**α_4β_3δ** GABA_A Receptors Characterized by Fluorescence Resonance Energy Transfer-derived Measurements of Membrane Potential*

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Selective modulators of γ-aminobutyric acid, type A (GABA_A) receptors containing α subunits may provide new treatments for epilepsy and premenstrual syndrome. Using mouse Ltk(-tk) cells, we stably expressed the native GABA_A receptor subunit combinations α_4β_2γ_2 and, for the first time, α_4β_3δ and characterized their properties using a novel fluorescence resonance energy transfer assay of GABA-evoked depolarizations. GABA evoked concentration-dependent decreases in fluorescence resonance energy transfer that were blocked by GABA_A receptor antagonists and, for α_4β_2γ_2 and α_4β_3γ_2 receptors, modulated by benzodiazepines with the expected subtype specificity. When combined with α_4 and β_3, δ subunits, compared with γ_2 confer greater sensitivity to the agonists GABA, 4,5,6,7-tetrahydroisoxazo[5,4-c]pyridin-3-ol (THIP), and muscimol and greater maximal efficacy to THIP. α_4β_3δ responses were markedly modulated by steroids and anesthetics. Alphaxalone, pentobarbital, and preganalone were all 3-7-fold more efficacious at α_4β_3δ compared with α_4β_2γ_2. The fluorescence technique used in this study has proven valuable for extensive characterization of a novel GABA_A receptor. For GABA_A receptors containing α subunits, our experiments reveal that inclusion of δ instead of γ subunits can increase the affinity and in some cases the efficacy of agonists and can increase the efficacy of allosteric modulators. Pregnanalone was a particularly efficacious modulator of α_4β_3δ receptors, consistent with a central role for this subunit combination in premenstrual syndrome.

γ-Aminobutyric acid (GABA)^1^ is the predominant inhibitory neurotransmitter in the central nervous system, and modulators of type A GABA (GABA_A) receptors are used to treat anxiety, insomnia, muscle spasms, and epilepsy. GABA_A receptors are pentameric ligand-gated chloride channels, mediating rapid inhibitory synaptic neurotransmission, and are composed of different combinations of subunits from a family including α_1-6, β_1-4, γ_1-3, δ, ε, θ, and π_1-2 (1). They are modulated by a plethora of clinically important drugs including benzodiazepines, barbiturates, steroids, and anesthetics. Subunit stoichiometry has been contentious (2–4), but the evidence is now convincing that receptors composed of α, β, and γ subunits contain two α, two β, and one γ subunit (5). The precise combination of subunits is an important determinant of receptor pharmacology: α subunits govern GABA affinity (6), α and γ subunits regulate benzodiazepine site pharmacology (6–9), and δ subunits control loreclezole and etomidate sensitivity (10).

α subunits comprise only a small percentage of neuronal subunits, concentrated in hippocampus, striatum, cerebral cortex, thalamus, and basal ganglia (11–15). They assemble with β_2/3 and γ_2 subunits in most areas of the brain (12), but also with β_2/3 and δ subunits in olfactory bulb, dentate gyrus, and thalamus (14–17). Of the 20–27% of thalamic GABA_A receptors that contain α subunits, approximately one-third contain γ_2 subunits, and two-thirds contain δ subunits (14). Compared with other GABA_A receptors, those containing α subunits differ in their rectification properties (18), affinity for GABA (19), and modulation by benzodiazepines (20). Receptors containing α and δ subunits lack benzodiazepine binding sites entirely, and those containing α, β, and γ_2 subunits have a benzodiazepine binding site that is atypical (6, 14, 21).

