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Original Paper

Apelin-13 Regulates Vasopressin-Induced Aquaporin-2 Expression and Trafficking in Kidney Collecting Duct Cells

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Key Words
Kidney • Aquaporin • Apelin • Vasopressin • Confocal microscopy • PCR • mpkCCD

Abstract
Background/Aims: Apelin and its G protein-coupled receptor APJ (gene symbol *Aplnr*) are strongly expressed in magnocellular vasopressinergic neurons suggesting that the apelin/APJ system plays a key role at the central level in regulating salt and water balance by counteracting the antiduretic action of vasopressin (AVP). Likewise, recent studies revealed that apelin exerts opposite effects to those of vasopressin induced on water reabsorption via a direct action on the kidney collecting duct. However, the underlying mechanisms of the peripheral action of apelin are not clearly understood. Here, we thus investigated the role of the apelin/APJ system in the regulation of water balance in the kidney, and more specifically its involvement in modulating the function of aquaporin-2 (AQP2) in the collecting duct. ***Methods:*** Mouse cortical collecting duct cells (mpkCCD) were incubated in the presence of dDAVP and treated with or without apelin-13. Changes in AQP2 expression and localization were determined by immunoblotting and confocal immunofluorescence staining. ***Results:*** Herein, we showed that the APJ was present in mpkCCD cells. Treatment of mpkCCD with apelin-13 reduced the cAMP production and antagonized the AVP-induced increase in AQP2 mRNA and protein expressions. Immunofluorescent experiments also revealed that the AVP-induced apical cell surface expression of AQP2, and notably its phosphorylated isoform AQP2-pS269, was

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considerably reduced following apelin-13 application to mpkCCD cells. ***Conclusion:*** Our data reinforce the aquaretic role of the apelin/APJ system in the fine regulation of body fluid homeostasis at the kidney level and its physiological opposite action to the antiduretic activity of AVP.

Introduction

The kidneys play a vital role in maintaining homeostasis by regulating salt and water balance. This function is finely tuned by an interplay of several regulatory mechanisms, including the recruitment of the renin-angiotensin-aldosterone and antidiuretic hormone, 8-arginine vasopressin (AVP) control systems. Water movement across renal epithelial cells is dependent on the presence of specific aquaporin water channels (AQP). Three AQP isoforms (AQP2, AQP3 and AQP4) are expressed in collecting duct principal cells and play a critical role in water reabsorption under the control of AVP. While AQP3 and AQP4 are constitutively expressed at the basolateral membrane of these cells, AQP2 facilitates water transport across the apical plasma membrane and represents the main water channel regulated by AVP. Binding of AVP to its V_2 receptor at the basolateral membrane of collecting duct principal cells stimulates AQP2 channel expression and translocation of AQP2-containing vesicles to move and fuse with the apical plasma membrane via the cAMP/PKA pathway, therefore enhancing membrane permeability and water reabsorption. Various studies have shown that AQP2 phosphorylation in the cytoplasmic C-terminal tail is involved in the trafficking of this water channel [1].

Apelin is a endogenous vasoactive peptide discovered in the late nineties as the natural ligand for the seven-transmembrane domain G protein-coupled receptor related to the angiotensin receptor AT1, named APJ (gene symbol *Aplnr*) [2]. The apelin gene encodes a 77-amino acid preproapelin which is subsequently cleaved into various biological active forms of apelin, including apelin-13 (apelin 65-77), apelin-17 (apelin 6177) and apelin-36 (apelin 42-77). The biological activity of apelin-13 is the highest among the three forms of this peptide. Upon binding, the apelin isoforms induce coupling of APJ to $G\alpha_{i/o}$ and negatively modulate the cyclic AMP (cAMP) production [3-6]. Apelin and its receptor have been identified in a diversity of tissues [7-9]. Several studies have revealed the existence of a functional cross-talk between apelin and vasopressin systems in magnocellular neurons suggesting that apelin is a key component in the maintenance of water balance at the central level [10]. Recent studies showed that tha APJ is expressed in the kidney and the apelin exerts vasoactive effects on afferent and efferent arterioles suggesting a role in the control of renal hemodynamic function. In addition, intravenous injection of apelin in lactating rats increases diuresis and reduced urine osmolality suggesting that apelin also has a direct action on the kidney. This effect was associated with a decrease of AQP2 immunolabeling in apical membranes of the collecting duct, although the mechanism of action responsible for this reduction is not clearly understood [11-14].

In light of the above, the present study aimed to unravel the underlying mechanisms involved in the regulatory action of apelin on collecting duct cell activity. APJ receptors were found to be expressed in mpkCCD cells, a well-characterized model system for the principal cells of the cortical collecting duct of mammalian kidney and a highly-suitable model to gain better understanding of water balance dynamics [15, 16]. Furthermore, apelin-13 was shown to reduce cAMP production and antagonize the hydroosmotic effect of AVP on AQP2 expression by reducing its mRNA and protein levels in mpkCCD cells. Finally, we demonstrated that this reduction in AQP2 abundance was associated with a large decrease in AQP2-pS269 density at mpkCCD apical membrane. Altogether, the present findings provide additional compelling evidence for the involvement of the apelinergic system in the control of body fluid homeostasis.

