Epithelial Transport

Characterization of a Madin-Darby canine kidney cell line stably expressing TRPV5

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Abstract  To provide a cell model for studying specifically the regulation of Ca²⁺ entry by the epithelial calcium channel transient receptor potential-vanilloid-5 (TRPV5), green fluorescent protein (GFP)-tagged TRPV5 was expressed stably in Madin-Darby canine kidney type I (MDCK) cells. The localization of GFP-TRPV5 in this cell line showed an intracellular granular distribution. Ca²⁺ uptake in GFP-TRPV5-MDCK cells cultured on plastic supports was threefold higher than in non-transfected cells. Moreover, apical Ca²⁺ uptake in GFP-TRPV5-MDCK cells cultured on permeable supports was eightfold higher than basolateral Ca²⁺ uptake, indicating that GFP-TRPV5 is expressed predominantly in the apical membrane. Patch-clamp analysis showed the presence of typical electrophysiological features of GFP-TRPV5, such as inwardly rectifying currents, inhibition by divalent cations and Ca²⁺-dependent inactivation. Moreover, the TRPV5 inhibitor ruthenium red completely inhibited Ca²⁺ uptake in GFP-TRPV5-MDCK cells, whereas Ca²⁺ uptake in non-transfected cells was not inhibited. The characterized GFP-TRPV5-MDCK cell line was used to assess the regulation of TRPV5. The protein kinase C activator phorbol 12-myristate 13-acetate and the cAMP-elevating compounds forskolin/3-isobutyl-1-methylxanthine, 8-Br-cAMP and PGE₂ stimulated TRPV5 activity in GFP-TRPV5-MDCK cells by 121±7, 79±5, 55±4 and 61±7%, respectively. These compounds did not affect Ca²⁺ uptake in non-transfected cells. In conclusion, the GFP-TRPV5-MDCK cell line provides a model to specifically study the regulation of TRPV5 activity.

Keywords  ECaC - Epithelial calcium channel - Ca²⁺ homeostasis - Kidney
Introduction

Active reabsorption of Ca\(^{2+}\) occurs in the distal convoluted tubule (DCT) and the connecting tubule (CNT) of the kidney. Reabsorption by these nephron segments accounts for 10–20% of total renal Ca\(^{2+}\) reabsorption. The transient receptor potential-vanilloid-5 (TRPV5) channel plays a key role in this Ca\(^{2+}\) transport, as knock-out mice lacking this channel experience severe hypercalciuria caused by diminished active Ca\(^{2+}\) reabsorption within the early part of the DCT/CNT [17]. TRPV5 is present in the apical membrane of these tubular cells, where it is constitutively active. To adapt to changes in Ca\(^{2+}\) balance, active renal Ca\(^{2+}\) reabsorption is under control of hormones such as parathyroid hormone (PTH), arginine vasopressin (AVP), prostaglandin E\(_2\) (PGE\(_2\)), calcitonin, 1,25-dihydroxy-vitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] and oestrogen [3, 10, 28–30]. Oestrogen, PTH and 1,25(OH)\(_2\)D\(_3\) regulate TRPV5 at the level of transcription [14, 24, 28]. Besides transcriptional control, short-term control mechanisms may also influence Ca\(^{2+}\) reabsorption.

As it is difficult to investigate transcellular Ca\(^{2+}\) transport in vivo, most studies have been performed in isolated perfused tubules or in cell cultures. For instance, Ca\(^{2+}\) transport by primary cultures of the rabbit DCT/CNT is activated by apical adenosine and basolateral PGE\(_2\) and AVP [13, 29, 31]. In isolated perfused tubules PTH also has a non-transcriptional stimulatory effect on Ca\(^{2+}\) reabsorption and a role for cAMP in this effect has been postulated [10]. To provide an in vitro cell model for studying Ca\(^{2+}\) transport, we have recently described a murine renal DCT cell line (mpkDCT) that approaches the endogenous physiological state of DCT in mice. Consistent with findings in other studies, Ca\(^{2+}\) transport by mpkDCT cells is stimulated by cAMP-elevating compounds and by deamino-Cys\(^\text{1}\) -d-Arg\(^\text{8}\) -vasopressin (dDAVP) [7]. However, as Ca\(^{2+}\) reabsorption comprises a sequence of processes involving apical Ca\(^{2+}\) entry via TRPV5 [15], translocation of Ca\(^{2+}\) through the cytosol by calbindins [9, 27] and extrusion over the basolateral membrane by the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and plasma membrane Ca\(^{2+}\) ATPase (PMCA) [15, 25], a general effect on Ca\(^{2+}\) transport does not provide detailed mechanistic information about which individual processes are affected.

