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Biochimica et Biophysica Acta 1758 (2006) 1100–1105
BIOCHIMICA ET BIOPHYSICA ACTA
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cAMP regulates vasopressin-induced AQP2 expression via protein kinase A-independent pathway

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Received 5 October 2005; received in revised form 31 May 2006; accepted 1 June 2006
Available online 7 June 2006

Abstract

The regulation of AVP-induced AQP2 expression was investigated in the present study. AVP administration induced AQP2 expression in a dose-dependent manner in association with an increase in intracellular cAMP concentration. PKA activity was stimulated by AVP but PKA inhibitors did not block the upregulation of AQP2 expression. However, AVP also activated both ERK and CREB pathways, and ERK inhibitor attenuated the upregulation of AQP2 expression. These results therefore indicate that the effect of AVP stimulation to upregulate AQP2 expression involves a PKA-independent pathway.

Keywords: Aquaporin-2; Arginine vasopressin; cAMP; ERK; CREB; PKA

The regulation of water balance by the kidney is one of the most fundamental homeostatic functions and is rigorously controlled by arginine vasopressin (AVP). Aquaporin-2 (AQP2) is exclusively expressed in the principal cells of the collecting tubule and collecting duct and is known as the vasopressin-regulated water channel [1,2]. Acute water reabsorption in the kidney collecting duct is mediated by the trafficking of AQP2 to the apical membrane from intracellular vesicles in response to AVP [3–5]. In addition to this regulation on the short-term, the long-term regulation of AQP2 expression plays an important role in water reabsorption in the collecting duct. The regulation of AQP2 by AVP involves upregulation of AQP2 transcription via cAMP responsive element (CRE), which results in a remarkable increase of AQP2 expression [6,7]. Further, it is thought that AQP2 transcription is mediated by the phosphorylation of the CRE binding protein (CREB). To date, the cellular and molecular mechanisms on the regulation of AVP-induced AQP2 expression have not been fully defined. To better elucidate this process, a cell line expressing intact *cis*- and *trans*-acting AQP2 is required. In this regard, an immortalized mouse cortical collecting duct cell line mpkCCD$_{C14}$ was used in the present study. This cell line, which is derived from microdissected cortical collecting ducts of an SVPK/Tag transgenic mouse, exhibits most of the major functional properties of collecting duct principal cells and natively expresses AQP2 [8,9]. By using this cell line, the regulation of AVP-induced AQP2 expression was investigated.

First, the reactivity and sensitivity of AVP to increase AQP2 expression was investigated. mpkCCD$_{C14}$ cells were seeded on transwell chambers with permeable support. After confluence, cells were exposed to serum-free medium with no growth factors for 24 h before use. Then, the cells were incubated with different concentrations of AVP. After 24 h of incubation, AQP2 protein expression was investigated by immunoblot analysis. As shown in Fig. 1A, AQP2 protein bands were detected as non-glycosylated, core-glycosylated, and fully glycosylated forms. AVP-induced AQP2 expression was observed at the concentration of 10^{-11} M and increased in a dose-dependent

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doi:10.1016/j.bbamem.2006.06.001