Materials and Methods

Cell culture

Immortalized mouse kidney cortical collecting duct cells (mpkCCD$_{c14}$) were grown in DMEM F12 (Dulbecco's modified Eagle's medium/Ham's F12 (v/v), Wisent, ON, Canada) supplemented with 5 µg/ml transferrin, 20 mM D-glucose, 2% Fetal Bovine Serum (FBS), 20 mM Hepes, 1% penicillin/streptomycin, 5µg/ml insulin, 50 nM dexamethasone, 60 nM sodium selenat, 1 nM triidothyronine and 10 ng/ml Epidermal Growth Factor (EGF). Cells were plated in 100 mm dishes at 37°C and 5% CO_2, 95% air atmosphere until 80% confluency, after which cells were transferred into 4.5 cm^2 semi-permeable filter-containing pores of 0.4 µm (Greiner-Bio-One, Germany) at a density of 80, 000 cells/cm^2. Upon reaching a transepithelial resistance of 4500 Ω.cm^2, (4-5 days after seeding), cells were treated for 24 hours with 10 nM dDAVP (Sigma-Aldrich, Oakville, ON, Canada) to induce endogenous aquaporin-2 expression and then treated with or without 200 nM apelin-13 in the continued presence of dDAVP, according to the experiment. All treatments were added to the basolateral compartment and culture medium was renewed daily. Apelin was generously provided by E. Marseault, IPS, FMSS, Sherbrooke University.

Western blot and total protein extraction

After each treatment, cells were washed 3 times with cold PBS and lysed during 25 min in 300 µl of cold lysis buffer containing (20 mM Tris-HCl, (pH 7.4), 5mM EDTA, 40 mM $_{\beta}$-glycerophosphate, 30 mM NaF, 1% Triton X-100), supplemented with 200 µM sodium orthovanadate and the complete™ EDTA-free protease inhibitor (Roche Diagnostics, Laval, Qc, Canada). Protein quantification assay was performed according to the Bicinchoninic Acid (BCA) procedure (ThermoFisher Scientific, Burlington, ON, Canada). Twenty-five µg protein samples were separated in 12% SDS-PAGE gels, and transferred onto Polyvinylidene difluoride (PVDF) membranes (Perkin Elmer, Woodbridge, ON, Canada). Membranes were stained with Ponceau S (Sigma-Aldrich, Oakville, ON, Canada) to confirm protein loading equivalence.

Nonspecific binding sites were blocked, for 2h at RT, in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) containing 6% skimmed milk; for detection of the phosphorylated form of AQP-2 at the serine 269 residue, membranes were incubated in TBST with 5% BSA. Membranes were then hybridized overnight at 4°C with the following primary antibodies: anti-AQP2 (rabbit monoclonal, 1/2000, Novus Biologicals, CA), anti-AQP2pS269 (rabbit polyclonal, 1/1000, Phosphosolution, Aurora, CO, USA), anti-Actin (mouse monoclonal, 1/10000, Millipore CA), anti αNa$^+$/K$^+$-ATPase (mouse monoclonal, 1/1000, Santa Cruz, Dallas, TX, USA), anti-$_{\beta}$-lamin (goat polyclonal, 1/1000, Santa Cruz, Dallas, TX, USA), anti-APJ SC-33823 Aplnr Antibody, H-300 (goat polyclonal, 1/1000, Santa Cruz, Dallas, TX, USA) and anti-GAPDH, Glyceraldehyde-3-phosphate dehydrogenase, (rabbit polyclonal, 1/1000, Abcam Cambridge, MA, USA). Membranes were washed thrice (10 min each) in TBST and hybridized (1h at RT with gentle shaking) with the appropriate HRP-conjugated secondary antibody 1/5000 (anti-mouse (NA931V) or antirabbit (NA934V) from Amersham, Missisauga, ON, Canada or anti-goat (sc-2352) from Santa Cruz). After five washes in TBST, proteins were subjected to Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Woodbridge, ON, Canada) and detected on Amersham Hyperfilm ECL (GE Healthcare, Missisauga, ON, Canada). Protein densitometric analysis was performed with Image J [17]. Antibody stripping protocol: PVDF membranes were incubated in a stripping solution(62.5 mM Tris-HCl, pH 6.8; 2% SDS; 0.8% β-mercaptoethanol) at 54°C for 25 min and washed for 1 hour in tap water, and then allowed to equilibrate in TBST for 15 min. Membranes were thereafter incubated in blocking solution for 1h, hybridized with the appropriate secondary antibody and proteins revealed on ECL film. This protocol was used to detect proteins of similar molecular weight on the same blot.

Quantification of cellular cAMP levels

cAMP dosage was achieved using the cAMP-Gs dynamic kit from Cisbio (Bedford, MA, USA), using a modified protocol for cell stimulation. Briefly, cells were used when they have reached a transepithelial resistance of 4500 Ω.cm^2. Culture media was replaced with HBSS (containing 1.25 mM $CaCl_2$, 1.25 mM, $MgCl_2$ and 20 mM HEPES from Wisent, St-Bruno, QC, Canada) in both apical and basolateral compartments. Cells were then incubated at 37°C for 30 minutes to equilibrate. HBSS was then removed and fresh HBSS containing the desired treatment was added to the basolateral compartment, cells were incubated at 37°C for 1 hour. After incubation, the media was removed and 50 µL of 1X lysis buffer was added to each insert. The plate was incubated at RT under agitation for at least 2 hours. Cell lysate was triturated by pipetting

up and down several times. 10 µL of lysate was then transferred into a shallow volume withe opaque 384-well plate (PerkinElmer, Missisauga, ON, Canada). Detection reaction and fluorescence measurement were performed as described previously [18]. Results were normalized relatively to the signal obtained after AVP treatment (100%) and signal of the cell treated with HBSS alone.

