Relative Potency of Three Homologous Natriuretic Peptides (ANP, CNP and VNP) in Eel Osmoregulation

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Relative Potency of Three Homologous Natriuretic Peptides (ANP, CNP and VNP) in Eel Osmoregulation

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ABSTRACT—Evidence has accumulated that atrial natriuretic peptide (ANP) plays important roles in seawater adaptation in eels. However, the roles of the other two natriuretic peptides (CNP and VNP) in osmoregulation have not been examined yet. In the present study, the effects of homologous ANP, CNP and VNP were compared on plasma Na^+ concentration (an indicator of plasma osmolality), hematocrit (an approximate indicator of blood volume) and drinking rate in freshwater- and seawater-adapted eels. In seawater eels, ANP and VNP, but not CNP, infused at 5 pmol/kg/min decreased plasma Na^+ concentration and drinking rate and increased hematocrit. In freshwater eels, ANP and VNP failed to decrease plasma Na^+ concentration but increased hematocrit to the same extent as in seawater eels. Inhibition of drinking was not detectable in freshwater eels because of little drinking before NP infusions. These results show that the effects of NPs on plasma Na^+ concentration, drinking rate and hematocrit are mediated by NPR-A, since only ANP and VNP that bind with higher affinity to NPR-A are effective in seawater eels. The mechanisms of regulation of plasma Na^+ concentration and hematocrit are unknown, but NPR-A is present in the responsible tissues for regulation of hematocrit in both freshwater and seawater eels. However, NPR-A may be absent in the tissues of freshwater eels that are responsible for regulation of plasma Na^+ concentration.

Key words: atrial natriuretic peptide, ventricular natriuretic peptide, C-type natriuretic peptide, drinking, plasma sodium concentration

INTRODUCTION

Accumulating data indicate that natriuretic peptides (NPs) are involved in cardiovascular and body fluid homeostasis in vertebrates (Farrell and Olson, 2000; Loretz and Pollina, 2000; Takei, 2000a). The NP family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) in tetrapods. ANP and BNP are circulating hormones secreted mainly from the heart, while CNP is basically a paracrine factor in the brain and periphery (Fowkes and McArdle, 2000; Takei, 2000a). Three NP receptors have been identified in mammals, of which NPR-A and NPR-B are members of the membrane-bound guanylyl cyclases and are involved in biological actions of the NP family. Another type, NPR-C, lacks the enzyme domain and may be involved in the clearance of NPs (Maack, 1992). ANP and BNP bind to NPR-A preferentially and CNP is specific for NPR-B, while all three NPs have high affinity to NPR-C.

In the eel, ANP, ventricular natriuretic peptide (VNP), CNP and four NP receptors (NPR-A, -B, -C, and -D) have been identified (Takei, 2000a). VNP is a teleost-specific NP with a long C-terminal sequence extending from the intramolecular ring (Takei *et al.*, 1991). VNP may be unique in its function because it has high affinity not only to NPR-A but also to NPR-B equivalent to 1/10 of CNP (Katafuchi *et al.*, 1994). NPR-D is a new type of guanylyl cyclase-deficient receptor and has been identified only in the eel thus far (Kashiwagi *et al.*, 1995).

Previous studies showed that ANP is secreted soon after transfer of freshwater eels to seawater (Kaiya and Takei, 1996), and acts on various osmoregulatory organs to decrease NaCl in the body, thereby promoting adaptation to seawater (Takei, 2000a). In contrast, expressions of CNP gene as well as plasma CNP level are greater in freshwater eels than in seawater eels (Takei *et al.*, 2001). NPR-B messages are more abundantly expressed in freshwater eels than in seawater eels (Katafuchi *et al.*, 1994) and CNP increases cyclic guanosine 3', 5'-monophosphate (cGMP) accumulation in the membrane fraction of freshwater eel gills but not of seawater eel gills (Mishina and Takei, 1997). These results indicate a role of CNP in freshwater adaptation. However, nothing is known about the role of VNP in osmoregulation except for the vascular action (Takei *et al.*, 1991).