To gain insight into the regulation of apical Ca\(^{2+}\) entry through TRPV5, a Madin-Darby canine kidney (MDCK) cell line was generated that expresses green fluorescent protein (GFP)-tagged TRPV5 stably. The generation and characterization of this cell line are described in this study and the signalling pathways affecting TRPV5-mediated Ca\(^{2+}\) entry addressed using this cell system.

Materials and methods

Constructs

The open reading frame from rabbit TRPV5 (GENBANK sequence number AJ133128) was sub-cloned as a PvuII-BamHI fragment into the PCINeo/IRE-GFP vector as described previously [32]. To generate a N-terminal GFP-tagged TRPV5 construct, rabbit TRPV5 was cloned into the p-EGFP-C1 vector (Clontech, Palo Alto, Calif., USA) by PCR (forwards primer 5'-
TCCGGACGGGGGGGATGGGGCCCTGTCCTGACCC-3′; reverse primer 5′-CCGGTGATCCCTGATCAG-3′)

**Transient transfection of HEK293 cells with TRPV5 and GFP-TRPV5 and Western blot analysis**

To compare the electrophysiological properties of untagged and N-terminus-GFP-tagged TRPV5, both proteins were expressed heterologously in human embryonic kidney (HEK293) cells. HEK293 cells were grown in DMEM (Bio Whittaker Europe, Vervier, Belgium) containing 10% (v/v) fetal calf serum (PAA, Linz, Austria), 13 mM NaHCO₃, 2 mM L-glutamine and 10 μg/ml ciproxin (Bayer, Mijdrecht, The Netherlands) at 37°C in a humidity-controlled incubator with 5% CO₂. The cells were transfected transiently with TRPV5-pCINeo/IRES-GFP or eGFP-TRPV5 using Lipofectamin 2000 (Invitrogen Life Technologies, Breda, The Netherlands) according to the manufacturers’ instructions. After 24–48 h, cells (0.1 cm² confluent) were treated with Laemmli sample buffer and subjected to SDS-PAGE and Western blotting. TRPV5 was detected with guinea-pig anti-TRPV5 [16] and peroxidase-coupled goat-anti-guinea-pig (Sigma, St. Louis, Mo., USA). For electrophysiological analysis, transfected cells were identified visually by their green appearance, as described previously [32].

**Electrophysiology**

Electrophysiological methods for measuring Na⁺ and Ca²⁺ currents carried by TRPV5 have been described in detail previously [32]. Patch-clamp experiments were performed on single cells in the whole-cell configuration (24–48 h after transfection for HEK293 cells transiently expressing TRPV5 and 2 h after seeding for GFP-TRPV5-MDCK cells), using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). Patch pipettes had DC resistances of 2–4 MΩ when filled with intracellular solution. A ramp protocol, consisting of linear voltage ramps from +100 mV to –100 mV within 450 ms, was applied every 2 s from a holding potential of +20 mV. Current densities, expressed per unit membrane capacitance, were calculated from the current at –80 and +90 mV during ramp protocols. The internal (pipette) solution contained (mM): 20 CsCl, 100 Cs-aspartate, 1 MgCl₂, 10 BAPTA, 4 Na₂ATP and 10 HEPES/CsOH, pH 7.2. The external solution contained (mM): 150 NaCl, 6 CsCl, 1 MgCl₂, 10 HEPES/NaOH (pH 7.4) and 10 glucose. To inhibit monovalent cation currents, 150 mM NaCl was replaced with an equimolar amount of N-methyl-D-glucamine-Cl (NMDG-Cl). Permeation of Ca²⁺ was measured in divalent (ion)-free solution (DFV) consisting of the NMDG-Cl-containing external solution with 10 mM Ca²⁺ but without the addition of other divalent cations. To completely abolish the effect of divalent cations on Ca²⁺ permeation, a buffer was used with the same composition as DFV but containing 100 μM EDTA to remove trace amounts of divalent cations. All experiments were performed at room temperature.

