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**Oxytocin-induced elevation of ADP-ribosyl cyclase activity, cyclic ADP-ribose or Ca<sup>2+</sup> 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 Gallgher, 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., 2007). Intracellular cADPR 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, 2007); 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 to 12 weeks old, 30-35g 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). Xestospongine 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<sup>+</sup>) as a substrate, with a slight modification (Higashida et al., 1997 and 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<sup>+</sup>, 50 mM Tris-HCl, pH 7.0, 100 mM KCl, 10 μM CaCl<sub>2</sub> 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-5300PC 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 x 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 nerve 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<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 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 1,000 x g, then the supernatant was further spun at 2,400 x 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% polyornithine 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 AM) of 5  $\mu$ M 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 1h 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, 2007) .

## 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  $\mu$ M vasotocin. For other signaling inhibition experiments, the nerve endings were pre-incubated within the Locke's solution containing 100 nM Calphosin C, 100  $\mu$ M 8-bromo-cADPR or 2  $\mu$ M Xestospongine C for 30 min at 37°C after perfusion. At the end of incubation, a solution with 100 nM Calphosin C, 100  $\mu$ M 8-bromo-cADPR or 2  $\mu$ M Xestospongine 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  $\mu$ M 8-bromo-cADPR or 2  $\mu$ M Xestospongine 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 Locke 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) – (OT concentration without OT) / (pre-stimulation of OT level=100 pM), 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  $\pm$  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  $158 \pm 7\%$  (n=5) of the pre-exposure levels in the hypothalamus, while it was  $278 \pm 57\%$  (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 antagonist, significantly inhibited the OT-induced increase of ADP-ribosyl cyclase activity. Vasotocin did not inhibit the basal activity, suggesting little or no oxytocin present in the reaction buffer together with membranes as an enzyme source.

In order to test whether ADP-ribosyl cyclase stimulation by OT required receptor coupling to endogenous G proteins, we examined the effect of exogenous GTP in well-washed membranes. Addition of 10 nM GTP with or without 10 nM OT resulted in a significant increase in enzymatic activity by  $33 \pm 4\%$  or  $41 \pm 3\%$  ( $p < 0.05$ , n=4) in the hypothalamic preparations, respectively. Application of 10 nM GTP alone resulted in only an increase of 10 %, while GTP together with 10 pM OT caused a significant

inhibition of  $47 \pm 7\%$  ( $p < 0.01$ ,  $n=4$ ) in pituitary nerve endings, suggesting a signal pathway via  $G_{q/11}$  or  $G_i$  (Gimpl and Fahrenholz, 2001).

Tissue cADPR concentrations ( $[cADPR]_i$ ) were measured by the enzyme recycling method.  $[cADPR]_i$  in the hypothalamus of male mice was  $228 \pm 53$  nmol/mg protein ( $n=4$ ) before stimulation.  $[cADPR]_i$  increased during incubation with OT for 5 min in a dose-dependent manner. The maximal increase to  $1050 \pm 72$  nmol/mg protein ( $n=4$ ) (4.6-fold higher than the control level) was obtained at the same concentration of 10 nM OT for the maximum activation of ADP-ribosyl cyclase (Fig. 2). It is interesting to measure the cADPR level in the pituitary in response to OT, which will be reported elsewhere.

### 3.2. Inhibition of OT-induced activation of the ADP-ribosyl cyclase

Next, we examined the possibility that ADP-ribosyl cyclase is also activated by protein kinases *via* the OT-signaling pathway. We first used staurosporine (PKI-STSP), one of the most potent and widely used cell permeable inhibitors of protein kinases, to measure ADP-ribosyl cyclase activity after OT stimulation for 5 min in the hypothalamus and pituitary. A significant inhibition was obtained with 5 nM PKI-STSP (Fig. 3 A and B) and no greater inhibition was observed at a higher dose of 25 nM in either tissue (data not shown). As PKI-STSP selectively inhibits PKC at the lower concentration of 5 nM and various protein kinases, including PKA, PKG, and CaMKII, at the higher concentrations of 25 nM (Tamaoki et al., 1986), our results indicate that PKC is likely involved in sustained activation of ADP-ribosyl cyclase. This was confirmed by using a more specific PKC inhibitor, calphostin C (100 nM), in both

tissues challenged by these two concentrations (Fig. 3). PKI-STSP or calphostin C alone had no essential effect on cyclase activity (n=3). Taken together, these results indicate that PKC may play a role in activating ADP-ribosyl cyclase by OT.

