Role of cAMP/PKA signaling cascade in vasopressin-induced trafficking of TRPC3 channels in principal cells of the collecting duct

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Goel M, Zuo CD, Schilling WP. Role of cAMP/PKA signaling cascade in vasopressin-induced trafficking of TRPC3 channels in principal cells of the collecting duct. Am J Physiol Renal Physiol 298: F988–F996, 2010. First published January 27, 2010; doi:10.1152/ajprenal.00586.2009.—Transient receptor potential channels TRPC3 and TRPC6 are expressed in principal cells of the collecting duct (CD) along with the water channel aquaporin-2 (AQ2) both in vivo and in the cultured mouse CD cell line IMCD-3. The channels are primarily localized to intracellular vesicles, but upon stimulation with the antidiuretic hormone arginine vasopressin (AVP), TRPC3 and AQ2 translocate to the apical membrane. In the present study, the effect of various activators and inhibitors of the adenylly cyclase (AC)/cAMP/PKA signaling cascade on channel trafficking was examined using immunohistochemical techniques and by biotinylation of surface membrane proteins. Both in vivo in rat kidney and in IMCD-3 cells, translocation of AQ2 and TRPC3 (but not TRPC6) was stimulated by [deamino

THE CANONICAL TRANSIENT RECEPTOR potential (TRPC) channels are expressed in a variety of mammalian cell types, including specific cells of the renal nephron (9). There are seven members of the TRPC channel family, designated TRPC1–TRPC7. These channels are activated following stimulation of G protein-coupled membrane receptors linked to phospholipase C and thus are thought to play an essential role in receptor-mediated Ca2+ signaling in most cell types (for a recent review, see Ref. 24). Although the actual mechanism by which these channels are activated downstream of PLC remains highly controversial, it is well established that TRPC3, TRPC6, and TRPC7 can be activated by exogenous application of diacylglycerol. Diacylglycerol may directly or indirectly activate TRPC3/C6/C7 channels constitutively expressed in the plasmalemma (12, 19, 25) and/or may stimulate the translocation of active channels to the surface membrane from an intracellular pool (4, 5, 20, 21), but the molecular details of channel activation and translocation remain unclear.

Our previous studies have shown that TRPC3 and TRPC6 are abundantly expressed in principal cells of the collecting duct (CD) (10). Principal cells play an important role in water reabsorption during periods of dehydration (for reviews, see Ref. 3). Specifically, principal cells express basolateral receptors for the antidiuretic hormone arginine vasopressin (AVP) and they also express the water channel aquaporin-2 (AQ2). In the normal hydrated state, AQ2 is primarily localized to intracellular vesicles. However, upon stimulation of AVP receptors of the V2 subtype, AQ2 channels translocate to the apical membrane where they greatly enhance water permeability. Two other water channels, AQP3 and AQP4, are constitutively expressed in the basolateral membrane of the principal cells. Thus the AVP-induced increase in water permeability of the apical membrane results in immediate net transepithelial water movement, driven by the osmotic gradient within the medullary interstitium. This represents the fundamental mechanism by which the kidneys conserve water and produce a concentrated urine during periods of dehydration.

Some of the molecular mechanisms by which stimulation of V2 receptors in the basolateral membrane results in translocation of AQ2 to the apical membrane are well established (2, 17, 22). The V2 receptor is a G protein-coupled membrane receptor that activates adenyl cyclase via Gs. The resultant increase in cAMP activates PKA, which in turn leads to phosphorylation of AQ2 on critical serine residues (7, 11, 14, 16, 23). Activation of PKA triggers the movement of intracellular vesicles via cytoskeletal elements and translocation of AQ2 to the apical membrane via an exocytotic mechanism. Interestingly, both TRPC3 and TRPC6 are primarily found in intracellular vesicles in principal cells of the CD, and our recent studies have shown that stimulation by AVP causes translocation of TRPC3 (but not TRPC6) channels to the apical membrane (10). The role of the V2 receptor or the adenyl cyclase (AC)/cAMP/PKA signaling cascade in the translocation of TRPC3 is unknown. Thus the purpose of the present study was to determine the effect of activators and inhibitors of...
the AC/cAMP/PKA signaling pathway on TRPC3 translocation in principal cell of the CD.

