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ORIGINAL PAPER

# Antagonistic effects of vasotocin and isotocin on the upper esophageal sphincter muscle of the eel acclimated to seawater

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**Abstract** The effects of isotocin (IT) and vasotocin (VT), which are fish analogues of mammalian oxytocin and vasopressin respectively, were examined in the isolated upper esophageal sphincter (UES) muscle. IT relaxed and VT constricted the UES muscle in a concentration-dependent manner. The relaxation by IT and the contraction by VT were completely blocked by H-9405 (an oxytocin receptor antagonist) and by H-5350 (a $V_1$-receptor antagonist), respectively, suggesting that the eel UES possesses both IT and VT receptors. Truncated fragments of VT did not show any significant effects, indicating that all nine residues are essential for the VT and IT actions. IT may relax the UES muscle through enhancing cAMP production, since similar relaxation was also observed after treatment with 3-isobutyl-1-methylxantine, forskolin and 8-bromoadenosine, 3′, 5′-cyclic mono-phosphate (8BrcAMP). Although 8-bromoguanosine, 3′, 5′-cyclic monophosphate also relaxed the UES, its effect was less than 1/3 of that 8BrcAMP, suggesting minor contribution of nitric oxide (NO) in the relaxation of the UES muscle. Both peptides seem to act directly on the UES muscle, not through release of other substances from the epithelial cells, since similar relaxation and contraction were observed even in the scraped UES preparations. When IT and VT were intravenously administrated (in vivo experiments), the drinking rate of the seawater eel was enhanced by IT and was inhibited by VT. These effects correspond to the in vitro results described above, relaxation by IT and contraction by VT in the UES muscle. The significance of the relaxing effect by IT is discussed with respect to controlling the drinking behavior of the eel.

**Keywords** Isotocin · Vasotocin · Upper esophageal sphincter muscle · Relaxation · Drinking behavior

**Abbreviations**

| | |
|---|---|
| Ach | Acetylcholine |
| 8BrcAMP | 8-Bromoadenosine, 3′, 5′-cyclic monophosphate |
| 8BrcGMP | 8-Bromoguanosine, 3′, 5′-cyclic monophosphate |
| FSK | Forskolin |
| GVC | Glossopharyngeal-vagal motor complex |
| H-3056 | [d$(CH_2)_5^1$, D-Ile$^2$, Ile$^4$, Arg$^8$, Ala-$NH_2^9$]-vasopressin |
| H-5050 | [d$(CH_2)_5^1$, Tyr(Me)$^2$, Arg$^8$]-vasopressin |
| H-9405 | [d$(CH_2)_5^1$, Tyr(Me)$^2$, Thr$^4$, Orn$^8$, Tyr-$NH_2^9$]-vasotocin |
| IBMX | 3-Isobutyl-1-methylxantine |
| IT | Isotocin |
| NO | Nitric oxide |
| OT | Oxytocin |
| UES | Upper esophageal sphincter |
| VP | Arginine vasopressin |
| VT | Arginine vasotocin |

## Introduction

The upper esophageal sphincter (UES) muscle is located between the pharynx and the esophageal body, and restricts movement of food and water by contraction. When the UES muscle is relaxed, therefore, foods or water enters into

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the esophageal body (swallowing). The seawater eels have been used as a model system to analyze the regulatory mechanisms for controlling drinking behavior, because their drinking behavior mainly consists of swallowing alone (Ando and Nagashima 1996; Takei 2000; Ando et al. 2000a, b; Kozaka and Ando 2003). All saltwater fish live in seawater and water passes the pharynx constantly to the gill for respiration. Therefore, the rate of drinking is limited by a contraction of the UES muscle in these fish. Our previous studies have demonstrated that the eel UES muscle, which consists of striated muscle (Kozaka and Ando 2003), is cholinergically innervated by the glossopharyngeal-vagal motor complex (GVC) of the medulla oblongata (Mukuda and Ando 2003), and is constricted robustly by acetylcholine (ACh) (Kozaka and Ando 2003). Similar cholinergic innervation to the striated UES muscle has been reported in many mammals (Diamant 1993; Neuhuber et al. 1994; Kuramoto et al. 1996). Such similarity also suggests the eel UES is a useful model system to understand regulatory mechanisms for controlling swallowing.

