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Nanomolar allopregnanolone potentiates rat cerebellar $GABA_A$ receptors

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Abstract

The ionophore function of γ-aminobutyric acid A ($GABA_A$) receptors was studied by whole-cell patch clamp electrophysiology in primary cultures of rat cerebellar cortex. Chloride currents elicited by 1 μM GABA were potentiated by allopregnanolone with a plateau of high affinity (EC_{50} = 14 nM) and a peak of potentiation around 1 μM allopregnanolone. Furosemide (0.1 mM) eliminated the high affinity phase and increased the EC_{50} to 685 nM. $GABA_A$ receptors of rat cerebellar synaptosomal membranes were labelled with [^{3}H]ethynylbicycloorthobenzoate (EBOB). Allopregnanolone displaced [^{3}H]EBOB binding with IC_{50} = 320 nM. The displacing potency of allopregnanolone was strongly enhanced (IC_{50} = 39 nM) in the presence of 400 nM GABA and 60 nM SR 95531. Nanomolar potentiation by allopregnanolone can be associated with cerebellar $GABA_A$ receptors containing α_6, β_{2-3} and δ subunits. This might be suitable for physiological modulation of tonic inhibitory neurotransmission via extrasynaptic $GABA_A$ receptors in cerebellar granule cells by neurosteroids.

Keywords: γ-Aminobutyric acid A receptor-ionophore; Cerebellar granule cells; Allopregnanolone; Furosemide; Displacement of [^{3}H]EBOB binding; $\alpha_6\beta_{2/3}\delta$ $GABA_A$ receptors

Ionophoric A type γ-aminobutyric acid ($GABA_A$) receptors have a predominant role in the inhibitory neurotransmission of mammalian brain. Five subunits out of α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π, ρ_{1-3} and θ subunits form a membrane channel for the penetration of dehydrated chloride and bicarbonate ions [14]. The pharmacological fine-tuning of GABAergic neurotransmission can be performed by allosteric agents such as neurosteroids. Allopregnanolone is a major neurosteroid formed from progesterone in brain. Fluctuations of cortical and plasma levels in the range of 10–30 nM allopregnanolone have been considered to modulate $GABA_A$ receptors during the menstrual cycle, pregnancy [5], prenatal [1] and postnatal development [18]. Submicromolar concentrations of allopregnanolone potentiate the chloride currents elicited by GABA with subunit-dependent potencies [2]. The enhancement by GABA of the K^+-evoked aspartate release from cerebellar synaptosomes was also potentiated by submicromolar, while micromolar concentrations of allopregnanolone enhanced this release in the absence of GABA [21]. The modulatory effects of neurosteroids on $GABA_A$ receptors can be characterized not only by ionophore function but also by receptor binding. Allosteric modulation of binding of radiolabelled cage convulsants such as [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) correlates with the ionophore function of $GABA_A$ receptors [9]. The displacing potency of allopregnanolone is about 100 nM for [^{35}S]TBPS binding [10]. These functional correlations can be extended to [^{3}H]*t*-butylbicycloorthobenzoate (TBOB) [16] and [^{3}H]ethynylbicycloorthobenzoate (EBOB) binding [15] here.

Most of the above in vitro effects required allopregnanolone concentrations higher than the physiological range [5]. Therefore, we examined cerebellar $GABA_A$ receptors displaying high affinity for agonists and great sensitivity for neurosteroids due to their α_6 and δ subunits [2,3,12,20,22,24,25].

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Primary cerebellar cultures were prepared from 4-day-old Wistar rats. After decapitation, dissected cerebella were washed with Ca^{2+}/Mg^{2+}-free HEPES-buffered solution (in mM: NaCl 137, KCl 5, $NaHCO_3$ 3, Na_2HPO_4 0.6, KH_2PO_4 0.4, D-glucose 5.6, HEPES 20, penicillin G 100 U/ml, streptomycin 100 mg/l, amphotericin B 5 μg/ml, pH 7.4) (all from Sigma), and incubated in 1× Trypsin-EDTA solution (Sigma) for 3 min. After trituration, cell suspensions were filtered through a 70 μm mesh and sedimented at 125 × *g* for 5 min. Pellets were resuspended in culture medium (D-MEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), nerve growth factor (Sigma) 20 ng/ml, KCl 20 mM, amphotericin B 2.5 μg/ml, penicillin G 100 U/ml and streptomycin 100 μg/ml. Cells were plated at a density of (1–2) × 10^5 cells/cm^2 on sterilized glass coverslips coated with poly-D-lysine (Sigma). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air, half of the medium was changed to fresh serum-free medium twice a week.