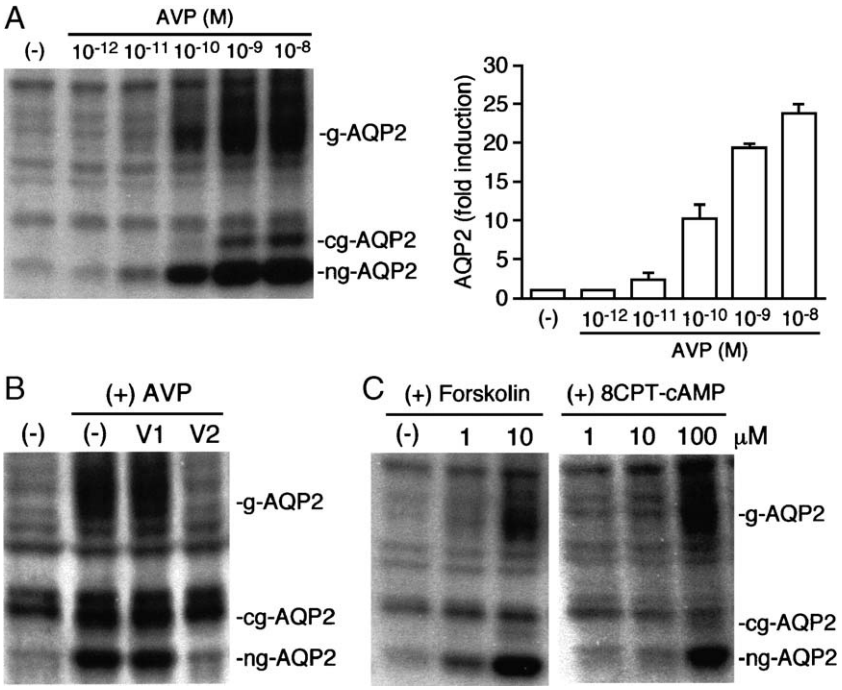

Fig. 1. (A) AQP2 expression is increased in an AVP dose-dependent manner in mpkCCD$_{C14}$ cells. Cells grown on Transwell chambers with permeable support were incubated with serum-free medium with no growth factors for 24 h before use. Cells were incubated with different concentrations of AVP (10^{-12} to 10^{-8} M) for 24 h. AVP was added to the basolateral side. Then, cells were washed with ice-cold PBS, suspended with 20 mM Tris (pH 7.5) containing 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail, and incubated for 30 min on ice. After centrifuged at 14,000×*g* for 10 min at 4 °C, the supernatant was collected, and protein concentration was measured using the Bradford protein assay method. The cell extract was resolved on a 12% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated with a polyclonal anti-AQP2 antibody. After washing, the membrane was incubated with anti-rabbit IgG horseradish peroxidase secondary antibody. The immunoreactive bands were visualized by enhanced chemiluminescence method. AQP2 protein bands were detected as non-glycosylated (ng-AQP2), core-glycosylated (cg-AQP2), and fully glycosylated (g-AQP2) forms. The bands on the film were scanned and analyzed by using the NIH image software. To confirm that equal amounts of protein samples were loaded, duplicate gels were stained with Coomassie Brilliant Blue (data not shown). AQP2 expression in basal medium was assigned as 1-fold. Values represent the mean±SE of three independent sets of experiments. (B) V_2R, but not V_1R, antagonist blocks AVP-induced AQP2 expression. Cells were pretreated without or with V_1R antagonist ([β-mercapto-β,β-cyclopentamethylenepropionyl^1, O-me-Tyr^2, Arg^8]-vasopressin; 1 μM) or V_2R antagonist ([adamantaneacetyl^1, O-Et-D-Tyr^2, Val^4, aminobutyryl^6, Arg^{8,9}]-vasopressin; 1 μM) for 30 min and then incubated without or with AVP (10^{-8} M) in the presence of antagonist for 24 h. Then, cells were harvested and total protein was analyzed by immunoblot. (C) AQP2 expression is regulated by increased intracellular cAMP concentration. Cells grown on Transwell chambers with permeable support were incubated with different concentrations of forskolin (1 or 10 μM) or 8-(4-chloro-phenylthio)-3′,5′-cyclicmonophosphate (8CPT-cAMP; 1, 10, or 100 μM) for 24 h. The cells were then harvested and total protein was analyzed by immunoblot.

manner. The maximal induction of AQP2 was achieved at 10^{-8} M AVP. This induction was shown when AVP was added only to the basolateral membrane side, but not the apical side. Further, there was no AQP2 induction in cells cultured on a plastic dish (data not shown). Thus, AQP2 expression is increased in an AVP dose-dependent manner.

The results in Fig. 1A indicated that AQP2 expression was associated with AVP action. In the collecting duct, AVP binds to both its V_1 and V_2 receptors (V_1R and V_2R) located on the basolateral membrane. The V_1R and V_2R elevate intracellular Ca^{2+} via G protein-coupled phospholipase C/ inositol 3,4,5-triphosphate signaling and cAMP via adenylyl cyclase, respectively. Therefore, both vasopressin receptor antagonists were tested whether they blocked AVP-induced AQP2 expression. The pretreatment with V_2R, but not V_1R, blocked AVP-induced AQP2 expression (Fig. 1B). Further, phospholipase C inhibitor did not block AVP-induced AQP2 expression (data not shown). This result therefore indicates that AVP-induced AQP2 expression is mediated via its V_2R.