METHODS

Antibodies and reagents. Two affinity-purified, rabbit polyclonal antibodies specific for TRPC3 (designated αA-TRPC3 and αB-TRPC3) and TRPC6 (designated αA-TRPC6 and αB-TRPC6) were generated and characterized as previously described (8, 9). Commercial antibodies used were from the following sources: goat anti-aquaporin-2, Santa Cruz Biotechnology; Alexa 488- and 594-conjugated anti-rabbit and anti-goat IgG (secondary antibodies), Molecular Probes. AVP, [deamino-Cys¹, d-Arg⁸]-vasopressin (dDAVP), [adamantaneacetyl¹, O-Et-d-Tyr²,

Fig. 1. Effect of arginine vasopressin (AVP) and [deamino-Cys¹, d-Arg⁸]-vasopressin (dDAVP) on the subcellular distribution of transient receptor potential channel TRPC3 and aquaporin-2 (AQP2) in medullary collecting duct of rat kidney. Anesthetized rats were injected with either saline control or saline containing AVP (25 ng), dDAVP (50 ng), or [adamantaneacetyl¹, O-Et-d-Tyr², Val⁴, Aminobutyryl⁶, Arg⁸⁺¹]-vasopressin (AEAVP; 60 ng) followed by AVP (25 ng). The kidneys were removed after 30 min and processed for immunohistochemical analysis using fluorescence confocal microscopy as described in METHODS. Thin sections were colabeled with primary antibodies against TRPC3 (green) and AQP2 (red). The panels on the far right show selected merged images at higher magnification. In this and all subsequent figures, the results shown are representative of at least 3 independent experiments.
Val<sup>4</sup>, aminobutyryl<sup>6</sup>, Arg<sup>8,9</sup>-vasopressin (AEAVP), and forskolin were purchased from Sigma-Aldrich. SQ22536, H89, and dibutyryl-cAMP were from Calbiochem. The myristoylated inhibitor of PKA (m-PKI) and Rp-cAMPS were from Biomol.

**Cell culture.** Mouse IMCD-3 cells were obtained from American Type Culture Collection and cultured in a 1:1 mixture of DMEM and Ham’s F12 medium containing 2 mM l-glutamine, 1% penicillin-streptomycin-neomycin (PSN) solution (GIBCO), 15 mM HEPES, 0.5 mM Na-pyruvate, 1.2 g/l Na-bicarbonate, and 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub>-air atmosphere. IMCD-3 cells were grown as confluent monolayers on glass coverslips.

**Animal protocol.** All experimental protocols involving the use of animals were approved by and performed in compliance with Case Western Reserve University Institutional Animal Care and Use Committee guidelines. Adult Sprague-Dawley rats were fed standard rat chow and were allowed water ad libitum. On the day of the experiment, rats (~0.2 kg) were anesthetized with pentobarbital sodium (60 mg/kg).

Fig. 2. Effect of AVP and dDAVP on the subcellular distribution of TRPC6 and AQP2 in medullary collecting duct of rat kidney. The protocol was identical to that described in the legend to Fig 1. Thin sections were colabeled with primary antibodies against TRPC6 (green) and AQP2 (red). The far right panels show selected merged images at higher magnification.
mg/kg ip) and injected via the tail vein with either saline solution (controls) or saline solution containing AVP (25 ng) or dDAVP (50 ng). After 30 min, the kidneys were perfusion-fixed for immunohistochemical analysis as described below. In some experiments, the rats were injected with AEAVP (60 ng) immediately before injection of AVP or dDAVP.

**Immunofluorescence.** Frozen sections (6 μm) from adult rat kidneys through both the cortex and medulla were mounted on glass coverslips and fixed in 4% paraformaldehyde for 30 min. IMCD-3 grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min. The coverslips were briefly rinsed in PBS and subsequently incubated with blocking solution containing 3% IgG-free BSA (Vector Laboratories), 10% normal donkey serum, and 0.1% Triton-X100 for 1 h at room temperature. Sections were washed three times with PBS for 5 min and mounted with Prolong Gold antifade medium (Molecular Probes). Confocal images were acquired using a Leica TCS SP2 confocal microscope. All images shown are representative merged confocal images taken before (control) and 30 min after addition of AVP (50 nM), dDAVP (100 nM), or forskolin (100 μM) to the bath solution at 37°C.

