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Involvement of Drinking and Intestinal Sodium Absorption in Hyponatremic Effect of Atrial Natriuretic Peptide in Seawater Eels

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ABSTRACT—Atrial natriuretic peptide (ANP) decreases plasma Na^+ concentration and promtes seawater (SW) adaptation in eels. The hyponatremia may most probably be caused by increased branchial extrusion of Na^+, but the mechanism has not been determined yet. The present study examined initially the effects of ANP on branchial Na^+ efflux *in vivo* using isotopic ^{22}Na. However, the efflux rate was not altered by infusion of a hyponatremic dose of ANP (5 pmol·kg^{-1}·min^{-1}). Therefore, we sought to examine whether the ANP-mediated hyponatremia is caused by a decrease in the uptake of Na^+ from the environment. Since a decrease in drinking was highly correlated with a degree of hyponatremia, conscious SW eels were infused with dilute SW into the stomach at a normal drinking rate to offset the antidipsogenic effect of ANP. Under this regimen, the hyponatremic effect of ANP was abolished. Then, we examined the site of Na^+ absorption in the alimentary tract by measuring the changes in ion composition of intraluminal fluid along the tract. Since Na^+ was absorbed at the esophagus and anterior/middle intestine, a sac was prepared at each site and the effects of ANP were examined *in situ* in conscious SW eels. ANP infusion did not alter Na^+ absorption at the esophagus, but it profoundly reduced the absorption at the intestine. Together with our previous finding that ANP does not alter renal Na^+ excretion, we propose that ANP reduces plasma Na^+ concentration in SW eels by inhibiting drinking and subsequent absorption of Na^+ by the intestine.

Key words: natriuretic peptides, osmoregulation, seawater adaptation, eel, *Anguilla japonica*

INTRODUCTION

Since plasma osmolality of marine teleosts is only one third that of seawater (SW), these fish face a constant threat of dehydration (Evans, 1993). Drinking of environmental SW, and its processing to achieve a net gain of free water, is an essential component to survival in the hyperosmotic media (Smith, 1930). Indeed, if eels in SW are not allowed to drink, they die within 5 days because of hypovolemia and hypernatremia (Takei *et al*., 1998). Ingested SW is diluted to a 'turning point osmolality' (*ca*. 350 mOsm kg^{-1}) during the passage through the anterior alimentary tract prior to absorption by the intestine (Smith, 1930; Hickman, 1968; Shehadeh and Gordon, 1969; Kirsch and Meister, 1982; Parmelee and Renfro, 1983). Water is then absorbed together with monovalent ions, which are excreted principally via the mitochondrion-rich cells of the gills (McCormick, 1995). Thus, the control of water and electrolyte economy at these osmoregulatory sites (the gills, drinking and alimentary tract) plays an important role in the adaptation of teleost fish to SW.

Accumulating evidence indicates that atrial natriuretic peptide (ANP) is one of the key hormones for SW adaptation in teleost fish (Loretz and Pollina, 2000; Takei and Hirose, 2002). Notably, the infusion of ANP depresses plasma Na^+ concentration in SW-adapted eels (Takei and Kaiya, 1998; Tsukada and Takei, 2001). However, the kidney, one of the osmoregulatory organs in fish, does not contribute to the hyponatremia, since ANP did not induce natriuresis in SW eels (Takei and Kaiya, 1998). Therefore, the major site of Na^+ economy in SW fish, the gill, is most likely to be involved in the ANP-mediated hyponatremia. In fact, human ANP stimulates ^{22}Na extrusion across the body surfaces, mostly through the gills, in three species of flatfishes *in vivo* (Arnold-Reed *et al*., 1991), and rat ANP inhibits unidirectional Cl^- efflux in the opercular epithelium, which con-

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tains mitochondrion-rich cells as the gills, in the killifish *in vitro* (Scheide and Zadunaisky, 1988). Concerning the route for Na^+ uptake from the environment, ANP potently inhibits drinking in eels (Tsuchida and Takei, 1998; Ando *et al.*, 2000) and decreases Na^+ influx across the intestinal epithelium *in vitro* in eels and other teleost species (O'Grady *et al.*, 1985; Ando *et al.*, 1992; Loretz, 1996). However, the involvement of each regulatory site in ANP-induced hyponatremia has not been examined in fish *in vivo*. In the present study, therefore, three experiments were performed to delineate the mechanism of ANP-mediated hyponatremia *in vivo* using conscious SW eels. In the first experiment, the effect of ANP on Na^+ efflux across the body surfaces, primarily via the gills, was examined using isotopic ^{22}Na. In the second experiment, the role of drinking in ANP-induced hyponatremia was examined by modifying the drinking rate during ANP infusion. In the third experiment, the effect of ANP on Na^+ absorption by the intestine was examined *in situ* to assess the role of intestine in ANP-mediated hyponatremia. Finally, we attempted a quantitative analysis of the Na^+ economy at the organismal level in SW eels, and to evaluate the role of ANP in the whole-body regulation that leads to hyponatremia using the present results in combination with previous data.

MATERIALS AND METHODS

Animals

Cultured, immature eels, *Anguilla japonica*, of both sexes were purchased from a local dealer. They were maintained in SW without feeding for at least 2 weeks before use. Water in the tank was continuously circulated, aerated, and regulated at 18°C. All conditions for fish maintenance and experiments conform to the Guidelines for Animal Experiments at the University of Tokyo. Eels weighed 185±3 g (n=65) at the time of surgery.

Experimental protocol

Eel ANP was synthesized by the Peptide Institute Inc. (Osaka). Isotonic 0.9% NaCl solution containing 0.01% Triton X-100 was used as a vehicle for ANP infusion.

