Na⁺/K⁺ATPase activity inhibition and isoform-specific translocation of protein kinase C following angiotensin II administration in isolated eel enterocytes

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Abstract

In the eel, angiotensin II (Ang II) has a role at the level of both gill chloride and kidney tubular cells, regulating sodium balance and therefore osmoregulation. The present study extends these findings to another important osmoregulatory organ – the intestine. Enterocytes were obtained from sea-water (SW)-acclimated eels to investigate the role of Ang II on the intestinal Na⁺/K⁺ATPase activity, because in SW-acclimated animals the intestine represents an important site of water and NaCl transport from the mucosal to the serosal side. This paper demonstrates that isolated enterocytes stimulated with increasing Ang II concentrations (0.01–100 nM) showed a dose-dependent inhibition of the Na⁺/K⁺ATPase activity. The threshold decrease was at 0.01 nM Ang II; it reached a maximum at 10 nM (81.5% inhibition) and did not decrease further with the use of higher hormone doses. These hormonal effects were blocked by a specific competitive antagonist of the AT1 receptor subtype, DuP-753 (100% inhibition at 10 µM), indicating that these effects are mediated by an AT1-like receptor. Isolated enterocytes

stimulated with 10 nM Ang II showed a transient increase in intracellular calcium ([Ca²⁺]_i), followed by a lower sustained phase. Removal of extracellular Ca²⁺ did not reduce the initial transient response and completely abolished the plateau phase. The inhibition of the Na⁺/K⁺ATPase activity was dependent on protein kinase C (PKC) activation since PKC antagonists (calphostin C and staurosporine) abolished the inhibitory effect of Ang II, and the PKC activator phorbol 12-myristate 13-acetate reduced transporter activity. Western blot analysis with antibodies to PKC α, βI, βII, γ, δ, ε, ι, η and ζ isoforms showed that eel enterocytes expressed the conventional isoforms (α and βI), the novel isoforms (δ and η) and the atypical isoforms (ζ and ι). Ang II stimulated the translocation from the cytosol to the plasma membrane of PKC α, PKC δ and PKC η isoforms. In conclusion, our results suggest that Ang II modulates intestinal Na⁺/K⁺ATPase in SW-acclimated eels via calcium mobilization and PKC activation.

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Introduction

In fresh water (FW), teleosts actively accumulate ions (mainly Na⁺ and Cl[−]) through the gills, eliminating water and retaining electrolytes through the kidney. In sea water (SW), teleosts balance the influx of ions and the efflux of water by excreting the excess of monovalent ions through the gills, absorbing fluid from ingested SW in the intestine, and excreting the excess of divalent ions through the kidney.

In osmoregulatory epithelia, the electrogenic cationic pump Na⁺/K⁺ATPase is essential for transcellular movement of water and ions. It was previously shown (Marsigliante *et al.* 1997, 2000) that angiotensin II (Ang II) modulates the activity of the Na⁺/K⁺ATPase in the eel gill and kidney, suggesting a role in NaCl homeostasis for

this hormone. Ang II also modulates the activity of Na⁺/K⁺ATPase in the eel kidney via mobilization of intracellular calcium ([Ca²⁺]_i) and protein kinase C (PKC) activation (Marsigliante *et al.* 2000).

The epithelium of eel intestine is a single layer of columnar cells (Clarke & Witcomb 1980). In SW-acclimated animals, it is an important site of NaCl and water transport from the mucosal to the serosal side (Ando *et al.* 1974, Marvão *et al.* 1994). These processes are tuned by the activity of the basolateral membrane protein Na⁺/K⁺ATPase (Ando 1983).

Given the importance of the intestine and Na⁺/K⁺ATPase in osmoregulation and the role of Ang II in modulating Na⁺/K⁺ATPase activity in gill and kidney, we extended our study to investigate the modulation of the Na⁺/K⁺ATPase activity by Ang II in the intestine and

the Ang II signal-transduction pathway in enterocytes from SW-acclimated animals.

Materials and Methods

Animals

All animals used were European yellow eels (*Anguilla anguilla*) raised in FW, weighing between 150 and 200 g, and were kept under environmental conditions of photoperiod and water temperature (16–20 °C) in FW (0.15 mM Na^+ , 0.06 mM K^+ , 0.18 mM Cl^- , 0.42 mM Ca^{2+} , 0.34 mM Mg^{2+} , pH 7.6) aquaria. The eels were transferred to SW aquaria (460 mM Na^+ , 10 mM K^+ , 540 mM Cl^- , 20 mM Ca^{2+} , 107 mM Mg^{2+} , pH 7.6) when required and stabilized for 15 days before use. Procedures were approved by the Ministero Università Ricerca Scientifica Tecnologica (MURST).

