TRPV5 Is Internalized via Clathrin-dependent Endocytosis to Enter a Ca\textsuperscript{2+}-controlled Recycling Pathway

Received for publication, August 20, 2007, and in revised form, November 21, 2007 Published, JBC Papers in Press, December 12, 2007, DOI 10.1074/jbc.M706959200

Stan F. J. van de Graaf\textsuperscript{1,1}, Ursula Rescher\textsuperscript{1}, Joost G. J. Hoenderop\textsuperscript{3,2}, Sjoerd Verkaart\textsuperscript{3}, René J. M. Bindels\textsuperscript{3}, and Volker Gerke\textsuperscript{3}

From the \textsuperscript{1}Institute of Medical Biochemistry, Center for Molecular Biology of Inflammation, University of Münster, von-Eschbach-Strasse 56, Münster 48149, Germany and the \textsuperscript{2}Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen 6500 HB, The Netherlands

The epithelial Ca\textsuperscript{2+} channel TRPV5 plays an essential role in transcellular Ca\textsuperscript{2+} transport and is one of the most Ca\textsuperscript{2+}-selective members of the transient receptor potential superfamily. Regulation of the abundance of TRPV5 at the cell surface is critical in body Ca\textsuperscript{2+} homeostasis. However, little is known about the mechanisms underlying TRPV5 endo- and exocytosis. Here, we show that TRPV5 is constitutively internalized in a dynamin- and clathrin-dependent manner. Internalized TRPV5 first appears in small vesicular structures and then localizes to perinuclear structures positive for Rab11a. TRPV5 has a half-life of more than 8 h and is stable even after internalization from the cell surface for more than 3 h. Disruption of cell surface delivery of newly synthesized TRPV5 by brefeldin A does not reduce TRPV5-mediated Ca\textsuperscript{2+} influx in cells, suggesting the presence of a stable intracellular pool of the channel capable of recycling back to the surface. Furthermore, the endocytic recycling kinetics is decreased upon treatment with Ca\textsuperscript{2+} chelator BAPTA-AM, indicating that the channel’s trafficking pathways are dynamically controlled by Ca\textsuperscript{2+}.

Transitory receptor potential (TRP)\textsuperscript{3} channels have a remarkably diverse set of functions ranging from roles in sensory processes like vision, osmosensation, hearing, and taste to ion homeostasis (1, 2). Most investigations regarding the regulation of the TRP family of ion channels have focused on channel gating and signaling cascades controlling channel activity at the plasma membrane. Regulation of the spatial dynamics of TRP channels is a relatively unexplored area. However, recent evidence shows that translocation of TRP channels to the cell surface is a significant factor determining their overall activity (3–9). The role of regulated trafficking to and from the cell surface is especially important for TRP channels with constitutive activity. TRPV5 is one of the most Ca\textsuperscript{2+}-selective members of the TRP superfamily and shows constitutive activation at physiological membrane potentials (10). A TRPV5 knock-out mouse model displays significant hypercalcemia (6-fold increase in total Ca\textsuperscript{2+} excretion) and severe bone abnormalities (11, 12). Together with the localization of TRPV5 in Ca\textsuperscript{2+}-transporting cells in the kidney and its pronounced regulation by calcitropic hormones, these data provide strong evidence for the role of this channel as a pivotal element in epithelial Ca\textsuperscript{2+} transport (13).

Recent evidence shows that factors affecting the number of TRPV5 channels at the cell surface have a major contribution to the physiological regulation of this Ca\textsuperscript{2+} channel. Novel channel-associated proteins have been identified, including S100A10-annexin 2 and Rab11a, which appear to play a role in the trafficking and/or cell surface stability of TRPV5 (14, 15). Furthermore, klotho, a protein with glucuronidase activity that has been linked to longevity, was postulated to slow down cell surface removal of TRPV5 via an alteration of the extracellular TRPV5 glycosylation (16). Another factor affecting turnover of TRPV5 at the plasma membrane is the serine protease kalikrein that was shown to cause accumulation of TRPV5 at the cell surface via activation of the bradykinin/diacylglycerol/protein kinase C pathway (17). Although these studies have provided new information on the regulation of TRPV5, the molecular mechanisms utilized in the endocytosis of TRPV5 are not known. Similarly, the dynamics of TRPV5 internalization and the fate of internalized TRPV5 are unclear. In this study we show that TRPV5 is constitutively internalized via a dynamin- and clathrin-dependent pathway and that internalized TRPV5 enters the Ca\textsuperscript{2+}-sensitive recycling pathway. These findings provide a framework to understand the physiological regulation of TRPV5 trafficking. Furthermore, our data present the first evidence for a role of clathrin-dependent endocytosis and Ca\textsuperscript{2+}-sensitive protein recycling in the regulation of TRP channel cell surface expression.

