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Modulation of the human $\rho 1$ GABA_A receptor by inhibitory steroids

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Abstract

Rationale—Modulators of the $\rho 1$ GABA_A receptor may be useful in the treatment of visual, sleep, and cognitive disorders. Neuroactive steroids and analogues have been shown to modulate $\rho 1$ receptor function but the molecular mechanisms are poorly understood.

Objectives—We employed electrophysiology and voltage-clamp fluorometry to compare the actions of several neuroactive steroids and analogues on the human $\rho 1$ GABA_A receptor.

Results—Results confirmed that P294S and T298F mutations affect modulation by steroids. The P294S mutation abolished inhibition by (3 α ,5 β)-3-hydroxypregnan-20-one (3 α 5 β P) while the T298F mutation eliminated inhibition by 17 β -estradiol. Voltage-clamp fluorometry demonstrated that steroids differing in the presence of a charged group on C3 or nature of substituent on C17 uniquely modified fluorescence changes elicited by GABA in the extracellular domain. The I307Q mutation reversed the inhibitory effect of 3 α 5 β P but was without effect on modulation by (3 α ,5 β)-3-hydroxypregnan-20-one sulfate or 17 β -estradiol. The effect of 3 α 5 β P on the fluorescence change generated at Y241C was dependent on whether the steroid acted as an inhibitor or a potentiator. Further, the effect was limited to uncharged 5 β -reduced steroids containing an acetyl group on C17.

Conclusions—The data demonstrate that steroids and analogues differ with respect to conformational changes elicited by these drugs as well as sensitivity to the effects of mutations. Steroids and analogues could be provisionally divided into three major groups based on their actions on the $\rho 1$ GABA_A receptor: 5 β -reduced uncharged steroids, sulfated and carboxylated steroids, and 17 β -estradiol. Further division among 5 β -reduced uncharged steroids was based on substituent at position C17.

Keywords

GABA receptor; ion channel; neurosteroid; mutagenesis; fluorescence

INTRODUCTION

Ionotropic $\rho 1$ GABA_A receptors are homopentameric membrane proteins that belong to the Cys loop family of ligand gated ion channels. Binding of the neurotransmitter GABA leads to opening of a channel permeable to Cl⁻ and other small anions. GABA_A receptors containing the ρ subunit are predominantly expressed in the retina where knockout of the $\rho 1$ subunit eliminates GABA-evoked responses in rod bipolar cells (McCall et al. 2002). Additionally, ρ subunits are expressed in the hippocampus (Alakuijala et al. 2005; Didelon et al. 2002), superior colliculus (Schlicker et al. 2009; Wahle and Schmidt 2009), visual cortex (Wahle and Schmidt 2009), cerebellum (Rozzo et al. 2002; Wegelius et al. 1998), and dorsal root ganglia (Wegelius et al. 1998). The physiological and pharmacological properties of ρ receptors differ in some aspects from the more common $\alpha\beta\gamma$ and $\alpha\beta\delta$ subtypes of the GABA_A receptor. Currents from ρ receptors are insensitive to bicuculline (Shimada et al. 1992; Wang et al. 1994), have a slower deactivation rate (Amin and Weiss 1994), and a significantly lower (sub-picosiemens) single-channel conductance (Pan et al. 1997; Wotring et al. 1999). Similar to the extrasynaptic $\alpha 4\beta\delta$ receptors, ρ receptors are highly sensitive to GABA and demonstrate little desensitization (Amin and Weiss 1994; Lu and Huang 1998). The ρ receptor is thus best suited to respond to ambient GABA and contribute to tonic inhibition (Alakuijala et al. 2006).

Drugs acting on the $\rho 1$ GABA_A receptor may be useful in the treatment of a variety of visual, sleep, and cognitive disorders (Johnston et al. 2003). A recent study demonstrated that *cis*- and *trans*-(3-aminocyclopentanyl)methylphosphinic acid, selective antagonists of ρ receptors, can prevent the development of myopia (Chebib et al. 2009). The same study indicated that following intraperitoneal injection, ρ antagonists enhanced learning and memory in rats in the Morris water maze task. Furthermore, receptors containing the ρ subunit have been implicated in apoptosis of hippocampal neurons (Yang et al. 2003), and regulation of hormone release in the pituitary gland (Boue-Grabot et al. 2000). 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), which is an agonist of the $\alpha\beta\gamma$ GABA_A receptor but antagonist of the ρ subtype, induces analgesia in rodents and humans (Asiedu et al. 2012; Kjaer and Nielsen 1983). It has been proposed that antagonism of the ρ receptor contributes to the analgesic effect of THIP (Johnston et al. 2003).

Among the modulators of the $\rho 1$ GABA_A receptor are several neuroactive steroids, which, depending on their structure, can potentiate or inhibit currents elicited by GABA. Typically, 5 α -reduced steroids, such as (3 α ,5 α)-3-hydroxypregnan-20-one and (3 α ,5 α)-3,21-dihydroxypregnan-20-one (5 α THDOC), potentiate the receptor whereas their 5 β -reduced analogues (3 α ,5 β)-3-hydroxypregnan-20-one (3 α 5 β P) and (3 α ,5 β)-3,21-dihydroxypregnan-20-one (5 β THDOC) have an inhibitory effect (Morris et al. 1999). Sulfated steroids, regardless of the configuration at C5, and the sex hormone 17 β -estradiol inhibit the $\rho 1$ receptor (Li et al. 2007).

Here, we have employed molecular biology, electrophysiology and voltage-clamp fluorometry to examine modulation of the human $\rho 1$ GABA_A receptor by several steroids and analogues. The data indicate significant differences in the molecular mechanisms of action despite the common - inhibitory - functional effect and related structure. The P294S

mutation (P2'S mutation in the second membrane-spanning domain) abolished potentiation by 3 α 5 β P but had no effect on inhibition by (3 α ,5 β)-3-hydroxypregnan-20-one sulfate (3 α 5 β PS) or 17 β -estradiol. When the receptors contained the T298F mutation (6' residue in the M2), channel inhibition by 17 β -estradiol, but not 3 α 5 β P or 3 α 5 β PS, was greatly reduced. Voltage-clamp fluorometry experiments indicated that 3 α 5 β P, 3 α 5 β PS and 17 β -estradiol differently affect structural changes taking place during activation by GABA in the extracellular domain.

