Vasopressin-induced membrane trafficking of TRPC3 and AQP2 channels in cells of the rat renal collecting duct

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The antidiuretic hormone arginine-vasopressin (AVP) controls water homeostasis and urine concentration by stimulating water reabsorption in the renal collecting duct (CD; for reviews see Refs. 7, 36, 47). Stimulation of the V2-vasopressin receptor on the basolateral membrane of CD principal cells activates adenylyl cyclase and causes an increase in cAMP, which, in turn, activates PKA, leading to the phosphorylation of the water channel aquaporin-2 (AQP2). In the absence of circulating AVP, the majority of the AQP2 channels are found in intracellular vesicles, and the water permeability of the apical membrane of the principal cells lining the CD is low. Under this condition, a dilute urine is produced. However, AVP-induced phosphorylation of AQP2 causes the water channel to “shuttle” to the apical membrane, dramatically increasing water flux from the lumen of the CD into the cell. Water permeability of the basolateral membrane is normally high and not rate limiting, reflecting the constitutive presence and activity of two additional aquaporins, AQP3 and AQP4. Water rapidly moves across the epithelium driven by the hypertonicity of the medullary interstitium, thus producing concentrated urine.

AVP also increases cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in principal cells of the CD (9, 12, 29, 44, 54, 55), and it has been reported that AQP2 translocation is blocked or greatly attenuated by loading of the cells with the Ca\(^{2+}\) chelator BAPTA (9, 54). Thus increases in both cAMP and [Ca\(^{2+}\)]i may be necessary for initiation of AQP2 translocation to the membrane (but see Ref. 29). In primary cultures of cortical CD cells isolated from rabbits, the AVP-induced rise in [Ca\(^{2+}\)]i is associated with a dramatic increase in transcellular Ca\(^{2+}\) reabsorption (48). The increase in [Ca\(^{2+}\)]i results from both release of Ca\(^{2+}\) from internal stores and influx of Ca\(^{2+}\) across the apical membrane from the extracellular space. Only the influx component is mimicked by activation of adenylyl cyclase with forskolin, suggesting that cAMP stimulates Ca\(^{2+}\) influx, but the mechanism for this effect remains unknown.

In this regard, apical Ca\(^{2+}\) influx may be mediated by the recently discovered transient receptor potential (TRP) family of ion channels (34). There are 32 members of the mammalian TRP superfamily, which can be divided into at least 6 distinct subfamilies (56). Of these, the TRP canonical (TRPC) subfamily has seven members, designated TRPC1–TRPC7. In humans, TRPC2 is a pseudogene (52). TRPC proteins form Ca\(^{2+}\)-permeable, nonselective cation channels activated by membrane receptors linked to phospholipase C (PLC). Based on primary sequence analysis, the TRPC subfamily can be further divided into two major groups, TRPC1–4 and -5, -6, and -7. There is growing evidence that TRPC1, TRPC4, and TRPC5 proteins form so-called store-operated channels, i.e., channels activated by depletion of Ca\(^{2+}\) from internal stores (for review, see Ref. 38). Although TRPC3 and TRPC7 can function as store-operated channels under some conditions (27, 39, 45, 46, 49), there is general agreement that TRPC3, -6, and -7 channels are activated by diacylglycerol (DAG) (13, 20, 37, 38, 51).

Our previous studies showed that TRPC3 and TRPC6 are abundant in AQP2-expressing principal cells of the cortical and
medullary CD; TRPC3 is primarily localized to the apical membrane, whereas TRPC6 is found in both the apical and basolateral domains (15). However, diffuse TRPC3 and TRPC6 immunofluorescence was also found in the cytoplasm, presumably localized to intracellular vesicles. There is evidence in other cell types for the localization of TRPC channels to intracellular vesicles and for their translocation to the plasma membrane in response to either receptor stimulation or depletion of internal Ca\(^{2+}\)/H\(^{+}\) stores (6, 8, 42, 43). Together, these results suggest that TRPC3 and TRPC6 may shuttle to the apical membrane in CD principal cells in response to AVP. To test this hypothesis, we examined the subcellular localization of TRPC3, TRPC6, and AQP2 in thin sections of rat renal medulla and in isolated vesicle preparations before and after infusion of AVP. Additionally, we examined the distribution of these channels in cultured mouse M1 (cortical) and IMCD-3 (medullary) CD cell lines. The results demonstrate that, like AQP2, TRPC3, but not TRPC6, is selectively targeted to the apical membrane in response to AVP. Furthermore, TRPC3, but not TRPC6, co-immunoprecipitates with AQP2 from the medulla, and from M1 and IMCD-3 cell lysates, suggesting that these two channel proteins interact and may form a signaling complex in the plasmalemma. Last, stimulation of apical P2Y receptors or application of DAG analogs caused a substantial increase in transepithelial \(45\text{Ca}^{2+}\)/H\(^{+}\) flux across polarized IMCD-3 monolayers that was increased by overexpression of full-length TRPC3 and attenuated by overexpression of a dominant-negative construct. These results suggest that TRPC3 targeting to the apical membrane in CD principal cells could contribute, at least in part, to the AVP-induced Ca\(^{2+}\) reabsorption in this region of the nephron.

**METHODS**

**Antibodies.** Two affinity-purified, rabbit polyclonal antibodies specific for TRPC3 (designated \(\alpha_{A}-\text{TRPC3}\) and \(\alpha_{B}-\text{TRPC3}\)) and TRPC6 (designated \(\alpha_{A}-\text{TRPC6}\) and \(\alpha_{B}-\text{TRPC6}\)) were generated and characterized as previously described (14, 15). Commercial antibodies used were from the following sources: goat anti-aquaporin-2 (Santa Cruz Biotechnology); anti-GAPDH (Chemicon); and Alexa 488- and 594-conjugated anti-rabbit and anti-goat IgG (secondary antibodies; Molecular Probes).