### *3.3. Role of kinases in OT-induced activation of the ADP-ribosyl cyclase*

In isolated nerve endings of the neurohypophysis, it is easy to identify oxytocinergic nerve endings from those of vasopressin because of their larger size (Hlubek et al., 2003; Sheikh et al., 1998; Troadec et al., 1998). This distinct morphology makes it possible to measure intracellular calcium mobilization upon OT stimulation over a period of 5 min. OT-induced changes in  $[Ca^{2+}]_i$  were measured in each single nerve ending with OGB-1 before, during, and after application of OT, to examine downstream  $Ca^{2+}$  signal transduction of elevated ADP-ribosyl cyclase activity. Application of the supramaximal concentration of 100 pM OT to the nerve endings for full activation resulted in two phases of  $[Ca^{2+}]_i$  increase in its time course: a rapid initial increase and a sustained elevation lasting for 5 min (Fig. 4A). OT elicited elevations of the maximum  $[Ca^{2+}]_i$  to  $145 \pm 21\%$  (n=4) 20s after application. Two  $\mu$ M Xestospongin C, an  $IP_3$  receptor antagonist, slowed down the initial increase with little or no effect on the sustained  $[Ca^{2+}]_i$  increases. Pretreatment with 10  $\mu$ M 8-bromo-cADPR, an antagonistic cADPR analog, markedly inhibited OT-mediated sustained  $[Ca^{2+}]_i$  increases with decreased initial increases, resembling the effect of vasotocin. In contrast, calphostin C had little effect on the initial phase followed by significant decreases at the sustained level, indicating that PKC is involved in the sustained  $[Ca^{2+}]_i$  elevation. Under  $Ca^{2+}$ -free conditions, the OT-mediated increase of  $[Ca^{2+}]_i$  showed slight but not statistically

significant changes in both phases. The intensity modulation in initial and sustained  $[Ca^{2+}]_i$  elevation at 20 and 180 s after OT stimulation are summarized quantitatively in Fig. 4B and C, respectively. These results clearly showed that  $IP_3$  and cADPR inhibitors act differentially on the two phases of  $[Ca^{2+}]_i$  elevations. In most of experiments, nerve endings were tested for identifying as oxytocinergic endings by OT immunostaining after  $[Ca^{2+}]_i$  measurements (data not shown).

### *3.4. Effects of OT on promoting OT release from isolated nerve-endings*

Finally, we examined OT-induced OT release from isolated nerve-endings during 5 min under various conditions. Following application of 100 pM OT, the release of OT was increased by 2.07-fold to the concentration of  $1.39 \pm 0.12$  arbitrary unit (n=4) for 5 min from the control level with vasotocin ( $0.67 \pm 0.05$  arbitrary unit, n=4; Fig. 5). The effect of OT-mediated OT release was significantly inhibited by addition of calphostin C and 8-bromo-cADPR to the level induced by vasotocin. Two  $\mu$ M Xestospongine C had a significant inhibitory effect on OT release, while the  $Ca^{2+}$ -free condition had a non significant inhibitory effect, consequently they changed to similar level. The sensitivity of antagonists on autoregulation of OT release (Fig. 5) resembles the inhibitory pattern obtained on sustained  $[Ca^{2+}]_i$  levels (Fig. 4C).

## **4. Discussion**

The results of the present study indicated that OT-stimulated ADP-ribosyl cyclase activity and increases in  $[Ca^{2+}]_i$  were susceptible to PKC in both hypothalamus and/or posterior pituitary and OT-induced elevation in  $[Ca^{2+}]_i$  and subsequent OT release were both sensitive to PKC and dependent on cADPR. Therefore, we showed here that OT-induced OT release, autoregulation of the positive feedback, is a PKC- and cADPR-dependent process in the hypothalamus and/or pituitary. As we used 10-12-week-old adult male mice, this PKC- and cADPR-dependent autoregulation of OT release is not related to female reproductive processes, but to social recognition or social behavior found in this mouse strain (Jin et al., 2007a; Liu et al., 2008). The positive feedback mechanism of OT release plays a critical and physiological role in causing uterus contraction during labor and triggering milk release from the breast tissue when infants are nursed (Moos et al., 1984; Neumann et al., 1994 and 1996). A recent series of studies showed that nasal infusion of OT increases trust (Kosfeld et al., 2005), mind-reading (Domes et al., 2007), and generosity (Zak et al., 2007) in humans, indicating an important role of OT in human social behavior. Furthermore, OT reduces repetitive behavior in adults with autism and Asperger's disorder (Hollander et al., 2003). Taken together, positive feedback of PKC- and cADPR-dependent OT release in the hypothalamus and pituitary may be important for correct and efficient social conduct in relation to social stimulation, although further validation is necessary.

