Novel compounds selectively enhance δ subunit containing GABA~A~ receptors and increase tonic currents in thalamus

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ABSTRACT

Inhibition in the brain is dominated by the neurotransmitter γ-aminobutyric acid (GABA); operating through GABA~A~ receptors. This form of neural inhibition was presumed to be mediated by synaptic receptors, however recent evidence has highlighted a previously unappreciated role for extrasynaptic GABA~A~ receptors in controlling neuronal activity. Synaptic and extrasynaptic GABA~A~ receptors exhibit distinct pharmacological and biophysical properties that differentially influence brain physiology and behavior. Here we used a fluorescence-based assay and cell lines expressing recombinant GABA~A~ receptors to identify a novel series of benzamide compounds that selectively enhance, or activate α4β3δ GABA~A~ receptors (*cf.* α4β3γ2 and α1β3γ2). Utilising electrophysiological methods, we illustrate that one of these compounds, 4-chloro-*N*-[6,8-dibromo-2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide (DS1) potently (low nM) enhances GABA-evoked currents mediated by α4β3δ receptors. At similar concentrations DS1 directly activates this receptor and is the most potent known agonist of α4β3δ receptors. 4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide (DS2) selectively potentiated GABA responses mediated by α4β3δ receptors, but was not an agonist.

Recent studies have revealed a tonic form of inhibition in thalamus mediated by the α4β2δ extrasynaptic GABA~A~ receptors that may contribute to the regulation of thalamocortical rhythmic activity associated with sleep, wakefulness, vigilance and seizure disorders. In mouse thalamic relay cells DS2 enhanced the tonic current mediated by α4β2δ receptors with no effect on their synaptic GABA~A~ receptors. Similarly, in mouse cerebellar granule cells DS2 potentiated the tonic current mediated by α6βδ receptors. DS2 is the first selective positive allosteric modulator of δ-GABA~A~ receptors and such compounds potentially offer novel therapeutic opportunities as analgesics and in the treatment of sleep disorders. Furthermore, these drugs may be valuable in elucidating the physiological and pathophysiological roles played by these extrasynaptic GABA~A~ receptors.

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1. Introduction

Inhibition in the brain is determined predominantly through the neurotransmitter γ-aminobutyric acid (GABA), which binds and activates a family of chloride permeable ion-channel GABA~A~ receptors (Connolly and Wafford, 2004). GABA~A~ receptors are multimeric proteins composed of five subunits drawn from a repertoire of several homologous groups of proteins (α1–6, β1–3, γ1–3, δ, ε, θ, and π). The majority of receptors in the CNS consist of two α, two β and one γ subunit, however less abundant populations of GABA~A~ receptors containing a δ subunit (δ-GABA~A~ receptor) are now attracting considerable attention. δ-GABA~A~ receptors are highly sensitive to GABA, exhibit little desensitization, but unusually GABA only acts as a partial agonist at such receptors (Brown et al., 2002; Bianchi and Macdonald, 2003; Storustovu and Ebert, 2006). These properties are important for the physiological role of δ-GABA~A~ receptors in neuronal signaling (see below).

There are three known δ-GABA~A~ receptor isoforms: α6βδ, unique to cerebellar granule cells (CGCs); α1βδ found in hippocampal interneurons of the molecular layer and α4βδ, expressed predominantly in hippocampal dentate gyrus granule cells (DGGCs) and thalamic relay neurons (Porcello et al., 2003; Belelli et al., 2005; Chandra et al., 2006; Glykys et al., 2007). δ-GABA~A~ receptors are sited extra- or peri-synaptically and may be activated by ambient GABA (Farrant and Nusser, 2005). Under voltage-clamp, GABA~A~

receptor antagonists not only block "phasic" miniature inhibitory post-synaptic currents (mIPSCs), mediated by synaptic GABA$_A$ receptors, but additionally decrease the "holding" current and neuronal input conductance by reducing the number of conducting extrasynaptic GABA$_A$ receptors (Farrant and Nusser, 2005). Physiologically, these receptors play an important role in influencing neuronal excitability and consequently neuronal network activity (Farrant and Nusser, 2005; Cope et al., 2005). Indeed, mutations of the δ subunit are associated with generalized epilepsy with febrile seizures and juvenile myoclonic epilepsy (Dibbens et al., 2004; Feng et al., 2006).