**Generation of a MDCK cell line stably expressing GFP-TRPV5**

MDCK type-I cells [26] were grown in DMEM containing 5% (v/v) fetal calf serum (HyClone, Logan, Utah, USA), 13 mM NaHCO₃, 2 mM L-glutamine and 10 μg/ml ciproxin (Bayer) at 37°C in a humidity-controlled incubator with 5% CO₂. For stable transfection of MDCK cells, 25 μg circular DNA was transfected using the calcium phosphate precipitation technique [5]. At 24 h after
transfection, the cells were trypsinized, divided over Petri dishes and cultured in medium containing 800 μg/ml G418 (Life Technologies Europe). Between 10 and 14 days after transfection, individual colonies were selected by means of cloning rings and expanded.

**Western blot detection of GFP-TRPV5 from GFP-TRPV5-MDCK cells**

GFP-TRPV5-MDCK cells and non-transfected MDCK cells were washed twice with PBS and subsequently lysed for 1 h on ice in lysis buffer containing (mM): 135 NaCl, 20 TRIS (pH 7.4), 5 EDTA, 0.5% NP-40 and 0.1% Triton X-100. The lysate was centrifuged at 16,000 g for 1 h at 4°C to pellet undissolved proteins and treated with Laemmli buffer for 30 min at 37°C. A lysate volume equivalent to 0.2 cm² cells was subjected to SDS-PAGE and Western blotting. GFP-TRPV5 was detected with rabbit-anti-GFP [4] and peroxidase-coupled goat-anti-rabbit (Sigma).

**GFP-TRPV5 localization by confocal laser scanning microscopy**

GFP-TRPV5-MDCK cells were seeded at a density of 1.5×10⁵ cells/cm² on 0.33 cm² polycarbonate filters (Costar, Corning Europe, Badhoevedorp, The Netherlands) and grown to confluence for 3 days. Cells were washed twice with ice-cold PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ and were fixed in PBS containing 3% (w/v) paraformaldehyde for 30 min on ice. After washing twice with PBS, filters were cut out and mounted on glass slides with Vectashield (Vector Labs, Burlingame, Calif., USA). Images were obtained with a Bio-Rad confocal laser scanning microscope (MRC-1024, Bio-Rad, Richmond, Calif., USA) using a 60× oil-immersion objective.

**⁴⁵Ca²⁺ uptake**

Cells were seeded in 24-well plates at a density of 3×10⁵ cells/cm² and cultured to confluency for 2 days. Cells were washed twice with Krebs-Henseleit buffer (KHB) containing (mM) 110 NaCl, 5 KCl, 1.2 MgCl₂, 20 HEPES, 10 sodium acetate, 2 NaH₂PO₄, 4 L-lactate, 10 D-glucose and 1 L-alanine, adjusted to pH 7.4 using 1 M TRIS. After incubating for 1 h in KHB, this buffer was removed and replaced by KHB with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma), 10 μM forskolin (Sigma) and 100 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 100 μM 8-Br-cAMP (Sigma), 100 nM PGE₂ (Sigma) or 10 μM ruthenium red (Fluka, St. Louis, Mo., USA).

Cells were preincubated for 15 min, after which the preincubation buffer was exchanged for ⁴⁵Ca²⁺ uptake buffer, which consisted of KHB supplemented with (mM) 0.1 CaCl₂, 2 NaH₂PO₄, 10 μM felodipine, 10 μM verapamil, 1 BaCl₂ and 1 μCi ⁴⁵CaCl₂/ml. After incubating for 15 min, cells were washed 3 times with ice-cold stop buffer consisting of KHB without NaH₂PO₄ supplemented with 0.5 mM CaCl₂ and 1.5 mM LaCl₃. Subsequently, cells were lysed in 0.1% (w/v) SDS and radioactivity of the lysate was measured using a liquid scintillation counter.

