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The dual modulatory effects of efavirenz on $GABA_A$ receptors are mediated via two distinct sites☆

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ABSTRACT

Efavirenz is a widely prescribed medicine used to treat type 1 human immunodeficiency virus (HIV-1), the most prevalent pathogenic strain of the virus responsible for the acquired immune deficiency syndrome (AIDS) pandemic. Under prescribed dosing conditions, either alone or in combination therapy, efavirenz-induced CNS disturbances are frequently reported. Efavirenz was recently reported to interact in a similar concentration range with a number of receptors, transporters and ion channels including recombinant rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors whose actions were potentiated (Gatch et al., 2013; Dalwadi et al., 2016). Now we report on the molecular mechanism of efavirenz on $GABA_A$ receptors as a function of concentration and subunit composition via whole-cell recordings of GABA-activated currents from HEK293 cells expressing varying subunit configurations of $GABA_A$ receptors. Efavirenz elicited dual effects on the GABA response; it allosterically potentiated currents at low concentrations, whereas it inhibited currents at higher concentrations. The allosteric potentiating action on $GABA_A$ receptors was pronounced in the $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$ and $\alpha4\beta2\gamma2$ configurations, greatly diminished in the $\alpha6\beta2\gamma2$ configuration, and completely absent in the $\alpha3\beta2\gamma2$ or $\alpha5\beta2\gamma2$ configuration. In stark contrast, the inhibitory modulation of efavirenz at higher concentrations was evident in all subunit configurations examined. Moreover, efavirenz-induced modulatory effects were dependent on GABA concentration ([GABA]), with a pronounced impact on currents activated by low [GABA] but little effect at saturating [GABA]. Mutation of a highly-conserved threonine to phenylalanine in transmembrane domain 2 of the $\alpha1$ subunit abolished the inhibitory effect of efavirenz in $\alpha1\beta2$ receptors. Finally, mutations of any of the three conserved extracellular residues in $\alpha1/2/4$ subunits to the conserved residues at the corresponding positions in $\alpha3/5$ subunits (i.e., R84P, M89L or I120L) completely eliminated the potentiating effect of efavirenz in $\alpha1\beta2\gamma2$ configuration. These findings demonstrate that efavirenz's positive allosteric modulation of the $GABA_A$ receptor is mediated via a novel allosteric site associated with the extracellular domain of the receptor.

1. Introduction

The neurotransmitter γ-aminobutyric acid (GABA) inhibits neuronal activity in the mammalian CNS via activation of a chloride (Cl^-) selective ligand-gated ion channel subtype called the $GABA_A$ receptor. The $GABA_A$ receptor is a known mediator of anxiety states, somnolence, and seizures/epilepsy. Diverse therapeutic and pharmacological agents, such as neurosteroids, carisoprodol, benzodiazepines, barbiturates, and anesthetics modulate $GABA_A$ receptor function (Huang et al., 2006; Gonzalez et al., 2009; Tan et al., 2011).

Efavirenz (Sustiva®, Stocrin®) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) widely used in combination with other antiretrovirals for the treatment of HIV-1. However, efavirenz also

Abbreviations: BDZ, benzodiazepine; CNS, central nervous system; DMSO, dimethyl sulfoxide; DZ, diazepam; EFAV, efavirenz; FLU, flumazenil; LGICs, ligand-gated ion channels; HEK293, Human embryonic kidney cell line; NNRTI, non-nucleoside reverse transcriptase inhibitor; PTX, picrotoxin; TM2, second transmembrane domain.

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known to exert neuropsychiatric adverse events in some patients (Barreiro et al., 2002; Treisman and Kaplin, 2002; Cespedes and Aberg, 2006; Sutterlin et al., 2006; Arendt et al., 2007; Treisman and Soudry, 2016). For instance, in a large clinical study (>1000 patients) CNS disturbances were reported in over half of all patients taking a standard dose of efavirenz including dizziness (28%), depression (19%), insomnia (16%), anxiety (9%), impaired concentration (8%), somnolence (7%), nervousness (7%), abnormal dreams (6%), and hallucinations (1.2%) (Sustiva package insert, 1998). Other reports indicated 40–70% of patients treated with efavirenz suffer from CNS symptoms (Blanch et al., 2001; Puzantian, 2002). The apparent psychoactivity of efavirenz may even be encouraging its diversion for recreational use (Gatch et al., 2013; Grelotti et al., 2014). In a previous report, $GABA_A$ receptors ($\alpha1\beta2\gamma2$) were identified as one of several CNS targets of efavirenz (Gatch et al., 2013). In the present study we investigated the concentration and subunit-dependency of efavirenz on $GABA_A$ receptors in a molecular mechanistic context.

