Angiotensin II modulates the activity of the Na⁺/K⁺ATPase in eel kidney

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Abstract

We have previously shown that angiotensin II (Ang II) has a role at the level of the eel gill chloride cell regulating sodium balance, and therefore osmoregulation; the purpose of the present study was to extend these findings to another important osmoregulatory organ, the kidney.

By catalytic histochemistry Na⁺/K⁺ATPase activity was found in both sea water (SW)- and freshwater (FW)-adapted eel kidney, particularly at the level of both proximal and distal tubules. Quantitation of tubular cell Na⁺/K⁺ATPase activity, by imaging, gave values in SW-adapted eels which were double those found in FW-adapted eels (Student's *t*-test: *P*<0.0001). This was due to a reduced number of positive tubules present in FW-adapted eels compared with SW-adapted eels. By conventional enzymatic assay, the Na⁺/K⁺ATPase activity in isolated tubular cells from SW-adapted eels showed values 1.85-fold higher than those found in FW-adapted eels (Student's *t*-test: *P*<0.0001). Perfusion of kidney for 20 min with 100 nM Ang II provoked a significant increase (1.8-fold) in Na⁺/K⁺ATPase activity in FW, due

to up-regulation of Na⁺/K⁺ATPase activity in a significantly larger number of tubules (Student's *t*-test: *P*<0.0001). The effect of 100 nM Ang II in SW-adapted kidneys was not significant. Stimulation with increasing Ang II concentrations was performed on isolated kidney tubule cells: Ang II provoked a dose-dependent stimulation of the Na⁺/K⁺ATPase activity in FW-adapted eels, reaching a maximum at 100 nM (1.82-fold stimulation), but no significant effect was found in SW-adapted eels (ANOVA: *P*<0.001 and *P*>0.05 respectively). Isolated tubule cells stimulated with 100 nM Ang II showed a significant generation of inositol trisphosphate (InsP₃) and an increment in calcium release from intracellular stores.

In conclusion, our results suggest that tubular Na⁺/K⁺ATPase is modulated by environmental salinity, and that Ang II has a role in regulating its activity in FW-adapted eels, probably through an InsP₃-dependent mechanism.

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Introduction

Previous studies have shown that angiotensin II (Ang II) modulates the activity of the Na⁺/K⁺ATPase in the eel gill, suggesting a role for this hormone in gill NaCl retention acting via two receptors differentially modulated by salinity (Marsigliante *et al.* 1997). The system responsible for osmoregulation in euryhaline fish is a complex one, and given the physiological requirement for precise sodium and chloride ion regulation, the actions of the gill chloride cell cannot be considered in isolation. In salt and water metabolism, in both freshwater (FW)- and sea water (SW)-adapted fish, the kidney (and the intestine) plays a crucial role in accumulation or loss of water and ions, eliminating water and conserving electrolytes in FW, while excreting ions in a minimum of urine in SW. The electrogenic cationic pump Na⁺/K⁺ATPase, present in the membranes of all vertebrate cells, plays a crucial role

not only in the functionality of the chloride cell, but also in that of the kidney epithelial tubular cells. As well as its actions in the gill chloride cell, the fish renin-angiotensin system (RAS) also plays a role in renal and cardiovascular adaptation to increased environmental salinities (Gray & Brown 1985), in the control of drinking during sea-water adaptation (Tierney *et al.* 1993), and in the control of arterial blood pressure and plasma cortisol concentrations (Perrott & Balment 1990). One action of Ang II in the fish glomerular kidney is a glomerular anti-diuretic effect via regulation both of the number of filtering glomeruli and also of single nephron glomerular filtration rates (Brown *et al.* 1980), although the action on Na⁺ excretion is less clear. In the aglomerular fish, *Lophius americanus* (Churchill *et al.* 1979) and *Opsanus tau* (Zucker & Nishimura 1981), Ang II gives mixed results on both urine flow rate and Na⁺ excretion.