A variety of animal models of epilepsy lead to changes in the level of expression of α and δ subunit protein and mRNA in hippocampal dentate gyrus (17, 22–26) and thalamic relay nuclei (27), and acute pentylenetetrazol-induced seizures, to which mice lacking δ subunits are more susceptible (56), lead to an increase in δ subunit expression in neocortex (28). Elevated levels of α subunits are also implicated in an animal model of alcohol dependence (29) and in steroid-withdrawal models of premenstrual syndrome and postpartum or postmenopausal dysphoria, particularly the increased anxiety and incidence of seizures (30–34). The association of these pathologies with changes in α and δ subunit expression and the observation that ligands with high affinity for α_4β_2γ_2 GABA_A receptors are amethystic (35, 36) suggest that novel selective modulators of these GABA_A receptors may, as well as leading to a better understanding of the properties and physiological roles of these subunits in the brain, have great therapeutic benefit. The development of such modulators has been held back on two counts. First, α_4β_3δ receptors cannot easily be expressed in transient recombinant systems, and so their properties remain unclear. Second, GABA_A receptor drug-development programs have depended until now on difficult and time-consuming electrophysiological techniques or less sensitive radio-ion flux and pH methods for determining the effects of compounds on GABA_A receptor function (6, 37–39). We have overcome these problems by creating a stable Ltk(-tk) mouse cell line in which expression of α_4β_3δ receptors is under the control of a deoxy-
methasone-induced promoter, and by developing an experimental system using fast ratiometric voltage-sensitive FRET (40) to measure GABA-evoked changes in membrane potential. Fluorescence measurements of GABA<sub>A</sub> receptor function offer significant advantages because they are safe, are sufficiently sensitive to detect small potenations and inhibitions, and can be miniaturized for future ultrahigh throughput applications. Furthermore, unlike high throughput radioligand binding assays, which have also been used for the development of GABA<sub>A</sub> receptor modulators, they can identify modulators regardless of their site of action. Here we describe the use of this novel fluorescence technique to characterize the pharmacological activation and modulation of GABA<sub>A</sub> receptors with the subunit combinations α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>δ.

EXPERIMENTAL PROCEDURES

Expression of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>3</sub>, and α<sub>1</sub>β<sub>2</sub>δ GABA<sub>A</sub> Receptors—Li<sup></sup>(tk) cells were stably transfected, using a pMSGneo vector, with combinations of human GABA<sub>A</sub> receptor subunits. Expression of α, β, and γ subunits was controlled by a dexamethasone-inducible promoter as described previously (14, 41), whereas expression of δ subunits was constitutive. Enzyme-linked immunosorbent assays using Myc-tagged subunits confirmed that δ subunits were only present at the cell surface if both α<sub>1</sub> and β<sub>2</sub> subunits were also present. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% serum (Fetalclone II) at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were passaged weekly and for experiments were transferred to 96-well black-sided microtiter plates at a density that gave confluent monolayers on the days of experiments. Receptor expression was induced 24 h before experiments by replacing 50% of the medium with medium containing dexamethasone (1 μM final concentration).

Fluorescence Measurements of Membrane Potential—All experiments were performed in a low Cl⁻ buffer (160 mM sodium D-glucuronate, 4.5 mM potassium D-glucuronate, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM D-glucose, 10 mM HEPES, pH 7.4). Cells were washed twice, leaving a 25-μl residual volume, and 55 μl of dye solutions were added to give final concentrations of 0.4 μM chlorocoumarin-2-dimyristoyl phosphatidylethanolamine (CC2-DMPE) and 1 μM bis(1,3-diethyl-2-thiobarbiturate)trimethineoxonol (DisBac<sub>2</sub>(3)). After a 30-min incubation at room temperature in darkness, cells were washed again, and 65 μl of dye solutions were added to give final concentrations of 1 μM DisBac<sub>2</sub>(3) and 0.5 mM trazarine. Micrortiter plates were then placed in a voltage/ion probe reader (VIPRTM; Aurora Biosciences Corp.), which performs automated additions of pharmacological stimuli and records fluorescence emission. Briefly, the VIPRTM consists of a Hamilton 2200 pipetter, an automated microtiter plate positioning stage, and a fiber-optic illumination and detection system capable of measuring two emission wavelengths from eight wells simultaneously (40). A 400DF15 filter was used in the excitation pathway, and 460DF45 and 580DF60 filters were used in the respective emission pathways. In all experiments, basal fluorescence was read for 8 s before addition of modulators, and then GABA was added 22 s later. Fluorescence emission from wells was recorded at 1 Hz.