EXPERIMENTAL PROCEDURES

DNA Constructs—Constructs containing FLAG\textsuperscript{TM} or hemagglutinin epitope (HA)-tagged or untagged full-length TRPV5 in
TRPV5 Is Internalized via Clathrin-dependent Endocytosis

pT7Ts, pClNeo-IREs-GFP, or pCB6 were obtained as described previously (18). TRPV5 constructs with epitope tags at predicted extracellular positions were prepared by mutagenesis according to the manufacturer’s protocol (Stratagene), resulting in the replacement of amino acids in TRPV5 by an HA or FLAG™ tag or in the insertion of these tags (Fig. 1A). For the construction of HA-TRPV5, the HA-tagged transmembrane domain of KCNE1 was obtained by PCR (forward, 5’-CGCGGTCGAGCCACCTATGGACCATGCGAGCTGCGAC-3’; reverse, 5’-GGGATCCGTCAGTGGAGGCTTCTCAGTCA-3’). The predicted extracellular positions were prepared by mutagenesis (forward, 5’-CACGTCAGCAGCTACGAGCTCAACTTCTGTCGAGAC-3’; reverse, 5’-GGGATCCGTCAGTGGAGGCTTCTCAGTCA-3’). All constructs were verified by sequence analysis.

Co-immunoprecipitation—HeLa cells transiently transfected with GFP-TRPV5 and HA-TRPV5 were lysed in sucrose buffer containing 20 mM Tris, pH 7.4, 5 mM EDTA, 135 mM NaCl, 0.5% (w/v) sucrose, incubated on ice for 60 min, and centrifuged for 30 min at 16,000 × g. Supernatants were incubated with monoclonal anti-HA antibodies (clone 6Es2, Cell Signaling, Boston, MA, or clone 12CA5, Sigma) immobilized on protein-A-Sepharose 4B beads (Sigma) for 16 h at 4 °C. Immunoprecipitated proteins were analyzed by immunoblot analysis using horseradish peroxidase-coupled anti-HA (Sigma) and rabbit anti-GFP antibodies (provided by J. Fransen, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands).

Cell Culture and Transfections—HeLa and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Stably transfected HeLa cells with inducible dynamin K44E expression vector were seeded into 6-cm dishes and transfected the same day with 1 mg/ml pcDNA-IREs-TRPV5 (as described previously (20)). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) or Effectene (Qiagen) following the manufacturers’ instructions. TRPV5 protein was expressed in Xenopus laevis as described previously (18). For transient knock-down experiments HEK293 cells were transiently transfected with HA-tagged TRPV5 and incubated in internalization medium (10 mm KCl, 140 mm NaCl, 1 mm CaCl2, 1 mm MgCl2, 20 mm Hepes, pH 7.4, 5.5 mm glucose, and 1% (w/v) bovine serum albumin for 30 min at 37 °C. To track TRPV5 internalization in pulse-chase experiments, cells were first incubated for 1 h at 4 °C in internalization medium with mouse (1:100) or rabbit (1:200) anti-HA antibodies. Cells were then washed with cold PBS and subsequently chased for various times in internalization medium at 37 °C, and internalized anti-HA visualized with Texas Red- or Alexa 594-coupled secondary antibodies. Images were acquired using a LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany). TRPV5-expressing cells were identified by GFP expression due to the bicistronic vector used. Anti-clathrin heavy chain antibodies were from BD Biosciences. Antibodies directed against Tfr (H68.4) and Rab11a were obtained from Zymed Laboratories Inc. (South San Francisco, CA).

Cell Surface Biotinylation and Internalization Assay—HEK293 cells stably transfected with HA-tagged TRPV5 were seeded on poly-L-lysine-coated 10-cm dishes (7 million cells per plate). After 1–2 days proteins present at the cell surface were biotinylated at 4 °C using sulfo-NHS-SS-biotin (only Fig. 6A) or sulfo-NHS-S-SS-biotin (0.5 mg/ml, Pierce) in PBS supplemented with 0.5 mM CaCl2 and 1.0 mM MgCl2 (PBS-CM) for 30 min. Unreacted biotin was quenched using 0.1% (w/v) bovine serum albumin in PBS-CM. The cells were incubated at 37 °C for the indicated times in culture medium or kept at 4 °C. To measure TRPV5 internalization, biotin that remained at the cell surface was removed by incubation with fresh 100 mM 2-Mercaptoethanesulfonic acid sodium salt (mesna) in 100 mm NaCl, 1 mm EDTA, 50 mm Tris–HCl, pH 8.6, 0.2% (w/v) bovine serum albumin 3 times for 20 min at 4 °C. Subsequently, cells were incubated for 10 min with 120 mM iodoacetic acid in PBS-CM. Finally, cells were washed with PBS-CM and PBS and then lysed in 150 mm NaCl, 5 mm EDTA, 50 mm Tris–HCl, pH 7.5, 1% (v/v) Nonidet P-40, 5 μg/ml leupeptin, 1 μg/ml pepstatin A at 4 °C. The lysate was centrifuged for 10 min at 16,000 × g, and biotinylated proteins were precipitated overnight from the supernatant using Neutravidin-coupled beads (Pierce) and analyzed by immunoblot analyses.