In the present study, the relative potency of three eel NPs: ANP, CNP and VNP, were compared in water and electrolyte

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metabolism in freshwater and seawater eels using an *in vivo* infusion system. The parameters measured were drinking rate, plasma Na^+ concentration and hematocrit. Drinking was measured because it is essential for adaptation of eels to hyperosmotic environments (Takei *et al.*, 1998). Plasma Na^+ concentration and hematocrit were used to assess the changes in two major parameters of body fluid balance: plasma osmolality and blood volume, respectively. The differences in potencies of three NPs in different actions and in different osmotic environments are discussed in relation to the involvement of different NP receptors.

MATERIALS AND METHODS

Animals: Cultured, immature eels, *Anguilla japonica*, of mixed sexes were purchased from a local dealer. They were maintained without feeding in seawater or freshwater tanks for at least two weeks before use. Water in the tank was continuously circulated, aerated, and regulated at 18°C. Eels weighed 198.3±7.6 g (n=5; seawater) and 200.7±8.2 g (n=6; freshwater) at the time of surgery.

Drugs: Three homologous NPs were dissolved in distilled water, aliquoted and frozen at –20°C until use. On the day of experimentation, the stock solution (10^{-4} M) was diluted with isotonic saline (0.9% NaCl) containing 0.01% Triton X-100 (Wako pure chemicals industries, Osaka).

Surgical procedures: Eels were anesthetized by immersion in 0.1% (w/v) tricaine methanesulfonate (Sigma, St. Louis, MO, USA) for 15 min. Vinyl tubes (o.d.: 1.5 mm, Becton Dickson Co., Sparks, MD, USA) were inserted into the esophagus and stomach of eels as described previously (Takei *et al.*, 1998). In addition, a polyethylene tube (o.d.: 0.8 mm, SP31, Natsume Seisakusho Co., Tokyo) was inserted into the ventral aorta for infusion of NPs and blood collection. After surgery, eels were placed in a plastic trough through which aerated seawater or fresh water continuously circulated at 18°C. The troughs were covered with a black vinyl sheet to minimize visual stress during the experiments.

Synchronized drop counter and pulse injector system: The catheter placed in the esophagus was connected to a drop counter for continuous measurement of drinking rate. It has been shown that seawater dropped from the catheter is diluted to 80% by esophageal desalination in seawater eels (Tsuchida and Takei, 1998). Thus 80% seawater of the same volume as a drop was injected into the stom-