The activation efficiency of OT on ADP-ribosyl cyclase activity differed markedly between the hypothalamus and pituitary (Fig. 1). The maximum peak obtained was 1,000-fold more sensitive in the pituitary, though basal activity was lower by 25%. The activation ratio was also higher in the pituitary than in the hypothalamus. For this, one reason may reside on cyclase species: the lower expression of CD38 in the

pituitary (Jin et al., 2007a) or contribution of other molecular species, as suggested in the embryonic brain (Ceni et al., 2006). The second possibility is utilization of different G proteins or accessory molecules. As shown in our results with GTP reproduced both stimulatory and inhibitory effects on cyclase, with the different mode in the hypothalamus and pituitary. Since higher concentrations of OT lost activation in the hypothalamus and pituitary tissues, as seen in myocardial cells (Higashida et al., 1999), stimulatory and inhibitory G proteins coupled to OT receptors may be involved at different OT concentrations. Third, close inspection of the dose-response curve of cADPR formation in the hypothalamus, one more highly sensitive component seems to be present in response to OT at around 100 nM (Fig. 2).

We have demonstrated that the rise of  $[Ca^{2+}]_i$  in the isolated nerve endings after OT stimulation was dually controlled by  $IP_3$  and cADPR, resembling human myometrium cells (Barata et al., 2004). The initial part of the increase in  $[Ca^{2+}]_i$  seemed to be composed of  $IP_3$ -mediated  $[Ca^{2+}]_i$  increases without  $Ca^{2+}$  influx, because the  $IP_3$  antagonist delayed the initial peak. In contrast, the cADPR was largely responsible for the sustained  $Ca^{2+}$  signal for up to 5 min because of blockade by the pretreatment of nerve endings with 8-bromo-cADPR, which was also mimicked by the PKC inhibitor. Thus, we postulated that the OT-induced  $Ca^{2+}$  elevation is due to release mediated by cADPR through ryanodine receptors in a PKC-dependent manner, followed by initial  $Ca^{2+}$  mobilization by activation of the  $IP_3$  receptors, which was not sensitive to PKC. A similar susceptibility pattern to inhibitors was obtained for OT-mediated OT release in the isolated nerve endings, indicating that OT release is dependent on the sustained phase of  $Ca^{2+}$  increases.

Involvement of CD38 in the cADPR-dependent signal cascade could be examined by using CD38 knockout mice as a tool. Since more than 90% of cyclase activity is lost in membrane fractions of the CD38 knockout mouse hypothalamus and pituitary, no or little increase in cyclase activity was confirmed in response to OT. Next, we measured  $[Ca^{2+}]_i$  in an isolated nerve-endings from CD38 knockout mice during 5 min under OT stimulation. In response to application of 100 pM OT,  $[Ca^{2+}]_i$  concentrations increased by  $12 \pm 6 \%$  (n=4) in CD38 knockout mice and by  $39 \pm 12 \%$  (n=4) in wild-type mice. These preliminary results suggest that OT receptor on stimulation can induce OT release by the initial  $Ca^{2+}$  increase, but CD38 and cADPR/dependent sustained  $Ca^{2+}$  increases can contribute facilitated OT release.

In conclusion, the results confirm OT-mediated OT release in male mice, and this process is cADPR and  $Ca^{2+}$  dependent, with the mediation of PKC, The involvement of PKC in ADP ribosyl cyclase activity stimulation,  $[Ca^{2+}]_i$  increases and OT release, however, the whole intracellular mechanism of the downstream signaling pathway involved in autoregulation related in mammalian social behavior should be clarified.