However, except for the presence of nicotinic receptor for ACh on the UES muscle, involvement of other regulators is not clear yet even in mammals (Kerr et al. 2000; Krysiak and Preiksaitis 2001). Therefore, the present study aimed to search for such regulators modulating the UES muscle contraction and controlling drinking behavior. As candidates for such regulators, we considered vasotocin (VT) and isotocin (IT), since these neurohypophysial peptides are fish analogues of the mammalian vasopressin (VP) and oxytocin (OT), respectively, and the former is well-known as a water retaining hormone and the latter as a muscle-contracting hormone in mammals (Bentley 1998). VT constricted and IT relaxed the eel UES muscle in a concentration-dependent manner, and their effects were completely blocked by a $V_1$-type vasopressin receptor antagonist and an oxytocin receptor antagonist, respectively. Combining these in vitro results with effects of peptides on the drinking rate (in vivo experiment), a role of IT in regulating drinking behavior is discussed.

## Materials and methods

Cultured Japanese eels *Anguilla japonica*, weighing about 200 g, were obtained from a commercial source. They were kept in seawater aquaria at 20°C for more than 1 week without food before use. After decapitation, the upper esophagus was excised and cut transversely into 2–3 mm lengths. The ring of the upper esophagus (UES preparation) was tied with two cotton threads, one being connected to the bottom of the experimental chamber, the other to a strain gauge (45196A, Sanei, Tokyo, Japan) for tension recording with a chart recorder (EPR-10B, TOA, Tokyo). Since this UES preparation consists of muscle cells, nerve terminals and epithelial tissue, some esophagus were everted and the epithelial tissue was scraped off by a razor (scraped UES preparations). The UES preparations were made only from the upper part (5 mm from the heart-attaching portion) in the esophagus. The lower part of the esophagus contracted spontaneously as described previously (Uesaka et al. 1991). When the spontaneous contraction was observed, such preparations were discarded. Therefore only one UES preparation was obtained from each animal. Details of the experimental system are as described previously (Uesaka et al. 1991; Kozaka and Ando 2003). The UES preparations were bathed in Krebs bicarbonate Ringer solution consisting of (in mM): 118.5 NaCl, 4.7 KCl, 3.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 24.9 $NaHCO_3$, 5.0 alanine, 5.0 glucose.

The bathing solution (2.5 ml) was bubbled with a 95% $O_2$:5% $CO_2$ gas mixture (pH 7.4) at 20°C. Although 0.5% $CO_2$ gas mixture (pH 7.9) was also applied in some preparations, most experiments were performed under 5% $CO_2$ (pH 7.4) for convenience, since similar results were obtained under both conditions. After preloading with 2.0 g tension, the preparation was incubated for more than 1 h to obtain a steady tension. During this incubation time, the tension gradually returned to the initial levels (almost zero tension). Such preloading procedure is usually applied in various muscles including mammalian esophagus (Storr et al. 2001; Kozaka and Ando 2003). Under steady states, various reagents were added to the bathing solution.

For measurement of water intake, after anesthesia with 0.1% methane tricaine sulfonate (MS-222, Sigma, St Louis, USA), the esophagus was cannulated with a vinyl tube (o.d. 2.0 mm). The cannula was connected to a drop counter (PG-602, Keyence, Osaka, Japan) for continuous recording of the drinking rate. Each drop (29.2 µl) was recorded as a spike on a chart recorder (EPR-121A, TOA, Tokyo, Japan). Another tube (o.d. 1.1 mm) was inserted into the intestine for application of 170 mM NaCl solution, determined from the concentration measured at the gastrointestinal junction in the seawater eels (Ando and Nagashima 1996). Perfusion of this cannula was done via a peristaltic pump (MS-1 Reglo 160, Ismatic, Turich, Switzerland) controlled by an electric stimulator (SEN-3201, Nihon Koden, Tokyo), which was triggered by the drop counter. With this system, the swallowed seawater can be reintroduced into the intestine. A third cannula (SP-10, Natume, Tokyo) filled with heparinized saline (100 IU/ml) was inserted into the posterior cardinal vein. Through this cannula, VT and IT were administered into the blood. Peptides were dissolved in 0.9% NaCl solution (vehicle), and 50 µl of their appropriate concentrations was injected slowly (22.6 µl/min) into the vein with a syringe pump (MF-9090, Bioanalytical Systems, IN, USA), followed by an injection of 25 µl vehicle

to push through peptide remaining in the cannula. After the operation, the incision was closed using silk suture and all cannulae were sutured to the body. Eels were then transferred to a plastic trough of the same size. Well aerated seawater was circulated continuously through the trough at room temperature (20–23°C). Experiments were started on the following day, when the drinking rate was relatively constant. Details of the experimental system are as described previously (Ando et al. 2000b).