Measurement of Na^+ efflux rate

Eels (n=6) were anesthetized in 0.1% (w/v) tricaine methanesulfonate (Sigma, USA) for 15 min and cannulated with a polyethylene tube (o.d.: 0.8 mm) into the ventral aorta. After surgery, they were kept in a 5-liter SW bucket that was aerated and regulated at 18°C. After more than 18 h post-operation, the bucket water was replaced by fresh 2 liter SW, and 1 kBq of ^{22}Na (22.3 GBq·mg^{-1}, PerkinElmer Life Sciences, USA) was injected in 20 s through the cannula into the ventral aorta in a volume of 50 µl followed by a flush with 50 µl vehicle. Subsequently, 5 ml medium was collected 0.5, 1, 2, 3, 4, 5, 6 and 24 h after administration. Since the time-course data of the radioactivity were nonlinear (Fig. 1A), the data were applied to first-order rate equation to determine the Na^+ efflux rate across the body surface as describe by Motais and Isaia (1972):

$$Q_{(t)} = Q_{eq}\ (1-e^{-kt})$$

where Q (cpm) is the total radioactivity in the external medium as a function of time (t) in hour, Q_{eq} is the radioactivity at equilibrium, and k is the turnover rate in h^{-1} (Fig. 1A).

The rate of ^{22}Na efflux at time zero (cpm·h^{-1}) was given by $k \cdot Q_{eq}$ (dQ(t)/dt at time 0). Then, the efflux rate of Na^+ (µmol·h^{-1}) was calculated based on the specific activity of ^{22}Na in the extracellular fluid of eel using the following equation:

$$F_{out} = k\ Q_{eq} \cdot A^{-1}$$

where A is the specific activity of ^{22}Na in the extracellular fluid of eel (cpm·µmol^{-1}). The chloride space was used as an estimate for extracellular space to determine the specific activity, and was assumed to be 25.8% of body weight as measured by Kirsch (1972) in eels. Concerning Na^+ concentrations in plasma and SW, the average values measured in this study, 170 mM and 520 mM, were used. The efflux rate was finally normalized by the body weight of the eel.

Effects of ANP on Na^+ efflux rate

As shown in Fig. 1, ^{22}Na efflux rate was highly linear in the ini-

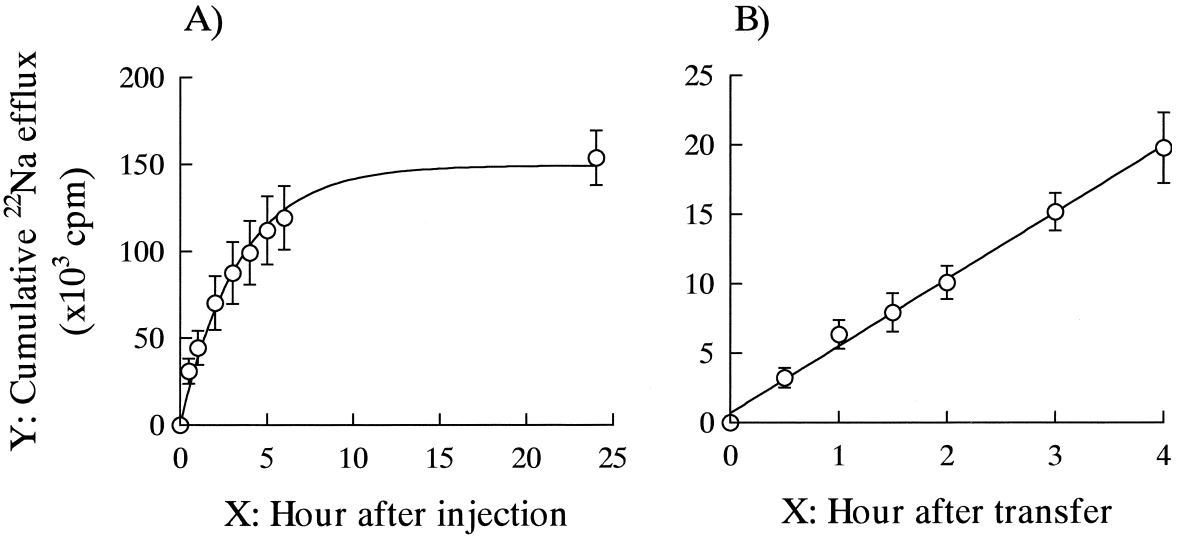

Fig. 1. (A) ^{22}Na efflux across body surfaces of seawater (SW) eels (n=6) after intra-arterial injection of ^{22}Na. The curve was fitted to a first-order rate equation of $Y=149406\ (1-e^{-0.29t})$ (r=0.994, p<0.001). The regression line within 4 h after injection was linear (Y=23424X + 14441, r=0.968, p<0.01). (B) Efflux from the ^{22}Na-equilibrated SW eels (n=6) after transfer to ^{22}Na-free SW. The regression line (Y=4824X+680) was highly linear (r=0.998, p<0.001) for 4 h.

tial 4 h after injection (r=0.967, p<0.01). Therefore, the effects of ANP on Na^+ efflux rate were examined during this period. The preliminary data showed that Na^+ efflux rate varied among individuals (data not shown). Thus the effect of ANP was compared with the control infusion of vehicle in the same fish. For this purpose, SW eels (n=4) were cannulated as previously described. After more than 18 h post-operation, they were placed in a bucket containing 2 liter SW, and 1 kBq of ^{22}Na was administered as described above. After equilibration for 0.5 h, hourly infusions were made into the fish in the order of vehicle-ANP (5 pmol·kg^{-1}·min^{-1})-vehicle, during which time water (5 ml) was collected from the bucket every 0.5 h for 4 h to measure radioactivity. This dose of ANP maximally decreased plasma Na^+ concentration in SW eels (Tsukada and Takei, 2001). The efflux rate was normalized by body weight. To remove possible error caused by non-linearity of ^{22}Na efflux rate, the rate during ANP infusion was compared with the average of vehicle infusions before and after ANP infusion.

We conducted another experiment to examine the ANP effect on ^{22}Na efflux in a more linear condition. To obtain linear ^{22}Na efflux, we initially equilibrated ^{22}Na in the extracellular compartment of the fish, and then transferred to ^{22}Na-free SW. For this purpose, SW eels (n=6) were cannulated in the ventral aorta as described above, then immersed in SW containing ^{22}Na (450 kBq/2 liter) for 24 h. Subsequently, they were transferred to a new bucket containing 2 liter of SW after rinsing in fresh SW to remove contamination of radioactivity. Medium water (5 ml) was collected at 0.5, 1, 1.5, 2, 3 and 4 h after transfer to measure ^{22}Na efflux. The efflux of ^{22}Na was highly linear (r=0.998, p<0.001) for 4 h after transfer (Fig. 1B).

