In mpkCCD cells, long-term regulation of aquaporin-2 by vasopressin occurs independent of protein kinase A and CREB but may involve Epac

Marleen L. A. Kortenoeven,1 Christiane Trimpert,1 Michiel van den Brand,1 Yuedan Li,1 Jack F. M. Wetzel,2 and Peter M. T. Deen1

1Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; and 2Department of Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted 8 July 2011; accepted in final form 2 March 2012

Kortenoeven ML, Trimpert C, van den Brand M, Li Y, Wetzel JS, Deen PM. In mpkCCD cells, long-term regulation of aquaporin-2 by vasopressin occurs independent of protein kinase A and CREB but may involve Epac. Am J Physiol Renal Physiol 302: F1395–F1401, 2012. First published March 14, 2012; doi:10.1152/ajprenal.00376.2011.—Urine concentration involves the hormone vasopressin (AVP), which stimulates cAMP production in renal principal cells, resulting in translocation and transcription of aquaporin-2 (AQP2) water channels, greatly increasing the water permeability, leading to a concentrated urine. As cAMP levels decrease shortly after AVP addition, whereas AQP2 levels still increase and are maintained for days, we investigated in the present study the mechanism responsible for the AQP2 increase after long-term regulation of AQP2. As cAMP levels decrease shortly after AVP addition, whereas AQP2 levels still increase and are maintained for days, we investigated in the present study the mechanism responsible for the AQP2 increase after long-term regulation of AQP2. As cAMP levels decrease shortly after AVP addition, whereas AQP2 levels still increase and are maintained for days, we investigated in the present study the mechanism responsible for the AQP2 increase after long-term regulation of AQP2. As cAMP levels decrease shortly after AVP addition, whereas AQP2 levels still increase and are maintained for days, we investigated in the present study the mechanism responsible for the AQP2 increase after long-term regulation of AQP2.

Interestingly, cAMP levels peak immediately after the addition of the stable AVP analog 1-desamino-8-D-arginine vasopressin (dDAVP) but are reduced to low levels within 120 min following addition in AQP2-transfected Madin-Darby canine kidney cells (5). AQP2 levels, however, continue to rise during the first 3 days of dDAVP exposure in mouse collecting duct (mpkCCD) cells (21). Therefore, the purpose of this study was to delineate whether cAMP and CREB are involved in the long-term maintenance of increased AQP2 levels with dDAVP.

As cAMP not only activates PKA, but also the exchange protein directly activated by cAMP (Epac), and both Epac1 and Epac2 are expressed in renal principal cells (20), Epac might have a role in AQP2 regulation as well. This is further suggested by a study by Yip (34), who showed that the Epac-specific activator 007 caused apical targeting of AQP2 in perfused inner medullary collecting ducts.

Here, we investigated the involvement of the PKA-CREB pathway as well as Epac in the long-term AQP2 regulation in the mpkCCD cell line.

MATERIALS AND METHODS

Cell culture. mpkCCD cells were cultured as described previously (11). Cells were seeded at a density of 1.5 × 10^5 cells/cm^2 on semipermeable filters (Transwell, 0.4-μm pore size; Corning Costar, Cambridge, MA) and cultured for 8 days. For the last 24 or 96 h, the cells were incubated with 1 nM dDAVP to the basolateral side to induce an increase in AQP2 expression. During this 96-h incubation period, the medium was refreshed daily. The PKA blocker H89 (10 μM; Calbiochem, San Diego, CA) or the Epac activator 007-AM (8-pCPT-2’-O-Me-cAMP-AM; kind gift of Dr. H. Rehmann, Universitätsmedizin Göttingen, Göttingen, Germany) was added to both sides of the filters for the last 24 h.

Transfection and generation of a stable mpkCCD cell line with a 2.0 AQP2 promoter-luciferase reporter construct were previously described (17). Transfection and generation of a stable mpkCCD cell line with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly placed CREs, were described previously as well (17).