**Apical and basolateral ⁴⁵Ca²⁺ uptake in GFP-TRPV5-MDCK cells**
Cells were seeded on Transwell permeable supports (Corning, N.Y., USA) at a density of $3 \times 10^5$ cells/cm$^2$. Confluent monolayers were obtained 3 days post seeding. Transepithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter (Millicell-D, Millipore, Denmark). Each cell monolayer exhibited a TEER $>1,330 \ \Omega \cdot \text{cm}^2$ prior to use in $^{45}\text{Ca}^{2+}$ uptake experiments. The apical and basolateral side of the filter was rinsed twice with KHB buffer. After 1 h incubation in KHB buffer this was exchanged for $^{45}\text{Ca}^{2+}$-uptake buffer, either at the apical or the basolateral side. After incubating for 15 min, cells were washed 3 times with ice-cold stop buffer consisting of KHB without NaH$_2$PO$_4$ supplemented with 0.5 mM CaCl$_2$ and 1.5 mM LaCl$_3$. Subsequently, cells were lysed in 0.1% (w/v) SDS and radioactivity of the lysate was measured using a liquid scintillation counter.

**Statistics**

Data are reported as means±SE. The significance of differences between means was determined using Student’s $t$-test. $P<0.05$ was considered significant.

**Results**

**Western blot and electrophysiological analysis of GFP-TRPV5 heterologously expressed in HEK293 cells**

The aim of the study was to generate a cell line that expresses TRPV5 stably in order to study the regulation of this channel. Expression of a GFP-tagged protein is preferred as it facilitates localization studies. The first question that must be addressed is whether the GFP tag influences TRPV5 channel properties. Untagged TRPV5 and N-terminus-tagged GFP-TRPV5 were expressed heterologously in HEK293 cells. Western blot analysis showed a major 60-kDa band for untagged TRPV5 (Fig. 1a) and a major 90-kDa band for GFP-TRPV5 (Fig. 1b). An additional band with a higher molecular weight was also observed for both proteins, possibly representing a post-translationally modified form. The current/voltage ($I/V$) curves in Fig. 1 show inwardly rectifying currents through TRPV5 and GFP-TRPV5. In a buffer containing Na$^+$ without divalent ions (DVF and EDTA) inwardly rectifying currents were only carried by sodium. These currents could be blocked by replacing Na$^+$ with NMDG$^+$ (NMDG). The currents carried by 10 mM Ca$^{2+}$ are much smaller than those carried by 150 mM Na$^+$. TRPV5 and GFP-TRPV5 conducted Na$^+$ and Ca$^{2+}$ currents with a similar amplitude. These results indicate that the GFP tag does not influence the electrophysiological properties of TRPV5.
Fig. 1a,b Western blot and electrophysiological analysis of the transient receptor potential-vanilloid-5 (TRPV5) channel and the green fluorescent protein-tagged TRPV5 (GFP-TRPV5). TRPV5 and GFP-TRPV5 were expressed heterologously in HEK293 cells. Western blot analysis was performed for non-transfected cells (NT), TRPV5- (a, upper panel) and GFP-TRPV5-transfected cells (b, upper panel). Electrophysiological properties of TRPV5 (a, lower panel) and GFP-TRPV5 (b, lower panel) were determined by patch-clamp analysis. Ionic currents (I) were determined during voltage (V) ramps in the absence of divalent cations [divalent ion-free (DVF) and EDTA], in the presence of N-methyl-D-glucamine (NMDG) and in the presence of 10 mM Ca^{2+} (Ca)

Generation of a MDCK cell line stably expressing TRPV5

To generate a polarized kidney epithelial cell line that expresses TRPV5 stably, MDCK-I cells were transfected with GFP-TRPV5. The GFP tag allowed direct screening of TRPV5-positive clones on basis of GFP fluorescence. A clone, all cells of which were GFP-positive, was selected for the experiments described in this study and named GFP-TRPV5-MDCK. To assess the localization of GFP-TRPV5, cells were grown to confluency on permeable filter supports, fixed and examined using confocal laser scanning microscopy. As shown in Fig. 2a, TRPV5 was present mainly in intracellular granular structures. A similar localization pattern was also observed in the partially GFP-TRPV5-positive clones (not shown). In an attempt to identify physiological triggers that induce trafficking of TRPV5 to the plasma membrane, filters were incubated (apically) for 10 and 45 min with 8-Br-cAMP (100 μM), 8-Br-cGMP (100 μM), forskolin (10 μM), PMA (100 nM) and the vasopressin-2 (V2) receptor agonist dDAVP (10 nM, basolateral) and the localization of TRPV5 studied by confocal laser scanning microscopy. Treatment with these compounds did not
induce a change in localization of GFP-TRPV5 (not shown). Western blot analysis of GFP-TRPV5-MDCK cells showed a major protein band at 90 kDa, which corresponds with the expected size of the tagged channel (60 kDa TRPV5 plus 26 kDa GFP) (Fig. 2b). Besides the 90-kDa protein an additional band with higher molecular weight was detected. This band might represent the complex glycosylated form of TRPV5 that has been reported previously [18].

