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UPREGULATION OF HIGH-AFFINITY $GABA_A$ RECEPTORS IN CULTURED RAT DORSAL ROOT GANGLION NEURONS

K. Y. LEE,[a] M. CHARBONNET[a] AND M. S. GOLD[a,b,c,d,e]*

[a]*Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA, USA*

[b]*Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA*

[c]*Department of Medicine (Division of Gastroenterology, Hepatology, and Nutrition), University of Pittsburgh, Pittsburgh, PA, USA*

[d]*Center for Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA*

[e]*Pittsburgh Center for Pain Research, University of Pittsburgh, Pittsburgh, PA, USA*

Abstract—Despite evidence that high-affinity $GABA_A$ receptor subunit mRNA and protein are present in dorsal root ganglia (DRG), low-affinity currents dominate those detected in acutely dissociated DRG neurons *in vitro*. This observation raises the possibility that high-affinity receptors are normally trafficked out of the DRG toward central and peripheral terminals. We therefore hypothesized that with time in culture, there would be an increase in high-affinity $GABA_A$ currents in DRG neurons. To test this hypothesis, we studied dissociated DRG neurons 2 h (acute) and 24 h (cultured) after plating with whole-cell patch-clamp techniques, Western blot, and semiquantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) analysis. $GABA_A$ current density increases dramatically with time in culture in association with the emergence of two persistent currents with EC_{50}'s of 0.25±0.01 μM and 3.2±0.02 μM for GABA activation. In a subpopulation of neurons, there was also an increase in the potency of GABA activation of the transient current from an EC_{50} of 78.16±10.1 μM to 9.56±1.3 μM with time in culture. A fraction of the high-affinity current was potentiated by δ-subunit agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP). δ-subunit immunoreactivity was largely restricted to the cytosolic fraction in acute, but the membrane fraction in cultured, DRG neurons, with no detectable change in δ-subunit mRNA. However, the emergence of a high-affinity current blocked by THIP and insensitive to bicuculline was detected in a subpopulation of cultured neurons as well in association with an increase in ρ_2- and ρ_3-subunit mRNA in cultured DRG neurons. Our results suggest that high-affinity δ-subunit-containing $GABA_A$ receptors are normally trafficked out of the DRG where they are targeted to peripheral and central processes. They also highlight that the interpretation of data obtained from cultured DRG neurons should be made with caution. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

*Correspondence to: M. S. Gold, Department of Anesthesiology, University of Pittsburgh, 3500 Terrace Street Room E1440 BST, Pittsburgh, PA 15213, USA. Tel: +1-412-383-5367; fax: +1-412-383-8663.
E-mail address: msg22@pitt.edu (M. S. Gold).

Abbreviations: DRG, dorsal root ganglia; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimal essential media; sqRt-PCR, semiquantitative reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; TACA, *trans*-4-aminocrotonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol.

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GABA is the major inhibitory neurotransmitter in the adult mammalian nervous system, mediating its principal effects via anion-selective ionotropic $GABA_A$ receptors. In the CNS, $GABA_A$ receptor activation leads to a net anion influx resulting in membrane hyperpolarization. Recent evidence indicates that $GABA_A$ receptors underlie at least two distinct modes of inhibition. Classical synaptic inhibition is phasic, reflecting rapid increases in GABA in the millimolar range, and is mediated by relatively rapidly desensitizing $GABA_A$ receptors, which have low affinity for GABA (Mody et al., 1994). A second mode of inhibition is tonic, mediated by high-affinity extrasynaptic $GABA_A$ receptors, which are activated by GABA concentrations in the tens of nanomolar to a few micromolar range (Farrant and Nusser, 2005). $GABA_A$ receptor subunit composition dictates the biophysical and pharmacological properties of the receptor as well as the surface distribution. For example, γ_2-subunit-containing receptors are transient low-affinity receptors targeted to synapses (Essrich et al., 1998), whereas δ-subunit-containing receptors are sustained high-affinity extrasynaptic receptors (Nusser et al., 1998; Belelli et al., 2009).

