γ -Aminobutyric Acid Type A $\alpha 4$, $\beta 2$, and δ Subunits Assemble to Produce More Than One Functionally Distinct Receptor Type

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ABSTRACT

Native γ -aminobutyric acid (GABA)_A receptors consisting of $\alpha 4$, $\beta 1$ – 3 , and δ subunits mediate responses to the low, tonic concentration of GABA present in the extracellular milieu. Previous studies on heterologously expressed $\alpha 4\beta\delta$ receptors have shown a large degree of variability in functional properties, including sensitivity to the transmitter. We studied properties of $\alpha 4\beta 2\delta$ receptors employing free subunits and concatemeric constructs, expressed in *Xenopus* oocytes, HEK 293 cells, and cultured hippocampal neurons. The expression system had a strong effect on the properties of receptors containing free subunits. The midpoint of GABA activation curve was 10 nM for receptors in oocytes versus 2300 nM in HEK cells. Receptors activated by the

steroid alfaxalone had an estimated maximal open probability of 0.6 in oocytes and 0.01 in HEK cells. Irrespective of the expression system, receptors resulting from combining the tandem construct $\beta 2$ – δ and a free $\alpha 4$ subunit exhibited large steroid responses. We propose that free $\alpha 4$, $\beta 2$, and δ subunits assemble in different configurations with distinct properties in oocytes and HEK cells, and that subunit linkage can overcome the expression system-dependent preferential assembly of free subunits. Hippocampal neurons transfected with $\alpha 4$ and the picrotoxin-resistant δ (T269Y) subunit showed large responses to alfaxalone in the presence of picrotoxin, suggesting that $\alpha 4\beta\delta$ receptors may assemble in a similar configuration in neurons and oocytes.

Introduction

γ -Aminobutyric acid (GABA)_A receptors are pentameric channel proteins that respond to the binding of the transmitter GABA with an opening of a channel pore that is permeable to Cl[−] and other small anions. GABA_A receptors containing $\alpha 4$, $\beta 1$ – 3 , and δ subunits are mainly located in the cell soma and mediate responses to tonic GABA (Farrant and Nusser, 2005; Belelli et al., 2009). These receptors have high affinity for GABA and, unlike synaptic GABA_A receptors, demonstrate little desensitization even during persistent presence of agonist, in accordance with their predicted role as mediators of a steady inhibitory tone on a neuron (Haas and Macdonald, 1999; Brown et al., 2002; You and Dunn, 2007; Karim et al., 2012).

Functional studies have shown ambiguity regarding the properties of recombinant $\alpha 4\beta\delta$ receptors. For example, there are conflicting reports on the GABA concentration–response relationship. Several studies had observed GABA activation curves for $\alpha 4\beta\delta$ receptors in *Xenopus* oocytes with half-maximal concentrations at 1–3 μ M (Storustovu and Ebert, 2006; You and Dunn, 2007; Hoestgaard-Jensen et al., 2010). In contrast, Karim et al. (2012) found that the GABA activation curve for $\alpha 4\beta 3\delta$

receptors expressed in *Xenopus* oocytes contained two components: one with the concentration of GABA producing a half-maximal response (EC₅₀) at ~10 nM, the other with the EC₅₀ at ~1 μ M. From inhibition by Zn²⁺ and comparison with properties of binary $\alpha 4\beta 3$ receptors, it was proposed that the micromolar component reflected the response of receptors containing only $\alpha 4$ and $\beta 3$ subunits, whereas the high-affinity component represented activity of receptors containing all three ($\alpha 4$, $\beta 3$, and δ) subunits. A single-component GABA activation curve with EC₅₀ at 24 nM was observed for $\alpha 4\beta 1\delta$ receptors (Karim et al., 2012).

Although the lack or incomplete inclusion of the δ subunit in surface receptor complexes could account for conflicting data, another possibility is that the stoichiometry of $\alpha 4\beta\delta$ GABA_A receptors is flexible, dependent on experimental conditions. Work on concatemeric receptors that demonstrate functionality of receptors formed by expression of constructs in which the δ subunit is in a different predicted position in the receptor (Kaur et al., 2009) supports this idea.

To attempt to resolve some of the ambiguities, we studied the activation properties of GABA_A receptors consisting of free $\alpha 4$, $\beta 2$, and δ subunits and various concatemeric constructs. Our data show a GABA activation curve with an EC₅₀ at 10 nM in *Xenopus* oocytes. However, estimated EC₅₀ value for activation by GABA was over 200-fold greater in HEK cells. Furthermore, receptors activated by the steroid alfaxalone (ALF) had an estimated maximal open probability of 0.6 in oocytes, but only

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ABBREVIATIONS: ALF, alfaxalone; DHEA, dehydroepiandrosterone; DS-2, 4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-*a*]pyridine-3-yl]benzamide; GABA, γ -aminobutyric acid; PEB, pentobarbital.

0.01 in HEK cells. The use of concatemeric receptors showed that subunit linkage could overcome the effect of the expression system, thereby suggesting that the preferred stoichiometry or subunit order of $\alpha 4\beta 2\delta$ receptors is different in HEK cells compared with oocytes. Finally, $\alpha 4\beta \delta$ receptors overexpressed in hippocampal neurons displayed similarities to receptors expressed in oocytes rather than HEK cells.

Materials and Methods

Clones, Constructs, Mutagenesis, and Expression. The experiments were conducted on combinations of free and concatemeric $\alpha 4$, $\beta 2$, and δ subunits of the GABA_A receptor. The cDNA for $\alpha 4$ was obtained from P. Whiting (Merck, Harlow, UK); $\beta 2$ was obtained from D. Weiss (University of Texas Health Science Center, San Antonio, TX); and the cDNA for δ was provided by R. Macdonald (Vanderbilt University, Nashville, TN). The species of origin for the $\alpha 4$ subunit is human, whereas the origin of $\beta 2$ and δ subunits is rat. In some experiments, mutated subunits were used. These were $\alpha 4$ (F63C), δ (F74C) (both in loop D at the “ α ” interface of the extracellular domain), and δ (T269Y) (T6’Y in the second membrane-spanning domain). The mutations to loop D residues were made to test the contributions of $\alpha 4$ and δ subunits to receptor activation by GABA (Petrini et al., 2011). The δ (T269Y) mutation was used to confer resistance to picrotoxin (Gurley et al., 1995). The mutations were made using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The δ subunit contained the FLAG epitope in the amino terminus of the subunit (Ueno et al., 1996).

The generation and initial characterization of δ - $\beta 2$ - $\alpha 4$ and $\beta 2$ - $\alpha 4$ concatemeric constructs have been described in an earlier publication (Shu et al., 2012). Additional concatemers, $\beta 2$ - δ - $\beta 2$ and $\beta 2$ - δ - $\alpha 4$, were made as follows. Using the δ - $\beta 2$ - $\alpha 4$ construct as a template, we generated a fragment containing part of δ and all of the $\beta 2$ subunit with additional sequence at the 3’ end encoding the restriction sites XbaI and ApaI for cloning purposes. This fragment and the DNA construct $\beta 2$ - δ were digested with the endonucleases SacII (in δ) and ApaI. The δ sequence was substituted with the δ - $\beta 2$ sequence through standard subcloning methods, generating the $\beta 2$ - δ - $\beta 2$ concatemer. Generation of the $\beta 2$ - δ - $\alpha 4$ construct was done using $\beta 2$ - δ - $\beta 2$ as the recipient vector and a polymerase chain reaction fragment containing part of δ and $\alpha 4$. Both of these were digested with SacII and XbaI, substituting the δ - $\beta 2$ sequence in the parent concatemer with the δ - $\alpha 4$ fragment using standard subcloning methods.