![Fig. 2a,b](http://www.springerlink.com/media/4NC6A0QUTG2UUXXUET/Contributions/R/1/4/G/R14G7R870T6T048V_html/fulltext.html)

**Fig. 2a,b** Localization and Western blot analysis of TRPV5 in GFP-TRPV5-MDCK cells. A MDCK cell line stably expressing GFP-TRPV5 was generated. **a** GFP-TRPV5 localization in cells cultured on filters was determined by confocal laser scanning microscopy. **b** Expression of GFP-TRPV5 was analysed by Western blotting. Non-transfected MDCK cells (NT) served as negative control (x, y, z axes).

### Increased Ca\(^{2+}\) uptake in GFP-TRPV5-MDCK cells

To determine the presence of functional TRPV5 channels in the apical membrane, GFP-TRPV5-MDCK cells and non-transfected MDCK cells were cultured to confluency in 24-well plates. Ca\(^{2+}\) uptake, calculated as picomoles/10\(^6\) cells, was determined at different times as described in Materials and methods. To prevent non-specific uptake through endogenous voltage-operated Ca\(^{2+}\) channels, the \(^{45}\)Ca\(^{2+}\) uptake buffer contained the inhibitors felodipine (10 \(\mu\)M) and verapamil (10 \(\mu\)M). BaCl\(_2\) (1 mM) was added to block potassium channels and so prevent changes in membrane potential. Figure 3 shows that Ca\(^{2+}\) uptake at 5 min was 4.4±0.2-fold higher in GFP-TRPV5-MDCK cells than in non-transfected cells. This difference persisted for the duration of the observation period and represents TRPV5 activity. These data point to the presence of functional TRPV5 channels in the apical membrane of GFP-TRPV5-MDCK cells.
Ca\textsuperscript{2+} is taken up via apical, ruthenium red-sensitive channels in GFP-TRPV5-MDCK cells

Previous studies have shown that ruthenium red inhibits TRPV5 activity with an IC\textsubscript{50} of 121±13 nM [23]. Ruthenium red sensitivity of Ca\textsuperscript{2+} currents might, therefore, indicate the presence of TRPV5 activity. Ca\textsuperscript{2+} uptake in GFP-TRPV5-MDCK was 356±17% higher than in non-transfected cells (Fig. 4a). Ruthenium red (10 \(\mu\)M) did not affect Ca\textsuperscript{2+} uptake in non-transfected MDCK cells, whereas it decreased Ca\textsuperscript{2+} uptake in GFP-TRPV5-MDCK cells to the level observed in non-transfected cells.

![Graph showing Ca\textsuperscript{2+} uptake](http://www.springerlink.com/media/4NC6A0QUTG2UUXUEET/Contributions/R/1/4/G/R14G7R870T6T048V_html/fulltext.html)
ruthenium red was 21±1 pmol/10⁶ cells and was regarded as 100%. The uptake by non-transfected MDCK cells (closed bars) and GFP-TRPV5-MDCK cells (open bars) is referred to this value. Means±SE (n=3). *P<0.05 vs. control. b GFP-TRPV5-MDCK cells were seeded on permeable supports and cultured for 3 days to form tight layers of polarized cells [trascathelial electrical resistance (TER) >1,330 Ωcm²]. After incubation for 15 min with ⁴⁵Ca²⁺ buffer either at the apical membrane or at the basolateral membrane, the supports were washed and Ca²⁺ uptake/10⁶ cells determined. Means±SE (n=8). *P<0.05 vs. apical Ca²⁺ uptake.