MATERIALS AND METHODS

Molecular biology and expression

The experiments were conducted on wild-type and mutant human $\rho 1$ (GenBank accession no. M62400) GABA_A receptors expressed in *Xenopus laevis* oocytes. Two sites in the membrane-spanning domain were mutated to electrophysiologically examine the effects of these mutations on modulation by steroids. The substitutions were: P294S and T298F (2' and 6' residues in the second membrane-spanning domain, respectively). For use in fluorometry experiments, four cysteine mutations were made in the extracellular domain. The sites were: K217 (Loop F), Y241 (Loop C), and S66 (subunit interface). A subset of these mutations were combined with the I307Q (second membrane-spanning domain) mutation.

The cDNAs were subcloned into the pGEMHE expression vector in the T7 orientation. The cDNAs were linearized by Nhe I (NEB Labs, Ipswich, MA) digestion, and the cRNAs were produced using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with 5–15 ng cRNA in a volume of 20–60 nl, and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 2.5 mM sodium pyruvate, and 5 mM HEPES; pH 7.4) at 16 °C for 2–5 days before labeling and recording.

Electrophysiological and fluorescence recordings

Electrophysiological measurements were conducted using an RC-1Z (Warner Instruments) recording chamber. Currents were recorded using standard two-electrode voltage clamp. Both voltage and current electrodes were patch-clamp electrodes filled with 3 M KCl and had resistances of 0.5 to 1.5 M Ω . The oocytes were clamped at –60 mV. The chamber was perfused continuously at approximately 5 ml/min. Bath solution was perfused between test applications. Solutions were applied from glass reservoirs via metal or teflon tubing to reduce adsorption, and switched via pClamp using a Warner Instruments VC-8T valve controller. The current responses were amplified with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digidata 1320 series digitizer (Molecular Devices) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices).

Voltage-clamp fluorescence measurements were conducted using Alexa Fluor 546 C5 maleimide (A5m; Invitrogen, Carlsbad, CA) as the fluorescence reporter. Labeling with A5m was carried out by incubation for 45–60 min at room temperature in the dark with 20 μ M A5m dissolved in OR2 (92.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES) at

pH 7.2. Following labeling, the oocytes were kept in bath solution (OR2, pH 7.5) before use in experiments.

For fluorescence recordings, we employed a custom made recording chamber (Akk and Steinbach 2011; Chang and Weiss 2002). The chamber consists of two compartments, separated by a 0.8 mm aperture on which the oocyte is placed. The oocyte is impaled in the top compartment which contains static bath. Part of the surface of the oocyte is exposed via the aperture to the lower compartment. The drugs (agonist and modulators) are added to the bath flowing through the lower compartment. The seal between the oocyte and the aperture is tight and there is little leakage between the two compartments. Accordingly, the fluorescence and peak current signals are measured from the same general population of receptors.

An inverted microscope (Nikon Diaphot TMD) fitted with a Nikon 20X LWD 0.4 NA objective was used to collect the fluorescence signal from the lower chamber. The microscope holds dichroic (565DCLP) and emission (D605/55m) filters (Chroma Technology Corp, Rockingham, VT) for fluorescence detection. The fluorescence measurement system was purchased from Photon Technology International (Birmingham, NJ). The system consists of a DeltaRAM monochromator, a liquid light guide that is used to pass the light (546 nm) into the microscope, a photomultiplier tube (R1527P Hamamatsu Photonics, Bridgewater, NJ) mounted on the side port of the microscope, BryteBox acquisition hardware, and FeliX32 software for control of excitation. A 75-watt Xenon short arc lamp (Ushio Inc., Tokyo, Japan) was employed as the light source.

Experiments and analyses

The functional effect of each steroid was determined as follows. A cell was initially exposed to a saturating concentration of GABA, followed by a washout. Several applications were then made with low (EC_{5-10}) GABA, until a stable response was obtained. This was followed by an application of low GABA + steroid. Another exposure to low GABA was conducted to verify reversibility of the effect of steroid. Control experiments were conducted to test direct activation by steroids. Drug applications lasted 20–40 s, followed by 1–3 min washouts. Previous work has revealed that steroid IC_{50} values are in the low micromolar range (Li et al. 2007). To reduce error arising from cell to cell variability and to focus on maximal effects, all experiments were conducted employing 20 μ M steroid.

Fluorescence changes were measured on oocytes labeled with Alexa 546 maleimide (A5m). The sequence of drug applications was similar to the one described above. The effects of steroids, however, were examined in the presence of higher concentrations of GABA (EC_{35-70}). Higher concentrations of GABA were necessary in this set of experiments to reliably elicit a robust fluorescence response. The use of higher concentrations of GABA resulted in a smaller magnitude of functional effect of steroid. We also tested the effect of steroid in the absence of GABA. In each case, the application of steroid alone was without effect on current or fluorescence signal.

Current and fluorescent transients were analyzed with Clampfit (Molecular Devices). Fluorescence baseline was corrected for bleaching when needed. The magnitude of the

current response was estimated as the peak response within the window of drug application. The magnitude of the fluorescence signal was estimated as the mean response within the saturated portion of the fluorescence response (Figure 1). The parameter measured is the “response ratio”, i.e., the ratio of the response to GABA + steroid to the response to GABA prior to steroid exposure. Statistical analyses were carried out using paired t-test (Excel, Microsoft, Richmond, WA).

Drugs

The salts used in the study were purchased from Sigma-Aldrich (St. Louis, MO). The steroid analogues (3 α ,5 β)-3-hydroxymethylpregnan-20-one (S84-1) and (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (B164) were prepared by us (Han et al. 1996; Jin et al. 2009). Remaining steroids were purchased from Sigma-Aldrich (St. Louis, MO) or Steraloids (Newport, RI). Stock solutions (20 mM) of the steroids were made in dimethyl sulfoxide (DMSO), dilutions to test concentrations were made on the day of the experiment. Stock solution of A5m was made in DMSO at 5 mM and stored in aliquots at -80°C . The structures of steroids used are given in Figures 3A and 6A.

