Respiratory Physiology & Neurobiology 205 (2015) 7–15

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Evidence for respiratory neuromodulator interdependence after cholinergic disruption in the ventral respiratory column

Clarissa Muere[a], Suzanne Neumueller[a], Justin Miller[a,1], Samantha Olesiak[a], Matthew R. Hodges[a], Lawrence Pan[b], Hubert V. Forster[a,c,*]

[a] Physiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA
[b] Physical Therapy, Marquette University, Milwaukee, WI 53201, USA
[c] Zablocki Veterans Affairs Medical Center, Milwaukee, WI 53295, USA

ARTICLE INFO

Article history:
Accepted 17 September 2014
Available online 26 September 2014

Keywords:
Neuromodulator interdependence
Muscarinic receptor
Control of breathing

ABSTRACT

Reverse dialysis of the muscarinic receptor antagonist, atropine (ATR, 50 mM), into the pre-Bötzinger Complex region of the ventral respiratory column (VRC) of awake and sleeping goats increases breathing frequency and serotonin (5-HT), substance P (SP), glycine, and GABA concentrations in the effluent dialysate. Herein, we report data from goats in which we reverse dialyzed 5 mM ATR or specific antagonists of M2 or M3 muscarinic receptors into the VRC. The effects on frequency of all three antagonists were not significantly different from time control studies. 5 mM ATR and the M3 antagonist increased SP sevenfold less than 50 mM ATR. The antagonists had no effect on 5-HT, glycine, and/or GABA, suggesting that the increases in glycine and GABA with 50 mM ATR were secondary to the larger increases in 5-HT and/or SP. These data are suggestive of neuromodulator interdependence, whereby attenuation of one neuromodulator is compensated for by local changes in other neuromodulators to stabilize breathing.

1. Introduction

The neuromodulator, acetylcholine (ACh), has been shown to play multiple roles in physiological function, including the neural control of respiration and sleep-state transitions (Bohmer et al., 1989; Burton et al., 1994; Dev and Loeschcke, 1979; Douglas et al., 2004; Haji et al., 1996; Nattie and Li, 1990). There are five known G-protein-coupled muscarinic ACh receptor (mAChR) subtypes that are preferentially coupled to either excitatory Gq/11 (M1, M3, M5) or inhibitory Gi/o (M2, M4) G-proteins (Brown, 2010; Eglen, 2006). These receptors differ in their CNS expression patterns and synaptic distribution, but are known to be present in brainstem nuclei related to respiratory control in several animal models (Baghdoyan et al., 1994; Buckley et al., 1988; Levey et al., 1994).

It has been found in reduced preparations that cholinergic receptor activation is excitatory to neurons within the pre-Bötzinger Complex (preBötC) (Shao and Feldman, 2000), a site critical to normal respiratory rhythm and pattern generation (Smith et al., 1991; Tan et al., 2008; Wenninger et al., 2004). We, therefore, in a previous study on awake and sleeping goats, tested the hypothesis that reverse dialysis of the non-selective mAChR antagonist, atropine (ATR, 50 mM), into the preBötC region of the ventral respiratory column (VRC) would decrease breathing (Muere et al., 2013). We found no significant decrease in breathing, but rather that dialysis of 50 mM ATR significantly increased ventilation, driven primarily by an increase in breathing frequency. This effect was state-dependent, since frequency increased to a greater extent in the awake state compared to NREM sleep. We also found that during 50 mM ATR dialysis, the concentrations of serotonin (5-HT) and substance P (SP) in the effluent dialysate were markedly elevated. We concluded that the increases in 5-HT and SP served to offset or compensate for a presumed reduction in post-synaptic mAChR excitation of respiratory neurons. This conclusion is consistent with the recently formulated concept of "neuromodulator interdependence", whereby attenuation of a single excitatory neuromodulator is rapidly compensated for by an increase in another excitatory neuromodulator to maintain a stable level of breathing (Doi and Ramirez, 2008, 2010). Shown in Fig. 1 is a hypothesized mechanism for mediation of such rapid compensation. Interestingly, the increase in 5-HT and SP with 50 mM ATR appears to have been over-compensatory, as indicated by the