To determine whether Ca²⁺ uptake in GFP-TRPV5-MDCK cells is mediated via channels in the apical or the basolateral membrane, cells were cultured on permeable supports until confluent monolayers were obtained with a TEER of >1,330 Ω cm², a value comparable with the TEER of non-transfected MDCK monolayers [26]. Cells were then exposed to an apical or basolateral buffer containing ⁴⁵Ca²⁺. After 15 min cells were washed and lysed and the uptake of ⁴⁵Ca²⁺ determined. Ca²⁺ uptake via the apical membrane was 8.0±1.9-fold higher than that via the basolateral membrane (Fig. 4b) and was inhibited by ruthenium red (not shown). These data indicate that the increased Ca²⁺ uptake in GFP-TRPV5-MDCK cells is mediated predominantly via ruthenium red-sensitive Ca²⁺ channels in the apical membrane.

**TRPV5 characteristics of ion currents in GFP-TRPV5-MDCK**

Several electrophysiological properties have been identified that are characteristic of TRPV5. The presence of these typical properties in GFP-TRPV5-MDCK cells was studied. The I/V diagram in Fig. 5a shows the inwardly rectifying TRPV5 current. In the presence of EDTA and in DVF solution, currents were carried by Na⁺ and were abolished by replacing Na⁺ with NMDG⁺. Ca²⁺ currents in the presence of 10 mM Ca²⁺ were much smaller than monovalent currents.

![Graph](image)

**Fig. 5a,b** Electrophysiological properties of GFP-TRPV5-MDCK cells. a The I/V relationships were determined in response to a voltage ramp in the absence of divalent cations (DVF and EDTA), in the presence of NMDG and in the presence of 10 mM Ca²⁺. b Development of the inwards current at –80 mV during ramps as described in Materials and methods after applying different solutions.

Increased cytosolic Ca²⁺ levels are known to inactivate TRPV5 channels. Inactivation by Ca²⁺ and recovery from inactivation was further studied by measuring the activation time course of the
inwards currents at –80 mV during a ramp protocol similar to that used in the experiments shown in Fig. 5a. Control currents were measured in DVF and EDTA solutions (Fig. 5b). Currents were completely absent when Na\(^+\) was replaced by NMDG\(^+\). Addition of 10 mM Ca\(^2+\) resulted in a peak current that declined in subsequent ramps. Replacement of the solution containing NMDG and Ca\(^2+\) by DVF resulted in a slow but full recovery to the control level. These experiments show clearly that the characteristic TRPV5 properties, such as inwardly rectifying currents, large monovalent cation currents in the absence of divalent cations and channel inactivation by Ca\(^2+\), are present in the GFP-TRPV5-MDCK cell line.

**Regulation of TRPV5-mediated Ca\(^2+\) uptake**

The data presented thus far show that GFP-TRPV5-MDCK cells express functional TRPV5 channels. This cell line can now be used as a model for unravelling the regulation of TRPV5-mediated Ca\(^2+\) entry. PKC and cAMP are thought to be potential signalling molecules involved in the regulation of TRPV5 activity [10, 12, 13]. We therefore examined the effects of the PKC activator PMA (100 nM), the adenylyl cyclase activator forskolin (10 \(\mu\)M) in combination with the phosphodiesterase inhibitor IBMX (100 \(\mu\)M), 8-Br-cAMP (100 M) and the stimulatory G protein (G\(s\))-activating prostaglandin PGE\(_2\) (100 nM) on Ca\(^2+\) uptake in the GFP-TRPV5-MDCK cells (Fig. 6). A 15-min preincubation with PMA increased Ca\(^2+\) uptake by 121±7\% \((P<0.05)\) compared with untreated GFP-TRPV5-MDCK cells. Preincubation with forskolin/IBMX increased Ca\(^2+\) uptake by 79±5\% \((P<0.05)\). A similar stimulation was achieved with 8-Br-cAMP and PGE\(_2\), which increased Ca\(^2+\) uptake by 55±4\% and 61±7\%, \((P<0.05)\), respectively. None of the tested compounds affected Ca\(^2+\) uptake in non-transfected cells. From these experiments, it can be concluded that PKC-activating and cAMP-elevating agents specifically activate TRPV5-mediated Ca\(^2+\) uptake in GFP-TRPV5-MDCK cells.
Fig. 6 Effect of PMA and cAMP-elevating compounds on $\text{Ca}^{2+}$ uptake in GFP-TRPV5-MDCK cells and non-transfected MDCK cells. Cells were preincubated with PMA (100 nM), forskolin/IBMX (10 $\mu$M/100 $\mu$M), 8-Br-cAMP (100 $\mu$M) or PGE$_2$ (100 nM) for 15 min, followed by incubation with $\text{Ca}^{2+}$ for 15 min. $\text{Ca}^{2+}$ uptake in non-transfected cells (open bars) and GFP-TRPV5-MDCK cells (closed bars) was measured. $\text{Ca}^{2+}$ uptake in non-stimulated non-transfected cells (17±1 pmol/10$^6$ cells) was regarded as 100% and all values are referred to this. Means±SE ($n$=4). *$P<0.05$ vs. control

