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HYPOCRETIN-1 (orexin A) PREVENTS THE EFFECTS OF HYPOXIA/HYPERCAPNIA AND ENHANCES THE GABAergic PATHWAY FROM THE LATERAL PARAGIGANTOCELLULAR NUCLEUS TO CARDIAC VAGAL NEURONS IN THE NUCLEUS AMBIGUUS

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Abstract—Hypocretins (orexins) are hypothalamic neuropeptides that play a crucial role in regulating sleep/wake states and autonomic functions including parasympathetic cardiac activity. We have recently demonstrated stimulation of the lateral paragigantocellular nucleus (LPGi), the nucleus which is thought to play a role in rapid eye movement (REM) sleep control, activates an inhibitory pathway to preganglionic cardiac vagal neurons in the nucleus ambiguus (NA). In this study we test the hypothesis that hypocretin-1 modulates the inhibitory neurotransmission to cardiac vagal neurons evoked by stimulation of the LPGi using whole-cell patch-clamp recordings in an *in vitro* brain slice preparation from rats. Activation of hypocretin-1 receptors produced a dose-dependent and long-term facilitation of GABAergic postsynaptic currents evoked by electrical stimulation of the LPGi. Hypoxia/hypercapnia diminished LPGi-evoked GABAergic current in cardiac vagal neurons and this inhibition by hypoxia/hypercapnia was prevented by pre-application of hypocretin-1. The action of hypocretin-1 was blocked by the hypocretin-1 receptor antagonist SB-334867. Facilitation of LPGi-evoked GABAergic current in cardiac vagal neurons under both normal condition and during hypoxia/hypercapnia could be the mechanism by which hypocretin-1 affects parasympathetic cardiac function and heart rate during REM sleep. Furthermore, our findings indicate a new potential mechanism that might be involved in the cardiac arrhythmias, bradycardia, and sudden cardiac death that can occur during sleep. © 2011 Published by Elsevier Ltd on behalf of IBRO.

Key words: hypocretin, rapid eye movement sleep, rostral ventral medulla, parasympathetic preganglionic neurons, bradycardia.

The hypocretin-1 and hypocretin-2 peptides, also called orexin-A and orexin-B, are exclusively synthesized in perifornical area and lateral hypothalamus neurons (de Lecea et al., 1998; Sakurai et al., 1998). Hypocretin has been strongly implicated in sleep-wake control (Chemelli et al., 1999; Kilduff and Peyron, 2000). Evidence from human and animal studies indicates that hypocretin regulates/maintains both waking state and rapid eye movement (REM) sleep (Chemelli et al., 1999; Kilduff and Peyron, 2000; Thannickal et al., 2000; Kiyashchenko et al., 2002), whereas deficiency in hypocretin neurotransmission results in narcolepsy, a disorder characterized primarily by excessive daytime sleepiness and REM sleep dysregulation (Chemelli et al., 1999; Kilduff and Peyron, 2000). In addition, compelling evidence links hypocretin to regulation of cardiovascular function (Ciriello et al., 2003; Dergacheva et al., 2005). Electrical or chemical stimulation of the lateral hypothalamic area has been shown to increase both heart rate and blood pressure and also to excite neurons in the lateral paragigantocellular nucleus (LPGi) (Sun and Guyenet, 1986; Allen and Cechetto, 1993). Hypocretin fibers have been found in nuclei that are well known to be involved in cardiovascular regulation, including the LPGi (Peyron et al., 1998; Ciriello et al., 2003). Intracisternal or intrathecal administration of hypocretin-1 as well as microinjection of hypocretin-1 into the rostral ventral medulla and the commissural nucleus of the nucleus tractus solitarius complex increases mean arterial pressure and heart rate of rats (Chen et al., 2000; Antunes et al., 2001; Smith et al., 2002). Activation of hypocretin-1 receptors facilitates inhibitory and diminishes excitatory spontaneous neurotransmission to cardiac vagal neurons in the nucleus ambiguus (NA) (Dergacheva et al., 2005). Thus, hypocretin neurons and receptors have been proposed to provide a link between central mechanisms that regulate arousal and sleep-wakefulness states and central control of autonomic functions (Young et al., 2005).