* Corresponding author at: Physiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA. Tel.: +1 414 955 8533.
E-mail addresses: cmuere@mcw.edu (C. Muere), sneumueller@mcw.edu (S. Neumueller), jumiller@mcw.edu (J. Miller), solesiak@mcw.edu (S. Olesiak), mhodges@mcw.edu (M.R. Hodges), lawrence.pan@marquette.edu (L. Pan), bforster@mcw.edu (H.V. Forster).
[1] Present address: Zablocki Veterans Affairs Medical Center, Milwaukee, WI 53295, USA.

http://dx.doi.org/10.1016/j.resp.2014.09.010
1569-9048/

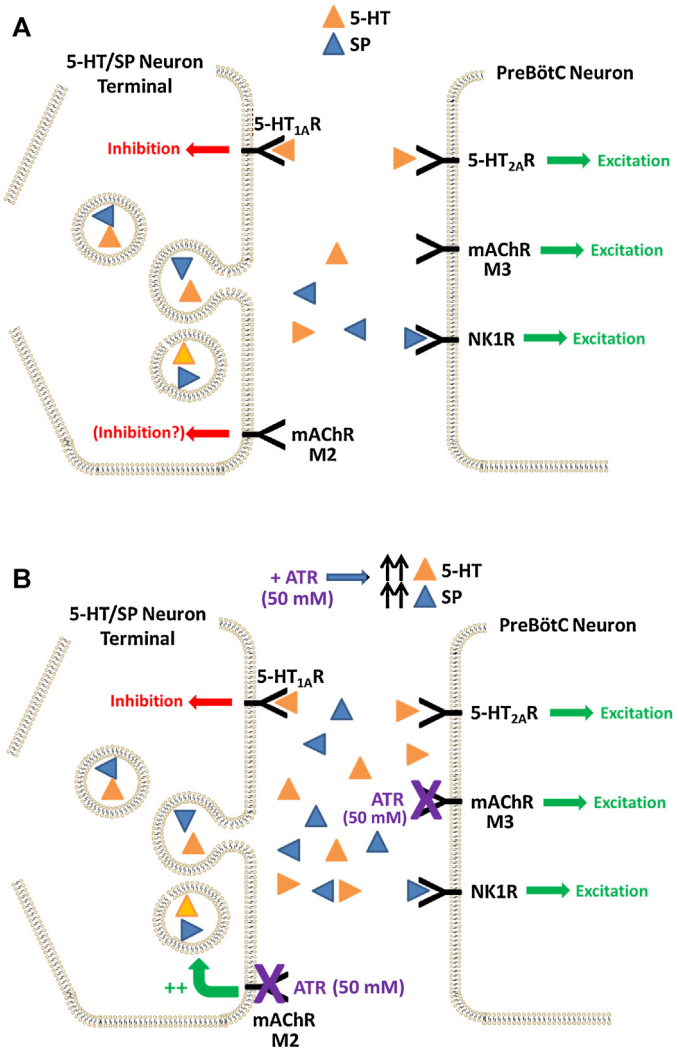

Fig. 1. Illustration of mechanism originally hypothesized to explain the increase in breathing frequency, 5-HT, and SP with dialysis of 50 mM ATR (Muere et al., 2013). Under normal conditions (A), 5-HT/SP-containing pre-synaptic terminals release 5-HT/SP into the extracellular space, where these neuromodulators act upon their respective receptors on a post-synaptic neuron, leading to excitation. Pre-synaptic terminals may express inhibitory mAChRs, the activation of which is thought to inhibit release of 5-HT/SP. Post-synaptic neurons may also express excitatory mAChRs, the activation of which would lead to post-synaptic excitation. For simplicity, ACh molecules have been omitted from the schematic. With dialysis of 50 mM ATR (B), post-synaptic excitation via mAChRs is dampened; however, this effect may be overridden by disinhibition of neurochemical release from pre-synaptic terminals due to blockade of inhibitory mAChRs. The net effect of ATR is thus an increase in the release of 5-HT/SP, leading to an increase in post-synaptic excitation and breathing frequency. 5-HT_{1A}R = 5-HT subtype 1A receptor; 5-HT_{2A}R = 5-HT subtype 2A receptor; mAChR = muscarinic ACh receptor; NK1R = neurokinin-1 receptor.

