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R-ISOVALINE: A SUBTYPE-SPECIFIC AGONIST AT $GABA_B$-RECEPTORS?

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Abstract—The R-enantiomer of isovaline, an analgesic amino acid, has a chemical structure similar to glycine and GABA. Although its actions on thalamic neurons are strychnine-resistant and independent of the Cl^- gradient, R-isovaline increases membrane conductance for K^+. The purpose of this study was to determine if R-isovaline activated metabotropic $GABA_B$ receptors. We used whole-cell voltage-clamp recordings to characterize the effects of R-isovaline applied by bath perfusion and local ejection from a micropipette to thalamic neurons in 250 μm thick slices of rat brain. The immunocytochemical methods that we employed to visualize $GABA_{B1}$ and $GABA_{B2}$ receptor subunits showed extensive staining for both subunits in ventrobasal nuclei, which were the recording sites. Bath or local application of R-isovaline caused a slowly developing increase in conductance and outward rectification in 70% (54/77) of neurons, both effects reversing near the K^+ Nernst potential. As with the $GABA_B$ agonist baclofen, G proteins likely mediated the R-isovaline effects because they were susceptible to blockade by non-hydrolyzable substrates of guanosine triphosphate. The $GABA_B$ antagonists CGP35348 and CGP52432 prevented the conductance increase induced by R-isovaline, applied by bath or local ejection. The $GABA_B$ allosteric modulator CGP7930 enhanced the R-isovaline induced increase in conductance. At high doses, antagonists of $GABA_A$, $GABA_C$, $glycine_A$, μ-opioid, and nicotinic receptors did not block R-isovaline responses. The observations establish that R-isovaline increases the conductance of K^+ channels coupled to metabotropic $GABA_B$ receptors. Remarkably, not all neurons that were responsive to baclofen responded to R-isovaline. The R-isovaline–induced currents outlasted the fast baclofen responses and persisted for a 1–2-h period. Despite some similar actions, R-isovaline and baclofen do not act at identical $GABA_B$ receptor sites. The binding of R-isovaline and baclofen to the $GABA_B$ receptor may not induce the same conformational changes in receptor proteins or components of the intracellular signaling pathways. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: isovaline, $GABA_B$ receptors, K^+ channels, thalamus, ventrobasal nuclei, analgesia.

Isovaline (2-amino-2-methylbutanoic acid) is structurally similar to the inhibitory neurotransmitters, glycine and GABA or 4-aminobutanoic acid. Isovaline produces antinociceptive effects in rodents following supraspinal, spinal, or i.v. administration (MacLeod et al., 2010). Both R- and S-enantiomers of isovaline have analgesic activity in pain models, such as counteracting the allodynia induced by strychnine. For several reasons, the antinociceptive effects are probably not caused by actions at ionotropic receptors for glycine-like amino acids or GABA. R-isovaline applied directly to thalamic slices blocks action potentials evoked by electrical stimulation of ventrobasal neurons (Cooke et al., 2009). This slowly developing blockade is neither Cl^--dependent nor sensitive to strychnine, an antagonist of receptors for glycine-like amino acids. The inhibition results from an outward current that shunts the Na^+ current needed for action potential generation. The current induced by R-isovaline reverses near the equilibrium potential for K^+ (E_K), exhibits a Nernstian dependence on the extracellular [K^+] and sensitivity to K^+ channel blockers. The independence from Cl^- receptor-channels, structural similarity to GABA, and dependence on K^+ led us to hypothesize that R-isovaline acts on G protein-coupled $GABA_B$ receptors.

$GABA_B$ receptors located in or around synapses exist as heterodimers that consist of $GABA_{B1}$ and $GABA_{B2}$ subunits (Bettler and Tiao, 2006). The $GABA_{B1}$ subunit contains the agonist recognition site, whereas the $GABA_{B2}$ subunit couples the receptor to G proteins, which inhibit adenylyl cyclase through $G_{\alpha i/o}$ subunits (Kaupmann et al., 1998; Galvez et al., 1999). Baclofen (4-amino-3-(4-chlorophenyl)butanoic acid) is a canonical agonist with actions at $GABA_B$ receptors similar to GABA (Bowery, 2010). Baclofen activation of the $GABA_B$ receptor evokes G protein-coupled inwardly rectifying K^+ (GIRK) currents (Bettler et al., 2004; Fowler et al., 2007), leak currents (Deng et al., 2009), and outwardly rectifying K^+ currents (Saint et al., 1990). $GABA_B$ receptors are common to presynaptic and postsynaptic sites, although coupling to K^+ channels appears more prevalent in postsynaptic membranes (cf. Thompson and Gähwiler, 1992; Ulrich and Huguenard, 1996).