Pulse-Chase Assay—HEK-TRPV5 cells were first incubated in Dulbecco’s modified Eagle’s medium without serum and methionine/cysteine (Invitrogen) for 30 min at 37 °C and then for 60 min in the same medium supplemented with 150 μCi/ml [35S]methionine/cysteine (Tran35S-label). After 3 washing steps in chase medium (Dulbecco’s modified Eagle’s medium supplemented with 3 g/liter methionine and 3 g/liter cysteine, 1 g/liter NaHCO3, 4 mm glutamine, and 10 mm Hepes-Tris, pH 7.4), cells were incubated at 37 °C for the indicated times, washed twice using PBS-CM, and lysed in buffer containing 20 mm Tris, pH 7.4, 5 mm EDTA, 135 mm NaCl, 0.5% (v/v) Nonidet P-40, and 10% (w/v) sucrose, incubated on ice for 60 min, and centrifuged for 30 min at 16,000 × g. TRPV5 was precipitated overnight using guinea pig anti-TRPV5 antibodies and visualized by autoradiography. Protein amounts were quantified using Image J.


**RESULTS**

**Internalization of Cell Surface TRPV5**—Studies into the mechanisms underlying the regulated endocytosis of TRPV5 had been hampered by the difficulty of detecting TRPV5 at the cell surface. Various attempts to create antibodies against regions of TRPV5 that face the extracellular milieu or to create functional TRPV5 channels with epitope tags in these regions were unsuccessful (Fig. 1A and data not shown). Finally, we successfully employed a different strategy where we added an additional transmembrane domain to the amino terminus to create a topology with an extracellular facing amino terminus tagged with HA (HA-TM-TRPV5, Fig. 1A). This construct yielded a functional TRPV5 channel, as small but distinct TRPV5-specific currents were detected in transiently transfected HEK293 cells (data not shown), and significant $^{45}\text{Ca}^{2+}$ uptake (63 ± 3% of untagged TRPV5) was measured in X. laevis oocytes ectopically expressing the channel construct. Furthermore, co-immunoprecipitation experiments demonstrated that HA-TM-fused TRPV5 is also able to form multimers with GFP-TRPV5, as expected from a channel which functions in a tetrameric configuration (18) (Fig. 1B). As a negative control, TRPV2-yellow fluorescent protein (YFP) could not be co-immunoprecipitated with HA-TM-TRPV5, in line with the previously described inability of TRPV2 and TRPV5 to form heteromultimers (21).

To visualize TRPV5 internalization, we incubated live HA-TM-TRPV5-expressing HeLa cells with anti-HA antibodies at 37 °C. Incubation with anti-HA antibodies at 4 °C only yielded a signal at the cell surface (Fig. 2B). Subsequent warming of the cells to 37 °C resulted in almost complete internalization of the antibody already within 5 min. The internalized TRPV5 first (5–10 min) appeared in numerous small vesicles in the periphery of the cell and then was found in a number of larger vesicles. Continuous labeling resulted in the signal in larger vesicles with some small peripheral vesicles remaining. Multiple z-sections of cells of this experiment are given in supplemental Fig. 1 showing similar results in all focal planes. This system is applicable to other cell types as anti-HA uptake was also observed in HA-TM-TRPV5-expressing HEK293 cells, commonly used for heterologous expression of TRPV5 (Fig. 2C) and in HA-TM-TRPV5-expressing Chinese hamster ovary cells (data not shown).