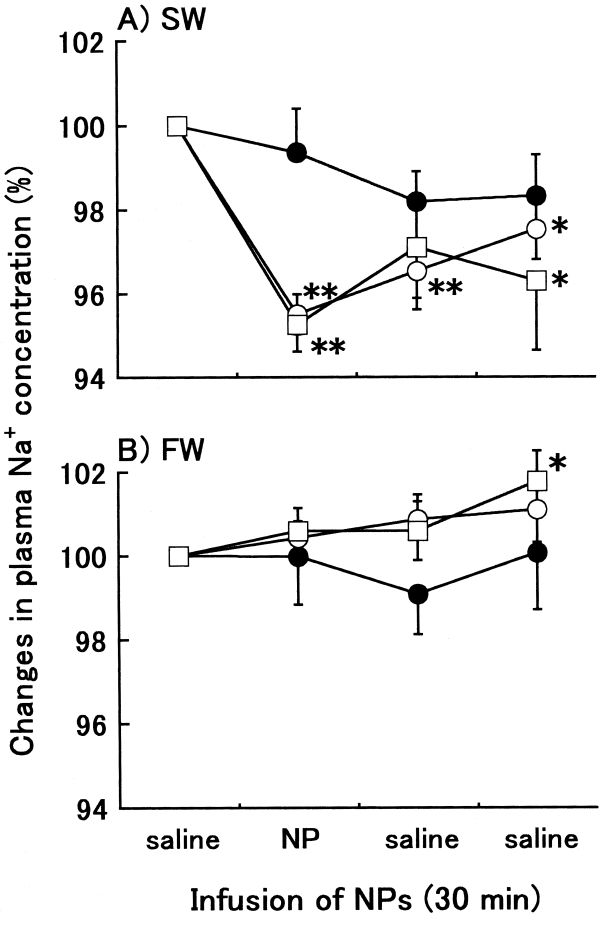

Fig. 1. The time course of changes in plasma Na^+ concentration during 30-min infusions of homologous ANP (○), CNP (●) and VNP (□) (5 pmol/kg/min) in seawater eels (A; n=5) and freshwater eels (B; n=6). Saline was infused before and after NP infusions. Data were expressed as percentage to the values before NP infusion. Values represent means±SE. *p<0.05, **p<0.01 compared with the value before NP infusion by Fisher's PLSD test.

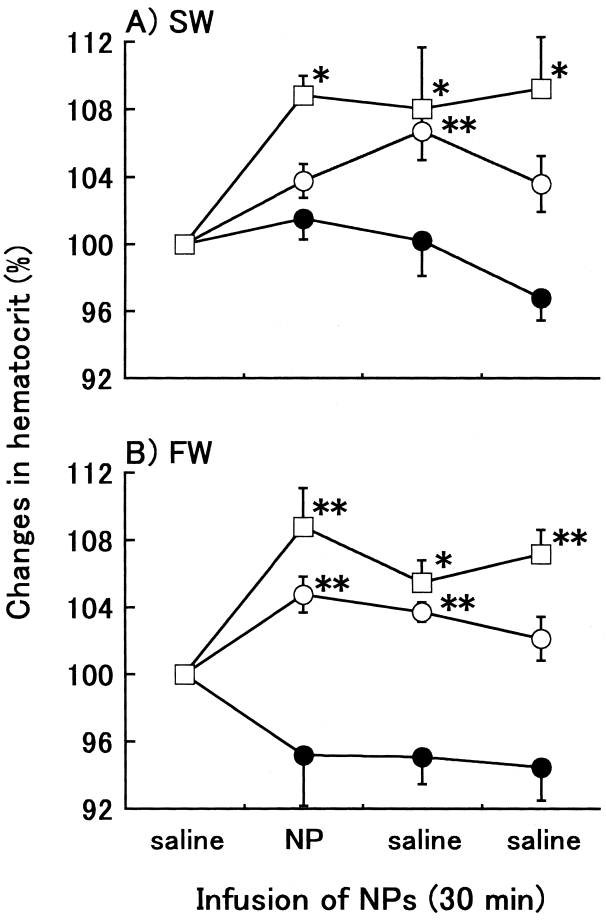

Fig. 2. The time course of changes in hematocrit during 30-min infusions of homologous ANP (○), CNP (●) and VNP (□) (5 pmol/kg/min) in seawater eels (A; n=5) and freshwater eels (B; n=6). Saline was infused before and after NP infusions. Data were expressed as percentage to the values before NP infusion. Values represent means±SE. *p<0.05, **p<0.01 compared with the value before NP infusion by Fisher's PLSD test.

ach by a pulse injector synchronized with the drop counter.

Experimental protocol: On the day following surgery (more than 12 hr later), eels were infused with one of three homologous NPs (ANP, CNP or VNP) at 5 pmol/kg/min for 30 min successively in random order. This dose was shown to be sufficient to ensure the renal and vascular NP actions (Takei and Kaiya, 1998). Before and after each NP infusion, a control infusion of saline was made for 30 min and 1 hr, respectively. A period of 1 hr without infusion was placed between different NP infusions to exclude the influence of previous infusion. Infusion rate was 100 μl/30min, whereas blood was collected into a capillary (40 μl) every 30 min during infusion for measurements of hematocrit and plasma Na^+ concentration. At the time of exchange of infusates, 50 μl of the next infusate was injected as a prime to obtain stable plasma NP levels quickly. The Na^+ concentration was determined in an atomic absorption spectrophotometer (Z5300 Hitachi, Tokyo). All measurements were made in triplicate.