Arginine vasotocin (VT), arginine vasopressin (VP), isotocin (IT), tocinoic acid, forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), 8-bromoadenosine, 3′, 5′-cyclic monophosphate (8BrcAMP), 8-bromoguanosine, 3′, 5′-cyclic monophosphate (8BrcGMP) were obtained from Sigma, St Louis, USA, and [d($CH_2$)$_5^1$, Tyr(Me)$^2$, Arg$^8$]-vasopressin (H-5350), [d($CH_2$)$_5^1$,D-Ile$^2$, Ile$^4$, Arg$^8$, Ala-$NH_2^9$]-vasopressin (H-3056), [d($CH_2$)$_5^1$, Tyr(Me)$^2$, Thr$^4$, Orn$^8$, Tyr-$NH_2^9$]-vasotocin (H-9405), and vasopressin fragment (VP(4–9)) were purchased from BACHEM, Torrance, USA. FSK was dissolved in dimethyl-sulfoxide (DMSO; Sigma), and the final concentration of DMSO was less than 0.1%.

Statistical analyses of the results were performed using a paired *t*-test. Results are given as means ± SEM and were considered significant at $P < 0.05$.

## Results

### Effect of vasotocin and isotocin on the intact UES muscle

The UES muscle did not constrict spontaneously as described previously (Kozaka and Ando 2003). When $10^{-8}$ M vasotocin (VT) was applied to the UES preparation, the muscle contracted gradually (Fig. 1a); peak tension 10.4 ± 4.2 mg ($n = 5$). However, higher concentration of VT ($10^{-7}$ M) induced a small transient contraction followed by a remarkable relaxation was usually observed (data not shown). In contrast, $10^{-7}$ M isotocin (IT) relaxed the UES immediately, without contraction (Fig. 1b); peak relaxation −76.1 ± 14.8 mg ($n = 6$). These effects of peptides were reproducible. The concentration-dependency of the effect of these peptides is summarized in Fig. 2. While VT constricted the UES muscle in a concentration-dependent manner below $10^{-8}$ M, with a threshold at $10^{-10}$ M, it relaxed the muscle at higher concentrations. In contrast, IT showed only relaxation in a concentration-dependent manner, with a threshold at $10^{-10}$ M. The VT fragments, VP (4–9) and tocinoic acid, did not show any significant effects. Arginine vasopressin (VP) only constricted the UES muscle concentration-dependently, with tenfold higher threshold than VT. Whereas oxytocin (OT) also relaxed the UES muscle, the degree of relaxation was almost 1/3 of that produced by IT.

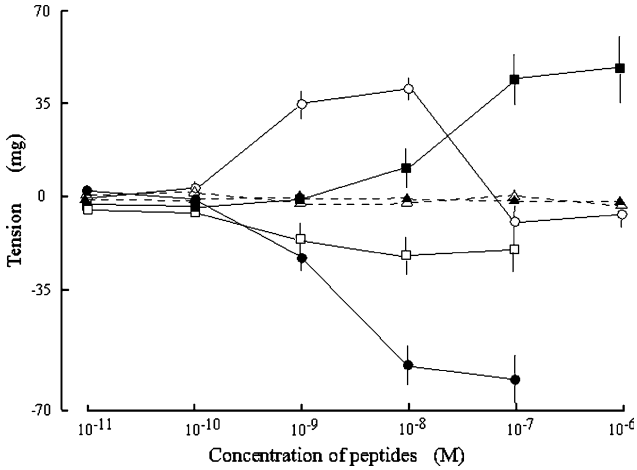


**Fig. 2** Concentration–response relationship of the effect of various peptides, such as VT (*open circle*, $n = 8$), IT (*filled circle*, $n = 5$), arginine vasopressin (VP *filled square*, $n = 5$), and oxytocin (OT *open square*, $n = 5$). For comparison, effects of VT fragments, VP (4–9) (*open triangle*, $n = 4$) and tocinoic acid (*filled triangle*, $n = 4$), were also examined. Each peptide was added into the bathing fluid successively from low concentration to the higher. *Plus signs* indicate contraction and *minus signs* mean relaxation of the UES muscle