The $\alpha 4$ and δ subunits within concatemeric constructs contained mutations to remove the putative dibasic retention signals in the M3-M4 intracellular loop. The wild-type $\alpha 4$ sequence was modified from MEKAKRKTS to MEAAAVATS. The wild-type δ sequence was modified from ISRRQG to ISAAQG. The mutations were introduced to the δ - $\beta 2$ - $\alpha 4$, $\beta 2$ - δ - $\beta 2$, $\beta 2$ - δ - $\alpha 4$, $\beta 2$ - δ , and $\beta 2$ - $\alpha 4$ constructs to enhance expression levels. We have previously shown that these mutations do not affect activation or basic pharmacological properties of the receptor (Bracamontes et al., 2014).

All cDNAs were subcloned into the pcDNA3 expression vector in the T7 orientation. For expression in *Xenopus laevis* oocytes, the cDNAs were linearized by XbaI (NEB Laboratories, Ipswich, MA) digestion, and cRNAs were produced using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with 3–15 ng cRNA per construct in a final 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 Na pyruvate, 5 mM HEPES, pH 7.4) at 16°C for 1–6 days before recording. The ratio of cRNAs used for injection was 5:1:5 for free $\alpha 4$, $\beta 2$, and δ subunits. It was 5:1 when only $\alpha 4$ and $\beta 2$ subunits were used, and 1:1 in the case of combinations of concatemeric constructs. A relative excess of δ and $\alpha 4$ cRNAs was used to enhance expression of the δ subunit and to reduce formation of $\beta 2$ homo-oligomers.

Transfection of HEK 293 cells was carried out using a calcium phosphate precipitation-based transient transfection technique (Akk,

2002). A total of 3 μ g cDNA in the ratio of 1:1:1 (α : β : δ) was mixed with 12.5 μ l 2.5 M CaCl₂, and dH₂O to a final volume of 125 μ l. In some experiments, a cDNA ratio of 1:1:5 was used. This mixture was added to 125 μ l 2 $\times$ *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffered solution. The combined mixture was incubated at room temperature for 10 minutes, followed by gentle mixing of the contents and an additional 15-minute incubation. The precipitate was added to the cells in a 35-mm dish for overnight incubation at 37°C, followed by replacement of medium in the dish the next day. The experiments were conducted in the course of the next 2 days after changing the medium.

Primary microcultures of hippocampal cells were prepared from 1- to 3-day postnatal Sprague Dawley rats, as described previously (Mennerick et al., 1995). The $\alpha 4$ and δ (T269Y) subunits were transiently expressed in hippocampal microculture neurons 8 days after plating. The cDNA amount was 0.6 μ g $\alpha 4$ and 0.3 μ g δ along with reporter plasmid (green fluorescence protein; Clontech Laboratories, Mountain View, CA) as a positive transfection marker. Lipofectamine2000 (Life Technologies, Carlsbad, CA) was used as transfection reagent, according to the manufacturer’s protocol. Electrophysiological experiments were performed 48–72 hours following transfection.

Electrophysiological Recordings and Data Analysis. Electrophysiological experiments in *Xenopus* oocytes were conducted using standard two-electrode voltage clamp. Voltage and current electrodes were patch-clamp electrodes that when filled with 3 M KCl had resistances of 0.5–1.5 M Ω . The oocytes were clamped at -60 mV. The chamber (RC-1Z; Warner Instruments, Hamden, CT) was perfused continuously at approximately 5 ml min⁻¹. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.4) was perfused between all test applications. Solutions were gravity applied from 30-ml glass syringes with glass Luer slips via Teflon tubing to reduce adsorption, and switched by pClamp using a Warner Instruments VC-8T valve controller. A typical drug application consisted of recording a 10-second baseline, followed by a 30-second drug application and a bath application (up to 10 minutes) until full recovery. 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). The traces were analyzed with Clampfit (Molecular Devices) to determine the peak amplitude of current response.

HEK cells expressing high levels of GABA_A receptors were identified using a bead-binding technique. The amino terminus of the δ subunit had been tagged with the FLAG epitope (Ueno et al., 1996). Surface expression of the FLAG epitope was verified using a mouse monoclonal antibody to the FLAG epitope (M2; Sigma-Aldrich, St. Louis, MO), which had been adsorbed to immunobeads with a covalently attached goat anti-mouse IgG antibody (Life Technologies).

Experiments on HEK cells were conducted on lifted cells using the standard whole-cell voltage clamp technique. The bath solution contained (in mM) the following: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 D-glucose, and 10 HEPES, pH 7.4. The pipette solution contained (in mM) the following: 140 CsCl, 4 NaCl, 4 MgCl₂, 0.5 CaCl₂, 5 EGTA, and 10 HEPES, pH 7.4. The drugs were applied onto the cells using a SF-77B fast perfusion stepper system (Warner Instruments). The solution exchange time, estimated from switching the bath solution from 100 to 75% Ringer solution, was 10–20 ms with a typical HEK cell. A typical drug application consisted of recording a 1-second baseline, followed by a 4- to 8-second drug application and a bath application (up to 1 minute) until full recovery. The cells were clamped at -60 mV. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices), low-pass filtered at 2 kHz, and digitized with a Digidata 1320 series interface (Molecular Devices) at 10 kHz. The analysis of current traces aimed at determining the peak current was conducted using pClamp 9.0 software (Molecular Devices).

Whenever feasible, the current responses are expressed in units of estimated open probability (P_o^{est}) of the channel. The latter is a useful parameter because it reflects a basic physiologic property of an ion channel and is unbiased by caveats inherent in normalization to the response to highest concentration of the activator. The theoretical range

of P_o spans from 0 to 1. The conversion of raw data to P_o^{est} involved determination of a drug combination whose response could no longer be enhanced by the δ -specific potentiator 4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-*a*]pyridine-3-yl]benzamide (DS-2) or the steroid ALF, and then normalization to the response to that drug combination. Such an approach has been described previously (Rusch et al., 2004). For example, in the case of $\alpha 4\beta 2\delta$ receptors, we observed that responses to saturating pentobarbital (PEB) were larger than responses to any other agonist employed, and that the responses to saturating (0.5 mM) PEB could not be enhanced by DS-2 or ALF. Accordingly, we considered the response to 0.5 mM PEB to have a P_o^{est} of ~ 1 . Peak responses to GABA or neurosteroids were then normalized to the response to 0.5 mM PEB for expression in units of P_o^{est} . The saturating concentration of PEB was determined for each receptor type individually. In oocytes expressing the combination of δ - $\beta 2$ - $\alpha 4$ and $\beta 2$ - $\alpha 4$ constructs, responses to PEB saturated at 2 mM. Furthermore, responses to saturating PEB could be further potentiated by 1 μ M DS-2. Accordingly, the response to 2 mM PEB + DS-2 was considered to have a P_o^{est} of ~ 1 , to which responses to GABA and steroid were normalized.

This approach was adopted to remove some of the ambiguity inherent when responses are simply normalized to the maximal response to particular agonists, and so to allow more straightforward comparisons among constructs. However, we note that there is one essential caveat: the accuracy of this normalization requires that the maximal (potentiated) response have an open probability approaching 1. Two factors could invalidate this assumption. One is experimental. If the peak response is experimentally underestimated due to slow perfusion (for example, for the rebound tail resulting from unblock), then the maximal P_o^{est} for GABA would be artifactually increased. The second reason arises from the possibility that the mechanism of potentiation might be affected in some constructs so that potentiators were inefficacious. In this case, again, the potentiated response would not necessarily approach a P_{open} of 1. In the case of $\alpha 4\beta 2\delta$ receptors expressed in HEK cells, direct evidence from single-channel recordings demonstrates a low-maximal P_{open} for GABA (Akk et al., 2004). In the case of other constructs, there is no direct evidence. However, we believe that the use of P_o^{est} provides a more consistent parameter for comparisons among different constructs.

