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Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5

Dimitra Gkika[1,3], Catalin N Topala[1,3], Qing Chang[1], Nicolas Picard[2], Stéphanie Thébault[1], Pascal Houillier[2], Joost GJ Hoenderop[1] and René JM Bindels[1,*]

[1]Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands and [2]INSERM, Unité 652 Institut Fédératif de Recherche 58 and René Descartes University Paris, Paris, France

The transient receptor potential vanilloid 5 (TRPV5) channel determines urinary Ca^{2+} excretion, and is therefore critical for Ca^{2+} homeostasis. Interestingly, mice lacking the serine protease tissue kallikrein (TK) exhibit robust hypercalciuria comparable to the Ca^{2+} leak in TRPV5 knockout mice. Here, we delineated the molecular mechanism through which TK stimulates Ca^{2+} reabsorption. Using TRPV5-expressing primary cultures of renal Ca^{2+}-transporting epithelial cells, we showed that TK activates Ca^{2+} reabsorption. The stimulatory effect of TK was mimicked by bradykinin (BK) and could be reversed by application of JE049, a BK receptor type 2 antagonist. A cell permeable analog of DAG increased TRPV5 activity within 30 min via protein kinase C activation of the channel since mutation of TRPV5 at the putative PKC phosphorylation sites S299 and S654 prevented the stimulatory effect of TK. Cell surface labeling revealed that TK enhances the amount of wild-type TRPV5 channels, but not of the TRPV5 S299A and S654A mutants, at the plasma membrane by delaying its retrieval. In conclusion, TK stimulates Ca^{2+} reabsorption via the BK-activated PLC/DAG/PKC pathway and the subsequent stabilization of the TRPV5 channel at the plasma membrane.

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Introduction

The overall Ca^{2+} balance is regulated by a homeostatic mechanism tightly controlling the concerted actions of intestinal Ca^{2+} absorption, exchange of Ca^{2+} from bone and renal Ca^{2+} reabsorption. In kidney, Ca^{2+} can cross the epithelial cells and reach the blood compartment via two pathways: passive (paracellular) and active (transcellular) Ca^{2+} reabsorption. Active Ca^{2+} reabsorption takes place in the distal convoluted (DCT) and the connecting (CNT) tubules (Hoenderop *et al*, 2005). Although it accounts only for ~15% of total renal Ca^{2+} reabsorption, it is generally considered the site for fine-tuning of urinary Ca^{2+} excretion. Furthermore, active Ca^{2+} reabsorption is the primary target for regulation by calciotropic hormones, including 1,25-dihydroxyvitamin D_3 and parathyroid hormone (PTH), enabling the organism to regulate Ca^{2+} reabsorption to the body's demand.

The transient receptor potential vanilloid 5 (TRPV5) channel is expressed along the apical membrane of DCT and CNT and represents the rate-limiting step in renal transcellular Ca^{2+} reabsorption (Hoenderop *et al*, 2005). Inactivation of TRPV5 in mice ($TRPV5^{-/-}$) abolishes active Ca^{2+} reabsorption in kidney resulting in severe hypercalciuria (Hoenderop *et al*, 2003). To compensate this renal Ca^{2+} leak, $TRPV5^{-/-}$ mice exhibit intestinal Ca^{2+} hyperabsorption. In addition, the bone structure of these mice is significantly disturbed as illustrated by a reduced trabecular and cortical bone thickness (Hoenderop *et al*, 2003). Hence, these data demonstrate the key function of TRPV5 in active Ca^{2+} reabsorption and its essential role in the body Ca^{2+} homeostasis.

Interestingly, hypercalciuria was recently observed in tissue kallikrein-deficient ($TK^{-/-}$) mice (Picard *et al*, 2005). TK is a serine protease produced in CNT (Figueroa *et al*, 1988), where it co-localizes with TRPV5 (Hoenderop *et al*, 2003). Proteolytic enzymes such as TK are synthesized as inactive precursors or zymogens, to prevent protein degradation and to enable spatial and temporal regulation of enzymatic activity. The precursor of TK is converted to the mature active form before entering the luminal tubular compartment. Trypsin, plasma kallikrein, plasmin and thermolysin can cleave *in vitro* the TK precursor, but the endogenous activator is still unknown (Margolius, 1995). Once activated, TK is excreted and can process low molecular weight kininogen to release kinin, which acts through kinin receptors such as the bradykinin (BK) 2 receptor (B2R) (Bhoola *et al*, 1992). Remarkably, recent studies show that TK can directly activate the B2R independently of BK release (Hecquet *et al*, 2000). However, the molecular events that link TK to Ca^{2+} balance are at present unknown.

