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# Oxytocin-induced elevation of ADP-ribosyl cyclase activity, cyclic ADP-ribose or $Ca^{2+}$ concentrations is involved in autoregulation of oxytocin secretion in the hypothalamus and posterior pituitary in male mice

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ABSTRACT

Locally released oxytocin (OT) activates OT receptors (2.1:OXY:1:OT:) in neighboring neurons in the hypothalamus and their terminals in the posterior pituitary, resulting in further OT release, best known in autoregulation occurring during labor or milk ejection in reproductive females. OT also plays a critical role in social behavior of non-reproductive females and even in males in mammals from rodents to humans. Social behavior is disrupted when elevation of free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and OT secretion are reduced in male and female CD38 knockout mice. Therefore, it is interesting to investigate whether ADP-ribosyl cyclase-dependent signaling is involved in OT-induced OT release for social recognition in males, independent from female reproduction, and to determine its molecular mechanism. Here, we report that ADP-ribosyl cyclase activity was increased by OT in crude membrane preparations of the hypothalamus and posterior pituitary in male mice, and that OT elicited an increase in $[Ca^{2+}]_i$ in the isolated terminals over a period of 5 min. The increases in cyclase and $[Ca^{2+}]_i$ were partially inhibited by nonspecific protein kinase inhibitors and a protein kinase C specific inhibitor, calphostin C. Subsequently, OT-induced OT release was also inhibited by calphostin C to levels inhibited by vasotocin, an OT receptor antagonist, and 8-bromo-cADP-ribose. These results demonstrate that OT receptors are functionally coupled to membrane-bound ADP-ribosyl cyclase and/or CD38 and suggest that cADPR-mediated intracellular calcium signaling is involved in autoregulation of OT release, which is sensitive to protein kinase C, in the hypothalamus and neurohypophysis in male mice.



## 1. Introduction

Oxytocin (OT), a peptide of the vasopressin and OT family, plays a critical role in social recognition and behavior in mammals from rodents to humans (Donaldson and Young, 2008; Neumann, 2008). This short polypeptide hormone is secreted dendritically from neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and spread to other areas of the brain (McGregor et al., 2008; Skuse and Gallagher, 2009). Locally released OT in the brain causes excitation of OT neurons by activating OT receptors, a class A family (2.1:OXY:1:OT: in IUPHA data base; http://www.iuphar-db.org/GPCR/ReceptorListForward?class=class%20A), expressed on both neurons of the PVN and SON and nerve-endings in the posterior pituitary (Adan et al., 1995; Freund-Mercier et al., 1994; Young et al., 1997). This excitation leads to facilitative OT release, known as autoregulation (Moos et al., 1984; Neumann et al., 1996). The autoregulation of OT-induced OT release occurs during uterine contraction in labor and milk ejection in lactation (Richard et al., 1991). However, it is not yet clear whether this autoregulation functions during non-productive daily life in males and in non-reproductive females.

OT receptors are seven-transmembrane domain proteins that couple with $G_{q/11}$ or $G_i$ and stimulate the production of inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) through the activation of phospholipase C (PLC) (Gimpl and Fahrenholz, 2001), resulting in activation of $Ca^{2+}$ signals and protein kinase C (PKC). This PLC- and $IP_3$-dependent $Ca^{2+}$ signaling may function in autoregulation (Lambert et al., 1994). On the other hand, another $Ca^{2+}$ signal pathway of cyclic ADP-ribose (cADPR) has recently been demonstrated in many tissues, including the nervous system (Lee, 2001; Higashida et al., 2007b). Intracellular cADPR

