Effect of the cGMP pathway on AQP2 expression and translocation: potential implications for nephrogenic diabetes insipidus

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Abstract

Background. Arginine vasopressin (AVP) binding to the V2 receptor (V2R) in renal collecting duct principal cells induces a cAMP signalling cascade resulting in the activation of protein kinase A (PKA), translocation of aquaporin-2 (AQP2) to the apical membrane and an increase in AQP2 expression. Consequently, concentration of urine is initiated. X-linked nephrogenic diabetes insipidus (NDI), characterized by the inability to concentrate urine in response to AVP, is caused by mutations in the V2R gene. Initiation of AQP2 translocation, while circumventing the V2R–cAMP–PKA pathway has been suggested as a putative therapy for these patients. In this respect, the activation of a cAMP-independent and cGMP-dependent pathway for AQP2 membrane insertion by different cyclic guanosine monophosphate (cGMP) pathway activators, such as atrial natriuretic peptide (ANP), L-arginine and 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), has been put forward. However, it is unclear whether they can increase AQP2 expression.

Methods. Mouse cortical collecting duct (mpkCCD) cells were incubated with ANP, L-arginine and 8-Br-cGMP for 2 h and subjected to immunocytochemistry and cell surface biotinylation assays to examine their effect on AQP2 translocation. To test the effect of cGMP pathway activators on AQP2 expression, the mpkCCD cells were treated with dDAVP, ANP and L-arginine for 4 days, or with 8-Br-cGMP for the last day. AQP2 protein levels were determined by immunoblotting.

Results. ANP, L-arginine and 8-Br-cGMP induced the translocation of AQP2 in the mpkCCD cells. However, in contrast to dDAVP, ANP, L-arginine and 8-Br-cGMP did not increase the expression of AQP2.

Conclusions. Our results suggest that while activators of the cGMP pathway are likely beneficial in the treatment of X-linked NDI, their ability to relieve NDI in the patients may be improved when combined with agents stimulating AQP2 expression.

Keywords: aquaporin-2; cGMP; mpkCCD cells; nephrogenic diabetes insipidus

Introduction

In mammals, maintenance of a proper water balance is of vital importance and, therefore, is tightly regulated. To maintain this balance, water is reabsorbed from the pro-urine by the principal cells of the renal collecting duct, which is under the control of the pituitary-derived antidiuretic hormone arginine vasopressin (AVP). In the state of dehydration or hypovolaemia, the release of AVP and its binding to the vasopressin type 2 receptor (V2R) in the basolateral membrane of principal cells increases intracellular cyclic adenosine monophosphate (cAMP) levels through Gs-mediated stimulation of adenylyl cyclase [1–3]. This increase in cAMP activates protein kinase A (PKA), resulting in the phosphorylation of aquaporin-2 (AQP2) water channels and, likely, other proteins [2–5]. Consequently, AQP2-bearing vesicles translocate to and fuse with the apical membrane, rendering the membrane water permeable. Due to an osmotic gradient, water will then enter the cells via AQP2 and leave to the interstitium via AQP3 and AQP4, which are constitutively present in the basolateral membrane. Upon correction of hypovolaemia, blood AVP levels drop and AQP2 is retrieved from the apical membrane, resulting in reduced water reabsorption.

In humans suffering from nephrogenic diabetes insipidus (NDI), the ability to reabsorb water and concentrate urine in response to AVP is severely disturbed. Congenital NDI can be caused by mutations in either the AQP2 gene or the AVPR2 gene. Mutations in the AQP2 gene are responsible for the autosomal dominant and recessive form of inheritance of NDI [6], whereas mutations in the AVPR2 gene, which account for ~90% of inherited NDI cases, are inherited as an X-linked recessive trait [7,8].

