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SPINAL 5-HT7 RECEPTORS AND PROTEIN KINASE A CONSTRAIN INTERMITTENT HYPOXIA-INDUCED PHRENIC LONG-TERM FACILITATION

M. S. HOFFMAN AND G. S. MITCHELL *

Department of Comparative Biosciences, University of Wisconsin, Madison, WI 53706, USA

Abstract—Phrenic long-term facilitation (pLTF) is a form of serotonin-dependent respiratory plasticity induced by acute intermittent hypoxia (AIH). pLTF requires spinal Gq protein-coupled serotonin-2 receptor (5-HT2) activation, new synthesis of brain-derived neurotrophic factor (BDNF) and activation of its high-affinity receptor, TrkB. Intrathecal injections of selective agonists for Gs protein-coupled receptors (adenosine 2A and serotonin-7; 5-HT7) also induce long-lasting phrenic motor facilitation via TrkB ''trans-activation.'' Since serotonin released near phrenic motor neurons may activate multiple serotonin receptor subtypes, we tested the hypothesis that 5-HT7 receptor activation contributes to AIH-induced pLTF. A selective 5-HT7 receptor antagonist (SB-269970, 5 mM, 12 μl) was administered intrathecally at C4 to anesthetized, vagotomized and ventilated rats prior to AIH (3, 5-min episodes, 11% O_2). Contrary to predictions, pLTF was greater in SB-269970 treated versus control rats (80 ± 11% versus 45 ± 6% 60 min post-AIH; $p < 0.05$). Hypoglossal LTF was unaffected by spinal 5-HT7 receptor inhibition, suggesting that drug effects were localized to the spinal cord. Since 5-HT7 receptors are coupled to protein kinase A (PKA), we tested the hypothesis that PKA inhibits AIH-induced pLTF. Similar to 5-HT7 receptor inhibition, spinal PKA inhibition (KT-5720, 100 μM, 15 μl) enhanced pLTF (99 ± 15% 60 min post-AIH; $p < 0.05$). Conversely, PKA activation (8-br-cAMP, 100 μM, 15 μl) blunted pLTF versus control rats (16 ± 5% versus 45 ± 6% 60 min post-AIH; $p < 0.05$). These findings suggest a novel mechanism whereby spinal Gs protein-coupled 5-HT7 receptors constrain AIH-induced pLTF via PKA activity.

Key words: respiratory plasticity, motor neuron, phrenic, respiratory control, long-term facilitation, hypoxia.

INTRODUCTION

Plasticity is a hallmark feature of the neural system controlling breathing, and is essential for its response to repeated or prolonged physiological or environmental challenges (Mitchell and Johnson, 2003). One well-studied model of respiratory plasticity is phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH; Mitchell et al., 2001; Feldman et al., 2003; Mahamed and Mitchell, 2007). AIH-induced pLTF is a progressive, serotonin-dependent increase in respiratory motor output lasting at least 60 min following episodic stimulation of the carotid sinus nerve (Millhorn et al., 1980a,b) or AIH (Hayashi et al., 1993; Bach and Mitchell, 1996; Baker and Mitchell, 2000). In our working model, AIH activates medullary raphe neurons (Erickson and Millhorn, 1994; Teppema et al., 1997) with spinal projections (Lalley, 1986) resulting in serotonin (5-HT) release in or near the phrenic motor nucleus (Pilowsky et al., 1990; Kinkead et al., 2001; Morris et al., 2001). Subsequent 5-HT2 receptor activation on phrenic motor neurons (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; MacFarlane et al., 2011) elicits signaling cascades that underlie pLTF (Dale-Nagle et al., 2010).

Activation of Gq protein-coupled serotonin type-2 ($5HT_2$) receptors (Kinkead and Mitchell, 1999; MacFarlane et al., 2011) on or near phrenic motor neurons (Basura et al., 2001) is necessary and sufficient to elicit pLTF (Fuller et al., 2001; MacFarlane and Mitchell, 2009). Moreover, episodic 5-HT receptor activation elicits LTF in synaptically isolated hypoglossal motor neurons of neonatal rat rhythmogenic brainstem slice preparations (Bocchiaro and Feldman, 2004), and in spinal respiratory motor output from brainstem–spinal cord preparations (cervical and thoracic LTF; Lovett-Barr et al., 2006). Cervical spinal injections of serotonin and selective 5-HT2 receptor agonists induce long-lasting phrenic motor facilitation (pMF) by a mechanism that requires NADPH oxidase activity (MacFarlane and Mitchell, 2008; MacFarlane et al., 2009).

Recently, spinal serotonin type-7 (5-HT7) receptors were also shown to elicit pMF in the absence of AIH (Hoffman and Mitchell, 2011). 5-HT7 receptors are necessary for full expression of AIH-induced ventilatory LTF in rats pretreated with chronic intermittent hypoxia (McGuire et al., 2004). To further understand serotonergic mechanisms giving rise to pLTF, we tested the hypothesis that serotonin released during AIH activates multiple serotonin receptor subtypes (e.g., 5-HT2 and 5-HT7), and that both contribute to

*Corresponding author. Address: Department of Comparative Biosciences, 2015 Linden Drive, Madison, WI 53706-1102, USA. Tel: +1-608-263-9826; fax: +1-608-263-3926.
E-mail addresses: mitchell@svm.vetmed.wisc.edu (G. S. Mitchell).
Abbreviations: AIH, acute intermittent hypoxia; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; MAP, mean arterial pressures; NADPH, nicotinamide adenine dinucleotide phosphate; $PETCO_2$, end-tidal carbon dioxide partial pressures; PKA, protein kinase A; pLTF, phrenic long-term facilitation; pMF, phrenic motor facilitation; ROS, reactive oxygen species.