Based on the above data, an additional experiment was performed to examine the ANP effect during the initial 4 h using ^{22}Na-equilibrated eels (n=8). The eels received hourly infusions at the same time course as described above and medium water (5 ml) was taken every 0.5 h to determine radioactivity. The efflux rate was normalized to the body weight of each eel. The efflux rate during ANP infusion was compared with those of control vehicle infusions as described above.

Effect of ANP on drinking

After anesthesia, polyethylene tubes were inserted into the ventral aorta of eels (n=19) for infusion and blood collection. Vinyl tubes (o.d.: 2.4 mm) were then inserted into the esophagus and stomach as described previously (Takei *et al.*, 1998). The esophageal catheter was connected to a drop counter for continuous measurement of drinking rate, and the stomach catheter to a pulse injector synchronized with the drop counter for reintroduction of ingested SW. Since ingested SW was diluted to 80% of its initial concentration prior to removal by the catheter, eighty percent SW was reintroduced into the stomach to maintain sodium balance in the test animal. After surgery, eels were placed in a plastic trough for recovery from anesthesia. The trough was covered with a black vinyl sheet to minimize visual stress during the experimentation. After more than 18 h post-operation, ANP was infused by catheter at 5 pmol·kg^{-1}·min^{-1} for 0.5 h in a volume of 0.1 ml. Control infusion of vehicle was made at the same rate for 1 h before and after ANP infusion. Blood (approx. 40 μl) was collected every 0.5 h after infusion for measurement of hematocrit and plasma Na^+ concentration.

Since a significant correlation was detected between drinking rate and plasma Na^+ concentration (see Results), we selected 9 of 19 eels that exhibited profound decreases in drinking for subsequent examination of the role of drinking in ANP-induced hyponatremia. For this purpose, vehicle-ANP (5 pmol·kg^{-1}·min^{-1})-vehicle infusions were made on the same regimen except that 80% SW was infused into the stomach during ANP infusion at a normal drinking rate of the fish to offset the antidipsogenic effect of ANP. Drinking rate and plasma Na^+ concentration were measured every 0.5 h.

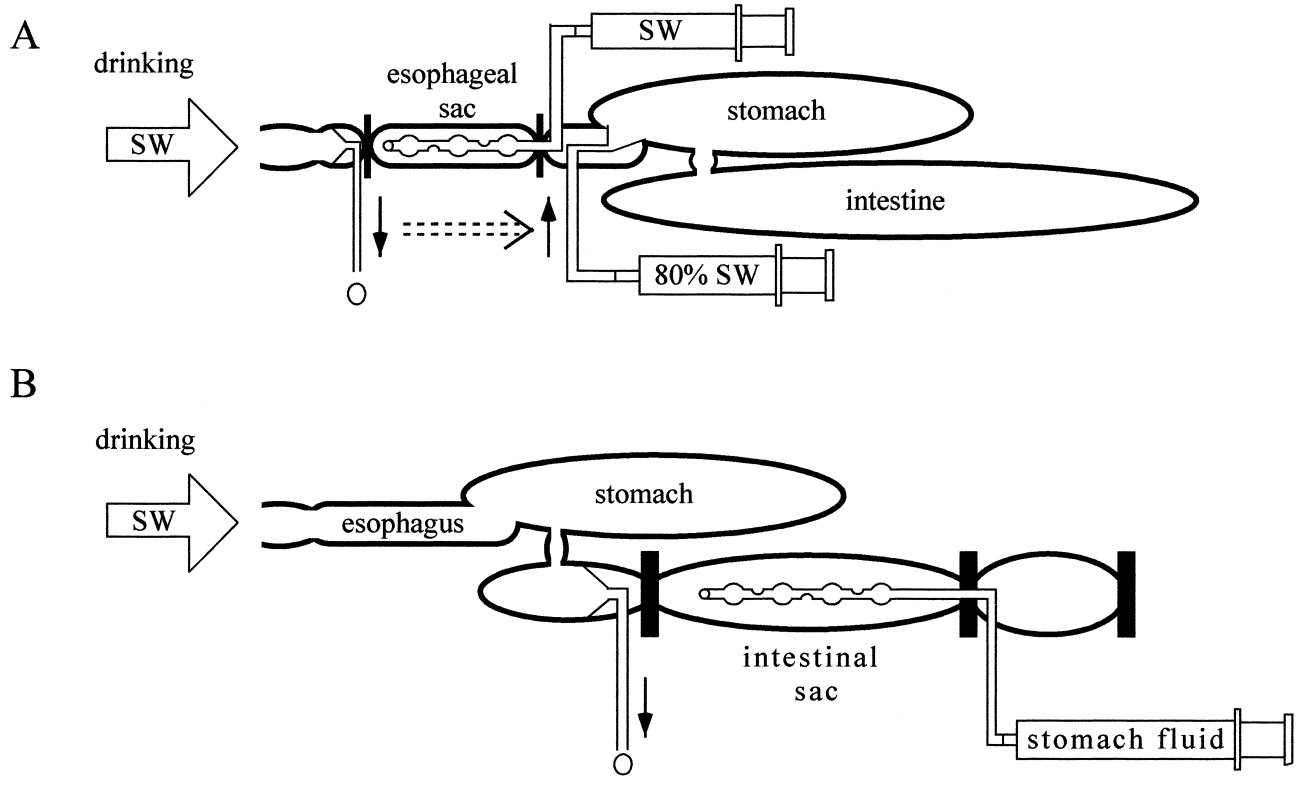

Fig. 2. Experimental setup for measurement of water and ion absorption by (A) esophagus and (B) intestine of SW eels. The fluid in the sac was collected after hourly infusion of ANP or vehicle. For details, see text.

Effects of ANP on the alimentary tract

Initially, changes in the ionic composition of ingested SW were measured along the alimentary tract of eels to identify the major site of Na^+ absorption in the tract. For this purpose, eels (n=9) were placed supine on an operation board after anesthesia, and the whole alimentary tract was exposed by a midline incision of the body wall. Each segment (esophagus, stomach, anterior/middle intestine and posterior intestine) was then tied at both ends, and luminal fluid from each segment was collected into a syringe. The anterior/middle and posterior intestine were divided at the sphincter separating the two. The fluid was transferred to a chilled tube, and centrifuged at 10,000xg for 5 min at 4°C. The concentrations of Na^+, Cl^-, Mg^{2+}, Ca^{2+} and osmolality of the supernatant were measured. To calculate Na^+ absorption, the data of fluid absorption are essential. To this end, we calculated fluid absorption using Mg^{2+} concentration as a marker since it is known that Mg^{2+} ions are scarcely absorbed by the teleost intestine (Smith, 1930; Parmalee and Renfro, 1983).