The result in Fig. 1B suggested that AVP-induced AQP2 expression was associated with intracellular cAMP concentration. Therefore, cAMP analog 8CPT-cAMP and adenylyl cyclase activator forskolin were tested whether they induced AQP2 expression. As shown in Fig. 1C, both stimulators strongly induced AQP2 expression in a dose-dependent manner. This result indicates that AVP-induced AQP2 expression is mediated by intracellular cAMP via V_2R and the increase in intracellular cAMP is critical for upregulation of AQP2 expression.

The result in Fig. 1C suggested that AQP2 expression was associated with increased intracellular cAMP concentration. Therefore, the intracellular cAMP concentration was measured by ELISA. As expected, intracellular cAMP concentration was increased in an AVP dose-dependent manner (Fig. 2A). Since PKA is the principal target of cAMP, the PKA activity was measured by using PKA substrate Kemptide. Similarly, PKA activity was significantly increased when the cells were incubated with AVP (Fig. 2B). The addition of forskolin to

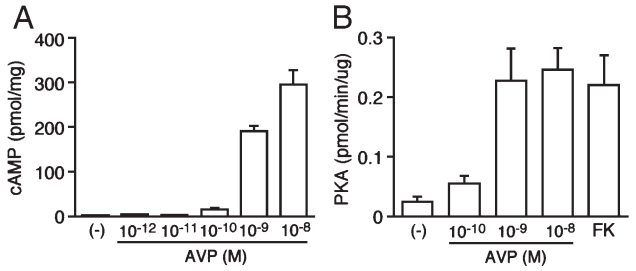

Fig. 2. Measurement of intracellular cAMP concentration and PKA activity in mpkCCD$_{C14}$ cells. (A) Intracellular cAMP. Intracellular cAMP level was measured using cAMP EIA kit (Cayman Chemical) according to the manufacturer's instructions. Cells were incubated with different concentrations of AVP (10^{-12} to 10^{-8} M) in the presence of 3-isobutyl-1-methylxanthine (IBMX; 100 μM) for 30 min. IBMX was used as an inhibitor of cAMP phosphodiesterase. Intracellular cAMP was extracted with 150 μl of 0.1 N HCl at room temperature for 20 min. Cells were scraped off and cell protein content was measured by the Bradford protein assay. cAMP concentration was expressed in picomoles per microgram protein. Values represent the mean±SE of three independent sets of experiments. (B) PKA activity. Cells were incubated with different concentrations of AVP (10^{-10} to 10^{-8} M) or forskolin (FK; 10 μM) in the presence of IBMX (100 μM) for 30 min. Then, cells were lysed in a buffer containing 50 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium orthovanadate, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μg/ml aprotinin, and 100 μg/ml leupeptin. The lysates were incubated with a reaction buffer containing 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 12.5 mM $MgCl_2$, 0.1 mM ATP, 200 μM Kemptide as a substrate peptide and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). The phosphorylation reaction was performed for 10 min at 30 °C and then stopped by spotting on to Whatman P81 papers. The papers were washed three times with 0.75% phosphoric acid, rinsed in acetone and air dried. The radioactivity was determined by scintillation counter. PKA activity was expressed in picomoles phosphate incorporated into Kemptide/min. Each cell extract was assayed in the absence of cAMP to determine endogenously activated PKA. Values represent the mean±SE of three independent sets of experiments.

the cells stimulated PKA activity as well. These results suggest that AVP can activate PKA in a cAMP dose-dependent manner.

Next, to investigate whether the activation of PKA was involved in AVP-induced AQP2 expression, cells were pretreated with different concentrations of PKA inhibitor Rp-cAMP or H89 and then incubated with AVP for 24 h. As shown in Fig. 3, AVP-induced AQP2 expression was not blocked by either PKA inhibitor tested. Moreover, PKA peptide inhibitor (14–22 amide) also had no effect on AVP-induced AQP2 expression (data not shown). This result indicates that AVP-induced AQP2 expression is mediated by a PKA-independent pathway.

