., 2007).

The inhibitory properties of the steroids were also determined by assuming that the response to 10 μM GABA had a peak P_{open} of 0.92. This was done to estimate the extent of potential error introduced by initially assigning a P_{open} of 0.999 to the reference response. The estimated values of $K_{steroid}$ and $c_{steroid}$ then were 2.44 ± 0.43 μM and 1.23 ± 0.02, respectively, for 3α5βP, 66.5 ± 37.6 μM and 3.06 ± 2.24 for 3α5βPS, and 14.8 ± 4.2 μM and 1.42 ± 0.06 for β-estradiol. We infer that the precise P_{open} of the reference response has an acceptably small effect (nominally up to 30%) on the estimated properties of the steroids.

The value of the parameter c, which characterizes the degree to which the steroid prefers a closed receptor over the open receptor, ranged from 1.25 for 3α5βP to 2.34 for 3α5βPS. Thus, the binding of steroid contributes 0.7–2.5 kcal/mol free energy toward stabilizing the closed state.

TABLE 1

Analysis of activation of the ρ1 receptor by GABA

The table gives the results of fitting the GABA conc.-P_{open} response data to eq. 1. The first column shows the constrained value of P_{open} at 10 μM GABA. The next columns show the calculated value of L and the fitted values of K_C and c (best-fit parameter ± S.D.). The number of binding sites for GABA was constrained to 5.

P_{open} at 10 μM GABA	L	K_C (μM)	c
0.999	1018	2.11 ± 0.46	0.085 ± 0.012
0.98	1039	1.73 ± 0.33	0.097 ± 0.011
0.96	1060	1.44 ± 0.26	0.110 ± 0.010
0.94	1083	1.24 ± 0.21	0.119 ± 0.010
0.92	1106	1.09 ± 0.19	0.128 ± 0.009

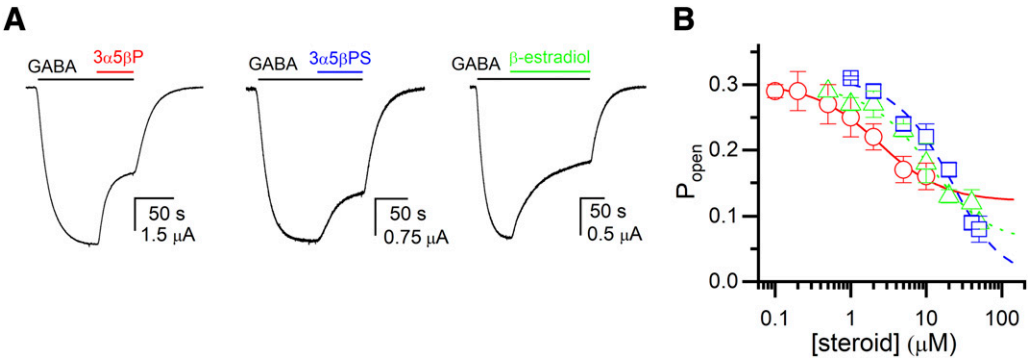

Fig. 3. Inhibition of the ρ1 wild-type receptor by neurosteroids. (A) Sample current responses to 0.4–0.8 μM GABA (P_{open} ~0.3) followed by a coappplication of GABA + 10 μM 3α5βP, 3α5βPS, or β-estradiol. The bars above the traces indicate the durations of applications of GABA and the steroids. (B) The graph shows the steroid concentration-P_{open} relationships for 3α5βP (circles and solid line), 3α5βPS (squares and dashed line) or β-estradiol (triangles and dotted line). The data points show mean ± S.D. from five to seven cells at each experimental condition. The curves were generated by fitting eq. 2 to the pooled data. The best-fit parameters for 3α5βP are: K_C = 2.85 ± 0.62 μM, c = 1.25 ± 0.02. The best-fit parameters for 3α5βPS are: K_C = 51.1 ± 25.3 μM, c = 2.34 ± 0.89. The best-fit parameters for β-estradiol are: K_C = 16.4 ± 4.8 μM, c = 1.47 ± 0.07.

The Effects of Coapplication of Multiple Inhibitory Steroids. A prior study comparing the effects of mutations in the second membrane-spanning domain on the ability of steroids to inhibit the ρ1 receptor found that the mutations differently affected inhibition by various steroids. Specifically, it was proposed that the steroids 3α5βP, 3α5βPS and β-estradiol interact with distinct, nonoverlapping sites (Li et al., 2007). This was indirectly confirmed by Eaton et al. (2014), who showed that these steroids elicit unique conformational changes in the extracellular domain of the receptor. Here, we employed the coagonist concerted transition model to investigate overlap between the steroid binding sites. To that end, we coapplied two or more inhibitory steroids and measured the net effect of the steroid combination on the amplitude of current generated by a low concentration of GABA (P_{open} ~0.35). The experimental observations were compared with the predicted open probabilities calculated using models in which the paired steroids interact to produce a larger functional effect, which we interpret as reflecting independent binding to sites that are physically distinct (eqs. 3 and 4), or interact to produce a smaller functional effect, which we interpret as reflecting mutual prevention of simultaneous binding to a shared, overlapping site (eq. 5).