sustained and significant increase in breathing frequency, rather than maintenance of a normal breathing frequency (Muere et al., 2013). Moreover, 50 mM ATR dialysis also significantly increased glycine (GLY) and *gamma*-aminobutyric acid (GABA) concentrations in the effluent dialysate, which may have been secondary compensatory responses to counter the increase in 5-HT and SP, and thus minimize the over-compensation and return breathing to normal levels. In line with this reasoning and the concept of neuromodulator interdependence, we hypothesize that dialysis of a lower concentration of ATR (5 mM) will slightly increase 5-HT and/or SP without changing GLY and GABA, such that breathing does not change during blockade of cholinergic transmission. One objective of the present study was to test this hypothesis.

A second objective of our current study was to gain insight into which mAChR subtypes were responsible for the effects of 50 mM ATR on breathing and neurochemical release. The M2 receptor has been reported to be the most abundant mAChR subtype in the brainstem and is known to be expressed pre-synaptically in cholinergic and non-cholinergic neurons at various locations in the CNS (Hamada et al., 2010; Levey et al., 1991; Wevers, 2011). These properties make the M2 receptor the ideal candidate for study, since its pre-synaptic localization would allow it to modulate the release of potentially multiple neurochemicals. On the other hand, the M3 receptor has been shown to play an excitatory role in controlling breathing frequency in brainstem slice preparations containing the preBötC (Shao and Feldman, 2000). Since the M2 and M3 subtypes have also been shown to be the predominant mAChRs expressed in the preBötC (Lai et al., 2001), we chose to study the contributions to breathing and neurochemical modulation of these two subtypes by dialyzing specific antagonists to the M2 and M3 receptors in the VRC. Consistent with the mechanism depicted in Fig. 1, we hypothesize that blockade of M2 mAChRs would recapitulate the effects of dialysis of 50 mM ATR in the VRC, and that blocking M3 mAChRs would depress breathing. Lastly, given that the effects of 50 mM ATR dialysis on breathing are state-dependent (Muere et al., 2013), we sought to test if dialysis of 5 mM ATR also had state-dependent effects on breathing. We hypothesize that 5 mM ATR would not change breathing in both the awake and NREM sleep states and have minimal effects on neurochemical concentrations in the effluent dialysate.

2. Methods

2.1. Goats

Data were obtained from a total of 19 adult, non-pregnant female goats weighing 46.1 ± 2.1 kg. The goats were housed and studied in an environmental chamber with a fixed ambient temperature and 12-h light/dark cycle (lights on 6 AM). Goats were allowed ad libitum access to food and water, except during study periods and during a 24-h fasting period before all surgeries. This study was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee before studies were initiated.

2.2. Surgical procedures

Prior to surgery, goats were anesthetized with ketamine (5.0 cm^3, IV) for intubation, after which they were mechanically ventilated with 2% isoflurane in 100% oxygen. A non-steroidal anti-inflammatory analgesic, flunixin meglumine (Banamine, 1 mg/kg, IM) was given once pre-operatively. Rectal body temperature (T_r), heart rate, respiratory rate, and blood oxygen saturation were monitored throughout and over the next 24 h after each surgery. To reduce the risk of infection, ceftiofur sodium (Naxcel, 4 mg/kg, IM) was administered daily and all surgical sites treated with triple antibiotic for ≥7 days post-operatively. Buprenorphine hydrochloride (Buprenex, 0.005 mg/kg, IM) was administered twice daily 48 h after surgery to minimize pain.

After a ≥3-day acclimatization period, two surgeries separated by two weeks were performed under sterile conditions. In an initial surgery, to monitor and score sleep state, electroencephalogram (EEG) and electro-oculogram (EOG) electrodes were implanted in the midline cranium and superior orbital ridge, respectively. The second surgery was later performed for chronic bilateral or unilateral implantation of stainless steel microtubules (MTs, 70 mm length, 1.27 mm outer diameter, 0.84 mm inner diameter) targeting the preBötC. This surgery required an occipital craniotomy, after which the dura mater was excised to expose the posterior