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Differences in acid-induced currents between oxytocin-mRFP1 and vasopressin-eGFP neurons isolated from the supraoptic and paraventricular nuclei of transgenic rats

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HIGHLIGHTS

- The differences between OXT-mRFP1 neurons and AVP-eGFP neurons in response to acid.
- Acid-induced currents were identified in isolated rat SON and PVN MNCs.
- The currents in the OXT-mRFP1 neurons were smaller than those in the AVP-eGFP neurons.
- The acid sensitivity of OXT neurons may be lower than that of AVP neurons.

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ABSTRACT

The hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN) consists of two types of magnocellular neurosecretory cells, oxytocin (OXT) and arginine vasopressin (AVP). We generated and characterized rats that express an OXT-monomeric red fluorescent protein 1 (mRFP1) and an AVP-enhanced green fluorescent protein (eGFP) fusion transgene. These transgenic rats enable the visualization of OXT or AVP neurons. Taking advantage of this, we examined the differences between OXT-mRFP1 neurons and AVP-eGFP neurons in response to acid. Acid-sensing ion channels (ASICs) are neuronal voltage-insensitive cationic channels that are activated by extracellular acidification. Although functional ASICs have been identified in AVP neurons, differences in acid-induced currents between OXT and AVP neurons in SON have not been reported. In the present study, we used the whole-cell patch-clamp technique to investigate differences between OXT-mRFP1 neurons and AVP-eGFP neurons reaction to acid in SON and PVN. In voltage clamp mode, lowering extracellular pH evoked inward currents in both OXT-mRFP1 neurons and AVP-eGFP neurons. In our findings, the acid-induced currents in the OXT-mRFP1 neurons were significantly smaller than those in the AVP-eGFP neurons. These acid-induced currents were inhibited by amiloride, a known blocker of ASICs. Further, to compare the response to acid between OXT-mRFP1 and AVP-eGFP neurons in the same transgenic rat, we used a double transgenic rat by mating an OXT-mRFP1 transgenic rat with an AVP-eGFP transgenic rat. The acid-induced currents of OXT-mRFP1 neurons were significantly smaller than those of AVP-eGFP neurons from the double transgenic rats. These currents were almost completely inhibited by amiloride. The difference of acid-sensitivity between OXT and AVP neurons might contribute to maintaining systematic order in hypothalamic function.

Oxytocin (OXT) and arginine vasopressin (AVP) are synthesized and secreted from magnocellular neurosecretory cells (MNCs) in the supraoptic nucleus (SON) and in the paraventricular nucleus (PVN) [1]. AVP plays a role in water homeostasis, cardiovascular regulation, controls osmolality, and blood pressure. In recent reports, the centrally released AVP has also been associated with regulation of circadian rhythm and/or social behavior [2,3]. The role

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of OXT in lactation and parturition has been well characterized. In addition, centrally released OXT is involved in maternal bonding, sexual behavior, and social affiliation [4–7].

Acid-sensing ion channels (ASICs) are widely expressed in the peripheral and central nervous system where they perform physiological functions [8]. The ASICs are voltage-insensitive cation channels that are activated by extracellular acidification.

Our recent electrophysiological and biochemical studies showed that functional ASICs are expressed in rat SON AVP neurons identified by the fluorescence generated from an enhanced green fluorescent protein (eGFP) fusion gene in the AVP-producing neurons [9,10]. However, the acid sensitivity of SON OXT neurons has not been assessed until now. In the present study, we used a whole-cell patch-clamp technique to identify the acid-mediated currents in isolated rat hypothalamic MNCs that express an OXT-monomeric red fluorescent protein 1 (mRFP1) transgene [11] and/or an AVP-eGFP transgene. Interestingly, acid-induced currents in the OXT-mRFP1 neurons were significantly smaller than those in the AVP-eGFP neurons.

All preparations and electrophysiological experiments were performed as previously reported [12]. Experiments were performed on transgenic rats at 3–5 weeks of age (young adults, weighing 80–150 g). These transgenic rats express chimeric OXT-mRFP1 [11] and/or AVP-eGFP gene products [9]. The animals were housed in standard plastic cages at 23–25 °C on a 12-h light (07.00–19.00 h)–12-h dark cycle. All experiments in this study were carried out in accordance with the guidelines of the Physiological Society of Japan under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

A modified Krebs–Henseleit solution (KHB) was used in the experimental steps where SON slice preparations were manipulated; the KHB solution contained: 124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 2 mM $CaCl_2$, 25.9 mM $NaHCO_3$ and 10 mM glucose. The solution was continuously oxygenated with a mixture of 95% O_2–5% CO_2. In the experiments with dissociated neurons, HEPES buffered solution (HBS) was used: 140 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH. This solution was continuously oxygenated with 100% O_2, and the osmolarity was adjusted to 300 ± 5 mosmol l^{-1} with mannitol. The pipette solution used in the recording electrodes contained 140 mM potassium gluconate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP, adjusted to pH 7.3 with Tris base. Acid solutions were buffered using a combination of 10 mM HEPES and 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (Sigma).

Young adult male transgenic rats were euthanized by decapitation. The brains were quickly removed and cooled in KHB at 4 °C for 1 min. A block containing the hypothalamus was cut from the brain and glued onto the stage of a vibrating blade tissue slicer (Linearslicer Pro 7, DSK, Kyoto, Japan). After removal of the meninges, 500 μm coronal slices containing the SON and PVN were sectioned in KHB at 4 °C. SON slices were excised from the coronal brain sections using a circular tissue puncher (inner diameter 1.8 mm) and then incubated in KHB at room temperature (22–24 °C) for 1 h before enzyme digestion.