Given the importance of the kidney in osmoregulation and the importance of Ang II in modulating gill chloride cell Na⁺/K⁺ATPase activity, we decided to extend our study to investigate the modulation of kidney Na⁺/K⁺ATPase activity by Ang II, and to determine the signalling pathways by which Ang II exerts its effects in kidney tubular epithelia.

Materials and Methods

Animals

All animals used were European yellow eels (*Anguilla anguilla*), raised in freshwater, weighing between 150–200 g, and kept unfed under environmental conditions of photoperiod and water temperature (16–20 °C) in freshwater (Na⁺ 0.15 mM, K⁺ 0.06 mM, Cl[−] 0.18 mM, Ca²⁺ 0.42 mM, Mg²⁺ 0.34 mM, pH 7.6) aquaria.

When required, animals were transferred to sea water aquaria (Na⁺ 460 mM, K⁺ 10 mM, Cl[−] 540 mM, Ca²⁺ 20 mM, Mg²⁺ 107 mM, pH 7.6) and stabilised for 15 days before use.

Kidney perfusion

After heparinising the animal, the unanaesthetised fish was decapitated and a catheter was inserted into the dorsal aorta and tied in place. The kidney was cleared of blood by perfusing with Fish Ringer's buffer (FRB) (in mM: 143.2 NaCl for FW-adapted eels and 164.2 NaCl for SW-adapted eels, with 4.2 KCl, 1.0 Na₂HPO₄, 1.2 MgSO₄, 2.3 CaCl₂, 13.1 NaHCO₃, 0.1 (NH₄)₂SO₄, 0.4 KH₂PO₄, 5.6 glucose, and 3% dextran) at a constant pressure of 50 cm H₂O. The kidney was then perfused for 20 min without Ang II (control) or with (Asn-Arg-Val-Tyr-Val-His-Pro-Phe) fish Ang II (Bachem AG, Bubendorf, Switzerland) at 100 nM.

Preparation of isolated kidney tubular cells

Kidneys were removed, dissected free of fat and minced in the corresponding (FW or SW) FRB without dextran and supplemented with 0.5% trypsin. Incubations were carried out for 20 min at 37 °C on an orbital shaker and subsequently drawn through a 10-ml pipette five times. Enzymatic reaction was stopped with 2 volumes ice-cold buffer and tissue was filtered through a 1-mm mesh. Dissociated extrarenal cells were separated from tubules by filtration through a 40-µm nylon mesh. Tubules were centrifuged for 5 min at 1000 g, diluted in a sodium citrate buffer (at room temperature) containing (in mM): Na-citrate 27, NaCl 96, NaH₂PO₄ 5.6, KCl 1.5 adjusted to pH 7.6 with Tris and gently stirred with a glass rod for 10 min to facilitate cell detachment. This procedure was repeated twice. The released cells were filtered through a 25-µm

nylon mesh and centrifuged at 100 g for 10 min. The supernatant was discarded and the pellet containing the cells re-suspended in FRB. The cell suspension obtained from four kidneys was divided into 20 aliquots, and incubations were performed in quadruplicate for 15 min at 25 °C in the presence of increasing concentrations of Ang II (0.1, 10, 100 and 500 nM) and were terminated by dilution with ice-cold FRB.

Measurement of Na⁺/K⁺ATPase activity in kidney homogenates and isolated tubular cells

Kidneys were removed, dissected free of fat and minced in the relative (FW or SW) FRB, without dextran, containing 1 µg/ml of each of the proteinase inhibitors aprotinin, leupeptin, soybean trypsin inhibitor, and 1 mM phenylmethylsulphonyl fluoride (all from Sigma Chemical Co. Milano, Italy). Kidneys were homogenised in an ice-cold bath using a Polytron homogeniser (3 cycles of homogenisation of 10 s each). Protein concentration in the homogenates was measured with the Bio-Rad Protein Assay (BioRad Laboratories, Segrate, Italy) Kit I (lyophilised bovine plasma γ-globulin as standard), and the homogenates were diluted to 2 mg protein/ml in FRB.