Preparation of isolated intestine cells

The eels were killed by decapitation. The intestines were rapidly removed, placed in 1.1% (w/v) ice-cold NaCl solution and then rinsed several times with the same buffer to remove food particles, mucus and other contaminants. Only the middle segment of the intestine was used for all the experiments described here because it has been shown that the activity of the Na^+/K^+ ATPase in this region is different from the activity expressed by the posterior part of the intestine (Vokler *et al.* 1987, Seidelin & Madsen 1999).

The intestinal mucosa was stripped away from the other tissues, using fine forceps. Cells were isolated at room temperature by gently stirring the intestine pieces with a glass rod for 10 min in a sodium citrate buffer (27 mM Na-citrate, 96 mM NaCl, 5.6 mM NaH_2PO_4 , 1.5 mM KCl adjusted to pH 7.6 with Tris-HCl). This procedure was repeated twice. The released cells were filtered in sequence through nylon membranes of different pore size (225, 100 and 50 μm respectively) and then centrifuged at 100 *g* for 10 min. The supernatant was discarded and the pellet containing the cells was resuspended in a physiological saline solution (PSS: 4 mM KCl, 2 mM CaCl_2 , 4 mM NaHCO_3 , 1 mM MgSO_4 , 0.5 mM NaH_2PO_4 , 150 mM NaCl, 30 mM Hepes, 10 mM D-glucose and 2 mM L-glutamine) adjusted to pH 7.6 with Tris-HCl. The cell suspension was filtered again through a 50 μm nylon mesh and diluted with PSS to a final concentration of 1 mg protein/ml. Cell suspensions from four intestines were divided into aliquots. Incubations were performed in quadruplicate for 15 min at 25 °C in the presence of increasing concentrations of Ang II (0.01, 0.1, 1, 10 and 100 nM) and were stopped with dilutions of ice-cold PSS.

Measurement of Na^+/K^+ ATPase activity in isolated enterocytes

The phosphorus released from the hydrolysis of adenosine triphosphate (ATP) was measured by the Fiske & Subbarow method (Higgins 1987) modified as previously described (Marsigliante *et al.* 2000). This method is based on the reaction between phosphate and molybdate, producing yellow molybdate phosphoric acid which contains a Mo(VI) that is then reduced to Mo(V) present in a blue-coloured heteropolyacid compound. This blue compound is directly measured by reading the absorbance at 700 nm. Briefly, two assay mixtures (solution A containing (in mM) MgCl_2 (10), NaCl (189), KCl (42), ATP- Na_2 (47), imidazole (50), 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 isolated enterocyte 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 expressed as μmol inorganic phosphate (Pi)/mg protein/h.

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 concentrations were measured with a protein assay kit (no. I; Bio-Rad, Hercules, CA, USA). Lyophilized bovine plasma γ -globulin was used as the standard.

Measurement of intracellular calcium ($[\text{Ca}^{2+}]_i$)

The method of measuring intracellular calcium ($[\text{Ca}^{2+}]_i$) was derived from one that was previously described (Jacobs & Mandel 1987), using fura-2, acetoxymethyl ester, with modifications (Poggioli *et al.* 1992). Isolated enterocytes were incubated for 30 min at 25 °C in 2 ml PSS buffer supplemented with 5 mM pyruvate, 0.1% fatty acid-free BSA, and 4 μM fura-2/acetoxymethyl ester (pH 7.4). After fura-2 loading, cells were pre-incubated at 25 °C for 10 min before resuspension in 1 ml medium in a 2 ml fluorescence cuvette in a water-jacketed cuvette holder maintained at 25 °C, and gassed with 5% CO_2 . A similar preparation of cells incubated without fura-2/acetoxymethyl ester was used for auto-fluorescence measurement. Values for $[\text{Ca}^{2+}]_i$ were calculated as described in Barker *et al.* (1995).

