Neuronal actions of oxytocin on the subfornical organ of male rats

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1Department of Physiology, School of Allied Health Sciences, Osaka University Faculty of Medicine, Suita, Osaka 565–0871, Japan; and 2Max-Planck-Institut für Physiologische und Klinische Forschung, W. G. Kerckhoff-Institut, D-61231 Bad Nauheim, Germany

Hosono, Takayoshi, Herbert A. Schmid, Kazuyuki Kanosue, and Eckhart Simon. Neuronal actions of oxytocin on the subfornical organ of male rats. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E1004–E1008, 1999.—The aim of this study was to investigate effects of oxytocin (OT) on electrical neuronal activities in rat subfornical organ (SFO) and compare its action with the well-described excitatory effects of blood-borne angiotensin II (ANG II) on the same SFO neurons. With the use of extracellular recordings from spontaneously active neurons in slice preparations of the SFO of male rats, 11.7% of tested neurons (n = 206) were excited and 9.7% were inhibited by superfusion with 10−6 M OT. Both excitatory and inhibitory effects of OT were dose dependent with similar threshold concentrations and were blocked by a specific OT-receptor antagonist but not by a vasopressin receptor antagonist. Blocking synaptic transmission with low calcium medium suppressed only inhibitory effects of OT. All but one of the OT-sensitive neurons were also excited by superfusion with ANG II at a concentration much lower than required for OT, suggesting that synaptically released OT rather than blood-borne OT alters the activity of SFO neurons in vivo. The results support the hypothesis that neurally released OT may modulate SFO-mediated functions by acting on OT-sensitive neurons.

angiotensin II; vasopressin; osmoregulation; extracellular recording

THE SUBFORNICAL ORGAN (SFO) is one of the circumventricular organs lacking the blood-brain barrier and has been implicated as a key structure for osmoregulation, especially in the induction of thirst elicited by circulating angiotensin II (ANG II). Direct injection of ANG II into the SFO causes thirst and salt appetite in rats, and these responses are absent after the SFO is lesioned (25). Electrical stimulation of the SFO also induces thirst (26) and causes the release of arginine vasopressin (AVP) and oxytocin (OT) from the neurohypophysis (27). Additional functions of the SFO have been suggested recently by showing that calcitonin, a peptide primarily involved in calcium homeostasis, strongly activates SFO neurons (23). These SFO-mediated functions are differently modified by many blood-borne peptides as well as by brain intrinsic hormones released locally as neurotransmitters (1, 8, 21, 24).

OT, which in the periphery facilitates milk letdown and stimulates uterine contractions in females, affects various regulatory behaviors that are centrally mediated in both sexes (18). As for dipsogenic behavior, intracerebroventricular injection of OT has been shown to inhibit water intake (2). Such injections also reduce salt appetite induced by ANG II (5) in both male and female animals (4), and treatments that inhibit salt appetite, such as hyperosmolality, stimulate central and pituitary release of OT (27). However, the exact sites of OT action for these responses are not yet clarified (12). The fact that oxytocinergic fibers are found in the SFO (7) suggests that OT may affect salt and water intake by acting on the SFO. The aim of this study is to investigate the effects of OT in comparison with those of ANG II on identical neurons of the rat SFO as a first step to clarify whether OT might affect SFO-mediated functions at this level.

METHODS

In this experiment, 65 adult male Wistar rats (190–250 g) were used to avoid possible effects of estrous cycles in females. The materials and methods are the same, with minor modifications, as previously described (23). Briefly, rats were decapitated, and their brains were quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF). The brain was trimmed to a square block containing the entire hypothalamus. A slice of the body of the fornix containing the entire SFO was cut by hand and preincubated in aCSF at 35°C for 2 h. The average thickness of the slices was ~500 μm. The slice was transferred to a gilded recording chamber made from brass and was fixed to its bottom with a small metal weight. It contained a fluid volume of 0.7 ml when perfused with aCSF. During recordings, the temperature in the chamber was kept constant at 37°C by means of a Peltier element. The chamber was perfused at a rate of 1.6 ml/min with aCSF. The SFO could be easily identified by its yellowish protrusion into the third ventricle and the blood vessels lining the organ on both sides. Extracellular recordings were made from SFO neurons with glass-coated platinum-iridium electrodes. The recorded action potentials were amplified and displayed on a storage oscilloscope (Gould, Valley View, OH) and were analyzed with custom-made software (Spike2, Cambridge Electronic Design, Cambridge, UK) on a personal computer after passing through a window discriminator (World Precision Instruments). Once a stable recording from a single neuron had been established, its responsiveness was tested by switching to the perfusion solution containing the drug under consideration. From the continuous recorded rate meter counts, the average discharge rate of each neuron was evaluated for 60 s before the stimulus. According to previous reports (1, 21), a neuron was considered sensitive to the applied substance if the average change of discharge rates during the entire response time was larger than ±20% and at least ±0.5 spikes/s. The aCSF was equilibrated with 95% O2-5% CO2 and had the following composition (in mM): 124...
NaCl, 5 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 1.2 CaCl₂, 26 NaHCO₃, 10 glucose, pH 7.4, with an osmolality of 290 mosmol/kg. The aCSF used for synaptic blockade contained less CaCl₂ (0.3 mM) and more MgSO₄ (9.0 mM).