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concentrations are known to be regulated in many different ways: in one such mechanism, ADP-ribosyl cyclase or CD38 seems to be coupled directly with neurotransmitter or hormone receptors such as muscarinic acetylcholine or metabotropic glutamate receptors via different G proteins on the membrane surface (Higashida et al., 1997, 1999, 2007a,b); or phosphorylation downstream of the G-protein-coupled receptor signaling pathway (Boittin et al., 2003; Sternfeld et al., 2003). Specifically, the activation of ADP-ribosyl cyclase or CD38 by cyclic GMP- or cyclic AMP-dependent protein kinases has been reported in *Aplysia californica* (Graeff et al., 1998), LAK cells (Rah et al., 2005) and artery smooth muscle cells (Boittin et al., 2003). However, there have been no previous reports regarding the mechanisms by which ADP-ribosyl cyclase and CD38 are activated after OT receptor stimulation in the hypothalamus, leading to secretion of OT. Here we address the above question of whether ADP-ribosyl cyclase/CD38-dependent cADPR and $[Ca^{2+}]_i$ signaling are involved in the autoregulatory positive feedback of OT release in the hypothalamus and posterior pituitary in adult male mice. We demonstrated that the activity of ADP-ribosyl cyclase was increased in response to OT, resulting in production of cADPR, increases in $[Ca^{2+}]_i$, and facilitation of OT release. The OT-induced reactions in the signal cascade were sensitive to PKC inhibitors, and the later two reactions after formation of cADPR were inhibited by a cADPR blocker.

## 2. Materials and methods

### 2.1. Mice

ICR mice (10–12-weeks-old, 30–35 g body weight) were kept in the animal center under standard conditions (24 °C; 12-h light/dark cycle, lights on at 8:00 a.m.) with food and water *ad libitum*. For preparing tissues, mice were first anesthetized with diethyl ether and mouse heads were cut off by a scissor, in accordance with the guidelines for the care and use of laboratory animals of Kanazawa University.

### 2.2. Materials

Most drugs were purchased from Sigma (St. Louis, MO, USA). Xestospongin C was obtained from Wako (Osaka, Japan).

### 2.3. ADP-ribosyl cyclase activity

ADP-ribosyl cyclase activity in the hypothalamus and pituitary were determined fluorometrically using nicotinamide guanine dinucleotide ($NGD^+$) as a substrate, with a slight modification (Higashida et al., 1997, 1999) of the method described previously (Graeff et al., 1994). The tissues were homogenized in 10 mM Tris–HCl buffer, pH 7.4. The fresh homogenates were mixed with reaction solution containing 60 μM $NGD^+$, 50 mM Tris–Hcl, pH 7.0, 100 mM Kcl, 10 μM $CaCl_2$ at 37 °C with constant stirring. The samples were then excited at 300 nm, and fluorescence emission was monitored continuously at 410 nm for 10 min in a Shimadzu RF-5300 PC spectrofluorometer (Kyoto, Japan). Protein content was determined using a Bio-Rad protein assay kit and bovine serum albumin as a standard. The specific ADP-ribosyl cyclase activity was calculated using cyclic guanosine diphosphate ribose (cGDPR) standards, and the results are presented as nM cGDPR per mg protein per minute.

### 2.4. Measurement of tissue cADPR concentrations

The cADPR content was measured by using a cyclic enzymatic assay as described previously (Graeff and Lee, 2002). The hypothalamus was treated with 0.5 ml of 0.6 M perchloric acid under sonication. After centrifugation at 20,000 × *g* for 10 min, perchloric acid was removed by mixing the aqueous sample with a solution containing 3 volumes of 1,1,2-trichlorotrifluoroethane to 1 volume of tri-n-octylamine. The contents of this preparation were then measured.

### 2.5. Preparation of isolated neurohypophysial nerve terminals

Isolated never terminals were prepared from ICR male mice as previously described (OuYang et al., 2004; Sasaki et al., 2005). Briefly, the posterior pituitary lobes were carefully removed and incubated in normal Locke's solution containing (in mM): NaCl, 140; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 2.2; glucose, 10; HEPES, 10; BSA, 0.1%, adjusted to pH 7.25 with Tris–HCl. The tissues were gently homogenized using a Teflon homogenizer in a solution containing (in mM): sucrose, 270; HEPES, 10; EDTA, 0.2, adjusted to pH 7.25 with Tris–HCl. The homogenate was first centrifuged for 1 min at 1000 × *g*, then the supernatant was further spun at 2400 × *g* for 4 min. After discarding the supernatant, the pellet was resuspended in normal Locke's solution and loaded onto cover glasses coated with 0.1% polyorinithine and left to stand at 37 °C for 5 min to settle.

