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High affinity, heterogeneous displacement of [^{3}H]EBOB binding to cerebellar $GABA_A$ receptors by neurosteroids and GABA agonists

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Abstract

Heterogeneous binding interactions of cerebellar $GABA_A$ receptors were investigated with GABA agonists and neurosteroids. $GABA_A$ receptors of rat cerebellum were labelled with [^{3}H]ethynylbicycloorthobenzoate (EBOB), a convulsant radioligand. Saturation analysis revealed a homogenous, nanomolar population of [^{3}H]EBOB binding. Both GABA and 5α-tetrahydrodeoxycorticosterone (5α-THDOC) displaced [^{3}H]EBOB binding heterogeneously, with nanomolar and micromolar potencies. The nanomolar phase of displacement by GABA was selectively abolished by 100 μM furosemide. Physiological concentrations of allopregnanolone (8 nM) and 5α-THDOC (20 nM) increased the displacing effects of nanomolar GABA. GABA (0.3 μM) and 5α-THDOC (0.3 μM) potentiated the micromolar population of displacement by the other. Taurine inhibited [^{3}H]EBOB binding also heterogeneously, with micromolar and millimolar potencies, and 0.3 μM 5α-THDOC potentiated this inhibition. 5β-THDOC did not affect [^{3}H]EBOB binding significantly but in 1 μM it antagonised selectively the nanomolar displacement by 5α-THDOC. [^{3}H]EBOB binding to hippocampal $GABA_A$ receptors was inhibited by GABA and allopregnanolone with low (micromolar) potencies and with slope values higher than unity referring to allosteric interaction. High affinity displacement of cerebellar [^{3}H]EBOB binding by GABA agonists and neurosteroids can be associated with constitutively open $\alpha_6\beta\delta$ $GABA_A$ receptors, tonic GABAergic inhibitory neurotransmission and its modulation by physiological concentrations of neurosteroids.

Keywords: $\alpha_6\beta\delta$ $GABA_A$ receptors; [^{3}H]EBOB binding; Allopregnanolone; THDOC epimers; Taurine; Furosemide

1. Introduction

γ-Aminobutyric acid is the major inhibitory neurotransmitter in mammalian brain. Its A-type ionotropic receptors belong to the Cys-loop superfamily of neurotransmitter receptors. Five subunits form a chloride-permeable channel. Combinations of the subunits $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, δ, ε, π, $\rho_{1\text{-}3}$ and θ contribute to great heterogeneity of $GABA_A$ receptors (Whiting et al., 1999). A huge variety of pharmacological agents such as benzodiazepines, barbiturates, neurosteroids, anaesthetics and convulsants bind to $GABA_A$ receptors and allosterically modulate the opening of the ionophore to result in pharmacological fine-tuning of GABAergic neurotransmission. An exponentially increasing number of reports deal with the pharmacological effects of neurosteroids such as allopregnanolone and 5α-THDOC which have been considered the major endogenous modulators of $GABA_A$ receptors (Lambert et al., 2001). Allopregnanolone is a major metabolite of progesterone synthesized in the brain, while 5α-THDOC

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derives mainly from adrenal deoxycorticosterone (Reddy, 2003; Schumacher and Baulieu, 1995). In nanomolar concentrations these 3α-hydroxy-pregnane neurosteroids potentiate GABA-elicited ionophore activity (Majewska et al., 1986), in micromolar concentrations they have own efficacy to open the chloride channel while in submillimolar concentrations they block it (Wohlfarth et al., 2002). Since allopregnanolone and 5α-THDOC concentrations in rat cerebral cortex and plasma vary between 4 and 30 nM (Concas et al., 1998), the question arises what is the physiological relevance of these modulatory effects?

The effects of neuroactive steroids depend on the structures of the ligands and $GABA_A$ receptor subunits (Belelli et al., 2002). Full potentiation requires 3α-hydroxy-5α-pregnane rings (Lambert et al., 2001). 5β-THDOC has partial agonist–antagonist efficacy (Xue et al., 1997). The δ subunit results in extrasynaptic location of $GABA_A$ receptors (Farrant and Nusser, 2005, Porcello et al., 2003), greatest sensitivity to 3-hydroxylated neurosteroids (Adkins et al., 2001; Belelli et al., 2002; Bianchi and Macdonald, 2003; Brown et al., 2002; Saxena and Macdonald, 1996; Wohlfarth et al., 2002) and this sensitivity is attenuated if δ subunits are deleted (Mihalek et al., 1999; Vicini et al., 2002).