RESULTS

Mutations to the membrane-spanning domain differently affect the actions of inhibitory steroids

Human ρ subunit-containing GABA_A receptors are susceptible to modulation by steroids. In particular, the $\rho 1$ receptor is inhibited by 5 β -reduced steroids, charged (sulfated or carboxylated) steroids, and the sex hormone 17 β -estradiol (Li et al. 2007; Morris et al. 1999). Our data demonstrate that wild-type $\rho 1$ receptors expressed in *Xenopus* oocytes exhibit strong inhibition of peak responses in the presence of 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol. The peak responses from wild-type receptors activated by 0.15 μM GABA (EC₅) were reduced to $40 \pm 8\%$ (mean $\pm$ S.D., 4 cells; $p < 0.001$, paired t-test) of control in the presence of 20 μM 3 α 5 β P. Application of 20 μM 3 α 5 β PS reduced the peak response to $26 \pm 5\%$ of control (4 cells; $p < 0.001$). When 20 μM 17 β -estradiol was coapplied with GABA, the peak response was reduced to $38 \pm 13\%$ of control (4 cells; $p < 0.01$). Sample recordings are shown in Figure 2A.

Introduction of the P-to-S mutation at position 294 (2' in the second membrane-spanning domain) affected modulation by steroids. Coapplication of 20 μM 3 α 5 β P with 0.75 μM GABA (EC₇) was without effect on the peak amplitude ($85 \pm 10\%$ of control, 4 cells; $p > 0.05$). The mutant receptors were inhibited by 20 μM 3 α 5 β PS ($30 \pm 4\%$ of control, 4 cells; $p < 0.001$) or 20 μM 17 β -estradiol ($59 \pm 7\%$ of control, 4 cells; $p < 0.01$). Thus, the P294S mutation enables distinction between the inhibitory effects of 3 α 5 β P vs. 3 α 5 β PS and 17 β -estradiol. The simplest explanation is that the sites and/or mechanisms of action of 3 α 5 β P differ from those of 3 α 5 β PS and 17 β -estradiol. Sample current traces are shown in Figure 2B.

We next tested the effect of the T-to-F mutation at position 298 (6' in the second membrane-spanning domain). There was no effect on peak responses when 20 μM 17 β -estradiol was

coapplied with 0.15 μM GABA (EC_{50}) (100 ± 6 % of control, 4 cells; $p > 0.05$). Coapplication of 20 μM 3 α 5 β PS with GABA reduced the peak amplitude of the mutant receptors to 20 ± 6 % of control (4 cells; $p < 0.001$). Interestingly, in the presence of 20 μM 3 α 5 β P the peak amplitude was enhanced to 217 ± 94 % of control (5 cells; $p < 0.05$). Sample recordings are shown in Figure 2C.

The effects of steroids were also tested on $\rho 1(\text{T298F})$ mutant receptors in the presence of 0.3 μM GABA (EC_{40-50}). The average peak response elicited by 0.3 μM GABA was reduced to 52 ± 15 % of control (4 cells; $p < 0.01$) or 17 ± 2 % of control (4 cells; $p < 0.001$) in the presence of 3 α 5 β P or 3 α 5 β PS, respectively. Thus, the polarity of effect of T298F on modulation by 3 α 5 β P is dependent on the concentration of GABA used to activate the receptor. The mean peak response in the presence of 20 μM 17 β -estradiol was 102 ± 2 % of control (4 cells; $p > 0.05$). Sample traces are shown in Figure 2D. A summary of the findings is presented in Figure 2E.

To probe direct activation by steroids, oocytes expressing $\rho 1(\text{P294S})$ or $\rho 1(\text{T298F})$ receptors were exposed to 20 μM 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol. In one case ($\rho 1(\text{P294S})$), exposure to 3 α 5 β P resulted in inward current that had a mean peak response of 0.05 ± 0.03 % (5 cells) of the peak response to saturating GABA. In all other steroid-receptor combinations, exposure to steroid led to apparent outward current that we interpret as steroid-induced closure or block of spontaneously active receptors. We note that these data exclude the possibility that enhancement of currents from $\rho 1(\text{T298F})$ receptors activated by a low concentration of GABA in the presence of 3 α 5 β P (Figure 2C) is due to receptors being directly activated by the steroid.

In sum, the P294S and T298F mutations selectively affect inhibition of the human GABA_A $\rho 1$ receptor by 3 α 5 β P, 3 α 5 β PS, and 17 β -estradiol. The P294S mutation, by eliminating inhibition by 3 α 5 β P, distinguishes the actions of 3 α 5 β P from those of 3 α 5 β PS and 17 β -estradiol. The T298F mutation removes inhibition by 17 β -estradiol, therefore differentiating the actions of 17 β -estradiol from 3 α 5 β P and 3 α 5 β PS. We infer that the three steroids act via distinct sites and/or mechanisms.

Inhibitory steroids elicit distinct conformational changes in the extracellular domain

Voltage-clamp fluorometry (VCF) entails the measurement of the fluorescence signal from a fluorophore linked to an introduced cysteine residue (Mannuzzu et al. 1996). A change in fluorescence signal (ΔF) in the presence of a drug is widely accepted as an indication of a structural rearrangement leading to altered polarity of the environment surrounding the fluorophore. This approach is particularly useful in comparing and contrasting the effects of drugs. Here, we have investigated the effects of the steroids 3 α 5 β P, 3 α 5 β PS, and 17 β -estradiol (structures in Figure 3A) on GABA-induced ΔF , to determine whether inhibition of channel function is associated with changes in GABA-elicited structural rearrangements.

We selected four residues in the extracellular domain for labeling. The residues were: K217 (within Loop F, contributes to the transmitter binding site), S66 (at the subunit-subunit interface, outside of the transmitter binding pocket), L166 (Loop E, top of the transmitter binding site), and Y241 (Loop C, critically important for activation). The selected residues

were mutated to cysteine and labeled with Alexa 546 maleimide. Previous work has demonstrated that these sites respond with a robust fluorescence change to receptor activation by GABA (Chang and Weiss 2002; Khatri et al. 2009).