Coapplication of 3α5βP and 3α5βPS strongly inhibited the response to GABA. In seven cells, the application of 0.5–0.6 μM GABA elicited a response with P_{open} of 0.31 ± 0.09. Coapplication of 10 μM 3α5βP and 20 μM 3α5βPS with GABA reduced the P_{open} of the response to 0.08 ± 0.03. The predicted open probability of the response, assuming five shared sites for the two steroids, was 0.14 ± 0.05, whereas the predicted P_{open}, assuming that the steroids act through nonoverlapping sites, was 0.08 ± 0.03. Sample current traces along with the predicted current levels for the two models are shown in Fig. 4A.

To provide quantitative insight into the goodness of fit for the two models, we calculated the Akaike weights for the shared site and the distinct site models. The Akaike weight (w) for a particular model expresses the probability or likelihood of the model among those considered (Wagenmakers and Farrell, 2004; Burnham et al., 2011). A value of w closer to one supports the idea that a given model gives a better description of the data. For the 3α5βP + 3α5βPS pair, the $w_{\text{shared sites}}$ was $<10^{-11}$ and $w_{\text{distinct sites}}$ was $1–10^{-11}$. We infer that a model with distinct sites for 3α5βP and 3α5βPS is better supported by experimental observations.

Next, we examined the combined effect of 3α5βP and β-estradiol (Fig. 4B). Coapplication of 10 μM 3α5βP and 20 μM β-estradiol inhibited the response to GABA. The P_{open} was 0.37 ± 0.07 (n = 7 cells) in the absence of the two steroids and 0.09 ± 0.02 in the presence of the two steroids. The predicted P_{open}, assuming the steroids acted through same sites, was 0.17 ± 0.05 (w ~10^{-9}) and different sites 0.09 ± 0.03 (w = $1–10^{-9}$).

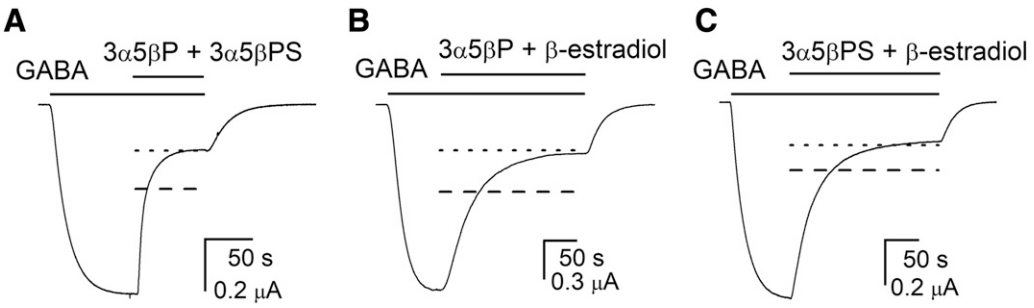

Fig. 4. Inhibition of the ρ1 wild-type receptor by combinations of neurosteroids. Sample current responses to 0.4–0.8 μM GABA (P_{open} ~0.3) followed by a coappplication of GABA + 10 μM 3α5βP + 20 μM 3α5βPS (A), GABA + 10 μM 3α5βP + 20 μM β-estradiol (B), or GABA + 20 μM 3α5βPS + 20 μM β-estradiol (C). The bars above the traces indicate the durations of applications of the drugs. The dashed and dotted lines show theoretical current levels simulated by the models assuming same or distinct binding sites, respectively, for the paired steroids.

Combination of 20 μM 3α5βPS and 20 μM β-estradiol reduced the open probability of the response to GABA from 0.35 ± 0.09 (n = 15 cells) to 0.08 ± 0.02 (Fig. 4C). Assuming that 3α5βPS and β-estradiol act through the same site, the predicted open probability in the presence of GABA and the two steroids was 0.13 ± 0.04 ($w < 10^{-10}$). The predicted open probability predicted by the model with different sites for the two steroids was 0.08 ± 0.02 ($w = 1–10^{-10}$).

Finally, we coapplied the triple combination of 5 μM 3α5βP, 10 μM 3α5βPS, and 10 μM β-estradiol with GABA. The P_{open} of the control response to GABA alone was 0.37 ± 0.02 (n = 6 cells). In the presence of GABA plus the three steroids, the open probability was 0.071 ± 0.010. The calculated P_{open} for the model with the same set of five binding sites mediating the effects of the three steroids was 0.17 ± 0.01 ($w \sim 10^{-9}$). Assuming different binding sites for each steroid, the calculated P_{open} was 0.086 ± 0.007 ($w = 1–10^{-9}$).