$GABA_B$ receptors mediate metabotropic inhibition in somatosensory nuclei of the thalamus (Potes et al., 2006; Andreou et al., 2010). In the present study, the objective was to determine if R-isovaline increased the K^+ conductance of ventrobasal neurons by activating postsynaptic $GABA_B$ receptors. We used whole-cell recording from neurons in slices of juvenile rat brain to elucidate R-isovaline actions and compared them with the effects of baclofen.

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Abbreviations: aCSF, artificial cerebrospinal fluid; DAPI, 4',6-diamidino-2-phenylindole; E_K, equilibrium potential for K^+; GDP-β-S, 5′-[β-thio]diphosphate; GTP, guanosine triphosphate; GTP-γ-S, tetralithium guanosine 5′-[γ-thio]triphosphate; PBS, phosphate buffered saline; TTX, tetrodotoxin; V_h, holding potential.

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The thalamus in adult rats expresses $GABA_{B1}$ and $GABA_{B2}$ receptor subunits (Kulik et al., 2002), whereas immunohistochemical studies on juvenile rat brain are absent. Hence we employed antibodies to verify the presence of colocalized $GABA_{B1}$ and $GABA_{B2}$ subunits required for functional receptors. Our results show that the effects of R-isovaline broadly resemble those of baclofen but differ in important aspects.

EXPERIMENTAL PROCEDURES

All experiments were approved by the Animal Care Committee at the University of British Columbia. Sprague-Dawley rats (P10–15) were obtained from the Centre for Disease Modelling. The rats decapitated under deep isoflurane anesthesia and their brains placed in oxygenated artificial cerebrospinal fluid (aCSF) at 4 °C, which contained (in mM): 124 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, and 25 dextrose. Hemispheres were sectioned sagittally into 250 μm thick slices using a vibroslicer (Campden Instruments, London, UK) and incubated for >1 h at 23–25 °C in aCSF saturated with a 95:5% mixture of O_2 and CO_2.

Electrophysiology

Slices were immobilized with polypropylene mesh in a Perspex recording chamber (volume, ~2 ml) and perfused at room temperature (22–24 °C) with oxygenated aCSF at a rate of ~2 ml/min. Thalamocortical neurons were visualized at 400× magnification with a differential interference contrast microscope (Axioscope II, Zeiss, Germany).

Recording pipettes were made from borosilicate glass tubing (World Precision Instruments, Sarasota, FL, USA) filled with a solution containing (in mM): 133 K-gluconate, 12 KCl, 4 NaCl, 0.5 $CaCl_2$, 10 EGTA, 1 HEPES, 3 Mg adenosine triphosphate, and 0.3 disodium guanosine triphosphate (GTP). In some experiments, GTP was omitted and replaced with trilithium guanosine 5′-[β-thio]diphosphate (GDP-β-S; 1 mM; Sigma Aldrich, St. Louis, MO, USA) or tetralithium guanosine 5′-[γ-thio]triphosphate (GTP-γ-S; 0.3 mM; Sigma Alrich, St. Louis, MO, USA). The pH of the solution was adjusted to 7.3–7.4 using 50% gluconic acid or KOH.

Whole-cell, patch clamp recordings were performed using a List EPC 7 amplifier (HEKA, Germany) in current- and voltage-clamp modes. Electrode resistances ranged from 4 to 7 MΩ. Series resistance ranged from 5 to 30 MΩ, and data were discarded if series resistance increased by more than 25%. Signals were filtered (3 kHz), digitized at 10 kHz, and analyzed using pCLAMP software (Axon Instruments, Sunnyvale, CA, USA). Before tetrodotoxin (TTX; Sigma Aldrich) blockade, neurons showed burst firing when depolarized from −80 mV and tonic firing at potentials depolarized from rest. TTX (1 μM) was present for all voltage clamp-recordings. Membrane potentials were corrected for a junction potential of −11 mV, yielding an average resting potential near the holding potential, V_h=−70 mV. Input conductance was averaged from 10 steady-state currents in response to −5 mV steps of 400 ms duration delivered from V_h. These small hyperpolarizing commands caused minimal activation of inward rectifier (I_h) currents seen in thalamocortical neurons (cf. Cooke et al., 2009). Current-voltage (I–V) relationships were obtained using 400 ms duration voltage commands in 10 mV increments from V_h to a range of −120 and −30 mV. Steady state currents on pulse injection were averaged near the end of 400 ms voltage steps. For the extracellular K^+ concentration of 2.5 mM and the estimated intracellular [K^+] of 135 mM, the K^+ Nernst potential was ~−103 mV.