At present, several approaches are under development to find a treatment for NDI. One of these approaches for
X-linked NDI is to bypass V2R and the cAMP activation pathway by the activation of the cGMP pathway. This approach is based on the findings of Bouley et al. that AQP2 insertion could also be accomplished by the activation of the cAMP-independent and cGMP-dependent pathway [9–11]. Nitric oxide (NO) donors, such as sodium nitroprusside (SNP) and NONOate, as well as the nitric oxide synthase (NOS) substrate l-arginine, appeared to induce AQP2 translocation from intracellular vesicles to the apical membrane by increasing cGMP levels in rat kidney slices and AQP2-transfected LLC-PK1 cells. In addition, atrial natriuretic peptide (ANP), which increases cGMP levels by activating membrane-bound guanylyl cyclase, stimulates AQP2 membrane insertion in the principal cell of ANP-infused rats [11].

In the absence of functional V2R, however, AQP2 expression levels are also reduced, because cAMP, generated through the activation of the V2R, stimulates AQP2 transcription through a cAMP-responsive element in its promoter [12–15]. At present, it is unclear whether the activation of the cGMP pathway also leads to an increased AQP2 expression. In the mouse cortical collecting duct cell line (mpkCCD), AVP increases endogenous AQP2 expression [16]. Therefore, we used this cell line to determine whether the activation of the cGMP-signalling pathway not only increases AQP2 translocation, but also increases AQP2 expression.

Materials and methods

Chemicals and reagents

ANP, l-arginine, 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), forskolin and (deamino-Cys9, D-arg1)-vasopressin (dDAVP) were purchased from Sigma (St. Louis, MO, USA). V2R antagonist (SR121463B) was kindly provided by Dr C. Serradeil-Le Gal (Sanofi Synthelabo Recherche, Toulouse, France).

Cell culture

Mouse cortical collecting duct (mpkCCD) cells (clone 14) [16] were grown in a modified defined medium [DMEM:Ham's F12 1:1 vol/vol; 60 nM sodium selenate, 5 µg/ml transferrin, 2 nM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 20 mM D-glucose, 2% fetal calf serum and 20 mM HEPES (pH 7.4)] at 37 °C in an air atmosphere of 5% CO2. The medium was replaced every 2 days.

Exponentially growing mpkCCD cells (at ~70% confluence) were trypsinized and seeded at a density of 1.5 × 105 cells/cm2 on semi-permeable filters (Transwell®, 0.4 µm pore size, Corning Costar, Cambridge, MA, USA). A total of 10 1.13 cm2 filters were used for immunocytochemistry or immunoblotting and 4.7 cm2 filters for biotinylation experiments. The cells remained in culture for 8 days before being analysed. Unless stated otherwise, the cells were treated for the last 4 days with dDAVP (1 nM) only on the basolateral side, to maximally induce AQP2 expression [16]. For internalization of AQP2, the cells were washed three times with a warm medium to remove dDAVP after which they were incubated with 1 µM of the V2R antagonist SR121463B for the indicated time points. The affinity of the V2R for dDAVP (K) in relation to AVP around 3 nM [17]) and SR121463B (K: 0.54 nM [18]) is similar, indicating that with the concentrations used, a 1000-fold excess of the antagonists was used. After again three washes, forskolin (10 µM), ANP (1 µM), l-arginine (10 mM) or 8-Br-cGMP (100 µM) were administered to both the apical and basolateral sides for the indicated time points. Biotinylation was performed as described [19].

Statistical analyses

Films and blots were scanned using a Bio-Rad (Hercules, CA, USA) 690c densitometer and chemidoc XRS, respectively, and analysed using the Bio-Rad software. Statistical comparisons were made using one-way analysis of variance (ANOVA) and Bonferroni’s post hoc correction. P-value <0.05 was considered significant.