Although it is thought that PKA is the main target of cAMP, the above results indicate that PKA activation by increased intracellular cAMP does not influence the regulation of AQP2 expression. It is known that AVP stimulates mitogen-activated protein kinase (MAPK) pathway in many cell types [10,11]. Therefore, to investigate the possibility of the involvement of MAPK pathway on AVP-induced AQP2 expression, extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK) inhibitors were tested. As shown in Fig. 4, only ERK inhibitor, U0126, blocked AVP-induced AQP2 expression; the decrease occurred in a dose-dependent manner. However, p38 inhibitor SB203580 and JNK inhibitor SP600125 did not attenuate AVP-induced AQP2 expression. These data indicate that AVP-induced AQP2 expression is mediated via cAMP/ERK rather than cAMP/PKA signaling pathway.

The results in Fig. 4 indicated that AVP-induced AQP2 expression was dependent on ERK activation. Therefore, to examine whether ERK pathway is activated by the addition of AVP in this cell line, different concentrations of AVP were tested. Indeed, ERK was activated by AVP in a dose-dependent manner (Fig. 5A). Next, to investigate whether AVP-stimulated ERK pathway is associated with PKA activation, cells were pretreated with ERK or PKA inhibitor and then incubated with AVP. The phosphorylation of ERK was examined by immunoblot. As shown in Fig. 5B, AVP-stimulated ERK activation was completely blocked by ERK inhibitor U0126, but not by PKA

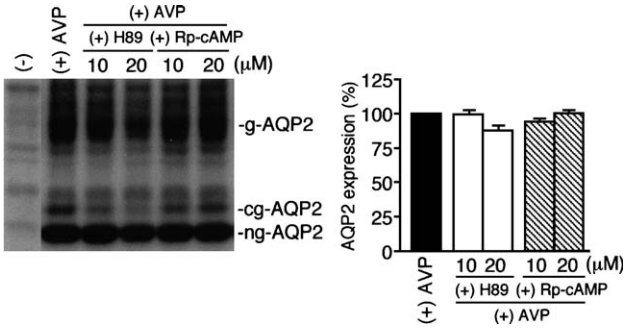

Fig. 3. PKA inhibitors do not block AVP-induced AQP2 expression. Cells were pretreated without or with PKA inhibitor Rp-adenosine 3′,5′-monophosphorothioate (Rp-cAMP; 10 or 20 μM) or N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89; 10 or 20 μM) for 30 min and then incubated without or with AVP (10^{-8} M) in the presence of inhibitor for 24 h. Then, cells were harvested and total protein was analyzed by immunoblot. Each protein blot was analyzed by densitometry. AVP-induced AQP2 expression in the absence of inhibitor was assigned as 100%. Values represent the mean±SE of three independent sets of experiments.

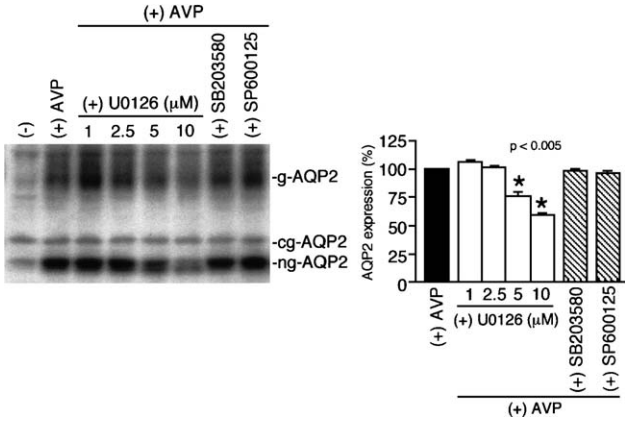

Fig. 4. AVP-induced AQP2 expression is attenuated by ERK inhibition. Cells were pretreated without or with ERK inhibitor U0126 (1, 2.5, 5, or 10 μM), p38 inhibitor SB203580 (10 μM), or JNK inhibitor SP600125 (10 μM) for 30 min and then incubated without or with AVP (10^{-8} M) in the presence of inhibitor for 24 h. Cells were harvested and total protein was analyzed by immunoblot. Each protein blot was analyzed by densitometry. AVP-induced AQP2 expression in the absence of inhibitor was assigned as 100%. Values represent the mean±SE of three independent sets of experiments. *$p<0.005$ versus AVP-induced AQP2 expression.