The phosphorus released from the hydrolysis of adenosine triphosphate (ATP) was measured by a modification of the method of Fiske and Subbarow (Higgins 1987). This method is based on the reaction between phosphate and molybdate to give the yellow molybdate phosphoric acid which contains a molybdate (Mo) (VI) which is then reduced to Mo (V) present in a blue coloured heteropoly-acid compound. This blue compound is directly measured by reading the absorbance at 700 nm. Briefly, two assay mixtures (solution A containing (in mM): 10 MgCl₂, 189 NaCl, 42 KCl, 47 ATP-Na₂, 50 imidazole, pH 7.5, and solution B made as A but also containing 2.4 mM ouabain), were made just prior to assay. The phosphate produced from ATP hydrolysis in 50 µl total kidney or isolated tubular cell homogenates (2 mg protein/ml) after 10 min at 25 °C, in the presence and absence of ouabain, was determined directly by reading the absorbance at 700 nm of a standard curve from 0 to 800 nmol phosphate. Na⁺/K⁺ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, expressed as nmoles inorganic phosphate (Pi)/mg protein/h.

Na⁺/K⁺ATPase determination by catalytic histochemistry

Kidneys were frozen in 2-methyl butane pre-cooled to liquid nitrogen temperature for 30 min and stored in a freezing vial at −80 °C until used. Cryosections of 5 µm were cut on an HM 500 OM microtome cryostat (Microm Laborgerate GmbH, Walldorf, Germany) at −25 °C and used for catalytic histochemistry. Sections were collected on poly-L-lysine-coated slides, air dried for 20 min and

fixed using 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 min at 4 °C. Detection of Na⁺/K⁺ATPase was performed by the method of Kobayashi *et al.* (1987) modified by Zemanova and Gossrau (1994) which uses *p*-nitrophenyl phosphate as substrate and cerium ions for inorganic phosphate capture. For the visualization of cerium phosphate, 3,3'-diaminobenzidine-H₂O₂-Ni-hexamonium sulphate was used. Briefly, cryo-sections were incubated for 45 min at 25 °C in a medium containing 2 mM *p*-nitrophenyl phosphate, 2 mM CeCl₃, Mg²⁺ and K⁺ as activators and the non-specific alkaline phosphatase inhibitors, levamisole and L-phenylalanine, in 50 mM Tricine buffer, pH 7.5. After incubation, the sections were treated for 15 min at 60 °C using the visualisation medium of Halhuber *et al.* (1988) containing 1.4 mM 3,3'-diaminobenzidine, 100 mM NaN₃ and 12 mM Ni(NH₃)₆SO₄ in 50 mM Tris-HCl buffer, pH 7.6, and 0.002% H₂O₂. Control sections were either incubated without substrate, or with 10 mM ouabain, or in medium in which potassium ions were replaced by equimolar concentrations of sodium ions.

Other enzyme and protein assays

The effect of salinity on the activity of alkaline phosphatase (a marker enzyme for brush border membrane) was measured as specific activity in the original homogenate by monitoring *p*-nitrophenyl phosphate hydrolysis spectrophotometrically (410 nm) at a constant pH of 10.0 (Berner *et al.* 1976).

Protein concentration was measured with the Bio-Rad Protein Assay Kit I (lyophilised bovine plasma γ-globulin as standard).

Phospholipase C assay

This was carried out by measuring the formation of [³H]inositol phosphates from isolated kidney tubular cells pre-labelled with 150 μCi myo-[2-³H]inositol for 3 h, in 1.5 ml FRB. After incubation, cells were washed twice with FRB containing 10 mM inositol and 10 mM LiCl and suspended in the same buffer to give a cell density of 10⁶ cells/ml. Labelled cells were stimulated with 0, 10 and 100 nM Ang II for 5 min and incubations were terminated by the addition of ice-cold perchloric acid (15%, v/v), centrifuged, and the supernatants neutralised by adding 1.5 ml Freon/trioctylamine (1:1, v/v). The [³H]inositol triphosphate (InsP₃) was analysed by anion-exchange chromatography and the elution profile for the different inositol phosphates verified by using ³H-labelled inositol phosphates.