Protein deglycosylation

After cell treatment and protein extraction, 25 µg of proteins were preincubated in glycoprotein denaturing buffer, G7 reaction buffer and 10% Nodidet P-40 at 37°C for one hour, according to the company procedure, in the absence or presence of 100 U of Peptide-N-glycosidase F (PNGase F, P0704S, NEB, Montreal, Canada). Proteins were subsequently loaded in 12% SDS-PAGE gels, as described in the Western blot section.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the TRIzol Reagent (Life Technologies, Burlington, ON, Canada) following the manufacturer's instructions. To remove potentially contaminating DNA, total RNA was treated with DNase (Promega) for 1h 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 mAQP-2 forward, 5'-ctggctgtcaatgctctccac3', and reverse, 5'-ttgtcactgcggcgctcatc-3', and for mGAPDH forward, 5'tggtgccaaaagggtcatc-3', and reverse, 5'-cttccacgatgccaaagttg-3'. Quantitative RT-PCRs were performed in triplicate in a MX3000P Real-Time device (Stratagene, La Jolla, CA, USA) using Brilliant II SYBR Green QPCR master mix. The mRNA expression levels were assessed as fold increase relative to the control condition, normalized to GAPDH expression, and calculated according to the Pfaffl mathematical model [19].

Confocal immunofluorescence microscopy

Prior to fixation, the cell culture medium was removed from both cell culture inserts and cell culture plate wells. Cells were fixed with 4% formaldehyde solution for 20 min at 4°C, and permeabilized with PBSTriton X-100 0.5% for 5 min at RT. Non-specific protein binding sites were blocked using 5% PBS-BSA for 60 min at RT. Filters were then incubated with either rabbit polyclonal anti-AQP2 primary antibody (1/500, Novus) or rabbit anti-AQP2-pS269 antibody (1/600, Phosphosolution). The latter were incubated with the mouse monoclonal anti-occludin primary antibody, (1/500, Santa Cruz, CA), overnight at 4°C. Samples were then incubated with goat anti-rabbit Alexa-Fluor568 or rabbit anti-mouse Alexa-Fluor488 secondary antibodies (Invitrogen, Burlington, ON) for 60 min at RT. Nuclei were stained 2 min at RT with 10 ng/ml 4'6-Diamidino-2-phenylindol dihydrochloride (DAPI)/PBS (pH 7.4). After a simple wash, membranes were detached from the insert housings using a scalpel and mounted onto microscopy slides using fluorescence mounting medium. Specimens were observed in the x-y and x-z planes using a confocal laser-scanning microscopy equipped with a Fluoview 1000 (FV1000) IX81-ZDC inverted microscope (Olympus Canada, Markham, ON, Canada). Apical AQP2 and AQP2-pS269 fluorescence intensities were measured using the MetaMorph Software (version 7.7 from Molecular Devices, LLC, Sunnyvale, CA, USA). Identical microscope settings were used for each condition. For quantitative analysis of AQP2 expression, immunofluorescence intensity was measured in filters of four independent experiments. Localization of AQP2 was expressed as the ratio of fluorescence signal intensity at the plasma membrane to intracellular fluorescence signal intensity.

Statistical analysis

Results are given as the mean ± SEM. Each experiment was performed on cells from the same passage and all experiments were performed at least three times. In each experiment we used 2 to 3 filters per condition. The number of experiments performed is indicated in figure legends. All statistical analyses were performed using Prism software (Graph Pad Software, La Jolla, CA, USA). Student's paired t-test and one-way or two-way ANOVA using Bonferroni's Multiple Comparison Test were used to analyze the data. Data were considered to be statistically significant at the 95% confidence level ($P < 0.05$).

Results

The APJ receptor is present in mpkCCD cells

To investigate whether the apelin peptide was able to modulate AVP-stimulated AQP 2 activity in mpkCCD cells, we first examined whether these kidney collecting duct cells expressed the APJ receptor. Western blot analyses revealed that the APJ receptor was highly endogenously expressed in mpkCCD$_{c14}$ cells comparatively to rat brain tissue, whereas it was not detected in HEK293 cells. As shown in Fig. 1, immunoblots performed on mpkCCD protein cell preparations enabled the detection of a band at 44 kDa, consistent with the molecular weight of the expected monomeric form of the APJ receptor [20].

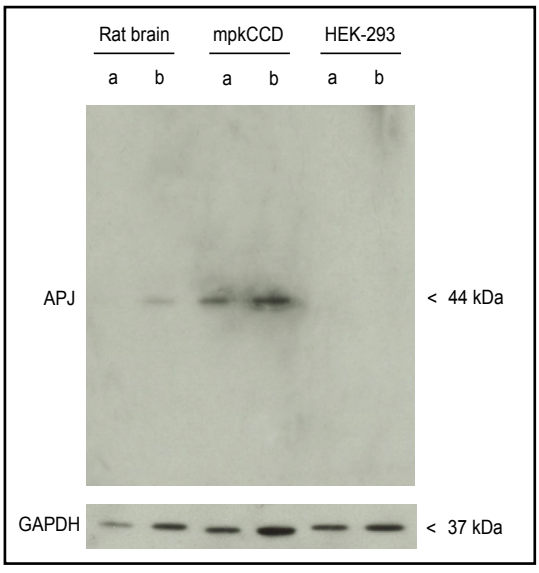

Fig. 1. Expression of APJ receptors in mpkCCD cells. Representative immunoblot of 4 independent experiments showing APJ receptor in both mpkCCD cells and rat brain tissue. Protein extracts from HEK293 cells were used as a negative control. In a and b, 5 and 10 µg of protein were loaded, respectively. Total protein levels were normalized to GAPDH levels, used as a control.

