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Benzodiazepine modulation of the rat $GABA_A$ receptor α4β3γ2L subtype expressed in *Xenopus* oocytes

Haitao You [1], Janna L. Kozuska, Isabelle M. Paulsen, Susan M.J. Dunn*

Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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ABSTRACT

The effects of benzodiazepines on $GABA_A$ receptors are dependent largely on the particular α subunit isoform that is present in the receptor pentamer. The inclusion of either the α4 or α6 subunit is generally thought to render the receptor insensitive to classical benzodiazepines. We expressed the rat α4β3γ2L subtype in *Xenopus* oocytes and observed that both diazepam and flunitrazepam significantly potentiated GABA-gated currents. This potentiation occurred at nanomolar concentrations similar to those seen at the most abundant "diazepam-sensitive" receptor i.e., the α1β2γ2 subtype. In the α4β3γ2L receptor, the effects of diazepam and flunitrazepam were inhibited by nanomolar concentrations of the benzodiazepine site antagonists, Ro15-1788 and ZK93426. The presence of the β3 subunit appears to be important for this modulation since diazepam did not affect GABA responses mediated by recombinant α4β1γ2L or α4β2γ2L receptors. Interestingly, when the α4β3γ2L receptor was expressed in HEK293 cells, diazepam and flunitrazepam displaced the relatively non-selective benzodiazepine site ligand, [^{3}H]Ro15-4513, only at high concentrations (>10 μM) demonstrating a lack of high affinity binding for these classical benzodiazepines. Functional studies of the cell-expressed receptors using whole cell recording techniques showed that neither diazepam nor flunitrazepam potentiated GABA-evoked currents although currents were enhanced by nanomolar concentrations of Ro15-4513. These results suggest that the observed benzodiazepine modulation of the α4β3γ2L subtype depends on the expression system used and may be specific for expression in *Xenopus* oocytes.

1. Introduction

Since their introduction in the 1960s, the benzodiazepines have been prescribed widely as anxiolytics, hypnotics and muscle relaxants (see Korpi et al., 1997). These drugs act as allosteric modulators of the γ-aminobutyric acid type A ($GABA_A$) receptor, the major inhibitory neurotransmitter receptor in the mammalian brain. The $GABA_A$ receptor belongs to the Cys-loop family of ligand-gated ion channel that includes the nicotinic, serotonin type 3, and glycine receptors (Sieghart et al., 1999). Each receptor in the family is a pentamer of homologous subunits that assemble to form a central ion channel. The anion-selective $GABA_A$ receptors are heterogeneous, and nineteen mammalian $GABA_A$ receptor subunits have been identified to date i.e., α1–6, β1–3, γ1–3, ρ1–3, π, δ, ε and θ (McKernan and Whiting, 1996; Barnard et al., 1998). The major receptor isoform in the mammalian brain is the α1β2γ2 combination where the subunits occur in a likely stoichiometry of 2:2:1 (Baumann et al., 2001, 2002; Farrar et al., 1999), oriented in an anti-clockwise arrangement of α–β–α–γ–β when viewed from the synaptic cleft (Baumann et al., 2002).

The pharmacology of benzodiazepines is determined mainly by the particular α subunit isoform that is present in the pentamer. $GABA_A$ receptors containing the α1, α2, α3 or α5 subunits, along with a β and γ subunit, are sensitive to classical benzodiazepines, such as diazepam and flunitrazepam. In contrast, receptors containing the α4 or α6 subunits are generally thought to be "diazepam-insensitive" although they retain high affinity for the imidazobenzodiazepines, Ro15-4513 and Ro15-1788 (Derry et al., 2004; Knoflach et al., 1996). The results of many mutagenesis and biochemical studies (Wieland et al., 1992; Duncalfe et al., 1996) have attributed diazepam sensitivity primarily to a single amino acid substitution. In the diazepam-sensitive α subunits, a histidine at position H101 (rat α1 subunit numbering) is replaced by an arginine in the equivalent position of the α4 or α6 subunits.

There is abundant evidence to suggest that a high affinity benzodiazepine site lies at the α – γ subunit interface (see Sigel and

* Corresponding author. Tel.: +1 780 492 0511; fax: +1 780 492 4325.
E-mail address: susan.dunn@ualberta.ca (S.M.J. Dunn).
[1] Present address: Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada.

