Apelin-13 Decreases Epithelial Sodium Channel (ENaC) Expression and Activity in Kidney Collecting Duct Cells

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Abstract

Background/Aims: Apelin and its G protein-coupled receptor APLNR (also known as APJ) are widely expressed within the central nervous system and peripheral organs including heart, lung and kidney. Several studies have shown that the apelin/APJ system is involved in various important physiological processes such as energy metabolism, cardiovascular functions and fluid homeostasis. In the kidney, the apelin/APJ system performs a wide range of activities. We recently demonstrated that apelin antagonises the hydro-osmotic effect of vasopressin on aquaporin-2 water channel (AQP-2) expression by reducing its mRNA and protein levels in collecting duct principal cells. The central role of these cells in water and sodium transport is governed by AQP-2 and the epithelial sodium channel (ENaC). The coordination of these channels is essential for the control of extracellular fluid volume, sodium homeostasis and blood pressure. This study aimed at investigating the role of apelin in the regulation of sodium balance in the distal nephron, and more specifically its involvement in modulating the expression and activity of ENaC in collecting duct principal cells. Methods: mpkCCD cells were incubated in the presence of aldosterone and treated with or without apelin-13. Transepithelial Na+ current was measured and the changes in ENaC expression determined by RT-PCR and immunoblotting. Results: Our data show that apelin-13 reduces the transepithelial sodium amiloride-sensitive current in collecting duct principal cells after 8h and 24h treatment. This effect was associated with a decrease in αENaC subunit expression and mediated through the ERK pathway as well as SGK1 and Nedd4-2. Conclusion: Our findings indicate that apelin is involved in the fine regulation of sodium balance in the renal collecting duct by opposing the effects of aldosterone, likely by activation of ENaC ubiquitination.
Introduction

Apelin is a vasoactive peptide isolated from bovine stomach recognised as the endogenous ligand for a putative receptor related to the angiotensin receptor AT1 (AP1), which belongs to the G protein coupled receptor family [1, 2]. It originates from a 77-aminoacid precursor, preproapelin, which encompasses several active fragments including apelin-13, -17, and -36 [3, 4]. Apelin and its receptor are widely expressed in a number of tissues such as central nervous system, heart and blood vessels [5, 6]. In the kidney, the apelin/AP1 system is expressed in glomeruli, vascular endothelial cells and all nephron segments including collecting tubules [7-9]. Recent studies show that a second peptide ligand of AP1, elabela, discovered in 2014, is localised in collecting duct principal cells [10-12]. A large body of evidence indicates that the apelin/AP1 system plays a crucial role in numerous pathophysiological and physiological processes [13-15]. In the kidney, it is a critical mediator of renal fibrosis, renal ischemia, diabetic nephropathy and hemodialysis [16-18]. In addition, the apelin/AP1 system exerts a central role in the regulation of blood pressure, glomerular haemodynamics and acts on the tubule to promote diuresis [8, 19, 20]. Recently, we have determined that the effect on diuresis is due to inhibition of aquaporin-2 (AQP-2) water channel insertion into the apical plasma membrane of the collecting duct cells [21]. The central role of these cells in water and sodium transport is governed by AQP-2 and epithelial sodium channel (ENaC).