**Cell culture.** Mouse M1 and IMCD-3 cells were obtained from ATCC 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, and 1.2 g/l Na-bicarbonate at 37°C in a humidified 5% CO\(_2\)-air atmosphere. Medium for M1 cells was supplemented with 0.005 mM dexamethasone, 5% heat-inactivated FBS, whereas that for IMCD-3 was supplemented with 10% FBS. LLC-PK1 cells were obtained from ATCC and cultured in M199 medium containing 2 mM l-glutamine, 1% PSN solution, and 3% FBS.

**Heterologous expression of TRPC3 and AQP2 in LLC-PK1.** Cells were seeded onto 35-mm culture dishes and maintained until they reached 90–95% confluence. A single dish of cells was transfected with 2 \(\mu\)g of pIRES2-enhanced green fluorescent protein (EGFP)-TRPC3 or pIRES2-EGFP-AQP2 cDNA using Lipofectamine 2000 (Invitrogen). Eight hours after transfection, the cells were dispersed with trypsin/EDTA and reseeded onto 12-mm glass coverslips (24 coverslips/35-mm dish). After 48 h of reseeding, cells were treated with 50 nM AVP at 37°C for 30 min, fixed with 4% paraformaldehyde and processed for immunostaining. Identical procedures were used for the transfection of HEK cells.

**Stable expression of TRPC3 constructs in IMCD-3 cells.** To generate IMCD-3 cell lines stably expressing TRPC3 or the NH\(_2\)-terminal...
dominant-negative TRPC3 construct, the respective cDNAs were subcloned into the bicistronic vector pIRES2-EGFP. This vector directs the production of a single mRNA that produces both the EGFP and TRPC3 proteins, allowing for the direct identification of transfected cells by fluorescence. The plasmids were selected for expansion based on green fluorescence, were serially cultured under continuous selection pressure. Control IMCD-3 cells were transfected with empty pIRES2-EGFP vector alone, and stable clones were similarly selected.

Animal protocol. All experimental protocols involving the use of animals were approved by, and performed in compliance with, the Case Western Reserve University Institutional Animal Care and Use Committee guidelines. Adult Sprague-Dawley rats were fed standard rat chow and were allowed to drink water ad libitum. On the day of the experiment, rats were anesthetized with pentobarbital sodium (70 mg/kg ip). The femoral artery was isolated by dissection and infused with either saline solution (controls) or saline solution containing AVP (50 ng/kg). After 20 min, the kidneys were removed and immediately used for immunohistochemical or biochemical assays as described below.

Preparation of tissue and whole-cell lysates. The medulla, isolated by dissection from rat kidneys, was minced and suspended in lysis buffer containing 150 mM NaCl, 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, and 0.1% deoxycholate. The tissue suspension was homogenized on ice using a Brinkman PT10/35 Polytron fitted with a 10-mm generator (3 × 10-s pulses at a power setting of 5). Homogenates/lysates were centrifuged at 6,000 g for 10 min at 4°C to remove tissue debris. The resulting supernatant was incubated at 4°C for 30 min and subsequently subjected to centrifugation at 200,000 g for 60 min at 4°C to remove unsolubilized membrane fragments. The resulting supernatants/lysates were used immediately for immunoprecipitation experiments. For cell lysates, IMCD-3 or M1 cells grown on 35-mm plastic culture dishes were incubated in 1 ml of lysis buffer containing 150 mM NaCl, 20 mM Tris·Cl (pH 7.5), and 1% Triton X-100 plus a protease inhibitor cocktail using a motor-driven teflon pestle and glass homogenization vessel (5 strokes at 1,250 rpm). The resulting homogenate was subjected to centrifugation at 400 g for 15 min, and the supernatant was reserved. The pellet was rehomogenized a second time and recentrifuged. The pooled supernatants were centrifuged at 20,000 g for 30 min. The resulting plasmalemmal pellets (low speed; LS) were collected and resuspended in buffer containing 150 mM NaCl, 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, and 0.1% deoxycholate. The supernatants were again subjected to centrifugation at 200,000 g for 60 min. The resulting vesicle pellets (high speed; HS) were resuspended in buffer containing 150 mM NaCl, 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, and 0.1% deoxycholate. Protein concentration in each fraction was determined by a Micro BCA protein assay kit (Pierce) using BSA as a standard. The preparations were used immediately for Western blot analysis.

Immunoprecipitation and immunoblotting. Tissue and cell lysates were precleared by adding control IgG together with protein A/G-agarose beads for 1 h at 4°C. Precleared lysates were incubated with primary antibodies, and the immunocomplexes were captured by incubation with protein A/G-agarose beads at 4°C for 12 h. Beads were washed, washed four times with lysis buffer, resuspended in 100 µl of 2× SDS sample buffer, and boiled for 3 min. Immunoprecipitated proteins were fractionated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (100 V for 1 h) in Tris-
glycine buffer. Blots were probed with the indicated primary antibody and detected, following incubation with horseradish peroxidase-conjugated anti-rabbit IgG, by SuperSignal West Pico chemiluminescent substrate (Pierce). The figures show representative results from experiments repeated at least three times.