Discussion

In this study, we describe the generation and characterization of an MDCK cell line that stably expresses GFP-TRPV5. Western blot analysis showed a major 90-kDa protein and an additional band with higher molecular weight that probably represents the complex glycosylated form of TRPV5 [18]. Functional TRPV5 channels are expressed in the plasma membrane as $\text{Ca}^{2+}$ uptake in GFP-TRPV5-MDCK cells was increased significantly compared with parental non-transfected MDCK cells, and TRPV5-specific electrophysiological properties could be demonstrated in GFP-TRPV5-MDCK cells. Moreover, ruthenium red inhibited this increased $\text{Ca}^{2+}$ uptake completely, whereas it had no effect on non-transfected cells. Exposure of GFP-TRPV5-MDCK cells to apically or basolaterally added $^{45}\text{Ca}^{2+}$ showed substantial $\text{Ca}^{2+}$ uptake across the apical membrane only. This points to a primary apical localization of $\text{Ca}^{2+}$ channels, which is consistent with the physiological localization of this channel in the apical membrane of DCT cells.

The intracellular localization pattern of TRPV5 in GFP-TRPV5-MDCK cells contrasts with the predominantly apical localization found in DCT2, but closely resembles the localization of TRPV5 at the end of CNT, where exclusively cytoplasmic staining is observed [19]. Intracellular localization is not restricted particularly to $\text{Ca}^{2+}$ channels, since the epithelial Na$^+$ channel is also localized mainly intracellularly, with only 1% of the channels present in the apical membrane [11]. It would be interesting to identify the physiological triggers that can direct a large portion of intracellular TRPV5 to the plasma membrane. This may provide more insight into membrane trafficking mechanisms. However, the compounds tested in this study (8-Br-cAMP, 8-Br-cGMP, forskolin, PMA, dDAVP) did not affect TRPV5 localization visibly (data not shown). This indicates that either shuttling of channels to the membrane is not induced by these compounds, or that the subset of channels routed to the membrane is too small to detect using confocal laser scanning microscopy. Alternatively, trafficking of TRPV5 channels to the plasma membrane might be sub-optimal due to the absence of certain regulatory factors in MDCK cells such as serum and glucocorticoid-inducible kinase-1 (SGK1) and Na/H exchanger-regulating factor-2 (NHERF2) [8]. However, localization studies in primary rabbit DCT/CNT cells have shown also that a large fraction of endogenous TRPV5 channels is localized intracellularly (unpublished data).

$\text{Ca}^{2+}$ reabsorption has been shown to be stimulated by PTH, AVP, PGE$_2$, calcitonin, 1,25(OH)$_2$D$_3$ and oestrogen [3, 10, 28, 29, 30]. While 1,25(OH)$_2$D$_3$, PTH and oestrogen stimulate $\text{Ca}^{2+}$ reabsorption via an increase of TRPV5 transcription [14, 24, 28] the other compounds may activate short-term stimulatory pathways. For example, $\text{Ca}^{2+}$ reabsorption by rabbit DCT/CNT primary cultures is activated by short incubations with apical adenosine, PGE$_2$ and AVP [13, 29, 31]. As these compounds raise cAMP via G$\beta$-coupled receptors, and the stimulatory effect can be mimicked by membrane-permeable cAMP analogues and forskolin in isolated perfused nephron tubules [10].
a role for cAMP in stimulation of Ca\(^{2+}\) reabsorption has been suggested. However, other studies have suggested a major role for (atypical) PKC, as the PKC inhibitor chelerythrine inhibits stimulatory effects of several cAMP-elevating hormones [10, 12].