ENaC is a protein complex consisting of three homologous subunits (α, β, γ) encoded by three different genes [22]. It is expressed at the apical membrane of a variety of tissues, such as the distal nephron of the kidney, the lungs and distal colon [23-25]. This channel is a major regulator of salt and water reabsorption and blood pressure in the aldosterone-sensitive distal nephron (ASDN) [26, 27]. Thus, ENaC is regulated in a very stringent manner, including the involvement of several hormonal factors and mechanisms. For instance, arginine vasopressin and its analogues bind to V2 receptors and increase the activity and translocation of ENaC to the apical membrane from intracellular stores through activation of protein kinase A [28]. Aldosterone stimulates ENaC activity and expression through different genomic responses [29, 30]. Aldosterone regulation of ENaC occurs through receptor-mediated modulation of intracellular signalling pathways involving various kinase cascades, such as inhibition of extracellular signal-regulated kinase (ERK1/2), stimulation of serum and glucocorticoid-regulated kinase (SGK1) or inhibition of the neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2), an E3 ubiquitin ligase inducing internalisation and degradation of ENaC [31-34]. The coordination of ENaC and AQP-2 channels is essential for the control of extracellular fluid volume, sodium homeostasis and blood pressure. The action of the apelin/AP1 system on the renal tubule in promoting diuresis is mediated by several signalling pathways, including those involved in the regulation of sodium homeostasis. In light of the above, we aimed to investigate the role of apelin in the regulation of sodium balance in the distal nephron, more specifically its involvement in modulating the expression and activity of ENaC in collecting duct principal cells, as well as elucidate the mechanisms implicated in this regulation.

Materials and Methods

Cell culture

Immortalised mouse kidney cortical collecting duct cells (mpkCCDc14) were maintained in DMEM-F12 (Dulbecco’s modified Eagle’s medium / Ham’s F-12 (v/v), Wisent) supplemented with 5 μg/ml transferrin, 20 mM D-glucose, 2% foetal bovine serum (FBS), 20 mM HEPES, 1% penicillin/streptomycin, 5μg/ml insulin, 50 nM dexamethasone, 60 nM sodium selenate, 1 nM triiodothyronine, and 10 ng/ml epidermal growth factor (EGF). Cells were plated in 100 mm dishes at 37°C and 5% CO₂, 95% air atmosphere until 80% confluency, after which cells were transferred into 4.5 cm² semi-permeable filter-containing pores measuring 0.4 μm (Greiner-Bio-One, Germany) at a density of 80 000 cells/cm². Upon reaching a transepithelial resistance
of 1200 Ω.cm² (4-5 days after seeding), cells were pretreated for 8h or 24h with 1μM aldosterone (Sigma-Aldrich, USA) to induce endogenous ENaC expression and subsequently treated, in the continued presence of aldosterone, with or without 200 nM apelin-13 for varying time intervals. All treatments were added to the basolateral compartment and culture medium was renewed daily. Apelin-13 was generously provided by P. Sarret, IPS, FMSS, Université de Sherbrooke.

**Transepithelial current measurement**

Transepithelial voltage (Vte) and resistance (Rte) of each filter were measured under sterile conditions with an EndOhm-6 coupled to an EVOM2 (WPI, Sarasota, FL, USA) before and after treatment. Briefly, Vte was measured by means of a set of 2 Ag-AgCl electrodes and determined with the apical electrode as reference. Rte was measured by passage of current through the cell monolayer and measurement of the resulting voltage gradient across the cells. The transepithelial current (Isc) was calculated using Ohm’s law. 10 μM amiloride was added in the apical compartment to determine the magnitude of ENaC-mediated sodium transport across the cell monolayer.

**RNA isolation and QPCR**

Total RNA was isolated using Trizol Reagent (Life Technologies, Burlington, ON, Canada) according to the manufacturer’s instructions. To remove potentially contaminating DNA, total RNA was treated with DNase (Promega, Madison, WI) for 1 hour at 37°C. RNA was reverse-transcribed according to the manufacturer’s procedure (Quantitect Qiagen, CA) and cDNA samples were amplified using the specific primers for αENaC forward, 5’CGGAGTTGCTAAACTCAACATC3’, and reverse, 5’TGGAGACGAGTACCAGCT3’, and for GAPDH forward, 5’CTTCGACGATGCCAAAGTTG3’, and reverse 5’CTTCGACGATGCCAAAGTTG3’. The resulting cDNAs were used as templates in duplicate qPCR reactions each in triplicate, using a Brilliant II SYBR Green qPCR Master Mix kit (Stratagene, Mississauga, Ontario) in a MX3000P Real-Time PCR system to assess changes in expression of several transcripts. Fold-change values were calculated in “relative change” compared to the control condition, normalised with the expression of mGAPDH, and calculated according to the Pfaffl mathematical model [35].