Since the esophagus and anterior/middle intestine were shown as the major sites for Na^+ absorption (see below), these segments were used to examine the effects of ANP on Na^+ absorption. To examine the effects of ANP on Na^+ absorption at these sites, SW eels (n=7 for esophagus and n=6 for intestine) were anesthetized and cannulated with a polyethylene tube in the ventral aorta for infusions. Then, an incision was made in the ventral skin along the esophagus or anterior/middle intestine, and a sac was prepared at each segment by ligating both ends. A polyethylene tube was inserted through the posterior end of the sac for filling and emptying the sac with a simulated luminal fluid (Fig. 2). The tube had balloon-shaped swellings and apertures to permit efficient re-collection of fluid. The major arteries and veins that ran along the alimentary tract were carefully isolated to avoid ligation. In addition, vinyl tubes were inserted at the entrance of esophagus and stomach in eels with esophageal sac for measurement of drinking rate and reintroduction of ingested SW as described above (Fig. 2A). In eels with an intestinal sac, a vinyl tube was inserted at the entrance of the anterior intestine to drain the ingested SW (Fig. 2B). The intestinal sac was filled with the luminal fluid of the stomach (see below) to maintain fluid balance after operation. After more than 18 h post-operation, the infusion was initiated with vehicle for 1 h, followed by ANP at 5 pmol·kg^{-1}·min^{-1} for 1 h, and ended with vehicle for 2 h. Before each infusion, 0.5 ml of SW (esophagus) or stomach fluid (intestine) was introduced into the sac. After each infusion, the fluid was collected, and its volume, ion concentrations and osmolality were measured. Before the next filling, the sac was washed 3 times with the new solution.

Measurements

The radioactivity of ^{22}Na was measured at the energy range of 450–600 keV for 20 min using a gamma-counter (COBRA QUANTUM 5003, Packard Instrument, USA). The cation concentrations were determined in an atomic absorption spectrophotometer (Z5300, Hitachi, Japan). The Cl^- concentration was determined in a chloridometer (Buchler Instruments, USA), and osmolality in a vapor pressure osmometer (Wescor, USA). All measurements were made in duplicate or triplicate.

Statistical analyses

The ^{22}Na efflux data were fitted to an exponential function (see above) and the correlation was examined by a regression analysis followed by an analysis of variance (ANOVA). The initial 4-h data were subjected to a linear regression analysis followed by ANOVA. The effects of ANP on Na^+ efflux were compared with the mean of vehicle injections before and after ANP infusion by paired t-test. ANOVA was used to analyze the effects of ANP on each of the Na^+-transporting sites, which was followed by Tukey test for sac experiments and by Steel test for other experiments. Significance was determined at $p<0.05$. All results were expressed as means±SE.

RESULTS

Effect of ANP on Na^+ efflux rate

The unidirectional Na^+ efflux rate across body surfaces was calculated to be 1315±285 μmol·h^{-1}·100 g^{-1} (n=6). The efflux rate was not altered by a hyponatremic dose of ANP infusion compared with that of control vehicle infusions

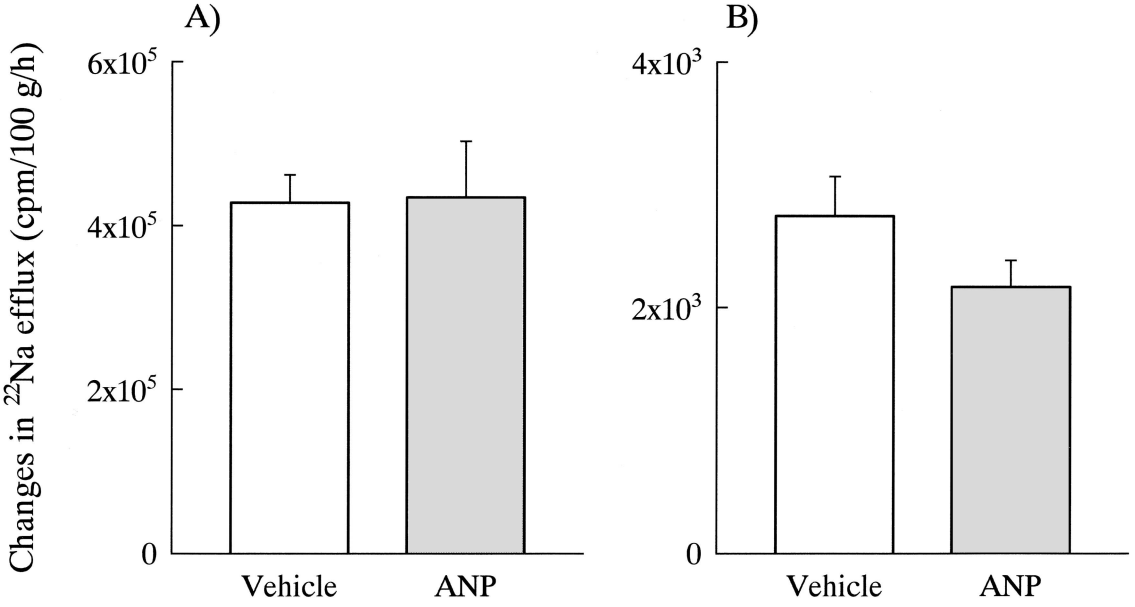

Fig. 3. Effect of ANP on ^{22}Na efflux rate (A) after intra-arterial injection of ^{22}Na (n=4) or (B) after transfer of ^{22}Na-equilibrated eels to fresh SW (n=8) in seawater eels. Control values were calculated as the average of efflux data during vehicle infusions made before and after ANP infusion. ANP does not increase ^{22}Na efflux compared with vehicle–infused controls.