We infer from these experiments that 3α5βP, 3α5βPS, and β-estradiol inhibit the ρ1 receptor through interactions with distinct, nonoverlapping binding sites. These findings are in agreement with previous studies employing mutational and fluorometric approaches (Li et al., 2007; Eaton et al., 2014).

Receptor Potentiation by 5αTHDOC and the Effects of Inhibitory Steroids on Potentiation. The ρ1 receptor is potentiated by 5α-reduced steroids (Morris et al., 1999). Here, we analyzed the potentiating effect of the steroid 5αTHDOC by coapplying 1–35 μM steroid with 0.2 μM GABA (P_{open} of 0.04–0.08). Analysis of the currents (five to six cells at each steroid concentration) using eq. 1 yielded a $K_{5\alpha THDOC}$ of 38.2 ± 28.6 μM and a $c_{5\alpha THDOC}$ of 0.58 ± 0.07. The number of steroid binding sites was constrained to five. A sample current trace and the steroid concentration-response curve are given in Fig. 5, A and B.

Examination of the effect on receptor function resulting from coapplication of 5αTHDOC with an inhibitory steroid can be used to determine whether the paired steroids interact with the same or distinct sites. The approach is analogous to that described above for combinations of inhibitory steroids in which the difference in predicted P_{open} values from the two models compared with experimental results enabled determination of the better model. For combinations of 5αTHDOC plus an inhibitory steroid, a reasonable separation between the two predicted P_{open} values was obtained only for 3α5βP, whereas for the 5αTHDOC + 3α5βPS and 5αTHDOC + β-estradiol combinations the two models predicted experimentally indistinguishable P_{open} values at steroid concentrations less than 50 μM.

Coapplication of the combination of 20 μM 5αTHDOC + 10 μM 3α5βP with 0.35 μM GABA decreased the steady-state P_{open} from 0.11 ± 0.03 to 0.09 ± 0.03 (n = 8 cells). The predicted P_{open}, assuming that different binding sites mediate the actions of the two steroids, was 0.14 ± 0.04 ($w = 10^{-8}$) whereas the predicted P_{open} from the same site model was 0.09 ± 0.03 ($w = 1–10^{-8}$). We infer that 3α5βP and 5αTHDOC act through overlapping sites.

A sample trace showing the effect of 5αTHDOC + 3α5βP is shown in Fig. 5C. Note that the steroid effect is biphasic: a rapid inhibition is followed by a slow increase in current level. We propose that the initial inhibition reflects the more rapidly developing effect of 3α5βP (e.g., as apparent in Fig. 3B), whereas the second, recovery phase represents the more slowly developing potentiation by 5αTHDOC (e.g., Fig. 5A).

Effect of the I307Q Mutation on Receptor Modulation by Potentiating and Inhibitory Steroids. The isoleucine-to-glutamine mutation at position 307 (15′ residue in the second membrane-spanning domain) in the ρ1 receptor switches the effect of 3α5βP from inhibition to potentiation (Morris and Amin, 2004; Eaton et al., 2014). The underlying mechanism for this switch is not clear. One possibility is that the mutation modifies the nature of the postulated site for 3α5βP and/or steroid interactions with the site. Alternatively, the I307Q mutation may unmask a novel site that mediates potentiation by 3α5βP, potentially by abolishing the actions of 3α5βP at the conventional inhibitory site. To attempt to distinguish between these possibilities, we examined modulation of the ρ1(I307Q) receptor by 3α5βP, 5αTHDOC, and the combination of the two steroids. We reasoned that if the mutation converts the existing site to potentiating for 3α5βP, then receptor behavior in the presence of 3α5βP + 5αTHDOC

