Signaling pathways of hypocretin-1 actions on pyramidal neurons in the rat prefrontal cortex

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We have investigated the direct excitatory effects of hypocretin-1 on acutely isolated prefrontal cortical pyramidal neurons and explored the signaling mechanisms of these actions. Puff application of hypocretin-1 caused an excitation in the recorded neurons. These effects of hypocretin-1 were abolished by a phospholipase C inhibitor D609, demonstrating that phospholipase C mediates the actions of hypocretin-1. A specific protein kinase C inhibitor, bisindolylmaleimide II, blocked the excitatory actions of hypocretin-1, suggesting that protein kinase C plays a key role. Finally, protein kinase A inhibitor applied intracellularly did not affect the responses. These results indicate that hypocretin-1 excites prefrontal neurons by activation of phospholipase C and protein kinase C pathways, but not protein kinase A. *NeuroReport* 16:1529–1533

Key words: Hypocretin-1; Phospholipase C; Prefrontal cortex; Protein kinase C; Wakefulness; Whole-cell patch-clamp

INTRODUCTION

The hypothalamic peptides hypocretin 1 and 2 (also know as orexin A and B) [1,2] have been implicated in the regulation of wakefulness [3,4]. This role is largely attributed to cortical activation induced by the stimulation of multiple subcortical arousal systems, such as the locus coeruleus, mesopontine and basal forebrain neurons, and the nonspecific thalamocortical projection system [5,6]. Interestingly, morphological studies have shown that hypocretinergic projections and hypocretin receptors are also distributed in the cerebral cortex [7,8]. Furthermore, hypocretin-1 has been found to elevate intracellular Ca^{2+} in cultured cortical neurons [9]. Electrophysiological experiments indicate a direct postsynaptic excitatory effect of hypocretins on primary somatosensory cortical neurons [10]. Collectively, these data suggest that hypocretins also participate in wakefulness consolidation through direct excitatory actions on the cerebral cortex.

The prefrontal cortex is a brain region whose activity is correlated with level of wakefulness [11–14] and which plays a critical role in many higher cognitive functions such as working memory and attention [15]. Previous studies have shown that hypocretin-2 exerts an excitatory modulation on layer V neurons of the prefrontal cortex by a presynaptic action but not by a direct postsynaptic action [14]. This may be attributed to hypocretin receptor-1 mRNA expression alone in prefrontal neurons [8]. Also, hypocretin-2 is less active than hypocretin-1 on hypocretin receptor-1 [2]. Therefore, we hypothesized that hypocretin-1 might have a direct postsynaptic effect on prefrontal cortical neurons. If the direct prefrontal modulation by hypocretin-1 was confirmed, questions would remain as to what intracellular signaling pathways underlie this electrical excitation.

To date, it has been identified that there are diverse ionic mechanisms responsible for the actions of hypocretins in varying cellular populations, such as an electrogenic pump and calcium current activation, blockage of potassium conductance, and activation of a nonselective cation conductance [16–18]. Similarly, the possibility that multiple signaling mechanisms mediate the excitatory effects of hypocretins on different cells should be considered. In fact, there is only limited report indicating that the activation of phospholipase C (PLC) and protein kinase C (PKC) signaling pathways is involved in the depolarizing effects of hypocretin-1 on nucleus tractus solitarius neurons [19]. It is uncertain whether the depolarizing effects induced by hypocretin-1 in different cells share a common signaling mechanism. Thus, further work is needed to delineate the exact signaling pathways underlying the hypocretin-1-induced excitation in prefrontal cortical neurons.

The aim of this study was to examine the postsynaptic effects of hypocretin-1 on acutely dissociated prefrontal pyramidal neurons, and explore the possible cellular mechanisms of these actions using whole-cell patch-clamp recordings and neuropharmacological tools.