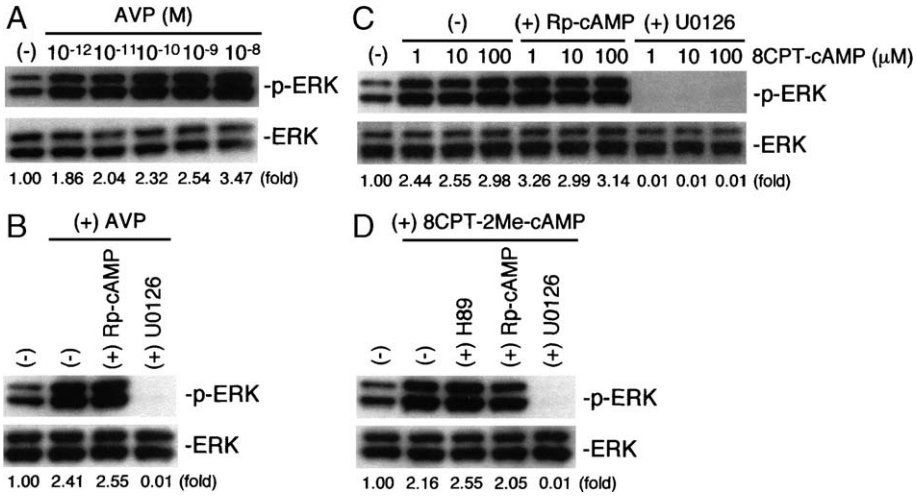

Fig. 5. (A) Cells were incubated with different concentrations of AVP (10^{-12} to 10^{-8} M) for 15 min. Then, cells were solubilized in lysis buffer (50 mM β-glycerophosphate, pH 7.2, 0.1 mM sodium orthovanadate, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μg/ml aprotinin, 100 μg/ml leupeptin, and protein phosphatase inhibitor cocktail). Cell lysate was analyzed by immunoblot with phospho-ERK (p-ERK) and ERK antibodies. Each protein blot was analyzed by densitometry. The ratio of p-ERK/ERK in basal conditions was assigned as 1-fold. (B) Cells were pretreated without or with PKA inhibitor Rp-cAMP (20 μM) or ERK inhibitor U0126 (10 μM) for 30 min and then incubated without or with AVP (10^{-8} M) for 15 min. Cells were solubilized in lysis buffer and cell lysate was analyzed by immunoblot with p-ERK and ERK antibodies. Each protein blot was analyzed by densitometry. The ratio of p-ERK/ERK in basal conditions was assigned as 1-fold. (C) Cells were pretreated without or with PKA inhibitor Rp-cAMP (20 μM) or ERK inhibitor U0126 (10 μM) for 30 min and then incubated without or with 8CPT-cAMP (1, 10, or 100 μM) for 15 min. Cells were solubilized in lysis buffer and cell lysate was analyzed by immunoblot with p-ERK and ERK antibodies. Each protein blot was analyzed by densitometry. The ratio of p-ERK/ERK in basal conditions was assigned as 1-fold. (D) Cells were pretreated without or with PKA inhibitor Rp-cAMP (20 μM) or H89 (20 μM) or ERK inhibitor U0126 (10 μM) for 30 min and then incubated without or with 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2Me-cAMP; 50 μM) for 15 min. Cells were solubilized in lysis buffer and cell lysate was analyzed by immunoblot with p-ERK and ERK antibodies. Each protein blot was analyzed by densitometry. The ratio of p-ERK/ERK in basal condition was assigned as 1-fold.

inhibitor Rp-cAMP. Similarly, the addition of cAMP analog to the cells stimulated ERK activation and PKA inhibitor Rp-cAMP did not block cAMP-stimulated ERK phosphorylation (Fig. 5C). These results provide further evidence that cAMP activated ERK pathway and this effect is independent of PKA pathway.