Pentobarbital blocks GABA_A receptors at millimolar concentrations, resulting in a characteristic response with a small initial peak, followed by a large tail or rebound response upon the termination of application of PEB (Akaike et al., 1987). The tail response reflects transient repopulation of the conducting state(s) of the channel. In situations in which the amplitude of the tail response was greater than the initial peak response (e.g., Fig. 2B), we used the tail or rebound response that reflects recovery from block, rather than peak response for comparing current amplitudes.

For recordings from neurons, the coverslips with hippocampal microculture neurons were transferred from culture medium to an extracellular recording solution consisting of (in mM) the following: 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH 7.25. Transfected hippocampal microculture neurons were selected for electrophysiology based on green fluorescence protein. Patch pipettes were filled with an internal solution containing (in mM) the following: 130 CsCl, 4 NaCl, 5 EGTA, 0.5 CaCl₂, and 10 HEPES, pH 7.25. When filled with this solution, pipette tip resistance was 3–6 M Ω . Cells were clamped at -70 mV. Drug applications were made with a multibarrel, gravity-driven local perfusion system. The estimated solution exchange times were 120 ± 14 ms (10–90% rise), measured by the change in junction currents at the tip of an open patch pipette. Currents were filtered at 5 kHz and recorded with an Axopatch 200B amplifier (Molecular Devices). All recordings were performed at room temperature.

Where applicable, the effects of drugs are presented in percentage of control, that is, 100% represents no effect. Parameters are shown as mean $\pm$ S.D. for responses. The Hill equation was fit to the mean data for a given situation (constructs, drugs), and the best-fitting parameter value is given $\pm$ the estimated S.E. of the fit provided by the fitting program.

Chemicals. GABA, pentobarbital, and the inorganic salts were purchased from Sigma-Aldrich (St. Louis, MO). DS-2 was purchased from R&D Systems (Minneapolis, MN). The steroids were from Sigma-Aldrich, R&D Systems, or Steraloids (Newport, RI).

Results

Activation of $\alpha 4\beta 2\delta$ Receptors by GABA in *Xenopus* oocytes. We commenced by examining activation of $\alpha 4\beta 2\delta$ GABA_A receptors by the transmitter GABA. Oocytes injected with cRNAs for $\alpha 4$, $\beta 2$, and δ subunits exhibited robust responses to GABA. In the presence of saturating GABA (300 nM), the peak response was 2265 ± 829 nA (mean $\pm$ S.D., 7 cells, range: 1427–3922 nA). The concentration-response relationship for GABA was examined by exposing oocytes to 1–300 nM GABA. Sample traces are shown in Fig. 1A. Curve fitting to the Hill equation yielded an EC₅₀ of 10.3 ± 0.1 nM and a Hill coefficient of 0.8 ± 0.1 (best-fitting parameter $\pm$ uncertainty of the fit; 7 cells; Fig. 1B). In three cells, the GABA concentration range was extended to 30 μ M. In these cells, the relative mean peak responses to 1, 3, 10, and 30 μ M GABA were 92 ± 14 , 94 ± 17 , 93 ± 23 , and $93 \pm 23\%$ (mean $\pm$ S.D.), respectively, of the mean peak response to 300 nM GABA. Incorporation of data from these cells had only a small

Fig. 1. Activation of $\alpha 4\beta 2\delta$ receptors expressed in *Xenopus* oocytes. (A) Representative current responses in the presence of 1, 3, 10, 100, and 300 nM GABA. All traces are from the same oocyte. (B) A GABA concentration-response relationship for receptors formed from free $\alpha 4$, $\beta 2$, and δ subunits. The data points show mean $\pm$ S.E.M. for data from seven cells. The data are in units of P_o^{est} that was estimated by comparing responses to GABA to a response to saturating PEB in the same cells (see Materials and Methods for more details). The line through the points shows the predictions of the Hill equation fit to the means, yielding an EC₅₀ of 10.3 ± 0.1 nM and a Hill coefficient of 0.8 ± 0.1 (best-fit parameter $\pm$ uncertainty of the fit). The dashed line shows a fit resulting from the inclusion of data (small circles) obtained from three cells for which the range of GABA concentrations was extended to 30 μ M. Best-fit parameters $\pm$ uncertainty were EC₅₀ = 7 ± 0.9 nM, $n_H = 0.9 \pm 0.1$. (C) Comparison of responses to saturating GABA (G; 300 nM) and saturating PEB (0.5 mM). Both traces are from the same oocyte. (D) Comparison of responses to 0.5 mM PEB in the absence and presence of 1 μ M DS-2. Both traces are from the same oocyte.

effect on the concentration-response relationship (the revised EC_{50} was 7 ± 0.9 nM, and the revised Hill coefficient was 0.9 ± 0.1 ; dashed line in Fig. 1B). We infer that the GABA concentration-response relationship for $\alpha 4\beta 2\delta$ receptors expressed in *Xenopus* oocytes contains a single, nanomolar component.

The δ subunit is notoriously difficult to express in heterologous expression systems (Meera et al., 2010), and receptors containing just $\alpha 4$ and β subunits are functional (Meera et al., 2011; Karim et al., 2012). The $\alpha 4\beta 2$ receptors may have contributed to, or even underlain, the observed current responses. To confirm the presence of δ subunit in surface receptors, we coapplied 1 μ M DS-2, a δ -specific potentiator (Wafford et al., 2009), with 300 nM GABA. In three cells, from among those included in the analysis of the concentration-response relationship, the mean peak response in the presence of DS-2 was $166 \pm 36\%$ of control, that is, of the response to GABA alone. We used a saturating, rather than subsaturating, concentration of GABA to reduce variability originating from cell-to-cell differences in the GABA EC_{50} .

Further confirmation of the presence of the δ subunit in receptor complexes became apparent from comparing the concentration-response relationships for oocytes injected with $\alpha 4$, $\beta 2$, and δ subunits versus $\alpha 4$ and $\beta 2$ subunits. In oocytes injected with cRNAs for only $\alpha 4$ and $\beta 2$ subunits, the GABA concentration-response relationship was shifted by 70-fold to higher GABA concentrations (Table 1). We estimate that for $\alpha 4\beta 2$ receptors the EC_{50} is 0.71 ± 0.32 μ M (4 cells).

Previous single-channel work has suggested that the maximal open probability of $\alpha 4\beta 2\delta$ receptors expressed in HEK cells in the presence of GABA is relatively low (0.1–0.2), whereas that in the presence of saturating pentobarbital (PEB) reaches ~ 1 (Akk et al., 2004; Bracamontes et al., 2014). To provide an estimate for the relative open probabilities in oocytes, we calculated a parameter P_o^{est} , as described in *Materials and Methods*. To do this, we determined the maximal response we could elicit from a cell using high concentrations of an agonist in the presence of a strong potentiator. We assumed that this response reflected a probability of being open close to 1. By normalizing to this maximal response, it was then possible to compare the efficacy of GABA more accurately among the various constructs studied. However, we note that there are some caveats associated with this procedure, as discussed in *Materials and Methods*. To determine the P_o^{est} of $\alpha 4\beta 2\delta$ expressed in oocytes, we compared peak responses

elicited by 300 nM GABA or 0.5 mM PEB (a saturating concentration; data not shown). In three cells, the relative response to GABA was $53 \pm 13\%$ of the response to PEB. Sample traces are shown in Fig. 1C. To confirm the P_o^{est} for PEB, we probed the effect of DS-2 and the steroid ALF on the peak response to 0.5 mM PEB. We reasoned that receptors that open with a P_o near 1 lack the dynamic range to be potentiated by drugs acting through changes in kinetics (Colquhoun, 1998; Rusch et al., 2004). Coapplication of 1 μ M DS-2 with 0.5 mM PEB was without effect on the peak response ($102 \pm 8\%$ of control, 5 cells; Fig. 1D). Coapplication of 10 μ M ALF with PB was also without effect on peak current ($99 \pm 14\%$, 5 cells). We infer that the maximal P_o^{est} in the presence of PEB is ~ 1 , and that the maximal P_o^{est} of GABA-activated $\alpha 4\beta 2\delta$ receptors in oocytes is ~ 0.5 .

