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Antidiuretic Effect of Eel ANP Infused at Physiological Doses in Conscious, Seawater-Adapted Eels, *Anguilla japonica*

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ABSTRACT—Atrial natriuretic peptide (ANP) is known as a potent natriuretic/diuretic hormone in vertebrates. However, eel ANP infused at doses that did not alter arterial blood pressure (0.3-3.0 pmol/kg/min) decreased urine volume and increased urinary Na concentration in seawater (SW)-adapted eels but not in freshwater (FW)-adapted eels. The renal effects were dose-dependent and disappeared after infusate was switched back to a vehicle (0.9% NaCl). Urinary Na excretion (volume x Na concentration) did not change during ANP infusion. ANP infusion increased plasma ANP concentration, but the increase at the highest dose was still within those observed endogenously after injection of hypertonic saline. Urinary Mg and Ca concentrations increased during ANP infusion in SW eels, but urinary Ca excretion decreased in FW eels. Plasma Na concentration profoundly decreased during ANP infusion only in SW eels, suggesting that ANP stimulates Na extrusion via non-renal routes. These results indicate that ANP is a hormone which specifically extrudes Na ions and thereby promotes SW adaptation in the eel. This is in sharp contrast with mammals where ANP is a volume regulating hormone that extrudes both Na and water.

INTRODUCTION

Atrial natriuretic peptide (ANP) has been established as a volume regulating hormone which is secreted in response to an increase in blood volume and acts to decrease it by inhibiting the uptake and stimulating the excretion of water and Na ions (Brenner *et al*., 1990; Ruskoaho, 1992). In the eel, however, ANP secretion is augmented by osmotic stimuli rather than by volemic stimuli (Kaiya and Takei, 1996), and ANP action seems to direct more specifically to extrusion of Na than of water (Takei and Balment, 1993a).

As its name indicates, ANP causes profound natriuresis and diuresis in most vertebrate species thus far tested (Evans, 1990; Takei and Balment, 1993b). Exceptions are the turtle, *Amyda japonica*, in which rat ANP fragment (atriopeptin III) has no effect on water and Na extrusion (Cho *et al*., 1988), and the spiny dogfish, *Squalus acanthias*, in which atriopeptin causes antidiuresis and antinatriuresis (Benyajati and Yokota, 1990). In teleost fish, mammalian or eel ANP causes diuresis and natriuresis in the trout, *Oncorhynchus mykiss* (Duff and Olson, 1986; Dunne and Rankin, 1992; Duff *et al*., 1997) and the toadfish, *Opsanus tau* (Lee and Malvin, 1987). In freshwater (FW) eels, however, low doses of eel ANP cause antidiuresis without changes in Na excretion (Takei and Balment, 1993a). In these studies, ANP was injected as a bolus which invariably decreased arterial blood pressure. Since glomerular filtration rate (GFR) is a major determinant of urine production in fish, and since GFR is most vulnerable to changes in systemic blood pressure (Nishimura, 1985), it is essential to examine renal actions of ANP in such a way that do not alter blood pressure in fish.

The present experiments were designed to examine the renal effect of homologous ANP infused at physiological doses in FW and seawater (SW)-adapted eels. Dorsal aortic pressure was continuously monitored to assess its influence on GFR. Blood was collected for measurements of plasma hormone and ion concentrations. The collected blood volume was identical to the infusion volume to minimize the changes in blood volume. Urine was collected via a bladder catheter for measurements of its volume and ion concentrations.

MATERIALS AND METHODS

Fish and maintenance

Cultured immature eels, *Anguilla japonica*, of both sexes were purchased from a local dealer. They were acclimated to laboratory conditions in a 1-ton FW tank for at least one week or in a 0.5-ton SW tank for more than two weeks without feeding. Water in the tank was continuously circulated, aerated, and regulated at 18°C. They weighed 180.4 ± 2.3 g (n = 14) at the time of surgery.

Drugs

Eel ANP was synthesized by Peptide Institute Inc. (Osaka) and kindly supplied to us for experiments. It was dissolved in distilled water, aliquoted and frozen at -20°C until use. On the day of experimentation, the stock solution was diluted with isotonic (0.9%) NaCl solution containing 0.01% Triton X-305 (Nakarai Kagaku, Kyoto). This solu-

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tion was used as a vehicle in the following experiments.