Immunoblotting. mpkCCD cells from 1.13-cm^2 filters were lysed in 200 μL Laemmli buffer and sonicated and heated for 30 min at 37°C. PAGE and blotting and blocking of the PVDF membranes were done.
as described previously (15). Membranes were incubated for 16 h at 4°C with 1:3,000-diluted affinity-purified rabbit R7 AQP2 antibodies (6), 1:2,000 anti-CREB antibody (Sigma, St. Louis, MO), 1:2,000 anti-phosphorylated CREB antibody (Sigma), 1:1,000 diluted mouse anti-Epac1 5D3 (25), or mouse anti-Epac2 3C12 antibodies (raised against the C terminus of Epac2 protein; kind gift of Dr. Johannes L. Bos, University of Utrecht, The Netherlands) in TBS Tween-20 (TBS-T) supplemented with 1% nonfat dried milk.

Blots were incubated for 1 h with 1:5,000-diluted goat anti-rabbit IgG’s or 1:2,000-diluted goat anti-mouse IgG’s (Sigma) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

Luciferase assay. Luciferase activity was measured using the luciferase assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Luminescence was measured for 10 s using an EG&G Berthold Lumat LB9507 luminometer. To verify that equal amounts of protein per sample were used for the luciferase assay, the protein concentration was determined using the Bio-Rad protein assay (München, Germany), according to the manufacturer’s instructions.

cAMP measurement. mpkCCD cells were seeded on filters for 8 days, the last 4 days with or without 1 nM dDAVP. The last day, the cells were incubated for 30 min with 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma). For short-term cAMP measurement, cells were treated for 30 min with IBMX with or without dDAVP. cAMP was measured using the cAMP enzyme immunoassay kit (Sigma) according to manufacturer’s instructions. Results were related to a standard curve based on the measurement of defined cAMP solutions done in triplicates.

Statistics. Students unpaired t-test was used when two groups with Gaussian distribution were compared. For multiple comparisons, Bonferroni correction was applied. A P value of <0.05 is considered significant. Data are presented as means ± SE.

RESULTS

Short- and long-term effects of dDAVP on cAMP levels and AQP2 transcription. At long-term incubation, dDAVP-induced AQP2 expression and plasma membrane accumulation are sustained, but cAMP levels drop to low levels within 120 min in MDCK cells (5). To investigate if cAMP levels are still increased after long-term dDAVP stimulation or return to basal levels, mpkCCD cells were grown for 8 days and incubated with or without 1 nM dDAVP. The last day, the cells were incubated for 30 min with 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma). For short-term cAMP measurement, cells were treated for 30 min with IBMX with or without dDAVP. cAMP was measured using the cAMP enzyme immunoassay kit (Sigma) according to manufacturer’s instructions. Results were related to a standard curve based on the measurement of defined cAMP solutions done in triplicates.

Although cAMP levels were lower after long-term dDAVP stimulation, AQP2 abundance was maximally increased after 4 days (Fig. 1A). Considering a half-life of 4 h for AQP2 in mpkCCD cells (31), the sustained expression of AQP2 over a period of 4 days indicates a sustained AQP2 gene transcription.

Previously, we showed that dDAVP increases luciferase activity and AQP2 protein expression in pooled colonies of mpkCCD cells stably transfected with a 3.0 kb AQP2 promoter luciferase construct (mpkCCD-AQP2–3.0-luc; Ref. 17). This dDAVP-induced luciferase activity in mpkCCD-AQP2–3.0-luc cells was due to AQP2 promoter-specific transcription, as
in mpkCCD cells stably transfected with the luciferase reporter construct without the AQP2 promoter, luciferase activity was ~100 times lower than the basal expression observed in mpkCCD-AQP2–3.0-luc cells, and this was not increased by dDAVP (17). Therefore, to test the long and short-term dDAVP effects on AQP2 transcription, mpkCCD-AQP2–3.0-luc cells were treated as above, except for the 30-min incubation, as this period was too short to observe changes in transcription (not shown). As shown in Fig. 1C, both 1 and 4 days of dDAVP application resulted in an increased luciferase activity compared with unstimulated cells, and there was no difference in the level of transcriptional activity after 1 or 4 days of dDAVP stimulation.