$GABA_A$ signaling in primary afferent neurons is distinct in several respects from that observed in the CNS. First, because of the differential regulation of Cl^-, $GABA_A$ receptor activation in primary afferents results in membrane depolarization (Sung et al., 2000). Second, there appears to be disagreement between mRNA and functional data, and anatomical evidence in support of presynaptic $GABA_A$ receptors, particularly on putative nociceptive afferents. On the one hand, a number of $GABA_A$ receptor subunits are expressed at relatively high levels in virtually all dorsal root ganglia (DRG) neurons (Persohn et al., 1991). These expression data are consistent with electrophysiological data indicating that there is a relatively high $GABA_A$ current density in ~100% of neurons tested (Oyelese et al., 1997; White, 1990). On the other hand, anatomical evidence of presynaptic $GABA_A$ receptors on putative nociceptive afferents is relatively sparse (Todd, 1996). Third, there is evidence that $GABA_A$ receptors are transported to the periphery in primary afferents (Carlton et al., 1999), despite the absence of a source for high concentrations of GABA such as that released at synapses from GABAergic neurons. These observations raise the distinct possibility that extrasynaptic $GABA_A$ receptors are the dominant $GABA_A$ receptor subtype in nociceptive primary afferents.

However, previous analysis of $GABA_A$ receptor-mediated currents in acutely isolated sensory neuron somata suggests

that these currents are dominated by relatively rapidly desensitizing low-affinity receptors (Oyelese et al., 1997; Sung et al., 2000). Even currents evoked with low concentrations of GABA (i.e. 2.5 μM) desensitize (White, 1990). There is also evidence that these currents are potentiated by benzodiazepines (Witschi et al., 2011), which act at γ-subunit-containing receptors that are normally targeted to synapses (McKernan et al., 1995). Furthermore, although it remains to be determined whether the amount of GABA released within the DRG is sufficient to activate low-affinity receptors, it has been suggested that GABA signaling within the ganglia constitutes a normal component of sensory processing (Vit et al., 2009). This raises the possibility that the $GABA_A$ currents present in acutely isolated sensory neurons are the currents normally present in sensory somata *in vivo*. Thus, despite evidence that high-affinity $GABA_A$ receptor subunits are expressed in DRG (Ma et al., 1993), there is little evidence that these receptors are detectable in acutely isolated DRG neurons. We therefore hypothesized that high-affinity $GABA_A$ receptors are normally trafficked out of the ganglia. A key prediction of this hypothesis is that high-affinity receptors should be detectable with time in culture, as receptors normally targeted for peripheral and central processes are inserted in the plasma membrane as is observed with other transducers (Kirillova et al., 2011). The present study was designed to test this hypothesis. The biophysical and pharmacological properties of GABA receptor-mediated currents were assessed in dissociated DRG neurons 2 h (acute) and 24 h (cultured) after plating with conventional whole-cell patch-clamp techniques. Semiquantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) and Western blot analysis were used to further asses the basis for culture-dependent changes in $GABA_A$ currents. Our results were generally consistent with our hypothesis that high-affinity $GABA_A$ receptors are normally trafficked out of the ganglia, but also suggested that there are additional changes in subunit expression, which appear to be associated with placing the neurons in culture.

EXPERIMENTAL PROCEDURES

Animals

Adult (180–280 g) male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were used for all experiments. Rats were housed in the University of Pittsburgh animal facility in groups of two on a 12-h light/dark schedule. Food and water were available *ad libitum*. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines as well as guidelines established by the International Association of the Study of Pain for the use of laboratory animals in research.