**Immunofluorescence.** M1, IMCD-3, and LLC-PK1 cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min. Frozen sections (6 μm) from adult rat kidneys were mounted on glass coverslips and fixed in 4% paraformaldehyde for 30 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 X-100 for 1 h at room temperature. Sections were incubated with the indicated primary antibody overnight at 4°C. After being washing three times for 5 min with PBS at room temperature, the sections were incubated with Alexa 488- or Alexa 594-conjugated anti-rabbit IgG 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 xy scans using a ×100 oil-immersion objective (1.4 NA). The scan mode was 1,024 × 1,024, zoom factor of ~2, which yields an xy pixel size of ~80 × 80 nm. Representative images are shown from a minimum of three kidneys under each condition. Identical profiles were obtained using either the αA- or αB-TRPC3 or -TRPC6 antibody preparations.

**Biotinylation of surface membrane proteins.** M1 and IMCD-3 cells were cultured to confluence on polylysine-coated 60-mm dishes. The cells were washed three times with PBS containing (in mM) 137 NaCl, 2.7 KCl, 10 Na2HPO4, 1.8 KH2PO4, pH 8.0, plus 5 glycine (PBS/glycine), then washed twice more with PBS alone. Ice-cold biotinylation reagent (Pierce Sulfo-Link NHS-LC-biotin, 0.5 mg/ml in PBS) was immediately added. Following a 10-min incubation period on ice, the reagent was removed and the cells were washed three times with PBS/glycine. The cells were lysed on the dish by incubation for 30 min in Tris-buffered saline (TBS) containing (in mM) 150 NaCl and 20 Tris·Cl, pH 8.0, plus 1% Triton X-100 and a protease inhibitor cocktail (lysis buffer). Lysates were subjected to centrifugation at 200,000 g for 60 min, and a 1-ml aliquot of the resulting supernatant was incubated at 4°C overnight in the presence of 50 μl of streptavidin-agarose beads (Pierce) to capture biotinylated proteins. The beads were washed three times with TBS, and 100 μl of SDS sample buffer was added. Samples were boiled for 2 min before separation by SDS-PAGE.

**Sequential pull-down assays.** IMCD-3 cells were first biotinylated as described above. Lysates from these cells were then subjected to immunoprecipitation using anti-TRPC3 or anti-AQP2 antibodies as described above. The beads were isolated by centrifugation and washed three times with lysis buffer. The beads were resuspended in 10% SDS, boiled for 2 min, and immediately returned to room temperature by addition of 500 μl lysis buffer. The beads were pelleted by centrifugation, and the supernatant was reserved. This procedure was repeated a total of two times, and the supernatants were pooled. Biotinylated proteins were subsequently captured using streptavidin-agarose beads and separated by SDS-PAGE as described.
above. For controls, cell lysates were subjected to the same procedures without biotinylation.

**Transepithelial $^{45}$Ca$^{2+}$ flux.** IMCD-3 cells were grown as confluent monolayers on Transwell filters (Polystyrene membrane, 12-mm diameter, 0.4-$\mu$m pore size). Transepithelial potential and resistance were measured using a Millicell-ERS (Millipore). The filter inserts were removed from the culture medium, and the apical and basolateral bath solutions were replaced with HEPES-buffered saline (HBS) containing (in mM) 140 NaCl, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, and 10 d-glucose, pH adjusted to 7.4 at room temperature with NaOH. For apical-to-basolateral flux, an aliquot of $^{45}$Ca$^{2+}$ (10 $\mu$Ci) was added to the apical bath, and the filter insert was transferred every 2 min to a new well (basolateral bath) of a 12-well plate. The radioactivity in duplicate aliquots of the basolateral solution was determined by the liquid scintillation technique, and cumulative flux normalized to membrane surface area was plotted as a function of time. For basolateral-to-apical flux, $^{45}$Ca$^{2+}$ (10 $\mu$Ci) was added to the basolateral bath and duplicate aliquots were removed from the apical bath as a function of time. Where indicated, ATP, 1-stearoyl-2-arachidonyl-sn-glycerol (SAG), 1,2-dioctanoyl-sn-glycerol (DOG), or 1-oleoyl-2-acetyl-sn-glycerol (OAG) were added to the apical bath solution at time 0. All flux assays were performed at room temperature. Figures show means ± SE of experiments performed at least three times.

**RESULTS**

Subcellular localization and targeting of TRPC3 and TRPC6 in cells of the CD. As mentioned at the beginning of this study, and as seen in Fig. 1 (top), a substantial amount of TRPC3 immunofluorescence in thin medullary sections from control rat kidneys appears to be localized to the cytoplasm of CD principal cells. At higher magnification, the fluorescence exhibited a punctate appearance. A similar profile was observed for AQP2 immunofluorescence, and TRPC3 and AQP2 colocalized throughout the cell, as indicated by the uniform yellow color seen in merged images (Fig. 1, top). These results suggest that TRPC3 and AQP2 may exist intracellularly in the same vesicles. In contrast, although TRPC6 was abundantly expressed in cells expressing AQP2 (Fig. 2, top), TRPC6 did not colocalize at the subcellular level with AQP2 to the same extent as that observed with TRPC3. In fact, merged images showed a substantial amount of green and red, suggesting that AQP2 and TRPC6 individually exist, at least in part, in a distinct population of vesicles.