Results

AQP2 internalization upon SR121463B treatment in the mpkCCD cells

To be able to test whether the activation of the cGMP pathway results in increased AQP2 expression levels in the mpkCCD cells, it was necessary to test whether this activation results in AQP2 translocation. For this, the mpkCCD cells were grown to confluence for 4 days and treated with 1 nM dDAVP for an additional 4 days to maximize endogenous AQP2 expression [22]. As anticipated, the cells stimulated with dDAVP showed clear AQP2 expression at the apical membrane (Figure 1a). To be able to study the effect of cGMP on AQP2 translocation, AQP2 needed to be internalized. Removal of AVP for 2 h or 4 h did not affect the localization of AQP2, but dDAVP wash-out for 8 h and 16 h resulted in increased intracellular AQP2 staining. However, dDAVP removal for 8 h and 16 h also decreased AQP2 expression to a large extent (data not shown).

Therefore, we tested whether the V2R antagonist SR121463B could be employed to internalize AQP2, while maintaining sufficient AQP2 expression. The dDAVP-treated mpkCCD cells were incubated with 1 µM SR121463B for 2 h, 4 h, 8 h or 16 h and subjected to immunocytochemistry or immunoblotting. CLSM demonstrated that after 2 h SR121463B, AQP2 was partially internalized (Figure 1b), which was more pronounced after 4 h of treatment (Figure 1c). After 8 h and 16 h, AQP2 appeared to be completely internalized, but the number of positive cells seemed reduced (Figure 1d and e). To analyse the expression quantitatively, we employed AQP2 immunoblotting, followed by semi-quantification (Figure 2a and b). At 2 h, SR121463B did not affect AQP2 expression. However, at 4-h and 8-h treatment, the AQP2 expression levels decreased significantly, whereas at 16-h treatment AQP2 expression was almost completely abolished. Based on these data, a 4-h treatment with SR121463B was chosen as the best condition.

Immunocytochemistry

Immunocytochemistry and confocal laser scanning microscopy (CLSM) of the cells grown on semi-permeable filters were performed as described [19]. To detect AQP2, the filters were incubated with affinity-purified rabbit anti-AQP2 antibodies ([20: 1:100] and goat anti-rabbit antibodies coupled with Alexa 488 (Molecular Probes, Eugene, OR, USA; 1:100).

Immunoblotting

Immunoblotting was done as described [21], for which affinity-purified rabbit anti-AQP2 antibodies (1:3000; [20]) were employed. Goat anti-rabbit antibodies coupled with horseradish peroxidase (HRP; Sigma, St. Louis, MO, U.S.A.; 1:5000) were used as secondary antibodies.
Fig. 1. Effects of V2R antagonist SR121463B on the cellular distribution of AQP2 in the mpkCCD cells. Confluent monolayers of the mpkCCD cells were stimulated with 1 nM dDAVP for 4 days and left untreated (a), or treated with 1 µM V2R antagonist for 2 h (b), 4 h (c), 8 h (d) or 16 h (e). Cells were subjected to immunocytochemistry, using rabbit AQP2 antibodies, followed by Alexa 488-conjugated anti-rabbit IgGs. Horizontal (XY) and vertical (XZ) confocal images are shown.

Fig. 2. Effects of V2R antagonist SR121463B on the expression of AQP2 in mpkCCD cells. (a) MpkCCD cells were grown to confluence, treated with 1 nM dDAVP for 4 days, left untreated or treated with 1 µM SR121463B for the indicated times and subjected to immunoblotting for AQP2. (b) The signals from a were scanned and the amount of AQP2 was semi-quantified in arbitrary units. Significant differences are indicated by asterisks.

Effect of activation of the cGMP pathway on AQP2 translocation in the mpkCCD cells

To test whether the mpkCCD cells also show cGMP pathway-mediated trafficking of AQP2, AQP2 was internalized with 4-h V2R antagonist treatment as described above, after which the medium was replaced, and the cells were left untreated or stimulated with forskolin (10 µM), ANP (1 µM), L-arginine (10 mM) or 8-Br-cGMP (100 µM) for 2 h. As mentioned before, the cells treated with SR121463B showed mainly internalized AQP2 expression (Figure 3a). Upon forskolin stimulation, AQP2 was completely translocated to the apical membrane (Figure 3b). Incubation with ANP (Figure 3c), the NOS substrate L-arginine (Figure 3d) and the cell-permeable analogue of cGMP, 8-Br-cGMP (Figure 3e), also induced translocation of AQP2 from intracellular vesicles towards the apical membrane, although not to the same extent as forskolin.

