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PRESYNAPTIC INHIBITION OF GABAERGIC SYNAPTIC TRANSMISSION BY ADENOSINE IN MOUSE HYPOTHALAMIC HYPOCRETIN NEURONS

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Abstract—Hypocretin neurons in the lateral hypothalamus, a new wakefulness-promoting center, have been recently regarded as an important target involved in endogenous adenosine–regulating sleep homeostasis. The GABAergic synaptic transmissions are the main inhibitory afferents to hypocretin neurons, which play an important role in the regulation of excitability of these neurons. The inhibitory effect of adenosine, a homeostatic sleep-promoting factor, on the excitatory glutamatergic synaptic transmissions in hypocretin neurons has been well documented, whether adenosine also modulates these inhibitory GABAergic synaptic transmissions in these neurons has not been investigated. In this study, the effect of adenosine on inhibitory postsynaptic currents (IPSCs) in hypocretin neurons was examined by using perforated patch-clamp recordings in the acute hypothalamic slices. The findings demonstrated that adenosine suppressed the amplitude of evoked IPSCs in a dose-dependent manner, which was completely abolished by 8-cyclopentyltheophylline (CPT), a selective antagonist of adenosine A1 receptor but not adenosine A2 receptor antagonist 3,7-dimethyl-1-(2-propynyl) xanthine. A presynaptic origin was suggested as following: adenosine increased paired-pulse ratio as well as reduced GABAergic miniature IPSC frequency without affecting the miniature IPSC amplitude. Further findings demonstrated that when the frequency of electrical stimulation was raised to 10 Hz, but not 1 Hz, a time-dependent depression of evoked IPSC amplitude was detected in hypocretin neurons, which could be partially blocked by CPT. However, under a higher frequency at 100 Hz stimulation, CPT had no action on the depressed GABAergic synaptic transmission induced by such tetanic stimulation in these hypocretin neurons. These results suggest that endogenous adenosine generated under certain stronger activities of synaptic transmissions exerts an inhibitory effect on GABAergic synaptic transmission in hypocretin neurons by activation of presynaptic adenosine A1 receptors, which may finely regulate the excitability of these neurons as well as eventually modulate the sleep–wakefulness.

Key words: sleep, wakefulness, adenosine, inhibitory postsynaptic currents, hypocretin

It is well known that in the CNS, adenosine, a product of cell energy metabolism, plays an important role in promoting sleep by inhibition of waking-active neurons in basal forebrain (Rainnie et al., 1994; Arrigoni et al., 2006) or excitation of sleep-active neurons in ventrolateral preoptic nucleus (Gallopin et al., 2005). Recently, abundant evidence has strongly suggested that hypocretin neurons in the lateral hypothalamus, a new wakefulness-promoting center (Adamantidis et al., 2007; Sakurai, 2007; Chen et al., 2008; Li et al., 2010a), may be another important target involved in the hypnogenic action of adenosine in the CNS. *In vivo*, local perfusion of adenosine receptor agonist in the lateral hypothalamus elicits sleep (Alam et al., 2009), whereas microinjection of adenosine receptor-specific antagonist in the same area can induce increase of wakefulness (Thakkar et al., 2008; Alam et al., 2009).

By activation of adenosine A1 and A2 receptors (Basheer et al., 2004), adenosine leads to directly postsynaptic hyperpolarization in waking-active neurons or depolarization in sleep-active neurons (Rainnie et al., 1994; Gallopin et al., 2005; Arrigoni et al., 2006), but more importantly, it inhibits the presynaptic release of excitatory and inhibitory neurotransmitters including glutamate and GABA in these brain regions (Arrigoni et al., 2001; Morairty et al., 2004). *In vitro*, an electrophysiological study by Liu and Gao has shown that adenosine significantly attenuates the frequency of action potentials in hypocretin neurons without a change in membrane potentials, while a depression of excitatory synaptic transmission to these neurons by activating presynaptic A1 receptors is thought to be a cellular mechanism for this inhibition (Liu and Gao, 2007).

In addition to excitatory glutamatergic synaptic transmissions, inhibitory GABAergic synaptic transmissions are also abundant in the hypothalamus and are the main inhibitory afferents to hypocretin neurons (Li et al., 2002; Sakurai et al., 2005; Henny and Jones, 2006; Kokare et al., 2006). The application of $GABA_A$ selective agonist muscimol or GABA into hypothalamic slices produces a hyperpolarization and a decrease of the firing rates in hypocretin

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Abbreviations: ACSF, artificial cerebral spinal fluid; APV, DL-2-amino-5-phosphonopentanoic acid; CPT, 8-cyclopentyltheophylline; DMPX, 3,7-dimethyl-1-(2-propynyl) xanthine; DNQX, 6,7-dinitroquinoxaline-2,3 (1H, 4H)-dione; EGFP, enhanced green fluorescent protein; EPSCs, excitatory postsynaptic currents; IPSCs, inhibitory postsynaptic currents; mIPSCs, miniature IPSCs; PPR, paired-pulse ratio; TTX, tetrodotoxin.

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neurons (Li et al., 2002; Eggermann et al., 2003; Xie et al., 2006). Importantly, local microdialysis of $GABA_A$ receptor antagonist into lateral hypothalamus will activate hypocretin neurons and lead to the suppression of sleep and induction of arousal (Alam et al., 2005, 2010), suggesting that the GABAergic system within lateral hypothalamus plays an important role in the regulation of sleep. It has been well documented that adenosine could regulate the GABAergic synaptic transmission in several areas in hypothalamus, such as supraoptic nucleus (Oliet and Poulain, 1999), paraventricular nucleus (Li et al., 2010b; Han et al., 2011), and histaminergic tuberomammillary nucleus (Yum et al., 2008), an important subcortical arousal system. Whether adenosine modulates the inhibitory GABAergic transmission in hypothalamic hypocretin neurons is still unknown.