The aim of this study was, therefore, to elucidate the molecular mechanism of hypercalciuria observed in $TK^{-/-}$ mice. To this end, the relation between TK expression and hypercalciuria was investigated *in vivo* using $TK^{-/-}$ and $TRPV5^{-/-}$ mice. Subsequently, the effect of TK on transcellular Ca^{2+} transport was examined in primary cultures of renal CNT/cortical collecting duct (CCD) cells. Finally, the signaling pathway through which TK acts on Ca^{2+} reabsorption and its effect on TRPV5 surface expression were delineated in TRPV5-expressing cells.

*Corresponding author. Department of Physiology, Radboud University Nijmegen Medical Centre, PO Box 9101, Nijmegen 6500 HB, The Netherlands. Tel.: +31 24 3614211; Fax: +31 24 3616413;
E-mail: r.bindels@ncmls.ru.nl
[3]These authors contributed equally to this work

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Results

TK expression levels inversely correlates with urinary Ca^{2+} excretion

The relation between urinary TK expression and urinary Ca^{2+} excretion was investigated by using wild-type (WT) ($TK^{+/+}$) and $TK^{-/-}$ mice fed a 0. 3% w/w (normal) and 3% w/w (high) Na^+ diet. Urinary Ca^{2+} excretion was significantly increased in $TK^{+/+}$ mice on high Na^+ diet compared to mice on normal diet ($P<0.05$, $n=12$ mice) (Figure 1A). Conversely, Ca^{2+} excretion in $TK^{-/-}$ mice on normal Na^+ diet was significantly higher ($140\pm13\%$) than in $TK^{+/+}$ mice ($P<0.05$, $n=12$ mice), but remained constant on a high Na^+ diet ($P>0.2$, $n=12$ mice) (Figure 1A). On the high Na^+ diet, there was no significant difference in urinary Ca^{2+} excretion between $TK^{+/+}$ and $TK^{-/-}$ mice. Body weight, food intake, urinary creatinine as well as plasma values of Ca^{2+}, creatinine and protein concentrations did not differ between mice genotypes on either diet (Table I).

TK is upregulated in $TRPV5^{-/-}$ mice

The amount of TK excreted in the urine was determined by trichloroacetic acid (TCA) protein precipitation of 24 h urine collected from WT ($TRPV5^{+/+}$) and $TRPV5^{-/-}$ mice fed either a 0.02% w/w (low) or a 2% w/w (high) Ca^{2+} diet. Immunoblot analysis of the TCA precipitate showed the specific TK band migrating at ~40 kDa (Figure 1B). Semiquantitative densitometry of the TK corresponding protein band demonstrated that urinary TK excretion increased in $TRPV5^{-/-}$ mice compared to $TRPV5^{+/+}$ littermates on low Ca^{2+} diet, whereas the high Ca^{2+} diet did not affect TK excretion between $TRPV5^{-/-}$ and $TRPV5^{+/+}$ genotypes (Figure 1C).

TK stimulates Ca^{2+} transport in renal primary cell cultures

The effect of TK on transcellular Ca^{2+} transport was evaluated in primary cultures of renal CNT/CCD cells. Application of 100 nM TK to the apical side of the monolayer significantly stimulated Ca^{2+} transport compared to non-treated monolayers, while no effect was observed when TK was added to the basolateral side only (Figure 2A). Importantly, apical addition of TRPV5 channel blocker ruthenium red (10 μM) completely inhibited baseline transepithelial Ca^{2+} transport and abolished the stimulatory effect of TK (Figure 2A). TK action was mimicked by apical addition of 100 nM trypsin, which like TK displays serine protease activity (Figure 2B), as well as 1 μM BK (Figure 2C). Interestingly, the stimulatory effect on Ca^{2+} reabsorption of both BK and TK was inhibited by apical application of 1 μM JE049, a B2R antagonist (Figure 2C). Basolateral treatment of trypsin, BK