Inhibition of ρ receptors by steroids is pseudocompetitive, i.e., the inhibitory effect is reduced at higher concentrations of the agonist (Li et al., 2007). Accordingly, steroid effects were examined at submaximal GABA concentrations while maintaining constant fractional activation (EC_{39-72} across the various mutants). Coapplication of $3\alpha 5\beta P$, $3\alpha 5\beta PS$ or 17β -estradiol with GABA reduced electrophysiological responses from labeled mutant receptors (Figure 3B–E). Peak responses during exposure to steroid ranged from $27 \pm 7\%$ (K217C in the presence of 17β -estradiol) to $79 \pm 14\%$ (Y241C in the presence of $3\alpha 5\beta PS$) of the response to GABA alone. A summary is given in Figure 3F.

Steroid effects on fluorescence varied. In the K217C receptor, coapplication of $20\ \mu M$ $3\alpha 5\beta PS$ with GABA reduced the F to $75 \pm 12\%$ ($p < 0.05$) of that observed in the presence of GABA alone. In contrast, exposure to $3\alpha 5\beta P$ or 17β -estradiol was without effect on F (Figure 3B, F). Thus, at the K217C site, modulation of the fluorescence signal distinguishes the actions of the sulfated steroid $3\alpha 5\beta PS$ from those of $3\alpha 5\beta P$ and 17β -estradiol.

Fluorescence changes in receptors labeled at the S66C site were inhibited by $3\alpha 5\beta PS$ and 17β -estradiol but not affected by $3\alpha 5\beta P$ (Figure 3C, F). The mean F was reduced to $60 \pm 13\%$ ($p < 0.01$) or $38 \pm 11\%$ of control ($p < 0.01$) in the presence of $20\ \mu M$ $3\alpha 5\beta PS$ or 17β -estradiol, respectively. In the presence of $3\alpha 5\beta P$, the mean F was $119 \pm 27\%$ of control. We infer that comparison of steroid effects on F at the S66C site enables separation of the effects of $3\alpha 5\beta PS$ and 17β -estradiol from $3\alpha 5\beta P$.

Receptors labeled at the L166C site exhibited sensitivity to 17β -estradiol, but not $3\alpha 5\beta P$ or $3\alpha 5\beta PS$ (Figure 3D, F). Application of 17β -estradiol reduced the mean F to $54 \pm 18\%$ of control ($p < 0.01$). The effects by $3\alpha 5\beta P$ and $3\alpha 5\beta PS$ were not statistically different from no effect. Therefore, we conclude that L166C differentiates the effects of 17β -estradiol from the effects exerted by $3\alpha 5\beta P$ and its sulfated analogue, $3\alpha 5\beta PS$.

Finally, all three steroids functionally inhibited receptors labeled at the Y241C site, but none modulated F (Figure 3E, F).

Overall, the data demonstrate that the actions of inhibitory steroids are associated with structural rearrangements in the extracellular domain of the $\rho 1$ receptor, and that movements in the presence of $3\alpha 5\beta P$, $3\alpha 5\beta PS$, and 17β -estradiol involve different regions within the extracellular domain.

Are the structural changes in the extracellular domain productively or casually linked to functional modulation?

We next sought to determine whether steroid-elicited structural rearrangements in the extracellular domain are linked to the functional effects of the steroid, or reflect associated, but non-productive rearrangements. In other words, are the conformational changes causal or casual?

An earlier study had found that the I307Q mutation reverses inhibition of the human $\rho 1$ GABA_A receptor by several 5β -reduced steroids (Morris and Amin 2004). We examined the effect of the I307Q mutation on fluorescence changes in the presence of the same three classes of steroids. We reasoned that if the changes in F result from the functional effect of the steroid, i.e., electrophysiological inhibition, then the presence of the mutation that reverses inhibition would also affect steroid-induced changes in F . Conversely, if F is associated with steroid binding per se, then the introduction of the I307Q mutation should not alter the F signal. This interpretation is dependent on an assumption that introduction of the I307Q mutation solely alters the modulatory effect of a steroid without eliminating the original binding site for the steroid, or introducing or unmasking a new site.

In initial experiments, we probed the effect of the I307Q mutation, alone and in the presence of the labeling mutation, on modulation of current responses by $3\alpha 5\beta$ P, $3\alpha 5\beta$ PS and 17β -estradiol. Each steroid was co-applied at $20\text{ }\mu\text{M}$ along with GABA at a concentration eliciting 5–10 % of the maximal response. Our findings confirm that the I307Q mutation converts $3\alpha 5\beta$ P from being an inhibitor to being a potentiator (Figure 4). This effect was observed in the absence and presence of a labeling mutation (K217C, S66C, or Y241C), however, the I307Q mutation did not reverse the inhibitory effects of $3\alpha 5\beta$ PS or 17β -estradiol (Figure 4). These data provide further support to the hypothesis that inhibitory steroids act through different mechanisms on the $\rho 1$ GABA_A receptor. Control experiments conducted in the presence of steroid alone demonstrated that direct activation by steroids does not result in significant current responses (data not shown).

We next tested the effects of steroids on current and fluorescence responses from double mutant receptors labeled with A5m. To obtain a reliable fluorescence signal, the experiments were conducted at GABA concentrations eliciting an approximately half-maximal response ($\text{EC}_{47\pm 15}$, a total of 46 cells). Currents from the $\rho 1$ (I307Q+K217C) receptor activated by GABA were potentiated by $20\text{ }\mu\text{M}$ $3\alpha 5\beta$ P (Figure 5A). The average peak response was $124 \pm 10\%$ of control ($p < 0.05$; 5 cells). The increase in current was not accompanied by changes in F . Thus, receptors labeled at the K217C site exhibit no changes in F , regardless of whether the action of $3\alpha 5\beta$ P is potentiating (in the presence of I307Q; Figure 5A) or inhibitory (in the absence of I307Q; Figure 3B). Due to the higher GABA concentration used, potentiation of peak current response was less in fluorescence experiments compared to data shown in Figure 4.

The actions of $3\alpha 5\beta$ PS and 17β -estradiol were inhibitory on the $\rho 1$ (I307Q+K217C) receptor, i.e., the presence of the I307Q mutation did not convert these steroids into potentiators. The I307Q mutation also did not affect how these steroids influenced F ; the application of $3\alpha 5\beta$ PS reduced the magnitude of F ($70 \pm 9\%$ of control, $p < 0.02$, 6 cells) while 17β -estradiol was without effect on F ($106 \pm 3\%$ of control, 3 cells). The data are summarized in Figure 5C.