Effect of apelin-13 on cAMP production in mpkCCD cells

To appreciate the functional coupling of the apelin receptor to intracellular signaling pathways, we evaluated the ability of activated APJ to inhibit the AVP-triggered cAMP production in mpkCCD$_{c14}$ cells. Intracellular cAMP levels were quantified after treating mpkCCD cells with either buffer, AVP, or AVP combined to apelin-13 for one hour in three independent experiments (Fig. 2). Our results showed an increase in cAMP levels following AVP stimulation, compared to non-stimulated cells. A significant decrease in cAMP levels (32±4.4%, n=3) was observed when cells were treated with a combination of AVP and apelin-13, demonstrating the functional coupling of endogenous apelin receptor to the G$\alpha_{i/o}$-cAMP signaling pathway in mpkCCD cells. Our results also demonstrate that apelin-13, through its action on APJ, is able to counteract AVP-stimulated cAMP formation.

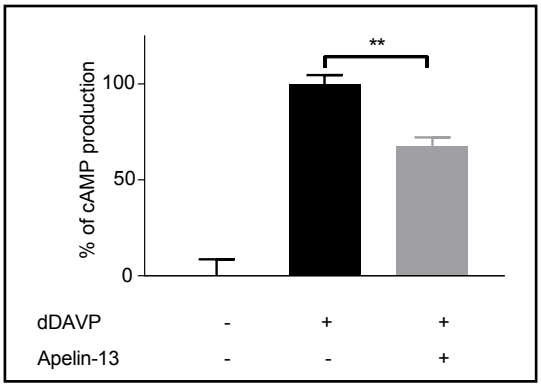

Fig. 2. Effect of AVP and apelin-13 treatment on intracellular cAMP levels. mpkCCD cells grown on permeable supports were treated with HBSS, 1 nM of AVP, or with a combination of 1nM AVP and 200 nM of apelin-13 for 1 hour at 37°C. Each data set represents the mean of three independent experiments, each performed in triplicate, and expressed as the mean ± S.E.M. *p<0.05 one-way ANOVA with a post-hoc Bonferroni multiple comparison test.

Effect of apelin-13 on AVP-induced AQP2 mRNA expression

We next evaluated whether apelin-13 treatment induced increase in AQP2 mRNA expression. To this end, mpkCCD cells were stimulated for 24 hours with dDAVP to induce endogenous AQP2 expression and then treated or not with apelin-13 for 8 or 24 hours in the continued presence of dDAVP. As determined by quantitative RT-PCR (Fig. 3), dDAVP treatment alone induced an 8.34±2.05 fold increase in AQP2 mRNA (n=5). Treatment of mpkCCD cells with apelin-13 for 8 or 24 hours resulted in respective 65% and 80% reductions in AQP2 mRNA, suggesting that apelin-13 antagonizes AVP-induced AQP2 mRNA expression, in part by reducing its mRNA levels.

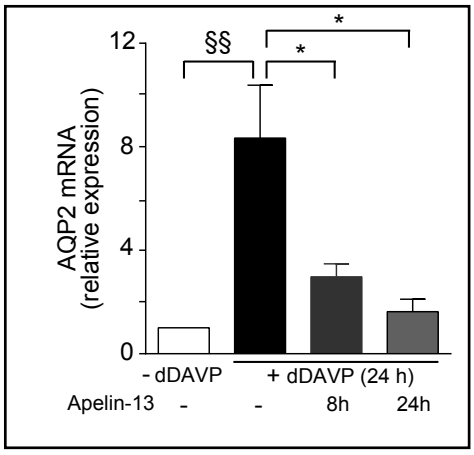

Fig. 3. Effect of apelin-13 on AQP2 mRNA expression. Upon reaching a transepithelial resistance of 4.5 kΩ.cm^2, mpkCCD cells were treated for 24 h with 10 nM dDAVP to induce endogenous AQP-2 expression (+ dDAVP) and then treated with 200 nM apelin13 for 8 h or 24 h in the continued presence of dDAVP. Left column represents the negative control (-dDAVP, -Apelin-13). RNA extraction and reverse transcription were performed as described in experimental procedures. Real-time qPCR was performed using specific primers for mouse AQP-2. Relative amounts of AQP2 mRNA were determined and normalized on the mGAPDH mRNA reported gene. Bars represent means ± SEM from five independent experiments, each performed in duplicate. §§: p <0.01; *: p <0.05.