Conventional whole-cell patch clamp recordings were made from cultured cerebellar cells 7–22 days after plating. Cultures in a recording chamber were superfused with the extracellular solution (e.s.) at 25 °C. The e.s. contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, HEPES-Na 5, glucose 20, pH 7.35. Patch electrodes (resistances: 5–10 MΩ) pulled from borosilicate capillary glass (GC120F-10, Harvard Apparatus) were filled with the intracellular solution (i.s.). The composition of i.s. was (in mM): CsCl 110, $MgCl_2$ 4.5, HEPES 9, BAPTA 10, ATP-Na_2 4, GTP 0.3, creatine phosphate 14, creatine phosphokinase 50 U/ml, pH 7.25. Osmolarities of the e.s. and i.s. were 310 and 290 mOsm, respectively. Tetrodotoxin (Latoxan, 0.3 μM in the e.s.) was used to block action potentials. The compounds diluted in the e.s. (dimethyl sulfoxide: 0.1%) were applied near the cells via multi-barrelled ejection pipettes controlled by electromagnetic valves. GABA (1 μM) was applied repetitively for 3 s at 30-s intervals with or without allopregnanolone (5α-pregnan-3α-hydroxy-20-one, Sigma) and 100 μM furosemide (Sigma). Inward currents were low-pass filtered (1 kHz) and recorded at a holding potential of −70 mV using an Axopatch 200A amplifier, digitized (Digidata 1200), captured and analyzed using pClamp 8.0 (Axon Instruments, Union City, CA).

For [^{3}H]EBOB binding, male Wistar rats were decapitated, the cerebella were homogenized in 0.32 M sucrose and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 45,000 × *g* for 30 min. The pellet was homogenized in distilled water, centrifuged at 45,000 × *g* for 30 min, washed by suspension in 50 mM Tris–HCl buffer (pH 7.4) and similar centrifugations twice and frozen. The thawed suspensions were centrifuged in 50 mM Tris–HCl containing 0.2 M NaCl at 10,000 × *g* for 10 min and washed by a similar centrifugation. For displacement studies triplicate membrane suspensions were incubated with 1 nM [^{3}H]EBOB (30 Ci/mmol, Dupont-NEN), allopregnanolone in the presence or absence of 400 nM GABA and 60 nM SR 95531 (2-(3-carboxypropyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide, donated by Prof. C.G. Wermuth, France) for 2 h at 25 °C. 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. The experimental protocols have been approved by the Veterinary and Food Control Station of Budapest, in agreement with the guidelines of the European Communities Council Directive (86/609/EEC).

Data are presented as means ± S.E.M. Potentiation was calculated from the current peak amplitude evoked by GABA compared to the baseline current in the presence and absence of allopregnanolone. Potentiation and displacement data were fitted with sigmoidal curves and variable slopes via GraphPad Prism 4.02 (San Diego, CA). IC_{50} data were compared in Student's paired *t*-test.

Cerebellar granule cells in culture responded to 1 μM GABA with chloride currents of 346 ± 40 pA (mean ± S.E.M. of 68 cells). Allopregnanolone potentiated this response in a concentration-dependent manner. Fig. 1 shows that potentiation was saturated first with a nanomolar phase followed by a peak around 1 μM allopregnanolone then potentiation returned to about the plateau of the high affinity phase (67% increase in the current amplitude). The first phase up to 0.3 μM allopregnanolone can be characterized with EC_{50} = 14.0 nM (95% confidence interval: 5–38 nM) and slope values of $n = 1.5 \pm 0.9$.

The diuretic furosemide is a selective blocker of $GABA_A$ receptors in cerebellar granule cells containing α_6 and β_{2-3} subunits [13] and decreases the displacing potency of GABA on rat cerebellar [^{3}H]EBOB binding [10]. Therefore, we examined the effects of 100 μM furosemide on potentiation by allopregnanolone. Furosemide decreased the peak ampli-

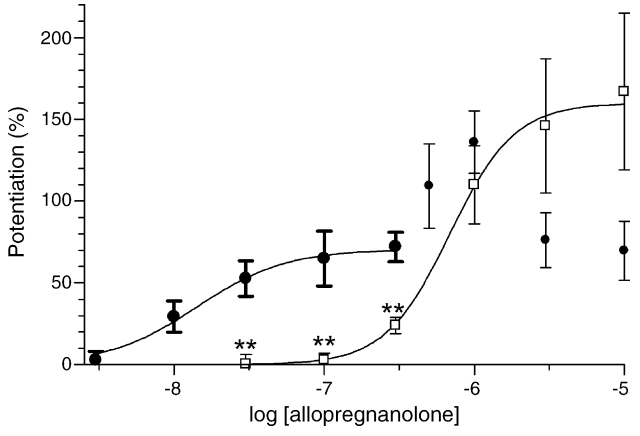

Fig. 1. Concentration dependent potentiation of 1 μM GABA-elicited chloride currents of rat cerebellar granule cells by allopregnanolone in the absence (●, means ± S.E.M. of 5–21 experiments) and presence of 100 μM furosemide (□, means ± S.E.M. of 4–10 experiments). Sigmoidal fitting to the cumulated data of the high affinity phase up to 0.3 μM allopregnanolone in the absence (bold ●) and up to 10 μM in the presence of furosemide. Fitted parameters are summarized in Table 1. $^{**}p < 0.05$, significantly different from control (without furosemide).