Heart rate and parasympathetic activity to the heart change dramatically during transition from non-REM sleep to REM sleep (Sei et al., 2002; Valladares et al., 2008). We have recently demonstrated stimulation of the LPGi, the nucleus which is thought to play a role in REM sleep control (Verret et al., 2005, 2006), evokes an inhibitory pathway to preganglionic cardiac vagal neurons in the NA (Dergacheva et al., 2010b). The LPGi-elicited inhibitory GABAergic pathway to cardiac vagal neurons likely constitutes a neurochemical mechanism underlying REM sleep-related reduction in parasympathetic cardiac activity (Dergacheva et al., 2010b). In the present study we extend this framework and propose that hypocretin-1 influences REM sleep-associated changes in parasympathetic cardiac activity by modulating GABAergic synaptic neuro-

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Abbreviations: LPGi, lateral paragigantocellular nucleus; NA, nucleus ambiguus; REM, rapid eye movement sleep.

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transmission to cardiac vagal neurons from the LPGi. Because hypercapnia activates hypocretin neurons (Williams et al., 2007; Kuwaki, 2010) and hypoxia/hypercapnia significantly influences the activity of cardiac vagal neurons in the NA (Dergacheva et al., 2010a,b) we also examined the effects of hypocretin-1 and hypoxia/hypercapnia on the GABAergic pathway from the LPGi to cardiac vagal neurons.

EXPERIMENTAL PROCEDURES

All animal procedures were performed in compliance with the institutional guidelines at George Washington University and are in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and any possible discomfort.

To identify cardiac vagal neurons *in vitro* a two-stage procedure was used. In an initial surgery, Sprague–Dawley rats (postnatal days 2–3; Hilltop, Scottdale, PA, USA) were anesthetized with hypothermia and received a right thoracotomy. The heart was exposed, and 0.05 ml of 1–5% rhodamine (XRITC, Molecular Probes, Eugene, OR, USA) was injected into the pericardial sac to retrogradely label cardiac vagal neurons. On the day of experiment (2–4 days later), the animals were anesthetized with isoflurane and killed by rapid cervical dislocation. The brain was submerged in cold (4 °C) buffer composed of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES and continually gassed with 100% O_2. Using a dissection microscope the cerebellum was removed and the hindbrain was isolated. A single slice of the medulla (400 μm thickness) that included the NA and the LPGi was obtained and submerged in a recording chamber, which allowed perfusion (5–10 ml/min) of artificial cerebrospinal fluid at room temperature (25 °C) containing 125 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES equilibrated with 95% O_2 and 5% CO_2 (pH 7.4).

Individual cardiac vagal neurons in the NA were identified by the presence of the fluorescent tracer. These identified cardiac vagal neurons were then imaged with differential interference contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution. Patch pipettes (2.5–3.5 MΩ) were filled with a solution consisting of 150 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP, pH 7.4. With this pipette solution, the chloride current induced by activation of GABA receptors was recorded as an inward current. Voltage clamp whole-cell recordings were made at a holding potential of −80 mV with an Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, CA, USA).

GABAergic inhibitory postsynaptic currents were isolated by continuous focal application of strychnine (1 μM), D-2-amino-5-phosphonovalerate (50 μM), and 6-cyano-7-nitroquinoxaline-2,3-dione (50 μM) to block glycine, *N*-methyl-D-asparate, and non–*N*-methyl-D-asparate glutamatergic receptors, respectively. Drugs were focally released using a picrospritzer and pressure ejected from a patch pipette positioned within 30 μm of the patched cardiac vagal neuron. The maximum range of drug application was determined previously to be 100–120 μm downstream from the drug pipette and was considerably less behind the drug pipette (Wang et al., 2002). Continual focal drug applications were performed using a pneumatic picopump pressure system (WPI, Sarasota, FL, USA). Hypocretin-1 (0.1 μM, 0.5 μM, and 1 μM) and the hypocretin-1 receptor antagonist SB-334867 (10 μM) were bath-applied. After the end of each experiment the GABAergic neurotransmission was abolished by focal application of gabazine (25 μM) to the patched cardiac vagal neuron. All drugs used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