**TRPC3 and AQP2 shuttle to the apical membrane in response to AVP.** Since TRPC3 and AQP2 appear to colocalize in intracellular vesicles, we reasoned that TRPC3 may translocate to the apical membrane in response to AVP. To test this hypothesis, a bolus intravenous injection of AVP was given to anesthetized rats. After 20 min, the kidneys were removed for immunohistochemical analysis. As seen in Fig. 1 (bottom), cytoplasmic labeling of both TRPC3 and AQP2 is reduced, whereas apical membrane labeling for both is increased following stimulation by AVP. In contrast, AVP had no discernable effect on the subcellular distribution of TRPC6 (Fig. 2, bottom). These results are consistent with the hypothesis that TRPC3, but not TRPC6, is present in AQP2-
containing vesicles and shuttles to the apical membrane in response to AVP.

Expression and targeting of native TRPC3 and TRPC6 in cultured CD cell lines. TRPC3 is also expressed in cultured mouse IMCD-3 cells (15). When grown as monolayers on glass coverslips, a substantial amount of TRPC3 is present in intracellular vesicles, where it colocalizes with AQP2 as indicated by the yellow coloring seen in merged images of cells colabeled for both channels (Fig. 3). Addition of AVP to the bathing solution caused a time-dependent shuttling of TRPC3 and AQP2 to the plasma membrane that was complete within 30 min. In sharp contrast to AQP2, TRPC6 subcellular distribution in IMCD-3 cells was unaffected by AVP as indicated by the segregation of red and green coloring seen in the merged images 30 min after AVP (Fig. 3, bottom). Identical results were obtained in M1 cells (data not shown). These results demonstrate that both M1 and IMCD-3 cells express functional AVP receptors and that the subcellular distribution and targeting of TRPC3, TRPC6, and AQP2 in response to AVP is identical to that observed in vivo.

Targeting of TRPC3 channels in a heterologous expression system. LLC-PK1 cells express V2 AVP receptors and have been used extensively to study regulation of AVP-induced translocation of heterologously expressed AQP2 (22, 23, 30). In preliminary studies, we found that this cell line does not express AQP1, AQP2, or AQP3 (data not shown). Additionally, the only TRPC channel detectable at the protein level in LLC-PK1 cells is TRPC1, and it appears to be expressed in very low abundance (data not shown). To determine whether TRPC3 trafficking to the plasma membrane is independent of AQP2, we compared the effect of AVP on the subcellular distribution of TRPC3 and AQP2 following heterologous expression in LLC-PK1 cells. As seen in Fig. 4, both TRPC3 and AQP2 localize to intracellular vesicles when individually expressed in LLC-PK1 cells grown on glass coverslips. Following stimulation with AVP for 30 min, both TRPC3 and AQP2 were localized exclusively near the plasma membrane. Thus the regulated trafficking of both proteins is recapitulated in the LLC-PK1 cell line following heterologous expression, and AQP2 does not appear to be necessary for regulated trafficking of TRPC3.

Vasopressin stimulates the membrane insertion of native TRPC3. To demonstrate that apical translocation of native TRPC3 and AQP2 immunofluorescence in vivo reflects a membrane insertion, we isolated membrane fractions from the medulla enriched in either plasmalemma or intracellular vesicles using a differential centrifugation technique previously described (32). In this procedure, the medulla is homogenized and first subjected to a LS centrifugation step. The resulting LS pellet is enriched in plasma membrane. The supernatant is then subjected to a very HS centrifugation step. The resulting HS pellet is highly enriched in intracellular vesicles. Proteins from each fraction were separated by SDS-PAGE and subjected to Western blot analysis. As seen in Fig. 5, TRPC3 and AQP2 were enriched in the HS vesicle fraction isolated from the medulla of control saline-treated rats. In contrast, TRPC6 was equally distributed between the HS and LS fractions. AVP treatment caused a shift in both TRPC3 and AQP2 from the HS vesicle fraction to the LS plasma membrane fraction. AVP had no effect on the fractionation pattern for TRPC6. Thus AVP stimulation causes the selective translocation of both TRPC3 and AQP2 from intracellular vesicles to the plasma membrane in cells of the kidney medulla.

Fig. 5. Distribution of channel proteins in subcellular membrane fractions. Kidney homogenates from control rats (top) or rats treated with AVP (bottom), as described in the legend to Fig. 1, were separated into plasmalemmal (LS) and vesicular (HS) fractions using a differential centrifugation technique as described in MATERIALS AND METHODS. Proteins in each fraction were separated by SDS-PAGE and subjected to Western blot analysis. Equal amounts of protein were loaded in each lane. The primary antibody used for probing is indicated above each blot. Both glycosylated (37–50 kDa) and nonglycosylated (~27 kDa) forms of AQP2 were present in these experiments.

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To determine whether the translocation observed in IMCD-3 cells also reflects a membrane insertion of TRPC3 and AQP2, surface membrane proteins in intact cells were biotinylated before and after stimulation with AVP. Biotinylated proteins were captured using streptavidin-conjugated agarose beads. As seen in Fig. 6, AVP produced a significant increase in the biotinylation of both TRPC3 and AQP2, consistent with an increase in the membrane density of both channel proteins. In contrast, AVP had no effect on the biotinylation of TRPC6. Importantly, no proteins were observed in the absence of biotinylation, and GAPDH, an abundant cytosolic protein, was not observed in the avidin pull-downs, demonstrating that biotinylation was restricted to plasmalemmal proteins. Together, these results confirm that translocation of TRPC3 and AQP2 in IMCD-3 cells reflects membrane insertion.