To analyse translocation of AQP2 by the activation of the cGMP pathway quantitatively, we treated the mpkCCD cells as mentioned above and performed apical cell surface biotinylation assays. As anticipated, immunoblotting of the biotinylated proteins revealed that forskolin treatment strongly increased apical membrane expression of AQP2.
cGMP in AQP2 expression and translocation

Fig. 3. Effect of the activation of the cGMP pathway on translocation of AQP2 in the mpkCCD cells. Confluent mpkCCD monolayers were stimulated with 1 nM dDAVP for 4 days, incubated with 1 µM V2R antagonist SR121463B for 4 h, and left untreated (a), or treated with 10 µM forskolin (b), 1 µM ANP (c), 10 mM L-arginine (d), or 100 µM 8-Br-cGMP (e) for 2 h. Cells were subjected to immunocytochemistry. Horizontal (XY) and vertical (XZ) confocal images are shown.

In addition, incubation with ANP, L-arginine or 8-Br-cGMP significantly increased cell surface expression of AQP2 in these cells, while the total amount of AQP2 remained equal (Figure 4). These data demonstrate that the mpkCCD cells also exert a cGMP-mediated translocation of AQP2 to the apical membrane and that these cells are suitable to analyse the effect of cGMP on AQP2 expression.

Effect of the activation of the cGMP pathway on AQP2 expression in the mpkCCD cells

To test the effect of cGMP pathway activation on AQP2 expression, the mpkCCD cells were grown for a total period of 8 days and incubated with dDAVP, ANP and L-arginine for the last 4 days or with 8-Br-cGMP for the last day. As anticipated, immunoblotting revealed that dDAVP treatment increased expression of AQP2 extensively (Figure 5). However, incubation with ANP, L-arginine or 8-Br-cGMP did not change AQP2 expression compared to untreated cells (Figure 5). These data show that ANP, L-arginine and 8-Br-cGMP are not able to induce AQP2 expression in the mpkCCD cells. While the compounds activating the cGMP pathway were not able to stimulate AQP2 expression by themselves, we also tested whether they would affect AQP2 expression in the presence of dDAVP. Following determination of a dDAVP concentration that resulted in sub-maximal...

Fig. 4. Effect of the activation of the cGMP pathway on apical membrane expression of AQP2 in the mpkCCD cells. Confluent monolayers of the mpkCCD cells were stimulated with 1 nM dDAVP for 4 days, incubated with 1 µM V2R antagonist SR121463B for 4 h, and left untreated, or treated with 10 µM forskolin, 1 µM ANP, 10 mM L-arginine, or 100 µM 8-Br-cGMP for 2 h. Then, the cells were subjected to a cell surface biotinylation assay. Biotinylated proteins were pulled-down and immunoblotted for AQP2 (AQP2). In addition, total lysates were immunoblotted for AQP2 (Total AQP2). The signals were scanned and the amount of protein was semiquantified in arbitrary units. Significant differences are indicated by asterisks. Biotinylation experiments were performed in triplicates in three independent experiments.
Fig. 5. Effect of the activation of the cGMP pathway on AQP2 expression in the mpkCCD cells. Confluent mpkCCD monolayers were left untreated, treated with 1 nM or 10 nM dDAVP, or treated with 1 nM dDAVP together with 1 µM ANP, 10 mM L-arginine (4 days) or 100 µM 8-Br-cGMP (1 day). Protein samples were immunoblotted for AQP2 and the blot was coomassie-stained to check for equal protein loading. The signals were scanned and the amount of AQP2 normalized for total protein amount was semiquantified in arbitrary units. Significant differences are indicated by asterisks. The effects on AQP2 expression were determined in triplicate in three independent experiments.