Recently, a new member of cAMP-dependent protein kinase was discovered, namely exchange protein directly activated by cAMP (Epac) [12,13]. It was hypothesized that this novel cAMP-dependent protein kinase, Epac, was involved in AVP-induced AQP2 expression. Therefore, it was investigated whether the Epac specific activator 8CPT-2Me-cAMP influenced the activation of ERK pathway in this cell. As shown in Fig. 5D, this Epac activator indeed stimulated ERK pathway. Both PKA inhibitors H89 and Rp-cAMP did not influence Epac activator-stimulated ERK phosphorylation, whereas ERK inhibitor U0126 completely blocked its phosphorylation. Therefore, these results suggest that AVP activates cAMP/Epac/ERK signaling cascade.

In the regulation of AQP2 expression, increased intracellular cAMP upregulates AQP2 expression through a cAMP response element (CRE) in the AQP2 gene [6,7]. The activation of CRE binding protein (CREB) phosphorylation is critical for upregulation of AQP2 transcription. In this regard the CRE in the AQP2 promoter is conserved among human, mouse and rat species [14]. It is thought that an increase in intracellular cAMP concentration by AVP stimulates the phosphorylation of CREB. Therefore, to investigate whether AVP stimulated the phosphorylation of CREB, cells were treated with different concentrations of AVP. The phosphorylation of CREB was examined by immunoblot. As shown in Fig. 6A, AVP indeed stimulated the phosphorylation of CREB. Moreover, the addition of forskolin, 8CPT-cAMP, or 8CPT-2Me-cAMP stimulated CREB phosphorylation as well. These results indicate that the activation of cAMP/Epac/ERK signaling cascade stimulates the phosphorylation of CREB. Next, the effect on AVP-stimulated CREB phosphorylation by ERK and PKA inhibitors was investigated. Cells were pretreated with ERK or PKA inhibitor and then incubated with AVP. AVP-stimulated CREB phosphorylation was attenuated by ERK inhibitor U0126, but not by PKA inhibitor Rp-cAMP (Fig. 6B). Taken together, these results indicate that AVP preferably stimulates cAMP/Epac/ERK/CREB, but not cAMP/PKA/CREB signaling cascade during the upregulation of AQP2 expression. A postulated regulatory mechanism of AVP-induced AQP2 expression therefore is as follows. AVP administration leads to the activation of G_s/adenylyl cyclase by binding to its V_2 receptor in the basolateral membrane. This increases intracellular cAMP concentration with activation of the cAMP/Epac/ERK/CREB signaling cascade.

It is clear that the second messenger cAMP produced by AVP action in the collecting duct principal cells is the critical factor in regulating AQP2 expression as well as its trafficking. cAMP regulates a variety of important biological processes such as cell growth, differentiation, secretion and division [15,16]. Elevated intracellular cAMP generally leads to the activation of cAMP-dependent protein kinases (cAPKs) such as PKA [17]. To study AQP2 trafficking in response to AVP, a number of transfected epithelial cell culture models have been generated [3–5]. Using