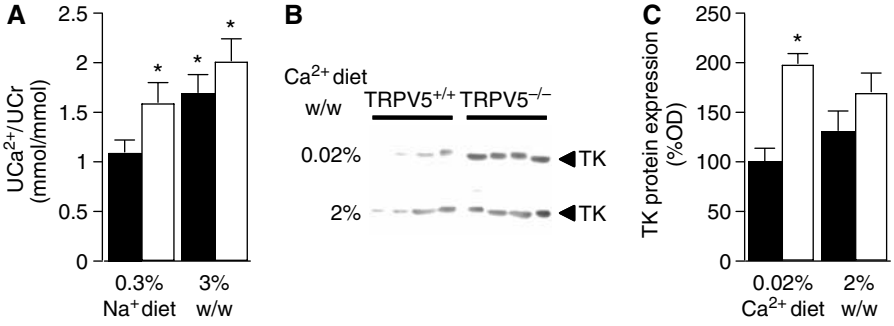

Figure 1 TK expression in relation to Ca^{2+} excretion. (**A**) Twenty-four hours Ca^{2+} excretion normalized for creatinine excretion (UCa^{2+}/UCr) in WT ($TK^{+/+}$, closed bars) and knockout ($TK^{-/-}$, open bars) mice fed a 0.3% w/w or 3% w/w Na^+ diet. Data are expressed as means±s.e.m. ($n=12$ mice). *$P<0.05$ versus $TK^{+/+}$ mice on 0.3% w/w diet. (**B**) Immunoblotting of urinary TK in WT ($TRPV5^{+/+}$) and knockout ($TRPV5^{-/-}$) mice fed a 0.02% or a 2% w/w Ca^{2+} diet. Each lane represents the protein TCA-precipitate of 24 h urine from one individual mouse. (**C**) The intensity of the immunopositive bands was quantified by densitometry and TK expression was depicted as a percent ratio to $TRPV5^{+/+}$ mice (closed bars) fed with 0.02% w/w Ca^{2+} diet. Data are expressed as means±s.e.m. ($n=4$ mice). *$P<0.05$ versus $TRPV5^{+/+}$ mice on the same diet.

Table I Phenotypic characterization of $TK^{+/+}$ and $TK^{-/-}$ mice on 0.3% w/w and 3% w/w Na^+ diets

	0.3% w/w Na^+		3% w/w Na^+	
	$TK^{+/+}$	$TK^{-/-}$	$TK^{+/+}$	$TK^{-/-}$
Weight (g)	23.8±1.0	24.5±0.8	24.6±1.0	23.8±0.4
Food intake (g/24 h)	2.68±0.64	2.09±0.5	1.91±0.32	2.18±0.34
Plasma values				
Total Ca^{2+} (mM)	2.05±0.06	1.96±0.07	2.01±0.08	2.07±0.08
Creatinine (μM)	10.5±1.0	9.3±0.9	9.5±2.1	9.0±2.2
Protein (g/l)	46.3±0.9	44.9±0.9	49.3±1.0	44.8±1.0
Urine values				
Volume (ml/24 h)	1.47±0.20	1.36±0.26	3.47±0.89*	2.18±0.34
Creatinine excretion (μmol/24 h)	4.94±0.42	4.56±0.37	4.79±0.39	4.71±0.38
UNa^+/UCr (mmol/mmol)	39.6±2.5	33.9±2.4	292.5±38.3*	223.2±23.8*

*$P<0.05$ versus same genotype on 0.3% w/w Na^+ ($n=12$ mice).

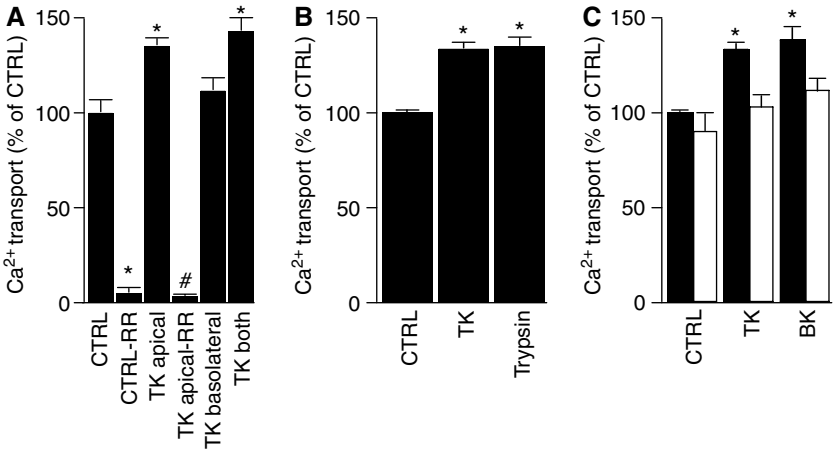

Figure 2 Stimulatory effect of TK on active Ca^{2+} reabsorption. (**A**) Monolayers of rabbit primary cultures of CNT/CCD cells were incubated with 100 nM TK for 90 min at the apical, basolateral or both sides. In one condition, cell monolayers were incubated for 90 min in the presence of the TRPV5 blocker ruthenium red (RR, 10 μM, apical side) in the presence or absence of TK (100 nM, apical side). Vehicle-treated cells were used as control (CTRL) and net apical to basolateral Ca^{2+} transport was measured. Data are expressed as means ± s.e.m. ($n = 6$ wells). $^{*}P < 0.05$ versus CTRL, $^{\#}P < 0.05$ versus TK-treated. (**B**) Primary cells were treated apically with 100 nM trypsin or 100 nM TK. (**C**) 1 μM BK and 100 nM TK were applied at the apical side of the monolayers in the presence (open bars) or absence (closed bars) of 1 μM JE049, a B2R antagonist. In all experiments, vehicle-treated cells were used as control (CTRL) and net apical to basolateral Ca^{2+} transport was measured. Data are expressed as means ± s.e.m. ($n = 4$ wells). $^{*}P < 0.05$ versus CTRL.