The expression of δ-GABA$_A$ receptors is not static, but subject to dynamic change, *e.g.* during the ovarian cycle, stress and puberty (Belelli and Lambert, 2005; Maguire et al., 2005; Maguire and Mody, 2007; Shen et al., 2005). Intriguingly, these situations are associated with perturbations in the production of neurosteroids (putative endogenous allosteric modulators of δ-GABA$_A$ receptors). Fluctuations in neurosteroid levels, coupled with the altered δ-GABA$_A$ receptor expression, influence neuronal excitability and consequently contribute to the changes in behavior associated with these conditions (Belelli and Lambert, 2005; Maguire et al., 2005; Maguire and Mody, 2007; Shen et al., 2005; Herd et al., 2008). In support, the behavioral effects of neurosteroids are blunted in δ$^{-/-}$ mice (Mihalek et al., 1999).

δ-GABA$_A$ receptors are emerging as an important pharmacological target. Although these receptors are not influenced by benzodiazepines, such as diazepam, some general anesthetics, *e.g.* etomidate and certain neurosteroids are highly effective and consequently produce a profound increase of the tonic conductance (Brown et al., 2002; Stell et al., 2003; Belelli et al., 2005). Low concentrations of ethanol are also reported to enhance GABA responses mediated by δ-GABA$_A$ receptors (Wallner et al., 2003; Lovinger and Homanics, 2007). 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP or gaboxadol) is a relatively selective agonist of δ-GABA$_A$ receptors (Brown et al., 2002; Storustovu and Ebert, 2006; Belelli et al., 2005) and highlighting the importance of these receptors, the behavioral effects (including sedation, ataxia and analgesia) of gaboxadol are blunted in either α4$^{-/-}$ (a δ subunit partner), or δ$^{-/-}$ mice (Boehm et al., 2006; Chandra et al., 2006). Indeed, activation of α4β2δ receptors in the thalamus may play a role in the generation, or maintenance of slow wave sleep and gaboxadol has undergone clinical investigation as a treatment for insomnia (Wafford and Ebert, 2006). Although relatively selective, gaboxadol is a directly acting agonist, indeed, there are no selective positive allosteric modulators of δ-GABA$_A$ receptors. Such compounds would be invaluable in discerning the role of δ-GABA$_A$ receptors in brain function.

Here we describe a novel series of compounds that at nanomolar concentrations selectively potentiate GABA-evoked responses mediated by δ subunit containing receptors, with little effect at other GABA$_A$ receptor subtypes. Furthermore, we demonstrate that an example of this chemical series specifically enhances the tonic current of thalamocortical neurons, without influencing inhibitory synaptic currents. Such compounds may inform the development of novel therapeutics as analgesics and in the treatment of insomnia and may help elucidate the role of these receptors in health and disease.

2. Methods

2.1. FLIPR

2.1.1. Chemicals

Fluorescence imaging plate reader (FLIPR) membrane potential dye was obtained from Molecular Probes. Solutions and chemicals for cell culture (GIBCO) were purchased from Invitrogen. Other chemicals were purchased from Sigma–Aldrich.

2.1.2. Cell cultures

Stable cell lines expressing human GABA$_A$-α4β3δ, GABA$_A$-α4β3γ2, or GABA$_A$-α1β3γ2 were generated using mouse L(tk$^-$) fibroblast cells, as described previously (Hadingham et al., 1992; Sur et al., 1999; Adkins et al., 2001; Brown et al., 2002). The α, β, and γ subunits were under the control of a dexamethasone inducible promoter. The c-myc tagged δ subunit was constitutively expressed from a human CMV promoter (pcDNA3.1Zeo, Invitrogen). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and then maintained in 95% O$_2$/5% CO$_2$ at 37 °C. Cells were plated in black-sided, clear bottomed Greiner 384-well plates 24 h before the FLIPR assay at a density of 5000 cells (in 100 μl) per well with 1 μM dexamethasone for induction of receptor expression (Adkins et al., 2001). Column 24 of the cell plates was left empty as "no cell" background control.

