

Some Factors Affecting Drinking Behavior and Their Interactions in Seawater-Acclimated Eels, *Anguilla japonica*

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ABSTRACT—Intravenous administration of eel angiotensin II (eANG II), histamine (HA), serotonin (5-HT), acetylcholine (ACh) or carbachol (CCh), mammalian substance P (mSP) and isoproterenol (β -adrenoceptor agonist) enhanced drinking rate in the seawater eels. The dipsogenic effects of HA and 5-HT seem to be due to ANG II synthesis, because these effects were completely blocked by captopril, an inhibitor of angiotensin converting enzyme (ACE). Captopril blocked eANG I effect, but not eANG II effect, suggesting existence of ACE in seawater eels. 800 μ l Hemorrhage also enhanced water intake, and this effect was completely blocked by captopril. Therefore, it is likely that blood withdrawal stimulates renin-angiotensin system (RAS) in seawater eels. Effects of ACh, CCh and mSP were not inhibited by captopril, suggesting separate action of these regulators from ANG II synthesis. Isoproterenol action was partially inhibited by captopril, suggesting existence of some β -adrenoceptors other than the RAS. On the other hand, intravenous eel atrial natriuretic peptide (eANP), arginine vasotocin (AVT), human vasoactive intestinal peptide (hVIP), mammalian bradykinin (mBK), eel intestinal pentapeptide (EIPP), cholecystokinin (CCK-8), and phenylephrine (α -adrenoceptor agonist) depressed the drinking rate. In the presence of mBK, HA and 5-HT enhanced water intake similarly as in the absence of mBK. Plasma hyperosmolarity also reduced drinking. Although the *in vivo* system is so complicated and many regulators are involved in the drinking behavior, a possible regulatory mechanisms are proposed. Compared to mammalian results, eels seem to be a suitable model for analyzing drinking mechanisms in vertebrates.

INTRODUCTION

Maintenance of blood homeostasis is essential for life of vertebrates. Especially, water intake is most important for terrestrial vertebrates or seawater teleosts. However, the mechanisms how the drinking is controlled are complicated and not clear yet even in mammals (Bourque *et al.*, 1994; Fitzsimons, 1998). Looking from another viewpoint, the obscurity in mammals may be due to complexity in their drinking behavior. After perception of thirst, they must seek for water, ingest water into their mouth, and finally swallow. For analyzing drinking mechanisms, more simple model may be needed. As a candidate for such model, fishes will be suitable, because they hold water constantly in their mouth for respiration. They can swallow immediately after perceiving thirst, and may skip seeking and ingestion.

Among fishes, eels are a suitable subject for study, because their drinking rate can be measured continuously using esophagus cannulations as developed by Hirano (1974). However, his system is for fresh-water eels and not for sea-

water eels. Seawater eels must absorb water from the diluted sea water in the intestine. Without water absorption across the intestine, the seawater eels die (Takei *et al.*, 1998). Therefore, a new system was developed by Takei *et al.* (1998). Simplifying their method, effects of various regulators on the drinking behavior were examined in this study. The present study was aimed to classify various regulators into dipsogens and antidipsogens. Most dipsogens in mammals also enhanced the drinking rate in eels. Furthermore, in the present study, interactions between these dipsogens were examined pharmacologically.

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 before use without food. For measurement of water intake, the esophagus was cannulated with a vinyl tube (o.d. 2.0 mm) as described previously (Ando and Nagashima, 1996). The cannula was connected to a drop counter (PG-602, Keyence, Osaka, Japan) for continuous recording of the drinking rate. Each drop was recorded as a spike on a chart recorder (EPR-121A, TOA, Tokyo, Japan). One drop was 27.8 μ l. Another tube (o.d. 1.1 mm) was inserted into the stomach to apply 0.2 mol l⁻¹ NaCl solution, which was determined from the NaCl concentration at the

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esophagogastric junction in seawater eels (Ando and Nagashima, 1996). The perfusion through the cannula was made with a peristaltic pump (MS-1 Reglo 160, Ismatic, Zurich, Switzerland) controlled by an electric stimulator (SEN-3201, Nihon Koden, Tokyo), which was connected to the drop counter. Through this system, the swallowed sea water can be reintroduced into the stomach.

A third cannula (SP-10, Natume, Tokyo) filled with heparinized saline (100 IU/ml) was inserted into the posterior cardinal vein. Through the cannula, various regulators were administered into the blood. All reagents were dissolved in 0.9% NaCl solution (vehicle), and 100 μ l of their appropriate concentrations was injected slowly (100 μ l/min) into the vein, followed by an injection of 50 μ l vehicle to push out whole reagent remaining in the cannula. In case of administration of hyperosmotic NaCl and sucrose solutions, 200 μ l was injected for 2 min. 200 μ l injection of vehicle at the same rate did not alter the drinking rate significantly. 800 μ l blood was withdrawn from the posterior caudal vein with microinjector (IM-1, Narishige, Tokyo) for 30 min.