MATERIALS AND METHODS

Acute dissociation: Deep layer (V–VI) prefrontal cortical pyramidal neurons (Fig. 1a) from postnatal 8 to 10-day-old

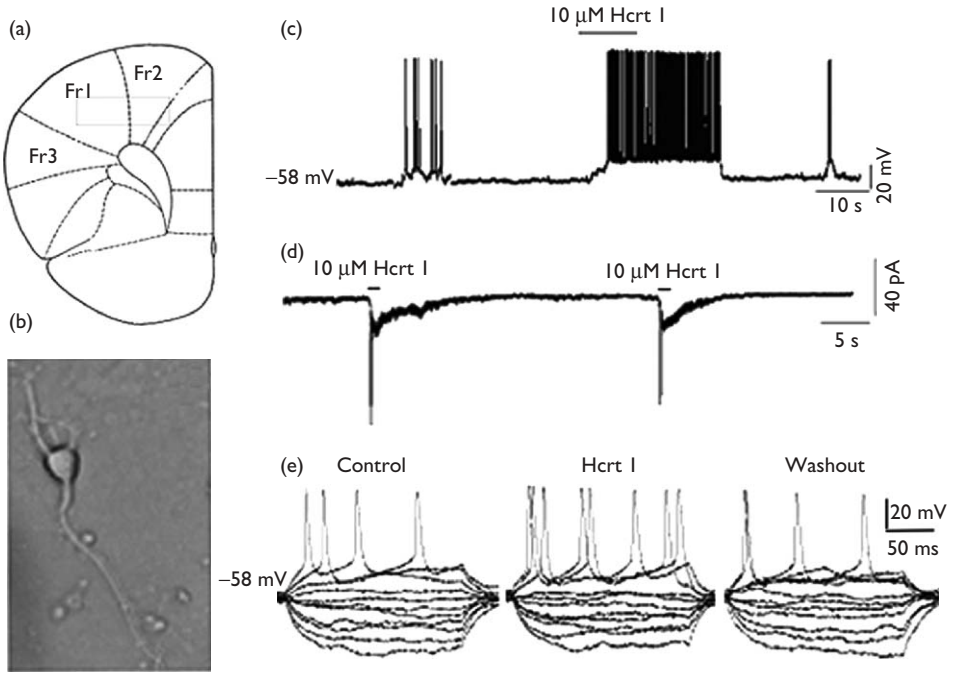

Fig. 1. Rat prefrontal cortical pyramidal neuron and excitatory effects of hypocretin-1 (Hcrt 1) on these neurons. (**a**) Schematic representation of the place of layers of V and VI of the rat frontal cortex (Fr). (**b**) Photomicrograph of an acutely isolated prefrontal pyramidal neuron that has a long apical dendrite and three basal dendrites. (**c**) Application of 10 μM hypocretin-1 produced rapid sustained depolarization accompanied by a rapid increase in firing frequency of action potentials. After washout of hypocretin-1, the membrane potential and action potential frequency returned to control levels. (**d**) Inward currents were evoked by hypocretin-1. (**e**) Application of hypocretin-1 increased the number of action potentials evoked by a series of current pulses of −60 to +40 pA. The effect was sustained when the membrane potential was clamped at the control level.

Wistar rats were acutely dissociated using procedures modified from those of previous reports [20]. In brief, rats were anesthetized with ether and decapitated. The brains were quickly removed, blocked, and then 400 μm slices were prepared with a vibratome tissue sectioner in ice-cold oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM) 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 glucose, pH 7.3–7.4. Coronal slices were incubated for 2 h at room temperature (22°C–24°C) in oxygenated aCSF before dissociation. Individual slices were then placed into an oxygenated, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered aCSF containing 1 mg/ml trypsin at 36°C for 35–40 min. The aCSF containing the enzyme consisted of (in mM) 150 NaCl, 5 KCl, 1.6 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose. After enzymatic treatment, the tissue was rinsed several times and kept in the enzyme-free aCSF. The prefrontal cortex was isolated under a dissecting microscope and triturated with a graded series of fire-polished Pasteur pipettes. The cell suspension was placed in a Lucite chamber, which was mounted on an inverted microscope (Nikon, Tokyo, Japan). Cells were then given 10–15 min to settle down before electrophysiological recording.

Electrophysiological procedures: Whole-cell recordings were performed at room temperature using standard techniques. The normal intracellular recording solution consisted of (in mM) 145 potassium gluconate, 2 $MgCl_2$, 5 K_2ATP, 0.5 ethyleneglycol-bis-(β-amino ethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid, and 5 HEPES, pH 7.2–7.3. Recordings were obtained with an Axon Instruments (Foster City, California, USA) 200B patch-clamp amplifier and controlled and monitored with a personal computer running pClamp (version 6.06) with a Digi-Data 1200 analog-to-digital interface (Axon Instruments), filtered at 2 kHz and digitized at 10 kHz. Electrode resistances were ~3–6 MΩ in bath. The average whole-cell capacitance of the recorded neurons was about 12 pF. The liquid junction potential (~3 mV) was not compensated, and membrane potentials were not corrected.