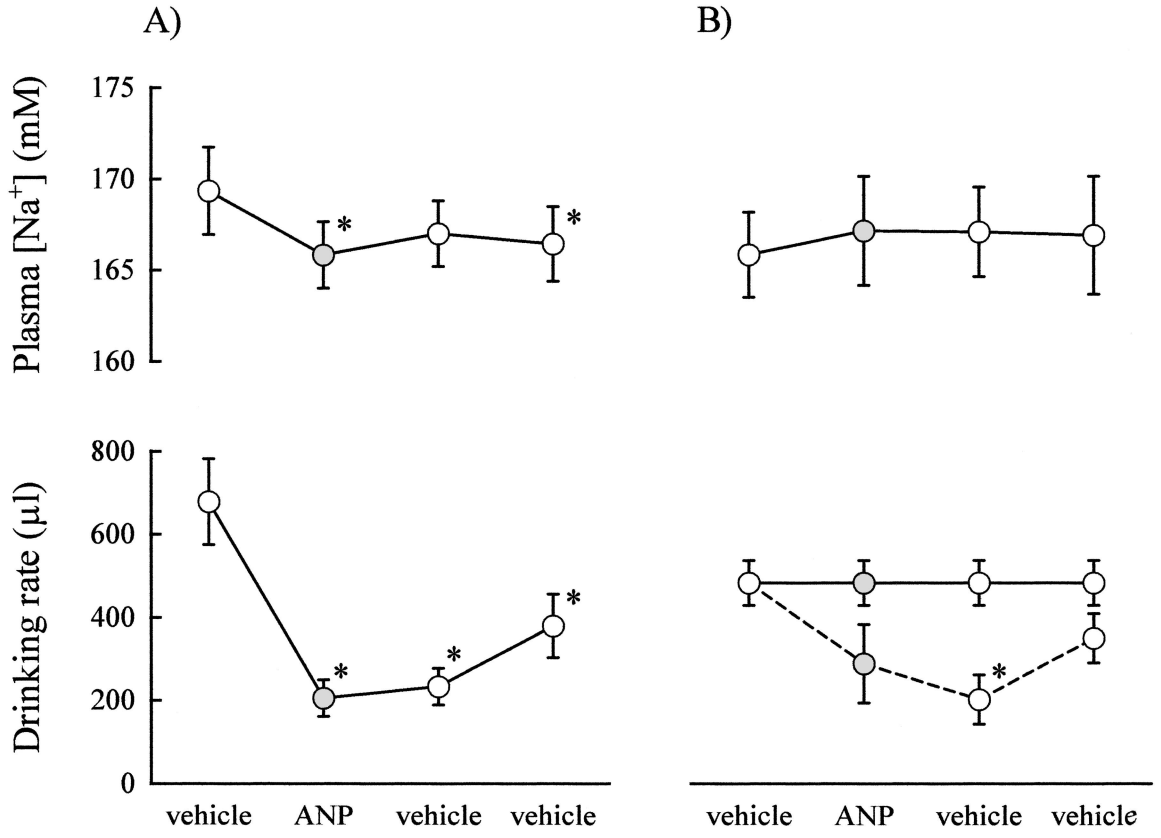

Fig. 4. Changes in plasma Na^+ concentration and drinking rate after ANP infusion: (A) eels in which ingested water was reintroduced into the stomach at the reduced drinking rate (n=9), and (B) eels in which the reintroduction was made at the initial drinking rate (n=9). In group (B), actual drinking rate decreased by ANP is shown by bars depicted with broken lines. Infusions were made for 0.5 h with vehicle or ANP (5 $pmol \cdot kg^{-1} \cdot min^{-1}$) in the order shown in the abscissa. *p<0.05.

(Fig. 3A). Neither did the rate change in ^{22}Na-equilibrated eels after ANP infusion (Fig. 3B). Thus, ANP did not change Na^+ efflux across the body surfaces.

Effect of ANP on drinking

Plasma Na^+ concentration and drinking rate decreased significantly after ANP infusion at 5 $pmol \cdot kg^{-1} \cdot min^{-1}$; this continued for 1 h after infusate was changed from ANP to vehicle (Fig. 4A). There was a positive correlation between decreases in plasma Na^+ concentration and in drinking rate after ANP infusion (r=0.78, p<0.001, n=19) (Fig. 5). Furthermore, the hyponatremic effect of ANP disappeared when the 80% SW was infused into the stomach at the pretreatment rate during ANP infusion (Fig. 4B).

Effects of ANP on Na^+ absorption in the alimentary tract

Ion concentrations and osmolality of the luminal fluid in each segment are summarized in Table 1. Both Na^+ and Cl^- concentrations of ingested SW decreased along the tract posteriorly, particularly in the esophagus and anterior/middle intestine. By contrast, the concentration of Mg^{2+}, which is scarcely absorbed in the alimentary tract, increased posteriorly, reflecting fluid absorption. Ca^{2+} concentration was relatively constant along the length of the alimentary tract (Table 1). The osmolality of luminal fluid in the anterior/middle intestine was similar to that of plasma. The Na^+ absorption at each segment was estimated by the Na^+ concentration and absorbed fluid volume. Na^+ absorption was predominant at the esophagus (41.3±4.8% of ingested Na^+) and anterior/middle intestine (55.8±4.9% of ingested Na^+),

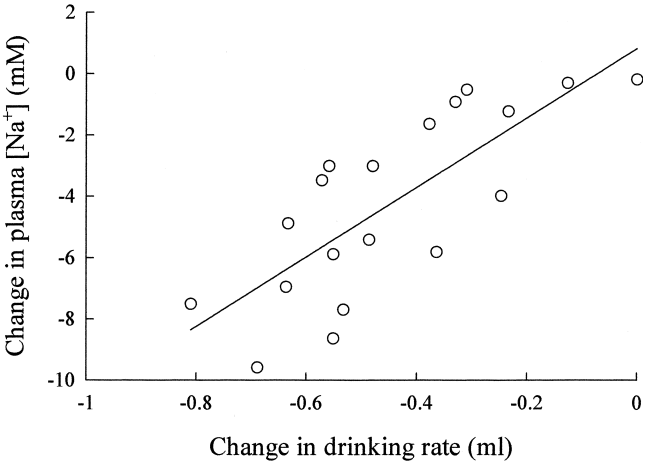

Fig. 5. Correlation between changes in plasma Na^+ concentration (Y) and drinking rate (X) in SW eels (n=19). The regression line (Y= 11.4X+0.9) was linear and correlation was significant (r=0.78, p<0.001).