Activation of $\alpha 4\beta 2\delta$ Receptors by GABA in HEK 293 Cells. Studies examining properties of $\alpha 4\beta 2\delta$ receptors in HEK cells have reported a GABA EC_{50} in the micromolar range (Bracamontes et al., 2014; Patel et al., 2014). To confirm these prior findings, we examined GABA activation properties of $\alpha 4\beta 2\delta$ receptors transiently expressed in HEK 293 cells. In four cells, the estimated EC_{50} was 2.3 ± 0.4 μ M and the Hill coefficient 1.3 ± 0.3 (Fig. 2, A and B). The presence of the δ subunit in surface receptors was demonstrated by potentiation of receptors activated by 50 μ M GABA by 1 μ M DS-2 ($160 \pm 23\%$ of control, 5 cells).

To determine the maximal P_o^{est} for GABA, we compared responses to 50 μ M GABA and 1 mM PEB (Fig. 2C). The mean ratio of the tail current observed upon the termination of the application of PEB to the peak response in the presence of GABA was 5 ± 3 (7 cells). Coapplication of 100 μ M PEB with 50 μ M GABA potentiated the peak response by 5 ± 2 (7 cells). These data suggest that the P_o^{est} in the presence of saturating GABA is 0.2. This is consistent with our previous single-channel work on $\alpha 4\beta 2\delta$ receptors in HEK cells (Akk et al., 2004).

We probed whether changes in transfection ratio affect activation properties of $\alpha 4\beta 2\delta$ receptors in HEK cells. Specifically, we wished to test whether a fivefold increase in cDNA for δ shifts the GABA concentration-response relationship to lower agonist concentrations and enhances P_o^{est} in the presence of saturating GABA. In cells transfected in the ratio of 1:1:5 ($\alpha 4$: $\beta 2$: δ), the estimated EC_{50} was 3.5 ± 0.3 μ M and the Hill coefficient 1.1 ± 0.1 (4 cells). The mean ratio of the tail current upon termination of the application of PEB to the maximal response to GABA was 4 ± 3 (4 cells). We infer that HEK cells transfected with $\alpha 4\beta 2\delta$ in the ratio of 1:1:1 and 1:1:5 have similar activation properties.

Activation of Concatemeric Receptors by GABA in *Xenopus* Oocytes. In the next set of experiments, we determined whether activation properties of free subunit-containing receptors can be replicated in concatemers that impose a specific order and stoichiometry of subunits. To imitate a receptor containing two $\alpha 4$ subunits, two $\beta 2$ subunits, and one δ subunit, arranged in the counterclockwise order $\delta\beta 2\alpha 4\beta 2\alpha 4$, we generated $\delta\beta 2\alpha 4$ and $\beta 2\alpha 4$ concatemeric constructs (Shu et al., 2012). Oocytes injected with cRNAs for $\delta\beta 2\alpha 4$ and $\beta 2\alpha 4$ constructs demonstrated peak currents of 186 ± 91 nA (range: 102–324 nA; 6 cells) in the presence of 100 μ M GABA. Responses to 100 μ M GABA were potentiated by 1 μ M DS-2 to $436 \pm 143\%$ of control (6 cells), indicating the presence of δ subunit in concatemeric receptor complexes and a low P_o in the presence of saturating GABA. Curve fitting of

TABLE 1

Activation properties of receptors expressed in *Xenopus* oocytes

The table gives the EC_{50} (best-fit parameter $\pm$ uncertainty of the fit) and Hill coefficients (n_H) of GABA concentration-response curves fitted to averaged data from four to six cells using the Hill equation. I_{GABA} (mean $\pm$ S.D.) was determined as the peak response to the highest GABA concentration tested in concentration-response measurements. Maximal P_o^{est} was calculated as ratio of I_{GABA} to the response to saturating concentration of pentobarbital in the absence or presence of DS-2, whichever generated the largest response. In the case of $\alpha 4\beta 2$ receptors that are not potentiated by DS-2, alfaxalone replaced DS-2. Maximal P_o^{est} provides an estimate of gating efficacy of GABA.

Receptor	GABA EC_{50}	n_H	I_{GABA}	Maximal P_o^{est}
	μ M		nA	
$\alpha 4\beta 2\delta$	0.010 ± 0.001	0.8 ± 0.1	2265 ± 829	0.53
$\delta\beta 2\alpha 4 + \beta 2\alpha 4$	3.5 ± 0.6	1.3 ± 0.2	186 ± 91	0.06
$\beta 2\alpha 4 + \delta$	4.6 ± 0.2	1.2 ± 0.1	325 ± 176	0.08
$\beta 2\delta\beta 2 + \beta 2\alpha 4$	1.2 ± 0.7	0.9 ± 0.4	8 ± 3	0.08
$\beta 2\delta\alpha 4 + \beta 2\alpha 4$	55 ± 17	1.3 ± 0.4	66 ± 73	0.13
$\beta 2\delta + \alpha 4$	0.17 ± 0.02	1.0 ± 0.1	1131 ± 221	0.46
$\alpha 4\beta 2$	0.71 ± 0.32	0.7 ± 0.2	770 ± 330	0.31

Fig. 2. Activation of $\alpha 4\beta 2\delta$ receptors expressed in HEK 293 cells. (A) Representative current responses in the presence of 0.5, 1, 2, 5, 20, and 50 μM GABA. All traces are from the same cell. (B) A GABA concentration-response relationship for receptors formed from free $\alpha 4$, $\beta 2$, and δ subunits. The data points show mean $\pm$ S.E.M. for data from four cells. The data are in units of P_o^{est} that was estimated by comparing responses to GABA to a response to saturating PEB in the same cells (see *Materials and Methods* for more details). The curve was fitted to the Hill equation yielding an EC_{50} of 2.3 ± 0.4 μM and a Hill coefficient of 1.3 ± 0.3 (best-fit parameter $\pm$ uncertainty of the fit). (C) Comparison of responses to saturating GABA (50 μM) and saturating PEB (1 mM). Both traces are from the same cell.

concentration-response data from four cells yielded an EC_{50} of 3.5 ± 0.6 μM and a Hill coefficient of 1.3 ± 0.2 (Fig. 3; Table 1).

We also tested receptors resulting from combining the $\beta 2$ - $\alpha 4$ construct with a free δ subunit. We presumed that such receptors assemble as two pairs of the tandem construct and a single free δ subunit per receptor, in a stoichiometry similar to the combination of δ - $\beta 2$ - $\alpha 4$ and $\beta 2$ - $\alpha 4$. For $\beta 2$ - $\alpha 4$ + free δ receptors, the GABA EC_{50} was 4.6 ± 0.2 μM and the Hill coefficient was 1.2 ± 0.1 (5 cells). Thus, GABA activation curves of concatemeric receptors expected to assemble as $\delta\beta 2\alpha 4\beta 2\alpha 4$ are right-shifted, compared with free $\alpha 4\beta 2\delta$ receptors, by over 300-fold.