Role of PKA in the dDAVP-induced increase in AQP2 transcription. Although the observed sustained, but lower, cAMP production after 4 days stimulation with dDAVP may provide an explanation for the long-term increased AQP2 expression, we wanted to investigate whether the cAMP signaling cascade is really important during long-term dDAVP stimulation. To study this, mpkCCD-AQP2–3.0-luc cells were grown as described above and incubated with dDAVP for the last 24 h or 4 days and with or without the PKA blocker H89 during the last 24 h. As luciferase has a half-life of 2–4 h (14, 19), the obtained luciferase activity will reflect the AQP2 transcription during the day incubated with H89. Analysis of luciferase-activity revealed that addition of H89 after 3 days of dDAVP stimulation did not reduce AQP2 transcription. In contrast, and in line with an essential role of PKA in initiating dDAVP-induced AQP2 gene transcription, H89 reduced AQP2 transcription when added at initiation of dDAVP stimulation (Fig. 2). These data indicate that, in contrast to initial stimulation of AQP2 transcription, PKA activity is not essential for AQP2 transcription after prolonged activation by dDAVP.

Effect of dDAVP on CREB- and CRE-mediated transcription. PKA is known to activate CREB through phosphorylation of its serine 133 (S133), which has been reported to increase AQP2 gene transcription via the CRE element in the AQP2 promoter (13, 22, 27).

To investigate CRE-mediated transcription after long-term dDAVP administration, we stably transfected mpkCCD cells with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly placed CREs. As luciferase has a half-life of 2–4 h (14, 19), luciferase activity derived from this promoter consisting only of intact CRE elements allows us to evaluate the role of CRE-mediated transcription during dDAVP stimulation for 1 or 4 days. While incubation with dDAVP for 1 day yielded a more than twofold increase in luciferase activity, luciferase activity was not different from untreated cells after 4 days of dDAVP stimulation (Fig. 3A). As CRE-mediated transcription is increased after 1 day dDAVP, but not after 4 days, these data suggest that CRE-mediated transcription is important to initiate, but not to maintain, dDAVP-induced transcription from the AQP2 promoter.

To test whether the difference in CRE-mediated transcription after long-term dDAVP stimulation could result from a change in the abundance and S133 phosphorylation of CREB, the effect of dDAVP on CREB was investigated by incubating mpkCCD cells with dDAVP for 30 min and 1 and 4 days to investigate the initial response and the effects of long-term dDAVP stimulation. Immunoblot analysis revealed that dDAVP application for 30 min strongly increased the abundance of pS133 CREB (Fig. 3B). However, after 1 and 4 days of dDAVP treatment, pS133-CREB decreased to a very low level, while after 4 days, total CREB was significantly decreased as well. These data indicate that after an initial activation of CREB by phosphorylation at S133, the reduced abundance of total and S133-phosphorylated CREB may underlie the absence of CRE-mediated transcription upon long-term dDAVP stimulation.

Expression of Epac1 and 2 in mpkCCD cells. As our data indicate that PKA and CREB are not involved in the long-term transcription of AQP2, and as cAMP levels were still increased after 4 days dDAVP stimulation, compared with untreated cells, we wanted to test whether the exchange protein directly activated by cAMP (Epac) 1, and/or its close relative Epac2, could be involved.

To test whether mpkCCD cells do express the Epac proteins and whether their abundance is influenced by dDAVP, mpkCCD cells were grown on filters for 8 days of which the last 4 days without dDAVP or for 30 min, 1 day, or 4 days with dDAVP. Immunoblotting for Epac1 and Epac2 revealed a band of the anticipated mass of 96 kDa for Epac1 and 116 kDa for Epac2 in untreated cells (Fig. 4). Interestingly, while no significant change in Epac1 or Epac2 expression was seen after 30 min, Epac1 and Epac2 levels were significantly increased and decreased, respectively, with 1 and 4 days of dDAVP stimulation.

Effect of Epac activity on AQP2 expression. To investigate whether Epac activation affects AQP2 promoter-dependent transcription and AQP2 abundance, mpkCCD-AQP2–3.0-luc cells were again grown 8 days in the presence or absence of 0.1 μM of the Epac-specific activator 007-AM during the last 24 h.
As positive controls, mpkCCD-AQP2–3.0-luc cells incubated with dDAVP for 1 or 4 days were taken along. Immunoblot analysis revealed that, in contrast to dDAVP, 007-AM treatment alone did not stimulate AQP2 abundance (Fig. 5A), nor AQP2 promoter driven luciferase activity (Fig. 5B). These data indicate that Epac activation alone is not enough to stimulate AQP2 gene transcription.