**TRPC3 and AQP2 physically interact.** Our results showing colocalization and coshuttling of TRPC3 and AQP2 in cells of the CD suggested that these two proteins may physically interact and exist as part of a signaling complex. To begin to test this hypothesis, detergent lysates from rat medulla were subjected to immunoprecipitation using either TRPC3 or AQP2 antibodies. As seen in Fig. 7, AQP2 coimmunoprecipitated with TRPC3, and TRPC3 coimmunoprecipitated with AQP2, but TRPC6 did not immunoprecipitate with either TRPC3 or AQP2. Additionally, TRPC3 did not coimmunoprecipitate with AQP1 from medulla lysates (not shown). These results suggest that AQP2 and TRPC3 either directly or indirectly interact. It is important to note that before immunoprecipitation, the detergent lysates were subjected to a high-speed centrifugation step (200,000 g × 60 min) to remove partially solubilized membrane fragments and small vesicles that may form during the isolation and lysis procedure. Thus the coimmunoprecipitation of TRPC3 and AQP2 proteins suggests that they strongly interact. To further test this hypothesis, we examined the association of TRPC3 and AQP2 in lysates from both M1 and IMCD-3 cells. The assays showed that AQP2 and TRPC3 reciprocally coimmunoprecipitated from both IMCD-3 and M1 cell lysates, whereas TRPC6 does not (Fig. 8). These results demonstrate that the protein-protein interactions seen in vivo are recapitulated in the cultured cell lines from the CD. It is possible that the interaction of TRPC3 and AQP2 occurs in vitro, i.e., after detergent solubilization, and may not be indicative of protein-protein interaction within the cell. To test this hypothesis, we individually expressed TRPC3 and AQP2 in HEK cells. The cells were harvested, and separate detergent lysates were made. The individual lysates from these cells were then mixed together, and the combined lysate was subjected to immunoprecipitation. If TRPC3 and AQP2 simply bind in solution, they should coimmunoprecipitate. However, as seen in Fig. 9, TRPC3 and AQP2 did not coimmunoprecipitate from the mixed lysate. In contrast, if TRPC3 and AQP2 are coexpressed in the same cells, then reciprocal coimmunoprecipitation is observed. The same result was obtained when TRPC3 and AQP2 were expressed in LLC-PK1 cells (Fig. 9). These results demonstrate that the interaction must occur before lysate formation; i.e., coimmunoprecipitation is indicative of protein-protein interaction within the cell.

**TRPC3 and AQP2 interact in the plasma membrane.** To determine whether the interaction between TRPC3 and AQP2 occurs in the plasmalemma, a sequential pull-down assay was performed. The different steps in the pull-down assay were as follows:

1. **Pull downs**
   - Avidin
   - TRPC3
   - Avidin
   - TRPC3

2. **AVP treatment**
   - -- + -- +
   - -- + -- +
   - -- + -- +
   - -- + -- +

3. **Biotinylated**
   - non-biotinylated
   - non-biotinylated
   - non-biotinylated
   - non-biotinylated

4. **TRPC3**
   - 250
   - 150
   - 100
   - 50
   - 25

5. **AQP2**
   - 75
   - 50
   - 37

6. **Lysate avidin pulldown**
   - 100
   - 75
   - 50
   - 37

7. **GAPDH**
   - 100
   - 75
   - 50
   - 37

8. **Biotinylated**
   - non-biotinylated

**Fig. 6.** Biotinylation of plasmalemmal proteins in IMCD-3 cells. Surface membrane proteins were biotinylated in control IMCD-3 cells or in cells treated with AVP (50 nM) for 20 min as indicated below each lane. Whole cell lysates were subjected to avidin pull-down using streptavidin-agarose beads or immunoprecipitation using anti-TRPC3 or -TRPC6 antibodies as indicated above the lanes. The recovered proteins were separated by SDS-PAGE and analyzed by Western blot analysis as described in MATERIALS AND METHODS. The antibodies used for immunoblotting are indicated to the left of each panel. To demonstrate that only surface membrane proteins are biotinylated, blots were probed with anti-GAPDH (bottom right). As additional controls, pull-down assays were performed on nonbiotinylated cells as indicated below the lanes.
performed (Fig. 10). IMCD-3 cells were first challenged with AVP to initiate translocation of both channels to the surface membrane. Next, surface membrane proteins were biotinylated in intact cells as described above. Following biotinylation, the lysates were first subjected to immunoprecipitation using either anti-TRPC3 or anti-AQP2 antibodies. The immunocomplexes were released from the beads by boiling in 10% SDS. Biotinylated proteins were subsequently extracted using streptavidin-agarose beads. Under these conditions, the final pull-downs should contain only integral membrane proteins that interact with either TRPC3 or AQP2. As seen in Fig. 10, both TRPC3 and AQP2 are found in the final avidin pull-downs in both control cells and in IMCD-3 cells pretreated with AVP. These results provide strong support for the hypothesis that TRPC3 and AQP2 interact in the plasma membrane.