Cannulation

Eels (6 FW and 8 SW eels) were anesthetized by immersion in 0.1% (w/v) tricaine methanesulfonate (Sigma, St. Louis, USA) for 15 min. The ventral aorta was cannulated at the exit of the heart with a polyethylene tube (o.d. = 0.8 mm, SP31, Natsume Seisakusho Co. Ltd., Tokyo) without stagnating the blood flow, and the dorsal aorta with a polyethylene tube (o.d. = 0.5 mm, Imamura Rubber Co. Ltd., Tokyo) via the pneumogastric artery (Takei, 1988). The urinary bladder was cannulated via the urogenital papilla with an SP31 tube whose tip was heat-flared and modified to ensure continuous flow of urine. The cannula was secured by a ligature around the papilla (Oide and Utida, 1968). The cannula in the dorsal aorta was connected, by way of 3-way stopcock, to a 1 ml syringe which was set on an infusion pump for continuous infusion of ANP, and to a pressure transducer for continuous monitoring of blood pressure (Fig. 1). The cannula in the ventral aorta was used for blood sampling. The cannula in the urinary bladder was connected to a drop counter, and each drop was collected into a 1.5 ml tube for 1 hr (Fig. 1). After surgery, eels were placed in a plexiglass trough through which aerated and thermoregulated (18°C) water continuously circulated. The eels in troughs were covered with a black vinyl sheet to minimize the stress during pressure measurements.

Experimental protocol

On the next day of surgery (usually more than 18 hr thereafter), the infusion was initiated with the vehicle for 2 hr, followed by increasing doses of ANP for 3 hr (0.3, 1.0 and 3.0 pmol/kg/min), and ended with the vehicle for 2 hr. The first 1-hr infusion of vehicle was used for stabilization of the system, and the blood sampling was initiated after the infusion. At the time of exchange of infusates, 50 μl of the next infusate was injected as a prime to obtain stable plasma ANP levels quickly. The infusion rate was 0.4 ml/hr and 0.4 ml blood was collected hourly to nullify the increase in blood volume. Blood was collected into a chilled 1 ml polyethylene syringe containing 2K-EDTA (1.2 mg/ml blood).

Measurements

Collected blood was aliquoted (50 μl) into a capillary for measurements of hematocrit, Na concentration and osmolality. The remaining blood was centrifuged at 11,000 × g, and plasma was used for measurements of eel ANP and ventricular natriuretic peptide (VNP), another cardiac natriuretic peptide of eels, by homologous radioimmunoassays (Takei *et al*., 1992, 1994). Urine volume was determined by the number of drops emerged from the catheter (0.03 ml/drop). Urine pooled for each hour was used for determination of Na, Ca and Mg concentrations. In SW eels, white precipitate often appeared in the urine during ANP infusion. In this case, urine was mixed with the same volume of 5M HCl to dissolve the precipitate, and ion concentrations were corrected accordingly. Plasma and urine ion concentrations were determined in an atomic absorption spectrophotometer (Model 180-50, Hitachi, Tokyo), and plasma osmolality by a vapor pressure osmometer (Model 5500, Wescor, USA).

Statistical analysis

Results were expressed as means ± S. E. of the mean. For statistical analysis, two-way analysis of variance was used for time course data, and paired t-test for comparison of each time point with the initial control value. Significance was determined at $p < 0.05$.

RESULTS

Low-dose infusion of eel ANP (0.3-3.0 pmol/kg/min) did not change arterial blood pressure in both FW and SW-adapted eels (Table 1). Plasma ANP concentration increased after ANP infusion, but the increase at 0.3 pmol/kg was within the normal variation of plasma ANP level. Even at 3.0 pmol/kg, the increase was 20-30 folds, which occurs endogenously after injection of hypertonic solutions (Kaiya and Takei, 1996). High plasma ANP level continued for 1 hr after infusate was replaced by the vehicle, but the level returned to normal in 2 hr (Table 1). Plasma VNP concentration did not change during