Immunoblotting analysis of PKC isozymes

For PKC immunoblots, enterocytes isolated from eight to ten animals were pooled and homogenized at 4 °C in lysis

buffer (25 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 5 mM dithiothreitol, 25 mg/ml aprotinin, 25 mg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride). After removal of nuclei by 5-min centrifugation at 2000 *g* at 4 °C, separation of the particulate and soluble fractions was performed by 1 h centrifugation at 100 000 *g* at 4 °C. Proteins (100 µg/sample) corresponding to the soluble and particulate fractions were subjected to SDS-PAGE (8% acrylamide gel) and transferred onto a 0.2 µm nitrocellulose membrane. Membranes were blocked for 1 h in 5% non-fat dry milk solution in Tris-buffered saline/Tween. After overnight incubations with rabbit polyclonal antibodies to human PKC α , β I, β II, γ , δ , ϵ , ι , η and ζ isoforms (Santa Cruz Biotechnology, CA, USA), membranes were washed and incubated for 1 h with a horseradish-peroxidase-labeled goat anti-mouse antibody (1:10 000; Amersham, Little Chalfont, Buckinghamshire, UK). The concentrations of the PKC isoform antibodies were as follows: PKC α , 1:5000; all others, 1:300.

To confirm the specificity of immunoreactive bands, a peptide block of PKC immune reactivity was performed by adding corresponding control peptides (Santa Cruz Biotechnology) in the PKC antibody solution before incubation with the nitrocellulose membrane.

Proteins were visualized by enhanced chemiluminescence (ECL; Amersham) for all Western blots. Semi-quantitative analysis of PKC isoform bands was performed by scanning densitometry.

Statistical analysis

Experimental data-points represent the means $\pm$ standard deviation (S.D.) of 3–5 replicates. Statistical analysis was carried out using the Student's *t*-test for unpaired samples and ANOVA. When indicated, post-hoc tests (Bonferroni/Dunn) were also performed. A *P* value less than 0.05 was considered as statistically significant.

Results

Na^+/K^+ ATPase activity in isolated enterocytes

The method for measurement of Na^+/K^+ ATPase activity used here was sensitive and reproducible. The coefficient of variation of a single homogenate was 4.5% ($n=7$) and the Na^+/K^+ ATPase activity increased linearly with increasing amounts of homogenates from 10 to 60 µg.

Na^+/K^+ ATPase activity in isolated intestine cells obtained from the middle segment was 3.07 ± 0.49 µmol Pi/mg protein/h (mean $\pm$ S.D., $n=12$) and 6.77 ± 0.67 µmol Pi/mg protein/h ($n=12$) in enterocytes from FW- and SW-acclimated eels respectively (the unpaired Student's *t*-test gave $P<0.0001$).

The activity of the alkaline phosphatase was unaffected by the water-salinity change: 70 ± 7 mU/mg protein

Figure 1 Effects of increasing Ang II concentrations on specific Na^+/K^+ ATPase activity in isolated enterocytes. The data are means $\pm$ S.D. of 6 experiments run in triplicate. ANOVA: $P<0.0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

($n=4$) and 66 ± 9 mU/mg protein ($n=4$) were the results for FW- and SW-acclimated intestine respectively.

Effects of increasing concentrations of Ang II on isolated enterocytes from SW-acclimated eels

For the isolated enterocytes, a significant dose-dependent decrement in the Na^+/K^+ ATPase activity was found in SW-acclimated eels (ANOVA: $P<0.0001$). A threshold decrease was observed at 0.01 nM Ang II, with a maximum at 10 nM Ang II (from 6.77 ± 0.67 to 1.25 ± 0.43 µmol Pi/mg protein/h, $n=10$ animals). No further decrease was shown at 100 nM Ang II (Fig. 1). The effect of 10 nM Ang II was time-dependent. There was a threshold decrease at 5 min, a maximal effect at 15 min and no further effects with longer incubation times (Fig. 2).

Figure 3 shows the effects of different concentrations of DuP-753, a specific competitive AT1 receptor subtype antagonist, on the Ang II-dependent reduction in the Na^+/K^+ ATPase activity. DuP-753 (at a concentration range spanning from 0.001 to 100 µM) produced a progressive reduction in Ang II action. A 100% inhibition was observed at 10 µM, indicating that Ang II effects are mediated by an AT1-like receptor.