or JE049 did not affect transcellular Ca^{2+} transport across the monolayer (data not shown).

TK enhances TRPV5 channel activity

The stimulatory effect of TK on Ca^{2+} transport was further examined in TRPV5-transfected human embryonic kidney (HEK) 293 cells. Incubation of these cells with TK concentrations ranging from 0.05 to 5000 nM enhanced $^{45}Ca^{2+}$ influx in a dose-dependent manner with a maximal stimulation at 500 nM and an EC_{50} of 6.00 ± 0.04 nM (Figure 3A), whereas no effect was observed in mock-transfected cells (data not shown). Further, TK action on TRPV5 channel activity was investigated by the whole-cell patch-clamp technique in TRPV5-expressing HEK293 cells. Preincubation with 100 nM TK increased the inward Ca^{2+} current in response to the step protocol, but the Ca^{2+}-dependent inactivation of the TRPV5 currents remained unchanged (Figure 3B). The current–voltage (*I–V*) relation of the currents in response to a voltage ramp did not alter after preincubation with 100 nM TK (Figure 3C), but the inward Ca^{2+} current at −80 mV increased by 167 ± 14% compared to non-treated control (CTRL) cells ($P < 0.05$, $n = 15$ cells) as depicted in Figure 3D. Likewise, in divalent free (DVF) solution, a significant increase of 149 ± 17% in Na^{+} inward currents at −80 mV ($P < 0.05$, $n = 15$ cells) was observed, while the *I–V* relation remained unchanged (Figure 3E and F).

TK increases TRPV5-mediated currents via the B2R

Since BK mimicked the TK action on Ca^{2+} transport in primary cultures of CNT/CCD cells, the effect of BK on TRPV5-expressing HEK293 cells was further studied. First, the expression of the B2R in HEK293 cells was confirmed by immunoblot analysis. B2R-transfected HEK293 cells were used as a positive control. In both mock and B2R-transfected HEK293 cells, the receptor was detected as a single immunopositive band of ~69 kDa (Figure 4A), confirming the presence of the endogenous B2R in this cell line. Subsequently, TRPV5-transfected HEK293 cells were incubated for 1 h with 1 μM BK and functionally characterized by patch-clamp measurements in comparison to non-treated (CTRL) cells. Interestingly, BK treatment resulted in a significant increase of in both Ca^{2+} (Figure 4D) and Na^{+} (Figure 4F) currents ($P < 0.05$, $n = 12$ cells) compared to CTRL cells, without affecting the Ca^{2+}-dependent inactivation (Figure 4B) or the *I–V* relation of the currents (Figure 4C and E). Because the different treatments of TRPV5-expressing HEK293 cells affected both Ca^{2+} and Na^{+} current amplitudes leaving unaffected the other current properties, only the Ca^{2+} currents were depicted to demonstrate the effect of particular compounds. Incubation of TRPV5-transfected cells with 1 μM JE049 reversed the stimulatory effect of TK on TRPV5-mediated currents. The Ca^{2+}-dependent inactivation of Ca^{2+} currents remained unchanged for cells treated with TK or TK and JE049 (Figure 4G). Ca^{2+} current amplitudes measured from the step protocol were significantly lower, 285 ± 50 pA/pF, for the JE049-treated cells ($P < 0.05$, $n = 11$ cells) compared to TK-treated cells (Figure 4H).