### 2.6. Measurement of $[Ca^{2+}]_i$ in nerve-endings

To monitor the changes of intracellular calcium concentrations after various treatments, the isolated nerve-endings were incubated with a cell-permeable acetoxymethylester form(Fura-2 a.m.) of 5 μM Oregon Green 488 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetate (BAPTA-1) (OGB-1; Molecular Probes, Invitrogen, Tokyo, Japan) diluted in normal Locke's solution for 1 h at 37 °C. Nerve-endings loaded with Oregon Green 488 BAPTA-1 were illuminated at wavelength of 485 nm, and the emission was detected at 538 nm using an Argus 50 (Hamamatsu Photonics, Hamamatsu, Japan). Image of nerve-endings were collected every 10 s for up to 5 min. The changes in fluorescence intensity of each nerve-ending were expanded into an X-t plane. Data are performed in change in fluorescence divided by resting fluorescence, i.e., $\Delta F/F_0$ (Higashida et al., 2007a).

### 2.7. OT release from nerve-endings

The prepared nerve-endings were perfused with normal Locke's solution at 0.2 ml/min for 45 min. At the end of perfusion, the perfusate during 5 min was collected three times to determine the basic OT release. Then, the perfusate was changed to normal Locke's solution containing 100 pg/mL OT with or without 2 mM extracellular calcium or 1 μM vasotocin. For other signaling inhibition experiments, the nerve-endings were pre-incubated within the Locke's solution containing 100 nM Calphosin C, 100 μM 8-bromo-cADPR or 2 μM Xestospongin C for 30 min at 37 °C after perfusion. At the end of incubation, a solution with 100 nM Calphosin C, 100 μM 8-bromo-cADPR or 2 μM Xestospongin C was perfused for periods of 5 min each and samples were collected three times to determine basal OT release. Then, the perfusate was changed to 100 pg/mL OT Locke's solution with 100 nM Calphosin C, 100 μM 8-bromo-cADPR or 2 μM Xestospongin C, respectively. All the samples were stored at −80 °C for later immunoassay. Amount of OT released during 5 min from isolated nerve-endings on the coated glass was determined by replacing incubation Lock solution with or without the known concentration of OT (for stimulation). Therefore, we used the following formula to calculate the ratio of OT release under various conditions of pre- and post-stimulation: OT release ratio = (OT concentration incubated with OT) − (pre-stimulation of OT level = 100 pM)/(OT concentration without OT), according to the method described previously (Jin et al., 2007b).

### 2.8. ELISA assay for OT

The concentration of OT was determined by using an enzyme-linked immunosorbent assay (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol, as described previously (Jin et al., 2007a). The inter- and intra-assay coefficients of variations were 10.7% and 12.2%, respectively, and the sensitivity was 11.7 pg/mL.

### 2.9. Statistical analyses

All data are shown as means ± SE ($n = 4$–6). The statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc. San Jose, CA). A $p$ value of less than 0.05 determined by Student's $t$-test was considered to be statistically significant.

## 3. Results

### 3.1. Effects of OT on activity of membrane-bound form of ADP-ribosyl cyclase and cADPR levels

First, we examined whether application of OT stimulates ADP-ribosyl cyclase activity in crude membranes prepared from the hypothalamus and posterior pituitary of adult male mice. The effects of various concentrations of OT were examined in crude membrane preparations by fluorometric measurement of cGDPR production as an assay for ADP-ribosyl cyclase enzyme activity. cGDPR production increased upon exposure to OT for 5 min (Fig. 1A and B). The maximum increase in ADP-ribosyl cyclase activity in response to 10 nM OT was 158 ± 7% ($n = 5$) of the pre-exposure levels in the hypothalamus, while it was 278 ± 57% ($n = 5$) at 10 pM OT in the pituitary. No or little activation was detected at higher concentrations of OT in the hypothalamus (100 nM) and the pituitary (1 nM). Simultaneous application of vasotocin, an OT receptor