In δ - $\beta 2$ - $\alpha 4$ + $\beta 2$ - $\alpha 4$ receptors, 1 μM DS-2 potentiated the maximal response to saturating PEB (2 mM) to $177 \pm 45\%$ of control (5 cells). From this, we calculate a P_o^{est} of 0.6 (i.e., 1.77^{-1}) for δ - $\beta 2$ - $\alpha 4$ + $\beta 2$ - $\alpha 4$ receptors in the presence of PEB. The mean peak response to 100 μM GABA was $10 \pm 4\%$ (14 cells) of the mean response to 2 mM PEB. Accordingly, the P_o^{est} of δ - $\beta 2$ - $\alpha 4$ + $\beta 2$ - $\alpha 4$ receptors activated by saturating GABA is 0.06. Employing a similar approach, we determined that the maximal P_o^{est} of $\beta 2$ - $\alpha 4$ + δ receptors is 0.45 in the presence of PEB, and 0.08 in the presence of GABA. The P_o^{est} values in the presence of both PEB and GABA are lower for linked $\delta\beta 2\alpha 4\beta 2\alpha 4$ than for free $\alpha 4\beta 2\delta$ receptors.

Concatemers in $\beta 3\delta\beta 3\beta 3\alpha 1$ and $\beta 3\delta\alpha 1\beta 3\alpha 1$ configurations produce functional receptors (Kaur et al., 2009). We created concatemeric constructs $\beta 2$ - δ - $\beta 2$ and $\beta 2$ - δ - $\alpha 4$ that when combined with the $\beta 2$ - $\alpha 4$ tandem construct were expected to assemble in the analogous $\beta 2\delta\beta 2\beta 2\alpha 4$ and $\beta 2\delta\alpha 4\beta 2\alpha 4$ configurations, respectively. Both combinations produced receptors that were activated by GABA, although the peak responses were smaller than with other concatemers or with free subunits. The maximal peak currents in the presence of saturating GABA were 8 ± 3 nA (6 cells) and 66 ± 73 nA (6 cells) in $\beta 2$ - δ - $\beta 2$ + $\beta 2$ - $\alpha 4$ and $\beta 2$ - δ - $\alpha 4$ + $\beta 2$ - $\alpha 4$, respectively. The GABA EC_{50} was 1.2 ± 0.7 μM and the Hill coefficient 0.9 ± 0.4 (from 6 cells) for $\beta 2$ - δ - $\beta 2$ + $\beta 2$ - $\alpha 4$. Receptors consisting of $\beta 2$ - δ - $\alpha 4$ + $\beta 2$ - $\alpha 4$ had an EC_{50} of 55 ± 17 μM and a Hill coefficient of 1.3 ± 0.4 (6 cells) in the presence of GABA. The concentration-response curves for the $\alpha 4\beta 2\delta$ and concatemeric receptors are shown in Fig. 3, and the data are summarized in Table 1.

Coapplication of 1 μM DS-2 with saturating PEB (5 mM) enhanced the maximal response to $174 \pm 42\%$ (4 cells) in $\beta 2$ - δ - $\beta 2$ + $\beta 2$ - $\alpha 4$. Responses elicited by 5 mM PEB from $\beta 2$ - δ - $\alpha 4$ + $\beta 2$ - $\alpha 4$ were potentiated to $123 \pm 18\%$ of control (5 cells) by

Fig. 3. GABA concentration-response relationship of receptors expressed in *Xenopus* oocytes. GABA concentration-response properties for receptors formed from a combination of the concatemeric $\beta 2$ - δ construct and free $\alpha 4$ subunit, a combination of δ - $\beta 2$ - $\alpha 4$ and $\beta 2$ - $\alpha 4$ concatemeric constructs, a combination of the concatemeric $\beta 2$ - $\alpha 4$ construct and free δ subunit, a combination of $\beta 2$ - δ - $\beta 2$ and $\beta 2$ - $\alpha 4$ concatemers, or a combination of $\beta 2$ - δ - $\alpha 4$ and $\beta 2$ - $\alpha 4$ concatemers. The data points show mean $\pm$ S.E.M. for data from four to six cells. The data are in units of P_o^{est} that was estimated by comparing the responses to GABA to a response to saturating PEB $\pm$ 1 μM DS-2, whichever generated the largest response (see *Materials and Methods* for more details). The curves were fitted to the Hill equation. Fitting results are summarized in Table 1. The dashed line replicates data for receptors formed from free $\alpha 4$, $\beta 2$, and δ subunits (Fig. 1B).

DS-2. In both cases, we focused on the rebound current rather than the small initial peak response due to the latter being inhibited by channel block at such a high PEB concentration. The P_o^{est} in the presence of saturating PEB was 0.6 for $\beta 2\text{-}\delta\text{-}\beta 2 + \beta 2\text{-}\alpha 4$ and 0.8 for $\beta 2\text{-}\delta\text{-}\alpha 4 + \beta 2\text{-}\alpha 4$ receptors. By comparing the peak responses to saturating GABA and PEB, we calculated the P_o^{est} for GABA as 0.08 and 0.13 in $\beta 2\text{-}\delta\text{-}\beta 2 + \beta 2\text{-}\alpha 4$ and $\beta 2\text{-}\delta\text{-}\alpha 4 + \beta 2\text{-}\alpha 4$, respectively. We infer that concatemeric receptors predicted to assemble in $\beta 2\delta\beta 2\beta 2\alpha 4$ and $\beta 2\delta\alpha 4\beta 2\alpha 4$ configurations do not functionally resemble receptors containing free $\alpha 4\beta 2\delta$ subunits expressed in oocytes.

Lastly, we tested the combination of a tandem construct $\beta 2\text{-}\delta$ and a free $\alpha 4$ subunit. An application of 3 μM GABA elicited responses with a mean peak current of 1131 ± 221 nA (6 cells). Curve fitting of current responses obtained at 10 nM to 10 μM GABA gave an EC_{50} of 0.17 ± 0.02 μM and a Hill coefficient of 1.0 ± 0.1 . The estimate for gating efficacy was obtained by first determining the effect of DS-2 on receptors activated by saturating PEB (2 mM), and then by comparing peak responses to saturating GABA and PEB + DS-2. Coapplication of 1 μM DS-2 enhanced the response to 2 mM PEB to $157 \pm 48\%$ of control (4 cells). The peak response to 3 μM GABA was $46 \pm 6\%$ of the response to PEB + DS-2 (6 cells). The maximal P_o^{est} of $\beta 2\text{-}\delta$ + free $\alpha 4$ combination is calculated as 0.64 or 0.46 in the presence of saturating PEB or GABA, respectively. With regard to apparent affinity and gating efficacy in the presence of GABA, the combination of the $\beta 2\text{-}\delta$ tandem and free $\alpha 4$ subunit produced a receptor that most closely resembled the receptor consisting of free $\alpha 4\beta 2\delta$ subunits in oocytes.

Right-shifted concentration-response curves have been observed for concatemeric GABA_A receptors previously (Baumann et al., 2002; Akk et al., 2009; Kaur et al., 2009). To determine whether the presence of the linker sequence could account for the difference in GABA EC_{50} observed for free $\alpha 4\beta 2\delta$ (10 nM) and $\beta 2\text{-}\delta$ + free $\alpha 4$ (170 nM), we created a free (nonlinked) $\beta 2$ subunit that had the linker sequence attached to its carboxy terminus. Expression of this subunit with wild-type $\alpha 4$ and δ subunits produced receptors with a GABA EC_{50} at 518 ± 74 nM (4 cells). We infer that the mere presence of a linker sequence on the $\beta 2$ subunit can affect properties of activation by GABA.

Activation of $\alpha 4\beta 2\delta$ Receptors by Neuroactive Steroids in *Xenopus* Oocytes. Neuroactive steroids, although best known as potentiators of GABA-activated receptors, can also directly activate the GABA_A receptor through allosteric binding site(s). Steroids are considered low-efficacy agonists of the GABA_A receptor (Shu et al., 2004; Hosie et al., 2006).