Data Analysis—For each time point and for each fluorescence emission wavelength, we subtracted background fluorescence recorded from wells without cells in the same microtiter plate and calculated the ratio of fluorescence at 460 nm to that at 580 nm. GABA-evoked depolarizations were then expressed as a fractional change in this ratio. Algorithms written as Excel 97 (Microsoft Corp.) macros were used for automated calculations of fluorescence ratio and GABA responses (39), and an iterative curve-fitting program (Prism, GraphPad Software Inc.) was used to fit concentration-effect relationships to a four-parameter logistic equation.

Materials and Methods—DisBac<sub>2</sub>(3) and CC2-DMPE were from Aurora Biosciences Corp. Dulbecco’s modified Eagle’s medium was from Life Technologies Inc., and Fetalclone II was from Hyclone (Logan, UT). Loreclezole was a gift from Janssen, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) was from Tocris (Baldwin, MO), and bretazenil was synthesized by Merck Sharp & Dohme Research Laboratories. Tartrazine, gluconate salts, and all other GABA<sub>A</sub> receptor modulators were obtained from Sigma. All other reagents were of the highest analytical grade available.

RESULTS

Previously, optical sensors of membrane potential operated through a slow redistribution of permeant ions or a rapid but insensitive perturbation of dyes attached to one face of the membrane (42–44). However, a recently developed membrane potential indicator, described in Fig. 1, uses FRET to provide a fluorescent readout of membrane potential that is both rapid and robust (45). Before using this technique to characterize cell lines expressing α<sub>4</sub> subunit-containing GABA<sub>A</sub> receptors, we first established its pharmacological utility using cells expressing the well-characterized subunit combination α<sub>4</sub>β<sub>2</sub>γ<sub>2</sub>. In low chloride medium, GABA-evoked depolarizations of cells expressing α<sub>4</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub> receptors and loaded with CC2-DMPE and DisBac<sub>2</sub>(3) were rapidly transduced into decreased FRET efficiency and, therefore, a decrease in the ratio of fluorescence emission at 460 nm to that at 580 nm.
dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) (Fig. 2c). These findings are highly consistent with those from electrophysiological experiments (38).

Having established that fluorescence measurements of GABAא receptor function appear to reliably report receptor pharmacology, we then examined GABA-evoked changes in FRET using L(−tk) cells expressing either αβγδ GABAא receptors or the previously uncharacterized subunit combination αβγδ. The kinetics of GABA-evoked depolarization were similar for these cells to those for cells expressing αβγδ GABAא receptors (Fig. 3c). GABA, muscimol, and THIP were between 3 and 6 times more potent at αβγδ receptors compared with αβγδ (Figs. 2b and 3b). The first detectable response to muscimol occurred 1 s earlier than that for GABA or THIP, but thereafter the three agonists evoked changes in fluorescence ratio with similar kinetics (Fig. 3c). Although less potent than GABA, THIP was a fully efficacious agonist at αβγδ receptors and a superagonist at αβγδ (Fig. 3b). Responses mediated by both αβγδ and αβγδ receptors were inhibited by pretreatment with picrotoxin and bicuculline. Whereas picrotoxin (30 μM) inhibited the responses to all concentrations of GABA, bicuculline (30 μM) inhibited only submaximal responses, causing a 30-fold shift in the GABA concentration-response curve (Fig. 3d).

We then examined the regulation of α4 subunit-containing GABAא receptors by a variety of known modulators of GABAא receptors, including benzodiazepines, steroids, and anesthetics (Table I). α4βγδ receptor-mediated responses were partially inhibited by pretreatment with DMCM, which had a similar efficacy to that at αβγδ. α4βγδ responses were potentiated by nanomolar concentrations of bretylium and Ro15-4513 but were insensitive to the classical benzodiazepine site agonists zolpidem and flunitrazepam (Fig. 4). αβγδ receptors were largely insensitive to the benzodiazepine site modulators used in this study with just two exceptions. First, Ro15-4513 inhibited αβγδ-mediated responses, although with an EC50 100 times higher than that for its potentiation of αβγδ receptors. Second, micromolar concentrations of ethyl-β-carboline-3-carboxylate (β-CCE) potentiated responses mediated by both αβγδ and αβγδ receptors (Fig. 4). Micromolar concentrations of furosemide, an inhibitor of GABAא receptors (20, 46), selectively inhibited αβγδ receptors with no discernible effect on αβγδ (Table I).