Statistical analysis: All data were expressed in terms of percentages from the value of initial saline infusion. The ANOVA was used to determine the changes in time course data, which was followed by the Fisher's Protected Least Significant Difference (Fisher's PLSD) test at each time point. The Mann-Whitney's U-test was used for comparison between different groups. Significance was set at $p<0.05$. All results were expressed as means±SE.

RESULTS

Plasma Na^+ concentration: Plasma Na^+ concentration was 171.0±4.9 mM in seawater eels and 153.1±1.3 mM in fresh-

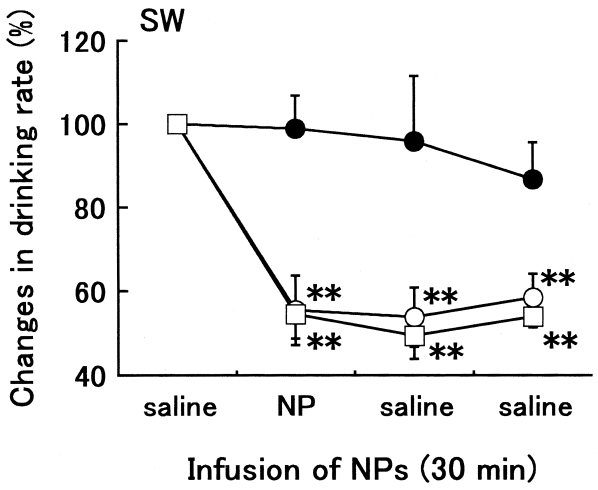

Fig. 3. The time course of changes in drinking rate during 30-min infusions of homologous ANP (○), CNP (●) and VNP (□) (5 pmol/kg/min) in seawater eels (n=5). Saline was infused before and after NP infusions. Data were expressed as percentage to the values before NP infusion. Freshwater eels did not drink before NP infusion. Values represent means±SE. *p<0.05, **p<0.01 compared with the value before NP infusion by Fisher's PLSD test.