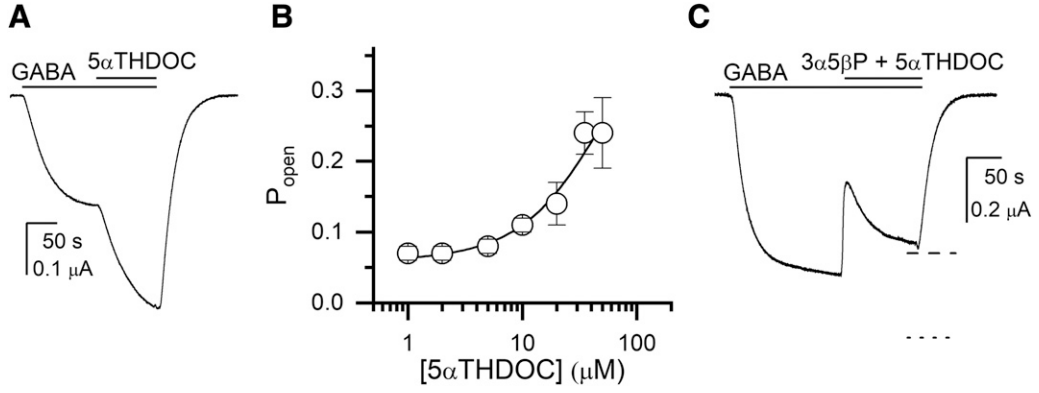

Fig. 5. Potentiation of the ρ1 wild-type receptor by 5αTHDOC in the absence and presence of the inhibitory steroid 3α5βP. (A) A sample current response to 0.2 μM GABA (P_{open} = 0.05) followed by a coappplication of GABA + 20 μM 5αTHDOC. The bars above the traces indicate the durations of applications of the drugs. (B) The graph shows the 5αTHDOC concentration-P_{open} relationship. The data points show mean ± S.D. from five to six cells at each experimental condition. The curve was generated by fitting eq. 2 to the pooled data. The best-fit parameters are: $K_{C,5\alpha THDOC}$ = 38.2 ± 28.6 μM, $c_{5\alpha THDOC}$ = 0.58 ± 0.07. (C) A sample current response to 0.35 μM GABA (P_{open} = 0.10) followed by a coappplication of GABA + 20 μM 5αTHDOC + 10 μM 3α5βP. The biphasic effect upon the application of steroids is likely due to a rapidly developing inhibitory effect of 3α5βP followed by a more slowly developing potentiating effect of 5αTHDOC. The dashed and dotted lines show theoretical current levels simulated by the models assuming same or distinct binding sites, respectively, for 5αTHDOC and 3α5βP.

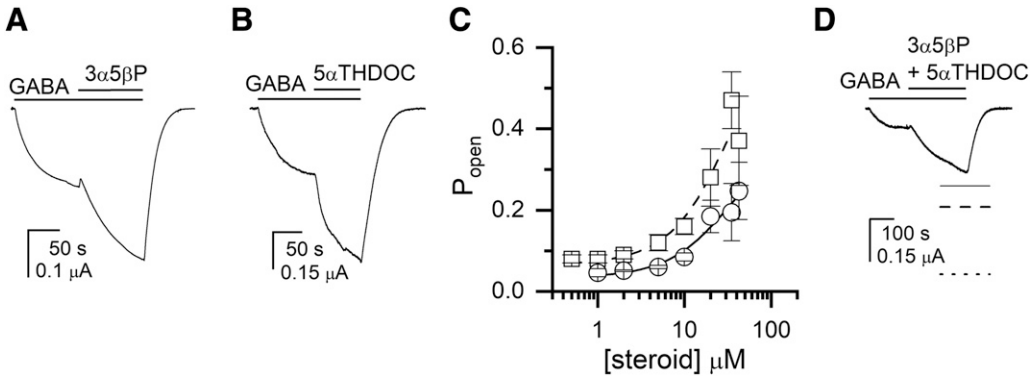

Fig. 6. Potentiation of the ρ1(I307Q) receptor by 3α5βP, 5αTHDOC, and the combination of the two steroids. (A) A sample current response to 0.11 μM GABA (P_{open} = 0.05) followed by a coappplication of GABA + 10 μM 3α5βP. The bars above the traces indicate the durations of applications of the drugs. (B) A sample current response to 0.2 μM GABA (P_{open} = 0.1) followed by a coappplication of GABA + 10 μM 5αTHDOC. (C) The graph shows the steroid concentration-P_{open} relationships for 3α5βP (circles and solid line) and 5αTHDOC (squares and dashed line). The data points show mean ± S.D. from five to six cells at each experimental condition. The curves were generated by fitting eq. 2 to the pooled data. The best-fit parameters for 3α5βP are: K_C = 20.5 ± 15.1 μM, c = 0.55 ± 0.05. The best-fit parameters for 5αTHDOC are: K_C = 28.6 ± 26.1 μM, c = 0.49 ± 0.08. (D) A sample current response to 0.15 μM GABA (P_{open} = 0.03) followed by a coappplication of GABA + 10 μM 3α5βP + 10 μM 5αTHDOC. The dashed and dotted lines show theoretical current levels simulated by the models assuming same or distinct binding sites, respectively, for 5αTHDOC and 3α5βP. The solid line shows the estimated steady-state response level in the presence of GABA + 3α5βP + 5αTHDOC from the fit of a single exponential function.

will continue to be described by a model in which the steroids compete for a shared site (eq. 5).