To determine whether Epac activation could stabilize expression of AQP2 initially stimulated by dDAVP, mpkCCD-AQP2–3.0-luc cells grown as above were incubated with dDAVP for 3 days, followed by 1 day with or without dDAVP, or without dDAVP, but in the presence of 007-AM. Interestingly, immunoblotting for AQP2 (Fig. 5C) revealed that 007-AM incubation significantly increased AQP2 abundance compared with cells left unstimulated, indicating that Epac positively affects AQP2 abundance. The AQP2 abundance of 007-AM treated cells, however, was significantly lower than of cells incubated with dDAVP during the last day. Moreover, luciferase activity analyses revealed that 007-AM incubation significantly increased AQP2 transcription compared with unstimulated cells (Fig. 5D), indicating that the increased abundance of AQP2 protein is likely partially due to a positive effect of Epac on AQP2 transcription.

DISCUSSION

Short and long-term dDAVP-incubation increase AQP2 transcription by different pathways. It has previously been shown that AVP increases AQP2 transcription by the cAMP-PKA-CREB pathway (11, 13, 22). This pathway is also activated in mpkCCD cells after dDAVP stimulation, as shown by the dDAVP-stimulated increase in cAMP levels, the PKA dependence of AQP2 transcription and the dDAVP-mediated increase in CREB phosphorylation as well as CRE dependent transcription (Figs. 1–3). However, our data indicate that there is a different pathway involved in the long-term regulation of dDAVP-induced AQP2 transcription. Although both short- and long-term dDAVP administration increased intracellular cAMP levels, this increase was clearly lower after long-term stimulation (Fig. 1). Long-term regulation of AQP2 transcription...
seemed to be independent of PKA, as H89 did not reduce AQP2 promoter-mediated transcription on the long term, whereas it did strongly reduce initial stimulation of AQP2 expression (Fig. 2). This cannot be explained by a lack of dDAVP stimulation after 4 days, as removal of dDAVP during the last day decreased AQP2 transcription (Fig. 5D) and likely also leads to decreased cAMP levels. The initial increase in AQP2 transcription observed after 1 day of dDAVP incubation was dependent on PKA, although the effect of the PKA blocker did not fully reduce luciferase activity (compare Figs. 2 and 5B). As the in vitro IC50 of H89 for PKA is ~50 nM (3), the 10 μM concentration used in our experiments is anticipated to block PKA completely. This suggests that PKA is not the only factor involved in the increased AQP2 transcription or H89 is not stable over the 24-h incubation as used in our experiments. Another difference between the long- and short-term regulation of AQP2 is the involvement of CREB. While acute dDAVP stimulation increased CREB-activating phosphorylation and CRE-dependent transcription, these effects were not seen after long-term dDAVP incubation (Fig. 3). Consistent with the

Fig. 5. Effect of the Epac activation on AQP2 expression. A: mpkCCD cells containing an AQP2-promoter-luciferase construct were grown for 8 days and were incubated with 007-AM or dDAVP during the last 24 h, or with dDAVP for 4 days. Cells were then lysed and subjected to immunoblotting for AQP2. B: cells were treated as in A. Cells were lysed and light emission was measured. C: mpkCCD cells containing an AQP2-promoter-luciferase construct were grown for 8 days and were incubated with dDAVP for 3 days, followed by 24 h unstimulated (−) or 007-AM or dDAVP stimulation. Cells were lysed and subjected to immunoblotting for AQP2. D: cells were treated as in C. Cells were lysed and light emission was measured. A–D: data are the means ± SE of 3 samples. *P < 0.05, significant differences.
absence of an important role of PKA and CREB in long-term AQP2 expression regulation, CREB expression and phosphorylation were reduced to nearly undetectable levels with long-term dDAVP stimulation. In agreement with this, long-term dDAVP incubation did not increase CRE-mediated transcription. These changes may be the consequence of a cellular desensitization to dDAVP. Continuous stimulation of CREB is known to lead to the synthesis of inducible cAMP-response element repressor (ICER), reducing CRE-dependent transcription (23). Furthermore, cAMP stimulation has been shown to desensitize PKA (4), potentially explaining the absence of CREB phosphorylation and role of PKA in long-term AQP2 regulation.

The temporary increase in CREB phosphorylation as seen in our experiments, is in agreement with previous studies (28, 29, 35), where forskolin treatment leads to a rapid phosphorylation of CREB within 15 min, which starts to decay already after 30 min and which returns to baseline levels within 8 h. In agreement with our results, dDAVP administration for 96 h has also previously been shown to decrease total CREB levels (21).