Apelin-13 decreases AVP-induced AQP2 protein expression

To assess whether apelin-13 was able to regulate the effect of AVP on AQP2 protein expression, mpkCCD cells were stimulated for 24 hours with dDAVP in the presence or not of apelin-13 for 8 or 24 hours. Cells treated with dDAVP showed a strong protein signal at 29 kDa and approximately 35 kDa, corresponding to the non-glycosylated and glycosylated forms of AQP2 protein, respectively (Fig. 4A, lane 2) [21, 22]. This overexpression of AQP2 was sharply decreased when apelin-13 was added to the cell medium on the basolateral side (Fig. 4B, n=5). Deglycosylating the protein extracts with PNGaseF eliminated the broad and diffuse bands around 35 kDa corresponding to the glycosylated AQP2 form, indicating the specificity of the antibody used to detect the AQP2 protein (Fig. 4C). These results suggest that apelin-13 exerts an opposite action to AVP on total AQP2 protein expression in principal collecting duct cells.

AQP2 translocation to the plasma membrane promoted by AVP stimulation is reduced by apelin-13 treatment

Previous studies have demonstrated that vasopressin increases water permeability of collecting duct by inducing translocation of AQP2 to the apical plasma membrane [23-25]. We therefore investigated whether AQP2 translocation to the plasma membrane could be reduced by apelin-13 treatment. Using immunofluorescence confocal microscopy, we revealed that AQP2 staining was mainly localized in the cytoplasm under basal conditions while dDAVP treatment induced a sharp accumulation of AQP2 at the apical plasma membrane. As illustrated in Fig. 5, apelin-13 treatment decreased the membrane localization of AQP2 by 2.61±0.19 fold and 3.51±0.12 fold at 30 and 60 minutes respectively, compared to dDAVP alone (n=4). These data strongly indicate that apelin-13 reduces AQP2 cell surface expression in mpkCCD cells.

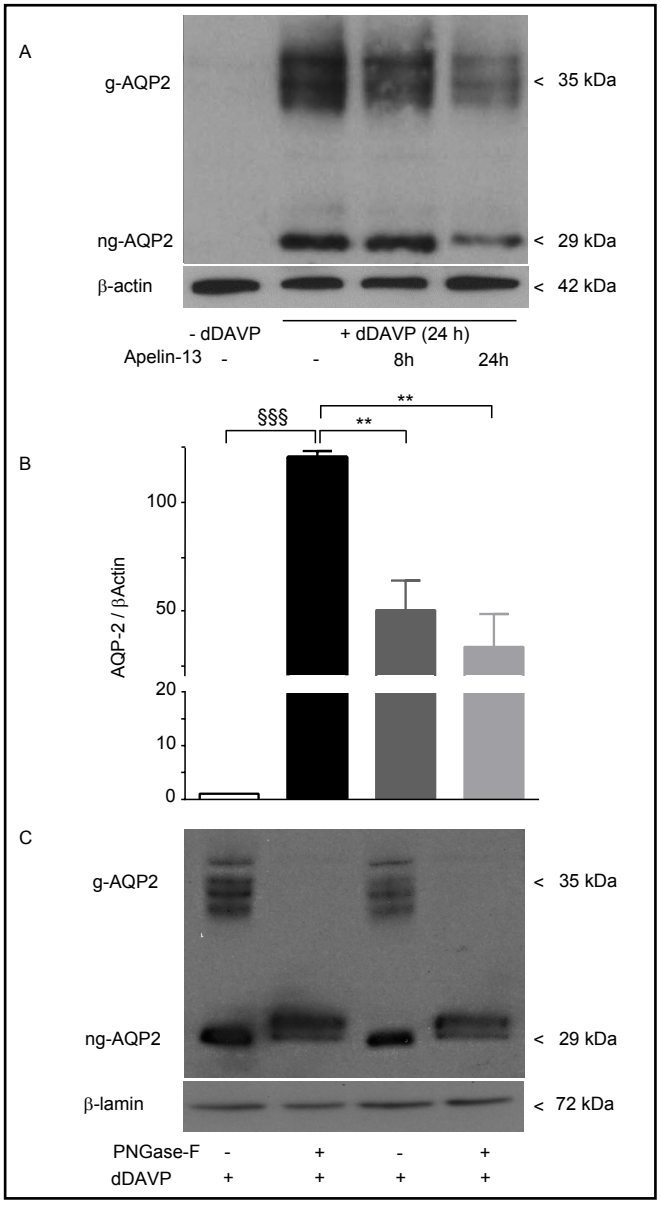

Fig. 4. Effect of apelin-13 on AQP-2 protein expression. (A) Representative immunoblot of five independent experiments showing the expression of non-glycosylated and glycosylated forms of AQP2 in mpkCCD cells treated or not with dDAVP (10 nM, 24 h) in the absence or presence of apelin-13 (200 nM) for 8 h or 24 h. β-actin was used as a loading control. (B) Densitometric analysis of AQP2 expression. §§§ $p<0.001$, ** $p<0.01$, compared to dDAVP treatment (5 independent experiments; two-way ANOVA with a posthoc Bonferroni multiple comparison test). (C) Representative immunoblot of five independent experiments performed with protein extract samples treated (+) or not (-) with 100U of peptide-N-glycosidase F for 1 hour showing that the diffuse band corresponds to the glycosylated AQP-2 protein form and not that of other non-specific binding. In these experiments, mpkCCD cells were grown and treated with dDAVP as described above (10 nM, 24 h) and treated in the absence (-) or presence of apelin-13 for 8 h (+). g-AQP2: glycosylated aquaporin, ng-AQP2: non glycosylated aquaporin.