Coapplication of 3α5βP with 0.11–0.15 μM GABA (P_{open} ~0.04) resulted in potentiation of the current response (Fig. 6A). Analysis of the responses using eq. 2 yielded a $K_{3\alpha5\beta P}$ of 20.5 ± 15.1 μM and a $c_{3\alpha5\beta P}$ of 0.55 ± 0.05. Coapplication of 5αTHDOC with 0.1–0.2 μM GABA (P_{open} ~0.07) also potentiated the response to GABA (Fig. 6B). Curve fitting of the concentration-P_{open} data yielded a $K_{5\alpha THDOC}$ of 28.6 ± 26.1 μM and a $c_{5\alpha THDOC}$ of 0.49 ± 0.08. These values are similar to the K and c in the wild-type receptor, indicating that the mutation minimally affects potentiation by 5αTHDOC. The concentration-P_{open} relationships are shown in Fig. 6C.

Coapplication of the combination of 10 μM 3α5βP + 10 μM 5αTHDOC potentiated the response to GABA (Fig. 5D). The P_{open} was 0.05 ± 0.03 (n = 7 cells) in the presence of GABA and 0.14 ± 0.07 in the presence of GABA plus the two steroids. The predicted P_{open}, assuming that 3α5βP and 5αTHDOC bind to different sites, was 0.33 ± 0.16 (w = 0.00002). The predicted P_{open} for the same site model was 0.21 ± 0.12 (w = 0.99998). We infer that 3α5βP and 5αTHDOC interact with overlapping sites and propose that the I307Q mutation alters the direction of effect of 3α5βP rather than generating a new binding site for the steroid. This idea is also supported by the finding that the I307S substitution enables potentiation of the ρ1 receptor by the structurally unrelated pentobarbital (Belelli et al., 1999).

Homology Modeling and Docking of Steroids in Putative Binding Sites. Two binding sites for neurosteroids have been identified in bacterial-$GABA_A$ chimeric subunits: an intersubunit site between the first and third membrane-spanning domains of neighboring subunits for potentiating steroids, such as 5αTHDOC and 3α5βP, and an intrasubunit site lined by the third and fourth membrane-spanning domains for the inhibitory steroid pregnenolone sulfate (Laverty et al., 2017; Miller et al., 2017). In the heteromeric α1β3 receptor, analogs of potentiating steroids additionally label intrasubunit sites near the interface between membrane-spanning and extracellular domains in the α1 and β3 subunits (Chen et al., 2019). It is conceivable that homologous sites in the ρ1 receptor mediate the modulatory effects of the tested steroids.

We generated a homology model of the ρ1 receptor based on the published structures of β3 and GLIC-α1 homomeric structures (Miller and Aricescu, 2014; Laverty et al., 2017) and docked 3α5βP, 3α5βPS, β-estradiol, and 5αTHDOC in each of the three putative binding sites for steroids. The docking scores, determined using AutoDock Vina software, indicate only small (up to 1 kcal/mol) differences for the four steroids in each of the binding sites. At the intersubunit site (Hosie et al., 2006), the ranking of docking scores was: 3α5βPS (−8.6 kcal/mol) > 3α5βP (−8.2 kcal/mol) > β-estradiol (−7.8 kcal/mol) > 5αTHDOC (−7.6 kcal/mol). At the intrasubunit site near the interface between membrane-spanning and extracellular domains (Hosie et al., 2006; Chen et al., 2019), the docking scores were: 3α5βP (−7.1 kcal/mol) > β-estradiol (−6.9 kcal/mol) > 3α5βPS (−6.8 kcal/mol) > 5αTHDOC (−6.6 kcal/mol). At the intrasubunit site originally identified for the inhibitory steroid pregnenolone sulfate (Laverty et al., 2017), the docking scores were: β-estradiol (−7.7 kcal/mol) > 3α5βP (−7.4 kcal/mol) > 3α5βPS (−7.0 kcal/mol) > 5αTHDOC (−6.9 kcal/mol). The structures of binding sites with docked steroids are shown in Fig. 7.

Discussion

We implemented the coagonist concerted transition model to analyze the activation of the human ρ1 $GABA_A$ receptor by the transmitter GABA and modulation of GABA-activated receptors by structurally related inhibitory and potentiating neurosteroids (Fig. 1) and combinations of neurosteroids. The data indicate that the GABA equilibrium dissociation constant in the resting receptor is 1.1–2.1 μM and that the binding of five GABA molecules contributes 6.1–7.3 kcal/mol free energy toward stabilization of the open state. A range, rather than a precise estimate, is due to the lack of an exact value for maximal open probability in the presence of GABA. In macroscopic recordings, the maximal P_{open} for a given agonist is typically determined by comparing a response to a saturating concentration of the agonist with a reference