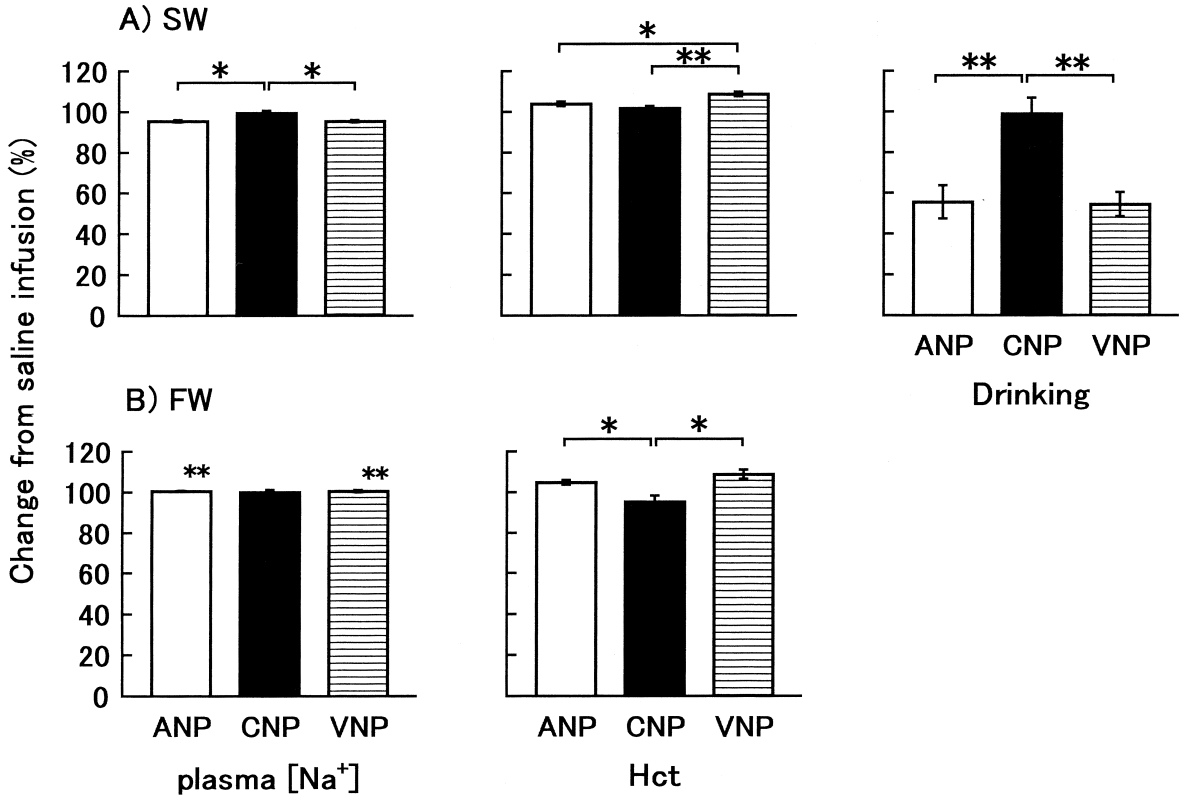

Fig. 4. Comparison of the effects of ANP (open bar), CNP (filled bar) and VNP (hatched bar) on plasma Na^+ concentration, hematocrit and drinking rate in seawater eels (A; n=5) and freshwater eels (B; n=6). Data were expressed as percentages from the value before NP infusions. Data on drinking rate in freshwater eels are not included because they did not drink before NP infusion. Values represent means±SE. *p<0.05, **p<0.01 compared among NPs by Mann-Whitney's U-test. The asterisks on the shoulder of the bar of freshwater eels show significant differences from the values of seawater eels.

water eels before infusion of NPs. Plasma Na^+ concentration decreased significantly during ANP or VNP infusion in seawater eels compared with the value of saline-infused controls before NP infusion (Fig. 1A). The levels were restored when infusate was changed to saline, but they were lower than the initial levels for 1 hr. By contrast, CNP infusion did not alter plasma Na^+ concentration. In freshwater eels, all NPs did not alter plasma Na^+ concentration except for VNP that increased it slightly after infusion (Fig. 1B).

Hematocrit: Hematocrit was 23.8±1.0% in seawater eels and 24.2± 0.8% in freshwater eels before NP infusion. Hematocrit increased significantly after infusion of ANP or VNP in seawater eels and the increase lasted for 30–60 min (Fig. 2A). However, CNP did not change hematocrit. Similarly, only ANP and VNP increased hematocrit in freshwater eels (Fig. 2B).

Drinking rate: Drinking rate was 46.2±4.5 μl/min in seawater eels and 0.8±0.4 μl/min in freshwater eels. Drinking rate was profoundly inhibited by ANP and VNP, but CNP had no effect in seawater eels (Fig. 3). The antidipsogenic effect continued for 1 hr after termination of ANP or VNP infusion. Since no appreciable drinking was observed in freshwater eels, it was impossible to detect any inhibition of drinking rate in these fish. In fact, 5 of 6 freshwater eels did not drink at all throughout the experiment (data not shown).