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Buhr, 1997) and, not surprisingly, the γ subunit has been shown to play a major role in benzodiazepine binding. Whereas the benzodiazepine site ligands, Ro15-4513, bretazenil and DMCM modulated GABA-mediated responses in the α4β3γ2 subtype expressed in mammalian cells, these effects were abolished by substitution of the γ2 subunit by the δ subunit (Brown et al., 2002). Receptors containing the α4-subunit represent a small percentage of the total $GABA_A$ receptor population in the mammalian brain. The α4 subunit is co-localized with γ2 subunits in the hippocampus and thalamus (Sur et al., 1999) and the co-assembly of these subunits appears to play an important role in epileptogenesis. Animal models of epilepsy suggest that there is a consistent up-regulation of the α4βxγ2 subtype in dentate granule cells which parallels a decreased expression of the α4βxδ subtype (Payne et al., 2006; Peng et al., 2004).

In the present study, we have re-examined the interactions of benzodiazepines with the rat α4β3γ2L subtype. We show that, at nanomolar concentrations, classical benzodiazepines can significantly potentiate GABA currents mediated by the α4β3γ2L receptor expressed in *Xenopus* oocytes and that this potentiation is inhibited by Ro15-1788 and ZK93426. However, consistent with previous reports (Ebert et al., 1996; Scholze et al., 1996), we did not detect high affinity binding of diazepam or flunitrazepam to this subtype expressed in HEK293 cells. Furthermore, neither of these ligands modulated GABA-mediated currents expressed in these mammalian cells, although Ro15-4513 potentiated agonist responses as has been reported previously (Kelly et al., 2002; Knoflach et al., 1996). These results suggest that benzodiazepine modulation of the recombinant α4β3γ2L subtype may be an oocyte-specific phenomenon.

2. Materials and methods

2.1. Chemicals

[^{3}H]Ro15-4513 was purchased from NEN Life Science Products (Boston, MA). GABA and flunitrazepam were obtained from Sigma-Aldrich (St. Louis, MO). Diazepam was a gift from Dr. G.B. Baker (Psychiatry, University of Alberta) and β-CCE from Dr. B.L. Jones (GlaxoSmithKline, Harlow, UK). Ro15-4513 and Ro15-1788 were provided by Hoffman-La Roche and Co. (Basel, Switzerland), and ZK93423 and ZK93426 were gifts from Schering. GABA was dissolved in frog Ringer's buffer (110 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4) and the other modulators were dissolved in DMSO to make concentrated stock solutions. The final concentration of DMSO in all experiments was constant at 0.01% (v/v) and this concentration was shown to have no effect on GABA responses.

2.2. Clones

The original cDNAs encoding the rat α4, β1 and β3 subunits were from Dr P. H. Seeburg's laboratory, and those encoding the γ2L and β2 subunits were provided by Dr D. L. Weiss. All cDNAs were subcloned into the pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA) except for the β2 subunit cDNA which was subcloned into pcDNA3.1(−). To improve the expression levels of α4β3γ2L receptors in HEK293 cells, the signal peptide sequence of β3 was substituted by that of β2. The coding regions of all subunit clones used in these studies were verified by sequencing.

2.3. Expression in Xenopus oocytes and two-electrode voltage clamp recordings

Capped cRNAs encoding rat $GABA_A$ receptor subunits were prepared as described (You and Dunn, 2007). Stage V-VI Xenopus laevis oocytes were isolated as described by Smith et al. (2004). Oocytes were injected with 50 nL of 1 μg/μL total subunit cRNA in a 1:1:1 ratio (α4:βx:γ2L). The injected oocytes were maintained in ND96 buffer (96 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) supplemented with gentamicin (1%, v/v) in 96-well plates at 14 °C for at least 48 h prior to functional analysis.

Oocyte recordings were carried out using standard two-electrode voltage clamp techniques at a holding potential of −60 mV. Oocytes were continuously perfused with frog Ringer's solution. Currents were recorded using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA). The microelectrodes were filled with 3 M KCl, and had a resistance between 0.5 and 3.0 MΩ. In studies of modulator effects, oocytes were pre-perfused with 10 ml of these ligands for ~2 min prior to activation of the receptor by perfusion with GABA at its EC_{20} concentration (or EC_{50} for β-CCE modulation of the α4β3γ2L subtype) and the same concentration of the modulator as used in the pre-perfusion. After each current measurement, oocytes were washed with buffer for >15 min to ensure complete recovery from desensitization.