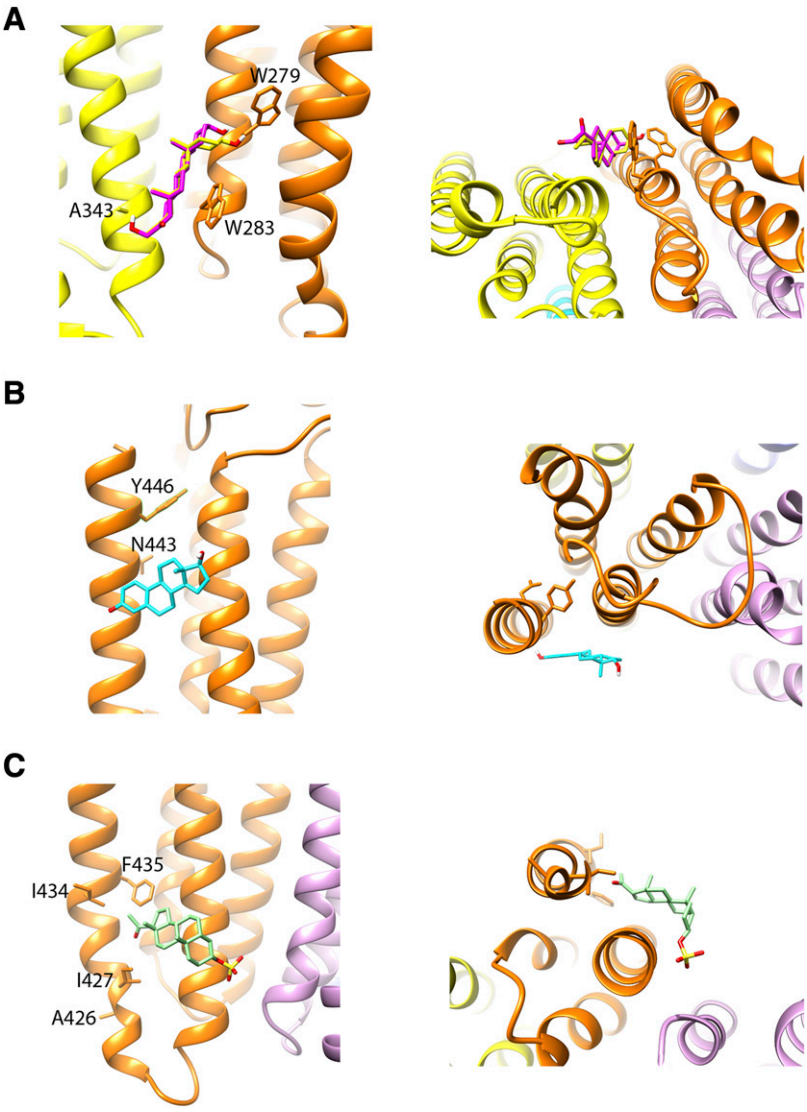

Fig. 7. Docking of steroids in putative binding sites. (A) The panels show a side view (left panel) and a view from the intracellular side of the receptor (right panel) at the putative intersubunit binding site for steroids (Hosie et al., 2006) with 3α5βP (yellow) and 5αTHDOC (magenta) docked in the site. The residues shown are W279 (corresponding to Q241 in α1), W283 (α1W245), and A343 (corresponds to T305 in the α1 or F301 in the β3 subunit) that have been implicated in the effects of potentiating steroids in the heteromeric $GABA_A$ receptor (Hosie et al., 2006; Akk et al., 2008; Chen et al., 2012). (B) The panels show a side view (left panel) and a view from the extracellular side of the receptor (right panel) at the putative intrasubunit binding site for potentiating steroids (Chen et al., 2019). The steroid β-estradiol is shown in the site. The residues shown are N443 and Y446 that correspond to N407 and Y410, respectively, in the α1 subunit and have been implicated in the actions of potentiating steroids in the heteromeric $GABA_A$ receptor (Hosie et al., 2006; Li et al., 2009). (C) The panels show a side view (left panel) and a view from the intracellular side of the receptor (right panel) at the putative intrasubunit binding site for inhibitory steroids (Laverty et al., 2017). The steroid 3α5βPS is shown in the site. The residues shown are A426, I427, I434, and F435 that correspond to K390, I391, I398, and F399, respectively, in the α1 subunit and have been shown to inhibit the actions of pregnenolone sulfate in the GLIC-α1 receptor (Laverty et al., 2017). The selection of docked steroids in (A–C) was not based on best docking scores (see *Results*) but rather on analogy with the presumed steroid selectivity in the heteromeric $GABA_A$ receptor.

response to a combination of the agonist and a potentiator. The latter is assumed to generate a response with P_{open} indistinguishable from one. Here, coapplication of the potentiating steroid 5αTHDOC with saturating GABA was without effect on the peak response, suggesting that the maximal P_{open} for GABA in the ρ1 receptor is near one. The absence of other suitable allosteric potentiators (Belelli et al., 1999) or orthosteric or allosteric agonists more efficacious than GABA (Chang et al., 2000) did not allow for a more precise estimate. The ambiguity in maximal P_{open} for GABA affects the estimated P_{open} values in Figs. 2, 3, and 5. Our calculations indicate that the resulting fitted values of $K_{steroid}$ and $c_{steroid}$ would differ from those presented in Table 2 by <30%.