The mechanism underlying the effects of Ang II on isolated intestine cells was also examined. Figure 4 shows the time-course effects of 10 nM Ang II on intracellular calcium. In the presence of 1 mM CaCl_2 in the medium, Ang II provoked a significant increase in $[\text{Ca}^{2+}]_i$ within 15 s after hormone addition; this was followed by a decline to a plateau phase. Conversely, when medium calcium was reduced by the addition of EGTA, Ang II still caused

Figure 2 Effects of increasing the incubation times of Ang II (10 nM) on Na^+/K^+ ATPase activity in isolated enterocytes ($n=6$). The data are presented as percentages of the control and are means $\pm$ S.D. of 6 experiments run in triplicate. ANOVA: $P<0.0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

an increase in $[\text{Ca}^{2+}]_i$, but the peak phase was not followed by a plateau. EGTA treatment did not alter basal $[\text{Ca}^{2+}]_i$.

Effects of Ang II on Na^+/K^+ ATPase activity: role of PKC

To test whether PKC was involved in Ang II-induced effects on Na^+/K^+ ATPase activity, enterocytes were incubated for 30 min with 100 nM calphostin C (a selective antagonist of PKC) or with 100 nM staurosporine (which interferes with the catalytic domain of protein

Figure 3 Effects of increasing DuP-753 concentrations on specific Na^+/K^+ ATPase activity in isolated enterocytes incubated with 10 nM Ang II. The data are means $\pm$ S.D. of 4 experiments run in triplicate. C, Na^+/K^+ ATPase activity control values (i.e. values obtained in the absence of Ang II and DuP-753); 0, Na^+/K^+ ATPase activity in enterocytes incubated with 10 nM Ang II for 15 min. ANOVA: $P<0.0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

Figure 4 Effects of 10 nM Ang II (+Ang II) on intracellular Ca^{2+} in isolated eel enterocytes (the data are means $\pm$ S.D. of 5 experiments). EGTA (5 mM) was added in order to reduce the Ca^{2+} concentration in the medium. EGTA treatment did not alter the basal Ca^{2+} concentration (+EGTA) and, after stimulation with Ang II, the peak phase was not followed by a plateau (+Ang II+EGTA). The arrow indicates the administration of Ang II.

kinases). As shown in Fig. 5, PKC antagonists did not significantly alter the basal activity of the Na^+/K^+ ATPase. If enterocytes were pre-incubated first with calphostin C or with staurosporine and then treated with 10 nM Ang II, no reduction in Na^+/K^+ ATPase activity was observed (Fig. 5).

Figure 5 Effects of 100 nM calphostin C and 100 nM staurosporine on the specific Na^+/K^+ ATPase activity in isolated enterocytes incubated in the presence of 10 nM Ang II (CC+Ang II and S+Ang II respectively) and in the absence of Ang II (CC and S respectively). The data are means $\pm$ S.D. of 4 experiments run in triplicate. C, Na^+/K^+ ATPase activity control values (i.e. values obtained in the absence of Ang II and inhibitors). Ang II, Na^+/K^+ ATPase activity in enterocytes incubated with 10 nM Ang II for 15 min. ANOVA: $P<0.0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

Figure 6 The effects of PMA on eel enterocyte Na⁺/K⁺ATPase activity. Cells were incubated with increasing concentrations of PMA or without PMA for the indicated time-periods. ANOVA: $P < 0.0001$ for all time-periods. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

To provide further evidence for a role for PKC in the inhibition of Na⁺/K⁺ATPase activity, we tested the effects of the PKC activator phorbol 12-myristate 13-acetate (PMA) on this activity. Incubation of enterocytes with PMA (at 0.01, 0.1, 1 and 10 μM) for 5, 15 and 25 min led to inhibition of Na⁺/K⁺ATPase activity. The maximum effect was observed after 25 min incubation with 10 μM PMA and was comparable to that elicited by Ang II (Fig. 6). This observation suggests the involvement of conventional and new, but not atypical, PKC isoforms (Dekker & Parker 1994).

PKC isoform expression in eel enterocytes

In enterocytes, the anti-α, -βII, -δ, -η, -ι and -ζ PKC antibodies recognized a single band with molecular masses

of 82, 82, 86, 92, 84 and 67 kDa respectively in both the cytosolic and membrane fractions (Fig. 7). Of these isoforms, the most abundant was PKC α (membrane-bound plus cytosolic PKC set as 100%). The relative abundance of the remaining isoforms were as follows: ι (88%), βII (57%), η (56%), ζ (51%) and δ (20%). However, no immunoreactivity was detected with the antibodies against PKC βI, γ and ε, even after prolonged exposure. This result indicates that eel enterocytes expressed the conventional isoforms α and βII, the novel isoforms δ and η, and the atypical isoforms ζ and ι.