**RESULTS**

**TRPC3 translocation is initiated by V2 receptor stimulation.** To determine whether membrane trafficking of TRPC3 channels is regulated by stimulation of the V2 vasopressin receptor, anesthetized rats were infused via the tail vein with saline alone, or with saline containing AVP or the V2-specific agonist dDAVP. Additionally, some rats were infused with the specific V2-receptor antagonist AEAVP immediately before AVP injection. After 30 min, the kidneys were isolated and prepared for chemical analysis as described below. In some experiments, the rats were injected with AEAVP (60 ng) immediately before injection of AVP or dDAVP.

**Immunofluorescence.** Frozen sections (6 μm) from adult rat kidneys through both the cortex and medulla were mounted on glass coverslips and fixed in 4% paraformaldehyde for 30 min. IMCD-3 grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min. The coverslips were briefly rinsed in PBS and subsequently incubated with blocking solution containing 3% IgG-free BSA (Vector Laboratories), 10% normal donkey serum, and 0.1% Triton-X100 for 1 h at room temperature. Sections were washed three times with PBS for 5 min and mounted with Prolong Gold antifade medium (Molecular Probes). Confocal images were acquired using a Leica TCS SP2 confocal microscope. All images shown are representative merged confocal images taken before (control) and 30 min after addition of AVP (50 nM), dDAVP (100 nM), or forskolin (100 μM) to the bath solution at 37°C.
for immunohistochemical localization of TRPC3 and AQP2 (Fig. 1) or TRPC6 and AQP2 (Fig. 2). As we previously described (10), TRPC3 (green) and TRPC6 (green) colocalize with AQP2 (red) in principal cells of the CD in control saline-injected rats, as indicated by yellow seen in merged images of kidney cross sections through the medulla (Figs. 1 and 2, control). Identical results were obtained in both the cortical and medullary CD. In control rats, the immunofluorescence was present throughout the cytoplasm, consistent with localization to intracellular vesicles. As we previously showed, injection of AVP caused translocation of AQP2 and TRPC3 to the apical membrane (Fig. 1) but had no effect on TRPC6 subcellular distribution (Fig. 2). Similarly, dDAVP, caused the translocation of TRPC3 and AQP2 but had no effect on TRPC6 subcellular distribution. The effect of AVP on translocation of TRPC3 and AQP2 was blocked by prior injection of the V2-receptor antagonist AEAVP. These results demonstrate that translocation of both AQP2 and TRPC3 in vivo are under control of the V2 vasopressin receptor.

To further test this hypothesis and to evaluate the signaling pathways involved, we examined the effect of AVP and dDAVP on AQP2, TRPC3, and TRPC6 subcellular distribution in the isolated mouse inner medullary CD cell line IMCD-3. As seen in Fig. 3, both AVP and dDAVP stimulated the translocation of AQP2 and TRPC3 from intracellular vesicles to the plasma membrane of IMCD-3 cells but had no effect on TRPC6 subcellular distribution. As was seen in vivo, the effect of both AVP and dDAVP was blocked by AEAVP, the V2-receptor antagonist (Fig. 4). Thus the translocation results observed in IMCD-3 cells recapitulates those obtained in vivo in rats.

Translocation of TRPC3 is under control of AC/cAMP/PKA signaling cascade. Previous studies have shown that the V2 receptor is linked to translocation of AQP2 via activation of the AC/cAMP/PKA signaling cascade (2, 17, 22). To determine whether the same is true of TRPC3, IMCD-3 cells were treated with forskolin for 30 min to activate AC independently of receptor stimulation. As seen in Fig. 3, forskolin caused the translocation of AQP2 and TRPC3 (but not TRPC6) to the surface membrane; the profile was essentially identical to that obtained with AVP and dDAVP, but importantly, the effect of forskolin was unaffected by blockade of the V2 receptor by AEAVP (Fig. 4). However, the effect of AVP, dDAVP, and forskolin on AQP2 and TRPC3 translocation in IMCD-3 cells was blocked by the presence of SQ22536, a specific inhibitor of AC (Fig. 5). Furthermore, the effect of each agonist was also prevented by pretreatment of the cells with H89, a specific inhibitor of PKA (Fig. 5). AQP2 and TRPC3 translocation to the membrane was also stimulated by incubation of the IMCD-3 cells with the membrane-permeable cAMP analog dibutyryl-cAMP (Fig. 6). Importantly, the translocation induced by dibutyryl-cAMP was unaffected by inhibition of AC by SQ22536, but was completed blocked by pretreatment of the cells with H89 (Fig. 6).