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AIH-induced pLTF. However, contrary to our hypothesis, spinal 5-HT7 receptor inhibition enhanced pLTF, suggesting that 5-HT7 receptor activation constrains (versus contributes to) normal mechanisms of pLTF. This enhancement is similar to that observed following intrathecal adenosine 2A receptor inhibition (Hoffman et al., 2010).

Because 5-HT7 (and adenosine 2A) receptors are coupled to Gs proteins, initiating an adenylate cyclase/cAMP/protein kinase A (PKA) signal transduction cascade (Lovenberg et al., 1993; Krobert et al., 2001), we hypothesized that PKA inhibition would enhance, whereas PKA activation would suppress pLTF. Indeed, our findings support a mechanism whereby PKA constrains pLTF via "cross-talk" inhibition of the signaling cascade initiated by Gq protein-coupled 5-HT2 receptors. A more comprehensive understanding of 5-HT receptor "cross-talk" may provide insights concerning fundamental mechanisms and the significance of respiratory plasticity.

EXPERIMENTAL PROCEDURES

Experimental studies

All experiments were performed on male Sprague–Dawley rats (Harlan colony 218a, $n = 56$; Harlan Inc., Indianapolis, IN, USA) aged 101 ± 14 days and weighing 384 ± 33 grams. Animals were doubly housed in a controlled environment (12-h light/dark cycle, daily humidity and temperature monitoring). All protocols were approved by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine at the University of Wisconsin, Madison.

In a first experimental series, we tested the hypothesis that spinal 5-HT7 receptors contribute to AIH-induced pLTF by the intrathecal application of a selective 5-HT7 receptor inhibitor. Since spinal 5-HT7 receptor antagonist injections enhanced pLTF, a second experimental series was designed to test whether the inhibition of "downstream" spinal protein kinase A contributed to the enhancement of pLTF; in this series we delivered a selective PKA inhibitor to the cervical spinal cord. In the final experimental series, we tested the hypothesis that increased PKA activity inhibits AIH-induced pLTF by intrathecally injecting a cAMP/PKA activator. Investigators were not blinded concerning the identity of intrathecal solutions applied during experiments.

Surgical preparation

Surgical procedures have been described in detail elsewhere (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004) and are summarized below. Anesthesia was induced with isoflurane; isoflurane was continued during surgical preparations initially with a nose cone, and then through a tracheal cannula (2.5–3.5% in 50% O_2, balance N_2). Once surgical procedures were complete, rats were slowly converted to urethane anesthesia over 15 min (1.75 μg/kg). Adequate anesthetic depth was tested by lack of any pressor or respiratory neural response to toe pinch with a hemostat. After conversion to urethane anesthesia, a continuous intravenous infusion was initiated (4–6.5 ml kg^{-1} h^{-1}) of a 1:4 mixture of 6% Hetastarch (artificial colloid composed dissolved in 0.9% normal saline) and lactated Ringer's to maintain blood volume, fluid balance and acid–base status. A tracheal cannula was placed in the neck to enable artificial ventilation (Rodent Respirator, model 683, Harvard Apparatus, Holliston, MA, USA; tidal volume = 2.5 ml). A rapidly responding flow-through carbon dioxide analyzer (Capnogard, Novametrix, Wallingford, CT, USA) was placed on the expired limb of a Y-tube connected to the tracheal cannula to enable measurements of end-tidal carbon dioxide partial pressures ($PETCO_2$). The vagus nerves were cut in the mid-cervical region to prevent the entrainment of respiratory neural activity with the ventilator. During ventilation, rats were paralyzed with pancuronium bromide (2.5 mg/kg). A polyethylene catheter (PE-50, Intramedic) was placed in the right femoral artery and blood pressure was monitored with a pressure transducer (Gould, P23ID). A 3-way stop-cock, attached to the arterial catheter, was used to withdraw blood samples (0.2–0.4 ml) for blood gas analysis (ABL-500, Radiometer; Copenhagen, Denmark); during an experiment, blood gas determinations were made during baseline conditions, the first hypoxic episode of an LTF protocol, and at 15-, 30- and 60-min post-AIH. Body temperature was monitored with a rectal thermometer (Fischer Scientific, Pittsburgh, PA, USA) and maintained (37.5 ± 1 °C) with a heated surgical table.

The left phrenic and hypoglossal nerves were isolated using a dorsal approach, cut distally, desheathed and placed on bipolar silver electrodes to record respiratory neural activity. Phrenic and XII nerve signals were amplified (100,000×), band-pass filtered (300–10,000 Hz Model 1800, A-M Systems, Carlsborg, WA, USA), rectified and integrated (Paynter filter; time constant, 50 ms, CWE Inc., MA-821; Ardmore, PA, USA). The resulting integrated nerve bursts were digitized (8000 Hz) and analyzed using a WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA).

To test whether spinal 5-HT7 receptor activation contributed to pLTF, the spinal column was exposed dorsally, followed by laminectomy and partial durotomy at cervical level 2 (C2). A soft silicone catheter (2 French; Access Technologies, IL, USA) was inserted caudally below the dura until the tip was located at approximately C4. The catheter was attached to a 50 μl Hamilton syringe containing either vehicle (saline and 20% dimethyl sulfoxide, DMSO) or drug (dissolved in vehicle).

In our studies, hypoglossal nerve activity served as an internal control to determine whether intrathecal injections near the phrenic motor nucleus resulted in unintended drug distribution to the brainstem, as described previously (Baker-Herman and Mitchell, 2002; MacFarlane et al., 2009). Since we did not observe any changes in hypoglossal nerve activity, at doses that augmented pLTF, we conclude that each drug exerted