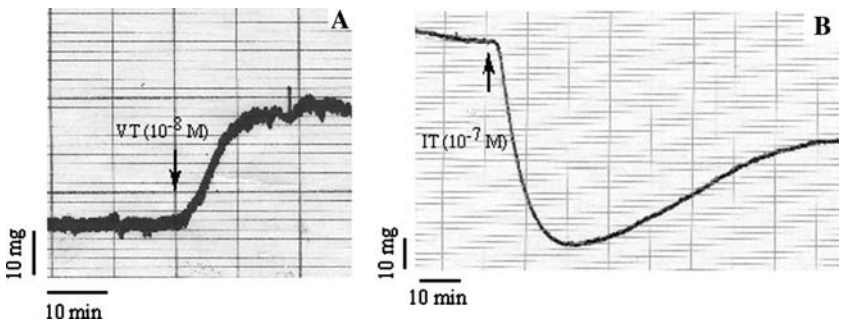


**Fig. 1** Effect of neurohypophysial peptides on the UES tension. a Effect of arginine vasotocin (VT). VT ($10^{-8}$ M) was added to the bathing solution at the *arrow*. Tracing is a representative of five experiments. b Effect of isotocin (IT), a representative of six experiments. IT ($10^{-7}$ M) was applied at the *arrow*. *Vertical bars* indicate the UES tension of 10 mg, upper and lower deflections mean contraction and relaxation, respectively. *Horizontal bar* shows 10 min

Characterization of VT- and IT-receptors

To characterize VT receptors, the effects of two receptor antagonists were examined. After blocking the $V_1$-type receptor with H-5350, known as a $V_1$ receptor antagonist of VP in mammals (Kruszynski et al. 1980; Drago et al. 1997), VT and VP did not constrict the UES muscle at all. Although a small relaxation was induced by VT, in the presence of H-5350 (Fig. 3a), such relaxation was not induced by VP (data not shown). Even in the presence of H-5350, however, relaxation induced by IT still remained (Fig. 3a). When a $V_2$ receptor antagonist (H-3056) was used, VT action (contraction) was not blocked (data not shown). The IT-induced relaxation was completely abolished by H-9405 (Fig. 3b), an OT receptor antagonist (Elands et al. 1988). In the presence of H-9405, however, the contraction induced by VT still remained (Fig. 3b).

Figure 4 shows effects of IBMX, FSK and 8BrcAMP. All these reagents relaxed the intact UES muscle, similarly to IT. Although 8BrcGMP also relaxed the UES muscle, the relaxing effect was significantly lower than that of 8BrcAMP ($P < 0.001$, Student $t$-test).

Effects of vasotocin and isotocin on the scraped UES preparation

To determine whether these peptides act directly on the UES muscle or indirectly via release of other substance(s) from the epithelial cells, epithelial layer was scraped off. Even in the scraped UES preparations, VT constricted

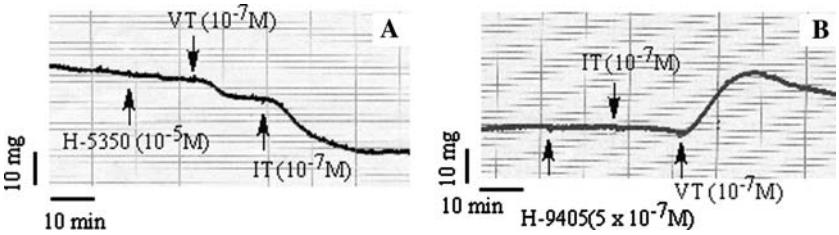


**Fig. 3** Characterization of VT and IT receptors. a Effect of $V_1$ receptor antagonist (H-5350 *first arrow*) on the effects of VT (*second arrow*) and IT (*third arrow*). Tracing is a representative of four experiments. b Effects of OT receptor antagonist (H-9405) on the effects of IT (*second arrow*) and VT (*third arrow*), a representative of five experiments. *Vertical bars* indicate 10 mg tension and *horizontal bar* shows 10 min