Our results show that after 1 day of dDAVP incubation, phosphorylated CREB is already reduced. However, CRE-dependent transcription, as measured by luciferase activity, is still increased after 24 h. This might be explained by the presence of luciferase transcribed in the beginning of the dDAVP stimulation, which is still available in the cell. However, the absence of increased phosphorylated CREB indicates that CRE-mediated transcription is very likely not increased after 24 h.

Long-term dDAVP induces a small cAMP response compared with short-term dDAVP stimulation. In our study, both short- and long-term dDAVP administration increases intracellular cAMP levels, although this increase was clearly lower after long-term stimulation (Fig. 1). Previously, it has been shown (5) that cAMP levels peak immediately after addition of dDAVP but are reduced to very low levels within 120 min following addition in AQP2-transfected MDCK cells.

An increased cAMP level after long-term dDAVP as found in our study is in agreement with a previous in vivo study (7), showing that in rats subjected to intramuscular dDAVP injections for 3 days, cAMP was still increased compared with untreated rats. Later, Dublineau et al. (8) also showed that in isolated collecting ducts of similarly treated rats addition of 1 nM AVP evoked a smaller cAMP response than in untreated rats. Both our studies and those of Dublineau point to desensitization of the cellular system to vasopressin.

A possible explanation for the lower cAMP production after 4 days dDAVP is a lower receptor expression on the cell surface, which is in line with earlier observations that V2R receptor activation leads to its internalization (26). Adenylate cyclase 6, which is expressed in the collecting duct, has also been shown to be desensitized upon continued activation of PKA (1, 12). Long-term dDAVP stimulation also results in an increased cAMP breakdown in vivo, as cAMP stimulation leads to an upregulation of cAMP phosphodiesterases (18). However, as our cAMP measurements were done in the presence of the phosphodiesterase inhibitor IBMX, an increased breakdown by increased phosphodiesterase activity cannot explain the difference in cAMP levels in our experiments.

Epac activation increases AQP2 transcription and abundance after pretreatment with dDAVP. In vivo, both Epac1 and Epac2 proteins are expressed in the collecting duct. Epac1 is mainly expressed in intercalated cells, although it is also found in principal cells in the outer medulla. Epac2 is highly expressed in all principal cells along the entire collecting duct (20). Yip (34) showed that Epac activation increases vasopressin-stimulated translocation of AQP2 to the apical membrane.

In agreement with in vivo, mpkCCD cells also endogenously express Epac1 and Epac2 (Fig. 4) and our results indicate that long-term regulation of AQP2 by dDAVP may involve Epac. In mpkCCD cells, Epac1 expression was stimulated by dDAVP, while Epac2 expression was reduced (Fig. 4), suggesting that dDAVP regulates Epac protein abundance. Moreover, Epac activity increases both AQP2 transcription and AQP2 protein abundance after sustained dDAVP stimulation (Fig. 5), although AQP2 abundance was not maintained at the same level as with dDAVP. As after prolonged dDAVP administration, cAMP levels are still increased compared with control cells, Epac activation by the increased cAMP levels may form part of the PKA independent pathway to maintain high AQP2 abundance levels at long-term dDAVP stimulation. The reduced level of maintaining AQP2 levels may be due to instability of the Epac stimulator over the 24-h period used or may indicate that Epac is not the only factor involved in stimulating long-term AQP2 expression.

Interestingly, although Epac and PKA can have synergistic effects, Cheng et al. (2) and Garay et al. (10) showed that Epac activation can also inhibit PKA and lead to a reduced CREB phosphorylation. As such, this may explain the absence of PKA-CREB activation seen in our studies after long-term dDAVP stimulation. The role of Epac in AQP2 regulation in vivo remains to be established.

In conclusion, our study shows that the PKA-CRE pathway is involved in the initial increase in AQP2 abundance after dDAVP stimulation but not in the long-term effect of dDAVP. Instead, long-term regulation of AQP2 may involve the activation of Epac.

ACKNOWLEDGMENTS

We thank Esther Nibbeling and Henrik Dimke (Nijmegen, The Netherlands) and Geurt Stokman and Leo Price (Leiden, The Netherlands) for help.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


16. Li Y, Shaw S, Kamsteeg EJ, Vandewalle A, Deen PM. Development of lithium-induced nephrogenic diabetes insipidus is dissociated from adenylyl cyclase cyclic.