To our surprise, several steroids elicited large currents from $\alpha 4\beta 2\delta$ receptors in oocytes. Receptors exposed to 10 μM ALF generated a peak response that was $51 \pm 18\%$ (27 cells) of the response to saturating PEB. Activation by 10 μM 3α -hydroxy- 5α -pregnan-20-one ($3\alpha 5\alpha\text{P}$) or 3α -hydroxy- 5β -pregnan-20-one ($3\alpha 5\beta\text{P}$) also resulted in prominent responses ($42 \pm 18\%$, 8 cells, and $45 \pm 19\%$, 4 cells, respectively). Ten micromolar is a saturating concentration for these steroids in terms of potentiation and activation (Shu et al., 2004), and is near the limit of solubility in aqueous solutions (Brewster et al., 1989). Testosterone, 17β -estradiol, dehydroepiandrosterone (DHEA), pregnenolone sulfate, and DHEA sulfate did not elicit current responses, although in some cases (e.g., in the presence of 10 μM DHEA sulfate) rebound currents following termination of steroid application were observed. Initial outward currents in the presence of DHEA sulfate and pregnenolone sulfate suggest

the presence of spontaneous channel activity that was blocked by the sulfated steroids. Contributions from spontaneously active receptors were not taken into consideration in data analysis. Sample traces are shown in Fig. 4A.

We examined the concentration-response relationship for ALF by exposing oocytes expressing $\alpha 4\beta 2\delta$ receptors to 10 nM–10 μM ALF (Fig. 4, B and C). For normalization, each cell was also exposed to 0.5 mM PEB. Curve fitting of combined data from six cells to the Hill equation yielded an EC_{50} of 0.9 ± 0.8 μM and a maximal fitted relative response of $61 \pm 13\%$ (maximal $P_o^{est} \sim 0.6$).

We considered the possibility that large steroid responses resulted from a population of receptors other than the intended $\alpha 4\beta 2\delta$. First, we tested omission of the δ subunit. Oocytes were injected with cRNAs for the $\alpha 4$ and $\beta 2$ subunits. Exposure to 2 mM PEB produced large currents with a P_o^{est} of nearly 1 (no potentiation by ALF). Exposure to 10 μM ALF alone, however, was essentially without effect ($0 \pm 5\%$ of the response to PEB, 8 cells). Sample currents are shown in Fig. 5. Homomeric $\beta 2$ receptors expressed in oocytes are activated by PEB (Cestari et al., 1996). Exposure of oocytes expressing the $\beta 2$ subunit to 10 μM ALF resulted in a small outward current, likely reflecting closure or blockade of spontaneous activity from $\beta 2$ GABA_A receptors. Pentobarbital elicited robust responses from the same set of cells (Fig. 5). We also tested the combination of $\alpha 4$ and δ subunits. In three oocytes injected with cRNAs for $\alpha 4$ and δ , no currents were observed in response to application of 0.5 mM PEB or 10 μM ALF (data not shown).

We further tested subunit specificity by replacing the $\alpha 4$ subunit with $\alpha 1$, and, in another set of experiments, by replacing δ with the $\gamma 2$ subunit. In oocytes expressing $\alpha 1\beta 2\delta$ receptors, 10 μM ALF elicited currents that were $31 \pm 4\%$ (4 cells) of the response to saturating (0.5 mM) PEB. In contrast, 10 μM ALF elicited a response that was $8 \pm 2\%$ (3 cells) of the response to saturating (2 mM) PEB in oocytes expressing $\alpha 4\beta 2\gamma 2\text{L}$ receptors. We infer that steroids selectively activate δ -containing receptors.

To determine whether currents elicited by ALF are mediated by the classic steroid binding site, we probed the effect of the $\alpha 4$ (Q246L) mutation. A previous study had shown that mutations to the homologous residue in the $\alpha 1$ subunit (Q241) reduce gating efficacy of neurosteroids on $\alpha 1\beta 2\gamma 2$ GABA_A receptors, possibly by eliminating a critical hydrogen bond (Hosie et al., 2006). Our experiments indicate that the $\alpha 4$ (Q246L) mutation reduces the gating efficacy of ALF in $\alpha 4\beta 2\delta$ receptors. The relative peak response to 10 μM ALF was $1 \pm 0.4\%$ (5 cells) of the response to saturating (1 mM) PEB in receptors containing the mutation. Incidentally, this also demonstrates the presence of the $\alpha 4$ subunit in receptors producing steroid responses.

Activation of Concatemeric Receptors by the Steroid ALF in *Xenopus* Oocytes. We next compared activation of concatemeric receptors by the steroid ALF. Oocytes expressing $\delta\text{-}\beta 2\text{-}\alpha 4 + \beta 2\text{-}\alpha 4$ constructs showed small responses to ALF. The mean response to 10 μM ALF was only $0.4 \pm 0.2\%$ (6 cells) of the response to 2 mM PEB. The combination of $\beta 2\text{-}\alpha 4$ tandem + a free δ subunit showed similar lack of sensitivity to ALF, having a relative ALF response of $1 \pm 1\%$ (4 cells). The $\beta 2\text{-}\delta\text{-}\beta 2 + \beta 2\text{-}\alpha 4$ receptors produced responses to 10 μM ALF that were $4 \pm 1\%$ (6 cells) of the response to saturating PEB. The relative steroid response from the $\beta 2\text{-}\delta\text{-}\alpha 4 + \beta 2\text{-}\alpha 4$ combination was $1 \pm 1\%$ (6 cells). Sample traces are shown in Fig. 5.

Fig. 4. Activation by steroids of $\alpha 4\beta 2\delta$ receptors expressed in *Xenopus* oocytes. Sample traces from oocytes expressing free $\alpha 4$, $\beta 2$, and δ subunits. (A) The oocytes were exposed to 10 μ M ALF, 3 α -hydroxy-5 α -pregnan-20-one (3 α 5 α P), 3 α -hydroxy-5 β -pregnan-20-one (3 α 5 β P), DHEA, DHEA sulfate (DHEAS), pregnenolone sulfate (PS), 17 β -estradiol (17 β -E), or testosterone (T). For comparison, a response to 0.5 mM PEB in the same oocyte is shown next to the steroid response. The initial outward currents during application of DHEAS and PS are most likely due to block of spontaneous activity. (B) Representative traces from an oocyte expressing free $\alpha 4$, $\beta 2$, and δ subunits, and exposed to 0.01–10 μ M alfaxalone. For comparison, a response to saturating (0.5 mM) pento-barbital from the same oocyte is also shown. (C) Alfaxalone concentration-response relationship for $\alpha 4\beta 2\delta$ receptors. The peak currents were normalized to response to 0.5 mM PEB in the same cells. The data points give mean $\pm$ S.E.M. for data from six cells. The curve was fitted to the Hill equation yielding an EC_{50} of 0.9 ± 0.8 μ M and a Hill coefficient of 0.7 ± 0.2 (best-fit parameter $\pm$ uncertainty of the fit). The maximal fitted response was 0.61 ± 0.13 .

Receptors generated from combining $\beta 2$ - δ tandems + free $\alpha 4$ subunits produced large currents in the presence of steroid (Fig. 5). The mean peak current in the presence of 10 μ M ALF was $32 \pm 22\%$ (7 cells) of the response to 2 mM PEB + 1 μ M DS-2, determined to elicit currents with P_0^{est} near 1. Thus, direct gating of free $\alpha 4\beta 2\delta$ by ALF is best replicated by the combination of $\beta 2$ - δ + free $\alpha 4$.