The mechanisms regulating renal Ca\(^{2+}\) reabsorption have been studied extensively. Transepithelial Ca\(^{2+}\) transport can be divided into three processes: Ca\(^{2+}\) entry at the apical side, intracellular Ca\(^{2+}\) translocation and extrusion at the basolateral side. Effects on transepithelial Ca\(^{2+}\) transport thus do not provide detailed information on the specific process influenced. Unravelling the regulatory mechanisms of TRPV5 has long been hampered by the lack of a cell model. Recently, we described a mpkDCT cell line that approaches the endogenous physiological state of DCT. In this cell line, forskolin stimulates ruthenium-red sensitive Ca\(^{2+}\) transport from the apical to basolateral side whereas PMA has no effect [7]. However, as mpkDCT cells express both TRPV5 and TRPV6, it is not possible to distinguish between stimulatory effects of forskolin on the modulation of TRPV5 or TRPV6 activity. To study the short-term modulation of TRPV5 activity specifically, we developed the GFP-TRPV5-MDCK cell line described in this study. The effect of the TRPV5-inhibiting compound ruthenium red on Ca\(^{2+}\) uptake was investigated [23]. Ca\(^{2+}\) uptake in GFP-TRPV5-MDCK cells was ruthenium red sensitive, whereas Ca\(^{2+}\) uptake in non-transfected MDCK cells was not affected by this compound. This shows that TRPV5-mediated Ca\(^{2+}\) entry can be studied specifically in the GFP-TRPV5-MDCK cell line with non-transfected cells as a negative control.

Using this cell model, we showed that Ca\(^{2+}\) uptake in GFP-TRPV5-MDCK cells is stimulated by cAMP-elevating compounds such as forskolin/IBMX, 8-Br-cAMP and PGE\(_2\). As these compounds did not affect basal Ca\(^{2+}\) uptake of non-transfected cells, this points to a specific stimulatory effect on TRPV5-mediated Ca\(^{2+}\) uptake. These results are consistent with findings of previous studies. However, whether this effect is mediated by atypical PKC isoforms, as previously found in rabbit primary DCT/CNT cultures [12], remains to be determined. A mechanism involving an atypical PKC isoform might be present in the GFP-TRPV5-MDCK cell line, as MDCK cells express PKC\(\zeta\) [1]. TRPV5 contains one putative PKA phosphorylation site which could be involved in a direct PKA-mediated stimulation [6]. However, as this site is not species-conserved a modulatory action of PKA via channel phosphorylation is unlikely. It has been shown that cAMP-elevating agents that induce PKA activity in renal Ca\(^{2+}\)-transporting cells also activate PKC. Moreover, their stimulatory effect on Ca\(^{2+}\) transport could be inhibited with the PKC inhibitor chelerythrine, suggesting that PKC mediates the stimulatory effect [12].

The finding that the PKC activator PMA stimulated TRPV5 activity twofold is in line with these results. However, in primary cultures of rabbit DCT/CNT phorbol ester-insensitive PKC isoforms appeared to be involved in stimulation of Ca\(^{2+}\) transport whereas phorbol-ester sensitive isoforms inhibited Ca\(^{2+}\) transport [2, 12]. The differences in PKC-mediated effects might be due to stimulation of PMCA or NCX by PKC [20, 34], which may enhance Ca\(^{2+}\) transport in primary cultures, but are not involved in TRPV5-mediated Ca\(^{2+}\) uptake in GFP-TRPV5-MDCK cells. Moreover, the latter studies were conducted in rabbit DCT/CNT primary cultures, which not only express TRPV5 but also TRPV6 [19, 22]. In this respect, it would be interesting to determine whether TRPV5 and TRPV6 are regulated differently. The stimulatory effect of PMA on TRPV5 activity in the GFP-TRPV5-MDCK cells might involve direct phosphorylation by PKC, as TRPV5 possesses three conserved putative PKC phosphorylation sites in both the N- and C-termini [6]. Alternatively, a co-factor could be phosphorylated that subsequently exerts its effect on TRPV5.
Taken together, the GFP-TRPV5-MDCK cell line developed and characterized in this study provides a new cell model for studying TRPV5 regulation specifically. Using this cell line, we showed that cAMP elevation and PKC activation stimulate TRPV5-mediated Ca\(^{2+}\) entry across the apical membrane. These findings will enable further studies with the aim of unravelling the mechanisms of TRPV5 activation.

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