**Western blot analysis**

Total cell lysates were collected in cold lysis buffer (20 mM Tris HCl, pH 7.4, 5 mM EDTA, 40 mM beta-glycerophosphate, 30 mM NaF, and 1% Triton X-100) supplemented with 200 μM sodium orthovanadate and complete™ EDTA-free protease inhibitors (Roche Diagnostics, Laval, QC, Canada). Protein quantification assays were determined using the BCA (bicinchoninic acid) procedure. Lysates were separated on 12% SDS-PAGE gel and transferred onto a PVDF membrane (Polyvinylidene difluoride; Perkin Elmer, Woodbridge, ON). The membrane was blocked in 5% BSA (Bovine Serum Albumin) in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 2 h and then incubated overnight at 4°C with the following primary antibodies: anti-αENaC (rabbit polyclonal, 1/2000, SPC403D, Biosciences Inc, Burlington, ON, Canada); anti-actin (mouse monoclonal, 1/10000, MAB1501, Millipore, CA); anti-p-SGK1 (rabbit monoclonal, ab-55281, 1/1000, Abcam, CA); anti-pNedd4-2 (rabbit polyclonal, 1/1000, ab-73386, Abcam, CA); anti-pERK (rabbit monoclonal, 1/1000, Cell Signaling 9100, CA); anti-ERK total (rabbit monoclonal, 1/1000, Cell Signaling 9100, CA). Membranes were then incubated with 1/1000 of suitable HRP-conjugated secondary antibody: anti-mouse (NA931V, Santa Cruz, CA); anti-rabbit (NA934V, Amersham, CA) for 2 hours at room temperature. Following the primary and secondary antibody incubations, the blot was washed in TBST three times for at least 10 min. Detection was performed using the Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer, Woodbridge, ON, Canada) and revelation carried out using the LICOR system. Densitometric protein analysis was performed using the STUDIO Image software.

**Statistical analyses**

Results are expressed as the mean ± SEM from independent experiments. Each experiment was carried out on cells having the same number of passages and all experiments were performed in quadruplicate. The number of experiments is indicated in the figure legends. All statistical analyses were performed using Prism 9 software (Graph Pad Software, San Diego, CA). A two-way ANOVA, with the Bonferroni multiple comparison test, were used to test the significance of the data. Data were considered to be statistically significant at the 95% confidence level (p <0.05).
Results

Apelin-13 reduces amiloride-sensitive sodium current transport in mpkCCD cells

We first investigated whether apelin affects the amiloride-sensitive sodium current. To achieve the latter, electrophysiological measurements of transepithelial sodium current ($I_{sc}$) were performed in monolayers of mpkCCD cells with high transepithelial resistance ($\geq 1200 \, \Omega \cdot \text{cm}^2$). The epithelial cell line was then pretreated for 8h or 24h with 1µM aldosterone to induce endogenous ENaC expression and subsequently treated, in the continued presence of aldosterone, with or without 200 nM apelin-13 for 8h or 24h. Aldosterone treatment produces a significant change in basal sodium $I_{sc}$ of 19% and 33% after 8h and 24h respectively ($p = 0.022$ and 0.010). Basal sodium $I_{sc}$ values were $79.31 \pm 6.29 \, \mu\text{A}$ (n = 72) in absence of aldosterone, and $94.32 \pm 3.39 \, \mu\text{A}$ (n = 60) and $105.61 \pm 4.55 \, \mu\text{A}$ (n = 60) in the presence of aldosterone 8h and 24h, respectively. Amiloride (10 µM) was added to the apical membrane at the end of the experiments to confirm that the transepithelial current was mediated by ENaC. In all experimental conditions, amiloride strongly decreased sodium current showing that the majority of basal sodium transport in mpkCCD cells is amiloride-sensitive. As illustrated in Fig. 1, treatment with 200 nM apelin-13, in the continued presence of aldosterone, produced a significant decrease in $I_{sc}$ amiloride-sensitive sodium current after 8h and 24h (76% ± 12% and 89% ± 6.3% respectively, n=6) compared to aldosterone alone (positive control). A similar decrease was observed when mpkCCD cells were treated with apelin-13 without aldosterone, suggesting that apelin is able to reduce, significantly, the transepithelial sodium current mediated by ENaC channels in the presence or absence of aldosterone (Fig. 1).