The findings indicate that the ρ1 $GABA_A$ receptor contains at least three classes of distinct, nonoverlapping sites for neurosteroids. There is one class of sites for both of the inhibitory neurosteroids 3α5βP and 3α5βPS and the neurosteroid/sex hormone β-estradiol. The site for 3α5βP can

TABLE 2

Analysis of modulation of the ρ1 receptor by steroids

The table summarizes the results of fitting the steroid conc.-P_{open} response data from ρ1 wild-type and I307Q mutant receptors to eq. 2. The experiments were conducted in the presence of GABA, and L was calculated as $(1-P_{open,GABA})/P_{open,GABA}$. The number of binding sites for steroids was constrained to five, and the maximal P_{open} for GABA was assumed to be 0.999.

Receptor	Steroid	K_C (μM)	c
ρ1 wild-type	3α5βP	2.85 ± 0.62	1.25 ± 0.02
	3α5βPS	51.1 ± 25.3	2.34 ± 0.89
	β-Estradiol	16.4 ± 4.8	1.47 ± 0.07
	5αTHDOC	38.2 ± 28.6	0.58 ± 0.07
ρ1(I307Q)	3α5βP	20.5 ± 15.1	0.55 ± 0.05
	5αTHDOC	28.6 ± 26.1	0.49 ± 0.08

alternatively bind the potentiating steroid 5αTHDOC. From the 5-fold symmetry of the homomeric ρ1 receptor, and for simplicity, we have assumed that there are five sites of each class in the receptor.

Our definition of distinct versus overlapping sites is based on functional effects of combinations of steroids, using parameters for steroid effects obtained in studies of one steroid in the absence of others. In the case of postulated distinct binding sites (3α5βP, 3α5βPS, and β-estradiol), we have shown that when used in combination, each steroid acts independently, and there is no indication that the binding of one steroid modifies the effect of another except through energetic additivity. This lack of interaction between the two steroid molecules, other than one mediated by stabilization of particular states of the receptor, is incompatible with the physical prevention by one steroid of access of the other to a binding site on the receptor. This, in turn, precludes the possibility that the interaction of the steroids with the receptor requires that the steroids associate with the same residues in the receptor or a shared subset of the residues.

In the case of postulated overlapping binding sites (3α5βP and 5αTHDOC), we have shown that in combination the two steroids display competitive interaction. For simplicity, and because of mutational and structural evidence that 5α- and 5β-reduced steroids act through a common site in heteromeric (Hosie et al., 2006; Akk et al., 2008; Chen et al., 2019) and α-homomeric $GABA_A$ receptors (Laverty et al., 2017; Miller et al., 2017; Chen et al., 2018), we have assumed that 3α5βP and 5αTHDOC compete for a shared site. We cannot, however, exclude a possibility that the two steroids bind to distinct but allosterically linked sites in the ρ1 receptor. We also note that the electrophysiological experiments provide no evidence about the actual physical location of the sites and also do not define the extent of overlap in the case of 3α5βP and 5αTHDOC or other details, such as the orientation of the bound steroids in the postulated site.

We tested the possibility that the three sites for potentiating and inhibitory steroids previously identified in the α1 subunit or the α1β3 receptor (Laverty et al., 2017; Miller et al., 2017; Chen et al., 2019) also mediate the actions of 3α5βP, 3α5βPS, β-estradiol, and 5αTHDOC in the ρ1 receptor. Docking of the steroids to homologous sites in a model generated using β3 and GLIC-α1 crystal structures, however, indicated little selectivity for different steroids. The docking scores were within 1 kcal/mol at each individual site and within 2 kcal/mol across all the steroid-site pairs. Although the estimated energies were similar, the poses of the docked steroids at a given site could vary even to the extent of having reversed orientations (see Supplemental Information). However, it was not possible to interpret the poses in terms of functional consequences since the homology model was built based on structures corresponding to the desensitized receptor, whereas the coagonist concerted transition model assumes that ligands have distinct affinities to different states. Thus, an inhibitory steroid, such as 3α5βP, 3α5βPS, or β-estradiol, is expected to have higher affinity to the resting or desensitized state, depending on the mechanism of inhibition, than the open state, whereas the potentiating steroid 5αTHDOC has higher affinity to the open state. In the present analysis, we have assumed that the inhibitory steroids act by stabilizing the resting state. In future work, once appropriate structures become available, it will be interesting to compare docking with different functional states. Alternatively, the tested steroids may bind elsewhere in the ρ1 receptor.