Measurement of intracellular calcium ([Ca²⁺]_i)

The method was derived from one previously described (Jacobs & Mandel 1987) using fura-2/acetoxymethyl ester,

with modifications (Poggioli *et al.* 1992). Renal tubular cells, isolated as described above, were loaded with fura-2 by incubation for 30 min at 25 °C, pH 7.4, in 2 ml FRB supplemented with 5 mM pyruvate, 0.1% fatty acid-free BSA, and 4 μM fura-2/acetoxymethyl ester. After fura-2 loading, cells were pre-incubated at 25 °C for 10 min before re-suspending in 1 ml medium in a 2 ml fluorescence cuvette in a water-jacketed cuvette holder maintained at 25 °C, and gassed with 5% CO₂. A similar preparation of cells incubated without fura-2/acetoxymethyl ester was used for auto fluorescence measurement. Values for intracellular calcium ([Ca²⁺]_i) were calculated as described in Barker *et al.* (1995).

Image analysis

Sections were mounted on a Zeiss Axioskop microscope linked to a colour video camera JVC KY-F30 (Tokyo, Japan) and the images obtained were digitalised and processed using Image Analyser Software Optilab 2.1 (Graftek, Voisins Le Bretonneux, France).

Image analysis was carried out as previously described (Muscella *et al.* 1997). To set a baseline for background signal, parallel sections were treated with 10 mM ouabain, an inhibitor of Na⁺/K⁺ATPase. The value for the mean grey level obtained from 50 of these negative sections was then subtracted from values obtained from grey values obtained in the absence of ouabain to give a final grey value which could be attributed to Na⁺/K⁺ATPase activity. Since Na⁺/K⁺ATPase activity may not be evenly distributed throughout the kidney, serial sections were cut (5 μm) and every twentieth section, spaced at 100 μm intervals through the kidney, was analysed. Values for kidney Na⁺/K⁺ATPase activity were the mean of results from analysis of 5 randomly chosen fields from each of 50 sections.

Statistical analysis

Experimental points represent the means ± standard deviation (s.d.) of 3–5 replicates. Statistical analysis was carried out using the Student's *t*-test for unpaired samples and the ANOVA; when indicated, *post hoc* tests (Bonferroni/Dunn) were also performed.

Results

Na⁺/K⁺ATPase activity in the whole kidney homogenates and in isolated tubule cells

The method used here for measurement of Na⁺/K⁺ATPase activity was sensitive and reproducible. The coefficient of variation of a single homogenate was 4.8% (*n* = 5) and Na⁺/K⁺ATPase activity increased linearly with increasing amounts of homogenates from 5 to 30 μl (2 mg protein/ml).

Figure 1 Na⁺/K⁺ATPase activity in SW-adapted eel kidney ($\times 400$). The reaction at the basolateral level is considerably reduced by ouabain treatment (right panel). The corresponding binary images are shown below.

Basal Na⁺/K⁺ATPase activity was detected in kidneys from both SW-adapted eels ($n=14$) and FW-adapted eels ($n=14$). Total Na⁺/K⁺ATPase activity was 738 ± 144 nmoles Pi/mg protein/h (mean $\pm$ s.d., $n=14$) in kidney homogenates obtained from SW-adapted eels, and 408 ± 140 nmoles Pi/mg protein/h ($n=14$) in FW-adapted eels ($P=0.009$, unpaired Student's *t*-test). The activity of the alkaline phosphatase was unaffected by the water salinity change: 70 ± 7 mU/mg protein ($n=4$) in FW-adapted kidney and 66 ± 9 mU/mg protein ($n=4$) in SW-adapted kidney. Na⁺/K⁺ATPase activity in isolated tubular cells was 2616 ± 204 nmoles Pi/mg protein/h ($n=10$) obtained from SW-adapted eels, and 1416 ± 132 nmoles Pi/mg protein/h ($n=10$) in FW-adapted eels ($P=0.007$, unpaired Student's *t*-test).