2.1.3. FLIPR assay

The cell plates were first washed with assay buffer for 4 cycles of adding buffer and removing buffer on an Embla cell washer (Molecular Device), and 30 μl of buffer residue was left in each well after the wash. The assay buffer consisted of (in mM); 160 sodium-D-gluconate, 4.5 potassium-D-gluconate, 2 CaCl$_2$, 1 MgCl$_2$, 10 D-glucose, and 10 HEPES. The assay buffer functioned in the range pH 7.4–7.7. 30 μl of FLIPR membrane potential dye solution (prepared with assay buffer, 1 vial per 100 ml) was added to each well with a Multimek automated 96-channel pipettor (Beckman–Coulter). The cell plates were then incubated at room temperature for 30 min before being transported to the FLIPR machine. The first solution addition in the FLIPR machine was the modulator (the GABA modulator, or antagonist), and the second addition was a low concentration of GABA (EC$_{30}$), which was added 2.5 min after the first addition to evaluate modulator, or antagonist activity (in the case of α4β3δ this was 100 nM). The fluorescence produced by membrane potential change was measured by a Molecular Device FLIPR$_{384}$. The effect of 9 concentrations (serial dilution at half log intervals) of the test compounds on basal, or GABA-stimulated membrane potential levels was measured. The effect on basal membrane potential levels was used to calculate any direct agonist action, and the effect on GABA-stimulated membrane potential levels used to calculate the modulator, or antagonist action. The assay process was fully automated by a Beckman–Coulter robot system.

2.1.4. Data analysis

All direct agonist activity was calculated as percent of the maximum GABA response with basal counts being subtracted. For the FLIPR assay, the agonist effect was an increase in the fluorescence, which was normalized to basal fluorescence signals. Antagonism of the GABA response was calculated by subtracting the compound-pretreated GABA effect from the control GABA effect, and dividing by the control GABA effect. The pre-first addition fluorescence level was used as baseline, and the agonist effect was measured as the peak before the second addition. The FLIPR data were analysed by non-linear regression with IDBS XL-fit software, or Prism data analysis software. All data are expressed as a percent of control (EC$_{30}$) GABA response and are mean ± SE.

2.2. Whole-cell voltage-clamp studies of stable L(tk$^-$)cells expressing human α4β3δ and α4β3γ2S and α1β3γ2 GABA$_A$Rs

Experiments were performed on the stable L(tk$^-$) cell lines expressing either α4β3γ2, α4β3δ or α1β3γ2 GABA$_A$ receptors following 24 h induction with 25 nM dexamethasone (Brown et al., 2002). Glass coverslips containing a monolayer of cells were placed in a chamber on the stage of a Nikon Diaphot inverted microscope. Cells were perfused continuously with artificial cerebral spinal fluid (aCSF) containing (in mM): 149 NaCl, 3.25 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, 11 D-glucose, 22 D(+)-sucrose, pH 7.4, and observed with phase-contrast optics. Fire-polished patch pipettes were pulled on a WZ, DMZ-Universal puller using conventional 120TF-10 electrode glass. Pipette tip diameter was approximately 1.5–2.5 μm, with resistances around 4 MΩ. The intracellular solution contained (in mM): 130 CsCl, 10 HEPES, 10 BAPTA·Cs, 5 ATP·Mg, 1 MgCl$_2$, pH adjusted to 7.3 with CsOH and 320–340 mOsm. Cells were voltage-clamped at −60 mV *via* an Axon 200B amplifier (Axon Instruments, Foster City, CA). Drug solutions were applied to the cells *via* a multi-barrel drug delivery system, which pivoted the barrels into place using a stepping motor. This ensured rapid application and washout of the drug. The measured agonist exchange time using this system was approximately 20–30 ms. GABA was applied to the cell for 5 s with a 30 s washout period between applications. Allosteric potentiation of GABA receptors was measured relative to a GABA EC$_{20}$ individually determined for each cell to account for differences in GABA affinity. Increasing concentrations of modulator were pre-applied for 30 s prior to co-application with GABA. Any direct effect of the compounds was measured relative to the maximum response produced by a saturating concentration of GABA. Data were recorded using P-clamp (version 9 – Axon Instruments, Foster City, CA). Curves were fitted using a non-linear square-fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^{n_H}]$, where x is the drug concentration, EC$_{50}$ is the concentration of drug eliciting a half-maximal response, and n_H is the Hill coefficient. EC$_{50}$s were calculated as geometric means and expressed with 95% confidence limits.

2.2.1. Brain slice preparation and electrophysiology

Thalamic slices were prepared from mice of either sex (P18–24) according to standard protocols (Belelli et al., 2005). Animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures) Act