Apelin-13 decreases αENaC mRNA expression

To assess whether apelin is able to regulate the expression of αENaC, we first tested the effect of apelin-13 on αENaC mRNA expression. mpkCCD cells were stimulated with aldosterone to induce endogenous ENaC expression and then treated or not with apelin-13 for 8h or 24h in the continued presence of aldosterone as described previously. As determined by quantitative RT-PCR (Fig. 2), treatment with aldosterone for 8h and 24h induced a 4.01 ± 0.31 and 3.96 ± 0.92 fold increase in αENaC mRNA compared to untreated cells. These results are consistent with previous findings showing that aldosterone stimulates ENaC transcription [30, 36, 37]. Treatment of mpkCCD cells with apelin-13 for 8h and 24h in the presence of aldosterone decreased αENaC mRNA by 61% and 60% (n=4), respectively, suggesting that apelin-13 induces a inhibitory effect on αENaC transcription in mpkCCD cells.

Fig. 1. Effect of apelin-13 on transepithelial amiloride-sensitive sodium current ($I_{sc}$) in mpkCCD cells. Cells were treated with aldosterone (1µM) for 8h or 24h in absence or presence of apelin-13 (200nM). The white bar represents the negative control (without aldosterone or apelin). Values were normalized to means of positive control measurements (pretreatment with aldosterone for 8h or 24h) and represent the means ± SEM of percentages of amiloride-sensitive current in 5 separate experiments. 10µM of amiloride was added to the apical membrane at the end of the experiment to determine the involvement of ENaC in the $I_{sc}$ current. Each experiment contained 3 to 4 filters per condition. Results were analyzed by two-way ANOVA with a Bonferroni post-hoc multiple comparison test.
Effect of apelin-13 on aldosterone-induced αENaC protein expression

We next assessed whether apelin-13 treatment induces a decrease in αENaC protein expression. To this end, mpkCCD cells were stimulated with aldosterone and then treated or not with apelin-13 for 8 h or 24 h in the continued presence of aldosterone. As shown in Fig. 3, there was no significant change in the expression of ENaC detected at a molecular weight of 95 kDa (as corrected for β-actin) induced after 8 h aldosterone treatment compared with unstimulated cells. However, a significant 31% decrease in the expression of the αENaC protein was observed when mpkCCD cells were treated with apelin-13 for 24 h (n=8). This reduction is consistent with the inhibitory effect of apelin on ENaC mRNA expression.

Effect of apelin-13 on pERK1/2 in mpkCCD cells

Aldosterone is known to regulate the function of ENaC through a variety of mechanisms, including an inhibition of extracellular signal-regulated kinase (ERK), an increase in expression and activation of SGK1 (a serum-and-glucocorticoid-induced kinase) [38] which in turn phosphorylates Nedd4-2, (a ubiquitin ligase) which interacts with ENaC and contributes to enhancing its expression at the plasma membrane [32, 39]. We therefore investigated whether the inhibitory effect of apelin on ENaC could be mediated by these signalling pathways.

To determine whether the inhibitory effect of apelin on ENaC is mediated by the ERK pathway, mpkCCD cells were treated with apelin-13 for 5, 10, 30 and 60 min in the presence of aldosterone. ERK total and its phosphorylated form (p-ERK) were detected using specific antibodies as described in the Methods section and assessed by Western blot. As shown in Fig. 4, treatment with apelin-13 induced significant ERK phosphorylation after 30 min whereas this phosphorylation could not be maintained until 60 min (n=5). These results suggest that the ERK pathway is involved in the apelin regulation of ENaC, in accordance with the mechanisms previously described in the action of the apelinergic system in the kidney [40, 41].

