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Electrophysiological effects of orexins/hypocretins on pedunculopontine tegmental neurons in rats: An in vitro study

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ABSTRACT

Orexin-A (ORX-A) and orexin-B (ORX-B) play critical roles in the regulation of sleep–wakefulness and feeding. ORX neurons project to the pedunculopontine tegmental nucleus (PPT), which regulates waking and rapid eye movement (REM) sleep. Thus, we examined electrophysiological effects of ORXs on rat PPT neurons with a soma size of more than 30 μm. Whole cell patch clamp recording in vitro revealed that ORX-A and ORX-B depolarized PPT neurons dose-dependently in normal and/or tetrodotoxin containing artificial cerebrospinal fluids (ACSFs), and the EC_{50} values for ORX-A and ORX-B were 66 nM and 536 nM, respectively. SB-334867, a selective inhibitor for ORX 1 (OX_1) receptors, significantly suppressed the ORX-A-induced depolarization. The ORX-A-induced depolarization was reduced in high K^+ ACSF with extracellular K^+ concentration of 13.25 mM or *N*-methyl-D-glucamine ($NMDG^+$)-containing ACSF in which NaCl was replaced with NMDG-Cl, and abolished in high K^+-$NMDG^+$ ACSF or in a combination of $NMDG^+$ ACSF and recordings with Cs^+-containing pipettes. An inhibitor of Na^+/Ca^{2+} exchanger and chelating intracellular Ca^{2+} had no effect on the depolarization. Most of PPT neurons studied were characterized by an A-current or both A-current and a low threshold Ca^{2+} spike, and predominantly cholinergic. These results suggest that ORXs directly depolarize PPT neurons via OX_1 receptors and via a dual ionic mechanism including a decrease of K^+ conductances and an increase of non-selective cationic conductances, and support the notion that ORX neurons affect the activity of PPT neurons directly and/or indirectly to control sleep–wakefulness, especially REM sleep.

1. Introduction

Novel neuropeptides, orexin-A (ORX-A) and orexin-B (ORX-B), also called hypocretin-1 and hypocretin-2, respectively, were newly identified in the perifornical region of the lateral hypothalamic area (LHA) [33,37]. The ORX binds to orexin 1 (OX_1) and orexin 2 (OX_2) receptors which belong to the G protein-coupled receptor superfamily. OX_1 receptors have a higher affinity for ORX-A than for ORX-B, whereas OX_2 receptors have an equal affinity for both ORX-A and ORX-B. The ORX was initially recognized as a regulator of feeding behavior because of the exclusive expression in the LHA known as the feeding center and the increase of food intake induced by intracerebroventricular administration of ORXs

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[37]. However, subsequent studies demonstrated that ORXs plays a crucial role in the regulation of sleep and wakefulness. For example, Lin et al. [27] found that canine narcolepsy, that is characterized by daytime sleepiness, cataplexy and the striking transition from wakefulness into rapid eye movement (REM) sleep, is caused by mutation of the OX_2 receptor gene. It is also reported that ORX knockout mice exhibit a phenotype strikingly similar to human narcolepsy patients as well as canarc-1 mutant dogs [5]. These results suggest that the intact ORX signaling system is required to maintain a proper wakefulness of animals including humans for survival [34], and deficiencies of ORX signaling are associated with the narcolepsy and cataplexy.

Sleep and wakefulness is regulated by dynamic interactions between multiple neurochemically distinct systems in the brain. Wakefulness is promoted by an ascending arousal system consisting of cholinergic and monoaminergic neurons in the forebrain, midbrain and brainstem, whereas the initiation and maintenance of REM sleep and non-REM (NREM) sleep are controlled by groups of neurons in the brainstem and preoptic area of the hypothalamus [16,31,39]. Cholinergic neurons in the pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT) of the brainstem are involved in not only the maintenance of wakefulness but also the generation of REM sleep [6,25,29,35,44,45]. Indeed, electrical stimulation of the PPT caused activation of the cortical electroencephalogram (EEG), i.e., EEG arousal [41], whereas extensive loss of cholinergic mesopontine neurons by kainic acid was associated with loss of REM sleep and absent or incomplete neck muscle atonia [20,49]. In addition, many cholinergic neurons in the PPT and LDT fire rapidly during wakefulness and REM sleep, whereas they are inactive during NREM sleep [6,8,36,40]. A distinct subpopulation of PPT and LDT cholinergic neurons also specifically increases their electrical activity just prior to and during REM sleep [6,8,40].

