Orexin depolarizes rat hypothalamic paraventricular nucleus neurons

TETSURO SHIRASAKA,1,2 SATOSHI MIYAHARA,3 TAKATO KUNITAKE,2 QING-HUA JIN,2 KAZUO KATO,2 MAYUMI TAKASAKI,1 AND HIROSHI KANNAN2

Departments of 1Anesthesiology, 2Physiology, and 3Neurosurgery, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki, 889–1692, Japan

Received 8 February 2001; accepted in final form 20 June 2001

Orexins or hypocretins, a novel hypothalamic peptide family (7, 20), were thought to be involved in food intake (8, 20), sleep (3, 16), and autonomic and neuroendocrine functions (6, 17, 28). Orexins include orexin-A and orexin-B, which are proteolytically derived from the same precursor protein (20). The expression pattern of mRNA encoding two orexin receptors (OX1R and OX2R) in the hypothalamus supports its proposed role in the regulation of food intake, but the central distribution of OX1R and OX2R is extensive and markedly differentiated (20, 27). The highest levels of OX1R were expressed in the ventromedial hypothalamic nucleus and locus ceruleus, whereas OX2R mRNA was found predominantly in the hypothalamic paraventricular nucleus (PVN) (20, 27). Orexin-containing fibers were also present abundantly in the PVN (6, 17). In a previous study, we demonstrated that intracerebroventricular administration of orexins increases heart rate, mean arterial pressure, renal sympathetic nerve activity, and plasma catecholamine levels in conscious rats (23). In spite of the fact that orexin and the orexin receptor have been identified in the PVN, which is thought to be involved in the control of the autonomic nervous system, cardiovascular function, and neuroendocrine system (24, 25), no in vitro evaluation of the effect of orexins on PVN neurons has ever been conducted. In the present study, using whole cell patch-clamp techniques, we examined the effects of orexin-B on PVN neurons in a hypothalamic slice.

MATERIALS AND METHODS

Slice preparation. The experimental procedure was mainly the same as previously described (22). Male Wistar rats (postnatal days 15–21) were anesthetized with isoflurane and then killed by decapitation. The brain was rapidly removed and placed in cooled (2–3°C) standard artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgCl2, 25 NaHCO3, 2 CaCl2, and 10 glucose, which had been oxygenated with a 95% O2-5% CO2 gas mixture. The osmolarity was maintained at 290 mosM with a pH of 7.4. This solution was also used as the extracellular solution in all experiments. Coronal slices 200 μm thick, which included the PVN, were prepared with a vibrating brain slicer (DSK-2000; Dosaka, Kyoto, Japan) and allowed to equilibrate for aCSF for at least 20 min at 34°C and for up to 1 h at room temperature (24–26°C) before recording was started.

Electrophysiological recording. The electrodes were made with a puller (PB-7; Narishige, Tokyo, Japan) made from thick-wall borosilicate glass (OD-1.5; Narishige). Their resistance was 3–5 MΩ in the bath, with access resistances in the range of 8 to 12 MΩ. The electrode solution contained (in mM): 120 K gluconate, 20 KCl, 10 EGTA, 2 MgCl2, 2 Na2ATP, 10 HEPES, and 0.3 GTP, with pH adjusted to 7.3 with KOH. Series resistance was compensated by 80%. Slices were transferred into the recording chamber and continuously perfused (1.5–2.0 ml/min) with aCSF at room temperature (24–26°C). Whole cell recordings were made from microscopically identified cells. Transmembrane voltage was recorded using a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) and stored on hard disk via an analog-to-digital converter for later analysis. The signals...
were also displayed on a thermal rectigraph (model 8M14; San-Ei, Tokyo, Japan). The stored data were analyzed using AxoGraph software (version 8.0, Axon Instruments). The membrane potential was low-pass filtered at 2 kHz. The liquid-junction potential between the pipette and aCSF was corrected at the beginning of the experiment. Once stable recording conditions were obtained, a PVN neuron was identified electrophysiologically as either a type 1 (putative magnocellular) or type 2 (putative parvoceellar) neuron according to previously established criteria (26). Type 1 neurons were characterized by a transient K+ current in the absence of a low threshold potential. Type 2 neurons were identified on the basis of the absence of a transient K+ current and the presence of a low threshold potential. The effects of orexins on membrane potentials were examined at the resting membrane potential (RMP). Drugs were applied after recording at least 100 s of control events. In evaluating concentration-dependent responses of the membrane potential, we tested the same neurons one to two times in another concentration. In these cases, the concentration of the applied drugs was selected at random, and subsequent drug applications were made after the effects of the first drug application had been fully reversed.

Drugs and chemicals. Orexin-B (Sigma, St. Louis, MO) and TTX (Sigma) were dissolved in aCSF. TTX was used to block sodium channels. Orexin-B was stored at −30°C until use. Orexin-B and other drugs were applied into the recording chamber by changing the perfusion line to the one that contained the drug.

