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Modulation of T-type Ca^{2+} channels by corticotropin-releasing factor through protein kinase C pathway in MN9D dopaminergic cells

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Abstract

Corticotrophin-releasing factor (CRF) is the main regulator of the body's stress axis and its signal is translated through G-protein-coupled CRF receptors (CRF-R1, CRF-R2). Even though CRF receptors are present in the midbrain dopamine neurons, the cellular mechanism of CRF action is not clear yet. Since voltage-dependent Ca^{2+} channels are highly expressed and important in dopamine neuronal functions, we tested the effect of CRF on voltage-dependent Ca^{2+} channels in MN9D cells, a model of dopamine neurons. The application of CRF-related peptide, urocortin 1, reversibly inhibited T-type Ca^{2+} currents, which was a major Ca^{2+} channel in the cells. The effect of urocortin was abolished by specific CRF-R1 antagonist and was mimicked by protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate. PKC inhibitors abolished the effect of urocortin. These results suggest that urocortin modulates T-type Ca^{2+} channel by interacting with CRF-R1 via the activation of PKC signal pathway in MN9D cells.

Keywords: Corticotrophin-releasing factor; Dopamine neuron; Voltage-dependent Ca^{2+} channel; MN9D cells; Urocortin; PKC; T-type Ca^{2+} channel; Phorbol 12-myristate 13-acetate

Corticotrophin-releasing factor (CRF) is a 41 amino-acid long neuropeptide hormone. It has been recognized as an important regulator of stress responses utilizing the hypothalamic-pituitary-adrenal axis in mammalian species [1,2]. Urocortin 1, a CRF family peptide, shows potent effects on CRF receptors. Recently, two other urocortins (urocortin 2 and urocortin 3) are identified in human genome database as well as in mouse genomic DNA [3]. CRF and its analogs act by binding to two subtypes of receptors, CRF-R1 and CRF-R2, which belong to the secretin-like family of G protein-coupled receptors [4,5]. CRF has higher affinity for CRF-R1 than CRF-R2 [6], while urocortin 1 has high affinities both for CRF-R1 and CRF-R2 [3]. Both of urocortin 2 and urocortin 3 are highly selective for CRF-R2, with little or no affinity for CRF-R1 [7]. CRF receptors are widely expressed in brain regions associated with the control of emotive processing and hormone regulation, as well as in regions centrally involved in drug abuse [8–11]. In dopamine neuron, CRF plays a key role in the modulation of drug addiction by stress [10–13]. However, the mechanism of CRF action on dopamine neuron is not clear yet. To elucidate the cellular signaling of CRF, we used MN9D cell as a model for dopamine neuron. MN9D cells are a fusion of embryonic ventral mesencephalic and neuroblastoma cells, which contains large amounts of dopamine and tyrosine hydroxylase [14].

Ca^{2+} channels have been traditionally classified into high voltage-activated (HVA) and low voltage-activated (LVA) subtypes [15]. LVA Ca^{2+} channels, also known as T-type, show more negative range of activation and inactivation, rapid inactivation, slow deactivation, and smaller single channel conductance. T-type Ca^{2+} channels are present in a variety of cell types where they appear to

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mediate low threshold spikes and rebound burst firing patterns, pacemaker activity, hormone secretion, cell growth, proliferation, and fertilization [16–19]. Ni^{2+} and amiloride are often described as effective blockers for T-type channels in many native cells, although the effect of these agents is quite variable in different cell types [16,20]. When we recorded voltage-activated Ca^{2+} current from MN9D cells using patch clamp method, majority of Ca^{2+} current was blocked by Ni^{2+} and amiloride, and showed low-threshold activation, indicating that the currents were carried by T-type Ca^{2+} channels. When urocortin 1 was applied, Ca^{2+} currents were reversibly inhibited. The effect of urocortin was abolished by 100 nM astressin, CRF-R antagonist, and by 100 nM antalarmin, a specific CRF-R1 antagonist. The effect of urocortin was blocked in the presence of protein kinase C (PKC) inhibitor, chelerythrine, while PMA mimicked the effect of urocortin. These results suggest that activation of CRF-R1 in dopamine neurons inhibits T-type Ca^{2+} channel via the activation for PKC signal pathway, thereby, may regulate burst firing patterns, pacemaker activity, and secretion of dopamine in dopamine neurons.

Materials and methods

All chemicals were purchased from Sigma unless indicated otherwise.

Cell culture. MN9D cells are a generous gift from Dr. A. Heller (University of Chicago, USA). MN9D cells were plated at a density of 2×10^5 cells on poly-D-lysine-coated plates. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 3.7 g/L $NaHCO_3$, and 1% penicillin/streptomycin (Gibco) for 3 days in an incubator with an atmosphere of 10% CO_2 at 37 °C. Differentiation of MN9D cells was performed by exposing cells to 1 mM butyric acid for more than 6 days. Medium was replaced every 48 h with fresh media.