In sum, the pattern of steroid-elicited effects on F in $\rho 1$ (I307Q+K217C) is identical to that in the K217C mutant. Most importantly, the reversal of inhibition caused by the introduction of the I307Q mutation did not result in changes in F in the presence of $3\alpha 5\beta$ P. We interpret

this as the Loop F residue reporting conformational changes that are not associated with or affected by the functional effect of 3 α 5 β P.

Fluorescence changes at the Y241C site were dependent on the presence of the I307Q mutation. In receptors containing the I307Q mutation, the application of 3 α 5 β P potentiated the peak response ($130 \pm 7\%$ of control, $p < 0.01$, 7 cells) and enhanced $\bar{F}$ ($161 \pm 18\%$ of control, $p < 0.05$). This finding is suggestive of the Y241C site “sensing” potentiation-related structural rearrangements. The sulfated steroid 3 α 5 β PS inhibited the peak response ($82 \pm 3\%$, $p < 0.001$, 9 cells) but was without effect on $\bar{F}$ ($103 \pm 7\%$ of control). This behavior is similar to that observed in the absence of the I307Q mutation. Lack of effect of the I307Q mutation on peak response by 3 α 5 β PS limits the interpretation. At minimum, the data indicate that sulfated steroids and 5 β -reduced steroids act via different mechanisms. Finally, 17 β -estradiol reduced the magnitude of $\bar{F}$ ($75 \pm 5\%$ of control, 6 cells, $p < 0.01$) but was without effect on the current response ($108 \pm 6\%$, $p > 0.3$). Sample traces are shown in Figure 5B, and a summary is given in Figure 5C. We believe that lack of inhibition of the peak response was due to a higher GABA concentration used in these experiments, because we had earlier found (Figure 4) that coapplication of 17 β -estradiol with EC₅₋₁₀ GABA results in inhibition. Reduction in $\bar{F}$ suggests that 17 β -estradiol modulates the conformational change at the Y241C site.

Additional members of the three groups of steroids

Electrophysiological and fluorescence data suggest that 3 α 5 β P, 3 α 5 β PS, and 17 β -estradiol act on the $\rho 1$ GABA_A receptor via distinct mechanisms. Further studies demonstrated that structurally related steroids or other inhibitory compounds act similarly to the prototypic representatives. For example, the P294S mutation abolishes inhibition of the peak response by 5 β THDOC and (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (B164), in addition to 3 α 5 β P (Li et al. 2007). The P294S mutation does not affect modulation by 3 α 5 β PS or other charged steroids such as (3 α ,5 α)-3-hydroxypregnan-20-one sulfate (3 α 5 α PS) and (3 α ,5 β)-20-oxopregnane-3-carboxylic acid (3 α COOH5 β P) suggesting that charged steroids act via similar mechanisms (Li et al. 2010; Li et al. 2007). Interestingly, the $\rho 1$ (T298F) mutation abolishes inhibition by 17 β -estradiol and picrotoxinin, placing these two compounds into a common class of inhibitors. The $\rho 1$ (T298F) mutation did not affect inhibition by 5 β -reduced steroids (3 α 5 β P, 5 β THDOC or B164) or charged steroids (3 α 5 β PS, 3 α 5 α PS or 3 α COOH5 β P).

Mutagenesis-based classification is generally maintained in voltage-clamp fluorescence. For example, $\bar{F}$ at the K217C site is reduced by the sulfated steroids 3 α 5 β PS and 3 α 5 α PS, but not by 5 β -reduced steroids (3 α 5 β P, 5 β THDOC) or 17 β -estradiol or picrotoxinin (Li et al. 2010). Receptors labeled at the S66C site exhibit $\bar{F}$ that is reduced by the sulfated steroids, and 17 β -estradiol and picrotoxinin (Li et al. 2010).

Fluorescence experiments have also revealed several cases where steroids closely related in structure induce distinct conformational rearrangements. In the S66C receptor, 3 α 5 β P is without effect on $\bar{F}$ whereas another 5 β -reduced steroid 5 β THDOC enhances the magnitude of fluorescence change (Li et al. 2010). The same pattern on fluorescence is observed in the $\rho 1$ (I307Q+S66C) receptor where both steroids potentiate functional

responses. We infer that enhancement of I_F in the presence of 5β THDOC is not related to function.

In $\rho 1$ (I307Q+Y241C) receptors, $3\alpha,5\beta$ P enhances I_F (161% of control) while 5β THDOC is without effect ($107 \pm 8\%$ of control, 6 cells, $p > 0.4$). The classic explanation is that potentiation by $3\alpha,5\beta$ P alters the nature of the conformational change in the vicinity of the Y241C site whereas potentiation by 5β THDOC does not. The two steroids differ in their C17 (D ring) substituents, where $3\alpha,5\beta$ P has a methylketone group (acetyl) and 5β THDOC has an α -hydroxymethylketone group. To further explore the relationship between steroid structure and conformational changes at the Y241C site, we examined the effects of the steroids etiocholanolone and B164 on I_F . Etiocholanolone contains a ketone group on C17 whereas B164 has a carbonitrile. Structures of the compounds are shown in Figure 6A. We found that although both etiocholanolone and B164 potentiate the functional responses, I_F is not affected. For etiocholanolone, the current response was potentiated to $123 \pm 5\%$ of control (3 cells, $p < 0.05$) while I_F was $104 \pm 6\%$ of control ($p > 0.6$). When B164 was coapplied with GABA, the current response was $124 \pm 6\%$ of control (4 cells, $p < 0.04$), but the effect on I_F ($119 \pm 15\%$ of control, $p > 0.25$) was not significant. The data are summarized in Figure 6B.

As a positive control we tested the effect of $(3\alpha,5\beta)$ -3-hydroxymethylpregnan-20-one (S84-1). This steroid analogue is similar to $3\alpha,5\beta$ P in structure except for the hydroxymethyl group on C3, instead of a hydroxy group (Figure 6A). We hypothesized that since both S84-1 and $3\alpha,5\beta$ P have a methylketone group on C17, S84-1 would also enhance I_F . Indeed, we observed that I_F was increased to $149 \pm 10\%$ of control (4 cells, $p < 0.02$). Also as expected, the presence of S84-1 enhanced the peak current response ($136 \pm 8\%$ of control, $p < 0.02$; Figure 6B).