TK stimulates TRPV5-mediated currents via the PLC-dependent PKC pathway

To elucidate in detail the mechanism through which TK enhances TRPV5 activity, the following strategies were followed. First, pretreatment with 10 μM of the PLC inhibitor U73122 for 10 min abolished the TK stimulatory effect on TRPV5-mediated Ca^{2+} currents, whereas 10 μM of its inactive analog U73343 had no effect ($P < 0.05$, $n = 10$ cells) (Figure 5A). Then, TRPV5-transfected cells were incubated for 1 h with 10 μM 1-oleoyl-acetyl-*sn*-glycerol (OAG), a synthetic DAG analog. OAG mimicked the stimulatory effect of TK on TRPV5-mediated Ca^{2+} currents ($P < 0.05$, $n = 10$ cells) (Figure 5B). The stimulatory effect of OAG was observed within 30 min after addition (Figure 5C). Next, the effect of TK on the sextuple PKC phosphorylation-deficient mutant was tested. In HEK293 cells expressing this mutant, the

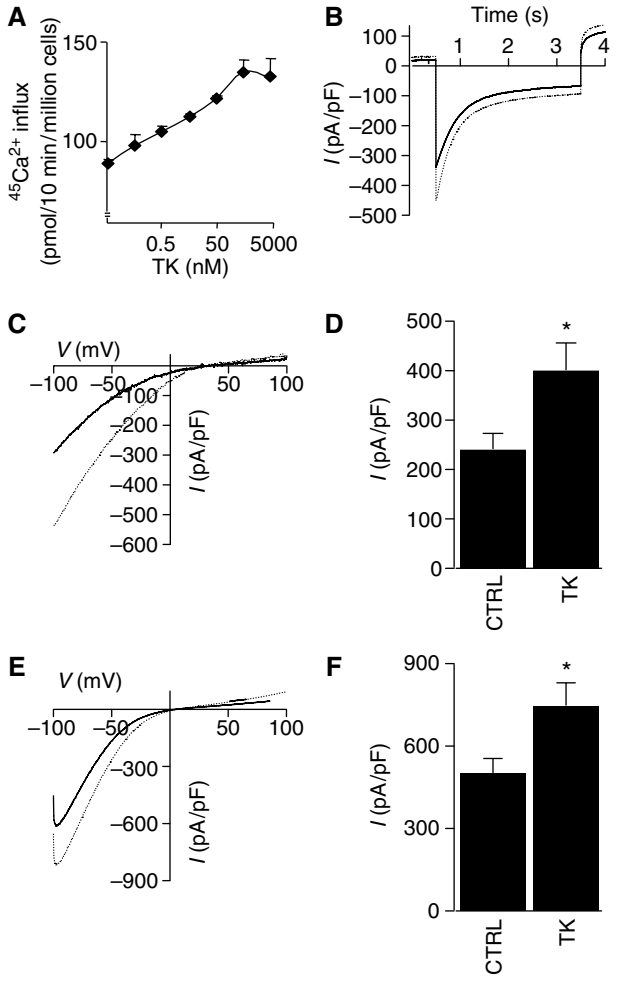

Figure 3 Effect of TK on TRPV5-transfected HEK293 cells. (**A**) HEK293 cells were transfected with TRPV5 and treated for 1 h with TK in concentrations ranging from 0.05 to 5000 nM and finally $^{45}Ca^{2+}$ uptake was measured. Values are expressed as means ± s.e.m. ($n=6$ wells). (**B**) Averaged Ca^{2+} currents measured with 10 mM Ca^{2+} in the extracellular solution during a 3-s step to −100 mV from a holding potential of +70 mV in TRPV5-transfected HEK293 cells treated (dotted trace) or non-treated (solid trace) with 100 nM TK. (**C**) *I–V* relations measured from 450 ms voltage ramps in 10 mM Ca^{2+}-containing extracellular solution from non-treated CTRL (solid trace) or TK treated (dotted trace) TRPV5-expressing HEK293 cells. (**D**) Average density of the Ca^{2+} current measured as in (C) was 400 ± 56 pA/pF ($n=15$ cells) for TK treated cells compared to 240 ± 33 pA/pF ($n=15$ cells) for CTRL cells. (**E**) *I–V* relations measured from 450 ms voltage ramps in nominally DVF solution in TRPV5-transfected HEK293 cells treated (dotted trace) or non-treated (solid trace) with TK. (**F**) Average Na^{+} current density at −80 mV in nominally DVF solution were 745 ± 85 pA/pF ($n=15$ cells) for TK treated cells compared to 500 ± 55 pA/pF for CTRL cells ($n=15$ cells). *$P<0.05$ versus CTRL.

stimulatory effect of TK was abolished (Figure 5D). Subsequently, the PKC phosphorylation sites were individually mutated into an alanine residue. TK increased Ca^{2+} currents of all single PKC mutants, except of S299A and S654A ($P<0.05$, $n=12$ cells) (Figure 5E). Furthermore, to determine which PKC isoforms are involved in this process, cells were incubated for 24 h with 1 μM of phorbol ester (phorbol 12-myristate 13-acetate (PMA)) to downregulate the PMA-sensitive PKC isoforms expressed in HEK293 cells as previously described (Camden *et al*, 2005). Then, these PMA-pretreated cells were treated as aforementioned with