Comparison of ANP, CNP and VNP: In seawater eels, CNP was less potent than ANP and VNP in all effects examined except for the effect on hematocrit (Fig. 4A). There were no differences in the effects of ANP and VNP on plasma Na^+ concentration and drinking rate. However, the effect of VNP on hematocrit was greater than that of ANP. In freshwater eels, no differences were detected in the effects of three NPs on plasma Na^+ concentration. However, CNP was less effective than ANP and VNP on increasing hematocrit (Fig. 4B). The antidipsogenic effect could not be expressed as percent changes because of no appreciable drinking before NP infusions. Comparing the effects between freshwater and seawater eels, the effects of ANP and VNP on decreasing plasma Na^+ concentration is stronger in seawater eels than in freshwater eels, whereas no difference was detected for the CNP effects (Fig. 4A, B). The effects of three NPs on hematocrit did not differ between freshwater and seawater eels.

DISCUSSION

Three members of the NP family (ANP, CNP and VNP) have been identified in the eel, but only the effect of ANP on seawater adaptation has been well established (Takei, 2000a). CNP is principally a local paracrine factor in mammals (Forkes and McArdle, 2000). However, recent study showed that CNP circulates in the blood of freshwater eels possibly by secretion from the heart and intestine (Takei *et al*., 2001). Therefore, it is possible that not only ANP and VNP but also CNP is a circulating hormone to regulate water and electrolyte balance in eels. However, effects of the three NPs have not been compared in detail with respect to the body fluid regulation except for the hemodynamic effects (Takei *et al*., 1991).

Effects on plasma Na^+ concentration

In seawater eels, ANP and VNP infused into the circulation decreased plasma Na^+ concentration, but CNP was without effect. It is known that ANP and VNP bind with higher affinity to NPR-A and increase greater cGMP accumulation compared with CNP (Kashiwagi *et al*., 1999). Thus, a decrease in plasma Na^+ concentration seems to be mediated by NPR-A. One possible route for the decrease is via Na^+ excretion by the gill since ANP and VNP increase the Na^+,K^+-ATPase activity in the gill cells isolated from seawater eels (Takei *et al*., 1999). Another possible route is through decreased oral intake of environmental seawater and decreased absorption of NaCl by the intestine. As shown in the present study, ANP and VNP greatly decreased drinking rate in seawater eels. Furthermore, ANP and VNP inhibit NaCl absorption by the intestine as shown in the Ussing's chamber *in vitro* (Ando and Hara, 1994; Loretz and Takei, 1997). In fact, we recently found that the hyponatremic effect is suppressed if eels are forced to drink environmental seawater at a normal rate during ANP infusion (T Tsukada and Y Takei, unpublished data). The hyponatremic effect of ANP is not due to increased Na^+ excretion by the kidney since ANP did not cause natriuresis in seawater eels (Takei and Kaiya, 1998).

In contrast to the result in seawater eels, ANP and VNP did not decrease plasma Na^+ concentration in freshwater eels. This may be due to the less important role of drinking and the subsequent intestinal absorption of NaCl and water for body fluid regulation in freshwater eels than in seawater eels. On the other hand, CNP did not alter plasma Na^+ concentration in this study, although the messages of CNP-specific receptor, NPR-B (Koller *et al*., 1991), are more abundantly expressed in the gills and other osmoregulatory organs of freshwater eels than in those of seawater eels (Katafuchi *et al*., 1994),

Effects on drinking

Drinking rate is generally high in seawater fish in order to compensate for water lost osmotically across the body surfaces (Hirano, 1974; Evans, 1993). Consistently, the seawater eels used in this study drank copiously and continuously throughout the experiment. Inhibition of drinking was apparent during ANP or VNP infusion, but CNP was without effect on drinking. Therefore, the antidipsogenic effect of NP is most probably mediated by NPR-A. This result is consistent with that obtained in the rat in which ANP inhibits drinking (Antunes-Rodrigues *et al*., 1985; Katsuura *et al*., 1986) but CNP slightly enhanced drinking (Samson *et al*., 1991).