After operation, the incision was closed using silk suture and all cannulae were fixed to the body with threads, then the eels were transferred to a plastic trough of the same size as the eel. Well-aerated sea water was circulated continuously through the trough at room temperature (20–23°C).

Eel angiotensin II ([Asp¹, Val⁵]ANG II, [Asn¹, Val⁵]ANG II), [Sar¹, Ile⁵, Ala⁶]ANG II, [Sar¹, Ile^{5,8}]ANG II, non-sulfated cholecystokinin-related peptide (CCK-8), human vasoactive intestinal peptide (hVIP), mammalian substance P (mSP), and mammalian bradykinin (mBK) were obtained from Peptide Institute Inc., Osaka, Japan. Tricain methanesulfonate (MS222), Captopril, cimetidine, cyproheptadine, 5-hydroxytryptamine creatine sulfate (5-HT), phenylephrine hydrochloride, carbamylcholine chloride (CCh), acetylcholine (ACh), and sheep prolactin (sPRL) were purchased from Sigma, St. Louis, USA. Histamine dihydrochloride, thioperamide maleate, PD 123319 ditrifluoroacetate, CGP 42112 were obtained from Research Biochemicals International, Natick, USA. Eel angiotensin I ([Asn¹, Val⁵, Gly⁹]ANG I) and angiotensin III ([Val⁴]ANG III) were purchased from Peninsula Laboratories, Belmont, USA. Heparin sodium (Katayama Chemical, Osaka), isoproterenol hydrochloride (Nakarai Tesque, Kyoto, Japan) and Atropine sulfate (Merck, Darmstadt, Germany) were purchased commercially. Losartan potassium (Banyu, Tokyo, Japan), Exp 3174 (Dupont, Wilmington, USA) and CV11974 (Takeda Chemical Industries, Tokyo) were kindly supplied. Eel atrial natriuretic peptide (eANP) was kindly gifted by Prof. Y. Takei, Ocean Research Institute, University of Tokyo.

Statistical analyses of results were performed using Mann-Whitney *U*-test. Results are given as mean \pm S.E.M. and considered significant at $P < 0.05$.

RESULTS

Effect of various eel angiotensins

The operated eels started drinking sea water immediately after recovery from anesthesia. The drinking rate was high initially but reached a relatively steady level after 20 hr. Thus all experiments were started 20 hr after operation. When 0.9% NaCl solution was injected into the vein, the drinking rate was not significantly altered (0.21 ± 0.08 ml/15 min vs 0.19 ± 0.05 ml/15 min, $n=5$).

Although eels have two kinds of angiotensin II (eANG II), [Asp¹]eANG II and [Asn¹]eANG II (Hasegawa *et al.*, 1983), both peptides had qualitatively similar effects. After administration of eANG II, the drinking rate was raised initially for 15–30 min, and then inhibited for more than 1 hr. Similar biphasic effect of ANG II has been reported in freshwater and 1/3 sea-

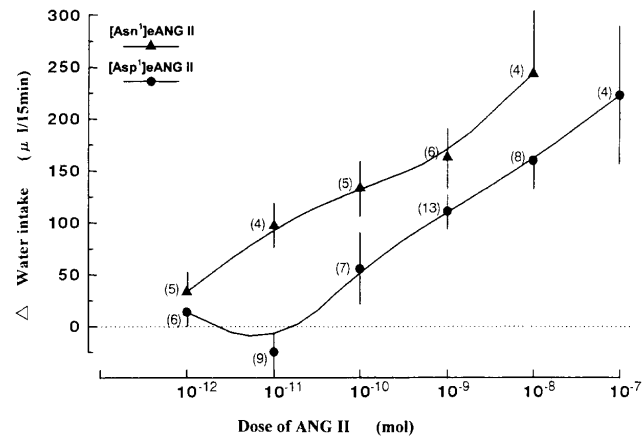


Fig. 1. Dose-response curve of the dipsogenic effect of eel angiotensins. Enhancement of water intake (Δ water intake) was plotted against dosage of two kinds of eel angiotensin II ([Asn¹]eANG II, \blacktriangle ; [Asp¹]eANG II, \bullet). Both eANG II were injected into the posterior cardinal vein. Water intake was obtained subtracting 15 min water intake before injection from 15 min water intake after injection. Each point and vertical bar indicate the mean and S.E.M.. Number of eels is shown in parentheses.

water eels (Takei *et al.*, 1979; 1988). Fig. 1 shows comparison between the dipsogenic effects of these 2 peptides. [Asn¹]eANG II was nearly 10 times potent than [Asp¹]eANG II. Eel ANG III (Arg-Val-Tyr-Val-His-Pro-Phe) also enhanced water intake initially, but the potency was 1/100 compared to [Asn¹]eANG II (data not shown). The dipsogenic effect of [Asn¹]eANG II was not inhibited by cimetidine nor atropine.