Catalytic histochemistry of Na⁺/K⁺ATPase in whole kidneys

Basal Na⁺/K⁺ATPase reactivity was detected in kidneys from both SW-adapted ($n=14$ eels, 7 perfused with 0 nM and 7 with 100 nM Ang II) and FW-adapted ($n=14$ eels, 7 perfused with 0 nM and 7 with 100 nM Ang II) animals. The reactivity was confined to the tubular cells and, to ensure the specificity of the reaction, experimental

controls were conducted in the absence of K⁺ or Na⁺: no reaction was visible in either case. The reaction was also completely inhibited at the basolateral membrane level of polarised epithelial cells (where the pump is usually located) by the specific Na⁺/K⁺ATPase inhibitor, ouabain (Fig. 1), thus proving its selectivity and specificity. Coefficients of variation (CV) were calculated using at least two serial sections from both SW- and FW-adapted kidneys, in the presence and in the absence of ouabain; CV ranged from 1% to 9.2%, mean $\pm$ s.d. was 5.9 ± 2.59 , median was 5.6 ($n=25$). The histochemical measurements of the Na⁺/K⁺ATPase were linear over the period of incubation (particularly from 15 to 60 min) in both SW- and FW-adapted kidney (Fig. 2). When the tubules present on all the slices used were counted (in 7 FW- and 7 SW-adapted eels), no statistically significant differences in their number between SW- and FW-adapted eels were found (SW: 210 ± 39 tubules/field at $\times 25$ magnification, ($n=125$ fields; 25 fields per animal), FW: 192 ± 32 tubules/field at $\times 25$ magnification, ($n=125$ fields, 25 fields per animal), unpaired Student's *t*-test: $P=0.7$). However, the number of tubules expressing detectable Na⁺/K⁺ATPase activity was significantly higher in SW- than in FW-adapted eels ($P<0.001$) (Fig. 3). In SW, virtually all of the

Figure 2 Histochemical measurements of kidney Na⁺/K⁺ATPase activity over the period of incubation (15 to 75 min) in FW-adapted ($n=4$) and SW-adapted ($n=4$) yellow eels.

proximal tracts (98%) expressed a very high Na⁺/K⁺ATPase activity.

Approximately 80% of the distal tracts and 6% of the collecting ducts and tubules were also 'positive' (i.e. displayed a cytochemically measurable Na⁺/K⁺ATPase activity). In contrast, in glomeruli and interstitial tissue containing well-developed haematopoietic cells, Na⁺/K⁺ATPase activity was undetectable and they were therefore referred to as 'negative'. In FW-adapted fish, Na⁺/K⁺ATPase activity was detectable in all regions of the nephron shown to be positive in SW-adapted fish; however, the frequency of positivity in FW was lower (proximal tracts: 50%, distal tracts: 40%, collecting ducts and

Figure 3 The number of the positive tubules (expressing detectable Na⁺/K⁺ATPase) in 25 microscopic fields analysed ($\times 25$) from kidneys taken from SW- and FW-adapted eels. The results obtained in FW-adapted eels which were perfused with 100 nM Ang II are also shown. Values are means $\pm$ S.D. of 4 SW- and 8 (of these 4 were perfused with Ang II) FW-adapted kidneys. $P<0.0001$ by unpaired Student's *t*-test between SW-adapted and FW-adapted, and between FW-adapted and FW-adapted perfused with Ang II.

tubules: 4%). No gradient along the kidney body was found, in terms of the number of tubules expressing measurable Na⁺/K⁺ATPase activity, in either SW- and FW-adapted kidneys (Fig. 4). The overall tubule-associated Na⁺/K⁺ATPase activity found by imaging in SW-adapted eels was twice that found in FW-adapted eels (unpaired Student's *t*-test: $P<0.0001$).

