Available online at www.sciencedirect.com
ScienceDirect
Neurochemistry International 51 (2007) 440–446
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Characterization of the interaction between fenamates and hippocampal neuron $GABA_A$ receptors

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Received 27 January 2007; received in revised form 30 March 2007; accepted 23 April 2007
Available online 3 May 2007

Abstract

Fenamate NSAIDs have several *central* effects, including anti-epileptic and neuroprotective actions. The underlying mechanism(s) of these actions are not presently understood. In this study, the effects of five members of the fenamate NSAID group were investigated on native ligand-gated ion channels expressed in cultured rat hippocampal neurons. All fenamates tested (1–100 μM) dose-dependently potentiated GABA-evoked currents; mefenamic acid (MFA) was the most potent and efficacious and was found to shift the GABA dose–response curve to the left without effect on the maximum amplitude or the GABA Hill Slope. The modulation of GABA receptors by MFA was not reduced in the presence of the benzodiazepine antagonist, flumazenil (10 μM) and was moderately voltage-dependent. MFA at concentrations ≥10 μM evoked dose-dependent currents in the absence of GABA. These currents were potentiated by diazepam (1 μM) and blocked by bicuculline (10 μM). The MFA (50 μM) current–voltage relationship and reversal potential were similar to that evoked by GABA. MFA (1–100 μM) had no effects on sub-maximal glycine, glutamate or NMDA evoked currents. These data show that fenamate NSAIDs are a highly effective class of $GABA_A$ receptor modulator and activators.

Keywords: Anticonvulsant; NSAID; Electrophysiology; Glutamate receptors; Glycine receptors

1. Introduction

NSAIDs are the most widely consumed medicines worldwide with an estimated 100 million prescriptions written per year in the USA. They are also a common source of self-poisoning with 100,000 hospitalizations annually from their adverse effects (Smolinske et al., 1990; Frolich, 1997; Pascucci, 2002). This chemically diverse group of drugs, prototypically represented by acetylsalicylic acid (*aspirin*), are used for their analgesic, anti-inflammatory, anti-pyretic and cardio-protective properties (Weissmann, 1991; Pascucci, 2002). The therapeutic properties of NSAIDs are thought to result, primarily, from their inhibition of cyclooxygenase (COX) isoenzymes and thereby inhibition of prostaglandin synthesis, although other mechanism are now under investigation (Vane, 1971; Vane and Botting, 1996; Arber and DuBois, 1999).

The fenamate NSAID, mefenamic acid (MFA) prevents convulsions and protects rats from seizure-induced forebrain damage evoked by pilocarpine (Ikonomidou-Turski et al., 1988) and is *anti-epileptogenic* against pentylenetetrazol (PTZ)-induced seizure activity, but at high doses induces seizures (Wallenstein, 1991). In humans, MFA overdose can lead to convulsions and coma (Balali-Mood et al., 1981; Young, 1979; Smolinske et al., 1990).

More recent data by Chen et al. (1998a,b) have shown that the fenamates, flufenamic, meclofenamic and mefenamic acid, protect chick embryo retinal neurons against ischaemic and excitotoxic (kainate and NMDA) induced neuronal cell death *in vitro* (Chen et al.,1998a, 1998b). MFA has also been reported to reduce neuronal damage induced by intraventricular amyloid beta peptide ($A\beta_{1–42}$) and improve learning in rats treated with $A\beta_{1–42}$ (Joo et al., 2006).

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0197-0186/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2007.04.017

The mechanisms underlying these anti-epileptic and neuroprotective effects are not well understood but together suggest that fenamates may influence neuronal excitability through modulation of ligand and/or voltage-gated ion channels. In the present study, therefore, we have investigated this hypothesis by determining the actions of five representative fenamate NSAIDs at the major excitatory and inhibitory ligand-gated ion channels in cultured hippocampal neurons.

2. Methods

2.1. Hippocampal neuron cultures

Rat embryonic (e17–19 days gestation) hippocampal neurons were isolated and cultured using methods described previously (e.g. Halliwell et al., 2002). Briefly the hippocampus was dissected free, chopped in to small fragments and enzymatically and mechanically dissociated in to a single cell suspension. Cells were plated onto 35 mm *Primaria* culture dishes (Falcon, Becton Dickinson, NJ, USA) in minimal essential medium, supplemented with fetal calf serum, rat serum (Harlan, Sera-Lab, Indianapolis, USA), penicillin/streptomycin, L-glutamine (2 mM, Gibco-Invitrogen, Carlsbad, CA, USA) and D-glucose (20 mM). Cells were then maintained at 37 °C, 95% air, 5% CO_2 and 100% relative humidity until required for electrophysiological study. Two thirds of the media was replaced every 5–7 days. Proliferation of non-neuronal cells was inhibited by adding 10 μM cytosine arabinoside (Sigma–Aldrich, St. Louis, MO, USA) to the culture media for 48 h after 7 days *in vitro*.