Eel ANG I (Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Gly-Leu) also enhanced drinking rate similarly as [Asn¹]eANG II. However, this effect of eANG I was completely blocked by captopril (10^{-6} mol), an inhibitor of angiotensin converting enzyme (ACE) (Fig. 2).

To determine receptor type bound with eANG II, various ANG II antagonists were pretreated before application of [Asn¹]eANG II. However, neither AT₁ receptor antagonists (saralasin, losartan, Exp 3174, CV 11974) nor AT₂ receptor antagonists (PD 123319, CGP42112) blocked the [Asn¹]ANG II effect (data not shown). These results confirm previous observations in fishes (Nishimura *et al.*, 1978; Tierney *et al.*, 1997).

Effect of other dipsogens

Beside angiotensins, histamine (HA), serotonin (5-HT), isoproterenol (β -adrenoceptor agonist), acetylcholine (ACh), carbachol (CCh, a cholinergic agonist), and mammalian substance P (mSP) also enhanced drinking rate (Table 1). Similar dipsogenic effect was observed after injection of sheep prolactin (sPRL, 10^{-9} mol) (data not shown). The dipsogenic effect of HA was dose-dependent from 10^{-12} to 10^{-7} mol, and was completely blocked by cimetidine, a H₂ receptor antagonist (Fig. 3b). Similar blockage was also observed after treatment with thioperamide, another H₂ receptor antagonist, but not with cyproheptadine, a H₁ receptor antagonist. These results indicate existence of H₂-type receptor in the eel. After treatment with captopril, the effect of HA was also blocked

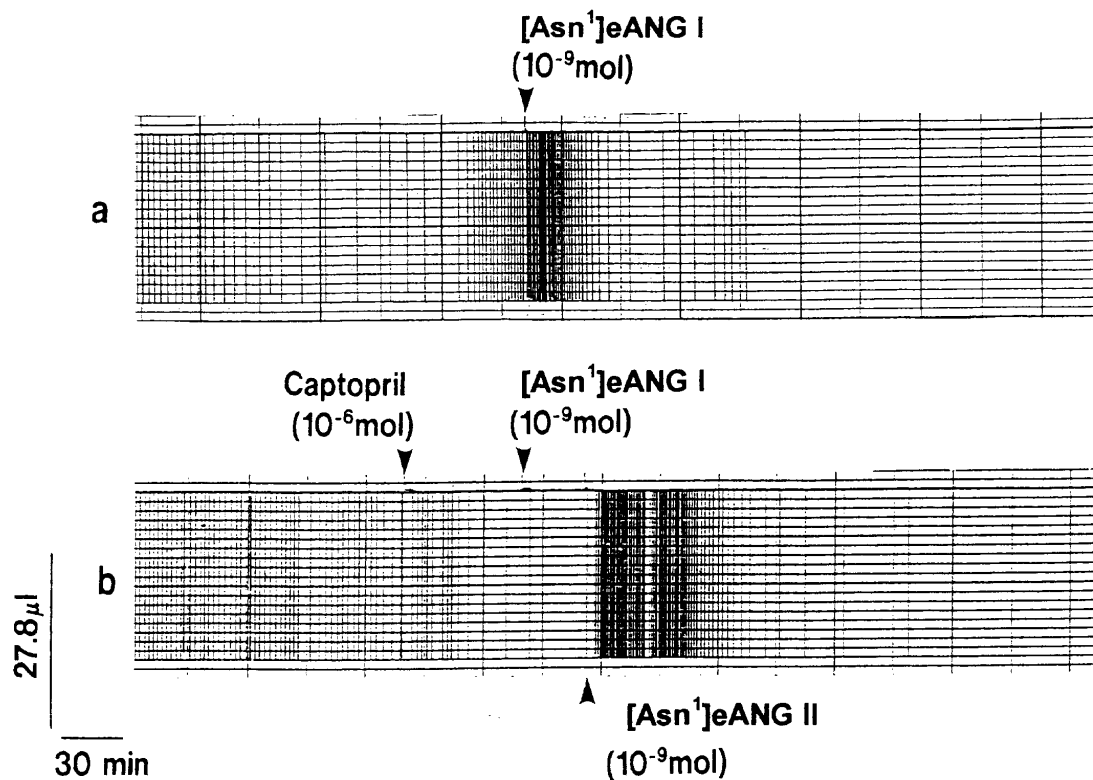


Fig. 2. Involvement of angiotensin converting enzyme in drinking. **a.** Effect of eel angiotensin I ([Asn¹]eANG I). After injection of [Asn¹]eANG I into the posterior cardinal vein (arrow head), the water intake increased initially then decreased as in case of [Asn¹]eANG II. **b.** Captopril blocks the effect of [Asn¹]eANG I, but not [Asn¹]eANG II. After pretreatment with captopril (first arrow head), [Asn¹]eANG I was administered intravenously (second arrow head). At the third arrow head, [Asn¹]eANG II was injected into the posterior cardinal vein.