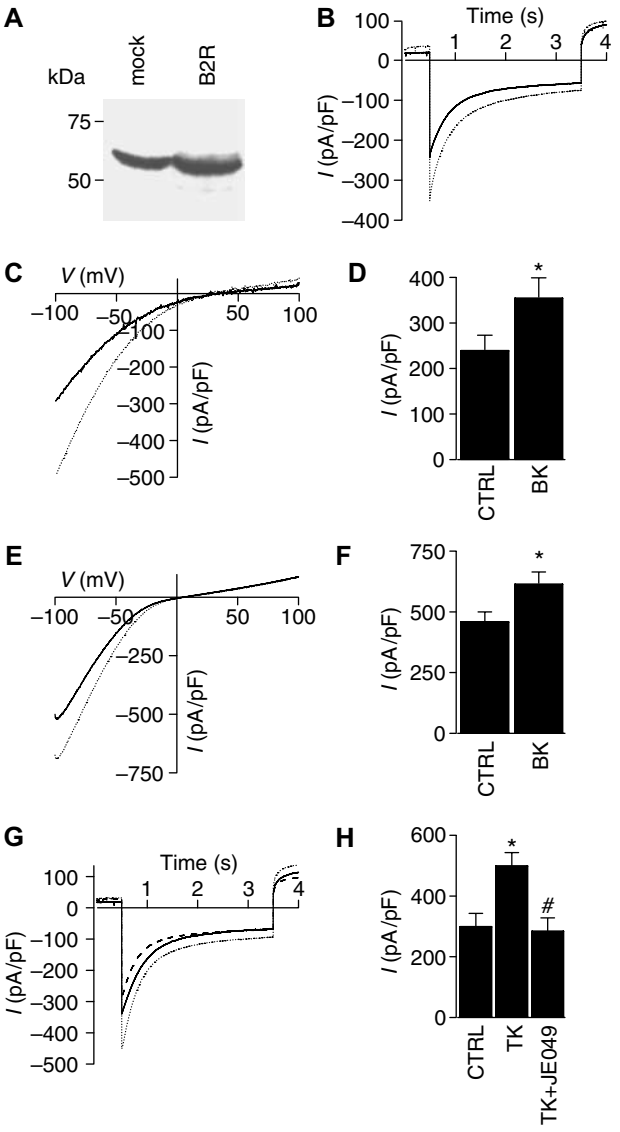

Figure 4 TK stimulation of TRPV5 activity is mediated by B2R. (**A**) B2R transfected and mock cells were lysed and calibrated for equally protein loading on gel. Immunoblot analysis of the samples shows the B2R expression. (**B**) Inward Ca^{2+} currents in TRPV5-transfected HEK293 cells treated (dotted trace) or non-treated (solid trace) with 1 μM BK for 1 h measured with the step protocol as in Figure 3B. (**C**) *I–V* relations measured from 450 ms voltage ramps in 10 mM Ca^{2+}-containing extracellular solution from non-treated CTRL (solid trace) or BK treated (dotted trace) TRPV5-expressing HEK293 cells. (**D**) Amplitude of Ca^{2+} current for BK treated cells was 363 ± 45 pA/pF and for control cells 240 ± 30 pA/pF ($n=12$ cells for each condition). (**E**) *I–V* relations measured from voltage ramps in nominally DVF extracellular solution from non-treated CTRL (solid trace) and BK (dotted trace) treated TRPV5-expressing HEK293 cells. (**F**) Average Na^{+} current for cells treated with BK was 615 ± 49 pA/pF and for CTRL cells 460 ± 46 pA/pF. (**G**) Inward Ca^{2+} currents measured with 10 mM Ca^{2+} in the extracellular solution during the voltage step to −100 mV of control non-treated cells (solid trace), cells treated with TK (dotted trace), and cells treated with both TK and 1 μM of the B2R antagonist JE049 (dashed trace). Inactivation of currents remained unchanged. (**H**) Average peak Ca^{2+} current of cells treated with TK and JE049 was 285 ± 45 pA/pF and for CTRL cells 300 ± 50 pA/pF compared to 500 ± 55 pA/pF measured from TK treated cells. *$P<0.05$ versus CTRL. #$P<0.05$ versus TK treated cells.