**Internalization of Cell Surface TRPV5 Is Dependent on Clathrin and Dynamin**—Anti-HA antibody uptake was employed to determine the role of dynamin in the endocytosis of TRPV5 using HeLa cells expressing the dominant negative dynamin K44A mutant in a tetracycline-inducible manner (HeLa K44A) (19). The anti-HA signal remained at the cell surface in most of...
TRPV5 is Internalized via Clathrin-dependent Endocytosis

**FIGURE 2.** Internalization of TRPV5 channels visualized using the HA-TM-TRPV5 reporter. A, HeLa cells transfected with HA-TM-TRPV5 were incubated with anti-HA for 1 h at 37 °C. Transfected cells were identified by GFP expression from the bicistronic HA-TM-TRPV5-ires-GFP vector that was employed. Cells displaying HA signal were always GFP-positive. B, time course of TRPV5 internalization. After labeling for 1 h at 4 °C, all the anti-HA signal is at the cell surface. TRPV5 internalization is induced by warming the cells to 37 °C. Internalized TRPV5 is first (5–10 min) present in a large number of small vesicles. TRPV5 internalization is also detected upon continuous labeling with anti-HA at 37 °C for 1 h. C, HA-TM-TRPV5 internalization is observed in transfected HeLa (left) as well as transfected HEK293 (right) cells incubated with anti-HA for 1 h at 37 °C. The bar indicates 10 μm.

To exclude that the dynamin-dependent internalization of TRPV5 was only a consequence of the anti-HA antibody addition, we employed as another model system HEK293 cells stably expressing TRPV5 (without the additional transmembrane domain of HA-TM-TRPV5). These cells show a low expression level of the channel that could still be detected using immunoblot analysis, but the cell surface expression of TRPV5 was too low to be detected by immunocytochemistry. Therefore, to probe for endocytosis of plasma membrane-resident TRPV5, cell surface molecules were labeled with sulfo-NHS-ss-biotin, and cells were then placed at 37 °C to permit constitutive internalization of biotinylated proteins. Biotin remaining at the cell surface was removed using cell-impermeable mesna. Maximum internalization was achieved within 15–30 min (supplemental Fig. 3), although the internalization signal, i.e. mesna-resistant TRPV5, always remained significantly lower than 100% (referred to as the “no mesna” control) even at incubation times up to 2 h. To elucidate the involvement of dynamin in constitutive TRPV5 internalization analyzed by this surface biotinylation assay, we used dynasore, a cell-permeable inhibitor of dynamin (22). The addition of 80 μM dynasore blocked TRPV5 uptake into a mesna-resistant structure, further confirming a role for dynamin in the endocytosis of TRPV5 (Fig. 3C).

We next analyzed the contribution of clathrin-dependent mechanisms to TRPV5 uptake. First, the addition of 450 mM sucrose to the medium (added after the biotinylation) was cho-
TRPV5 Is Internalized via Clathrin-dependent Endocytosis

TRPV5-expressing cells. To this end HeLa cells were transfected with clathrin siRNA and the next day again co-transfected with clathrin siRNA and HA-TM-TRPV5. Immunocytochemistry using anti-clathrin antibodies showed a significant reduction of clathrin heavy chain protein in 40–60% of these cells (Fig. 4D). Furthermore, Tf uptake (20 min, 37 °C) was clearly inhibited in cells showing the clathrin heavy chain depletion (Fig. 4E). Likewise, HA-TM-TRPV5 internalization was inhibited in the clathrin knock-down cells as revealed by the anti-HA signal remaining at the cell surface as opposed to the control cells showing HA-TM-TRPV5 internalization and accumulation of the signal in the perinuclear region (Fig. 4, F and G).

Internalized TRPV5 Is Not Degraded but Targeted to a Recycling Compartment—We next addressed the fate of internalized TRPV5. To avoid effects due to unphysiologically high overexpression, we used our model characterized by a relatively low and stable expression of TRPV5. To determine the stability of surface-expressed TRPV5, biotinylated cells were placed back at 37 °C for 0 or 3 h, and the amount of biotinylated channel protein was determined by immunoblot analysis using anti-TRPV5 antibodies. To test for possible targeting of internalized TRPV5 to the degradation machinery, inhibitors sen as an unspecific tool to disrupt the formation of clathrin-coated vesicles, thereby blocking clathrin-dependent endocytosis (23). This led to a complete block of TRPV5 uptake (Fig. 3C). To test the role of clathrin more directly, we depleted cells of clathrin heavy chain by siRNA transfection and first tested the effect on the internalization of TRPV5 by analyzing the uptake of cell surface-biotinylated channels. Although only partial depletion of clathrin was obtained in this transient approach (clathrin heavy chain expression was decreased by ~60%), TRPV5 internalization was reduced in the clathrin siRNA-transfected cells as compared with control cells (Fig. 4, A and B). TfR internalization in the clathrin siRNA-transfected cells was also partially reduced. That TfR internalization is clathrin-dependent (24) suggests the remaining internalized signal of both TRPV5 and TfR mainly originates from cells in the population showing poor or no depletion of clathrin. Therefore, we also tested the role of clathrin at the single cell level, employing the antibody internalization assay and HA-TM-TRPV5.