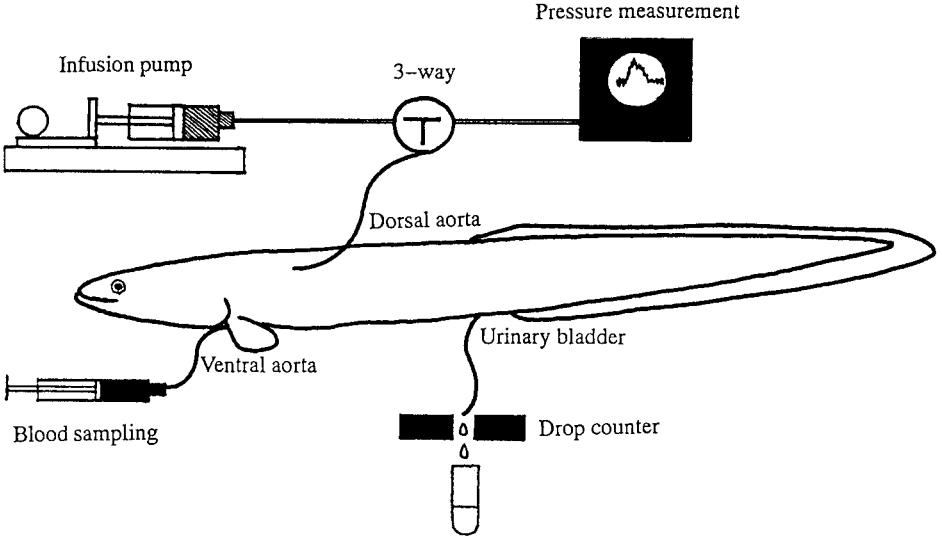

Fig. 1. Experimental setup for examination of the renal effect of ANP in the eel. ANP was slowly (6.7 μl/min) infused into the dorsal aorta, while arterial blood pressure was continuously monitored at the same aorta via a 3-way stopcock. Urine volume was measured by the number of drops (0.03 ml/drop) that emerged from the catheter in the urinary bladder. Urine was collected every hour for ion analyses. Blood was collected every hour through a catheter in the ventral aorta for determination of plasma hormone and ion concentrations.

Table 1. Changes in arterial blood pressure (mmHg) and plasma atrial and ventricular natriuretic peptide (ANP and VNP) concentrations (fmol/ml) during ANP infusion in eels

Parameter	Dose of hourly ANP infusion (pmol/kg/min)					
	0	0.3	1.0	3.0	0	0
Freshwater eels (n = 6)						
Arterial pressure	28.4 ± 3.8	30.1 ± 3.6	29.5 ± 3.8	28.9 ± 3.8	30.1 ± 3.4	30.2 ± 3.4
Plasma ANP conc.	97.9 ± 8.8	156.0 ± 21.5*	709.4 ± 156.7*	1827.9 ± 641.5*	604.8 ± 151.6*	84.1 ± 13.0
Plasma VNP conc.	19.3 ± 1.4	18.7 ± 2.6	19.1 ± 2.5	19.7 ± 2.3	17.5 ± 2.0	17.8 ± 3.4
Seawater eels (n = 8)						
Arterial pressure	33.9 ± 2.9	36.1 ± 2.8	36.2 ± 2.9	37.3 ± 2.8	38.2 ± 3.0	39.4 ± 3.0
Plasma ANP conc.	69.3 ± 16.0	150.4 ± 60.8	545.2 ± 135.7*	2381.4 ± 693.0*	514.9 ± 95.4*	72.7 ± 10.1
Plasma VNP conc.	43.0 ± 3.4	37.5 ± 5.4	39.8 ± 4.5	40.3 ± 4.9	37.5 ± 4.8	38.1 ± 4.9

Values are mean ± SE of the mean. *p < 0.05 compared with the level before ANP infusion.