To confirm the translocation results observed by immunofluorescence, IMCD-3 surface membrane proteins were biotinylated before and after the various treatment protocols with activators and inhibitors of the AC/cAMP/PKA signaling cascade. Biotinylated proteins, captured from whole-cell lysates using streptavidin beads, were separated by SDS-PAGE, and probed for TRPC3. In parallel, whole-cell lysates were subjected to immunoprecipitation using TRPC3-specific antibodies to determine the total amount of TRPC3 present under each assay condition. As seen in Fig. 7, both the AVP- and dDAVP-induced increase in biotin-TRPC3 was blocked by the V2-receptor antagonist AEAVP. Similarly, the increase in biotin-TRPC3 induced by AVP, dDAVP, and forskolin was blocked by SQ22536 and H89. However, the increase in biotin-TRPC3 seen with dibutyryl-cAMP was unaffected by SQ22536, but was significantly attenuated by H89. The various treatment protocols had no effect on the total amount of TRPC3 as determined by immunoprecipitation (Fig. 7) or on the amount of TRPC6 found in the avidin pulldowns (not shown). These results are consistent with TRPC3 translocation observed by immunofluorescence and demonstrate that TRPC3, but not TRPC6, is translocated and inserted into the surface membrane in response to activation of the AC/cAMP/PKA signaling cascade.

Fig. 4. Effect of the V2-antagonist AEAVP on AVP- and dDAVP-stimulated translocation of TRPC3 and AQP2 in IMCD-3 cells. The protocol was identical to that described in the legend to Fig 3. IMCD-3 cells were pretreated with AEAVP (100 nM) for 30 min before addition of AVP, dDAVP, or forskolin to the bath solution. The cell monolayers were fixed and labeled with primary antibodies against TRPC3 (green) and AQP2 (red). The figure shows representative merged confocal images of cells with and without pretreatment with AEAVP as indicated.
To obtain additional evidence for the involvement of PKA, two additional inhibitors were examined, specifically Rp-cAMPS and the myristoylated inhibitor of PKA (m-PKI). As seen in the immunofluorescence images (Fig. 8A), both inhibitors blocked AVP- and forskolin-induced translocation of TRPC3 and AQP2. Similarly, both inhibitors substantially blocked insertion of TRPC3 into the surface membrane, as estimated from the biotinylation assays (Fig. 8B). Based on the quantification of the biotinylation assay (Figs. 7B and 8C), AVP- and forskolin-induced translocation of TRPC3 was blocked 95.2 ± 1.0 and 92.0 ± 2.5%, respectively, by inhibition of PKA by H89, Rp-cAMPS, or m-PKI. These results demonstrate that activation of PKA is necessary for the AVP-induced trafficking of TRPC3.

DISCUSSION

Although TRP proteins are typically thought to function as plasmalemmal ion channels, in some cell types they are predominantly found in the cytoplasm localized to intracellular...
vesicles. In response to specific receptor stimulation, the cytoplasmic TRP channels can redistribute to the surface membrane presumably via an exocytotic process. This was first shown for the channel originally called growth factor-regulated channel or GRC, now known as TRPV2 (13). In response to stimulation by insulin-like growth factor, this channel was translocated to the surface membrane through a process that required phosphatidylinositol 3-kinase. There is also evidence in the literature that TRPC3, TRPC4, TRPC5, and TRPC6 can be inserted into the surface membrane in response to receptor stimulation (1, 5, 15, 18, 20, 21), but the molecular details remain poorly described. Pertinent to the present study, Fleming et al. (6) recently showed that epoxyeicosatrienoic acids stimulate the translocation of TRPC6 and TRPC3 to the surface membrane of vascular endothelial cells via a mechanism that involves cAMP, but the role of PKA in this response was not clear.