Table 1. Effects of various dipsogens on water intake in seawater eels. Water intake was measured for 15 min before and after administration of dipsogens

Dipsogens (dosage)	No. of eels	Water intake (ml/15min)		
		before	after	enhancement
[Asp ¹]eANG II (10 ⁻⁹ mol)	13	0.14 ± 0.02	0.25 ± 0.03***	0.11 ± 0.02
[Asn ¹]eANG II (10 ⁻⁹ mol)	10	0.17 ± 0.02	0.34 ± 0.03***	0.18 ± 0.02
HA (10 ⁻⁹ mol)	5	0.12 ± 0.03	0.32 ± 0.03***	0.20 ± 0.02
5-HT (10 ⁻⁸ mol)	4	0.12 ± 0.05	0.41 ± 0.08**	0.29 ± 0.03
CCh (10 ⁻⁸ mol)	4	0.04 ± 0.02	0.35 ± 0.10*	0.31 ± 0.04
mSP (10 ⁻⁹ mol)	7	0.13 ± 0.03	0.30 ± 0.02**	0.17 ± 0.04
Isoproterenol (10 ⁻⁹ mol)	4	0.11 ± 0.04	0.45 ± 0.08***	0.34 ± 0.09

Mean ± S.E.M.. *, **, *** P < 0.05, P < 0.01, P < 0.001 compared to before value (Mann-Whitney U-test).

completely (Fig. 3c). Even in the presence of cimetidine, dipsogenic effect of [Asn¹]eANG II still remained, suggesting that ANG II does not act through HA release.

After administration of 5-HT (10⁻⁸–10⁻⁷ mol), the drinking rate was enhanced initially, followed by an inhibition (Fig. 4a). This dipsogenic effect of 5-HT was also completely blocked by captopril (Fig. 4b), suggesting that 5-HT acts through ANG II synthesis. The 5-HT effect was not inhibited by cimetidine (Fig. 4c).

The effect of CCh (10⁻⁹–10⁻⁸ mol) was not inhibited by captopril (10⁻⁶ mol), but inhibited by atropine (10⁻⁶ mol) (data not shown), suggesting existence of muscarinic ACh receptor in the eel. The dipsogenic effect of mSP (10⁻⁹–10⁻⁸ mol) was not inhibited by captopril (10⁻⁶ mol), and the effect of isoproterenol (10⁻⁹–10⁻⁸ mol) was only partially inhibited by captopril (10⁻⁶ mol) (data not shown).