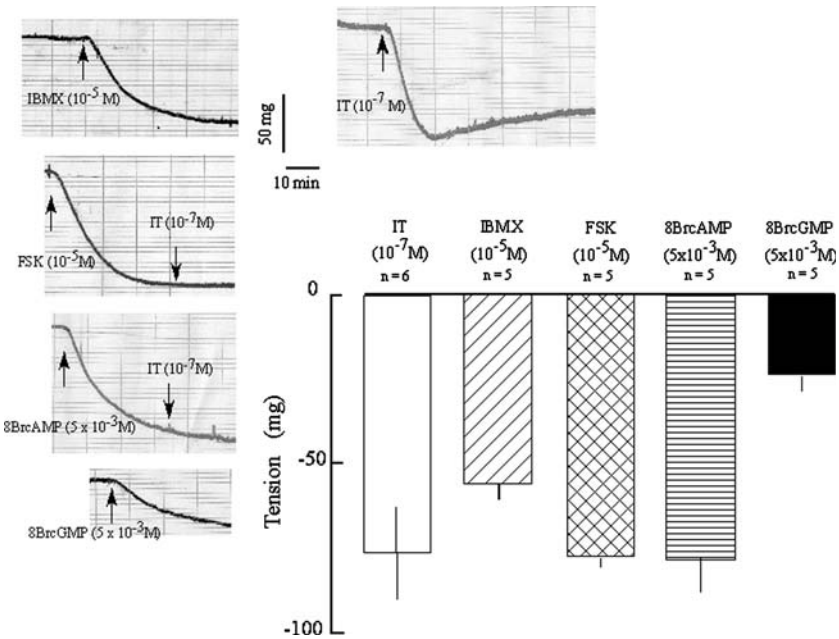


**Fig. 4** Effects of various cAMP analogues on the UES muscle. At the *first arrows*, IBMX ($10^{-5}$ M), forskolin (FSK, $10^{-5}$ M), 8-Bromoadenosine, 3′,5′-cyclic monophosphate (8BrcAMP, $5 \times 10^{-3}$ M), or 8-bromoguanosine, 3′, 5′-cyclic monophosphate (8BrcGMP, $5 \times 10^{-3}$ M) was applied to the UES muscle. After pretreatment with FSK or 8BrcAMP, IT ($10^{-7}$ M) was added to the bathing solution (the *second arrows*). The relaxing effect of 8BrcGMP was significantly lower than that of 8BrcAMP ($P < 0.001$). *Vertical bar* indicates 50 mg tension and *horizontal bar* shows 10 min. As a reference, effect of IT ($10^{-7}$ M) is also shown on the *upper right*. Mean value of each treatment (Mean ± SEM) is presented in the *lower right*

(9.0 ± 3.3 mg, $n = 5$) and IT relaxed (−35.0 ± 7.2 mg, $n = 5$) the UES muscle. Figure 5 shows representative of such effects of VT and IT on the scraped UES preparations.

### Effects on drinking behavior

Figure 6 shows effects of VT and IT on the drinking rate of the seawater eel. When VT was injected into the posterior cardinal vein, the drinking rate was depressed drastically (Fig. 6a) as reported previously (Ando et al. 2000a). On the other hand, IT stimulated the drinking rate (Fig. 6b). Table 1 summarizes these effects of VT and IT. In both peptides, dosage of $5 \times 10^{-10}$ mol showed the maximal effect ($P < 0.001$ and $P < 0.05$, respectively).

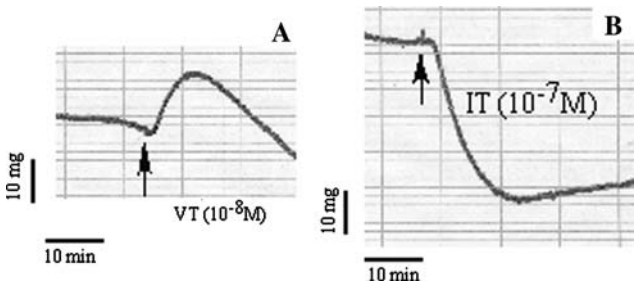


**Fig. 5** Effects of VT (a) and IT (b) on the scraped UES. Each tracing is a representative of five experiments. At each *arrow*, VT ($10^{-8}$ M) or IT ($10^{-7}$ M) was added to the bathing fluid. *Vertical bar* indicate 10 mg tension and *horizontal bar* shows 10 min