**TRPC3 channels play an important role in transepithelial Ca²⁺ flux.** Our previous studies revealed that TRPC3 is constitutively present in the apical membrane of M1 and IMCD-3 cells when grown as high-resistance monolayers on Transwell filters (15). Preliminary experiments similarly showed that AQP2 is constitutively present in the apical membrane of these cell monolayers. Furthermore, the apical distribution of TRPC3 and AQP2 was unaffected by AVP (data not shown). Thus there appear to be factors present in the growth medium that induce the accumulation of both channel proteins in the apical membrane when grown on permeable supports. The constitutive...
The presence of TRPC3 in the apical membrane of polarized M1 and IMCD-3 cells, however, allows us to determine the impact of channel activity on transepithelial Ca\(^{2+}\) flux. An aliquot of \(45^{\text{Ca}}\) was added to either the apical or the basolateral bathing solution of IMCD-3 cells grown on Transwell filters, and the appearance of the radioisotope in the opposite bath was measured as a function of time. As seen in Fig. 11 (top), both SAG and DOG, two agents known to activate TRPC3 channels, produced an increase in apical-to-basolateral Ca\(^{2+}\) flux when added to the apical bath. DOG produced a slightly larger increase in flux compared with SAG. Similar results were obtained with OAG (not shown). Pretreatment of the monolayers with AVP had no effect on either basal or stimulated flux, consistent with the observation that TRPC3 and AQP2 are already present in the apical membrane of these polarized IMCD-3 cells. Recent studies have identified the 3,5-bis(trifluoromethyl)-pyrazole derivative BTP2 as a potent inhibitor of the Ca\(^{2+}\) release-activated channel (CRAC) channel (57). This compound has also been reported to potently inhibit TRPC3 channel activity (18). Preincubation of the IMCD-3 monolayers with 10 \(\mu\)M BTP2 for 10 min attenuated basal transepithelial \(45^{\text{Ca}}\) flux and completely inhibited both DOG- and SAG-induced \(45^{\text{Ca}}\) flux (Fig. 11, top). These results suggest that TRPC3 may exhibit some constitutive activity that can be modestly enhanced by DAG analogs.

Previous studies indicate that principal cells of the CD have metabotropic purinergic receptors of the P2Y subtype associated with the apical membrane (24, 33, 53). To determine whether TRPC3 can be activated by receptor stimulation, IMCD-3 monolayers were challenged with ATP added to the apical bath. As seen in Fig. 11 (middle), addition of ATP to the apical bath produced a massive increase in transepithelial Ca\(^{2+}\) flux. Net Ca\(^{2+}\) flux stimulated by ATP was 50- to 100-fold greater than that observed with either DOG or SAG. Preincubation of IMCD-3 monolayers with 10 \(\mu\)M BTP2 for 10 min inhibited ATP-induced Ca\(^{2+}\) flux by 85%. In parallel experiments, we examined the effect of BTP2 on the subcellular distribution of TRPC3, AQP2, and TRPC6 in IMCD-3 cells grown on glass coverslips. Addition of BTP2 alone had no effect on the subcellular distribution of TRPC3, AQP2, or TRPC6. Similarly, BTP2 had no effect on the ability of AVP to cause translocation of either TRPC3 or AQP2, nor did BTP2...
cause a change in the distribution of TRPC3 or AQP2 when added after AVP (data not shown).

To determine whether this transepithelial Ca\(^{2+}\) flux reflects the activation of store-operated channels, we examined the effect of the SERCA pump inhibitor thapsigargin (Fig. 11, bottom). Addition of 200 nM thapsigargin to the apical bath had no significant effect on apical-to-basolateral \(^{45}\)Ca\(^{2+}\) flux relative to control, and BTP2 produced a small inhibition of \(^{45}\)Ca\(^{2+}\) flux in either the presence or absence of thapsigargin. Thus store-operated channels do not contribute to stimulated transepithelial Ca\(^{2+}\) flux in IMCD-3 cells. It is possible that transepithelial Ca\(^{2+}\) flux occurs, at least in part, via the paracellular pathway. However, the Ca\(^{2+}\) concentration in the basolateral and apical solution used for the flux assay is the same. Thus asymmetrical Ca\(^{2+}\) flux via the paracellular pathway can only occur if there is an electrical driving force, i.e., a transepithelial electrical potential (TEP). We measured the TEP and the transepithelial resistance (TER) under ionic conditions identical to those used for the flux measurements. TEP and TER were 0.83 ± 0.08 mV and 1,151 ± 1.3 \(\Omega\)·cm\(^2\) (n = 4), respectively. Neither the TEP nor the TER changed over the time course of the flux assay, and neither was affected by ATP. Thus the net apical-to-basolateral \(^{45}\)Ca\(^{2+}\) flux observed cannot occur via the paracellular pathway.

Several studies have shown that overexpression of the NH\(_2\)-terminal domain of TRPC3 acts a dominant negative (2, 16, 26, 41, 50). To obtain additional evidence that TRPC3 plays a role in transepithelial Ca\(^{2+}\) flux, we generated IMCD-3 cell lines stably expressing 1) full-length TRPC3, 2) the NH\(_2\) terminus of TRPC3, or 3) a control GFP-only construct. As seen in Fig. 12, transepithelial \(^{45}\)Ca\(^{2+}\) flux in response to apical stimulation by ATP was significantly (P < 0.001) enhanced two- to fourfold in two clonal cell lines overexpressing TRPC3 relative to control. Stable expression of the GFP-control construct had no effect, but stable expression of the TRPC3 NH\(_2\)-terminal domain-negative construct significantly (P < 0.001) attenuated flux ~30% in two clones relative to control. These results provide strong support for the hypothesis that TRPC3 channels play an important role in Ca\(^{2+}\) reabsorption via CD principal cells. Note that both basal and ATP-stimulated fluxes were similarly affected by expression of the TRPC3 constructs (Fig. 12). Thus it would appear that TRPC3 exhibits some constitutive activity under these assay conditions that can be further enhanced by receptor stimulation.