Table 1. Ion concentrations and osmolality of luminal fluid in the alimentary tract

Segment	n	Osmolality mOsm kg^{-1}	Na^+ mM	Cl^- mM	Mg^{2+} mM	Ca^{2+} mM
SW	5	1019.8±14.4	520.2±7.6	567.6±8.4	57.7± 0.9	13.7±0.2
esophagus	4–6	449.3±34.4	211.3±18.2	225.8±16.7	40.1± 1.4	8.1±0.5
stomach	8	460.1±28.5	222.2±15.2	255.1±13.2	40.6± 2.6	9.5±0.7
A/M intestine	8	294.8± 8.7	40.3± 7.4	68.4±10.1	150.4±12.3	11.9±1.6
posterior intestine	9	300.1± 6.0	3.6± 1.1	59.2± 8.2	187.7± 6.6	14.1±1.2

Values are means±SE.

while it was relatively low at the stomach and posterior intestine (Fig. 6).

In the intestinal sac, Na^+ and Cl^- concentrations were greatly increased after ANP infusion, consistent with an inhibition of Na^+ and Cl^- absorption (Fig. 7). Na^+ and Cl^- absorptions, which were calculated from the ion concentrations and fluid volume collected from the sac, showed that the inhibitions of Na^+ and Cl^- absorption were nearly equal (64% inhibition for Na^+ and 62% inhibition for Cl^-, compared with the control value). Mg^{2+} and Ca^{2+} concentrations were decreased after ANP infusion (Fig. 7), probably because of an inhibition of fluid absorption. The fluid osmolality was maintained at *ca.* one third of SW throughout the experiment even after ANP infusion due to the profound inhibition of Na^+ and Cl^- absorption (Fig. 7). In the esophageal sac, however, ANP infusion did not alter any ionic and other parameters compared with vehicle-infused controls (data not shown).

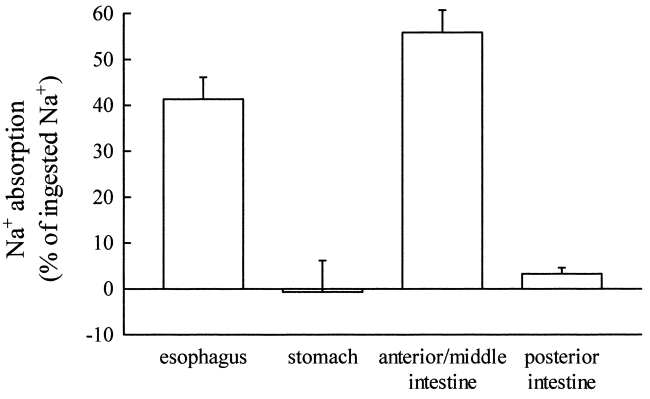

Fig. 6. Na^+ absorption in each segment of the alimentary tract in SW eels (n=6). The Na^+ absorption was calculated based on changes in Na^+ concentration and in fluid volume estimated by changes in Mg^{2+} concentration. Values are presented as percentage of ingested Na^+ (means±SE).

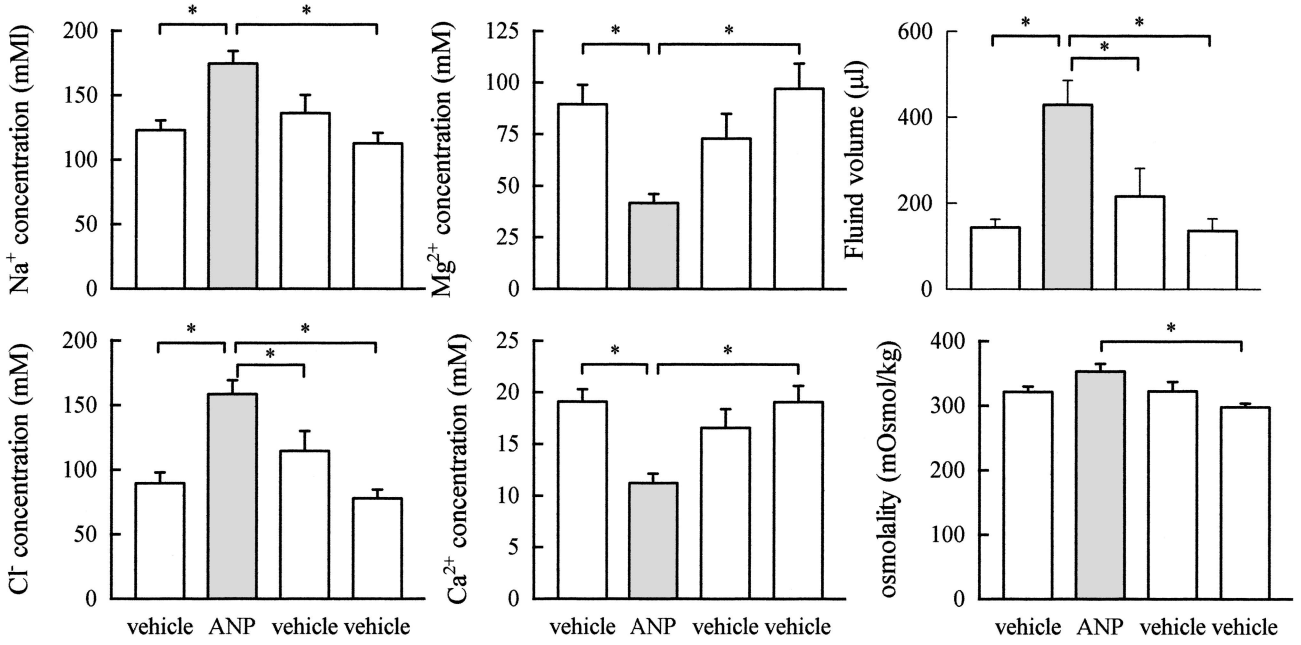

Fig. 7. Changes in ion concentrations, fluid volume and osmolality in the anterior/middle intestine of SW eels (n=6) after ANP infusion. The infusion was made for 1 h with vehicle and ANP (5 pmol·kg^{-1}·min^{-1}) in the order shown in the abscissa. Values are presented as means±SE. *p<0.05.