The cage convulsant [^{35}S]t-butylbicyclophosphorothionate (TBPS) has become the major radioligand of $GABA_A$ receptors because its binding kinetics has shown several correlations between allosteric modulation of receptor binding and ionophore function (Hawkinson et al., 1994a; Maksay and Simonyi, 1986; Maksay, 1996). Other cage convulsant radioligands [^{3}H]4′-ethynyl-4-n-propylbicycloorthobenzoate (EBOB) and [^{3}H]t-butylbicycloorthobenzoate (TBOB) have been used less frequently (Cole and Casida, 1992; Maksay and Van Rijn, 1993). Interestingly, displacement of [^{35}S]TBPS binding has been homogenous by 5α-pregnane neurosteroids such as allopregnanolone and 5α-THDOC but heterogeneous by 5β-pregnanediols such as 5β-THDOC (Hawkinson et al., 1994a,b; McCauley et al., 1995).

$GABA_A$ receptors in cerebellum and in its granule cells have been thoroughly studied. They are responsible for tonic inhibition and tuning motor activity in cerebellum (Farrant and Nusser, 2005; Hamann et al., 2002; Stell et al., 2003). A major fraction of cerebellar $GABA_A$ receptors contains $\alpha_6\beta\delta$ subunits (Pöltl et al., 2003) responsible for alcohol-induced impairment of motor coordination (Hanchar et al., 2005). These are constitutively open extrasynaptic receptors (Amin, 2004; Nusser et al., 1998). Although subunits α_6 and δ impart great sensitivity to GABA agonists and neurosteroids, no clear binding heterogeneity has been revealed for cerebellar $GABA_A$ receptors with nanomolar affinities of GABA and neurosteroids in physiological concentrations. Therefore we used extensively washed synaptic membranes of rat cerebellum for [^{3}H]EBOB binding to $GABA_A$ receptors and unmasked high affinity (nanomolar) binding of $GABA_A$ agonists and neurosteroids. These affinities enable persistent activation of $GABA_A$ receptors by nanomolar GABA and modulation by physiological concentrations of neurosteroids. This might contribute to the tonic activity of extracellular cerebellar $GABA_A$ receptors.

2. Materials and methods

2.1. Materials

[^{3}H]EBOB (30 Ci/mmol) was purchased from Dupont-NEN and freshly diluted in buffer for daily binding experiments. Allopregnanolone, 5α- and 5β-tetrahydrocorticosterone (THDOC, 5α- and 5β-pregnan-3α,21-diol-20-one) were obtained from Steraloids Inc. (Newport, USA). They were dissolved in DMSO which did not exceed 0.3% in the binding incubation. Taurine, furosemide and picrotoxinin were obtained from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Membrane preparation

The experimental protocol has been approved by the Veterinary and Food Control Station of Budapest (25-113/2001). Male Wistar rats were decapitated, brain regions dissected and homogenized in 0.32 M sucrose using a Teflon pestle and glass homogenizer and centrifuged at $1000\times g$ for 10 min. The supernatant was centrifuged at $45{,}000\times g$ for 30 min. The pellet was homogenized in double-distilled water by Ultra-Turrax for 2×10 s and centrifuged at $45{,}000\times g$ for 30 min. The pellet was washed by suspension in 50 mM Tris–HCl buffer (pH 7.4) by Ultra-Turrax and similar centrifugations twice, suspended and frozen in aliquots.

2.3. Receptor binding and displacement studies

Membrane suspensions were thawed and centrifuged in 50 mM Tris–HCl containing 0.2 M NaCl at $10{,}000\times g$ for 10 min and washed by a similar centrifugation. For saturation of [^{3}H]EBOB binding triplicate membrane suspensions were incubated with 0.4–7 nM [^{3}H]EBOB for 2 h at 25 °C. For displacement studies 1 nM [^{3}H]EBOB was incubated with varying concentrations of neurosteroids or GABA agonists. For nonspecific binding 50 μM picrotoxinin was applied. Samples of 1 ml were filtered on Whatman GF/B filters under vacuum with a Brandel Harvester and washed with 3×3 ml ice-cold buffer. Radioactivity of the filters was measured in Optiphase Hisafe using a scintillation spectrometer (Wallac Winspectral, Turku, Finland). Protein content was determined with the Bio-Rad