Drugs

Drugs for bath application were either freshly prepared or diluted from stock solutions just before use. Strychnine, picrotoxin, R(+)-baclofen, CGP35348, and CGP52432 were purchased from Sigma Aldrich (St. Louis, MO, USA). CGP7930 was obtained from Tocris Bioscience (Ellisville, MO, USA). Naloxone was obtained from Endo Pharmaceuticals (Newark, DE, USA). Curare and TTX were purchased from City Chemical Corporation (West Haven, CT, USA) and Alomone Labs (Jerusalem, Israel). R-isovaline was synthesized by BioFine International (Vancouver, BC, Canada).

For local application, R-isovaline was ejected from a micropipette placed within ~25 μm of a soma of a recorded neuron, using pressure pulses (2–5 p.s.i.) of N_2 lasting 1 s duration (Picospritzer II, General Valve Corporation, Fairfield, NJ, USA). In view of the observed irreversibility of responses to R-isovaline, dose–response relationships for micropipette applications were constructed from responses to single R-isovaline concentrations (i.e. one application per neuron).

Data analysis

Data were analyzed and graphed using GraphPad Prism (San Diego, CA, USA), and CorelDraw software (Ottawa, ON, Canada). Neurons were deemed unresponsive to R-isovaline at its approximate ED_{50} when the conductance change was <10%. Differences between treatment groups were analyzed using Student's *t*-test, paired *t*-test, one-way ANOVA, or two-way ANOVA, with Bonferroni's post hoc tests for comparison at specific data points. Fisher's exact test was used to evaluate whether the ratio of responders to non-responders was different between control and treated groups. All data were expressed as mean±SEM, with *n* being the number of neurons.

Tissue preparation for immunohistochemistry

Sprague–Dawley rats (P12) were anesthetized with pentobarbital (40 mg/kg) and transcardially perfused with cold 0.1% phosphate buffered saline (PBS), followed by 4% formaldehyde. Brains were dissected and post-fixed in 4% formaldehyde for 2 h at 4 °C, followed by submersion in 30% sucrose for 24–48 h at 4 °C. The tissue was embedded with Tissue-Tek embedding medium (Sakura Finetek, Torrence, CA, USA) and frozen in liquid N_2. Sagittal sections were made at 14 μm thickness and stored at −20 °C.

Immunohistochemistry was performed on sections post-fixed in 4% formaldehyde for 10 min. The sections were permeabilized with 0.1% Triton X-100, blocked with 0.5% bovine serum albumin (BSA) in PBS and incubated in primary antibody overnight at 4 °C in PBS solution containing 0.1% BSA. The primary antibodies were mouse anti-$GABA_BR1$ subunit (1:100; ab55051; Abcam, Cambridge, MA, USA) and rabbit anti-$GABA_BR2$ subunit (1:100; ab75838; Abcam, Cambridge, MA, USA). Sections were incubated in goat anti-mouse Alexa 546 and goat anti-rabbit Alexa 488 secondary antibodies for 1 h at room temperature (Invitrogen, Burlington, ON, Canada). To identify somata, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Sections were coated with Prolong Gold (Invitrogen) and allowed to cure overnight before imaging.

High magnification images were captured using an Olympus Fluoview 1000 confocal microscope (10× and 60×/1.4 oil Plan-Apochromat objectives). Five pixels were used to define a punctum. For co-localization, 665 overlapping puncta in $GABA_{B1}$ and $GABA_{B2}$ channels were quantified using ImageJ software (NIH, Bethesda, MD, USA). Random co-localization was assessed by rotating a staining channel by 90°. Perisomatic puncta were quantified by outlining the soma using the magnetic lasso tool and cropping out other pixels in Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The subunit labeling for each fluorescence