Activation of $\alpha 4\beta 2\delta$ Receptors by ALF in HEK Cells. In HEK cells, direct activation by ALF was examined on two forms of the $\alpha 4\beta 2\delta$ receptor. First, we studied free $\alpha 4\beta 2\delta$ subunits. This combination gave rise to small currents in the presence of 10 μ M ALF. The mean peak response was only $0.6 \pm 0.9\%$ (6 cells) of the response to saturating PEB.

We also tested receptors resulting from the combination of the $\beta 2$ - δ tandem and a free $\alpha 4$ subunit. This combination produced relatively large steroid currents. The mean peak response to 10 μ M ALF was $23 \pm 5\%$ (5 cells) of the response to 2 mM PEB. We infer that the combination of $\beta 2$ - δ + free $\alpha 4$ has similar properties when expressed in oocytes and HEK cells.

Configuration of the $\alpha 4\beta 2\delta$ Receptor. A previous study of concatemeric GABA_A receptors proposed that the δ subunit may substitute for α at the negative side of the GABA-binding interface (Kaur et al., 2009; Karim et al., 2012). To explore the configuration of the $\alpha 4\beta 2\delta$ receptor, we mutated a conserved phenylalanine residue in loop D to cysteine. This mutation in the $\alpha 1$ subunit ($\alpha 1F64C$) has been shown to effectively abolish activation by GABA without affecting gating by PEB in $\alpha 1\beta 1\gamma 2$ receptors (Petrini et al., 2011).

HEK cells expressing $\alpha 4\beta 2\delta$ receptors containing the $\alpha 4$ (F63C) mutation demonstrated essentially no currents in the presence of GABA up to 10 mM. The ratio of the current amplitude in the presence of 10 mM GABA to that in the presence of 3 mM PEB was $\sim 0.5\%$. In contrast, the $\alpha 4(F63C)\beta 2\delta$ receptors expressed in oocytes demonstrated robust currents with a midpoint of the GABA concentration-response curve at 198 ± 69 μ M (5 cells). The maximal P_0^{est} in the presence of GABA (0.29 ± 0.15) was slightly reduced compared with that of the wild-type $\alpha 4\beta 2\delta$ receptor.

Fig. 5. Alfaxalone selectively activates the $\alpha 4\beta 2\delta$ and $\beta 2$ - δ + $\alpha 4$ receptors in *Xenopus* oocytes. Representative traces from oocytes expressing free $\alpha 4$, $\beta 2$, and δ subunits and various combinations of concatemeric and free subunits in the presence of 10 μ M ALF. For comparison, a response to a drug mix (PEB in the absence or presence of DS-2) eliciting a P_0^{est} of 1 in the same cell is shown next to the steroid response. The concentration of DS-2 was 1 μ M. Comparison of the peak responses within each pair provides insight into gating efficacy of alfaxalone. The steroid activated free $\alpha 4\beta 2\delta$ subunits and the combination of $\beta 2$ - δ + free $\alpha 4$ with high efficacy.

To explore a potential contribution of the δ subunit to the “-” interface of the GABA binding site, we made the F-to-C mutation at the homologous site in the δ subunit. Expression of the δ (F74C) subunit with wild-type $\alpha 4$ and $\beta 2$ in HEK cells produced receptors that showed increased sensitivity to GABA. The EC_{50} of the GABA concentration-response curve was $1.1 \pm 0.2 \mu\text{M}$, and the P_o^{est} in the presence of saturating GABA was 0.35 (5 cells). In contrast, $\alpha 4\beta 2\delta$ (F74C) receptors expressed in *Xenopus* oocytes had a right-shifted GABA concentration-response curve, with the midpoint at $0.32 \pm 0.04 \mu\text{M}$ (6 cells). The maximal P_o^{est} in the presence of GABA was not affected by the mutation (0.72 ± 0.16).

In sum, the F-to-C mutation in the $\alpha 4$ subunit eliminates activation by GABA in HEK cells while showing a drastic rightward shift in the GABA activation curve in oocytes. When the analogous F-to-C mutation is made in the δ subunit, it has a gain-of-function effect in HEK cells but a loss-of-function effect in oocytes.

Properties of Overexpressed $\alpha 4\beta \delta$ Receptors in Hippocampal Neurons. The data above demonstrate that gating of $\alpha 4\beta 2\delta$ receptors by ALF depends on the expression system. When free $\alpha 4\beta 2\delta$ receptors are expressed in HEK cells, steroid-activated receptors have a maximal P_o^{est} of less than 0.01. In oocytes, saturating ALF elicits a response with a P_o^{est} of nearly 0.6. To gain initial insight into which type of behavior more closely resembles properties of $\alpha 4\beta \delta$ receptors in neurons, we transiently expressed $\alpha 4$ and δ (T269Y) subunits in cultured hippocampal neurons. Electrophysiological experiments were conducted in the presence of $100 \mu\text{M}$ picrotoxin to block GABA_A receptors not containing δ (T269Y), including native receptors.

In four cells, the average peak response to 200 nM GABA (a concentration producing a half-maximal response when this receptor is expressed in oocytes) was $14 \pm 10 \text{ pA}$. Coapplication of $1 \mu\text{M}$ DS-2 enhanced the peak response to $254 \pm 102 \text{ pA}$, indicating the presence of the δ subunit in receptors generating the responses. Application of $10 \mu\text{M}$ ALF in the absence of GABA produced a mean peak response of $148 \pm 69 \text{ pA}$, about 60% of the response to GABA + DS-2.

To verify that none of the responses resulted from receptors lacking the δ (T269Y) subunit, we conducted an identical set of experiments on untransfected neurons (in the presence of $100 \mu\text{M}$ picrotoxin). In nine cells, the average peak response to GABA was $4 \pm 4 \text{ pA}$. Coapplication of DS-2 had no effect ($4 \pm 3 \text{ pA}$). The mean response to ALF was $1 \pm 4 \text{ pA}$. Representative traces are shown in Fig. 6. We infer that picrotoxin effectively blocks receptors lacking the δ (T269Y) subunit, and that receptors containing the mutated δ subunit are efficaciously activated by ALF in hippocampal neurons.

Discussion

We have examined activation properties of GABA_A receptors containing $\alpha 4$, $\beta 2$, and δ subunits. Receptors were expressed as free subunits or concatemeric constructs in which two or three individual subunits were joined with short linkers to constrain subunit order and stoichiometry. We were motivated by earlier reports of ambiguity in the incorporation of the δ subunit in heterologously expressed receptors (Kaur et al., 2009; Meera et al., 2011) and a lack of clarity in basic activation properties of recombinant $\alpha 4\beta \delta$ receptors (Meera et al., 2011; Karim et al., 2012).

Our findings demonstrate that the properties of $\alpha 4\beta 2\delta$ receptors are dependent on the expression system. In the *Xenopus* oocyte expression system, receptors assembled from free $\alpha 4\beta 2\delta$ subunits demonstrated high, nanomolar sensitivity to GABA and high-efficacy activation by the steroid analog ALF. In contrast, in HEK cells, receptors assembled from free $\alpha 4\beta 2\delta$ subunits exhibited low, micromolar sensitivity to GABA and low-efficacy activation by ALF.

It is unlikely that post-translational modifications underlie differences in receptor properties in oocytes and HEK cells, because high-efficacy activation by ALF was observed in both oocytes and HEK cells expressing the combination of $\beta 2$ - δ tandem + free $\alpha 4$. Furthermore, it was possible to express receptors in oocytes with properties similar to those assembled from free subunits in HEK cells by using several different combinations of concatemers (e.g., δ - $\beta 2$ - $\alpha 4$ + $\beta 2$ - $\alpha 4$). One simple explanation is that $\alpha 4\beta 2\delta$ receptors assemble in different configurations with distinct activation properties in oocytes and HEK cells. However, both expression systems permit assembly into the alternative configuration if necessary constraints, for example, subunit linkage in concatemers, are applied. Data from hippocampal neurons transfected with $\alpha 4$ and a picrotoxin-resistant δ subunit suggest that neurons favor the assembly of, at least recombinant, $\alpha 4\beta \delta$ receptors in the form characterized by large steroid currents.