Statistical analysis. All data are expressed as means ± SE, and statistical analysis was performed using a Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

A total of 88 PVN neurons were recorded with the whole cell patch-clamp technique. The PVN was distinguished from surrounding tissue by its translucent appearance in the slice. The approximate location of each recorded cell with respect to the PVN was visually estimated from the placement of the recording electrode. Before the orexin-B application, all cells were electrophysiologically classified according to previously established criteria (26). Type 1 and type 2 neurons appeared from this estimation to be located within the PVN. Type 1 neurons have relatively large somata and are located in the PVN magnocellular subregions. Type 2 neurons have relatively small somata and are located in the PVN parvocellular subregions. These neurons (n = 50) had a mean RMP of −56.7 ± 1.5 mV and a mean input resistance of 822 ± 12.5 MΩ. No significant difference in RMP and input resistance was observed between type 1 and type 2 neurons. A 1-min bath application of orexin-B (0.01–1.0 μM) depolarized 80.8% (21 of 26) of type 1 and 79.2% (19 of 24) of type 2 neurons in normal aCSF. Orexin-B (1 μM) evoked the depolarization of type 1 (7.47 ± 0.61 mV; n = 8) and type 2 (10.1 ± 0.64 mV; n = 7) neurons with a latency of 56.1 ± 9.4 s after the peptide entered the recording chamber; then, the depolarization returned to a baseline level after 4–10 min of washing (Fig. 1A). Similar amplitude responses could be obtained from the same neuron without desensitization after an additional 10-min wash. These depolarizing responses were accompanied by an increase in the firing of action potentials (Fig. 1A). To elucidate whether these responses are due to a direct effect of orexin on PVN neurons, twelve additional cells were tested with orexin-B (1 μM) during a blockade of synaptic transmission by a bath application of TTX (1 μM). Orexin-B elicited a reversible depolarization in type 1 (7.1 ± 0.61 mV; n = 6) and type 2 (8.17 ± 0.65 mV; n = 6) neurons, indicating that these depolarizing actions were mediated by a postsynaptic orexin receptor (Fig. 1B). Addition of Cd2+ (1 mM) to the aCSF containing TTX (1 μM) induced a significant decrease in orexin-B (1 μM)-induced depolarization in type 2 (7.84 ± 0.51 mV; n = 8, P < 0.05) neurons...
but not in type 1 (6.25 ± 0.62 mV; n = 6, P > 0.05) neurons. The result suggests that there is a primarily excitatory input to type 2 neurons of the PVN that is probably the result of a local glutamatergic relay. The increase in the depolarization induced by orexin-B was dose dependent in both types of neurons, and the changes in membrane potential were larger in type 2 neurons than in type 1 (Fig. 2). These results suggest that orexin-B depolarizes type 2 neurons via not only postsynaptic but also presynaptic action. To monitor the input resistance during the response evoked by orexin-B, hyperpolarizing current pulse injections (50 pA, 200 ms duration) were applied every 10 s (Fig. 3A). Orexin-B (1 μM) did not significantly affect input resistance in type 1 (815.3 ± 38.7 to 821.2 ± 51.3 MΩ; n = 8, P > 0.05) or type 2 (827.3 ± 44.7 to 838.6 ± 36.2 MΩ; n = 8, P > 0.05) neurons (Fig. 3B).