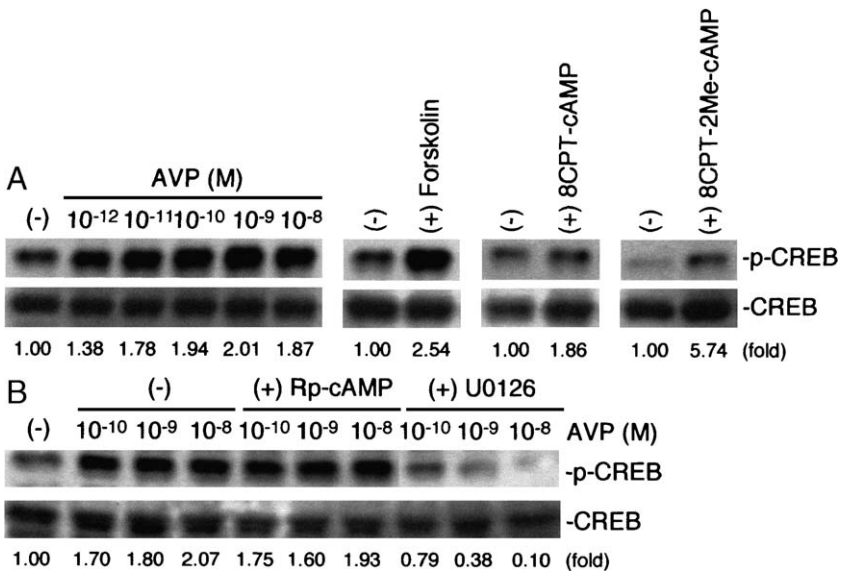

Fig. 6. (A) Cells were incubated with different concentrations of AVP (10^{-12} to 10^{-8} M) for 15 min. Cell were also incubated with forskolin (10 μM), 8CPT-cAMP (100 μM), or 8CPT-2Me-cAMP (50 μM) for 15 min. Then, cells were solubilized in lysis buffer and cell lysate was analyzed by immunoblot with phospho-CREB (p-CREB) and CREB antibodies. Each protein blot was analyzed by densitometry. The ratio of p-CREB/CREB in basal conditions was assigned as 1-fold. (B) Cells were pretreated without or with ERK inhibitor U0126 (10 μM) or PKA inhibitor Rp-cAMP (20 μM) for 30 min and then incubated without or with AVP (10^{-10} to 10^{-8} M) for 15 min. Then, cells were solubilized in lysis buffer and cell lysate was analyzed by immunoblot with p-CREB and CREB antibodies. Each protein blot was analyzed by densitometry. The ratio of p-CREB/CREB in basal conditions was assigned as 1-fold.

such cultured cell models, a series of studies have demonstrated that AQP2 translocation into the apical membrane occurs via cAMP/PKA pathway, whereby the serine 256 at the C-terminus of AQP2 is phosphorylated by the activation of PKA. It has been also reported that in normal rats phosphorylated AQP2 is already present in both the apical membrane and intracellular vesicles, and there is no significant increase in phosphorylated AQP2 after the treatment of vasopressin [18]. Further, it was shown by oocyte expression experiments that at least three phosphorylated AQP2 in each tetramer were required for the insertion into the membrane [19]. These results may explain why although phosphorylated AQP2 is already present in intracellular vesicles of the cells, a very small PKA-induced phosphorylation or the phosphorylation by another protein kinase besides PKA may be needed to AQP2 trafficking.

Recent studies have indicated that cAMP does not always mediate the activation of PKA. This suggests the possibility of a PKA-independent regulation by cAMP. In this regard, a second enzyme target of cAMP was discovered in 1998 [12,13]. It was demonstrated that cAMP-activated guanine nucleotide exchange factors (cAMP-GEFs/Epacs) directly activated Rap-1 upon cAMP binding, which is clearly mediated by PKA-independent activation [12,13]. However, the signaling cascade occurring with the activation of the Epac/Rap-1 pathway is not fully understood. Recently, a role for Epac I was identified in rat primary cortical collecting duct cells [20]. Laroche-Joubert et al. showed that calcitonin-induced H,K-ATPase activity was mediated by cAMP/Epac I/Rap-1/B-Raf/ERK cascade. Thus, cAMP targets the activation of Epac as well as PKA. To date, the possibility of the involvement of Epac in the regulation of AQP2 has not been investigated. In the present study it was demonstrated that AVP-induced AQP2 expression was regulated in a PKA-independent manner. In this regard, AVP-induced AQP2 expression was not blocked by PKA inhibitors in other mouse cortical collecting duct cell line $mCCD_{c1}$ (unpublished data). It is hypothesized that Epac, which is another target of cAMP signaling, may be involved in the regulation of AVP-induced AQP2 expression. Therefore, an Epac specific activator 8CPT-2Me-cAMP was tested in the present study. This cAMP analog does not stimulate PKA activity [21]. It was demonstrated in the present study that this Epac activator stimulated both ERK and CREB pathways. Increased cAMP concentration after AVP administration therefore activated Epac/ERK/CREB signaling cascade in a PKA-independent manner. Thus, we propose that AVP-induced AQP2 expression is mediated via cAMP/Epac/ERK/CREB pathway.

Acknowledgement

This work was supported by the National Institutes of Health Grant DK19928.

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