Finally, accumulating evidence suggests that phosphorylation of AQP2 is required for its intracellular trafficking. Indeed, five phosphorylation sites (threonine 244 and serines 256, 261, 264 and 269) have been described to be critical for this regulating process. Particularly, the AQP2-pS269 form could reflect the density of total AQP2 present at the apical plasma membrane [23, 26-29]. Accordingly, we examined the effect of apelin-13 on AQP2-pS269 cellular distribution. Results revealed that AQP2-pS269 was targeted to the apical membrane of collecting duct principal cells following 24h treatment with dDAVP.

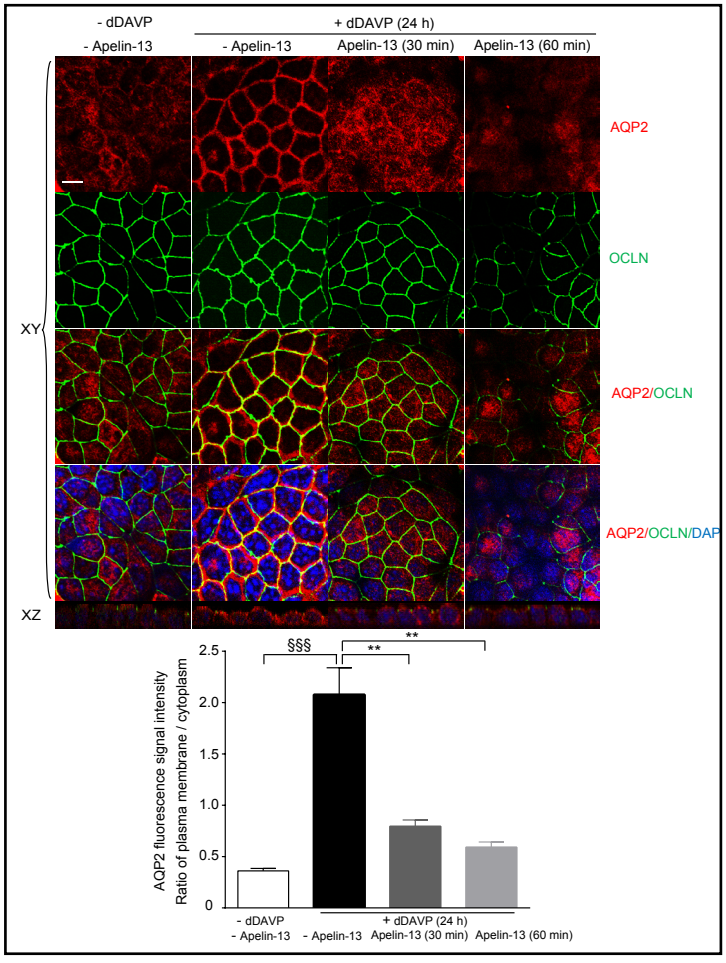

Fig. 5. AQP2 immunolocalization in response to AVP and apelin-13 treatment. mpkCCD$_{c14}$ cells grown on permeable supports were left untreated (negative control, 1st column) or treated with dDAVP for 24h (positive control, 2nd column) or with a combination of dDAVP and apelin-13 (for 30 or 60 min, 3rd and 4th columns). Double immunofluorescence labeling was performed, using an antibody recognizing all forms of AQP-2 (red) for total-AQP2 and an antibody targeting occluding (OCLN, green). OCLN, located at the tight junctions in sub-apical membrane, was used as a cytoplasm membrane marker while nuclei were stained with DAPI (blue). Confocal images are shown in both XY and XZ plans. Scale bar = 5 µm. Localization of AQP2 was expressed as the ratio of fluorescence signal intensity at the plasma membrane to intracellular fluorescence signal intensity. Analysis of AQP2 represents the means ± SEM of four independent experiments. §§§ p< 0.001; **p<0.01; compared to dDAVP treatment (two-way ANOVA with a posthoc Bonferroni multiple comparison test).

Indeed, AQP2-pS269 immunoreactivity was dramatically increased after dDAVP treatment whereas this phosphorylated isoform was almost completely absent from the apical membrane in non-treated mpkCCD cells. Of note, the redistribution of AQP2-pS269 to the membrane was drastically decreased when apelin-13 was co-incubated for shortterm periods with dDAVP. As shown in Fig. 6, apelin-13 treatment decreased AQP2pS269 membrane fluorescence intensity by 9.43±0.29 fold (30 min) and 28.42±0.83 fold (60 min), compared to dDAVP alone (n=4). These results strongly indicate that apelin-13 counteracts the effect of AVP on the translocation of AQP2 into the plasma membrane and thus reduces water intake through the collecting duct cells.

Discussion

Apelin and its receptor are expressed in a large variety of tissues including brain, heart and kidneys. During the past decade, several studies have reported key findings regarding the physiological and pathophysiological roles of the apelinergic system, although the underlying mechanisms of these effects are not clearly understood [30-32]. For instance, the apelin-APJ axis has been shown to exhibit a major role within the cardiovascular system, inducing drop in mean arterial blood pressure and powerful positive inotropic effects [33-35]. Accordingly, we previously demonstrated that the APJ receptor is expressed in cardiomyocytes and that its endogenous ligand modulates the gating and amplitude of sodium currents, thus supporting

inotropic effects of apelin [20]. Several studies also showed that apelin plays a critical regulatory role of fluid homeostasis at a central level and that this effect appears to counteract the central actions of vasopressin and the renin angiotensin system [14, 36-39]. In addition to its central action, recent data have shown that apelin also exerts peripheral effects including direct action at the kidney level [10].