Fig. 6. Effect of dDAVP combined with activators of the cGMP pathway on AQP2 expression in the mpkCCD cells. (a) Confluent mpkCCD monolayers were left untreated, treated for 4 days with 0.7 nM alone, or with 0.7 nM dDAVP together with 1 µM ANP, 10 mM L-arginine (4 days) or 100 µM 8-Br-cGMP (1 day). (b) Confluent mpkCCD monolayers were treated for 4 days with 0.7 nM dDAVP or 1.4 nM dDAVP. Subsequently, protein samples were immunoblotted for AQP2. Protein samples were immunoblotted for AQP2. The effect on AQP2 expression was determined in triplicate. Data from three independent experiments are shown.

Discussion

ANP, L-arginine and 8-Br-cGMP induce translocation of AQP2 in the mpkCCD cells

Bouley et al. demonstrated that elevation of cGMP levels by NO donors, such as SNP and NONOate, and the NO synthase substrate L-arginine results in membrane insertion of AQP2 in AQP2-transfected LLC-PK1 cells and kidney slices [9]. In addition, ANP, which activates the cGMP cascade, also induces the plasma membrane targeting of heterologously-expressed AQP2 in LLC-PK1 cells and ANP-infused rats [9,11]. In this study, we show that the mpkCCD cells represent a proper model to study the effects of the cGMP pathway on AQP2 functioning, because ANP, L-arginine and 8-Br-cGMP also induced the translocation of AQP2 to the apical membrane of these cells (Figures 3 and 4). The higher potency of forskolin compared to the other agonists may explain the increased translocation and plasma membrane expression of AQP2 with this drug. It remains to be established whether the translocation of AQP2 through activation of the cGMP pathway involves PKA phosphorylation of AQP2 or whether this is mediated by another kinase.

For these analyses in the mpkCCD cells, we needed to internalize AQP2 with the V2R antagonist SR121463B, because removal of dDAVP for 4 h did not result in clear internalization of AQP2, while removal for longer time points (8, 16 h) resulted in reduced AQP2 levels.

ANP, L-arginine and 8-Br-cGMP do not affect AQP2 expression in the mpkCCD cells

cGMP can replace cAMP in re-distributing AQP2 water channels from intracellular storage vesicles to the apical membrane. However, V2R-induced cAMP levels not only induce AQP2 translocation, but also increase AQP2 transcription and thus protein expression. We demonstrated
that, in contrast to dDAVP, ANP, L-arginine and 8-Br-cGMP could not induce AQP2 expression (Figure 5).

Although direct evidence for decreased AQP2 levels in principal cells of NDI patients is lacking, several studies suggest that AQP2 expression is reduced in NDI patients. For instance, the urinary excretion of AQP2 in patients with NDI is decreased [20,23] and patients with central NDI, who lack endogenous AVP, also showed decreased basal excretion of AQP2 [23]. However, as urinary AQP2 is predominantly derived from exosomes, which are released into urine following translocation of AQP2 from storage vesicles to the apical membrane, these studies cannot provide an answer whether reduced urinary AQP2 levels in urine of NDI-V2R patients is due to a reduced AQP2 expression, reduced AQP2 trafficking or both.

More information is present from animal models. In pups of mice completely lacking the V2R and in adult female mice heterozygous for a deletion of the V2R gene, the AQP2 expression levels were not changed [24]. These data are in line with the knowledge that AQP2 expression is only increased after birth (pups) and that carriers of a V2R gene mutation are generally without symptoms.

In untreated Brattleboro rats, which are AVP-deficient, AQP2 protein levels were found to be ~50% lower than in untreated Wistar rats [25]. However, treatment of these rats with the V2R-specific antagonist SR121463B still reduced the AQP2 protein and mRNA levels considerably (40–50%), indicating that the V2R in Brattleboro rats is still active and, as such, is not the ideal animal model for NDI patients with non-functional V2Rs. This V2R-mediated stimulation of AQP2 expression may be due to the basal activity of the V2R (i.e. low level of V2R activity without agonist binding) or, possibly, through activation by oxytocin.