FIGURE 4. Clathrin-dependent endocytosis of TRPV5. A, immunoblot analysis showing a reduced clathrin heavy chain expression upon clathrin siRNA transfection of HEK293 cells stably expressing TRPV5. B, biochemical quantification of the amount of (mesna-protected) TRPV5 internalized during 30 min at 37 °C in siRNA-transfected cells. Constitutive endocytosis of TRPV5 was partially inhibited by clathrin down-regulation (upper panel), whereas total cellular expression of TRPV5 was unaffected (lower panel). NT indicates non-transfected cells. Similar results were obtained for TRR, used here as a marker for clathrin dependent internalization. The role of clathrin in TRPV5 endocytosis was further analyzed in a single-cell based assay (D–G). HeLa cells were cotransfected with HA-TM-TRPV5 and control siRNA or siRNA directed against clathrin heavy chain. D, RNA-mediated interference depletion of clathrin was determined by immunocytochemistry using anti-clathrin heavy chain antibodies, showing a strong down-regulation in a subpopulation of cells in the clathrin siRNA transfected population but not in the control siRNA-transfected cells. E, Tf uptake, analyzed as a control, was inhibited in the clathrin siRNA-transfected cells compared with control siRNA transfected cells. F and G, TRPV5 endocytosis was determined by incubation of the transfected cells for 1 h at 37 °C with rabbit anti-HA. F, clear RNA-endocytosis was observed in cells with prominent clathrin expression. G, clathrin siRNA transfection resulted in a significant inhibition of TRPV5 internalization in cells with reduced clathrin levels, with a large fraction of the signal remaining at the cell surface. Depletion of clathrin heavy chain was confirmed by staining using anti-clathrin antibodies. HA-TM-TRPV5-transfected cells were identified by the GFP signal resulting from the independent IRES-mediated translation (left inset). The bar indicates 10 μm.
this hypothesis, we performed colocalization studies of internalized HA-TM-TRPV5 with Rab11 or CD63, markers of a recycling or degradative compartment, respectively. Early after internalization, when HA-TM-TRPV5 localized to numerous small peripheral vesicles, little colocalization was observed between Rab11 and internalized anti-HA (Fig. 5C). However, after incubation of the HA-TM-TRPV5-expressing cells with anti-HA antibodies at 37 °C for 1 h, a significant co-localization of internalized TRPV5 with endogenous Rab11 was observed. Co-localization was most prominent in larger perinuclear structures and less in the small peripheral TRPV5-positive punctae (Fig. 5D). In contrast, no obvious co-localization with CD63 was observed, even after prolonged incubation with anti-HA antibodies, again suggesting a limited targeting of the channel to late endosomes and then to the degradative lysosomal pathway (Fig. 5E).