In the present study, we demonstrate for the first time that apelin receptors are expressed by mpkCCD cells, a highly suitable mammalian kidney model to study fluid homeostasis. These results are consistent with previous findings showing that APJ mRNAs are expressed all along the nephron and with immunohistochemical staining demonstrating that the apelin-17 peptide is present in human collecting tubules of the kidney [7, 40].

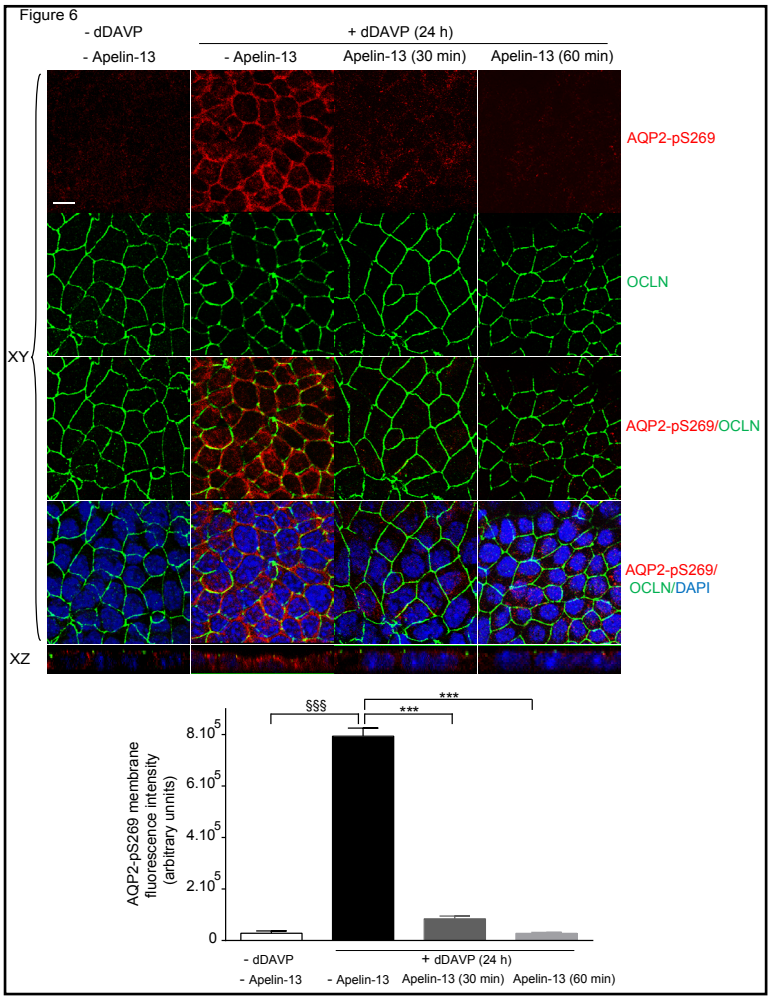

Fig. 6. Representative immunofluorescence images showing the effect of apelin-13 on AQP2-pS269 localization in mpkCCD$_{c14}$ cells. Cells grown on permeable supports were left untreated (negative control, 1st column) or treated with either dDAVP for 24h (positive control, 2nd column) or with a combination of apelin (for 30 or 60 min, 3rd and 4th columns) and dDAVP. Double immunofluorescence labeling of AQP2-pS269 (red) and occludin (green) was performed using an anti-AQP2-pS269 and anti-OCLN. Nuclei were stained with DAPI (blue). Confocal images are shown in both XY and XZ plans. Scale bar = 5 µm. Localization analysis of AQP2-pS269 represents the means ± SEM of four independent experiments compared to dDAP treatment. §§§ p< 0.001; ***p<0.001 two-way ANOVA with a post-hoc Bonferroni multiple comparison test.

Herein, we further show that AVP treatment of mpkCCD cells enhanced both mRNA and protein expressions of AQP2, thus supporting previous data described above and confirming the suitability of mpkCCD cell model for our study [25, 41-44]. On the other hand, immunoblots also revealed multiple bands between 30 to 50 kDa corresponding to non-glycosylated and glycosylated forms of AQP2 as described previously [45, 46]. Glycosylated and non-glycosylated forms of AQP2 are induced by AVP stimulation and both are present at the cell membrane [41, 47]. As reported previously, AQP2 glycosylation may induce the stabilization of the protein and consequently reduce its degradation [48]. The present data show that the application of apelin-13 on mpkCCD cells, in the continuous presence of dDAVP, reduced the expression of AQP2 suggesting an

anti-vasopressinergic effect of apelin. These findings confirm previous data reported by Hus-Citharel and colleagues showing that the intravenous injection of apelin-17 increases diuresis in lactating rats characterized by high circulating AVP levels. This is also consistent with more recent findings demonstrating that apelin agonists as well as ELABELA the second endogenous ligand of APJ, stimulate urinary output, thus acting as potent regulators of fluid homeostasis and kidney function [14, 49]. This increase in diuresis was observed simultaneously with a significant decrease in urine osmolality without any change in sodium or potassium excretion [12].