Effects of 100 nM Ang II on Na⁺/K⁺ATPase activity

Perfusion of kidneys with Ang II provoked different effects depending upon the environmental adaptation. In FW-adapted eels we found, by catalytic histochemistry followed by imaging, a significant increment in Na⁺/K⁺ATPase activity (Fig. 5) in all animals used ($n=7$), ranging from a minimum of a 1.5-fold to 2.2-fold stimulation (Fig. 6), with a mean value of 1.8-fold (ANOVA: $P<0.01$). This increment in the activity of the Na⁺/K⁺ATPase was due to the appearance of an activity in a larger number of tubules (Fig. 3). In SW-adapted eels, the effect was not significant (ANOVA: $P>0.05$) (Fig. 6). Similar results were also obtained assaying the Na⁺/K⁺ATPase activity on isolated tubular cells (results shown as percentage of the control in Fig. 6).

Time course of Na⁺/K⁺ATPase activity following seawater transfer

When FW-adapted eels were transferred to seawater there were readjustments of the renal Na⁺/K⁺ATPase activity.

Figure 4 Catalytic histochemistry of Na⁺/K⁺ATPase performed in a SW-adapted (upper panels) and in a FW-adapted (lower panels) eel kidney. Twelve images ($\times 25$) have been joined by computer to show an 11-mm long piece of kidney; the corresponding binary images are shown below with the reactive tubules against a white background. Generally, a whole picture of a kidney was made up of four such digital images.

The time course of the effects of salinity on Na⁺/K⁺ATPase activity was examined by comparing the data obtained in isolated tubular cells in FW and 1, 3, 5, 10 and 15 days after seawater transfer. On day 1, Na⁺/K⁺ATPase activity increased significantly at a maximum level (from 1416 ± 132 nmoles Pi/mg protein/h to 2244 ± 300 nmoles Pi/mg protein/h) and remained unchanged for the entire 15-day experimental period.

Effects of increasing concentrations of Ang II on isolated tubular cells

Using isolated tubular cells, a significant dose-dependent increase in the Na⁺/K⁺ATPase activity was found in those obtained from FW-adapted eels (ANOVA: $P < 0.0001$), with a threshold increase at 0.1 nM Ang II, a maximum (1.82-fold; from 1416 ± 132 to 2574 ± 366 nmoles Pi/mg protein/h, $n = 10$ animals) at 100 nM Ang II, and no further increase with 500 nM Ang II (Fig. 6, upper panel). Conversely, in cells obtained from SW-adapted eels no significant variation in Na⁺/K⁺ATPase activity was observed (Fig. 6, lower panel).

The mechanism underlying the effect of Ang II on isolated kidney tubular cells was also examined. When [³H]inositol-loaded tubular cells, obtained from FW- or

from SW-adapted eels, were stimulated with Ang II for 5 min, a significant increase (ANOVA: $P < 0.01$) in [³H]InsP₃ production was observed (Fig. 7). Figure 8 shows the time course for the effect of 100 nM Ang II on intracellular calcium in both SW- and FW-adapted cells. In the presence of 1 mM CaCl₂ in the medium, Ang II provoked a significant increase in [Ca²⁺]_i within 15 s of hormone addition, followed by a decline to a plateau phase in both SW- and FW-adapted eels. Conversely, when medium calcium was reduced by the addition of 5 mM EGTA, Ang II still caused an increase in [Ca²⁺]_i, but the peak phase was not followed by a plateau. EGTA treatment did not alter basal [Ca²⁺]_i.

Discussion

In previous studies we have demonstrated that the eel renal tubular epithelial cell expresses a specific Ang II receptor which binds radiolabelled Ang II and focuses at pI 6.5 by isoelectric focusing (Marsigliante *et al.* 1994), and that Ang II modulates branchial Na⁺/K⁺ATPase activity (Marsigliante *et al.* 1997). The purpose of the present experiments was to extend these studies on the role of Ang II in teleost osmoregulation by investigating the effects of

Figure 5 Variations in specific Na⁺/K⁺ATPase activities observed by enzymatic assay (enz) and by catalytic histochemistry (his) in 14 FW-adapted (upper panel) and 14 SW-adapted animals (lower panel) perfused in saline with and without 100 nM Ang II (+Ang II). C, control animals ($n=7$ each), i.e. eels perfused in the absence of hormone. ANOVA: $P<0.001$ and $P>0.05$ for FW- and SW-adapted eels respectively.