We used a human $\alpha 4$ and rat $\beta 2$ and δ subunits in this study. Species differences were unlikely to mediate the differences in activation properties in oocytes and HEK cells. Nanomolar sensitivity to GABA has been observed previously in all human $\alpha 4\beta 1\delta$ and $\alpha 4\beta 3\delta$ receptors expressed in oocytes, whereas micromolar sensitivity to GABA has been observed in all rodent $\alpha 4\beta 2\delta$ receptors expressed in HEK cells (Karim et al., 2012; Patel et al., 2014).

Our data are consistent with free $\alpha 4\beta 2\delta$ subunits in HEK cells assembling in the subunit order $\delta\beta 2\alpha 4\beta 2\alpha 4$ (counterclockwise when viewed from the extracellular side). The properties of $\alpha 4\beta 2\delta$ (GABA EC_{50} in the micromolar range and low-efficacy activation by ALF) are observed with concatemers predicted to assemble as $\delta\beta 2\alpha 4\beta 2\alpha 4$ (e.g., δ - $\beta 2$ - $\alpha 4$ + $\beta 2$ - $\alpha 4$) in oocytes. Furthermore, introduction of the F63C mutation to the $\alpha 4$ subunit effectively eliminates activation by GABA for receptors in HEK cells, in agreement with previous data showing that the presence of the mutation in

Fig. 6. Hippocampal neurons transfected with $\alpha 4$ and δ (T269Y) subunits demonstrate prominent responses to the steroid alfaxalone. (A) Representative traces in the presence of 200 nM GABA, 200 nM GABA + $1 \mu\text{M}$ DS-2, or $10 \mu\text{M}$ alfaxalone from a hippocampal neuron transfected with $\alpha 4$ and δ (T269Y) subunits. The currents were recorded in the presence of $100 \mu\text{M}$ picrotoxin to block GABA_A receptors not containing the mutated δ subunit. (B) Representative traces in the presence of 200 nM GABA, 200 nM GABA + $1 \mu\text{M}$ DS-2, or $10 \mu\text{M}$ alfaxalone from an untransfected hippocampal neuron. Lack of current responses indicates that $100 \mu\text{M}$ picrotoxin is sufficient to block GABA_A receptors not containing the δ (T269Y) subunit.

both GABA binding sites renders the receptor inactive in the presence of GABA (Petrini et al., 2011). The $\delta\beta 3\alpha 4\beta 3\alpha 4$ order of $\alpha 4\beta 3\delta$ receptors was proposed previously in a study employing atomic force microscopy imaging complexes between epitope-tagged receptors and antibodies in HEK cells (Barrera et al., 2008).

In oocytes, the combination of the $\beta 2$ - δ tandem with free $\alpha 4$ subunit effectively captured the properties (high sensitivity to GABA and high efficacy of ALF) of free $\alpha 4\beta 2\delta$ subunits. Our results do not directly address the subunit order of receptors formed by free subunits or by the combination of $\beta 2$ - δ and free $\alpha 4$. Some evidence, however, suggests that in such receptors the δ subunit may contribute to the negative interface of the transmitter binding site. The F-to-C mutations in loop D (F63C in $\alpha 4$ and F74C in δ) differently affected receptor activation in HEK cells and oocytes. In HEK cells, the $\alpha 4$ (F63C) mutation abolished activation by GABA, whereas δ (F74C) resulted in receptors with increased sensitivity to GABA. In contrast, in oocytes, both mutations resulted in reduced sensitivity to GABA. We speculate that $\alpha 4\beta 2\delta$ receptors in oocytes contain one $\beta 2$ - $\alpha 4$ and one $\beta 2$ - δ interface that are involved in binding GABA.

Involvement and contribution of the δ subunit to transmitter binding have been proposed previously. In concatemeric receptors containing $\alpha 1\beta 3\delta$ subunits, introduction of the $\beta 3$ (Y205S) mutation shifted the GABA activation curves to higher concentrations regardless whether the β subunit was paired to an α or a δ subunit (Kaur et al., 2009). In the $\beta 3$ subunit, a conserved arginine residue (R207) lines the transmitter-binding pocket and has been shown to affect receptor affinity to GABA (Wagner et al., 2004). Mutation of an arginine in the homologous position in the δ subunit (R218A) strongly reduced sensitivity of $\alpha 4\beta 3\delta$ receptors to GABA (Karim et al., 2012). Both these examples, as well as our data on δ (F74C), suggest that the δ subunit may be involved in binding of GABA in some variants of the $\alpha 4\beta 3\delta$ receptor.

Large ALF-gated, DS-2-sensitive currents were observed in hippocampal neurons overexpressing $\alpha 4$ and δ subunits. The δ subunit contained the T269Y mutation, which rendered receptors containing the exogenous δ subunit resistant to picrotoxin. Prominent ALF-gated currents, in the presence of picrotoxin, from these receptors indicate that the behavior of recombinant $\alpha 4/\delta$ receptors in neurons may more closely resemble that of $\alpha 4\beta 2\delta$ receptors in oocytes. It remains to be determined whether native $\alpha 4/\delta$ receptors show high level of activation in the presence of steroids. If so, physiologic levels of many neuroactive steroids (or combinations of neuroactive steroids) can be expected to activate native $\alpha 4\beta 3\delta$ receptors. Indeed, a recent study found GABA-independent tonic currents in dentate granule cells (Włodarczyk et al., 2013) that, we speculate, may be elicited by endogenous neuroactive steroids.

A prior study showed that overexpression of the δ subunit increases incorporation of the δ subunit into surface $\alpha 4\beta 2\delta$ receptors in HEK cells (Wagoner and Czajkowski, 2010). The increase in δ was accompanied by a reduction in $\beta 2$, whose average number per receptor dropped to ~ 1 ; however, the functional properties of receptors with different stoichiometries were not studied. Our data indicate that the GABA concentration-response relationship in HEK cells is not affected by a fivefold increase in the amount of cDNA for δ subunit used in transfections. The data from oocytes (present work; Kaur et al., 2009; Karim et al., 2012) suggest that the δ subunit can

replace an $\alpha 4$ at the GABA binding site. Further work will be required to determine how many δ subunits can be incorporated in a functional receptor and at which positions in the pentamer.

Previous studies of other members of the pentameric ligand ion channel family have demonstrated flexibility in subunit assembly. Neuronal nicotinic $\alpha 3$ and $\beta 4$ subunits assemble in different subunit stoichiometries, with distinct conductances and kinetic properties, in *Xenopus* oocytes and sympathetic neurons (Sivilotti et al., 1997). Receptors containing different stoichiometries of the nicotinic $\alpha 4$ and $\beta 2$ subunits show different pharmacological properties, as a result of intersubunit binding sites in addition to the canonical acetylcholine-binding sites (Harpsoe et al., 2011; Mazzaferro et al., 2011). We have extended these studies to a new subtype of receptor and reconcile previous disparities in reported properties of tonic GABA_A receptors containing δ subunits.

Authorship Contributions

Participated in research design: Eaton, Mennerick, Steinbach, Akk.
Conducted experiments: Eaton, Bracamontes, Shu, Li.
Contributed new reagents or analytic tools: Bracamontes.
Performed data analysis: Eaton, Bracamontes, Li, Mennerick, Akk.
Wrote or contributed to the writing of the manuscript: Eaton, Mennerick, Steinbach, Akk.

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