We previously showed that TRPC3, along with AQP2, is primarily located intracellularly in principal cells of the cortical and medullary CD of the rat and that stimulation with AVP results in the translocation and insertion of both TRPC3 and AQP2 in the apical membrane (10). Previous studies by several groups have shown that the translocation of AQP2 results from a V2 receptor-induced activation of the AC/cAMP/PKA signaling cascade (2, 17, 22). The results of the present study show that this is also true for AVP-induced translocation of TRPC3. First, TRPC3 translocation in vivo in principal cells of both the cortical and medullary CD was activated by dDAVP, a specific agonist, and blocked by AEAVP, a specific antagonist of the V2 receptor. This result was recapitulated in the cultured IMCD-3 cells grown as confluent monolayers on glass coverslips. Second, translocation of TRPC3 in IMCD-3 cells could be initiated in a V2 receptor-independent fashion by direct stimulation of AC with forskolin or by application of the membrane-permeable cAMP analog dibutyryl-cAMP. Third, translocation was prevented by specific inhibition of AC by SQ22536, and by inhibition of PKA by H89, Rp-cAMPS, and by m-PKI. Importantly, the specific inhibitors of this signaling cascade blocked only upstream agonists, i.e., AEAVP blocked dDAVP- and AVP-induced translocation, but had no effect on the

![Fig. 7. Biotinylation of plasmalemmal-associated TRPC3 in IMCD-3 cells treated with activators and inhibitors of the adenyl cyclase (AC)/cAMP/PKA signaling cascade. A: surface membrane proteins were biotinylated in control IMCD-3 cells or in cells treated with the agents indicated above each lane. Whole cell lysates were subjected to avidin pulldown using streptavidin-agarose beads or immunoprecipitation (IP) using anti-TRPC3 antibodies as indicated to the left of each lane. The recovered proteins were separated by SDS-PAGE and analyzed by Western blotting for TRPC3 as described in METHODS. For each condition, the top avidin-pulldown band and the bottom TRPC3 IP band are from the same lysate and were run on the same gel. B: bands from 3 independent experiments were quantified by densitometry. The intensity of the TRPC3 bands from the avidin pulldowns were normalized to the value obtained for the corresponding TRPC3 IP bands under each condition.](http://ajprenal.physiology.org/)

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forskolin-induced response; similarly, SQ22536 blocked AVP, dDAVP, and forskolin, but had no effect on TRPC3 translocation stimulated by dibutyryl-cAMP. Together, these results provide substantial evidence that TRPC3 translocation reflects a V2 receptor-mediated activation of the AC/cAMP/PKA signaling cascade.

There is now substantial evidence that cAMP can, in parallel with PKA, activate another signaling pathway via interaction with Epac, the exchange protein activated directly by cAMP. In iso-

lated, perfused CD from rats, Yip (26) showed that application of an Epac-specific cAMP analog increased Ca\(^{2+}\) oscillations, stimulated exocytosis, and increased membrane accumulation of AQP2. However, it remains unknown whether Epac is activated following stimulation by AVP. In the present study, AVP- and forskolin-induced translocation and membrane insertion of TRPC3 were inhibited on average 92–95% by H89, Rp-cAMPS, or m-PKI. Additionally, AVP had no effect on cytosolic free Ca\(^{2+}\) concentration in the IMCD-3 cells examined under conditions identical to those used for translocation assays (Goel M and Schilling WP, unpublished observations). Thus AVP-induced activation of PKA is necessary and perhaps sufficient for translocation of TRPC3 to the surface membrane in IMCD-3 cells.

Based on sequence analysis of rat, mouse and human proteins, TRPC3 and TRPC6 exhibit 71, 73, and 74% identity, and 78, 84, and 80% similarity at the amino acid level, respectively, yet their trafficking in response to AVP in vivo in rat kidney, and in mouse IMCD-3 cells, is very different. We previously showed that AVP stimulates the translocation of TRPC3 to the surface membrane when heterologously expressed in LLC-PK\(_1\) cells (10). This cell line expresses V2 receptors, but lacks AQP2. Thus AVP-induced translocation of TRPC3 is not dependent upon AQP2, suggesting that direct PKA-dependent phosphorylation of TRPC3 and/or some other regulatory protein is necessary for the receptor-mediated translocation event. Interestingly, TRPC6 expressed in LLC-PK\(_1\) cells is primarily localized to the cytoplasm in the absence of receptor stimulation. However, in contrast to TRPC3, the subcellular distribution of TRPC6 is unaffected by AVP (Supplementary Fig S1; all supplementary material for this article is available on the journal web site). Thus, despite similar structure and function, TRPC3 and TRPC6 are clearly sorted into different vesicle populations in kidney epithelial cells. The structural and biochemical basis for this difference remain unknown.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


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