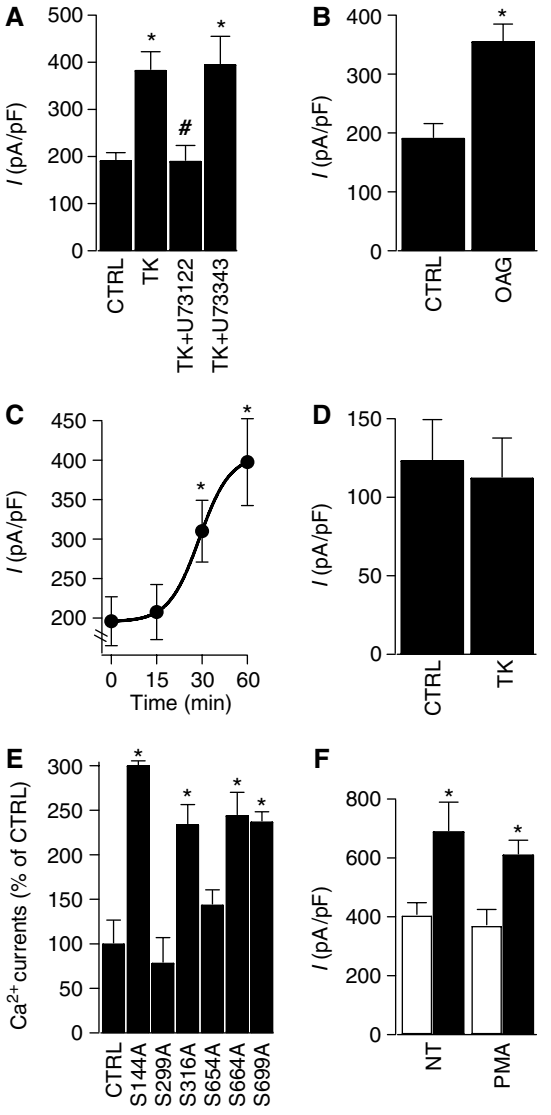

Figure 5 TK action is mediated by the DAG/PKC-dependent PLC signaling pathway. TRPV5 expressing cells were incubated for 1 h with 100 nM TK and subsequently treated as described below. (**A**) Ten minutes incubation with 10 μM of the PLC inhibitor U73122 reverted the average Ca^{2+} currents amplitude of the TK treated cells to values similar to CTRL cells, contrary to its inactive form U73343, which let the TK effect unaltered ($n=10$ cells). (**B**) Ca^{2+} current amplitude of cells pretreated for 1 h with 10 μM OAG was 355 ± 30 pA/pF and for CTRL cells was 191 ± 25 pA/pF. (**C**) Time course of TRPV5 current stimulation by OAG. Points represent Ca^{2+} peak current measured at −100 mV using the step protocol from cells pretreated with 10 μM OAG for 15, 30 and 60 min or non-treated cells. $^{*}P<0.05$ versus non-treated cells at 0 min. (**D**) Average Ca^{2+} currents for cells expressing TRPV5 mutated for all its six PKC phosphorylation sites were 123 ± 36 pA/pF and TK treated cells were 113 ± 25 pA/pF ($n=10$ cells for each condition). (**E**) Point mutation of the PKC phosphorylation sites S299 and S654 did not affect Ca^{2+} currents in comparison with the other four PKC mutants upon TK treatment. Data are expressed as percentage of Ca^{2+} currents measured of cells expressing PKC-TRPV5 point mutants treated with 100 nM TK normalized to non-treated CTRL cells ($n=12$ cells for each mutant). (**F**) At 24-h incubation with 1 μM PMA was used to downregulate the PMA-sensitive PKC isoforms in TRPV5-transfected cells. In both cases, non-treated (NT), or 24 h incubation with PMA, TK (black bars) was still able to significantly increase TRPV5-mediated Ca^{2+} currents compared to control cells (white bars) ($n=14$ cells for each condition). $^{*}P<0.05$ versus CTRL cells. $^{\#}P<0.05$ versus TK treated cells.

100 nM TK for 1 h. TK was still able to significantly increase ($164\pm14\%$ of control, $P<0.05$, $n=14$ cells) the TRPV5-mediated Ca^{2+} currents (Figure 5F). Vehicle-incubated (NT) cells were used as control for the effect of PMA on TRPV5 currents and PMA incubation left TRPV5-mediated currents unaffected.

Subsequently, the possible involvement of other G-protein coupled receptors in addition to the B2R was investigated. To this end, purinergic receptors were activated by the addition of extracellular ATP (1 mM for 1 h) to TRPV5-expressing HEK293 cells. Indeed, as shown in the Figure 6, ATP treatment enhanced the inward Ca^{2+} currents in these cells, while leaving the *I–V* relationship and Ca^{2+}-dependent inactivation unchanged. Moreover, after pretreatment with the B2R antagonist JE049, ATP was still able to stimulate the TRPV5 currents, indirectly demonstrating the specificity of TK stimulation via the B2R.