this hormone on the activity of the Na⁺/K⁺ATPase in the eel kidney nephron. The eel kidney is a heterogeneous organ, made up of nephrons, interstitial tissue containing haematopoietic cells, interrenal tissue containing steroidogenic and chromaffin cells, blood vessels and blood cells; therefore the classical biochemical assay performed on tissue homogenates measures contributions of Na⁺/K⁺ATPase activity from all these different cellular types. For this reason, the major advantage of catalytic histochemistry compared with the classical assay is that it makes it possible for these various contributions to be defined.

Using catalytic histochemistry, renal tubular Na⁺/K⁺ATPase activity was found to be uniformly distributed along the kidney from the cranial to the caudal part and from the inner to the outer side, therefore excluding a gradient in its distribution. In FW-adapted eels, Ang II

Figure 6 Effects of increasing Ang II concentrations on specific Na⁺/K⁺ATPase activity in isolated tubular cells obtained from FW- ($n=4$) and SW- ($n=4$) adapted kidneys. ANOVA: $P<0.0001$ and $P>0.5$ for FW- and SW-adapted eels respectively. Bonferroni/Dunn *post hoc*: control vs 0.1 nM: $P=0.0046$; control vs 10 nM: $P<0.0001$ (FW).

was shown to cause a significant and dose-dependent increase in Na⁺/K⁺ATPase activity of whole kidney sections and isolated cells. This increment was due to an induction of the Na⁺/K⁺ATPase in those FW-adapted

Figure 7 InsP₃ generation in isolated kidney tubular cells from both SW- and FW-adapted eels ($n=5$ for both) incubated with increasing concentrations of Ang II, evaluated by measuring the radioactivity associated with [³H]InsP₃ separated by anion-exchange chromatography. ANOVA: $P<0.0001$ for both SW- and FW-adapted eels. Bonferroni/Dunn *post hoc*: control vs 1 nM: $P<0.0001$; control vs 10 nM: $P<0.0001$ (SW). Control vs 1 nM: $P=0.0006$; control vs 10 nM: $P<0.0001$ (FW).

tubules which did not display measurable activity under basal conditions. This provides strong evidence that Ang II plays an important role in modulating Na⁺/K⁺ATPase in FW-adapted fish. In contrast, kidneys from SW-adapted eels were not affected in a similar way by Ang II, but did, however, express a twofold higher level of renal tubular Na⁺/K⁺ATPase activity than FW-adapted animals.

Tubular Na⁺/K⁺ATPase is involved in the translocation of Na⁺ at basolateral membranes, which, in SW-adapted animals, is strongly linked to renal water re-absorption. The higher Na⁺/K⁺ATPase activity found in SW-adapted animals has been shown to be related to the high activity of the Na⁺/H⁺ exchanger (Zonno *et al.* 1994) and to the presence of the Cl⁻/HCO₃⁻ exchanger (Vilella *et al.* 1997). This activity is, therefore, important in body fluid homeostasis. Thus, the higher Na⁺/K⁺ATPase activity observed in SW-adapted kidney provides a means by which more water can be re-absorbed, with plasma Na⁺ being excreted by the gill. Since the level of circulating Ang II in SW-adapted *Anguilla anguilla* is at least threefold higher than in FW-adapted eels (Tierney *et al.* 1995), it is conceivable that in SW the Na⁺/K⁺ATPase is not susceptible to further activation by Ang II. Conversely, in FW the effects of Ang II on the activity of the Na⁺/K⁺ATPase paralleled those observed in the gill

Figure 8 Effects of 100 nM Ang II (All) on intracellular Ca²⁺ in isolated kidney tubular cells from SW-adapted (upper panel, $n=4$) and FW-adapted (lower panel, $n=4$) eels. 5 mM EGTA was added in order to reduce the calcium concentration in the medium. EGTA treatment did not alter basal calcium concentration (EGTA) and after stimulation with AngII the peak phase was not followed by a plateau (100 nM All+EGTA).