Drugs and statistical analysis: All drugs were obtained from Sigma (St Louis, Missouri, USA). Hypocretin-1 was added by puff application to neurons, as described previously [4]. Other drugs were applied by switching perfusion from aCSF to a solution containing the desired drug. In the case of puff application, hypocretin-1 (10 μM) was ejected by pressure from a micropipette (tip diameter ~2–5 μm; duration of application ~10–15 s) positioned adjacent to the recording microelectrode. Then, hypocretin-1 peptide (40 μl) would diffuse in the solution in a chamber (volume 2 ml). Owing to this, though the concentration in the pipette was 10 μM, the final concentration of hypocretin-1 was only about 200 nM after diffusion. Puff application of aCSF (without hypocretin-1) with a similar duration did not influence the membrane potential of the recorded neurons. Protein kinase A (PKA) inhibitor (5–24) (1 μM) was added to the standard internal pipette solution. Drugs used to directly inhibit or stimulate cytosolic signaling molecules are membrane permeable. Neurons

were required to maintain a stable baseline for at least 2 min before application of the test agents.

A response to hypocretin-1 was arbitrarily defined as a sustained change in membrane potential of >5 mV. All values are given as the mean $\pm$ SEM. The number of neurons examined is given in parentheses. Statistical comparisons were made using the unpaired or paired Student's *t*-test, as appropriate. A minimum *p*-value of <0.05 was selected to determine significance.

RESULTS

Acutely isolated prefrontal pyramidal neurons were readily distinguished by their distinct morphological features similarly described previously [20]: a pyramidal-shaped soma (≥ 20 μm diameter) and a prominent apical dendrite (50–100 μm) (Fig. 1b). All recordings were restricted to the soma of the identified cells with such typical morphological properties. Whole-cell recordings were obtained from a total of 79 prefrontal pyramidal cells. Resting membrane potential and input resistance measured at the soma were -59.7 ± 4.4 mV and 2.0 ± 0.4 MΩ, respectively. All of the tested cells generated action potentials with a mean amplitude of 73.7 ± 6.2 mV, and spontaneous activities, especially, were observed in 57.9% of the cells during long current-clamp recordings.

Puff application of 10 μM hypocretin-1 resulted in rapid sustained depolarization (29.1 ± 10.9 mV, $n=20/22$), accompanied by a rapid increase in firing frequency of action potentials (Fig. 1c). After washout of hypocretin-1 with aCSF, both the membrane potential and action potential frequency recovered to the control level. In voltage-clamp experiments, application of 10 μM hypocretin-1 also evoked inward currents in all neurons tested (19.4 ± 11.2 pA; $n=14/14$). In addition, these responses were repeatable because the same cell responded to additional application of 10 μM hypocretin-1 (Fig. 1d). In current-clamp experiments, the number of evoked action potentials in response to current pulses increased after quick application of 10 μM hypocretin-1. After washout of hypocretin-1 with aCSF, the number of evoked action potentials returned to the control level ($n=8/8$) (Fig. 1e). Together, these data indicate that hypocretin-1 exerts a direct excitatory effect on prefrontal pyramidal neurons.

We next determined whether the excitatory effects of hypocretin-1 on prefrontal pyramidal neurons depend on PLC. As indicated in Figs 2a and 3, application of a PLC inhibitor D609 (10 μM) obviously prevented 10 μM hypocretin-1-induced depolarizations of the recorded neurons (0.7 ± 0.5 mV, $n=9/9$; $p<0.01$). Thus, these results confirm that PLC is involved in mediating the depolarizing effects of hypocretin-1 on prefrontal cortical neurons.