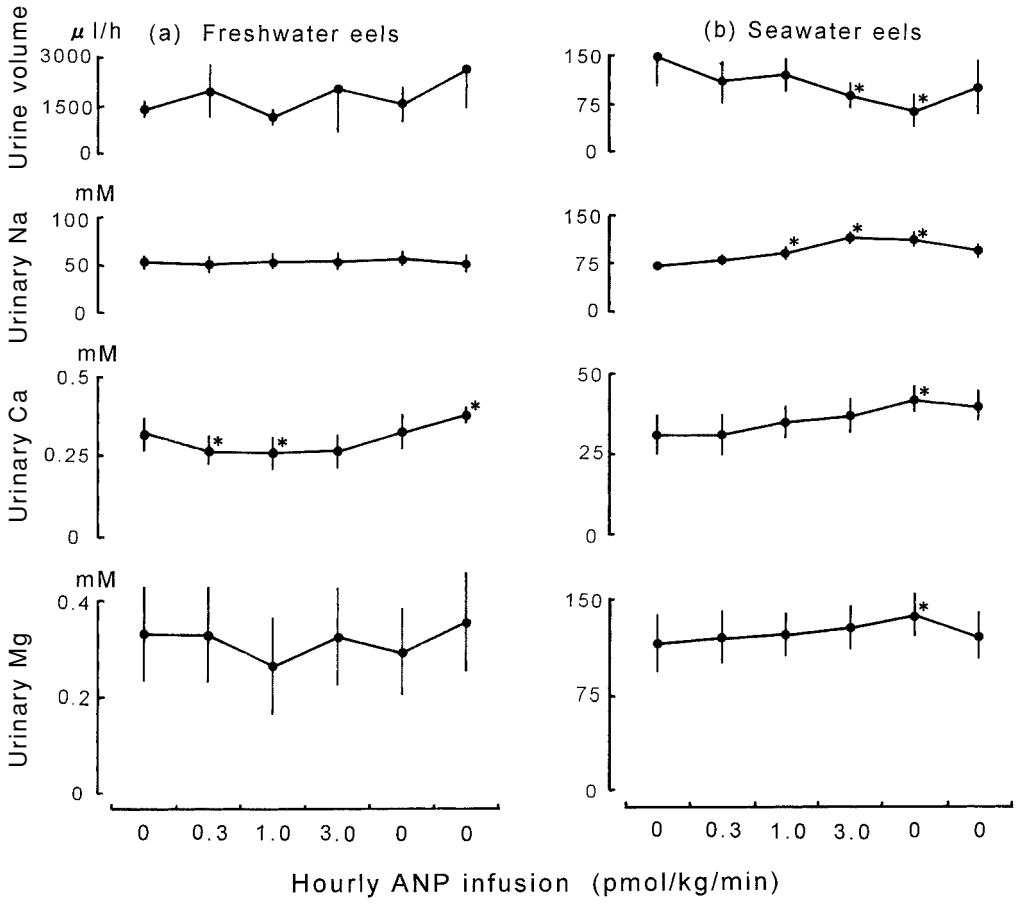

Fig. 2. Changes in urine volume and urinary ion concentrations during ANP infusion in (**a**) freshwater eels (n = 6) and (**b**) seawater eels (n = 8). Vertical bars indicate SE of the mean. *p < 0.05 compared with the level before ANP infusion.

ANP infusion in both FW and SW eels.

Urine volume was 10 fold larger in FW eels than in SW eels (Fig. 2). Urine volume did not display consistent changes during ANP infusion in FW eels. In SW eels, however, it decreased gradually during increasing doses of ANP infusion and the decrease became significant at 3.0 pmol/kg. The antidiuretic effect continued for 1 hr after vehicle infusion during which plasma ANP level was still high (Table 1), and returned to the initial level after 2 hr. Urinary Na concentration was slightly higher in SW eels than in FW eels, and ANP further increased it in SW eels but not in FW eels (Fig. 2). The urinary Na excretion (product of volume and concentration) was not altered by ANP infusion. Ca and Mg concentrations in SW eel urine were much higher than those of FW eels, and they gradually increased during ANP infusion in SW eels (Fig. 2). In FW eels, however, urine Ca concentration decreased during ANP infusion and increased after 2 hr of vehicle infusion. The increase in SW eels became significant 1 hr after changing