DISCUSSION

The $\rho 1$ GABA_A receptor is modulated by a wide range of steroids and analogues. A general rule is that 5α -reduced steroids potentiate currents from $\rho 1$ receptors whereas 5β -reduced steroids, charged steroids, and the steroid hormone 17β -estradiol inhibit the receptor (Li et al. 2007; Morris et al. 1999). In this study, we have focused on the actions of inhibitory steroids on the human $\rho 1$ GABA_A receptor. Comparison of the effects of P294S and T298F mutations (2' and 6' residues in the second membrane-spanning domain, respectively) have enabled division of inhibitory steroids into three classes: 5β -reduced steroids, charged (sulfated or carboxylated) steroids, and a separate class that includes 17β -estradiol and another GABA_A receptor modulator, picrotoxinin. By using voltage-clamp fluorometry we have shown that representatives of the three classes of steroids elicit a distinct pattern of conformational changes in the extracellular domain of the $\rho 1$ GABA_A receptor. Classification of the compounds is generally similar using the two approaches.

Mutations made in the second membrane-spanning domain distinctively influence the actions of inhibitory steroids. The P294S and T298F mutations reveal different molecular mechanisms involved in inhibition, separating $3\alpha,5\beta$ P, $3\alpha,5\beta$ PS, and 17β -estradiol into different classes of inhibitory steroids. The P294S mutation eliminates inhibition by $3\alpha,5\beta$ P,

thereby selectively affecting modulation by a 5 β -reduced steroid but not charged steroids or 17 β -estradiol.

The T298F mutation abolishes inhibition by 17 β -estradiol, identifying a mechanism for 17 β -estradiol that is distinct from those of charged steroids and 5 β -reduced steroids. Interestingly, the effect of this mutation on modulation by 3 α 5 β P depended on the concentration of GABA. It has previously been reported that inhibition by 3 α 5 β P is not affected in the T298F mutant (Li et al., 2007). We could replicate this finding but only when a relatively high concentration (EC₄₀₋₅₀) of GABA was used to activate the receptor. We found that 3 α 5 β P enhanced currents elicited by EC₅₋₁₀ GABA. The mechanism of apparent potentiation of currents is unclear, but does not involve direct activation of the mutant receptor by steroids. It is possible that besides the inhibitory effect that dominates at higher concentrations of GABA, 3 α 5 β P additionally potentiates monoliganded ρ 1(T298F) receptors, and that such potentiation successfully compensates for the inhibitory effect of steroid.

Block by 17 β -estradiol is removed when the T298F mutation is present, regardless of GABA concentration. Inhibition by 3 α 5 β PS is also not affected by GABA concentration. Despite the disparity, it is intriguing, and potentially useful, that the T298F mutation provides distinction between all three classes of inhibitory steroids at low GABA concentrations.

To explore whether inhibition of the current response is associated with structural changes in the extracellular domain, we employed voltage-clamp fluorometry. Sites previously shown to exhibit robust fluorescence changes during activation were selected for labeling by Alexa 546 maleimide. First and foremost, the data indicate that coapplication of steroids with GABA can modulate the magnitude of I_F , indicating that steroid interactions with the receptor modify GABA-elicited conformational changes in the extracellular domain. Steroids applied alone were without effect on I_F . The location of the steroid binding site(s) in the ρ 1 receptor is unknown but, by analogy to the α 1 β 2 γ 2 GABA_A receptor (Hosie et al. 2006), is likely to be within the membrane-spanning domain. If so, the distance to which steroid-induced modification of conformation is transmitted is remarkable.

The data do not support a notion that inhibitory steroids act on the ρ 1 receptor as classic open channel blockers. The pore blocking mechanism can be expected to be without effect on I_F produced in the extracellular domain. Previous work has shown that competitive inhibitors can elicit I_F at positions near the transmitter binding site (Chang and Weiss 2002; Khatri et al. 2009; Muroi et al. 2006). The lack of effect of inhibitory steroids on I_F when applied in the absence of GABA suggests that these steroids do not act as competitive antagonists of the ρ 1 receptor. We propose that inhibitory steroids act through an allosteric mechanism.

The data indicate that inhibitory steroids act through different molecular mechanisms. We define a common molecular mechanism as one where compounds bind to the same docking site and cause indistinguishable conformational changes. The finding that distinct patterns of effects on I_F emerge in the presence of different steroids indicates that the conformational changes are dissimilar. Specifically, we have shown that 3 α 5 β PS, but not 3 α 5 β P or 17 β -

estradiol, diminish I_F produced by GABA at the K217C position. When the L166C residue was labeled, 17β -estradiol but not $3\alpha,5\beta$ P or $3\alpha,5\beta$ PS reduced I_F . At the S66C position, exposure to $3\alpha,5\beta$ PS or 17β -estradiol reduced I_F while $3\alpha,5\beta$ P was without effect. None of the drugs tested influenced I_F at position Y241C.

A change in fluorescence is interpreted as a conformational change in the vicinity of the fluorophore, however further interpretation is limited. A change in fluorescence signal contains no information about the physical direction or the extent of the conformational change. Fluorescence intensity is dependent on the polarity of the environment surrounding the fluorophore and so the direction of I_F is indicative of whether the environment has become more (reduction in I_F) or less polar (increase of the magnitude of I_F). It is possible, however, for two ligands to induce different conformational changes yet show comparable fluorescence signals if the final microenvironment around the fluorophore is similar. Likewise, modification of I_F in the presence of steroid only provides an indication that the GABA-elicited conformational change has been altered, without providing additional data on the character of the alteration. Nonetheless, this approach is highly valuable when comparing the effects of related drugs or conformational changes at homologous sites in different subunits.

To determine whether steroid-induced modifications are linked to the functional effect of a steroid, we examined the effect of the I307Q mutation on the pattern of fluorescence changes in $\rho 1$ receptors. This mutation is known to reverse inhibition of the $\rho 1$ receptor by several 5β -reduced neurosteroids (Morris and Amin 2004). We reasoned that if the I_F is associated solely with steroid binding then the presence of the I307Q mutation would not alter the I_F signal. Conversely, if the I_F is associated with the functional effect of a steroid, then, for steroids whose actions are converted from inhibitory to potentiating, the presence of the I307Q mutation may lead to changes in the properties (magnitude or direction) of the I_F signal. In practice, a positive result would have been a case where a steroid increases or decreases the I_F in the absence of I307Q, and has an opposite effect (a decrease or increase, respectively) in the presence of the I307Q mutation. Alternatively, an instance where an absence of change in I_F is converted to a change in I_F (or vice versa) following introduction of the I307Q mutation would also indicate a positive result.