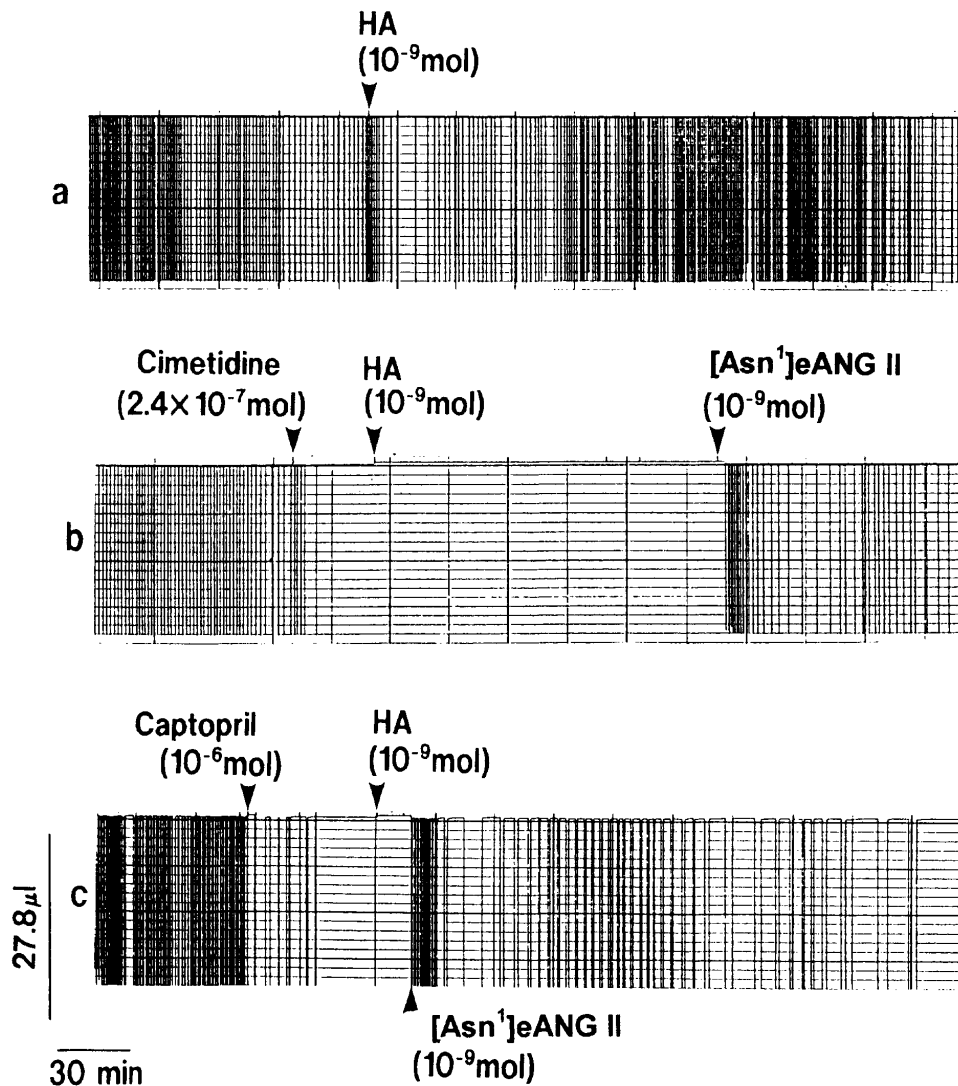


Fig. 3. Effect of histamine (HA) on drinking. HA was injected into the posterior cardinal vein (a). After intravenous administration of cimetidine, the dipsogenic effect of HA was completely blocked but the effect of [Asn¹]eANG II was still remained (b). After pretreatment with captopril, HA effect was also blocked but eANG II effect remained (c).

Effect of blood volume and osmolarity

When 800 μ l blood was withdrawn from the posterior cardinal vein, drinking rate was enhanced. However, the effect of blood withdrawal was inhibited by a pretreatment with captopril (10^{-6} mol). Figure 5 shows effect of hemorrhage in the absence or presence of captopril. In the presence of captopril, hemorrhage did not enhance the water intake at all.

When concentrated NaCl or sucrose solution was injected into the vein, the water intake decreased. The decrease was dose-dependent (Fig. 6).

Effect of antidipsogens

Eel atrial natriuretic peptide (eANP, 10^{-10} – 10^{-8} mol), arginine vasotocin (AVT, 10^{-10} – 10^{-8} mol), and eel intestinal pentapeptide (EIPP, Gly-Phe-Trp-Asn-Lys, 10^{-9} – 10^{-8} mol) isolated from eel intestine (Uesaka *et al.*, 1991) inhibited the drinking rate (Fig. 7). Similar antidipsogenic effect was observed after cholecystokinin (CCK-8, 10^{-9} – 10^{-8} mol), phenylephrine (α -adrenoceptor agonist, 10^{-9} – 10^{-8} mol), human vasoactive intestinal peptide (hVIP, 10^{-10} – 10^{-8} mol), and mammalian bradykinin (mBK, 10^{-10} – 10^{-8} mol). Even after pretreatment

with 10^{-8} mol mBK, HA and 5-HT enhanced the drinking rate similarly as in the absence of mBK (data not shown).

DISCUSSION

The drinking rate in seawater eels was enhanced by eANG II, HA, 5-HT, ACh (CCh), mSP, sPRL and isoproterenol, while inhibited by eANP, AVT, hVIP, EIPP, mBK, CCK-8 and phenylephrine (Table 2). Since these dipsogens, except for SP, are also known to increase water intake in mammals (Fitzsimons, 1998), the drinking behavior in the seawater eels may be controlled similarly as in mammals. ANP is also known to inhibit water intake induced by dehydration or ANG II in the rat (Antunes-Rodrigues *et al.*, 1985). However, the effect of ACE inhibitor (captopril) is reverse: inhibitory in eels (present study) and stimulatory in mammals (Fitzsimons, 1998).

Among dipsogens examined in the present study, HA and 5-HT seem to act through ANG II synthesis, since the effects of HA and 5-HT were completely blocked by a pretreatment with captopril, an ACE inhibitor. Although captopril is also