Extracellular recording studies demonstrate that cholinergic neurons in the LDT and basal forebrain (BF) that constitute the ascending arousal system are excited by ORXs [4,7,42]. Whole cell patch clamp recording studies indicate that the excitatory effect of ORXs in LDT cholinergic neurons is both presynaptic and postsynaptic, and mediated postsynaptically by an activation of noisy cation channels [4,24]. A recent in vitro study also shows that septohippocampal cholinergic neurons which contribute to hippocampal arousal are depolarized postsynaptically through a dual ionic mechanism including an inhibition of K^+ channels and an activation of Na^+/Ca^{2+} exchanger (NCX) [51]. In contrast to these cholinergic neurons, however, the effects of ORXs on PPT cholinergic neurons have not been described. Therefore, the purpose of the present study was to examine the electrophysiological effects of ORXs on putative PPT cholinergic neurons with a soma size of more than 30 μm in rat brain slices, and to determine the ionic mechanism involved. PPT neurons that were responsive and non-responsive to ORXs were also characterized by the electrophysiological membrane properties, and some of them were labeled with biocytin and then stained with nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), a reliable marker for mesopontine cholinergic neurons [26,47,48].

2. Materials and methods

2.1. Animals

Male Wistar rats 1–2 weeks of age were used (Sankyo Lab., Shizuoka, Japan). They were housed with their mothers in a light-controlled room (light on: 06:00–18:00) at a temperature of 23 ± 2 °C for several days before the experiments. The animals and experimental procedures used were approved by the Institutional Animal Care and Use Committee of the University of Toyama.

2.2. Slice preparations

Rats were decapitated after sevoflurane anesthesia and the brain was rapidly removed from the skull. The brain was then submerged in ice cold, oxygenated (95% O_2–5% CO_2) normal artificial cerebrospinal fluid (ACSF), composition in mM: NaCl 126, KCl 3, $CaCl_2$ 2.4, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10 with a pH of 7.4. Coronal brainstem slices 300 μm thick were cut by a microslicer (ZERO 1, Dosaka EM, Kyoto, Japan). The PPT was identified on the basis of its anatomical location adjacent to the superior cerebellar peduncle. Two or three slices including the PPT were selected from each animal, and cut with a scalpel along the midline so that two PPT slices, each including the left and right side PPT, were obtained from one coronal slice. The slices containing the PPT were then preincubated in a chamber with oxygenated normal ACSF for about 1 h at room temperature.

2.3. Whole cell patch clamp recording

After preincubation, slices were transferred into a whole cell patch clamp recording chamber fixed to the stage of an upright microscope (BX-50WI, Olympus, Tokyo, Japan). The recording chamber was perfused with oxygenated normal ACSF at 1 ml/min and at 34 °C. PPT neurons were visualized on a television screen through an infrared charge coupled device (CCD) camera (C2741-79, Hamamatsu Photonics, Hamamatsu, Japan) and a real-time digital video microscopy processor (XL-20, Olympus, Tokyo, Japan). Electrodes were filled with a standard internal pipette solution containing in mM: K-gluconate 120, KCl 20, HEPES 10, $MgCl_2$ 2.0, $CaCl_2$ 0.5, EGTA 1.0, Na-ATP 4.6, and Na-GTP 0.4, pH adjusted to 7.3 with KOH, and electrode resistances were 5–9 MΩ. Neurons were recorded in ''I-clamp normal'' current clamp or voltage clamp modes using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA, USA). An Ag/AgCl reference electrode was placed near the intermediate position between the inlet and outlet of the chamber. Membrane potentials and currents recorded via the electrodes were fed into the amplifier. Series resistance compensation and capacitive compensation were performed as much as possible by the amplifier. The output of the amplifier was digitized using an A/D converter board (Digidata 1200, Axon Instruments, Union City, USA) with a sampling rate of 10 kHz, and recorded on a hard disk by data acquisition and analysis software (pCLAMP 8, Axon Instruments, Union City, USA). Membrane potentials were low-pass filtered at 5 kHz. Furthermore, membrane potentials less than −60 mV were set to about −60 mV by current injections. Whole cell liquid