We began by examining the functional effects of several steroids on $\rho 1$ receptors. Our data confirm that introduction of the I307Q mutation reverses inhibition by $3\alpha,5\beta$ P, and further show that the mutation similarly modifies the effect of several other 5β -reduced non-sulfated steroids. The I307Q mutation, however, did not reverse inhibition by the sulfated steroid $3\alpha,5\beta$ PS or by the steroid hormone 17β -estradiol. This finding corroborates the hypothesis that these steroids act via distinct mechanisms on the $\rho 1$ GABA_A receptor, but it may have limitations in how to interpret the data with respect to causality of changes in I_F .

Two sites (K217C and Y241C) were labeled and examined with respect to I_F in the presence of the I307Q mutation. Introduction of the I307Q mutation reversed inhibition of K217C by $3\alpha,5\beta$ P, but that did not lead to changes in I_F . We infer that I_F from K217C does not report the functional effect of $3\alpha,5\beta$ P. The application of $3\alpha,5\beta$ PS inhibited currents and I_F from both K217C and $\rho 1$ (I307Q+K217C) mutant receptors. Thus the data do not exclude

the possibility that K217C reports structural changes associated with inhibition by sulfated steroids. The application of 17 β -estradiol reduced peak current but was ineffective at altering I_{F} , indicating that inhibition by 17 β -estradiol does not lead to structural changes at the K217C site.

The data obtained from receptors labeled at the Y241C site were more complex. None of the steroids tested had affected I_{F} from a receptor containing just the Y241C mutation, i.e., inhibition by 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol did not affect GABA-elicited structural changes. With the addition of the I307Q mutation, 3 α 5 β P and 5 β THDOC were rendered potentiating whereas 3 α 5 β P, but not 5 β THDOC, also enhanced the I_{F} . This suggests that potentiation by 3 α 5 β P, but not 5 β THDOC, affects the structural change at the Y241C site. The structures of the two steroids differ in their C17 substituents (methylketone in 3 α 5 β P and α -hydroxymethylketone in 5 β THDOC). Further investigations revealed that steroids with a ketone (etiocholanolone) or carbonitrile (B164) group at C17 did not modify I_{F} in the ρ 1(I307Q+Y241C) receptor, indicating that, of the steroids studied, 3 α 5 β P uniquely modifies conformational changes at the Y241C site. The steroid analogue S84-1 was then tested because of its similarity to 3 α 5 β P at C17 (methylketone). This compound, like 3 α 5 β P, enhanced I_{F} from the ρ 1(I307Q+Y241C) receptor, supporting the idea that the nature of the C17 substituent defines the effect at the Y241C site. Overall, these data suggest that the labeled Y241C residue reports productive conformational changes, i.e., conformational changes associated with the functional effect of a steroid, for 5 β -reduced steroids containing a methylketone group at position C17 (3 α 5 β P and S84-1).

The underlying assumption in our interpretations is that the I307Q substitution does not generate or unmask a novel steroid interaction site. Rather it alters the type of effect exerted by a 5 β -reduced non-sulfated steroid. Indeed, a previous study proposed that the I307 residue plays a role in signal transduction between the transmitter binding sites and the channel gate, or interacts with a sensor domain that translates steroid interaction with the receptor into a functional effect (Morris and Amin 2004). Our data could also be accounted for by a model where potentiation and inhibition are mediated by two independent sites and mechanisms, and the nature of the ρ 307 residue controls the balance between two opposing modulatory effects. In this model, any effect on I_{F} may result from steroid interactions with one of the two sites, regardless of whether that interaction is productive with regards to function.

In sum, the data demonstrate the complexity of molecular mechanisms of how different inhibitory steroids act on the human ρ 1 GABA_A receptor. Despite a common macroscopic functional effect, we count at least four (5 β -reduced steroids with methylketone at C17, 5 β -reduced steroids without methylketone at C17, sulfated steroids, and 17 β -estradiol) mechanisms when taking into consideration how selected mutations modulate inhibition and the structural changes that accompany the actions of steroids. We expect that future studies will refine the map of the GABA receptor-channel to illustrate in greater detail the conformational changes taking place in the presence of modulators.

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Figure 1. Analysis of current and fluorescence responses

The figure shows current (**A**) and fluorescence (**B**) responses from a *Xenopus* oocyte injected with cRNA encoding for the human $\rho 1(I307Q+Y241C)$ receptor. The abscissae depict time (in seconds) in each trace. The ordinates are current (in nA) and fluorescence intensity (in arbitrary fluorescence units). The left traces show raw data, while the right traces show the data after filtering (9 Hz) and baseline-correction. The oocyte was exposed to bath solution for 10 s, followed by a 30 s pulse of 30 mM GABA (a saturating concentration), and a 40 s washout period. The current response was determined as the peak response within “window 1”. In cases where there was no clear peak or the signal-to-noise ratio was low, the current response was determined as the mean current within a window covering a portion of the trace with no apparent change in amplitude. The fluorescence change (F) was determined as the ratio of signal (mean amplitude within window 3) to baseline (mean amplitude within window 2), and is expressed in %.