Moreover, we investigated the effects of a specific PKC inhibitor, bisindolylmaleimide II (BIS-II) (1 μM), on the hypocretin-1-induced depolarization of prefrontal pyramidal neurons. After preincubation with BIS-II for 1 h, hypocretin-1 (10 μM) did not increase the membrane potential and frequency of action potentials. As illustrated in Figs 2b and 3, the depolarizations of neurons caused by hypocretin-1 and hypocretin-1 with BIS-II (1.1 ± 0.3 mV; $n=11/11$) are significantly different ($p<0.01$). These data suggest that the excitations of prefrontal pyramidal neurons induced by hypocretin-1 are dependent on PKC.

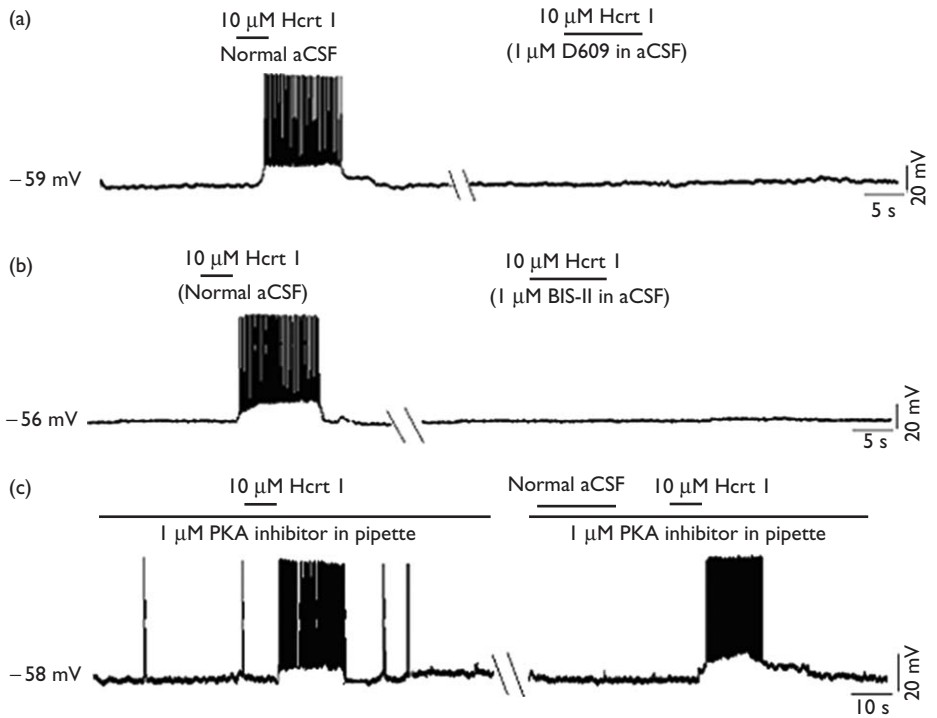

Fig. 2. Signaling mechanisms of excitatory effects of hypocretin-1 (Hcrt 1) on prefrontal pyramidal neurons. (**a**) Whole-cell recording from a prefrontal neuron with D609 in artificial cerebrospinal fluid (aCSF) indicated that application of hypocretin-1 did not depolarize this cell. (**b**) Preincubation of bisindolylmaleimide II (BIS-II) for 1 h, spontaneous firing activities remained unchanged before and during application of hypocretin-1. (**c**) When the pipette contained protein kinase A (PKA) inhibitor, hypocretin-1 evoked depolarizing response in a recorded neuron, and the response was reversible. In contrast, normal aCSF had no effect on the same cell.