Immunochemistry. Both undifferentiated and differentiated MN9D cells were plated on glass cover. Cells on glass coverslip were washed with phosphate based saline (PBS) buffer and fixed in 2.5% paraformaldehyde for 10 min at room temperature. Then cells were washed two times with PBS buffer. To permeate, MN9D cells were incubated with 0.1% Triton X-100 for 30 min and 5% BSA was added in PBS for 1 h. Cells were incubated with CRF receptor antibody (Santa Cruze) and tyrosine hydroxylase (TH, Chemicon) antibody for overnight at 4 °C and washed out three times with PBS buffer. CRF receptor was detected by anti-goat conjugated rhodamine (Molecular probe) and TH was detected by anti-rabbit-conjugated FITC (Molecular probe).

Electrophysiology. Voltage-dependent Ca^{2+} currents were recorded using whole-cell mode. The experiments were performed at 20–22 °C. The currents were filtered at 5 kHz, and recorded using an Axonpatch-200B amplifier (Axon Instruments). Patch pipettes were pulled from borosilicate glass (MTM 150F-4 from WPI) and fire polished. To ensure the voltage clamp quality, electrode resistance was kept from 2.5 MΩ to 3.5 MΩ. Eliciting Ca^{2+} current was done by using the voltage protocol, which consisted of 10 mV step pulses of 150-ms long from −80 mV to +40 mV from a holding voltage of −80 mV. Commend potentials and data acquisition were controlled by pClamp 7.0 software (Axon instrument). To measure Ca^{2+} currents, barium was used as a charge carrier to prevent calcium induced inactivation of the channel. For Ca^{2+} current recordings the bath solution (external solution) contained (in mM) 15 $BaCl_2$, 106 TEA-Cl, 10 Hepes, 1 $MgCl_2$, 5 KCl, and 19 Glucose (pH was adjusted to 7.2 with BaOH). Pipette solution (internal solution) contained (in mM) 140 CsCl, 2 $MgCl_2$, 10 Hepes, 2 Na–ATP, and 1 Na–GTP (pH was adjusted to 7.4 with CsOH).

Data analysis. Analysis of data was performed using Clamfit 7.0 (Axon Instruments). To analyze the inhibition of Ca^{2+} currents via urocortin, peak value of the obtained currents from −10 mV was used.

Results and discussion

To investigate the cellular mechanism of CRF action in dopamine neurons, we utilized MN9D cells as a model for dopamine neurons. It is recently reported that both undifferentiated and differentiated MN9D cells express several different kinds of voltage-activated channels [21]. When differentiation was induced by treating MN9D cells with 1 mM butyric acid for more than 6 days, they showed neurite formation and exhibited hyperpolarization-activated I_h currents similar to native dopamine neurons (data not shown). MN9D cells also expressed voltage-activated Ca^{2+} currents as shown in Fig. 1A. Ca^{2+} current densities were not different between undifferentiated and differentiated cells. To separate low voltage-activated Ca^{2+} current (LVA) and high voltage-activated Ca^{2+} current (HVA), we used double-pulse protocol as shown in the bottom of Fig. 1B. Cells were held at −80 mV, and total voltage-activated Ca^{2+} currents (LVA + HVA) were elicited by the first voltage pulse to −10 mV, which was followed by a holding voltage at −50 mV for 200 ms. Then, only HVA channels were activated by the second voltage pulse to −10 mV. Most of MN9D cells, either undifferentiated or differentiated, exhibited significant fast-inactivating Ca^{2+} currents by the first voltage pulse, while very small currents were elicited by the second voltage pulse. This result may indicate that most of Ca^{2+} channels in MN9D cells are LVA. We tested the effect of 50 μM Ni^{2+}, which is a selective blocker for some of T-type Ca^{2+} channels [16,20]. As shown in Fig. 1B, large part of fast-inactivating Ca^{2+} currents elicited by the first voltage pulse were blocked by Ni^{2+}, while Ca^{2+} currents elicited by the second voltage pulse was not blocked. The time-dependent effect of Ni^{2+} on the fast-inactivating Ca^{2+} currents is plotted in Fig. 1C, where Ni^{2+} inhibited the current by $69.4 \pm 3.8\%$ ($n = 10$). The inhibitory effect of Ni^{2+} was reversible, excluding the possibility of run-down of the current. The fast-inactivating Ca^{2+} currents were also sensitive to 500 μM amiloride, a T-type Ca^{2+} channel blocker (Fig. 1D; $68.5 \pm 4.5\%$, $n = 5$). We also observed that 10 μM mibefradil, which is a selective blocker for low-threshold Ca^{2+} channel [22], blocked the fast-inactivating current by $69.3 \pm 6.1\%$ (Fig. 1D; $n = 8$). However, the fast-inactivating Ca^{2+} currents were not blocked by 10 μM nifedipine, a specific L-type Ca^{2+} channel blocker (data not shown). These results confirm that most of Ca^{2+} channels in MN9D cells are LVA T-type.

Since intracellular Ca^{2+} controls a multitude of neuronal process, ranging from membrane excitability to plasticity and formation of synapses [23], we tested whether CRF might be involved in the regulation of Ca^{2+} channels in MN9D cells. First, we used antibody staining to identify the expression of CRF receptors in MN9D cells. Left-top