αβγδ and αβγδ responses were potentiated by the anesthetics propofol and lorazepam and inhibited by the steroid pregnenolone (5-pregnene-3β-ol-20-one). These compounds were of similar potency and efficacy at the two receptor types (Fig. 5 and Table I). The steroid pregnenolone (5β-pregnen-3α-ol-20-one) inhibited, and alpaxalone (5α-pregnen-3α-ol-11,20-dione) potentiated responses at both γδ- and δ-containing receptors. These agents had 3–4 times greater efficacy at αβγδ compared with αβγδ (Fig. 5). Alpaxalone, at concentrations above 3 μM, also directly activated GABAא receptors, evoking a depolarization of both cell types, again with greater efficacy at αβγδ receptors (Fig. 5c). In contrast, applications of pregnenolone and pregnanolone, at concentrations of up to 30 μM, did not affect membrane potential directly. The barbiturate pentobarbital was another more efficacious (7-fold) potentiator of αβγδ receptors (Fig. 4). Micromolar concentrations of furosemide, an inhibitor of GABAא receptors (20, 46), selectively inhibited αβγδ receptors with no discernible effect on αβγδ (Table I).

In this study we have developed a novel fluorescence technique that provides rapid and sensitive measurements of GABAא receptor function, and have used it to characterize a novel cell line expressing GABAא receptors with the composition αβγδ. Our initial experiments, using cell lines expressing the previously characterized GABAא receptor subunit combinations αβγδ and αβγδ, demonstrated that GABAא receptor-mediated chloride fluxes were rapidly and reliably transduced into decreased FRET. In contrast to traditional fluorescence assays of membrane potential utilizing oxonol redistribution, GABA-evoked depolarization of cells loaded with O2-DMPE and DisBac(f3) and excited with 410 nm light leads to a change in fluorescence emission that occurs within seconds rather than minutes. As previously reported, substitution of α4...
subunits for α₃ did not affect GABA potency, which was similar to that previously reported for the same subunit combinations expressed in mammalian cells (6, 19). GABA-evoked responses were blocked by picrotoxin and bicuculline, and at α₂β₂γ₂ and α₂β₂δ₂ receptors, the efficacies and potencies of the benzodiazepines tested were very similar to published values (6, 19).

The rank order for agonist potency muscimol > GABA > THIP was unchanged, but THIP acted as a superagonist at α₂β₂δ receptors, evoking substantially larger changes in FRET than either GABA or muscimol. Partial agonists at other GABAₐ receptor subtypes have been described, but no agonist has shown greater efficacy than GABA. An equally valid interpretation of this data, therefore, is that δ subunits, when combined with α₂ and β₃, confer partial agonism to GABA. The different potency, and perhaps efficacy, of GABA at α₂β₂γ₂ and α₂β₂δ receptors suggest quite different physiological roles for these receptor isoforms containing δ subunits. Receptors containing δ subunits in combination with α₄, as occur in situ, have never been characterized. We therefore created a novel L(−tk) cell line in which expression of α₄β₂δ GABAₐ receptors was under the control of a dexamethasone-inducible promoter, and used FRET-derived measurements of membrane potential to directly compare them to α₂β₂γ₂ receptors. We found that δ subunits, compared with γ₂, conferred higher affinity for all the agonists tested.

While GABAₐ receptors composed of α, β, and γ subunits have been studied extensively, relatively little is known about the functional and pharmacological properties of receptor isoforms containing δ subunits. Receptors containing δ subunits may be suited to synapses where GABA is plentiful and a rapid dissociation rate is beneficial to high frequency signaling.
lorepalone site present only on \( \beta_2 \) and \( \beta_3 \)-containing receptors (10). Therefore the potentiation of both \( \alpha_2 \beta_3 \gamma_2 \) and \( \alpha_2 \beta_2 \delta \) responses by \( \beta \)-CCE was almost certainly mediated by the binding site for lorepalone and does not indicate benzodiazepine sensitivity.