In order to investigate the action of Ang II on PKCs in eel enterocytes, translocation of PKC isoforms from the cytosol to cellular membranes was analyzed. Quantitative Western blot analysis was performed using enterocytes exposed to 0.1 and 10 nM Ang II for 15 min (Fig. 8). A translocation of PKC α, δ and η to enterocyte membranes was observed. Densitometric analysis revealed that after 15 min Ang II incubation there was an increase in membrane-associated PKC above their control value (set as 100%) to 190% (0.1 nM Ang II) and 285% (10 nM Ang II) for PKC η, to 160% (0.1 nM Ang II) and 262% (10 nM Ang II) for PKC δ and to 125% (0.1 nM Ang II) and 165% (10 nM Ang II) for PKC α (Fig. 8). The remaining PKC isoforms did not translocate from the cytosol to the membranes after 15 min incubation with the hormone.

PMA, which induces PKC (conventional and novel) activity, was used as a positive control. Western blot quantitative analysis was performed using eel enterocytes exposed to PMA (1 and 10 μM) for 25 min (Fig. 8). After 25 min PMA treatment at 1 and 10 μM, a translocation of PKC α and δ (but not of PKC ζ) was observed from the cytosol to enterocyte membranes. Densitometric analysis revealed that treatment with PMA caused an increase in membrane-associated PKC α above its control value (set as 100%) to 135% at 1 μM PMA and to 175% at 10 μM PMA. The membrane-associated PKC δ was increased above its control values to 140% at 1 μM PMA and to

Figure 7 Western blot analysis revealed PKC isoforms in the soluble cytosolic fractions (C) and associated with the membrane fractions (M) of eel enterocytes. The results are representative of three independent experiments.

Figure 8 Densitometric analysis of PKC- α , PKC- δ and PKC- η isoforms. The results are expressed as percentages of PKC signals corresponding to bands from membranes of control cells (treated with vehicle only). Data are means $\pm$ S.D. of 3 experiments run in duplicate. ANOVA: $P < 0.0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests. An asterisk indicates an overall effect of the treatment.

185% at 10 μM PMA. The isoform PKC η showed a somewhat different activation pattern. PMA treatment caused an increase above control values to 200% and 400% at 1 and 10 μM respectively after 20 min exposure (Fig. 8).

Discussion

Ang II modulates branchial (Marsigliante *et al.* 1997) and renal (Marsigliante *et al.* 2000) Na^+/K^+ ATPase activity, thereby providing strong evidence that it plays an important role in osmoregulation. Here, we extend our study on

the role of Ang II in teleosts and its effects on the activity of the Na^+/K^+ ATPase in eel enterocytes.

We found that the basal activity of Na^+/K^+ ATPase in enterocytes isolated from FW-acclimated eels was significantly lower than that in SW-acclimated eels. These data are consistent with data on the whole intestine by other authors (Vokler *et al.* 1987).

It is known that, in teleosts, the water-transport capacity of intestine increases after FW to SW transfer and during smoltification (Veillette *et al.* 1993); since a major fraction of water-transport capacity is inhibited by ouabain (Veillette *et al.* 1993), this shows that intestinal Na^+/K^+ ATPase plays a primary role in the extraction of ions, and consequently of water, from the lumen of SW-fish intestine.

In other words, the intestine of the SW-acclimated eel represents an important site of water and NaCl transport from the lumen to the blood. In order to clarify the role of Ang II in the modulation of Na^+/K^+ ATPase, we used enterocytes isolated from the middle part of SW-acclimated eel intestine because in both FW- and SW-acclimated teleosts the Na^+/K^+ ATPase activity is higher in the middle than in the posterior part of the intestine (Vokler *et al.* 1987, Seidelin & Madsen 1999). This may be due to the fact that there are functional differences between middle and posterior intestine with regard to the transport of nutrients, ions, and fluid (Garg & Sastry 1976, Buddington *et al.* 1987, Reshkin *et al.* 1989).