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## Figurer legends

**Fig. 1.** OT increases ADP-ribosil cyclase activity in mouse hypothalamus and posterior pituitary. ADP-ribosyl cyclase activities were measured as the rate of cyclic GDP-ribose formation (pmol/min/mg protein) by homogenates isolated from mouse hypothalamus (A) and posterior pituitary (B). Data were presented as activities measured for 5 min under various concentrations of OT with (red line) or without 1  $\mu$ M oxytocin receptor antagonist vasotocin (blue line). The data shown are means  $\pm$  SE (n=5). \*  $p < 0.05$  from the presence of vasotocin.

**Fig. 2.** cADPR content in mouse hypothalamus. Values were obtained during incubation for 5 min with various concentrations of OT. Data are presented as the means  $\pm$  SE (n=4). \*  $p < 0.05$ , \*\*  $p < 0.01$ .

**Fig. 3.** Inhibition of OT-induced activation of the ADP-ribosyl cyclase. Data are presented as percentages of control cyclic GDP-ribose formation activity, as shown in Fig. 1. Activities were measured in the presence or absence of OT (4 concentrations), PKI-STSP (5 nM) or PKC-inhibitor calphostin C (100 nM) indicated in hypothalamus (A) and pituitary (B). Data are shown as means  $\pm$  SE (n=4). \*  $p < 0.05$ , \*\*  $p < 0.01$  from OT stimulation.

**Fig. 4.** OT mediated changes in  $[Ca^{2+}]_i$  in single nerve endings isolated from the posterior pituitary. (A) Average time courses of changes in  $[Ca^{2+}]_i$  elicited with 100 pM OT with or without 1  $\mu$ M oxytocin receptor antagonist vasotocin, 100 nM

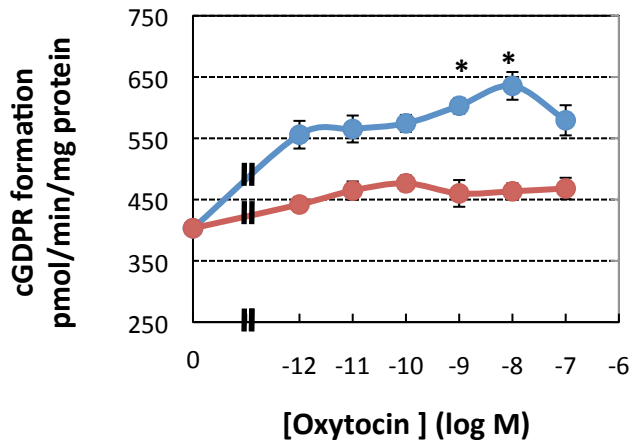
PKC-inhibitor calphostin C, 2  $\mu\text{M}$  IP<sub>3</sub>-inhibitor Xestospongin C, 100  $\mu\text{M}$  8-bromo-cADPR, and extracellular Ca<sup>2+</sup>. Average increases in [Ca<sup>2+</sup>]<sub>i</sub> measured at 20 s (B) and 180 s (C) after OT stimulation. Data are shown in change in fluorescence divided by resting fluorescence, i.e.,  $\Delta F/F_0$ , as means  $\pm$  SE (n=4-6). \*  $p < 0.05$  in comparison with OT stimulation.

**Fig. 5.** OT released from isolated nerve endings of posterior pituitary. OT concentrations are presented in OT release ratio (arbitrary unit) in an isolated nerve endings under 100 pM OT stimulation (5 min) with or without 1  $\mu\text{M}$  oxytocin receptor antagonist vasotocin, 100 nM PKC-inhibitor calphostin C, 2  $\mu\text{M}$  IP<sub>3</sub>-inhibitor Xestospongin C, 100  $\mu\text{M}$  8-bromo-cADPR, and extracellular Ca<sup>2+</sup>. Data are shown as means  $\pm$  SE (n=4-5). \*  $p < 0.05$ , \*\*  $p < 0.01$  from OT stimulation.