2. Materials and methods

2.1. Expression of cloned receptors

Human embryonic kidney cell lines (HEK293) stably expressing recombinant human or rat $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$ or $\alpha6\beta2\gamma2$ $GABA_A$ receptors were studied in the present investigation (short isoform of the $\gamma2$ subunit in all cases). Cells transiently expressing various wild type human or rat $GABA_A$ subunit configurations were also studied. A human HEK293 cell line was transiently transfected with recombinant receptor subunits using PolyJet™ DNA *In Vitro* transfection reagent (SignaGen Laboratories, Rockville, MD). Briefly, HEK293 cells were washed and placed in fresh Dulbecco's modified eagle medium containing 10% FBS and antibiotics (penicillin 100 U/mL). For human or rat $\alpha_x(x = 1, 4, 5)\beta2\gamma2$ $GABA_A$ receptors, a 1:1:3 ratio (total cDNA: 2.5 μg) of α_x, β2 and γ2 subunits was added to cells growing exponentially on poly-L-lysine coated coverslips placed in a 35-mm culture dish. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection.

2.2. Mutagenesis

Mutations of receptor cDNA were generated using a commercially available site-directed mutagenesis kit (QuickChange, Strategene, La Jolla, CA) and commercially produced mutagenic primers (MWG Biotech, NC). All mutants were verified by DNA sequencing (MWG Biotech, NC).

2.3. Electrophysiology

Whole-cell patch recordings were made at room temperature (22–25 °C) at a holding potential of −60 mV. Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 3–5 MΩ. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. A coverslip containing cultured cells was placed in a small chamber (~1.5 mL) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5–8 mL/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 $MgCl_2$, 3.0 $CaCl_2$, 10 HEPES, 10 D-glucose, pH 7.3. GABA-evoked currents from the whole-cell configuration were obtained using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) equipped with a CV201A headstage. The currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer for subsequent analysis. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored the current response to a 5 mV voltage pulse on our digital oscilloscope. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data were not included in the analysis. Current-voltage (I-V) relationship of GABA currents was assessed from a single cell using a ramp stimulus protocol. A transmembrane voltage ramped from −60 to +60 mV over 0.5 s time course was applied prior to (passive conductance phase) and during GABA application. The difference of these two current ramps (pA) represented the GABA-induced current, and was plotted as a function of applied holding potential (mV) to yield the I-V curve of GABA-induced currents.

2.4. Experimental protocol

GABA was prepared in extracellular solution and was applied to cells via gravity flow using a Y-shaped tube positioned near the target cell. With this system, the 10–90% rise time of the junction potential at the open tip is 60–120 ms (Huang and Dillon, 1999). Once a control response was determined, the effect of efavirenz was examined by co-applying it with a roughly EC_{30} concentration of GABA. Because recovery from the drug-induced effect was readily obtained upon wash out, the effects of multiple concentrations of efavirenz could generally be obtained from same cell. Efavirenz was tested at concentrations below 100 μM, because high concentrations resulted in a non-specific cytotoxicity as indicated by loss of patch during drug application.

2.5. Chemicals

GABA stock was made in doubly distilled H_2O. Efavirenz, diazepam, picrotoxin and flumazenil were solubilized in dimethyl sulfoxide (DMSO) and added to cells at a final concentration of less than 0.05% (v/v) DMSO. DMSO (0.05%) had no effect on GABA response. Efavirenz was purchased from Sequoia Research Products Limited (Pangbourne, UK) or provided by NIH via NIH AIDS Reagent Program (https://www.aidsreagent.org/). All other drugs were purchased from Sigma Aldrich or Tocris Biosciences (Minneapolis, MN).

2.6. Data analysis

All data were recorded on a chart recorder, and stored on a computer for subsequent off-line analysis (pClamp 6.0, Axon Instruments; and Origin 5.0, Microcal Software, Inc). GABA concentration–response profiles were fitted to the following equation: $I/I_{max} = [GABA]^n/(EC_{50}^n + [GABA]^n)$, where I and I_{max} represent the normalized GABA-induced current at a given concentration and the maximal current induced by a saturating concentration of GABA, respectively, EC_{50} is 50% effective GABA concentration, and n is the Hill coefficient. Concentration-response profiles for the positive modulatory actions of efavirenz were generated using the equation $I/I_{max} = [efavirenz]^n/([efavirenz]^n + EC_{50}^n)$, where I is the normalized current amplitude at a given concentration of efavirenz, I_{max} is the maximum GABA current induced by efavirenz, EC_{50} is the half-maximal effective concentration of efavirenz, and n is the Hill coefficient. The concentration-response relationship for the inhibitory action of efavirenz was fitted with the equation: $I/I_{max} = [efavirenz]^n/([efavirenz]^n + IC_{50}^n)$, where I is GABA current amplitude normalized to control, IC_{50} is the half-blocking concentration, and n is the Hill coefficient. Since efavirenz substantially enhanced current decay, during analysis of efavirenz-mediated