It is well established that AVP exerts its antidiuretic effect by generating AQP2 expression to the apical plasma membrane principal cells of the collecting duct. Our hypothesis was hence that apelin may counteract the stimulatory effect of AVP on AQP2 apical translocation. Accordingly, we observed that the application of apelin-13 for 30 or 60 minutes induced a delocalization of AQP2 to the cytoplasm and perinuclear areas. AQP2 is subjected to a number of signaling pathways and regulatory modifications, including phosphorylation and ubiquitination, which are crucial for AQP2 function, cellular localization and degradation. Apelin directly activates endothelial APJ and eNOS production through Akt and PLC pathways, leading to NO release and vascular smooth cell relaxation. AVP, upon binding to its V_2R, activates the cAMP/PKA pathway in which CREB and AP-1 bind to *cis* elements of the AQP2 promoter cAMP, enhancing AQP2 gene transcription [45, 50, 51]. On the other hand, AVP induces membrane translocation of AQP2 through Akt/AS160 and cAMP/PKA pathways [46, 52]. Recently, Hus-Citharel and colleagues showed that apelin 17 reduces cAMP activation and disrupts intracellular calcium distribution. While previous studies have demonstrated that intracellular calcium plays a major role in AQP2 exocytosis in collecting ducts [53], the mechanism underlying this effect remains unclear.

AQP2 contains five phosphorylation sites in the carboxyl-terminus at threonine 244 and serine 256, 261, 264 and 269. PKA induces AQP2 phosphorylation at serine256 which is necessary for the translocation and accumulation of AQP2 at the plasma membrane. Phosphorylation at S256 can be detected both in intracellular vesicles and in the apical membrane. AQP2 phosphorylated at serine256 leads to further phosphorylation at serine269 [26, 29]. In addition, the AQP2-S269 form prevents the internalization of AQP2 and its interaction with endocytosis machinery. Thus, the amount of AQP2-S269 form could reflect the density of total AQP2 present at the apical plasma membrane [27, 28]. Accordingly, our data showed that the application of apelin-13 exerted a significant reduction in AQP2-pS269 density at the apical membrane of collecting duct cells, thus enabling the internalization of AQP2.

Conclusion

Apelin, through APJ stimulation, may inhibit downstream effectors of AVP to induce AQP2 down-regulation, internalization and increase its degradation. cAMP/PKA and PI3K/Akt could represent candidate pathways for AQP2 regulation by apelin (Fig. 7). Further studies are needed to identify the exact mechanisms involved in these actions of apelin. In the present study, using a collecting duct cell model, we provide additional evidence of the direct implication of the apelin-APJ system in water balance at the kidney level, indicating that apelin agonists may represent potential therapeutic agents for diseases inducing hyperhydratation.

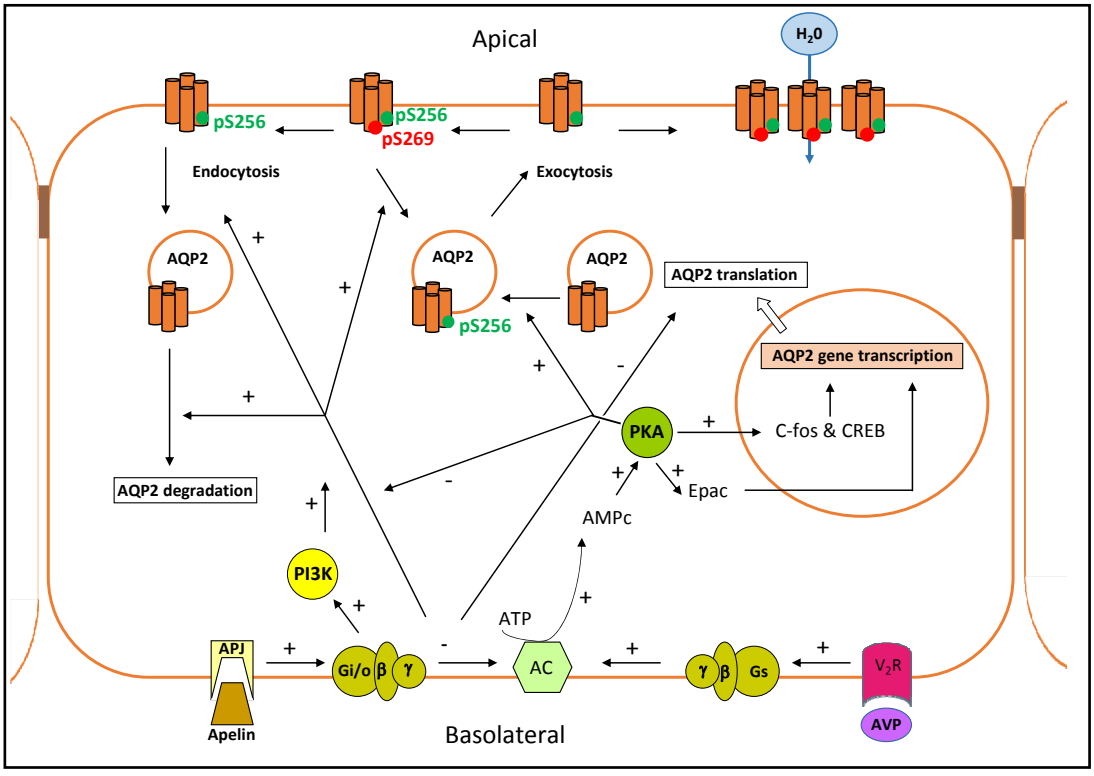

Fig. 7. Signaling cascades and cross-talk regulation of AQP2 expression and trafficking by apelin-13 and vasopressin in renal collecting duct cells.

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Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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