OT, ANG II, and AVP (all from Sigma, Deisenhofen, Germany) were stored in concentrations of 10⁻⁴ or 10⁻¹ M at −25°C in aliquots of 100 µl and were diluted by the aCSF shortly before the application. Usually, 10 ml of the aCSF containing the drug were superfused per stimulus, and the volume, and thus the superfusion time, was reduced when clearly sensitive responses occurred early after the start of the drug perfusion. To block oxytocinergic responses, 20 ml of solutions containing OT-receptor antagonist OT-A; (d(CH₂)₅ securities, 10⁻² M to 10⁻¹ M, and concentrations of 10⁻² and 10⁻¹ M were used normally to test the responsiveness of a neuron. The concentration of the OTR-A and MC was 10 times higher than the coapplied OT concentration. ANG II responsiveness was tested with 10⁻⁸ and 10⁻⁷ M. Drugs were applied in the order from low to high concentration. Neurons were regarded as OT sensitive when 10⁻⁶ M or lower concentrations of OT caused responses fulfilling the above criteria. In the case of ANG II, we used the responses to 10⁻⁷ M to determine the sensitivity of a neuron (24).

Average values of the parameters are presented as means ± SE. Statistical significance was estimated by paired t-test and χ² test at a level of P < 0.05 for the null hypothesis.

**RESULTS**

We tested 206 neurons for responsiveness to OT. The mean spontaneous firing rate of all neurons was 5.5 ± 0.2 spikes/s, which coincided with previous reports (1, 20). Among the tested neurons, 21.4% (n = 44) were OT sensitive; 11.7% (n = 24) were excited by OT application (Fig. 1A) and 9.7% (n = 20) were inhibited (Fig. 1B). Both excitatory and inhibitory responses showed similar dose dependence, and threshold concentrations ranged between 10⁻⁷ M and 10⁻⁶ M OT (Fig. 1, insets). Neurons that were excited by OT increased their firing rate by 1.3 ± 0.3 spikes/s, and those that were inhibited decreased their firing rate by −1.6 ± 0.3 spikes/s in response to 10⁻⁶ M OT. The absolute values of discharge rates of the neurons excited or inhibited by OT were not significantly different. The average latency from OT application to the onset of the response was 68 ± 18 s and did not differ between excitatory and inhibitory responses.

The excitatory responses to OT persisted during perfusion of low Ca²⁺ and high Mg²⁺ solution to block synaptic transmission in each of five neurons tested (Fig. 2A). In contrast, the inhibitory responses to OT disappeared during synaptic blockade in four of five neurons tested (Fig. 2B). In the low calcium medium, the fraction of neurons in which the change in discharge rate in response to OT application did not exceed ±0.5 spikes/s (Fig. 2, insets) was significantly larger in OT-inhibited than OT-excited neurons (χ² = 6.667, P < 0.01). Synaptic blockade with the low calcium medium changed the spontaneous activity in 9 out of 10 neurons; spontaneous activity was increased in six (by 49 ± 19%) and decreased in three neurons (by −59 ± 29%). These increases or decreases in spontaneous activity were not correlated with an excitation or inhibition by OT.

Coapplication of OT-A blocked both excitatory and inhibitory effects of OT in each of the 10 neurons tested, 5 of which were excited and 5 of which were inhibited by OT. In the presence of OT-A 10-fold in excess of OT, the excitatory responses to OT were reduced from 1.2 ± 0.2 to 0.1 ± 0.1 spikes/s (P < 0.05). Likewise, the inhibitory response to OT was reduced from −1.6 ± 0.6 spikes/s to −0.3 ± 0.2 spikes/s during superfusion with OT-A (P < 0.05). In 13 OT-sensitive neurons, 10 were also sensitive to AVP; four of them were excited (Fig. 3A) and three of them were inhibited by both OT and AVP.
AVP, one was excited by OT and inhibited by AVP, and two were inhibited by OT and excited by AVP (Fig. 3B). Coapplications of MC 10-fold in excess of OT did not block the response to OT of five neurons tested; two were excited and three were inhibited. In five AVP-responsive neurons, OTR-A did not block the response, which was, however, blocked by the potent AVP-receptor antagonist (MC). Therefore, the responses of SFO neurons to OT are mediated by OT-specific receptors. Functional evidence for OT receptors in the SFO has been previously provided by showing that application of OT on cultured SFO neurons increased intracellular Ca2+ detected by fluorescence measurement with fura 2 dye (14). It was also

