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An interaction between benzodiazepines and neuroactive steroids at $GABA_A$ receptors in cultured hippocampal neurons

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Abstract

Neurosteroids are modulators of several receptors and ion channels and are implicated in the pathophysiology of several neuropsychiatric diseases including hepatic encephalopathy (HE). The neurosteroid, allopregnanolone, a positive allosteric modulator of $GABA_A$ receptors, accumulates in the brains of HE patients where it can potentiate $GABA_A$ receptor-mediated responses. Attenuation of the effects of neurosteroids on GABA-ergic neurotransmission is therefore of interest for the management of HE. In the present study, we determined the effect of the benzodiazepine partial inverse agonist, Ro15-4513, and the benzodiazepine antagonist, flumazenil on modulation of the $GABA_A$ mediated chloride currents by allopregnanolone and on spontaneous synaptic activity in cultured hippocampal neurons using the patch-clamp technique. Allopregnanolone (0.03–0.3 μM), dose-dependently potentiated GABA-induced currents, an action significantly reduced by Ro15-4513 (10 μM). In contrast, flumazenil (10 μM) had no effect on the ability of allopregnanolone to potentiate $GABA_A$ currents but it blocked the effects of Ro15-4513. The frequency of spontaneous synaptic activity was significantly reduced in the presence of allopregnanolone (0.1 μM) from 1.5 ± 0.7 to 0.1 ± 0.04 Hz. This action was partially reversed by Ro15-4513 (10 μM) but was not significantly influenced by flumazenil (10 μM). These findings suggest that the beneficial affects of Ro15-4513 in experimental HE result from attenuation of the effects of neurosteroids at $GABA_A$ receptors. Our results may provide a rational basis for the use of benzodiazepine inverse agonists in the management and treatment of hepatic encephalopathy in patients with liver failure.

Keywords: Allopregnanolone; Ro15-4513; Flumazenil; $GABA_A$ receptors; Patch-clamp electrophysiology; Hippocampal neurons; Benzodiazepines

1. Introduction

Recent evidence suggests that allopregnanolone may be involved in the pathophysiology of several neuropsychiatric disorders such as anxiety (Ströhle et al., 2002; Brambilla et al., 2003), acute stress (Paul and Purdy, 1992; Droogleever Fortuyn et al., 2004), chronic fatigue (Murphy et al., 2004) and hepatic encephalopathy (Ahboucha et al., 2005). Steroidal hormones that are synthesized in various peripheral organs, such as the adrenal glands and the gonads readily cross the blood–brain barrier, while some steroids are synthesized in the brain and are the so-called ''neurosteroids''. Pregnenolone, the neurosteroid precursor, can be converted within glial cells to progesterone, which in turn is sequentially metabolized to 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone or allopregnanolone (Rupprecht, 1997). Allopregnanolone is a potent allosteric modulator of $GABA_A$ receptors and consistent with this action has anxiolytic, anticonvulsant and antidepressant actions (Rupprecht and Holsboer, 1999). Notably, increased brain levels of allopregnanolone are reported in animals (Norenberg et al., 1997) and humans (Ahboucha et al., 2005) with liver failure.

The benzodiazepines target $GABA_A$ receptors and selectively modulate the action of GABA at its receptor (Barnard et al., 1998). This drug class includes a spectrum of ligands for $GABA_A$ receptors that normally have full agonist, partial agonist, antagonist, partial inverse agonist, or full inverse agonist activities (for review see Haefely, 1994). Benzodiazepine

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agonists such as diazepam, are used successfully in the treatment of anxiety disorders and act by potentiating the effects of GABA at its receptor (Michael Kaplan and DuPont, 2005). In contrast, benzodiazepine inverse agonists dampen GABA-ergic activity and have pro-convulsant and anxiogenic properties that limit their clinical use. Benzodiazepine partial inverse agonists display weak negative intrinsic efficacy and act as mild non-competitive $GABA_A$ receptor antagonists (Haefely, 1994). Such properties may be therapeutically useful, for example, to increase arousal, attention and to improve cognitive function. Consistent with this idea the benzodiazepine partial inverse agonist, Ro15-4513, reverses the anxiolytic-like effects of allopregnanolone (Brot et al., 1997), and ethanol (Suzdak et al., 1986; Becker and Hale, 1991; June and Lewis, 1994). Moreover, Ro15-4513, but not the benzodiazepine antagonist, flumazenil, induced beneficial effects in behavioral and electrophysiological measures in animals with acute (van der Rijt et al., 1990; Steindl et al., 1991; Püspök et al., 1993) or chronic (Meyer et al., 1998) liver failure.

Here we hypothesize that the beneficial the effects of Ro15-4513 in experimental hepatic encephalopathy result from a direct reduction of the effects of allopregnanolone on the $GABA_A$ receptor complex. To address this issue, we used the patch-clamp technique to determine the effects of Ro15-4513 and flumazenil on the modulation of spontaneous activity and GABA-induced chloride currents by allopregnanolone in rat hippocampal neurons.