**DISCUSSION**

Our previous studies showed that TRPC3 and TRPC6 are abundantly expressed in CD principal cells (15). In the present study, we examined the subcellular distribution and targeting of these channels in vivo and in cultured CD cell lines. Several lines of evidence lead to the conclusions that TRPC3 and TRPC6 exist in different populations of intracellular vesicles and that TRPC3, but not TRPC6, is inserted into the apical membrane along with the water channel AQP2 in response to stimulation by AVP. First, intravenous injection of AVP caused the accumulation of both TRPC3 and AQP2 immunofluorescence in the apical membrane of CD cells, whereas the distribution of TRPC6 was unaffected. AVP also caused an accumulation of TRPC3 and AQP2 immunofluorescence in the plasmalemma of cultured M1 and IMCD-3 cells when grown...
as nonpolarized monolayers. To demonstrate that the accumulation of immunofluorescence actually reflects a membrane insertion, intracellular vesicles and plasmalemmal enriched fractions were obtained from control and AVP-treated rat medulla. A clear redistribution of both TRPC3 and AQP2 from vesicle to plasmalemma was observed following treatment with AVP. Similarly, both TRPC3 and AQP2 exhibited an increased availability for biotinylation in M1 and IMCD-3 cells following stimulation with AVP, consistent with increased surface membrane expression. Taken together, these experiments provide strong support for the hypothesis that TRPC3, but not TRPC6, is targeted to the apical membrane of CD principal cells in response to AVP.

It is well established that AVP acts via the V2 vasopressin receptor, a G protein-coupled membrane receptor linked primarily to adenyl cyclase via $G_{\alpha}\text{os}$ (7, 36, 47). The associated increase in cAMP and PKA activity leads to the phosphorylation of AQP2, which triggers, through currently unknown mechanisms, the translocation and insertion of AQP2 into the apical membrane. Presumably, a similar signaling cascade initiates the translocation of TRPC3. It is important to emphasize that although the present experiments suggest that TRPC3 and AQP2 may coexist in the same intracellular vesicle population, they do not eliminate the possibility that the two proteins are in different vesicles and that both are simultaneously targeted to the apical membrane in response to AVP. In this regard, a recent proteomic analysis of proteins associated with AQP2 vesicles isolated from medulla failed to identify TRPC3 (4). Although there are a number of possible explanations for this result, it is consistent with the hypothesis that TRPC3 and AQP2 are in different vesicle populations. However, if TRPC3 and AQP2 are in the same intracellular vesicles, the targeting of TRPC3 might depend on AQP2. To begin to test this hypothesis, we expressed TRPC3 in LLC-PK1 cells. Interestingly, AVP caused the translocation of TRPC3 from intracellular vesicles to the plasmalemma of LLC-PK1 cells in the absence of AQP2 expression. Thus TRPC3 targeting appears to be independent of AQP2 and presumably reflects a phosphorylation event associated with TRPC3 directly or some other protein coexpressed in the TRPC3-containing vesicles. Whether TRPC3 and AQP2 exist in the same vesicle population in vivo and the specific role of the cAMP/PKA signaling cascade in TRPC3 targeting awaits further investigation.

The parallel targeting of TRPC3 and AQP2 in response to AVP suggested that these two proteins may physically interact. Indeed, there is growing evidence that TRPC channels in general are part of large macromolecular signaling complexes localized to membrane microdomains (1). These so-called signalplexes may play important roles in compartmentalized signaling within the cell. With this in mind, we performed reciprocal immunoprecipitation assays from protein lysates obtained from both medulla and cultured M1 and IMCD-3 cell lines. The results clearly showed that TRPC3 and AQP2 physically associate. All immunoprecipitations were performed in reciprocal fashion. Thus association between TRPC3 and AQP2 is not dependent on the antibody used for precipitation. Furthermore, the interaction appears to occur in the plasmalemma since both proteins could be biotinylated in intact IMCD-3 cells and both proteins were present following sequential pull-down (i.e., immunoprecipitation followed by avidin pull-down). It is important to note that TRPC3 did not coimmunoprecipitate with AQP1 and that TRPC6 did not coimmunoprecipitate with either TRPC3 or AQP2, despite the fact that TRPC6 is constitutively expressed in the plasma membrane of CD principal cells. Thus the interaction between TRPC3 and AQP2 appears to be specific and is not related to some technical artifact such as mutual micellar association. The lack of interaction between TRPC3 and TRPC6, however, was unexpected. We previously reported that TRPC3 and TRPC6 coimmunoprecipitate from both rat brain cortex and cerebellum lysates (14). Similarly, Bandyopadhyay et al. (3) reported the coimmunoprecipitation of TRPC3 and TRPC6 from MDCK cell lysates. Thus, although these channels can potentially form heteromultimers, they apparently do not always form heteromultimers under native conditions, at least in CD principal cells.