2.2. Electrophysiology

Agonist-evoked currents were recorded from cultured hippocampal neurons using the whole-cell configuration of the patch-clamp technique as described previously (Halliwell et al., 2002). Briefly, patch electrodes were made from borosilicate glass pipettes (Harvard, Holliston, MA, USA) on a Narishige PB-7 electrode puller (East Meadow, NY, USA) and had tip resistances of 1–4 MΩ when filled with internal solution. Currents were recorded using an Axopatch 200 B amplifier and headstage (Axon Instruments, Foster City, USA) and low pass filtered at 10 kHz before digitization via a National Instruments DAQ card and a National Instruments BNC-2090 interface board (Austin, TX, USA) and storage on a PC running WinWCP software (University of Strathclyde, UK). Whole cell currents were monitored on the desktop PC. Series resistance and pipette and whole-cell capacitance were cancelled electronically. Cells were perfused with a bath solution containing the following (in mM): NaCl (140.0), KCl (2.8), $MgCl_2$ (2.0), $CaCl_2$ (1.0), HEPES (10.0). Tetrodotoxin (0.5 μM) was also added to the bath solution to block spontaneous voltage-dependent sodium channel activity. When recording *N*-methyl-D-aspartate (NMDA)-activated currents, magnesium ions were omitted and glycine (1 μM) was added to the bath solution. In all experiments potassium currents were suppressed by dialyzing the cell interior with a CsCl-based internal solution containing the following (in mM): CsCl (140.0), $MgCl_2$ (2.0), $CaCl_2$ (0.1), EGTA (1.1), HEPES (10.0) MgATP (2.0). Bath and internal solutions were titrated to pH 7.2 and filtered before use (using 0.22 μm Whatman, Maidstone, Kent UK). Neurons were normally voltage-clamped at a holding potential of −60 mV. For determination of agonist-evoked current–voltage relationships, the membrane potential was either stepped between −140 and 40 mV in 20 mV increments or a ramp protocol was used to clamp a cell between −140 and 40 mV over 500 ms. All experiments were carried out at ambient room temperature (23–25 °C).

2.3. Drugs and their application

All drugs were obtained from Sigma–Aldrich (St. Louis, MO, USA) except TTX which was purchased from Tocris Bioscience (Ellisville, MO, USA). Agonists and drugs were applied directly to neurons under voltage-clamp from the tip of a 250 μm pipette connected to a Y-tube (fabricated in-house). Fresh bath solution was also perfused through the culture dish (at 2 mL/min) using a gravity-feed system manufactured in-house to ensure there was no build up of drug solutions in the bath. At least three control responses were recorded before application of drugs. When a clear asymptotic drug effect was observed, it was washed off. During the drug wash-out phase, currents were monitored until the control responses were re-established which usually occurred within 5 min.

2.4. Data analysis

Sub-maximal (approximating $EC_{10–15}$) agonist-evoked currents were measured at their peak amplitude. Agonist-evoked responses in the presence of drugs are expressed as a percentage of the control response (± S.E.M. of *n* experiments). For concentration–response data, GABA-evoked currents were normalized to the maximal response evoked by a saturating concentration of agonist (1 mM GABA). These data were fitted, by a least squares, non-linear regression analysis (GraphPad Prism 4™ San Diego, CA, USA) to the logistic equation. The GABA reversal potential was determined for each cell from the data line intercepting the X axis at 0 current on the current to voltage (*I–V*) plot. For statistical comparisons, the Students *t*-test for repeated measures, or one-way ANOVA and Dunnett's multiple comparison tests were used where appropriate.

3. Results

3.1. Fenamate NSAIDs potentiate neuronal $GABA_A$ receptors

We first investigated five fenamate NSAIDs on native $GABA_A$ receptors. Addition of either flufenamic acid (FFA), meclofenamic acid (MCFA), mefenamic acid (MFA), tolfenamic acid (TFA) or niflumic acid (NFA) (all at 10, 30 and 100 μM) potentiated sub-maximal GABA (3 μM)-activated currents in a concentration-dependent manner recorded from hippocampal neurons. The order of efficacy, on the basis of maximal potentiation of I_{GABA}, was MFA > TFA > MCFA = FFA > NFA. The effects of all fenamates were rapid in onset and readily reversible upon drug washout (see Fig. 1).

Further investigation showed that MFA (1–100 μM) potentiated sub-maximal GABA (3 μM)-evoked currents in a concentration-dependent manner with a maximum potentiation to 698 ± 135% at 100 μM MFA (Fig. 2). The EC_{50} for modulation was 37 μM ($n = 4$). MFA alone at ≥10 μM evoked concentration-dependent inward currents which we subtracted from its efficacy to potentiate the GABA response (Fig. 2B). We next determined that potentiation of GABA-activated currents by MFA (10 and 100 μM) was not reduced by the benzodiazepine antagonist, flumazenil (10 μM) whereas potentiation by chlordiazepoxide (10 μM) was blocked by 10 μM flumazenil (Fig. 2A and C).

3.2. Mefenamic acid activates neuronal $GABA_A$ receptors

Application of MFA (10–1000 μM, 30 s, at approx 2 min intervals) to voltage-clamped hippocampal neurons evoked inward currents in the absence of exogenous GABA (Fig. 3) with an EC_{50} of 138 μM [95% CI = 79–239] and a Hill Slope of 1.7 ± 0.7 ($n = 4$). Sub-maximal MFA (50 μM) activated currents were blocked by bicuculline (10 μM) to 16 ± 4% ($n = 5$) of control and potentiated by diazepam (1 μM) to 189 ± 14% ($n = 5$) of control (see Fig. 3C). These effects were fully reversible upon washout of bicuculline and diazepam. The