The stability of (internalized) TRPV5 was also determined on a longer time scale by pulse-chase analysis. Immediately after metabolic labeling using [35S]methionine/cysteine (0 h), the TRPV5 signal was detected in SDS-PAGE mainly at ~110 kDa, most likely reflecting the core-glycosylated state of the channel. A minor background signal at ~70 kDa was also visible that was already seen in the non-transfected cells (Fig. 6A). After 1 h, most of the signal had shifted into the high molecular mass TRPV5 band, which runs at 85–100 kDa (Fig. 6, A and B). This shift in molecular weight likely reflects processing of the glycosylation moiety of TRPV5 in the Golgi compartment. Analysis of the total TRPV5 and only the upper, fully glycosylated-TRPV5 band revealed that TRPV5 was stable for 8 h but showed a large reduction in the signal at 16 h (Fig. 6, B and C). In comparison to the fully glycosylated form, it seems that the core-glycosylated TRPV5 disappears slightly more rapidly.
Recycling of Internalized TRPV5 Is Ca2+–dependent—Using the Ca2+-dye Fura2, we demonstrated that expression of TRPV5 in HEK293 cells resulted in a ~2-fold increase in the resting intracellular Ca2+ concentration ([Ca2+]i) (Fig. 7C). This difference was due to TRPV5-mediated Ca2+ influx as [Ca2+]i, decreased to control levels when extracellular Ca2+ was omitted, and EDTA was added (Fig. 7C). Subsequently, we tested whether the cytosolic Ca2+ concentration affects the endocytic trafficking of TRPV5. However, [Ca2+]i, was not significantly altered when we lowered the extracellular [Ca2+]i to values as low as 10 μM (not shown). Further reduction of the extracellular Ca2+ concentration using 2 mM EGTA resulted in massive cell detachment during the mesna-based internalization assay. Therefore, we tested whether lowering the cytosolic Ca2+ concentration using the Ca2+ chelator BAPTA would affect the endocytic trafficking of TRPV5. The addition of BAPTA (−AM) to cells has been performed in studies on TRPV5 function, as this treatment decreases the Ca2+–dependent channel inactivation. In vivo this Ca2+–buffering function is mediated by calbindins (26, 27). Interestingly, the amount of mesna-protected TRPV5 increased ~4-fold (408 ± 98%) upon treatment of the cells with BAPTA-AM (Fig. 7, A and B). The amount at the cell surface (no mesna condition) at the start of the chase was not significantly affected (100 ± 26 versus 113 ± 29% of control, p = 0.7), and the expression of TRPV5 was similar in all conditions (Fig. 7A, lower panel). In control experiments the effect of Ca2+ chelation on TIR trafficking was analyzed. Preincubation with BAPTA-AM for 30 min before cell surface biotinylation resulted in a similar increase in the amount of internalized biotinylated TIR compared with the effect of BAPTA-AM on the amount of internalized TRPV5.
TRPV5 Shows Constitutive Dynamin- and Clathrin-dependent Internalization and Enters a Recycling Pathway—Several lines of evidence show that TRPV5 is internalized by clathrin-mediated endocytosis. First, using the HA-TM-TRPV5 construct we observed a rapid and constitutive internalization of anti-HA antibodies. The internalization was specific for TRPV5-expressing cells and did not occur upon incubation with nonspecific antibodies, showing that general fluid phase uptake, e.g. by macropinocytosis, could be ruled out as internalization mode. Second, the role of dynamin in the internalization of TRPV5 was demonstrated by blocking TRPV5 endocytosis by overexpression of the dominant-negative dynamin K44A mutant and by chemical inhibition of dynamin using the cell permeable inhibitor dynasore (22). Third, hypertonic medium (450 mM sucrose) and, more specifically, depleting cells of clathrin using clathrin heavy chain siRNA also blocked TRPV5 internalization.

Once internalized, TRPV5 is rather stable and shows virtually no degradation within the first 3 h. To test whether this was due to internalized channels entering the recycling pathway, we subjected cells to BFA treatment for different time points. BFA has been used before to demonstrate internalization-dependent degradation of the epithelial Na⁺ channel and of ROMK, a K⁺ channel expressed in the kidney (25, 30). For both epithelial Na⁺ channel and ROMK, prolonged incubation (3–4 h) with BFA strongly reduced their activity, indicative of a reduced number of channels at the cell surface, and this process was attenuated by inhibition of endocytosis. In contrast, BFA treatment did not affect TRPV5 activity even after 4 h, suggesting that transport of newly synthesized TRPV5 from the Golgi to the plasma membrane does not play a significant role within this time frame. Therefore, an endosomal intracellular pool of TRPV5, which replenishes the constitutively internalized TRPV5 at the cell surface, is likely to exist. In addition, the pulse-chase results indicate an almost complete processing of TRPV5 to the fully-glycosylated form with considerable stability, suggesting that a significant pool of TRPV5 is present in a post-endoplasmic reticulum compartment for a significant amount of time. We postulate that this pool is located in a recycling compartment continuously exchanging TRPV5 with the plasma membrane. This is in line with the prominent colocalization of Rab11a with internalized TRPV5 (Fig. 5) and corroborated by previous data on endogenously expressed TRPV5 in primary renal epithelial cells (15).

Modulation of TRPV5 Recycling by the Intracellular Ca²⁺ Concentration—Previous studies indicated that regulatory pathways controlling the abundance at the cell surface of membrane proteins can target distinct steps in their trafficking. Two recent examples include the cargo-regulated rate of clathrin-coated vesicle formation (31) and cellular signaling-regulated sorting of the internalized material (32, 33). Our findings show constitutive clathrin/dynamin-dependent internalization of TRPV5, rendering it likely that control of this step could enable the cell to accumulate the channel at the cell surface, as observed with klotho or kallikrein treatment. Another mechanism to regulate protein trafficking in the endosomal pathway is modulation of endocytic recycling. Until recently, recycling was considered to be a constitutive, non-regulated process that
Using electropermeabilized cells, Knight (37) previously exchange inactivated cell surface TRPV5 with the active channel. An increase in TRPV5 recycling would enable the cell to rapidly restore individual TRP channels. Furthermore, this knowledge provides a framework to understand and further study the regulatory mechanisms underlying the kinetics of these processes could be tailored to individual TRP channels. Furthermore, this knowledge provides a framework to understand and further study the regulation of TRPV5, which is one of the most Ca\(^{2+}\)-selective members of the TRP family. It is known that the intracellular Ca\(^{2+}\) concentration \([Ca^{2+}]_i\) has evident effects on channel properties of TRPV5, showing rapid inactivation upon increases in \([Ca^{2+}]_i\), as determined by patch-clamp analysis (26). Furthermore, recovery of TRPV5 from a Ca\(^{2+}\)-induced inactivated state is a relatively slow process, with half-maximal recovery reached only after ~96 s (26). This timescale could allow a role of endocytic recycling, where inactivated TRPV5 at the cell surface is replaced with active TRPV5 from the intracellular TRPV5 pool in this recovery process. The effect of \([Ca^{2+}]_i\) on TRPV5 recycling described in this study suggests a physiological mechanism where a Ca\(^{2+}\)-dependent increase in TRPV5 recycling would enable the cell to rapidly exchange inactivated cell surface TRPV5 with the active channel from a recycling compartment to restore Ca\(^{2+}\) influx.