In contrast to seawater eels, freshwater eels used in the current study scarcely drank during the experiment. Thus, it was impossible to detect an inhibitory effect of NPs on their drinking. It is generally thought that fish in fresh water drink little to cope with an osmotic influx of water across the gills (Evans, 1993). However, ANP and VNP are potently antidipsogenic in freshwater eels when infused at low doses into the circulation (Tuchida and Takei, 1998). The low drinking rate in freshwater eels in the present study may be due to little bleeding during surgery because eels are highly responsive

to hypovolemia by drinking (Hirano, 1974; Takei, 2000b).

Effects on hematocrit

In both seawater and freshwater eels, ANP and VNP increased hematocrit, but CNP had no effect. Therefore, NPR-A may be present in the target tissues of eels that are responsible for regulation of water permeability across the vascular wall. The effects of ANP, BNP and CNP on hematocrit have been compared in the dog (Woods and Jones, 1999). The results show that ANP and BNP increased hematocrit but CNP was without effect, showing the involvement of NPR-A. Thus, similar mechanisms may be in function for regulation of capillary permeability in eels.

The hemoconcentrating effects of ANP have been extensively studied in mammals (Renkin and Tucker, 1996). It is possible that an increase in hematocrit, i.e., the extravasation of plasma fluid, is caused by an increase in the hydraulic conductivity across the endothelial cells and/or by enlargement of the functional capillary surface area by recruitment of non-perfused capillaries. However, Valentin *et al.* (1997) reported that sodium nitroprusside, which causes similar changes in the vasculature as does ANP through increased accumulation of cGMP, has no effect on hematocrit in the nephrectomized rat. Furthermore, if only water is exuded into the interstitial space, resultant increase in plasma oncotic pressure would re-withdraw interstitial water into plasma immediately as observed after administration of furosemide (Renkin and Tucker, 1996). It is also shown that ANP contributes to the development of pulmonary edema in the rat (Tamura *et al.*, 2000). It is more likely, therefore, that ANP and VNP increase permeability of plasma proteins through the intercellular pathway toward interstitial compartment. In addition, increases in hematocrit are achieved by an increased supply of red cells from blood-storing organs such as spleen and liver, and by expansion of red cell volume (Nikinmaa and Huestis, 1984). The exact mechanisms are not fully understood yet, but the hemoconcenting effects of NPs occur from fish to mammals.

Comparison of ANP, CNP and VNP

In mammals, comparative studies of ANP, CNP and BNP have been performed with respect to cardiovascular and renal actions. The results show that ANP and BNP are generally equipotent but CNP is much less potent in all actions examined except for the effects on the mesenteric vasoconstriction in the dog (Woods and Jones, 1999) and on the inhibition of aldosterone secretion in the sheep (Charles *et al.*, 1996). The hypotensive actions of ANP, CNP and VNP have also been compared in the eel, which showed the potency order of VNP=ANP>CNP (Takei *et al.*, 1991). This result is similar to the effects on drinking, plasma Na^+ concentration and hematocrit as observed in this study. Since the potency order is similar to the order of affinity to NPR-A (Kashiwagi *et al.*, 1999), the effects on drinking, plasma Na^+ concentration, and hematocrit may be mediated by NPR-A.

Comparison of seawater and freshwater eels

It is highly probable that ANP is an important hormone for promoting seawater adaptation (Takei, 2000a). In fact, our present study showed that ANP and VNP decreased plasma Na^+ concentration, which allows eels to survive in the high NaCl environment. Although the precise mechanisms for the hyponatremia are not understood yet, the close relationship between decreased drinking rate and hyponatremia in seawater eels suggests a role of drinking in regulation of plasma Na^+ concentration as mentioned above. Although the effects on drinking, plasma Na^+ concentration and hematocrit share the same receptor, NPR-A, the effects differ between freshwater and seawater eels probably because of the difference in the target tissues or in the density of the receptor in the same target tissue.

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