## Discussion

### Role of IT in regulating UES muscle contraction

The present study is the first report of relaxing effect of IT. Although OT is an IT homologue in mammals, OT is well-known to constrict the smooth muscle in the uterus and mammary tissues (see Gimple and Fahrenholz 2001). To our knowledge, no relaxation by OT is reported so far. In the equine esophagus, OT has no effect on both smooth and skeletal muscles (Wooldridge et al. 2002). The OT receptor is generally known to be a member of the class 1 protein-coupled receptor superfamily that activate $G_q$ and $G_i$, which in turn stimulate phospholipase C-mediated hydrolysis of phosphoinositide (Gimple and Fahrenholz 2001; Antunes-Rodrigues et al. 2004). The increased intracellular $Ca^{2+}$ levels ($[Ca^{2+}]_i$) constricts the smooth muscle in the uterus and mammary gland of rodents and humans (Gimple and Fahrenholz 2001; Antunes-Rodrigues et al. 2004; Mathew et al. 2004). This peptide also causes contraction of the aorta, basilar arteries, cremaster muscle of rats, carotid arteries of dogs, and urinary bladder of rabbit (see Wooldridge et al. 2002). Although vasodilatation in pulmonary vasculature and cerebral arteries of mammals is induced by OT, this response is associated with nitric oxide (NO) release or $V_1$-vasopressinergic receptor stimulation (see Wooldridge et al. 2002). Although NO is well-known to relax the arterial smooth muscle via cGMP, this process may not be so dominant in the eel UES, because the relaxing

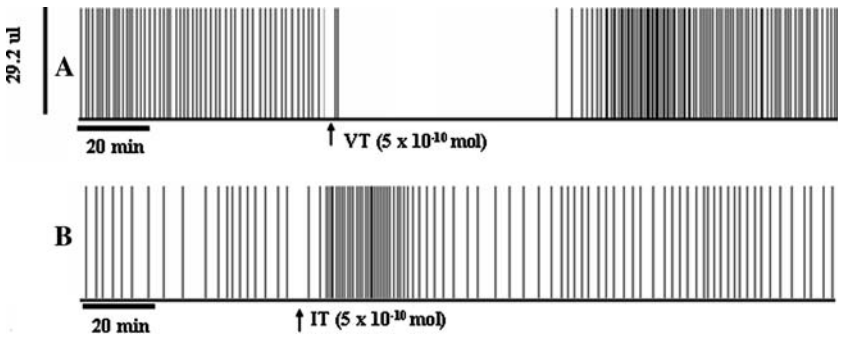


**Fig. 6** Effect of vasotocin (VT) and isotocin (IT) on water intake. At each *arrow*, $5 \times 10^{-10}$ mol VT (a) or IT (b) was administrated into the posterior cardinal vein. Each spike represents 29.2 µl. *Horizontal bar* shows 20 min

**Table 1** Effects of vasotocin (VT) and isotocin (IT) on water intake in seawater eels

| Peptides (dose) | Number of eels | Water intake (ml/30 min) | | |
|---|---|---|---|---|
| | | Before | After | Difference |
| VT | | | | |
| ($5 \times 10^{-11}$ mol) | 4 | 0.54 ± 0.08 | 0.20 ± 0.04 | −0.34 ± 0.12 |
| ($5 \times 10^{-10}$ mol) | 4 | 0.57 ± 0.06 | 0.14 ± 0.07** | −0.43 ± 0.03 |
| ($5 \times 10^{-9}$ mol) | 4 | 0.61 ± 0.11 | 0.35 ± 0.08 | −0.26 ± 0.14 |
| IT | | | | |
| ($5 \times 10^{-11}$ mol) | 5 | 0.38 ± 0.04 | 0.55 ± 0.05 | 0.17 ± 0.08 |
| ($5 \times 10^{-10}$ mol) | 5 | 0.36 ± 0.06 | 0.62 ± 0.08* | 0.26 ± 0.09 |
| ($5 \times 10^{-9}$ mol) | 5 | 0.53 ± 0.11 | 0.51 ± 0.09 | −0.01 ± 0.04 |

Water intake was measured for 30 min before and after administration of peptides. Minus signs in the difference indicate decrease in water intake and plus means enhancement

Mean ± SEM; *, ** $P < 0.05$, $P < 0.001$ compared to before value (paired $t$-test)

effect of 8BrcGMP is much less than that of 8BrcAMP (Fig. 5). The cAMP production might be through prostaglandin $E_2$ synthesis, since OT is known to cause a rapid increase in $[Ca^{2+}]_i$, to activate mitogen-activated protein kinase, and to stimulate prostaglandin $E_2$ synthesis by acting cyclooxygenase (Flint et al. 1986; Moore et al. 1988). Participation of prostaglandin E2 remains to be clarified in the future.