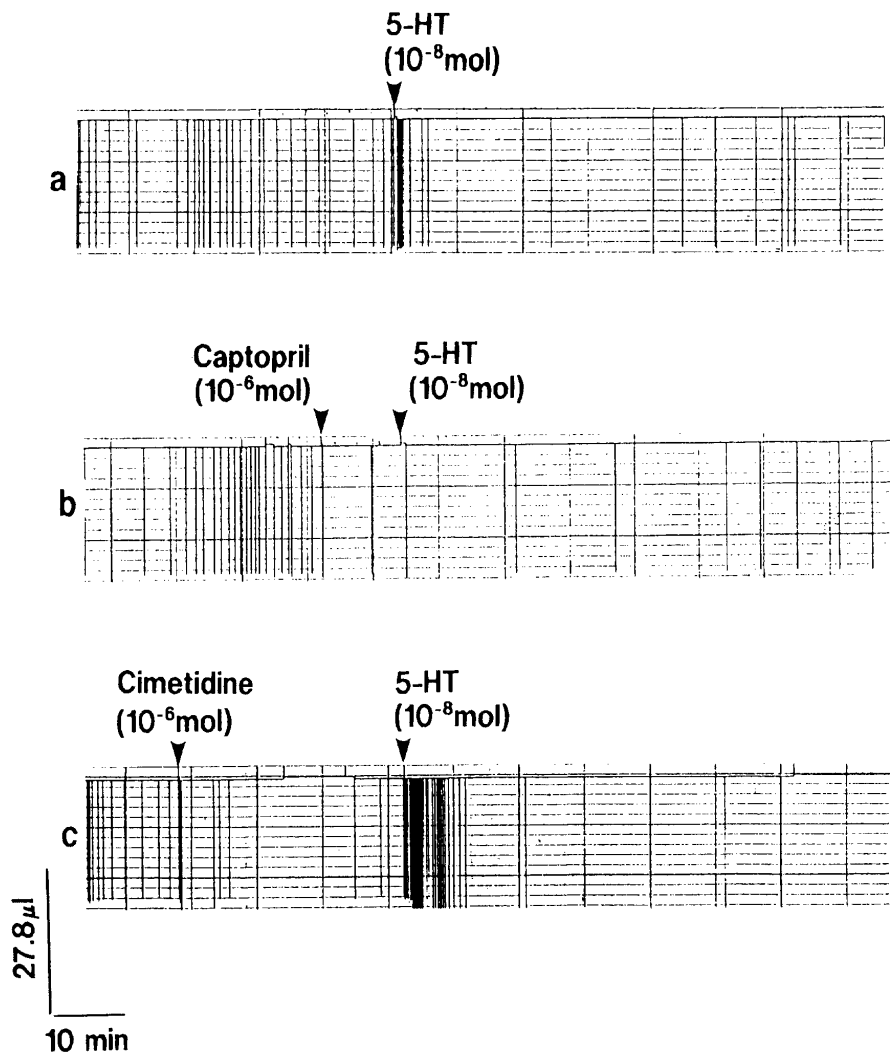


Fig. 4. Effect of serotonin (5-HT) on drinking. 5-HT was injected into the posterior cardinal vein (a). In the presence of captopril, the dipsogenic effect of 5-HT was completely blocked (b). After blocking histamine receptor(s) with cimetidine, 5-HT effect was not inhibited (c).

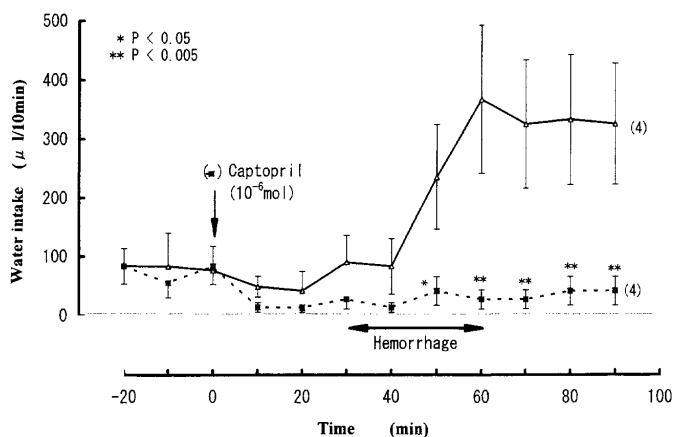


Fig. 5. Effect of blood withdrawal on drinking. Water intake was measured every 10 min. Blood withdrawal (800 μl hemorrhage, horizontal arrow) was performed between 30 and 60 min (△, ■). After intravenous administration of captopril (■, first arrow), water intake was not enhanced by hemorrhage. Each point and vertical bar indicate the mean value and S.E.M. (n=4). *P<0.05, **P<0.005 compared to the control hemorrhage without captopril (Mann-Whitney U-test).