In normal or Brattleboro rats treated with V2R antagonists may be more appropriate models, as these correspond to ‘functional inactivation’ of V2Rs. While normal adult adult rats treated with the V2R-selective antagonist OPC-31260 showed a ‘significant’ decrease in AQP2 expression [15], use of the V2R-specific antagonist SR121463B in Brattleboro rats thus reduced AQP2 mRNA and protein levels to ~25% of the levels observed in normal rat with water ad libitum (50% of 50%) [25]. The strong reduction in AQP2 levels in animals lacking functional V2R is further supported by recent data from Dr Wess and colleagues, who have been able to generate mice whose V2R gene can be conditionally knocked out. With excision of the loxP-flanked V2R gene at adulthood, these mice developed severe NDI and had only 20% of the basal AQP2 expression observed in the non-excised V2R gene littermates receiving water ad libitum (J. Wess, NIH, Bethesda, MD, USA, personal communications; manuscript under review). Altogether, these data reveal that a lack of functional V2R leads to strongly reduced AQP2 expression levels, which may, besides the absence of AQP2 translocation, be an additional factor fundamental to X-linked NDI.

In conclusion, our data reveal that the activation of the cGMP pathway does not induce AQP2 expression in vitro, but, together with data of others, does induce AQP2 translocation to the apical membrane in vitro and ex vivo. Assuming similar low AQP2 levels and translocation of AQP2 with compounds activating the cGMP pathway in X-linked NDI patients, our data suggest that the beneficial effect of such compounds to relieve NDI may be improved when combined with agents that stimulate AQP2 expression.

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Conflict of interest statement. None declared.

References
Given the phenotype of peristalsis in the proximal ureter.

**Methods.**

**Background.** Congenital pelvi-ureteric junction obstruction (PUJO) affects 0.3% of human births. It may result from aberrant smooth muscle development in the renal pelvis, resulting in hydronephrosis. Mice that are null mutations for the *Teashirt3* (*Tshz3*) gene exhibit congenital PUJO. To evaluate this possibility, we used *Tshz3* gene knockouts to analyse the three mammalian *Tshz* genes in index cases with non-syndromic PUJO. We considered that these genes in other groups of patients with renal tract malformations.

**Abstract**

**Background.** Congenital pelvi-ureteric junction obstruction (PUJO) affects 0.3% of human births. It may result from aberrant smooth muscle development in the renal pelvis, resulting in hydronephrosis. Mice that are null mutants for the *Teashirt3* (*Tshz3*) gene exhibit congenital PUJO with defective smooth muscle differentiation and absent peristalsis in the proximal ureter.

**Methods.** Given the phenotype of *Tshz* mutant mice, we considered that *Teashirt* genes, which code for a family of transcription factors, might represent candidate genes for human PUJO. To evaluate this possibility, we used in situ hybridization to analyse the three mammalian *Tshz* genes in mouse embryonic ureters and determined whether *TSHZ3* was expressed in the human embryonic ureter. *TSHZ2* and *TSHZ3* were sequenced in index cases with non-syndromic PUJO.

**Results.** *Tshz2* and *Tshz3* genes were detected in mouse ureters and *TSHZ3* was expressed in the human embryonic renal pelvis. Direct sequencing of *TSHZ2* and *TSHZ3* did not identify any mutations in an initial cohort of 48 PUJO index cases, excluding these genes as a major cause of this condition. A polymorphic missense change (E469G) was identified at a residue highly conserved throughout evolution in all *Teashirt* proteins, although subsequently no significant difference between the E469G allele frequency in Albanian and Macedonian PUJO index cases (3.2%) versus 633 control individuals (1.7%) was found (*P* = 0.18).

**Conclusions.** Mutations in *TSHZ2* and *TSHZ3* are not a major cause of PUJO, at least in Albanian and Macedonian populations. Expression of these genes in the human fetal ureter emphasizes the importance of analysing these genes in other groups of patients with renal tract malformations.