The field of TRP channel trafficking is still relatively unexplored and has focused on exocytic events (39, 40). Until recently, exploration of TRP channel internalization has remained very sparse (41–43). This study presents the elucidation of the internalization pathway utilized to extract TRPV5 from the cell surface and of the subsequent targeting to the recycling machinery. Clathrin-dependent internalization and endocytosis might represent a phenomenon shared among several TRP channels, although the regulatory mechanisms underlying the kinetics of these processes could be tailored to individual TRP channels. Furthermore, this knowledge provides a framework to understand and further study the regulation of TRPV5 abundance at the plasma membrane.

Acknowledgments—We thank Vera Konietzko, Carsten Ludwig (University of Münster, Münster, Germany), and Jack Fransen (Nijmegen Centre for Molecular Life Sciences, Nijmegen, the Netherlands) for assistance with the confocal microscope; Stephanie Thebault and Qing Chang (Nijmegen Centre for Molecular Life Sciences) for performing functional analyses on HA-TM-TRPV5; and Siegfried Waldegger (Philips University of Marburg, Marburg, Germany) for discussion about the construction of HA-TM-TRPV5 and for providing the KCNE1 construct. HeLa cells with inducible dynamin K44A expression were provided by Sandra Schmid (The Scripps Research Institute, La Jolla, CA). Rabbit anti-GFP antibodies were generously provided by Jack Fransen (Nijmegen Centre for Molecular Life Sciences). Dynasore was synthesized by Henry Pelish and generously provided by Thomas Kirchhausen (Harvard Medical School, Boston, MA).