Fig. 2. Discharge rates of 2 subfornical organ neurons recorded from slice preparations showing responses excited (A) and inhibited (B) by OT influenced by synaptic blockade in low Ca2+ medium. At periods indicated by bars with low Ca2+ and high Mg2+, slices were superfused with solution containing 0.3 mM Ca2+ and 9.0 mM Mg2+. Control medium contained 1.2 mM Ca2+ and 1.3 mM Mg2+. Insets: average changes of discharge rates during entire response time in control medium and low Ca2+ medium; shaded areas, range of discharge rates within ±0.5 spikes/s, beyond which a neuron was regarded as sensitive to OT.

Responsiveness to both ANG II and OT could be successfully tested in 82 neurons (Fig. 4). As in previous reports (23, 24), ANG II caused exclusively excitatory responses in 70.7% of all neurons tested. Table 1 shows that 13 (92.9%) of the 14 OT-sensitive neurons were also ANG II sensitive. The proportion of OT-sensitive neurons was significantly higher in ANG II-sensitive neurons than in ANG II-insensitive neurons \( (\chi^2 = 3.992, P < 0.05) \).

DISCUSSION

This is the first study investigating the effects of OT on neuronal electrical activity in the rat SFO. In this study, 21.4% of all SFO neurons responded to 10^{-6} M OT, with similar fractions being excited and inhibited. Both excitatory and inhibitory responses were dose dependent and reversible with similar threshold concentrations and were blocked with a potent and selective OTR-A, which shows little affinity to AVP (17). The responses to OT were, however, not blocked by the potent AVP-receptor antagonist (MC). Therefore, the responses of SFO neurons to OT are mediated by OT-specific receptors. Functional evidence for OT receptors in the SFO has been previously provided by showing that application of OT on cultured SFO neurons increased intracellular Ca2+ detected by fluorescence measurement with fura 2 dye (14). It was also

Fig. 3. Discharge rates of 2 subfornical organ OT-sensitive neurons recorded in vitro from slice preparations showing effect of arginine vasopressin (AVP), OT-receptor antagonist (OTR-A), and AVP V1-receptor antagonist Manning compound (MC). A: neuron excited by OT. B: neuron inhibited by OT.
reported that OT-receptor mRNA is strongly distributed in the anteroventral third ventricle (AV3V) region of which SFO is a part, without, however, mentioning the SFO explicitly (30), although there are no autoradiographic studies in which OT-binding sites in the SFO have been explicitly reported (3).

In contrast to the uniformly excitatory action of ANG II on a large fraction of SFO neurons (21, 23), OT affected only a minority of SFO neurons exciting or inhibiting similar fractions. However, OT responsiveness was restricted almost exclusively to ANG II-responsive neurons, indicating that the OT receptors are not randomly distributed among the SFO neurons. The fact that only the inhibitory responses could be blocked by low Ca$^{2+}$ and high Mg$^{2+}$ solution suggests that the inhibitory responses were due to local inhibitory synaptic inputs from neurons excited by OT. In this respect, the action of OT resembled that of AVP (1). Although transmitters of the inhibitory synapses have not been determined in this study, $\gamma$-aminobutyric acid was reported to exist in SFO, according to histological evidence (20), and to cause inhibitory responses in SFO neurons, demonstrated by electrophysiological intracellular recordings (11). It has also been reported that nitric oxide synthase exists in the SFO and that nitric oxide acts as an inhibitory messenger in the SFO (21).

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Table 1. Numbers of subfornical organ neurons responsive to ANG II and OT

<table>
<thead>
<tr>
<th>Response to ANG II</th>
<th>Excitation</th>
<th>Inhibition</th>
<th>No response</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation</td>
<td>7</td>
<td>6</td>
<td>45</td>
<td>58</td>
</tr>
<tr>
<td>Inhibition</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No response</td>
<td>0</td>
<td>1</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>7</td>
<td>68</td>
<td>82</td>
</tr>
</tbody>
</table>

ANG II, angiotensin II (10$^{-7}$ M); OT, oxytocin (10$^{-6}$ M).
tricular regions are required, together with electrophysiological studies on neurons of those regions. The SFO neurons responding to OT may also be a part of a neuronal feedback loop between the SFO and PVN because electrical stimulation of the SFO or local application of ANG II stimulates neuronal activity of PVN neurons and OT secretion (6, 8, 22). Lesions of the SFO or disconnections of SFO efferent fibers abolish the effect of the electrical stimulation of the SFO on OT secretion (6). A reciprocal neuronal circuit interconnecting PVN and SFO has also been demonstrated histologically (19). This might include brain intrinsic feedback pathways with OT as a synapticly released neuropeptide, which has also been discussed for AVP (1).

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