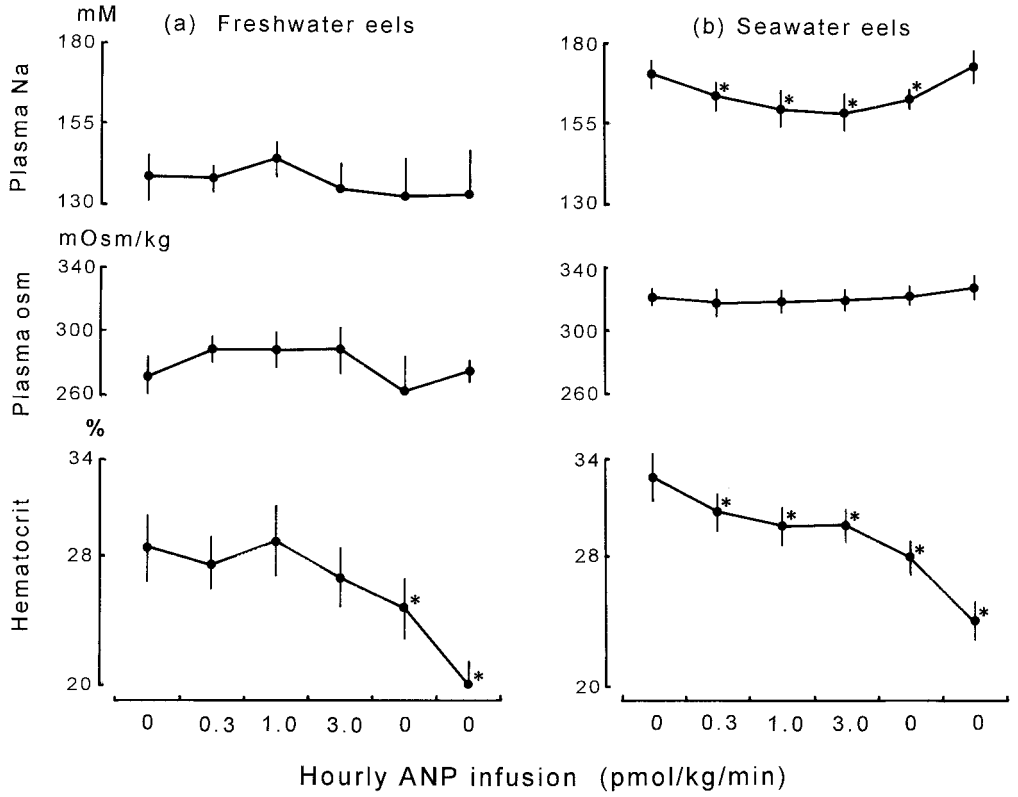

Fig. 3. Changes in plasma Na concentration, plasma osmolality and hematocrit during ANP infusion in (**a**) freshwater eels (n = 6) and (**b**) seawater eels (n = 8). Vertical bars indicate SE of the mean. *p < 0.05 compared with the level before ANP infusion.

infusate to a vehicle. The delay may be due to the dead space of bladder catheter (ca. 60 μl) which is nearly equal to the hourly urine output of SW eels.

Plasma Na concentration and osmolality did not change in FW eels, but it decreased during ANP infusion in SW eels as analyzed by ANOVA (Fig. 3). The decrease in plasma Na concentration was profound and apparently due to ANP because plasma Na concentration recovered after infusate was replaced by a vehicle. Hematocrit decreased gradually as the infusion progressed, but it was apparent that the hematocrit was maintained during ANP infusion in both FW and SW eels (Fig. 3).

DISCUSSION

The urine volume and urinary ion concentrations of eels used in this study were comparable to those reported in European eels (Chester Jones *et al.*, 1969) and other teleost species (Hickman and Trump, 1969). The infusion of homologous ANP into these eels at low, nondepressor doses induced antidiuresis and an increase in urinary Na, Ca and Mg concentrations in SW-adapted fish. No apparent renal effects occurred in FW-adapted eels except a decrease in Ca concentration. The infusion of ANP increased plasma ANP level, but the increase was within the range observed *in vivo* after injection of hypertonic saline (Kaiya and Takei, 1996). In fact, no cardiovascular effects were detected during ANP infusion at any doses. Thus the renal response to ANP observed in this study may be a physiological one.

In fish, urine volume is primarily regulated by glomerular filtration rate (GFR), and the GFR is affected profoundly by the systemic arterial pressure (Nishimura, 1985). In the previous study, we observed antidiuretic effect after a bolus injection of 0.2 nmol/kg of eel ANP or VNP into FW eels (Takei and Balment, 1993a). Although the dose injected was low, it was mildly hypotensive, which might have decreased GFR. In the present study, however, since blood pressure and blood volume were scarcely altered during ANP infusion, the antidiuretic effect observed in SW eels may be caused by a direct action of ANP on the glomerulus and/or renal tubules. In other teleost species, diuretic and natriuretic effects are generally resulted after a bolus injection of rather high doses (5-30 nmol/kg) of rat ANP. In the trout, ANP induces unique vasopressor effect, which may mediate the diuretic effect (Olson and Duff, 1992). In the aglomerular toadfish, however, the diuretic and natriuretic effects should be mediated by direct tubular actions because of the lack of glomerulus in this fish (Lee and Malvin, 1987). ANP was antidiuretic and antinatriuretic in the spiny dogfish, but the effect was mediated by the vasodepressor effect of ANP (Benyajati and Yokota, 1990).