Tissue preparation

Rats were deeply anesthetized with a subcutaneous injection (1 mg/kg) of a cocktail containing ketamine (55 mg/kg), xylazine (5.5 mg/kg), and acepromazine (1.1 mg/kg) (ketamine was from Fort Dodge Animal Health, Fort Dodge, WI, USA; xylazine and acepromazine were from Phoenix Scientific Inc., St. Joseph, MO, USA). L4-5 DRG were harvested, enzymatically treated, mechanically dissociated, and plated on laminin- and ornithine-coated (Life Technologies, Grand Island, NY, USA) coverslips as previously described (Lu et al., 2006). After plating for 2 h, cells were either flooded with an L-15 (Invitrogen)-based media containing 10% fetal bovine serum, 5 mM HEPES, 5 mM glucose, and 1000 U of penicillin–streptomycin (Life Technologies) or flooded with Minimal Essential Media (MEM, Life Technologies) containing 10% fetal bovine serum, 1000 U of penicillin–streptomycin, and 1× MEM vitamins (Life Technologies). Those flooded with L-15-based media were stored at room temperate and studied over the next 5–6 h, whereas those flooded with MEM-based media were placed in a CO_2 (3%) incubator at 37 °C for 24 h before study: the former were considered acutely cultured, and the latter were considered cultured neurons.

Electrophysiology

Whole-cell patch-clamp recordings were performed with a HEKA EPC10 amplifier (HEKA Eletronik GmbH, Lambrecht, Germany). Unless otherwise noted, data were acquired at 10 kHz and filtered at 2 kHz. Borosilicate glass (WPI, Sarasota, FL, USA) electrodes had a resistance of 2–3 MΩ when filled with the following solution (mM): CsCl 140, $MgCl_2$ 1, EGTA 11, HEPES 10, Mg–ATP 2, and GTP 1; pH was adjusted to 7.2 with Tris-base, and osmolality was adjusted to 310 mOsm with sucrose. The bath solution contained the following (in mM): NaCl 130, KCl 3, $CaCl_2$ 2.5, $MgCl_2$ 0.6, HEPES 10, and glucose 10; pH was adjusted to 7.4 with Tris-base, and osmolality was adjusted to 320 mOsm with sucrose. Neurons were held at -60 mV. Currents were evoked by 3 or 60 s of focal application of GABA or $GABA_A$ receptor agonists at different concentrations. To rule out the possibility that the KCl included in the bath solution contributed to the current evoked in response to $GABA_A$ receptor activation, $GABA_A$ currents were measured in bath solution with and without K^+. The results of this experiment indicate that there was no detectable influence of K^+ flux to the current evoked by GABA (1, 10, and 100 μM). Current amplitudes were 12.1±1.0, 68.5±3.1, and 128.4±12.0 pA compared with 12.6±5.7, 49.7±6.8, and 139.3±22.3 (n=4) in the presence and absence of 3 mM K^+ in the bath solution, respectively.

Western blot

For the separation of membrane and cytosol fraction, the cells or tissue were ground in Radio-immunoprecipitation assay buffer, were allowed to sit on ice for 20 min, and then spun down in a table-top centrifuge at 13,000 rpm for 10 min. The supernatant was then loaded onto a sucrose buffer and spun in an ultracentrifuge at approximately 100,000 g for an hour. The top three-quarters of the supernatant was used as the cytoplasm-enriched fraction. A pellet of membrane-enriched protein formed at the bottom of the tube. One microliter of 10% sodium dodecyl sulfate (SDS) was added to the remaining supernatant and used to resuspend the pellet. Protein was separated on a 7.5%–10% SDS-polyacrylamide gel and blotted to nitrocellulose membrane (GE Healthcare Biosciences, Piscataway, NJ, USA) with a Trans-Blot Transfer Cell system (Bio-Rad, Hercules, CA, USA). Blots were blocked with 5% milk in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4) at room temperature for 30 min. After decanting the blocking buffer, the blot was incubated with an antibody against the δ-subunit of the $GABA_A$ receptor (AB9752, Millipore, Billerica, MA, USA; 1:500). The specificity of this antibody has been determined previously (Korpi et al., 2002). Membranes were incubated with the δ-subunit antibody overnight at 4 °C at a 1:200 dilution. Immunoreactivity was detected using Enhanced Chemiluminescence (ECL, GE Healthcare Biosciences). Chemiluminescence was captured with a charge coupled device camera (Las-3000, Fujifilm, Tokyo, Japan) and analyzed with Fuji software Multi Gauge.

RT-PCR

Total RNA was extracted from two groups of cells (2 h [acute] and 24 h [cultured] after plating) using Trizol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using 1 μg total RNA with SuperScript II reverse transcriptase and an anchored oligo(dT) primer. SYBR