DISCUSSION

The present study confirmed our previous observation that ANP causes profound hyponatremia in SW-adapted eels (Takei and Kaiya, 1998; Tsukada and Takei, 2001). The most likely sites of action of ANP in producing the hyponatremic effect are the mitochondrion-rich cells in the gills and the opercular epithelium, since more than 95% of Na^+ efflux occurs via those sites in SW eels (Fig. 8). However, the present study demonstrated that the hyponatremia was not due to the facilitated Na^+ efflux across body surfaces in SW eels. This result is consistent with the absence of ANP effect on transepithelial potential difference across the opercular epithelium of winter flounder *in vitro* (O'Grady *et al*., 1985), but is inconsistent with the stimulation of Na^+ or Cl^- efflux through the gills and/or opercular epithelia of killifish *in vivo* (Scheide and Zadunaisky, 1988) and flatfishes *in vivo* (Arnold-Reed *et al*., 1991). In the flatfish, a similar method was used to measure ^{22}Na efflux, but these authors injected mammalian ANP (*ca*. 60% sequence identity with fish ANP) as a bolus into fish at 10 $ng \cdot kg^{-1}$ (Arnold-Reed *et al*., 1991). Despite technical variations, the differing responses among fishes may be due to a species difference. A radioligand-binding assay showed that ANP receptors exist in the gills, but the predominant receptors are clearance-type NPR-C in eels (Sakaguchi *et al*., 1993) and toadfish (Donald *et al*., 1994). Further, Mishina and Takei (1997) showed that the NPR-A type is not detectable in the gills of SW eels as shown by the absence of cGMP accumulation after addition of ANP to isolated gill cells. These results provide correlative support for the absence of ANP effects on the gills of SW eels.

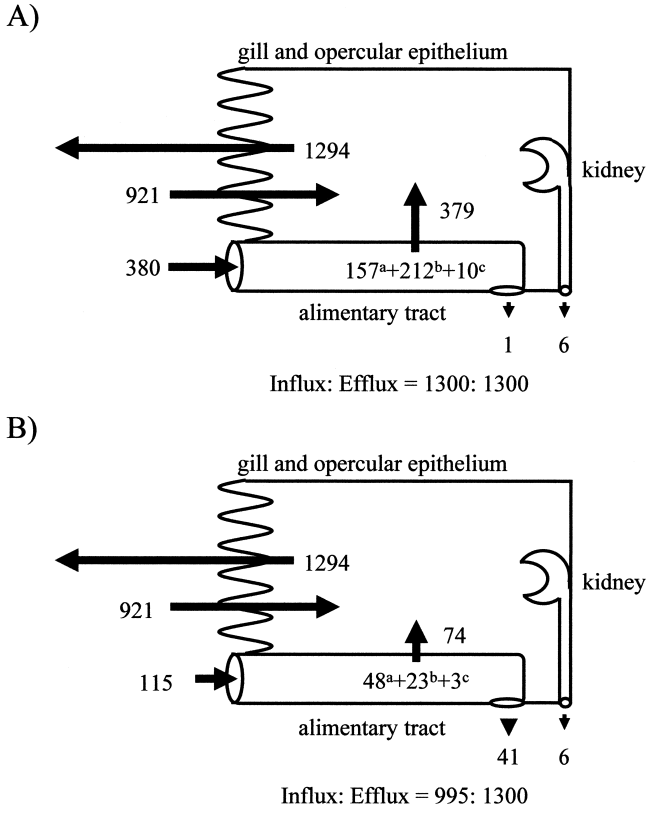

Fig. 8. Approximate influx and efflux of Na^+ across the body surfaces in intact SW eels (A), and in eels that were infused a hyponatremic dose of ANP (5 $pmol \cdot kg^{-1} \cdot min^{-1}$) for 1 h (B). Average values in $\mu mol \cdot 100\ g^{-1} \cdot h^{-1}$ obtained in the present and previous studies were used for the calculation. The absorption at [a]esophagus, [b]anterior/middle intestine, and [c]other segments of alimentary tract were calculated based on the data shown in Fig. 6. For more details, see text.

Based on the current findings and the previously-demonstrated absence of ANP-induced natriuresis in the kidney of SW eels (Takei and Kaiya, 1998), it seems that ANP-mediated hyponatremia is not the result of an increase in Na^+ extrusion but to decrease in Na^+ uptake from the environment. In fact, infusion of 80% SW into the stomach at a normal drinking rate during ANP infusion completely abolished the hyponatremic effect of ANP. Although we infused 80% SW to maintain Na^+ balance, Na^+ concentration of the luminal fluid in the stomach is actually less than 50% of SW (Table 1). The smaller dilution of SW in the esophagus in our experimental system results from continuous gravity drainage in the measurement of drinking rate, which shortens the time of ingested SW in the esophagus. In natural conditions, ingested SW may stay for some time in the esophagus before it is sent to the stomach by relaxation of sphincter muscle between the two segments.

The current study confirmed the previous data showing that the esophagus and anterior/middle intestine are the principal sites for Na^+ and Cl^- absorption in teleost fish as shown *in vivo* (Shehadeh and Gordon, 1969; Kirsch and Meister, 1982) and *in vitro* (Hirano and Mayer-Gostan, 1976; Parmalee and Renfro, 1983). In these segments, Na^+ and Cl^- were both absorbed, but in the posterior intestine, only Na^+ was absorbed in the SW eels. In fish intestine, Na^+ and Cl^- can be absorbed together through the apical Na^+/Cl^- and/or $Na^+/K^+/2Cl^-$ cotransporters (Loretz, 1995; Schettino and Lionetto, 2003). Thus, some other Na^+-specific channels/transporters may play a role in the posterior intestine of SW eels. The anterior/middle intestine was also the principal site for fluid absorption in the eel as shown in previous studies (Hickman, 1968; Hirano and Mayer-Gostan, 1976; Kirsch and Meister, 1982), since Mg^{2+} concentration of luminal fluid dramatically increased at the site. In SW teleosts, Mg^{2+} was conveniently used as a marker for estimating fluid absorption in the alimentary tract, since Mg^{2+} is scarcely absorbed in the tract (Smith, 1930). In fact, we found a high correlation ($r=0.949$, $p<0.001$) between absorbed fluid volume actually measured in the intestinal sac and that calculated from changes in Mg^{2+} concentration (data not shown). However, it has been reported that 10–15% of Mg^{2+} ingested is absorbed from the posterior part of intestine (Hickman, 1968; Parmalee and Renfro, 1983) and that intestinal secretion of bicarbonate ions precipitates excess divalent ions in the posterior intestine of SW fish as $CaCO_3$ and $MgCO_3$ (Wilson, 1999). Thus the fluid absorbed by the posterior

intestine may be somewhat greater than the volume calculated from the changes in Mg^{2+} concentration (Table 1). In contrast to the intestine, fluid was scarcely absorbed or slightly secreted in the esophagus as shown previously (Smith, 1930; Hirano and Mayer-Gostan, 1976; Parmalee and Renfro, 1983).