The autoradiographic and radioligand binding studies using ^{125}I-eel ANP demonstrate dense ANP receptors in the kidney glomerulus (Sakaguchi *et al.*, 1996; Mishina and Takei, 1997). Most of the receptors may be clearance type NPR-C

as characterized by the binding assay, but the presence of guanylyl cyclase-coupled receptors, probably NPR-A, is evident because addition of eel ANP to isolated glomeruli from FW *Anguilla anguilla* increased cGMP production (Perrott *et al*., 1993). In the present study, however, renal effects were demonstrated only in SW eels whose glomeruli are fewer in number and more degenerated than those of FW fish (Brown *et al*., 1993). We did not measure GFR in this study; however, it is evident that the renal effects observed in SW eels are caused by the ANP action on renal tubules. The decrease in urine volume and the increase in urine ion concentrations are due to the increased water reabsorption and/or decreased ion uptake by renal tubules. In fact, marked urine concentration occurred during ANP infusion as evidenced by the accumulation of white precipitate in the urine. Involvement of the urinary bladder in this concentration process is unlikely because urine that enters the bladder is immediately introduced into the catheter by a negative pressure.

In other nonmammalian species, ANP caused only weak renal effects despite profound hypotension in the chicken (Gregg and Wideman, 1986) and in the turtle (Cho *et al*., 1988). Furthermore, transgenic mice over-expressing ANP gene show normal urine output and ion excretion although arterial pressure decreases (Field *et al*., 1991). Therefore, it seems that the cardiovascular action of ANP is more sensitive and potent than the renal action throughout vertebrate species. In this study, however, renal action is evident without effects on blood pressure in SW-adapted eels. In mammals, higher doses are necessary to manifest consistent renal effects (Seymour *et al*., 1986). Specific renal actions observed in the eel are partly due to the use of homologous ANP which allows a specific action on a single receptor type. Among biologically meaningful guanylyl cyclase-coupled receptors, ANP specifically binds type A receptor (NPR-A) and has low affinity to NPR-B (Hagiwara *et al*., 1995). Thus the current data may suggest that NPR-A is a predominant renal ANP receptor in the eel.

It is likely that the renal effect of ANP is variable depending on the hydration state of fish. Diuresis was induced in the toadfish in which isotonic saline was loaded to ensure constant urine flow (Lee and Malvin, 1987). ANP was diuretic in the trout which is on the edge of overhydration because of hypotonic environmental water. Interestingly, the antidiuretic effect of ANP is reversed to diuretic effect in the spiny dogfish when fish are hydrated in 90% seawater (Benyajati and Yokota, 1990). Therefore, it is possible that ANP is fundamentally a natriuretic hormone and the effect on water economy changes depending on the hydration state of the fish. It is not known why diuresis was not induced in FW eels in this study but this may be due in part to the lack of natriuretic effect because the diuretic effect of ANP always accompanies natriuretic effect in all fish thus far studied.

The most striking result obtained in this study was a profound decrease in plasma Na concentration during ANP infusion in SW eels. One possible cause of the decrease is decreased drinking of seawater and subsequent absorption of Na ions by the intestine as ANP is a potent inhibitors of drinking (Takei and Balment, 1993a; Tsuchida and Takei, 1998) and intestinal absorption of water and Na (O'Grady *et al*., 1985; Ando *et al*., 1992; Loretz, 1995) in fish. Another possibility is branchial extrusion of Na and Cl; it is reported that ANP increases extrusion of ^{22}Na into the environment in the flatfish (Arnold-Reed *et al*., 1991), and ANP stimulates Cl secretion from the opercular epithelia rich in chloride cells in the killifish, *Fundulus heteroclitus* (Scheide and Zadunaisky, 1988). It is not known whether ANP acts directly on the chloride cells to activate Na^+,K^+-ATPase or via stimulation of cortisol secretion (Arnold-Reed and Balment, 1991; Balm *et al*., 1995). Cortisol is known to activate Na^+,K^+-ATPase in fishes (McCormik, 1995). In summary, the current results provide evidence to support the notion that ANP is a hormone which specifically remove Na ions and promote SW adaptation in fish as suggested by Evans (1990).

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