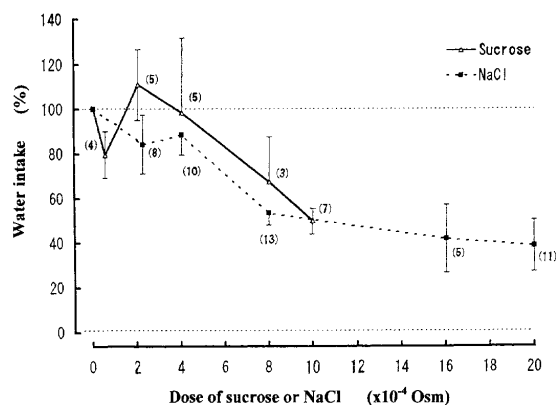


Fig. 6. Dose-response curve of effect of plasma hyperosmolarity on water intake. Plasma osmolarity was increased by intravenous injection (200 μl) of hypertonic NaCl (■) or sucrose (△). The dosage is presented as osmol (Osm). Water intake is shown as a ratio (%) of the 15 min drinking after injection of sucrose or NaCl to those before administration. Each point and vertical bar indicate the mean value and S.E.M.. Number of eels is shown in parentheses.

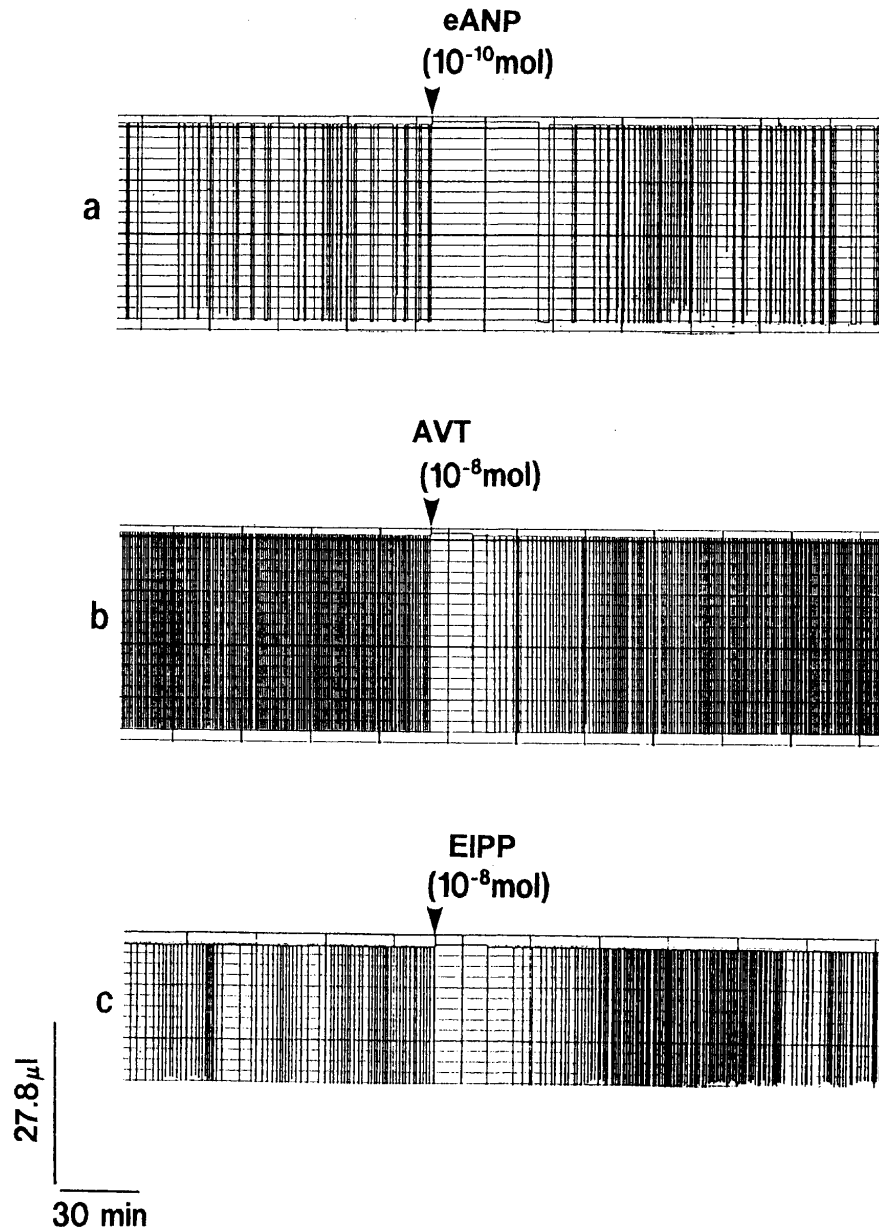


Fig. 7. Effect of various antidipsogens. **a.** Effect of eel atrial natriuretic peptide (eANP). **b.** Effect of arginine vasotocin (AVT). **c.** Effect of eel intestinal pentapeptide (EIPP).