Regardless of the mechanisms how IT increases cAMP, the relaxing effect of IT in the UES is very important to accelerate the drinking rate in the eel. And the present study demonstrates that intravenous IT administration enhances the drinking rate (Fig. 6b, Table 1). Although the concentration of these peptides employed in this study is relatively higher for hormones, higher concentrations might be required in these UES preparations, since circulation is completely stopped in the isolated organs and peptides may be degradated by various peptidases during diffusing through the tissues to their receptors. Therefore, it is likely that these neurohypophysial peptides may regulate the UES contraction hormonally, thus modulate drinking rate of the eel.

## Structure–activity relationships of the effects of VT and IT

From the structure–activity relationships, vasopressin (VP) family appears to constrict the UES muscle, while oxytocin (OT) family seems to relax it. For these effects, whole sequence, nine amino acid residues, may be essential, since *N*- and *C*-terminal fragments, VP (4–9) and tocinoic acid, have no effect on the UES contraction (Fig. 2). The disulfide bond between position 1 and 6 and basic amino acid such as arginine at position 8 seems to be essential to constrict the UES muscle. VP might be used as a specific agonist for the VT receptor in the eel UES, because no relaxation is observed by VP (Fig. 2), and vasopressin $V_1$ receptor antagonist (H5350) blocks the VP response (data not shown). Although amino acid sequence of OT is closely similar to VT, $Arg^8$ being replaced with $Leu^8$, the effect of OT is opposite to that of VT, i.e. OT relaxes and VT constricts the UES. These results suggest that neutral amino acid at position 8 may relax the UES. Indeed, IT, where $Leu^8$ and $Gln^4$ in OT are replaced with $Ile^8$ and $Ser^4$ respectively, relaxes the UES more pronouncedly (Fig. 2). Since IT is an OT analogue in teleosts, IT may physiologically relax the eel UES and enhance the drinking rate in the seawater eel (Fig. 6b, Table 1).

## Mode of action of VT and IT

VT seems to act on the $V_1$-type receptor in the UES, since the contraction evoked by VT is completely blocked by $V_1$ receptor antagonist (Fig. 3a). Since the $V_1$ receptor is well known to increase the $[Ca^{2+}]_i$ in various cells (Antunes-Rodrigues et al. 2004), VT may enhance the muscular $[Ca^{2+}]_i$, then constrict the UES muscle in the seawater eel.

On the other hand, IT relaxes the UES, presumably through producing cAMP, because IBMX, FSK and 8BrcAMP show similar relaxation as IT (Fig. 4), and IBMX and FSK are well-known to increase intracellular cAMP, and 8BrcAMP is a cAMP analogue. The observation of no relaxation by IT after pretreatment with FSK or 8BrcAMP (Fig. 4) also supports the hypothesis that IT enhances the intracellular cAMP levels.

## Biphasic action of VT

The present study demonstrates that there are pharmacologically distinct receptors for vasotocin (VT) and isotocin (IT) in the UES preparation of the eel. In the white sucker *Catostomus commersoni*, distinct cDNAs encoding VT- and IT-receptors, respectively, have been cloned (Mahlmann et al. 1994; Housmann et al. 1995). In the eel UES preparation, these two receptors appear to be present in muscle cells and/or nerve terminals, since similar effects of VT and IT still remain after scraping the epithelial tissue (Fig. 5). VT constricts the UES muscle only at low concentrations, but relaxes at high concentrations (Fig. 2). This biphasic effect of VT can be explained, if some VT molecules also bind to the IT-receptor at high concentrations, since molecular structure of VT is similar to that of IT, and since VP-receptors are closely related to the OT- receptors (Thibonnier et al. 1999). Cross action of VT on the IT-receptor has also been demonstrated in the teleost IT-receptor expressed in the oocytes (Housmann et al. 1995). Such cross action of VT on the IT-receptor may be also present in the eel UES preparation, since the relaxing effect of IT disappears after pretreatment with high VT (data not shown), and since VT constricts the UES muscle even at high concentrations when the IT-receptor has been blocked beforehand (Fig. 3b). Such biphasic action of VT, acting both VT and IT receptors, may complicate the osmoregulatory role of VT in teleosts (Warne et al. 2002; Balment et al. 2006), since VT- and IT-receptors may act oppositely as demonstrated in the present study.

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