REFERENCES

1. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) Cell 108, 595–598
2. Clapham, D. E. (2003) Nature 426, 517–524
3. Singh, B. B., Lockwich, T. P., Banerjipadhyay, B. C., Liu, X., Bollimuthu, S., Bzator, S. C., Combs, C. D., Des, S., Leyenders, A. G., Sheng, Z. H., Knepper, M. A., Ambudkar, S. V., and Ambudkar, I. S. (2004) Mol. Cell 15, 635–646
4. Bezerides, V. J., Ramsey, I. S., Kotecha, S., Greka, A., and Clapham, D. E. (2004) Nat. Cell Biol. 6, 709–720
5. Cayouette, S., Lussier, M. P., Mathieu, E. L., Bousquet, S. M., and Boulay, G. (2004) J. Biol. Chem. 279, 7241–7246
6. Xu, X. Z., and Sternberg, P. W. (2003) Cell 114, 285–297
7. Morenilla-Palao, C., Planelles-Cases, R., Garcia-Sanz, N., and Ferrer-Montiel, A. (2004) J. Biol. Chem. 279, 25665–25672
8. Zhang, X., Huang, J., and McNaughton, P. A. (2005) EMBO J. 24, 4211–4223
9. Oancea, E., Wolfe, J. T., and Clapham, D. E. (2006) Circ. Res. 98, 245–253
10. Verhees, R., Hoenderop, J. G., Preven, J., Stuiver, M., Willems, P. H., Droogmans, G., Nilius, B., and Bindels, R. J. (2003) J. Biol. Chem. 278, 3963–3969
11. Hoenderop, J. G., van Leeuwen, J. P., van der Eerden, B. C., Kersten, F. F., van der Kemp, A. W., Merillat, A. M., Waarsing, J. H., Rossier, B. C., Vallon, V., Hummler, E., and Bindels, R. J. (2003) J. Clin. Investig. 112, 1906–1914
12. van der Eerden, B. C., Hoenderop, J. G., de Vries, T. J., Schoemaker, T., Buurman, C. J., Uitterlinden, A. G., Pols, H. A., Bindels, R. J., and Van Leeuwen, J. P. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 17507–17512
13. Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2005) Physiol. Rev. 85, 373–422
14. Van der Graaf, S. F., Hoenderop, J. G., Gikka, D., Lamers, D., Preven, J., Rescher, U., Gerke, V., Staub, O., Nilius, B., and Bindels, R. J. (2003) EMBO J. 22, 1478–1487
15. Van der Graaf, S. F., Chang, Q., Mensenkamp, A. R., Hoenderop, J. G., and Bindels, R. J. (2006) Mol. Cell. Biol. 26, 303–312
16. Chang, Q., Hoefs, S., van der Kemp, A. W., Topala, C. N., Bindels, R. J., and Hoenderop, J. G. (2005) Science 310, 490–493
17. Gikka, D., Topala, C. N., Chang, Q., Picard, N., Thebault, S., Houillier, P., Hoenderop, J. G., and Bindels, R. J. (2006) EMBO J. 25, 4707–4716
18. Hoenderop, J. G., Voets, T., Hoefs, S., Weidema, F., Preven, J., Nilius, B., and Bindels, R. J. (2003) EMBO J. 22, 776–785
19. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
20. Den Dekker, E., Schoeber, J., Topala, C. N., van der Graaf, S. F., Hoenderop, J. G., and Bindels, R. J. (2005) Pfluegers Arch. Eur. J. Physiol. 450, 236–244
21. Hellwig, N., Albrecht, N., Harteneck, C., Schultz, G., and Schaefer, M. (2005) J. Cell Sci. 118, 917–928
22. Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006) Dev. Cell 10, 839–850
23. Heuser, J. E., and Anderson, R. G. (1989) J. Cell Biol. 108, 389–400
24. Hinrichsen, L., Harborth, J., Andrees, L., Weber, K., and Ungewickell, E. J. (2003) J. Biol. Chem. 278, 45160–45170
25. Staub, O., Gauthsi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. 16, 6325–6336
26. Nilius, B., Preven, J., Verhees, R., Hoenderop, J. G., Bindels, R. J., and Droogmans, G. (2001) Cell Calcium 29, 417–428
27. Lambers, T. T., Mahieu, F., Oancea, E., Hoofd, L., De Lange, F., Mensenkamp, A. R., Voets, T., Nilius, B., Clapham, D. E., Hoenderop, J. G., and Bindels, R. J. (2006) EMBO J. 25, 2978–2988
28. Basu, S. K., Goldstein, J. L., Anderson, R. G., and Brown, M. S. (1981) Cell 24, 493–502
29. Quon, M. J., Guerre-Millo, M., Zarnowsky, M. J., Butte, A. J., Em, M., Cushman, S. W., and Taylor, S. I. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 5587–5591
30. Zeng, W. Z., Babich, V., Ortega, B., Quigley, R., White, S. J., Welling, P. A., and Huang, C. L. (2002) Am. J. Physiol. Renal Physiol. 283, 630–639
31. Putheveedu, M. A., and von Zastrow, M. (2006) Cell 127, 113–124
32. Bao, J., Alroy, I., Waterman, H., Schejter, E. D., Brodie, C., Gruenberg, J.,
TRPV5 Is Internalized via Clathrin-dependent Endocytosis

and Yarden, Y. (2000) J. Biol. Chem. 275, 26178–26186
33. Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006) Cell 124, 997–1009
34. Koenig, J. A., and Edwardson, J. M. (1997) Trends Pharmacol. Sci. 18, 276–287
35. Le, T. L., Yap, A. S., and Stow, J. L. (1999) J. Cell Biol. 146, 219–232
36. Le, T. L., Joseph, S. R., Yap, A. S., and Stow, J. L. (2002) Am. J. Physiol. Cell Physiol. 283, 489–499
37. Knight, D. E. (2002) Traffic 3, 298–307
38. Sainte-Marie, J., Lafont, V., Pecheur, E. I., Favero, J., Philippot, J. R., and Bienvenue, A. (1997) Eur. J. Biochem. 250, 689–697
39. Ambudkar, I. S. (2007) Handb. Exp. Pharmacol. 179, 541–557
40. Cayouette, S., and Boulay, G. (2007) Cell Calcium 42, 225–232
41. Wegierski, T., Hill, K., Schaefer, M., and Walz, G. (2006) EMBO J. 25, 5659–5669
42. Lockwich, T., Singh, B. B., Liu, X., and Ambudkar, I. S. (2001) J. Biol. Chem. 276, 42401–42408
43. Itagaki, K., Kannan, K. B., Singh, B. B., and Hauser, C. J. (2004) J. Immunol. 172, 601–607