2.4. Transient transfection in mammalian cells and radioligand binding assays

Human embryonic kidney (HEK-293) cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich), supplemented with 10% bovine growth serum (Hyclone) at 37 °C in a 5% CO_2 incubator. Transient transfection was carried out using the calcium phosphate method described previously (Derry et al., 2004; Hansen et al., 2005). cDNAs encoding α4, β3, γ2L subunits were added in a 1:1:1 mass ratio to an appropriate volume of 250 mM $CaCl_2$, followed by the addition of an equal volume of *N*, *N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) buffer (pH 7.04). The solutions were mixed well and added dropwise to the cells. 44–48 hours after transfection, cells were harvested by scraping into ice-cold harvesting buffer (50 mM Tris, 250 mM KCl, pH7.4) supplemented with benzamidine (1 mM), bacitracin (0.1 mg/ml), trypsin inhibitor (0.01 mg/ml) and phenylmethylsulfonyl fluoride (0.5 mM). The cells were then homogenized, and following centrifugation (Sorvall SS34 rotor, 18,000 rpm, 30 min), the pellets were resuspended in harvesting buffer and stored at −80 °C.

Radioligand binding experiments were performed as described previously (Newell and Dunn, 2002; Derry et al., 2004). For saturation assays, cell homogenates were incubated in duplicate with increasing concentrations (1–80 nM) of [^{3}H]Ro15-4513 in Tris-HCl buffer (50 mM Tris, 250 mM KCl, 0.02% NaN_3, pH 7.4) in a final volume of 0.5 ml at 4 °C for 1 h. Non-specific binding was determined in the presence of a high concentration (250 μM) of unlabeled Ro15-4513. For competition binding assays, membranes were incubated with a constant concentration of [^{3}H]Ro15-4513 at a concentration equal to its K_D value and increasing concentrations of unlabeled flunitrazepam or diazepam. Following incubation, the membrane preparations were filtered through GF/B filters (Whatman, Maidstone, UK) using a cell harvester (Brandel, Gaithersburg, MD) and washed twice with 3 ml aliquots of ice-cold Tris-HCl buffer. Filters were transferred to scintillation vials and subjected to scintillation counting (LS6500 Scintillation System, Beckman Instruments Inc., Fullerton, CA) after addition of 5 mL scintillation fluid.

2.5. Whole-cell patch clamp recordings of receptors expressed in HEK293 cells

The rat α4β3γ2L receptor was expressed in HEK293 cells as described above. Cells were plated on 35 mm dishes and whole cell recordings were made 24–48 hours after transfection. During recordings, plates were continuously perfused with an extracellular recording solution containing 140 mM NaCl, 2.8 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, buffered to pH 7.4 with NaOH. Patch pipettes were pulled on a Flaming/Brown micropipette P87 puller (Sutter Instrument Co.) using thin walled electrode glass (cat. 5964, A-M Systems Inc.) and fire polished. Pipette open resistance was between 3–5 MΩ when filled with intracellular recording solution composed of 140 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, buffered to pH 7.4 with CsOH. Cells were voltage clamped at −60 mV using an Axon 200B amplifier (Axon Instruments) and GABA was applied to a clamped cell via a second drug- delivery pipette. Potentiation of $GABA_A$ receptor responses were measured relative to the response elicited by an EC_{20} concentration of GABA. Diazepam, flunitrazepam or Ro15-4513 was bath perfused for 2 min prior to subsequent challenges with GABA at the same concentration.

2.6. Data and statistical analysis

All electrophysiological and radioligand binding data were analyzed by non-linear regression techniques using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

The potentiation of GABA-induced currents by modulators was compared to the current evoked by the application of a control concentration of GABA (at its EC_{20} or EC_{50} value as described above). The extent of modulation is expressed as a percentage of the control current using the following equation:

$$\text{Potentiation}\ (\%) = [(I_{\text{GABA+modulator}}/I_{\text{GABA}}) \times 100] - 100$$

Normalized data were then used to construct concentration-response curves using the equation:

$$P = (P_{\max}[M]^n)/(\text{EC}_{50}^n + [M]^n)$$

where P is the normalized potentiation elicited by modulator; $[M]$ is the modulator concentration, EC_{50} is the concentration of the modulator producing 50% of the maximal response (P_{max}), and n is the Hill coefficient.

In experiments estimating antagonist affinities, IC_{50} values were calculated from the equation:

$$I/I_{\max} = [B]^n/([B]^n + \text{IC}_{50}^n)$$

where IC_{50} is the concentration of antagonist, $[B]$, that reduces the amplitude of benzodiazepine potentiation of GABA-evoked current by 50% and n is the Hill coefficient.