Figure 2. Effects of P294S and T298F mutations on human $\rho 1$ receptor modulation by $3\alpha 5\beta P$, $3\alpha 5\beta PS$, and 17β -estradiol

(A) Sample current responses from wild-type $\rho 1$ receptors activated by $0.15 \mu M$ GABA ($<EC_{10}$) in the absence and presence of $20 \mu M$ $3\alpha 5\beta P$, $3\alpha 5\beta PS$, or 17β -estradiol. The peak response is inhibited in the presence of steroids. (B) Sample current responses from $\rho 1$ receptors containing the P294S (2' in the second membrane-spanning domain) mutation activated by $0.75 \mu M$ GABA ($<EC_{10}$) in the absence and presence of $20 \mu M$ $3\alpha 5\beta P$, $3\alpha 5\beta PS$, or 17β -estradiol. The peak response is inhibited in the presence of $3\alpha 5\beta PS$ or 17β -estradiol, but not $3\alpha 5\beta P$. (C) Sample current responses from $\rho 1$ receptors containing the T298F (6' in the second membrane-spanning domain) mutation activated by 0.10 – $0.15 \mu M$ GABA ($<EC_{10}$) in the absence and presence of $20 \mu M$ $3\alpha 5\beta P$, $3\alpha 5\beta PS$, or 17β -estradiol. The peak response is enhanced by $3\alpha 5\beta P$ and inhibited by $3\alpha 5\beta PS$. The application of 17β -estradiol has no effect on the peak current. (D) Sample current responses from $\rho 1$ receptors containing the T298F mutation activated by $0.3 \mu M$ GABA (EC_{40-50}) in the absence and presence of $20 \mu M$ $3\alpha 5\beta P$, $3\alpha 5\beta PS$, or 17β -estradiol. The peak response is inhibited in the presence of $3\alpha 5\beta P$ or $3\alpha 5\beta PS$, but not 17β -estradiol. (E) Summary of the findings. The data show the effects of $20 \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol ($\beta E2$) on responses elicited by GABA alone. Data show mean $\pm$ SD from 4–5 cells. Statistical analysis was done with paired t-test (Excel, Microsoft, Redmond, WA) and applies to comparison to no effect. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Figure 3. Effects of steroids on the current and fluorescence responses from human $\rho 1$ receptors labeled with A5m

(A) Structures of the steroids $3\alpha 5\beta P$, $3\alpha 5\beta PS$, and 17β -estradiol. (B) Current and fluorescence responses from K217C receptors activated by $1\ \mu M$ GABA in the absence and presence of $20\ \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol (17β -E2). The top traces show currents (I) and the lower traces show the concomitant changes in fluorescence (F). (C) Current and fluorescence responses from S66C receptors activated by $5\ \mu M$ GABA in the absence and presence of $20\ \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol (17β -E2). (D) Current and fluorescence responses from L166C receptors activated by $60\ \mu M$ GABA in the absence and presence of $20\ \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol (17β -E2). (E) Current and fluorescence responses from Y241C receptors activated by $20\ mM$ GABA in the absence and presence of $20\ \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol (17β -E2). In each case, the application of steroids reduced the peak amplitude of GABA-elicited current response. Drug induced effects on fluorescence are shown with dotted lines. The traces are reproduced with permission from an earlier publication (Li et al. 2010). (F) Summary of the effects of steroids. The data show the effects of $20\ \mu M$ $3\alpha 5\beta P$ (P), $3\alpha 5\beta PS$ (PS), or 17β -estradiol ($\beta E2$) on responses elicited by GABA alone. Data show mean $\pm$ SD from 3–6 cells. Unfilled columns show the effects of steroids on the peak current response. Shaded columns show the effects of steroids on fluorescence change. Statistical analysis was done with

paired t-test (Excel, Microsoft, Redmond, WA) and applies to comparison to no effect. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Figure 4. The effect of the I307Q mutation on modulation by 3α5βP, 3α5βPS, and 17β-estradiol
 The graph shows the effects of 20 μM 3α5βP, 3α5βPS, or 17β-estradiol on peak currents elicited by GABA. GABA concentrations were selected to produce 5–10% of the maximal response (EC₅₋₁₀). Data show mean ± SD from 3–5 cells. Statistical tests (paired t-test) apply to comparison to no effect. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The data indicate that the I307Q mutation, regardless of the presence of a secondary Cys mutation used for labeling, reverses the inhibitory effect of 3α5βP, but does not reverse inhibition by 3α5βPS or 17β-estradiol. No statistical analysis was conducted to compare the effects of steroids among mutations due to small differences in the fractional responses to GABA and uncertainty about the maximal open probability for the mutants. Some receptor-steroid combinations consistently exhibited apparent outward currents when exposed to GABA + steroid, indicative of the presence of spontaneous activity that was inhibited when a steroid was co-applied with GABA. These cases are marked with #, and were not subjected to statistical analysis.

Figure 5. Effects of steroids on the current and fluorescence responses from human $\rho 1$ receptors containing the I307Q mutation labeled with A5m

(A) Current and fluorescence responses from $\rho 1$ (I307Q+K217C) receptors activated by 2 μ M GABA in the absence and presence of 20 μ M 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol. The top traces show currents (I) and the lower traces show the concomitant changes in fluorescence (F). (B) Current and fluorescence responses from $\rho 1$ (I307Q+Y241C) receptors activated by 3 mM GABA in the absence and presence of 20 μ M 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol. The application of 3 α 5 β P enhanced the peak amplitude of GABA-elicited current response while 3 α 5 β PS and 17 β -estradiol reduced it. Drug induced effects are shown with dotted lines. (C) Summary of the effects of steroids. The data show the effects of 20 μ M 3 α 5 β P, 3 α 5 β PS, or 17 β -estradiol on responses elicited by GABA alone. Data show mean $\pm$ SD from 3–9 cells. Unfilled columns show the effects of steroids on the peak current response. Shaded columns show the effects of steroids on fluorescence change. Statistical analysis was done with paired t-test (Excel, Microsoft, Redmond, WA) and applies to comparison to no effect. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

Figure 6. Summary of the effects of steroids on currents and fluorescence from $\rho 1(I307Q + Y241C)$ receptors

(A) Structures of the steroids (3 α ,5 β)-3,21-dihydroxypregnan-20-one (5 β THDOC), (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (B164), etiocholanolone (Etio), and (3 α ,5 β)-3-hydroxymethylpregnan-20-one (S84-1). The structure of 3 α 5 β P is given in Figure 3A. (B) Summary of the effects of steroids on currents and fluorescence changes in $\rho 1(I307Q + Y241C)$ receptors. Data show mean $\pm$ SD from 3–6 cells. Unfilled columns show the effect of 20 μ M 3 α 5 β P, 5 β THDOC, B164, Etio, or S84-1 on the peak current response. Shaded columns show the effects of steroids on fluorescence change. Statistical tests (paired t-test) apply to comparison to no effect. *, $p < 0.05$; ns, not significant.