Neurons were dissociated by an enzymatic digestion method [13]. Briefly, slices were incubated in 4 ml HBS containing DNase I (Sigma, 0.5 mg ml^{-1}) and papain (20 U ml^{-1}; Worthington Biochemical Corp., Lakewood, NJ, USA) in a 50 ml centrifuge tube containing 100% O_2 for 50 min at 30 °C. The slices were then transferred to normal HBS and washed for at least 1 h prior to mechanical dissociation by trituration with fire-polished glass pipettes. The cell suspension was plated onto coverslips individually placed into the wells of multiwell culture plates with 2 ml of culture medium and maintained in a humidified incubator at 37 °C with 5% CO_2 for 1 day. The culture medium was Neurobasal-A medium (Invitrogen) supplemented with 0.5 mM L-glutamine, B27 (Invitrogen), 5 ng ml^{-1} fibroblast growth factor (Sigma), and penicillin–streptomycin (Invitrogen). The osmolarity of the culture medium was adjusted to 300 ± 5 mosmol l^{-1} with NaCl.

The coverslip with the plated neurons was placed in a glass-bottomed chamber and continuously perfused with HBS at a rate of 1.5 ml min^{-1} using an eight-head peristaltic pump (MP-8, Gilson nucleus, Villiers le Bel, France). The volume of the recording chamber was 1 ml. The solution level was kept constant by a low-pressure aspiration system. Isolated MNCs expressing OXT-mRFP1 and AVP-eGFP were identified by their red and green fluorescence, respectively. The electrodes used in this study were triple-pulled from a glass capillary with a puller (P-87, Sutter Instrument Co., Novato, CA, USA). The pipettes had a final resistance of 5–8 MΩ when filled. An Axopatch 200B amplifier (Axon Instruments) was used to record ionic currents in the tight-seal, whole-cell configuration. Acidic-solution and the non-specific ASIC blocker, amiloride (Sigma–Aldrich, St. Louis, MO), were pressure-applied via a Y-tube system [14], which is a rapid drug exchange technique that allows the external solution surrounding a neuron to be exchanged within 20 ms. The Y-tube tip was positioned 1.5 mm from the recording electrode in the cell body. Membrane voltages were controlled and recorded with a computer running pCLAMP10.3 software (Molecular Devices, Sunnyvale, CA, USA). Recording commenced at least 3 min after membrane rupture when the currents reached a steady state. To avoid current variances due to differences in cell size, current density (pA/picofarads; pF) was set as peak current amplitude (pA) divided by membrane capacitance (pF) from the same cell [15]. Recordings included in the data analysis were collected during periods of stable series resistance. All experiments were carried out at room temperature (22–24 °C).

Results are expressed as the mean ± S.E.M. Statistical comparisons were performed with Student's *t*-test or Welch's *t*-test. In all cases, $p < 0.05$ was considered significant.

When neurons were held at −70 mV, fast reduction of the extracellular pH from 7.4 to 5.5 induced rapidly desensitizing inward currents in both SON OXT neurons and SON AVP neurons. Peak amplitude of current induced by pH 5.5 in the OXT-mRFP1 neurons was 8.79 ± 2.2 pA/pF ($n = 18$, Fig. 1a and b). On the other hand, peak amplitude of current induced by pH 5.5 in the AVP-eGFP neurons was 73.1 ± 7.6 pA/pF ($n = 10$, Fig. 1c and d, $p < 0.0001$ vs. OXT neurons). Co-application of 10 μM amiloride significantly reduced pH 5.5-induced current to 2.25 ± 0.62 pA/pF ($p = 0.0029$) and 14.1 ± 3.9 pA/pF ($p < 0.0001$) in OXT-mRFP1 neurons and AVP-eGFP neurons, respectively (Fig. 1). Similar results have been observed in double transgenic rats by mating an OXT-mRFP1 transgenic rat with an AVP-eGFP transgenic rat (Fig. 2a and b). That is, the peak amplitude of current induced by pH 5.5 in the OXT-mRFP1 neurons and AVP-eGFP neurons were 9.72 ± 2.1 pA/pF ($n = 20$) and 53.6 ± 5.5 pA/pF ($n = 21$), respectively ($p < 0.0001$, Fig. 2c–e). Application of 10 μM amiloride significantly reduced pH 5.5-induced current to 2.94 ± 0.56 pA/pF ($p = 0.0008$) and 8.59 ± 1.5 pA/pF ($p < 0.0001$) in OXT-mRFP1 and AVP-eGFP neurons, respectively (Fig. 2c–e). We confirmed the reaction to acidic condition in the magnocellular PVN neurons with the same experimental manner of the SON neurons. As a result, the peak amplitude of current induced by pH 5.5 in the OXT-mRFP1 neurons and AVP-eGFP neurons were 16.2 ± 3.6 pA/pF ($n = 4$) and 64.8 ± 10 pA/pF ($n = 4$), respectively ($p = 0.0045$). Application of 10 μM amiloride significantly reduced pH 5.5-induced current to 4.86 ± 1.5 pA/pF ($p = 0.0272$) and 8.56 ± 1.8 pA/pF ($p = 0.0129$) in OXT-mRFP1 and AVP-eGFP neurons, respectively (Fig. 2f).

Furthermore, to examine whether the other acid evoked the similar inward currents in both SON OXT neurons and SON AVP neurons, we have been observed the reaction to the other acid