A

Hypothalamus



B

Posterior pituitary

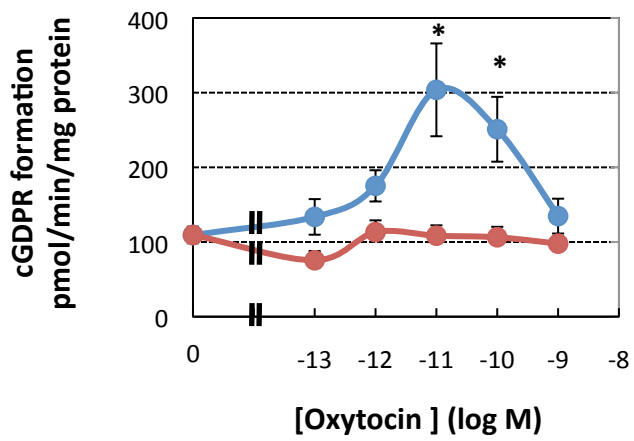


Figure 1

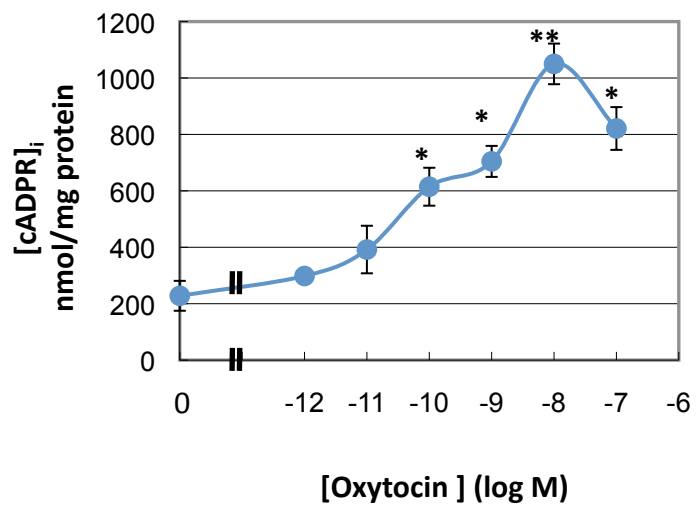
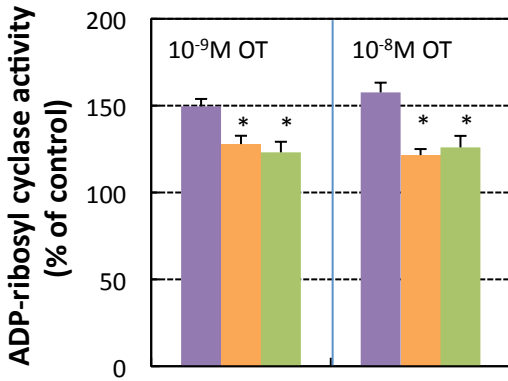