The functional implications of TRPC3-AQP2 channel interaction and their parallel targeting to the apical membrane is at present unknown. One possibility is that TRPC3 may play a role in Ca\textsuperscript{2+} reabsorption in the CD. The calcium ion is tightly regulated and actively reabsorbed in the distal portions of the renal nephron. In recent years, investigations have focused on the distal convoluted (DCT) and connecting tubules (CNT), which are major sites of Ca\textsuperscript{2+} reabsorption under control of parathyroid hormone and vitamin D (19, 25). In these segments of the nephron, transepithelial Ca\textsuperscript{2+} flux is controlled by the activity of an apically located channel called ECaC1 (also known as TRPV5). However, the expression of TRPV5 is restricted to portions of the DCT and CNT (28). TRPV6, another Ca\textsuperscript{2+} channel abundant in intestinal epithelium, has been reported in cortical CD (35), but its activity and role in
Ca\(^{2+}\) reabsorption in this segment are unknown. In fact, relatively little information is available concerning transsepithelial Ca\(^{2+}\) flux in the CD. Using microcatheterization of inner medullary CD, Bengele et al. (5) provided clear evidence for net Ca\(^{2+}\) reabsorption in this segment and estimated that the fraction reabsorbed was ~65% of that delivered or ~1.4% of filtered Ca\(^{2+}\). Similarly, net absorption of Ca\(^{2+}\) in the CD of rats was found by microperfusion techniques (31). More recently, vasopressin and dDAVP have been shown to stimulate a rise in [Ca\(^{2+}\)]\(_i\) and an increase in transepithelial Ca\(^{2+}\) reabsorption in primary cultures of cortical CD cells isolated from rabbits (48). To begin to evaluate the role of TRPC3 in Ca\(^{2+}\) reabsorption, we measured transepithelial 45Ca\(^{2+}\) flux in polarized IMCD-3 monolayers. Under our current culture conditions, both TRPC3 and AQP2 are constitutively expressed in the apical membrane of these polarized cell monolayers and basal, nonstimulated apical-to-basolateral Ca\(^{2+}\) flux is relatively low. Interestingly, basal Ca\(^{2+}\) flux is unaffected by thapsigargin, but is attenuated by BTP2, suggesting that TRPC3 may exhibit a slight constitutive activity under basal conditions. BTP2 is a potent blocker of CRAC, TRPC3, and TRPC5 channels, but it appears to have little effect on TRPV6 (18, 57). The fact that thapsigargin has no effect on transepithelial Ca\(^{2+}\) flux suggests that store-operated channels are not involved. However, transepithelial Ca\(^{2+}\) flux could be increased severalfold by application of DAG analogs, as would be expected if TRPC3 is in fact the channel responsible for apical influx. Consistent with this hypothesis, the DAG-activated Ca\(^{2+}\) flux was completely inhibited by BTP2. In contrast to the effect of the DAG analogs, ATP produced a large increase in apical-to-basolateral Ca\(^{2+}\) flux that was also substantially attenuated by BTP2, again implicating the involvement of TRPC3 channels in this response. The dramatic difference in the response observed for DAG vs. ATP is consistent with recent studies suggesting that DAG does not directly activate TRPC3 channels but rather requires some additional factors for full efficacy (43, 50). It is important to note that we cannot eliminate a contribution of homomeric TRPC6 channels to the transepithelial Ca\(^{2+}\) flux observed since both TRPC3 and TRPC6 are present in the apical membrane of polarized IMCD-3 cell monolayers (15) and it is well established that TRPC6 channels can also be activated by DAG and receptor stimulation (13, 20, 21). Apparently, TRPC6 channels are also sensitive to BTP2 (18).

What is the possible physiological role for AVP-induced Ca\(^{2+}\) reabsorption? Although reabsorption of Ca\(^{2+}\) in the CD is generally not thought to play a significant role in whole-body Ca\(^{2+}\) homeostasis, an obvious consequence of the dramatic water reabsorption that occurs during AVP-induced antidiuresis is the production of highly concentrated urine within the lumen of the CD. For most physiological ions and solutes, this is not problematic. However, a rise in tubular Ca\(^{2+}\) concentration above a critical level will increase the probability of Ca-phosphate and Ca-oxalate crystal formation. Urinary Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_U\)) has been shown to be a predictor of urinary stone disease (USD), but the difference in [Ca\(^{2+}\)]\(_U\) between patients with and without a history of USD is only ~0.6 mM (4.29 vs. 3.72 mM, respectively) (10). Based on rat micropuncture data, luminal [Ca\(^{2+}\)] at the end of the early distal tubule is 0.42 mM (11). Decreasing urinary volume by even a modest factor of 10 during antidiuresis would elevate luminal [Ca\(^{2+}\)] into the range seen in USD patients. One possible mechanism to prevent Ca\(^{2+}\) precipitation in the luminal fluid would be to increase Ca\(^{2+}\) reabsorption in parallel with water flux. The effect of antidiuresis on Ca\(^{2+}\) excretion has been examined in rats by Roman et al. (40). These investigators found that Ca\(^{2+}\) excretion was about fivefold greater in water-loaded rats compared with dehydrated rats. During water diuresis, [Ca\(^{2+}\)]\(_U\) averaged 0.37 mM, urinary flow rate was 188 µl/min, and urine osmolality was 98 mOsm. In contrast, the [Ca\(^{2+}\)]\(_U\) in antidiuretic rats averaged 2.8 mM, urinary flow rate was 4.3 µl/min, and urine osmolality was dramatically increased to 2,326 mOsm. Importantly, if Ca\(^{2+}\) excretion rate during antidiuresis had remained high, the [Ca\(^{2+}\)]\(_U\) would have increased to 16.3 mM, a value well above the solubility product. These results clearly indicate that AVP stimulates Ca\(^{2+}\) reabsorption in rats. A preliminary report of a similar study in humans has recently been published (17). In this study, the urinary Ca\(^{2+}\) and phosphate concentrations were determined before and after 16 h of water deprivation. The urinary concentrations of both ions decreased by >50%, suggesting a marked stimulation of Ca\(^{2+}\) and phosphate reabsorption. These authors concluded that antidiuresis in normal subjects is not a risk factor for precipitation of Ca\(^{2+}\)-phosphate. Thus AVP-induced Ca\(^{2+}\) reabsorption in the CD may represent a fundamental mechanism that has evolved to allow dramatic water reabsorption while limiting the rise in tubular Ca\(^{2+}\), and thus limiting the risk of nephrocalcinosis and urolithiasis.

REFERENCES

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