Effect of apelin-13 on SGK-1 phosphorylation in mpkCCD cells

We subsequently investigated whether the SGK pathway is involved in the inhibition of ENaC by apelin. mpkCCD cells were accordingly treated with apelin-13 for 5, 10, 30 and 60 min in the presence of aldosterone; the non-phosphorylated and phosphorylated forms of SGK-1 were assessed by Western blot. Cells treated with aldosterone alone showed a
strong increase in p-SGK/SGKtotal ratio (Fig. 5) in accordance with previously described data showing the central role of SGK-1 in the regulation of ENaC by aldosterone [42, 43]. This large activation of the SGK pathway was significantly reduced when cells were treated with apelin-13 for 5 and 10 min suggesting that the decrease in p-SGK/SGKtotal ratio could be responsible for the reduction in ENaC activity by apelin.

**Effect of apelin-13 on Nedd4-2 phosphorylation in mpkCCD cells**

An acknowledged central mechanism by which Sgk1 stimulates ENaC is the phosphorylation of the ubiquitin ligase Nedd4-2 which is thus prevented from ubiquitinating this channel [32, 44]. In order to verify whether the inhibitory effect of apelin on ENaC is in part due to a decrease in Nedd4-2 phosphorylation, experiments were performed to detect the presence of phosphorylated and non-phosphorylated forms of Nedd4-2 following treatment of mpkCCD cells for 5, 10, 30, and 60 min. As illustrated in Fig. 6, apelin-13 treatment in the presence of aldosterone showed a progressive decrease in the phosphorylated form of Nedd4-2 compared to that obtained after treatment with aldosterone alone. The p-Nedd4-2/Nedd4-2 ratio was respectively 1.7 and 0.9 when cells were treated with aldosterone in the absence or presence of apelin-13 for 60 min.

These data suggest that the inhibition of ENaC by apelin may be explained, in part, by the stimulation of ENaC ubiquitination.
Discussion

The apelinergic system is expressed in a wide variety of tissues including the brain, heart and kidneys. Its involvement in several physiological functions including energy metabolism, cardiac contractility and fluid homeostasis has been widely studied over the past decade. Several studies have reported key findings regarding the role of the apelinergic system in disorders of water and sodium balance and its interaction between vasopressin and the renin-angiotensin-aldosterone system (RAAS) [45-49]. We, and others, have recently demonstrated that apelin inhibits vasopressin-induced translocation of aquaporin 2 (AQP-2) channels to the apical membrane of collecting duct principal cells and therefore prevents water reabsorption [20, 21]. The function of AQP-2 is tightly coordinated with that of ENaC, a channel which constitutes the rate-limiting step for transepithelial sodium transport in this section of the nephron. In this setting, we hypothesised that apelin-13 could also play an important role in the downregulation of ENaC function and expression. Our current results provide evidence that aldosterone prompts an increase in amiloride-sensitive sodium current in a collecting duct cell line, thus supporting previously-described findings [50, 51]. This activation was significantly reduced by apelin-13 (Fig. 1).
In addition, our data show that the application of apelin-13 on mpkCCD cells, in the continuous presence of aldosterone, reduced the expression of the αENaC subunit (Fig. 2 and 3). Diverse mechanisms, including variation in channel synthesis, regulation of intracellular channel trafficking, membrane insertion and post-translational modifications that alter channel-open probability (Po) or increase their endocytosis are crucial in controlling channel function [52]. These mechanisms are not mutually exclusive and their combination can be employed to achieve sodium transport mediated by ENaC according to the equation: $I_{Na} = g_{Na} \cdot N \cdot Po \cdot (E - E_{Na})$. Thus, we believe that the differences observed on the current measurements and channel expression do not reflect any discrepancy. The vectorial movement of sodium establishes an osmotic gradient that facilitates the movement of water in the same direction while the inhibition of ENaC is associated with an imbalance of sodium and water [53]. Thus, our findings are consistent with recent data showing that the apelinergic system reduces water reabsorption [20, 21].