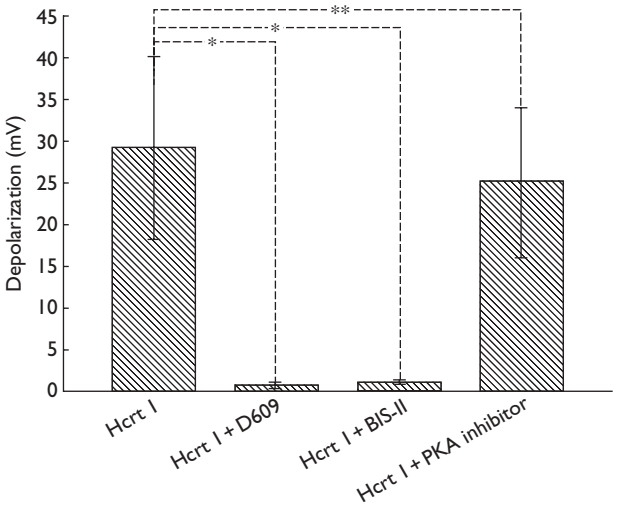

Fig. 3. This bar graph illustrates mean depolarization of prefrontal cortical neurons in response to 10 μM hypocretin-1 (Hcrt 1) (mean depolarizations, 29.1 ± 10.9 mV, n=20/22), 10 μM hypocretin-1 with 10 μM D609 in aCSF, 10 μM hypocretin-1 with 1 μM BIS-II in aCSF, and 10 μM hypocretin-1 recorded in the presence of 1 μM protein kinase A (PKA) inhibitor peptide (5–24). Error bars indicate SE. *$p < 0.05$, **$p > 0.05$.

As shown in Figs 2c and 3, recording with 1 μM PKA inhibitor peptide in internal solution illuminated the fact that 10 μM hypocretin-1 still depolarized the prefrontal pyramidal neurons. The mean depolarizations (n=14/15; 25.0 ± 8.9 mV) of the tested neurons induced by hypocretin-1 with PKA inhibitor are not markedly different from those induced by 10 μM hypocretin-1 ($p > 0.05$). These observations show that PKA is not associated with the depolarizations of prefrontal cortical neurons induced by hypocretin-1.

DISCUSSION

In the present study, the results confirm that hypocretin-1 has a direct postsynaptic excitatory effect on rat prefrontal cortical pyramidal neurons. Also, hypocretin-2 shows a presynaptic excitatory effect on these neurons [14]. Thus, we conclude that hypocretins can excite the prefrontal cortex not only by a presynaptic action but also by a direct postsynaptic action. In addition, Bayer *et al.* [10] demonstrate that hypocretins have a direct excitatory effect on primary somatosensory cortical neurons. Altogether, these data emphasize that the hypocretin system may play a powerful role in the regulation of arousal state by a direct excitatory action on the final target of arousal systems – the cerebral cortex – in parallel with its excitation of the multiple subcortical arousal regions.

Furthermore, we are interested in the signaling mechanisms responsible for the excitatory effects of hypocretin-1 on prefrontal pyramidal neurons. The biological actions of hypocretins are transduced via two hypocretin receptors, which belong to the G-protein-coupled receptor family [2]. Up to now, much attention has been paid to the identification of ionic basis involved in hypocretin-induced excitability via G-protein-coupled receptors. Only limited information exists, however, about the signaling mechanisms triggering the multiple ionic mechanisms. Yang *et al.* [19] reported that the excitatory effects of hypocretin-1 on nucleus tractus solitarius neurons are mediated through the activation of PLC and PKC pathways. Xu *et al.* [21] demonstrated that hypocretins increase the L-type Ca^{2+} current via a PKC-mediated pathway in ovine somatotropes. Despite this, the possible signaling mechanisms underlying the hypocretin-1-induced depolarization of prefrontal cortical neurons are not understood. In this study, we provide direct evidence that the excitatory effects of hypocretin-1 on prefrontal pyramidal neurons are mediated by activation of PLC and PKC pathways, but not by PKA. Notably, the signaling pathways mediating the hypocretin-1-induced excitation of prefrontal cortical neurons are consistent with those in nucleus tractus solitarius neurons. In all, the excitatory actions of hypocretin on different systems appear to share a common intracellular signaling mechanism. Additionally, it has been well established that the hypocretin receptor couples to a G_q protein [2]. Activation of G_q triggers the stimulation of PLC, which can activate PKC [22]. Thus, we can suppose that the working model of these two signaling molecules is that the stimulation of G_q by the binding of hypocretin-1 to the hypocretin receptors on the cortical neurons causes the activation of PLC, which triggers the activation of PKC. Our studies, however, only lead to a partial understanding of the initial steps of intracellular signaling cascades, and the possible ionic mechanisms underlying the excitatory actions of hypocretin on cortical neurons mediated by PLC and PKC signaling molecules need to be further investigated.

CONCLUSION

The chief finding of this study is that hypocretin-1 has a direct postsynaptic excitatory effect on prefrontal pyramidal neurons, which is mediated by activation of the PLC and PKC signaling pathways.

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