DISCUSSION

The present result demonstrates that orexins excite type 1 and type 2 neurons of the PVN mediated by a depolarization. These excitatory effects of orexins are in agreement with the previous reports that orexin-A or -B activated locus ceruleus (10, 12, 13), hypothalamus (28), and arcuate nucleus (19) neurons. The action of orexins appears to be mediated by OX1R and/or OX2R (20). OX2R is predominantly expressed in the hypothalamic PVN and is nonselective for the ligands of either orexin-A or -B (20). In a supplementary study, orexin-A (1 μM) also depolarized type 1 (7.25 ± 0.9 mV; n = 5) and type 2 (8.8 ± 1.7 mV; n = 5) neurons (unpublished data). The functional significance of the depolarizing effects of orexins on PVN neurons has not been established; however, our previous study demonstrated that intracerebroventricular administration of orexins produces increases in mean arterial pressure, heart rate, renal sympathetic nerve activity, and plasma catecholamine level in conscious rats (23). It has been suggested that the PVN is involved in the regulation of the autonomic nervous and neuroendocrine systems and, in particular, cardiovascular functions and body fluid balance (24, 25). Both electrical and chemical stimulation of the PVN have been demonstrated to increase blood pressure and renal sympathetic nerve activity in conscious rats (14). Thus it is possible that the cardiovascular and sympathetic responses induced by orexins are, at least in part, mediated by the excitatory effects of orexins on PVN neurons. Excitatory responses induced by orexin-B were maintained in the presence of TTX, which indicates that the peptide has a postsynaptic site of action that was observed in both type 1 (putative magnocellular) and type 2 (putative parvocellular) neurons. Both the magnocellular and parvocellular groups of neurons can
be further subdivided on the basis of peptide expression, projection targets, and/or location in the nucleus (25). Magnocellular neurons primarily synthesize arginine vasopressin (AVP) and oxytocin and secrete these hormones into the circulation from nerve terminals in the posterior pituitary. Parvocellular neurons can be either neuroendocrine or preautonomic neurons. The neuroendocrine parvocellular neurons regulate the anterior pituitary by secreting releasing or inhibiting hormones, such as corticotropin-releasing hormone, thyrotropin-releasing hormone, somatostatin, and dopamine, which control the secretion of the anterior pituitary hormones, adenocorticotropic, thyroid-stimulating hormone, growth hormone, and prolactin, respectively. The preautonomic neurons project to autonomic centers in the brain stem and spinal cord that are involved in the regulation of heart rate and blood pressure (24). Therefore, the initiation of a cellular signal by orexin may have diverse physiological consequences. The change in membrane potentials evoked by orexin-B was larger in type 2 neurons than in type 1 neurons. Excitatory inputs to parvocellular as well as magnocellular neurons of the PVN are mediated mainly by an intrahypothalamic glutamatergic pathway (5, 11). Blocking Ca$^{2+}$ channels with addition of Cd$^{2+}$ to the aCSF significantly reduced the depolarizing effects of orexin-B in type 2 neurons. These results suggest that orexin-B-evoked depolarization was produced in part by Cd$^{2+}$-sensitive Ca$^{2+}$ channels, which contribute to the release of glutamate from presynaptic nerve terminals, and that orexin-B also excites type 2 neurons via, at least in part, a glutamatergic transmission. It may be possible that orexin-B depolarizes type 2 neurons via not only postsynaptic but also presynaptic action. Monitoring input resistance before and during the depolarization induced by orexin-B could not reveal a clear conductance change. Orexin-B (hypocretin-2) has been reported to decrease potassium conductance (13) or reduce afterhyperpolarization (12) in locus ceruleus neurons. It is possible that the orexin-induced conductance change was too small to be detected in the present study. Another possibility is that orexin-B excites the type 1 and type 2 neurons of the PVN by an increase in depolarizing conductance and a decrease in hyperpolarizing conductance, leading to no changes in conductance. Although we could not detect any changes in conductance and further studies are required, it may be possible that orexin-B excites the type 1 and type 2 neurons of the PVN by a depolarization mediated by a different mechanism.

In conclusion, this study provides the first evidence that orexin-B activates magnocellular and parvocellular neurons in the PVN. These findings suggest that orexin may have a functional role in the regulation of the neuroendocrine, cardiovascular, and autonomic nervous systems at the PVN level.

**Perspectives**

Several initial lines of evidence suggest that the hypothalamic peptide orexin has a role in the regulation of food intake (6, 20). However, relatively widespread expression of OX1R and OX2R in brain regions and data from recent studies support further functional roles, such as arousal, the sleep/wake cycle (3), analgesia (2), and blood pressure regulation (21, 23). Intracerebroventricular administration of orexin induces activation of hypothalamic-pituitary-adrenal axis and stimulates hypothalamic corticotropin-releasing factor and AVP neurons in the PVN in conscious rats (1), results that are in very close agreement with our in vitro observation that orexin activates both magnocellular and parvocellular neurons of the PVN. Central orexin also increase renal sympathetic nerve activity, plasma catecholamines, and cardiovascular responses in conscious rats (23). Orexin may be relevant to multiple homeostatic functions, such as body fluid balance and blood pressure control, through the regulation of sympathetic nerve activity and humoral mechanisms such as vasopressin secretion. Although the pathophysiological role of the sympathoexcitatory effects of orexin is not clear, the close relationship among obesity, hypertension, and altered cardiovascular responses has been documented in a number of studies (15). In this regard, leptin, the protein product of the ob/ob gene (30), is produced and secreted by adipocytes (9) to regulate body weight homeostasis. Leptin induces weight loss by decreasing food intake and increasing energy expenditure (4). Intracerebroventricular administration of leptin elevates c-Fos immunoreactivity within the PVN (29), and direct application of leptin to PVN neurons in rat brain slices results in dose-related depolarization (18). In the arcuate nucleus, leptin inhibited electrical activity of orexin-sensitive neurons (19). Therefore, these neuropeptides, leptin and orexin, which are involved in the control of energy balance, may be chemical mediators in the brain that are responsible for the generation and maintenance of hypertension.

This study was carried out as a part of “Ground Research Announcement for Space Utilization,” promoted by the Japan Space Forum, and was supported by grants-in-aid for scientific research (10557009 and 11470019) from the Ministry of Education, Science, Sports, and Culture, Japan.

**REFERENCES**

Orexin Excites Paraventricular Nucleus Neurons