Our current results provided the first direct evidence to show that ANP profoundly inhibits intestinal Na^+ absorption *in vivo* except in the dog where ANP inhibits it in the jejunum (Matsushita *et al*., 1991). There have been *in vitro* studies showing the inhibitory effect of ANP on short-circuit current in teleost intestine (O'Grady *et al*., 1985; Ando *et al*., 1992; Loretz, 1996). In the flounder, ANP inhibits $Na^+/K^+/2Cl^-$ cotransporters through cGMP accumulation (O'Grady *et al*., 1985), but in mammals, the ANP effect is mediated by Na^+/glucose cotransporters (Gonzalez-Bosc *et al*., 2000). In the present study, glucose was not added to the luminal fluid in the intestinal sac. Furthermore, since Na^+ and Cl^- absorption in the intestinal sac were inhibited to the same extent, a Na^+ and Cl^--coupled transporter may be involved in this process. Together with Na^+ and Cl^-, fluid absorption by the intestine was inhibited by ANP as shown in the present study.

To assess the relative contribution of each osmoregulatory site to whole-body Na^+ balance of SW eel, approximate influx and efflux of Na^+ at each site were determined based principally on the method reported by Maetz (1974) (Fig. 8A). To this end, we conveniently used the average values obtained in the present and previous studies on eels, although the flux values vary between fish species and even among different individuals of the same species. The amount of Na^+ absorbed by the alimentary tract was calculated from the data in Fig. 6 and oral Na^+ intake calculated from drinking rate in Fig. 4A. Assuming that unidirectional influx and efflux of Na^+ are balanced at the organismal level, the unidirectional Na^+ influx from the gill and opercular epithelium was determined by subtracting the absorption of the alimentary tract from the total influx. Net Na^+ loss by the kidney was calculated from the previous data (Takei and Kaiya, 1998). The Na^+ efflux from the gill and opercular epithelium was determined by subtraction of the renal loss from the total unidirectional Na^+ efflux from the body.

Plasma Na^+ concentration of fish is regulated by the balance between influx and efflux of Na^+ across the body surfaces. The present study showed that the total Na^+ unidirectional efflux is *ca*. 1300 $\mu mol \cdot h^{-1} \cdot 100\ g^{-1}$, which is similar to or slightly higher than the value of European eels (Kirsch and Mayer-Gostan, 1973; Maetz, 1974). The oral Na^+ intake was *ca*. 380 $\mu mol \cdot h^{-1} \cdot 100\ g^{-1}$ as calculated from the normal drinking rate shown in Fig 4A. Since almost all Na^+ ingested is absorbed during passage through the alimentary tract, the Na^+ uptake by the alimentary tract accounts for *ca*. 30% of total Na^+ unidirectional influx for an eel in normal Na^+ balance. Since the branchial surface is more than 10 times larger than skin surfaces in the eel (Byczkowska-Smyk, 1958), and since permeability of branchial epithelia is much higher than that of the skin, the remaining 70% of Na^+ influx may be attributable to the gills. Concerning the efflux from the body, Na^+ excretion by the kidney is only 6 $\mu mol \cdot h^{-1} \cdot 100\ g^{-1}$ in SW eels (Takei and Kaiya, 1998). Therefore, more than 99% of Na^+ efflux is accounted for by the mitochondrion-rich cells in the gills and opercular epithelium. These Na^+ fluxes in SW eel were similar to those reported by Maetz (1974).

To evaluate the role of oral drinking of SW and subsequent Na^+ absorption by the intestine in ANP-mediated hyponatremia in SW eels, changes in plasma Na^+ concentration were re-calculated based on the inhibitory effects of ANP on these parameters (Fig. 8B). For this purpose, percent decreases in drinking rate and intestinal Na^+ absorption were applied to the normal values (Fig. 8A) for calculation. ANP inhibited the oral Na^+ intake from 380 to 115 $\mu mol \cdot h^{-1} \cdot 100\ g^{-1}$ as shown in this study (Fig. 4A), and the intestinal Na^+ absorption was further inhibited by 36% during ANP infusion. Therefore, ANP decreased net influx across the alimentary tract from 379 to 74 $\mu mol \cdot h^{-1} \cdot 100\ g^{-1}$ (Fig. 8B). This decrease is sufficient to decrease Na^+ concentration in the extracellular fluid including plasma from 169 mM to 166 mM, showing that ANP-induced hyponatremia in SW eels is caused principally by the combined inhibitory effects on drinking and intestinal Na^+ absorption. The inhibition of oral and intestinal uptake of Na^+ is accompanied by a reduction in water uptake from the environment, which is disadvantageous for adaptation to dehydrative SW environments. It is known that fishes are always in danger of over-drinking because of its aquatic habitat, and thus inhibitory mechanisms are predominant for drinking compared with terrestrial species (Takei, 2000). It seems that ANP is a primary factor for inhibition of drinking to maintain water and ion balance in SW, because removal of ANP from plasma by immunoneutralization increased drinking rate and plasma Na^+ concentration in SW eels (Tsukada and Takei, unpublished data). In particular, ANP may be involved in limiting the copious drinking that occurs just after encountering SW in response to Cl^- (Hirano, 1974), since plasma ANP concentration increases transiently for a few hours after transfer of eels from FW to SW (Kaiya and Takei, 1996). In this way, ANP appears to damper sudden increases in plasma Na^+ concentration and promote survival on encountering SW.

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