In addition to the above, we also endeavoured to determine the mechanisms by which apelin modulates the amiloride-sensitive sodium current. ERK, SGK1 and Nedd4-2 appear to be among the promising pathways for investigation. Indeed, several studies have demonstrated that ERK is a crucial factor in the regulation of ENaC activity, trafficking and transcription. Whereas the long-term effect of ERK is thought to involve an inhibition of ENaC transcription [54], the short-term inhibition is likely to be mediated by phosphorylation of ENaC subunits [34].

Numerous evidence supports an inhibitory effect on ENaC, whether tonic or in response to epidermal growth factor (EGF) [34, 55, 56]. A study by the Marunaka group showed that a major effect of hypotonic stress on transcriptional regulation of ENaC is caused by ERK inactivation (dephosphorylation) and that this dephosphorylation is mediated through the p38-dependent induction of MKP-1 [57]. Furthermore, aldosterone stimulates sodium transport in part by increasing GILZ1 expression, which inhibits ERK signalling, and hence acts in concert with SGK1 to enhance ENaC-mediated sodium current [56]. On the other hand, several studies show that the ERK1/2 signaling pathway is activated by apelin treatment in a time-dependent manner [58-62]. Indeed, Wang et al. shown that the treatment of LX2-cells with apelin-13 displayed an increased ERK1/2 phosphorylation within 45 minutes and this effect persisted up to 1h [61]. However, using HEK293 cells, Sun and colleagues shown that a considerable increase in ERK1/2 phosphorylation is obtained after 2 and 5 minutes of apelin treatment and that this phosphorylation is reduced to control values after 10 minutes of treatment [62]. Similar effect was found by Bai and colleagues, but the peak level of ERK1/2 phosphorylation was at 10 minutes of apelin treatment following by a decrease to control values at 20 and 30 minutes [58]. In our study, treatment of mpkCCD cells with apelin-13 induced significant ERK1/2 phosphorylation after 30 min whereas the phosphorylation return to control values after 60 min of apelin treatment. The dissimilarity of response of apelin on ERK1/2 phosphorylation could be attributed, in part, to the cells used in these different studies.

On the other hand, aldosterone also acts through the mineralocorticoid receptor (MR) to rapidly increase SGK1 gene transcription and, thus, SGK1 protein levels [63]. Importantly, this kinase must also be activated by two phosphorylation events that control its inherent activity [64]. Activated SGK1 in turn phosphorylates and regulates various targets, most notably the ubiquitin ligase Nedd4-2 [32]. Thus, SGK phosphorylates ENaC and inhibits its internalisation from the plasma membrane and subsequent degradation, this double negative (inhibiting the inhibitor) thus resulting in ENaC accumulation at the apical plasma membrane [23, 65]. Our results support these aforementioned studies and provide further evidence that aldosterone activates a phosphorylation of SGK and Nedd4-2. We also show that this phosphorylation was considerably reduced following the application of apelin-13 to mpkCCD cells. The above findings suggest that apelin may represent an important activator of ENaC ubiquitination which may explain the decrease in sodium amiloride sensitive current.
Conclusion

The present experiments show that apelin decreases the amiloride-sensitive sodium current. This decrease is associated with modulation of the ERK, SGK and Nedd4-2 pathways, suggesting the activation of ENaC ubiquitination. Further studies are needed to confirm this hypothesis. These results corroborate our recently published data showing that apelin induces downregulation of AQP2 in mpkCCD cells and provide further evidence that the apelinergic system is directly involved in the regulation of sodium and water balance in the distal nephron of the kidney.

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Author Contributions

HA and AC conceived the study and experiments, performed and analysed the data. HA contributed to the writing of the manuscript. AC wrote the paper and supervised the project.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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Ayari et al.: Apelin Decreases ENaC Activity and Expression in mpkCCD Cells


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