(Marsigliante *et al.* 1997), and would be expected to stimulate increased Na⁺ and Cl⁻ reabsorption. Thus, in euryhaline fish the primary physiological mechanism by which the organism faces the challenge of environmental salinity changes may be by using Na⁺/K⁺ATPase rather than the antiports and symports as a means for regulation of transcellular sodium transport.

The data suggest that in FW-adapted eels, Ang II may act as a rapid up-regulator of the Na⁺/K⁺ATPase activity in both the gill (Marsigliante *et al.* 1997) and the kidney, effects which are fundamental to salt and water re-absorption. A similar up-regulation of Na⁺/K⁺ATPase also occurs in SW; however, this latter effect is only apparent in the gill, where the activity can still be enhanced. In SW-adapted kidney, while the

Na⁺/K⁺ATPase is higher than in FW-adapted eel kidney, this may be independent of the effects of Ang II.

Measurement of InsP₃ showed that there was a significant effect of Ang II on InsP₃ output together with a rapid rise in intracellular calcium in both SW- and FW-adapted eels, in accordance with the finding of others who showed that Ang II increases intracellular calcium in rat proximal tubular cell (Poggioli *et al.* 1992), and enhances Na⁺/K⁺ATPase activity (Aperia *et al.* 1994). The use of EGTA to reduce extracellular calcium confirmed that the peak phase of calcium mobilisation resulted from release of calcium from intracellular stores. It is well established that Ang II can activate phospholipase C leading to InsP₃-induced calcium release from intracellular stores (Barker 1997) acting via a G-protein-coupled receptor known as the AT1 receptor. It may be possible, therefore, that an AT1-like receptor, expressed in eel kidney tubules, mediates these effects.

The fact that renal tubular cells from both FW- and SW-adapted eels were equally sensitive to Ang II stimulation of intracellular calcium while no increase in ATPase activity was observed in SW-adapted animals further supports the view that Na⁺/K⁺ATPase levels are already maximally stimulated. This might also be the result of additional effects, such as those of cortisol, which has been shown to modulate Na⁺/K⁺ATPase alpha-subunit levels in silver seabream branchial tissue (Deane *et al.* 1999). However, since Ca²⁺ mobilisation can cause activation of both protein kinase (PK) C and Ca²⁺/calmodulin-dependent protein phosphatase (PP2B; calcineurin) this could lead to either phosphorylation or dephosphorylation of the Na⁺/K⁺ATPase. Phosphorylation/dephosphorylation have been reported to have different effects on Na⁺/K⁺ATPase activity in different systems. PKC activates the Na⁺/K⁺ATPase in smooth muscle cells and proximal tubule cells, while the PKC- or PKA-induced phosphorylation inactivates the enzyme in rat and shark kidneys (Aperia *et al.* 1991, Bertorello *et al.* 1991, Nowicki *et al.* 1997, Pedemonte *et al.* 1997); moreover, the effects of PKC depend upon the PKC isoforms involved (Efendiev *et al.* 1999, Li *et al.* 1999). This may provide an additional or alternative explanation for our observations; however, further studies will be necessary to discover the exact nature of the increment in the Na⁺/K⁺ATPase activity operated by Ang II in this cell. Other factors, such as cortisol, growth hormone and insulin-like growth factor, have also been implicated in the regulation of Na⁺/K⁺ATPase in the brown trout (Madsen *et al.* 1995). However, these effects were at the level of the trout gill while renal Na⁺/K⁺ATPase activity was unaffected. In common with our study, SW adaptation of the brown trout led to increased Na⁺/K⁺ATPase activity preferentially in gill.

In conclusion, in a comparative and evolutionary sense, our findings demonstrate the antiquity of the RAS as a means of electrolyte (and water) regulation. Additionally,

given the ubiquity of angiotensin receptors in a wide variety of epithelia (in the mammalian system at least) it may even be that RAS and epithelial transport functions are invariably linked, although further work is needed to elucidate Ang II involvement in Na⁺/K⁺ATPase activity regulation in the gut.

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