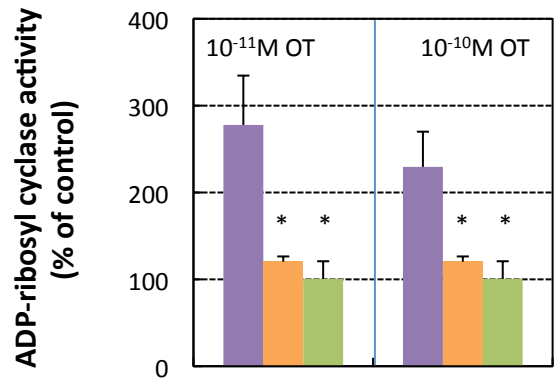
Figure 2

**A Hypothalamus**



OT	+	+	+	+	+	+
PKI-STSP (5nM)	-	+	-	-	+	-
Calphostin C (100nM)	-	-	+	-	-	+

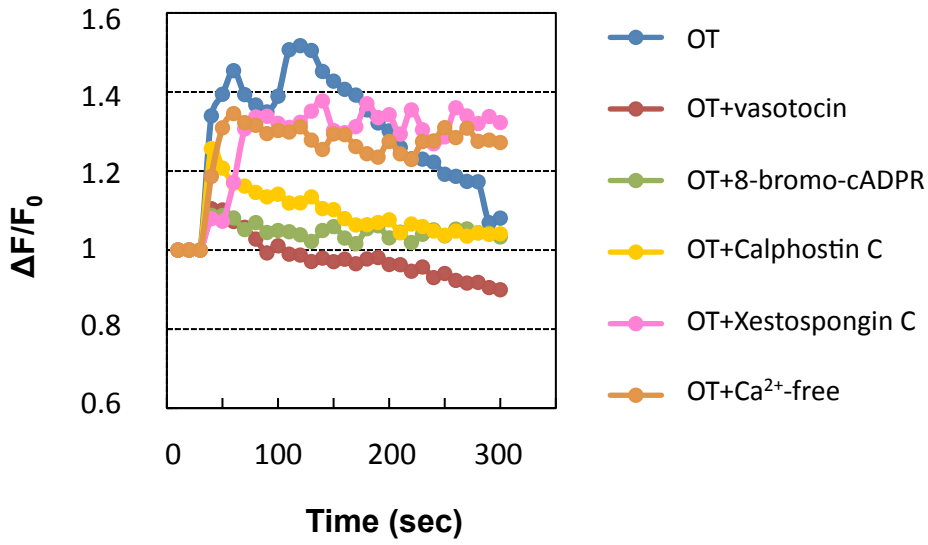
**B Posterior pituitary**



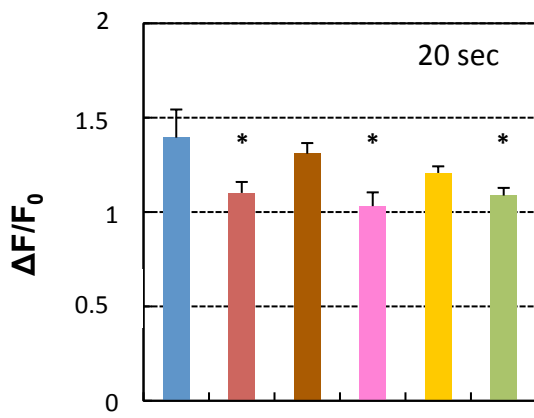
OT	+	+	+	+	+	+
PKI-STSP (5nM)	-	+	-	-	+	-
Calphostin C (100nM)	-	-	+	-	-	+

**Figure 3**

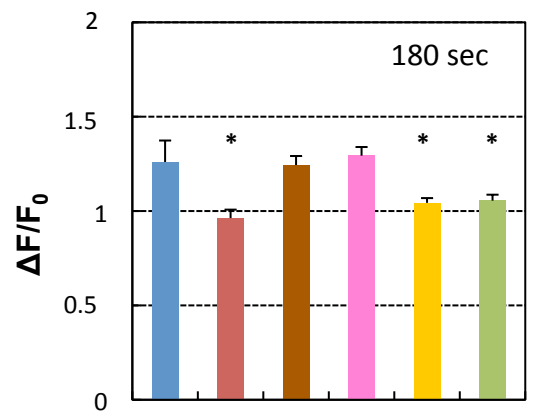
**A**



**B**



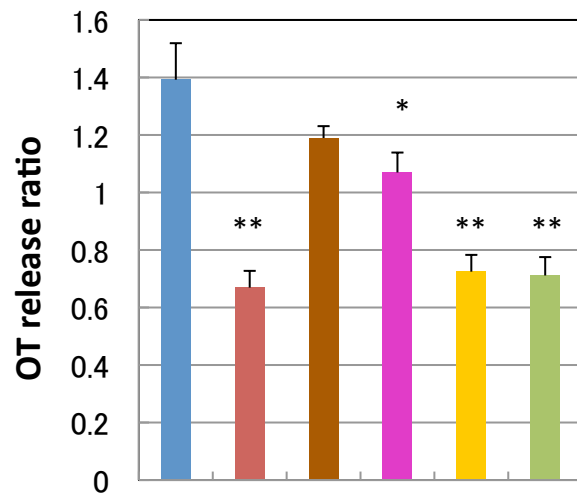
**C**



OT	+	+	+	+	+	+
Vasotocin	-	+	-	-	-	-
Ca <sup>2+</sup> -free	-	-	+	-	-	-
Xestospongin C	-	-	-	+	-	-
Calphostin C	-	-	-	-	+	-
8-bromo-cADPR	-	-	-	-	-	+

OT	+	+	+	+	+	+
Vasotocin	-	+	-	-	-	-
Ca <sup>2+</sup> -free	-	-	+	-	-	-
Xestospongin C	-	-	-	+	-	-
Calphostin C	-	-	-	-	+	-
8-bromo-cADPR	-	-	-	-	-	+

**Figure 4**



OT	+	+	+	+	+	+
Vasotocin	-	+	-	-	-	-
Ca <sup>2+</sup> -free	-	-	+	-	-	-
Xestospongin C	-	-	-	+	-	-
Calphostin C	-	-	-	-	+	-
8-bromo-cADPR	-	-	-	-	-	+

Figure 5