In experiments that examined the role of hypoxia/hypercapnia in modulation of GABAergic response in cardiac vagal neurons, slices were exposed to hypoxia/hypercapnia by changing control artificial cerebrospinal fluid to an identical solution bubbled with 85% N_2, 6% O_2, and 9% CO_2. After 1 h equilibration with 85% N_2, 6% O_2, and 9% CO_2 the pH of the solution was 7.1. Slices were exposed to hypoxia/hypercapnia for 10 min and then slices were reoxygenated during 10 min by returning the perfusate to initial control artificial cerebrospinal fluid.

The location of the LPGi was identified using stereotaxic coordinates (Paxinos, 1997) in addition to the location relative to fluorescently identified cardiac vagal neurons in the NA. The LPGi was stimulated with electrical stimuli of 1 ms duration using a stimulus isolator (A.M.P.I., Jerusalem, Israel). Stimulus intensity was 1.5 times of the minimum intensity that evoked a response in cardiac vagal neurons. Synaptic events were measured using pClamp 8 software (Molecular Devices, Sunnyvale, CA, USA). The responses to a series of 10 consecutive stimulations in each neuron were averaged in all series of experiments. The mean value from each neuron in the population was then averaged for the population of neurons to create a summary of results for each condition.

Results are presented as mean±SE and statistically compared using ANOVA with repeated measurement and Dunnett's post-test. Only one experiment was performed per preparation.

RESULTS

In agreement with previously published data (Dergacheva et al., 2010b), electrical stimulation of the LPGi evoked a GABAergic current in cardiac vagal neurons in the NA (see Figs. 1–3). Application of hypocretin-1 at a concentration of 0.1 μM did not significantly alter this GABAergic response (−217±26 pA vs. −220±29 pA, n=9, P>0.05, Fig. 1A). Washout of hypocretin-1 (0.1 μM) also did not change this LPGi-evoked GABAergic current (n=9, Fig. 1A). Similarly, at a concentration of 0.5 μM hypocretin-1 did not alter GABAergic current in cardiac vagal neurons (−197±27 pA vs. −209±24 pA, n=9, P>0.05, Fig. 1B). However, 5-min washout of hypocretin-1 (0.5 μM) significantly enhanced the LPGi-evoked GABAergic response (from −197±27 pA to 257±36 pA, n=9, P<0.001, Fig. 1B). This GABAergic response remained elevated for at least 30 min of hypocretin-1 washout (n=9, Fig. 1B). Application of hypocretin-1 at a concentration of 1 μM elicited a significant facilitation of LPGi-evoked GABAergic current in cardiac vagal neurons (from −214±28 pA to −284±45 pA, n=9, P<0.05, Fig. 1C). The LGPi-evoked GABAergic current remained elevated for at least 30 min of hypocretin-1 washout (n=9, Fig. 1C).

Since the temperature of the rat brain is higher than the room temperature (25 °C) at which most experiments for this study were performed we conducted six additional experiments at 37 °C. Hypocretin-1 at a concentration of 1 μM evoked a small but not significant facilitation of LPGi-evoked GABAergic current at 37 °C (−228±49 pA vs. −269±45 pA, n=6, P>0.05). However, similar to results obtained at 25 °C, 5-min washout of hypocretin-1 at 37 °C evoked a significant increase in the GABAergic current (from −228±49 pA to −349±69 pA, n=6, P<0.01) which remained elevated (−344±64 pA, n=6, P<0.05) for at