2. Materials and methods

2.1. Hippocampal neuron cell culture

Rat embryonic (17–19 days gestation) hippocampal neurons were isolated and cultured using a method described previously (Halliwell et al., 2002) with slight modifications as detailed below. Briefly, time-mated Wistar rats were killed by cervical dislocation and the embryos removed under aseptic conditions. Embryonic brains were quickly excised and placed into chilled Hank's balanced salt solution (HBSS, Gibco). Hippocampi were isolated, chopped into small fragments and transferred into an enzyme solution containing the following (in mM): NaCl (116.0), KCl (5.4), $NaHCO_3$ (26.0), NaH_2PO_4 (1.0), $CaCl_2$ (1.5), $MgSO_4$ (1.0), EDTA (0.5), glucose (25.0), DL-cysteine (1.0, Sigma) and papain (20 units/ml, Sigma) and incubated at 37 °C, 5% CO_2, 100% relative humidity for 1 h. Subsequently, the tissue fragments were washed in HBSS containing bovine serum albumin (BSA) and ovomucoid (1 mg/ml each, both from Sigma), transferred to another sterile test tube containing the same solution, and then gently triturated into a single cell suspension. The dissociated cells were layered on to HBSS containing BSA and ovomucoid (at 10 mg/ml each) and centrifuged at 100 g for 10 min. After discarding the supernatant, the cells were re-suspended in growth media containing 88% (v/v) minimal essential medium, 5% (v/v) heat-inactivated fetal calf serum, 5% (v/v) heat-inactivated rat serum (Harlan, Sera-Lab), penicillin/streptomycin (5000 i.u./ml/5000 μg/ml), L-glutamine (2 mM) (Gibco) and glucose (20 mM). Cells were plated at a density of approximately 1–2 × 10^5/35 mm on *Primaria* culture dishes (Falcon, Becton Dickinson) containing poly-D-lysine coated glass coverslips, and incubated in 1.5 ml of culture media, at 37 °C, 95% air, 5% CO_2 and 100% relative humidity. Approximately two-thirds of the culture media was replaced with fresh media every 5–7 days. Proliferation of non-neuronal cells was inhibited by adding 10 μM cytosine arabinoside (Sigma) to the culture media for 48 h after 7 days in vitro.

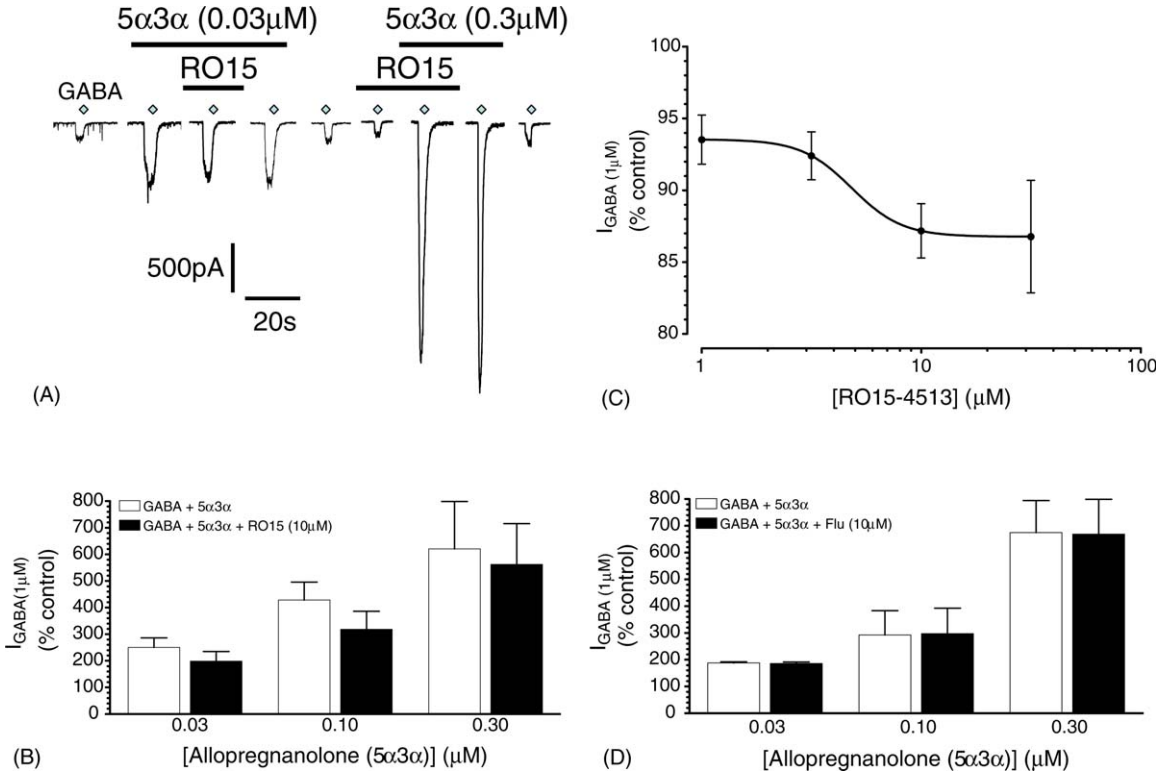

Fig. 1. Ro15-4513 attenuates the potentiation of GABA-evoked currents induced by allopregnanolone. (A) A discontinuous record of 1 μM GABA-evoked currents (◇) in the absence and presence of allopregnanolone (0.03 and 0.3 μM) and allopregnanolone + Ro15-4513 (10 μM). The holding potential (Vh) was −60 mV. (B) A histogram summary of similar experiments conducted on six cells. (C) The dose antagonist plot for Ro15-4513 (1–30 μM) against GABA-evoked currents ($n = 4$ cells). (D) A histogram of the potentiation of GABA-evoked currents by allopregnanolone (0.03–0.3 μM) in the absence and presence of flumazenil (10 μM, $n = 4$–5 cells).