The classification of three distinct sites for inhibitory steroids in the ρ1 receptor is also in agreement with previous mutagenesis and fluorometrical data. The P294S mutation (2′ residue in the second membrane-spanning domain) selectively eliminates inhibition by 3α5βP, whereas the T298F mutation (6' in the second membrane-spanning domain) eliminates inhibition by β-estradiol (Li et al., 2007). Furthermore, the steroids 3α5βP, 3α5βPS, and β-estradiol differently modify fluorescence changes caused by GABA in the extracellular domain of the receptor (Eaton et al., 2014). Here, we used an activation model–based approach to analyze ρ1 receptor modulation by inhibitory steroids. In previous work, the model has been most notably employed in studies of the actions of agonists and agonist combinations on heteromeric α1β γ2 $GABA_A$ receptors.

The electrophysiological data indicate that a common site mediates the actions of the inhibitory steroid 3α5βP and the potentiating steroid 5αTHDOC. To the best of our knowledge, this is the first demonstration of different steroids or analogs interacting with a common site eliciting opposite modulation of the $GABA_A$ receptor. Divergent modes of action have been reported previously for agonists and inverse agonists at the benzodiazepine site in the heteromeric αβγ $GABA_A$ receptor (Sigel and Ernst, 2018). In the α1β2γ2 $GABA_A$ receptor, the competitive antagonists bicuculline and gabazine inhibit activation by allosteric agonists and cause distinct conformational changes in a fluorescence assay (Ueno et al., 1997; Muroi et al., 2006; Akk et al., 2011). Finally, the different conformational changes elicited near the transmitter binding site by GABA and competitive antagonists may be considered a special case of this phenomenon. In the ρ1 receptor, the competitive antagonist 3-aminopropyl(methyl)phosphinic acid elicits conformational changes in the extracellular domain of the receptor that differ from those observed in the presence of GABA (Chang and Weiss, 2002).

Although energetic additivity arising from steroid interactions with distinct binding sites strongly enhances the net inhibitory effect when 3α5βP, 3α5βPS, and β-estradiol are coapplied, the low affinity of the steroids to the ρ1 receptor suggests little functional modulation under physiologic conditions. Using eq. 2, we estimate that the combination of 0.1 μM 3α5βP, 1 μM 3α5βPS, and 0.01 μM β-estradiol [approximate physiologic concentrations; (Bixo et al., 1995; Cheney et al., 1995; Weill-Engerer et al., 2002)] reduces the response to physiologic, ambient GABA (~300 nM) from a P_{open} of 0.08 to 0.07. In a reverse calculation, to estimate the concentrations of the individual steroids needed to elicit a more "meaningful" reduction in P_{open}, we find that a 5-fold increase in 3α5βP (to 0.5 μM) and 3α5βPS (to 5 μM) is sufficient to reduce the open probability from 0.08 to 0.045. A more drastic increase in the concentration of β-estradiol is needed for further reduction in P_{open} (coapplication of 1 or 5 μM β-estradiol with 0.5 μM 3α5βP + 5 μM 3α5βPS reduces the P_{open} to 0.041 or 0.030, respectively). Incidentally, this suggests that targeting of the β-estradiol binding site is the most efficient way to pharmacologically modulate function of the ρ1 receptor that is exposed to physiologic concentrations of 3α5βP and 3α5βPS.

In summary, we have shown that the ρ1 $GABA_A$ receptor contains three classes of functionally defined nonoverlapping binding sites for neurosteroids: one each for sulfated steroids

and β-estradiol and a shared site for 3α5βP and 5αTHDOC. Although interaction with distinct sites strongly enhances the net effect of combined drugs, the relatively low affinities and weak efficacies of the tested steroids suggest minimal modulation of the human ρ1 receptor by neurosteroids under physiologic conditions. This work has extended the applicability of the concerted transition model in two ways: by demonstrating that it can be used to analyze modulation by inhibitory allosteric agents as well as potentiate and by applying it to an additional member of the pentameric transmitter-gated ion-channel family.

Acknowledgments

We thank Mr. Daniel J. Shin for help with electrophysiology.

Authorship Contributions

Participated in research design: Germann, Evers, Steinbach, Akk.
Conducted experiments: Germann, Burbridge, Pierce.
Contributed new reagents or analytic tools: Reichert.
Performed data analysis: Germann, Burbridge, Pierce, Akk.
Wrote or contributed to the writing of the manuscript: Germann, Reichert, Evers, Steinbach, Akk.

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