Table 2. Regulators affecting drinking behavior in seawater eels

Dipsogens	Antidipsogens
eANG II	eANP
HA	AVT
5-HT	hVIP
ACh (CCh)	EIPP
mSP	mBK
sPRL	CCK-8
Isoproterenol	Phenylephrine

known to inhibit kininase thus increase kinin levels (Campbell, 1987, Olson, 1992), high concentration of mBK did not inhibit HA and 5-HT actions in the eels. The existence of ACE in the seawater eels is supported by a result that eANG I effect is blocked by captopril but eANG II effect not. Renin-angiotensin system (RAS) in teleosts has been demonstrated previously (see Olson, 1992). Since RAS exists in eels (Sokabe and

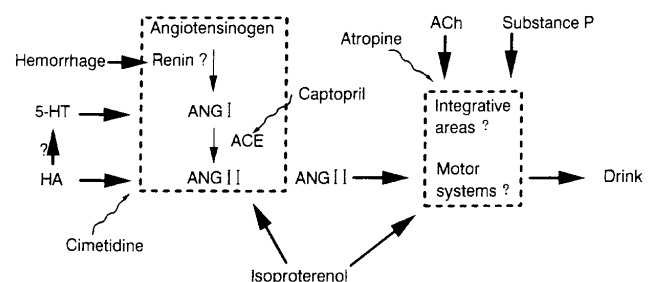


Fig. 8. A possible model for regulating drinking behavior in seawater-acclimated eels. Left dotted box shows renin-angiotensin system (RAS) in the periphery, right dotted box means integrative area or motor system in the brain. Arrows indicate the site to which dipsogens act. Waved arrows show acting site of inhibitors.

Ogawa, 1974; Henderson *et al.*, 1976; Tierney *et al.*, 1995), the hemorrhage may activate the RAS, thus activate ANG II synthesis, and the synthesized ANG II may enhance drinking (Fig. 8). The blockage of hemorrhage-induced drinking by

captopril supports this explanation. Increased plasma ANG II level after hemorrhage is already observed in eels (Takei, 1988). ACh and SP seem to act separately from RAS, since the effects of these regulators are not inhibited by captopril. β -Adrenoceptors may exist in RAS and in others, since the effect of isoproterenol is only partially inhibited by captopril. Although the present results appear to suggest that captopril inhibits RAS, other sites for captopril action can not be ruled out.

ANG II receptor in eels seems to be distinct from mammalian types, since mammalian AT_1 and AT_2 -receptor antagonists did not inhibit the eANG II action. Similar no antagonistic action of salarasin or losartan has been observed in eels (Nishimura *et al.*, 1978) or elasmobranchs (Tierney *et al.*, 1997). N-terminal Asn in the eel ANG II seems to be important for the dipsogenic action of the peptide, since replacement of Asn with Asp([Asp¹]eANG II) or deletion of Asn (eANG III) lowers dipsogenic potency.

The effects of eANG II were always biphasic, initial dipsogenic and secondary antidipsogenic. Because *in vivo* system is so complicated, many factors may be involved in the biphasic phenomena. ANG II may stimulate catecholamine release and make hypertension as in other teleosts (Platzack *et al.*, 1993; Perry *et al.*, 1999), and the hypertension may inhibit the drinking as observed by Hirano and Hasegawa (1984). However, it can be explained simply by a secretion of antidipsogens. Antidipsogenic effect of ANP has been reported in eels previously (Takei and Balment, 1993; Tsuchida and Takei, 1998). In addition, eel plasma ANP level is elevated after infusion of ANG II (Tsuchida and Takei, 1999).

In contrast to mammalian (Fitzsimons, 1979), avian (Kaufman and Peters, 1980; Kobayashi and Takei, 1982) and reptilian (Fitzsimons and Kaufman, 1977) cases, plasma hyperosmolarity reduces water intake in seawater eels. Similar inhibition by hyperosmolarity has been reported in freshwater and 1/3 seawater eels (Takei *et al.*, 1979; 1988). The mechanisms how the plasma hyperosmolarity depresses drinking rate in the eel are not known yet. Administration of hypertonic solution into eels increases plasma ANG II level (Takei *et al.*, 1988). However, enhanced secretion of antidipsogens, such as ANP, AVT, VIP, BK, catecholamines or EIPP, might explain this phenomenon simply. In fact, plasma hyperosmolarity increases plasma ANP level in eels (Kaiya and Takei, 1996). Although the mechanisms are not clear yet, this phenomenon seems to be significant physiologically, especially in seawater eels, because the lowered drinking rate accelerates desalination of the ingested sea water through their esophagus, more diluted sea water enters into the gastrointestinal tract (Ando and Nagashima, 1996), which enhances water absorption across the intestine (Skadhauge, 1969).

Previously, we demonstrated that intestinal Cl^- reduced drinking rate in seawater eels and suggested involvement of humoral mediator(s) released from the intestine and acting on the brain (Ando and Nagashima, 1996). EIPP can be a candidate for such mediators, because the peptide is isolated

from the eel intestine (Uesaka *et al.*, 1991) and reduces drinking rate (present study).

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