Here, we have demonstrated that in enterocytes isolated from SW-acclimated eels, physiological doses (Tierney *et al.* 1995) of Ang II caused a significant dose- and time-inhibition of Na^+/K^+ ATPase. The data suggest that in SW-acclimated eels Ang II may act as a rapid down-regulator of the Na^+/K^+ ATPase activity in the gut. This effect may be of fundamental importance to the control of salt and water re-absorption. The reduced activity of the Na^+/K^+ ATPase is consistent with the effect of Ang II in the gills, where the activity is significantly increased at physiological Ang II concentrations (Marsigliante *et al.* 1997). In SW, the diffusional influx of ions is offset by the extrusion of NaCl via the chloride cell and the reduction of ion uptake (due to the operation of a $2\text{Cl}^-/\text{Na}^+/\text{K}^+$ symport at the apical barrier and the Na^+/K^+ ATPase together with a Cl^-/K^+ symport at the basolateral barrier) in the gut. Therefore, the changes in the activity of the Na^+/K^+ ATPase induced by Ang II would indeed stimulate Na^+ and Cl^- extrusion in the gills and reduce ion uptake in the gut. Ang II also has a role in the regulation of the activity of Na^+/K^+ ATPase in mammalian intestine. Low doses of Ang II stimulate, through the AT₂ receptor, the transport of both water and electrolytes. High doses of Ang II inhibit the absorption via the AT₁ receptor which is coupled to an increase in PGE₂ production (Jin *et al.* 1998). We have shown here (by analogy with the situation in mammals) that the effects of Ang II are blocked by

DuP-753, a specific AT1 receptor subtype competitive antagonist. That DuP-753 is able to bind to the teleostean Ang II receptor is not a novel finding, since it was found that it inhibits the binding of ¹²⁵I-Asp Val and ¹²⁵I-Asn Val-Ang II to trout glomeruli (Cobb & Brown 1993) and antagonizes other Ang II actions in teleosts (Pamantung *et al.* 1997, Cobb *et al.* 1999, Qin *et al.* 1999). Therefore, the effect of Ang II in eel enterocytes seems to be mediated by a receptor related to the mammalian AT1 type.

Isolated enterocytes provide a good model for investigating the Ang II signal-transduction pathway in intestine. We have previously shown that Ang II leads to the generation of inositol 1,4,5-trisphosphate and the elevation of intracellular Ca²⁺ in the kidneys (Marsigliante *et al.* 2000). Here, we show that Ang II also causes a rapid increase in the cytosolic Ca²⁺ in enterocytes, and that the inhibitory effect of Ang II on Na⁺/K⁺ATPase activity clearly involves PKC isoforms. Indeed, the actions of Ang II were completely abolished by calphostin C and staurosporine, which were unable to influence basal Na⁺/K⁺ATPase activity. In mammalian cells, the isozymes of the PKC family mediate several functions. Each isoform displays distinctive tissue distribution, substrate specificity and regulation patterns. The presence of multiple isoforms, already demonstrated in other teleost fish (Mericko & Burnett 1998, Maler 1999, Reader *et al.* 1999, Gur *et al.* 2000, Jung *et al.* 2000), implies that the complexity of signal-transduction pathways in vertebrates is highly conserved.

To provide further evidence for a role for PKC in the inhibition of Na⁺/K⁺ATPase activity, we showed the direct effects of PMA on this activity. The PMA sensitivity and the Ca²⁺ mobilization suggest the involvement of conventional (α , β I, β II, γ), but not new (δ , ϵ , θ and η) or atypical (ζ and ι) PKC isoforms (Dekker & Parker 1994). Activation of PKC involves its translocation (stimulated by endogenous diacylglycerol, DAG) from the cytosol to the membrane, where it becomes tightly associated. This activation can be induced by PMA, which binds to the DAG-binding sites of PKC (Nishizuka 1986, Burns & Bell 1991). Here, we show that PMA induced a calphostin-C-sensitive reduction in Na⁺/K⁺ATPase activity (as also shown in the Atlantic cod; Crombie *et al.* 1996) comparable to that observed with Ang II, and a translocation of the PKC α , δ and η isoforms. Western blot analysis showed that these three isoforms also translocated from the cytosol to the membrane fraction in response to Ang II; of these, PKC η was the major isoform involved in this hormonal effect on intestinal Na⁺/K⁺ATPase activity.

In conclusion, these studies suggest that Ang II acts as a down-regulator of the Na⁺/K⁺ATPase activity in SW-acclimated eel intestine, through an AT1-like receptor-mediated mechanism involving calcium mobilization and activation of three PKC isoforms.

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