In the present study, therefore, a possible role for adenosine in regulating the inhibitory synaptic transmission in hypocretin neurons was investigated by performing perforated patch-clamp recordings in hypothalamic slices from hypocretin-enhanced green fluorescent protein (EGFP) transgenic mouse.

EXPERIMENTAL PROCEDURES

Hypothalamic slice preparation

Transgenic mice (kindly provided by Dr. T. Sakurai, Kanazawa University, Ishikawa, Japan) expressing EGFP under control of the hypocretin promoter (Li et al., 2002; Yamanaka et al., 2003) were used in this study. The animals use and methods were carried out in according with the guidelines of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committer (Shanghai, China). In brief, both male and female transgenic mice (21–28 day old) were killed by decapitation after sodium pentobarbital (1%) anesthesia, and the brain was quickly removed and placed in ice-cold oxygenated (95% O_2 and 5% CO_2) solution containing (in mM) 220 sucrose, 2.5 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.3–7.4 with NaOH. Coronal slices (250 μm) of the hypothalamus containing hypocretin neurons were cut with a Microm HM 650 V vibratome (Thermo Scientific Richard-Allan, Microm, Heidelberg, Germany) and then transferred to an incubating chamber at room temperature in continuously oxygenated artificial cerebral spinal fluid (ACSF) for ~1 h before use. The composition of the ACSF was (in mM): 125 NaCl, 2.5 KCl, 1.3 Na_2HPO_4, 25 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, and 10 glucose saturated with 95% O_2 and 5% CO_2.

Perforated patch-clamp recordings

A hypothalamic slice was transferred to a recording chamber where it was submerged and continuously superfused with 32 °C ACSF at a flow rate of 2 ml/min. Recordings were obtained with perforated patch-clamp configuration using amphotericin B (250 $\mu g\ ml^{-1}$) as the pore-forming agent (Oyamada et al., 1998) dissolved in pipette solutions before each experiment. Hypocretin neurons were visually identified using a fluorescence microscope (DM LFS, Leica, Wetzlar, Germany) fitted with infrared differential interference contrast (IR-DIC) optics. Patch-clamp recording pipettes (3~5 MΩ) were filled with a solution containing (in mM) 135 KCl, 4 $MgCl_2$, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.2 ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.4 Na-ATP, 0.3 Na_2-GTP, and 4 Na_2-phosphoreatin. Data acquisition and analysis were conducted with Axon Multi 700A amplifier and Clampex 9.0 software (Molecular Devices, Sunnyvale, CA, USA). Series resistance (<50 MΩ) was monitored online at regular intervals throughout the course of the experiments with voltage pulses (−10 mV), and cells were excluded from data analysis if a >20% change occurred during the course of the experiment.

For presynaptic stimulation, either a bipolar stainless steel or a glass-stimulating electrode was placed on the surface of the slice close to the dorsolateral border of the ipsilateral hypothalamus (Wollmann et al., 2005). An isolated constant-current source, Master-8 (A.M.P.I., Jerusalern, Isreal), was used to generate square wave pulses of direct current (40~100 μA; 100~200 μs duration). The stimulation trigger was controlled by Clampex 9.0 software. For recording of inhibitory postsynaptic currents (IPSCs), hypocretin neurons were voltage clamped at −70 mV. IPSCs were induced at a frequency of 0.033 Hz and represented as averages of 10 current traces, unless otherwise stated.

Drugs

Drugs used were adenosine, 6,7-dinitroquinoxaline-2,3(1H, 4H)-dione (DNQX), DL-2-amino-5-phosphonopentanoic acid (APV), 8-cyclopentyltheophylline (CPT), and 3,7-dimethyl-1-(2-propynyl) xanthine (DMPX) from Sigma (St. Louis, MO, USA). Amphotericin B was purchased from Calbiochem (Novabiochem, La Jolla, CA, USA), bicuculline methiodide (bicuculline) from BIOMOL International L.P. (Plymouth Meeting, PA, USA), and CGP 55845 hydrochloride (CGP 55845) from Tocris Bioscience (Bristol, UK). Tetrodotoxin (TTX) was obtained from Hebei Fisheries Research Institute (Qinghuangdao, China).

Statistical analysis

Results are presented as mean±SEM. The data for amplitude of inhibitory synaptic currents were normalized relative to baseline. Significance was determined using Student's *t*-test and nonparametric Kolmogorov–Smirnov test (K–S test). For all tests, $P<0.05$ was considered statistically significant.

RESULTS

Adenosine suppresses evoked inhibitory synaptic transmissions in hypocretin neurons

Green fluorescent neurons, in the hypothalamic slices from hypocretin-EGFP transgenic mice, were identified under fluorescence microscope (Fig. 1A, left panel) and subsequently subjected to perforated patch-clamp recording (Fig. 1A, right panel). The basic membrane properties of hypocretin neurons in our experiment were not different from those described in previous reports (Li et al., 2002; Yamanaka et al., 2003; Muraki et al., 2004). As seen in Fig. 1B (top panel), hyperpolarization-activated currents could be induced after currents (from −100 pA, 800 ms, 40 pA increment) injected into a hypocretin neuron. The lower panel in Fig. 1B showed that little spike frequency adaptation was seen in response to a higher depolarizing current (200 pA) in a hypocretin neuron. Electrical stimulation of the dorsolateral border of the lateral hypothalamus induced reproducible excitatory postsynaptic currents (EPSCs) in hypocretin neurons recorded in the presence of $GABA_A$ receptor antagonist bicuculline (10 μM). Application of specific α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonist DNQX (10 μM) completely blocked EPSCs in all neurons tested