Barbiturates are thought to potentiate the response of \( \text{GABA}_A \) receptors irrespective of their subunit composition (47). Both \( \alpha_2 \beta_2 \gamma_2 \) and \( \alpha_2 \beta_2 \delta \) responses were potentiated by pentobarbital. However, \( \delta \) subunits, compared with \( \gamma_2 \), conferred 7 times higher efficacy to pentobarbital. At micromolar concentrations, barbiturates have a second effect, directly activating \( \text{GABA}_A \) receptors (50, 51). \( \alpha_2 \beta_2 \gamma_2 \) receptors were activated by pentobarbital, but this effect was abolished when \( \gamma_2 \) subunits were substituted with \( \delta \). We conclude that \( \delta \) and \( \gamma_2 \) subunits affect both the modulation and activation of \( \text{GABA}_A \) receptors by barbiturates. There may also be a role for \( \beta \) subunits since \( \alpha_2 \beta_2 \gamma_2 \) and \( \alpha_4 \beta_2 \gamma_2 \) receptors are activated by pentobarbital (47), whereas the effect does not occur on \( \alpha_2 \beta_2 \gamma_2 \) (20).

\( \alpha_2 \beta_2 \gamma_2 \) and \( \alpha_2 \beta_2 \delta \) receptors were differentially modulated by steroids. In contrast to the stimulatory effect at other \( \text{GABA}_A \) receptors (52), receptors containing \( \alpha_4 \) subunits were inhibited by the naturally occurring neurosteroid pregnanolone. Furthermore, both pregnanolone and the synthetic anesthetic alpaxalone (52, 53) were more efficacious at \( \alpha_2 \beta_2 \delta \) compared with \( \alpha_2 \beta_2 \gamma_2 \). These data demonstrate that \( \delta \) subunits are a critical determinant of neurosteroid efficacy, possibly accounting for the reduced behavioral effects of alpaxalone and pregnanolone in mice lacking \( \delta \) subunits (55). During the menstrual cycle and pregnancy in normal women, levels of pregnanolone correlate with those of progesterone from which it is synthesized (54). In addition to their effects on \( \alpha_4 \) subunit expression (30–34), endogenous neuroactive steroids may therefore also modulate the function of \( \text{GABA}_A \) receptors, particularly those containing \( \delta \) subunits, and thereby contribute to the increased incidence of anxiety and seizures in premenstrual syndrome and postpartum and postmenopausal dysphoria. Our data imply that \( \alpha_2 \beta_2 \delta \) receptors may have a central role in these disorders and that new therapies might be developed by selective targeting of the steroid binding site of \( \text{GABA}_A \) receptors containing \( \delta \) subunits.

FRET-derived measurements of membrane potential provide the most robust and reliable high throughput assay of \( \text{GABA}_A \) receptor function yet developed and will be an invaluable tool for characterizing novel subunit combinations and identifying new therapeutic modulators. When applied to cell lines expressing \( \text{GABA}_A \) receptors with the subunit combinations \( \alpha_2 \beta_2 \gamma_2 \) and \( \alpha_2 \beta_2 \delta \), this novel fluorescence technique revealed that \( \delta \) subunits are an important determinant of the efficacy and potency of agonists and allosteric modulators. Of particular importance was the finding that \( \alpha_2 \beta_2 \delta \) receptors were markedly more sensitive to inhibition by pregnanolone, suggesting that this receptor subtype could be targeted for the treatment of premenstrual syndrome.

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Higher affinity \( \delta \) subunit-containing receptors may, in an extrasynaptic location where \( \text{GABA} \) is at lower concentrations (48, 49), have a modulatory role for which rapid responses are not required and for which a lower conductance is more appropriate.

\( \delta \) subunits were an important determinant of the effects of a variety of allosteric modulators, including benzodiazepines, steroids, and barbiturates. Substitution of \( \gamma_2 \) subunits with \( \delta \) abolished sensitivity to modulators acting at the benzodiazepine binding site. Although \( \beta \)-carbolines, such as \( \beta \)-CCE and methyl-\( \beta \)-carboline-3-carboxylate, inhibit \( \text{GABA}_A \) receptors with high potency via the benzodiazepine binding site, they also potentiate \( \text{GABA} \) responses, with lower potency, via the

![Figure 5](image-url)