TK increases cell surface expression of TRPV5 by delaying channel retrieval

The effect of TK on the amount of TRPV5 channel expressed at the plasma membrane was investigated in HEK293 cells. TRPV5-transfected cells were incubated with 100 nM TK alone or in combination with 1 μM JE049 for 1 h and then subjected to cell surface biotinylation. Biotinylated cell lysates were precipitated with neutravidin-agarose beads and immunoblotted for TRPV5. Mock-transfected and non-biotin-treated cells were used as negative controls. TK treatment enhanced TRPV5 expression in the biotinylated fraction, whereas JE049 abolished this effect (Figure 7A, left panel). Importantly, TRPV5 was equally expressed in total cell lysates of all tested conditions (Figure 7A, right panel). The observed increase in cell surface expression of TRPV5 by TK could be due to either an enhanced trafficking from the Golgi apparatus to the cell surface or a reduction in channel retrieval from the plasma membrane. The kinetics of cell-surface retrieval was measured in HEK293 cells using half-life cell-surface biotinylation. After a 1 h TK treatment, the retrieval of TRPV5 channel from the plasma membrane was decreased by 71, 46 and 17% ($P<0.05$, $n=3$ blots) at the time points of 1, 3 and 6 h, respectively (Figure 7B). TRPV5 expression in cell lysates was identical in all tested conditions (Figure 7C). These results suggested that TK increases cell surface expression of TRPV5 by delaying channel retrieval from the plasma membrane.

TRPV5 PKC mutants S299A and S654A are insensitive to TK treatment

The effect of TK on the TRPV5 PKC mutants S299A and S654A was investigated in HEK293 cells by half-life analysis. TRPV5 WT, S299A and S654A transfected cells were incubated with or without 100 nM TK for 1 h and then the kinetics of cell-surface retrieval was measured in HEK293 cells using half-life cell-surface biotinylation. Without TK treatment (Figure 8A), the plasma membrane expression of TRPV5 WT, S299A and S654A was decreased to a similar extent at the time points of 1, 3, 6 and 12 h. Interestingly, with TK treatment (Figure 8B), the retrieval of TRPV5 WT channel from the plasma membrane was decreased, but retrieval of S299A and S654A was not altered. TRPV5 WT, S299A and S654A expression in cell lysates was identical in all tested conditions (data not shown). These results indicated that the

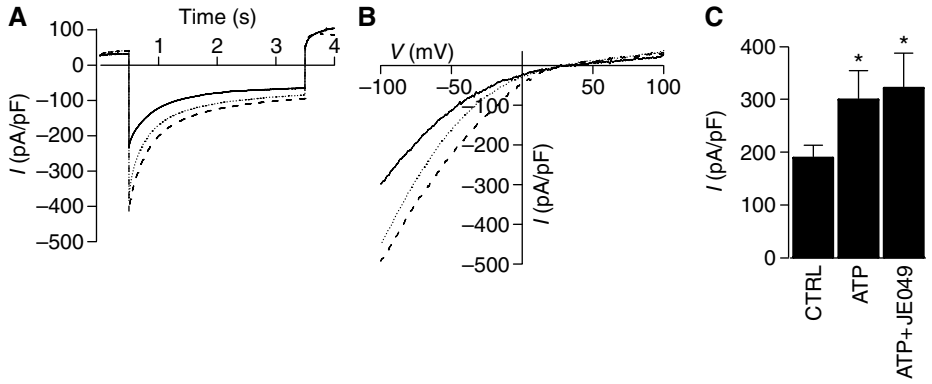

Figure 6 B2R confers specificity to TK-dependent stimulation of TRPV5 activity. (**A**) Averaged Ca^{2+} currents measured with 10 mM Ca^{2+} in the extracellular solution during a 3 s step to −100 mV from a holding potential of +70 mV in TRPV5-transfected HEK293 cells treated with 1 mM ATP (dotted trace), treated with 1 mM ATP and 1 μM of the B2R antagonist JE049 (dashed line) or non-treated CTRL (solid trace). (**B**) *I–V* relationships measured from 450 ms voltage ramps in 10 mM Ca^{2+}-containing solution in TRPV5-transfected HEK293 cells treated with ATP (dotted trace), treated with ATP and JE049 (dashed trace) or non-treated (solid trace) with ATP. (**C**) Average density of the Ca^{2+} peak current measured as in (B) was 300±54 pA/pF for ATP-treated cells and 322±65 pA/pF for ATP and JE049, compared to 190±23 pA/pF ($n=15$ cells) for CTRL cells. $^*P<0.05$ versus CTRL.

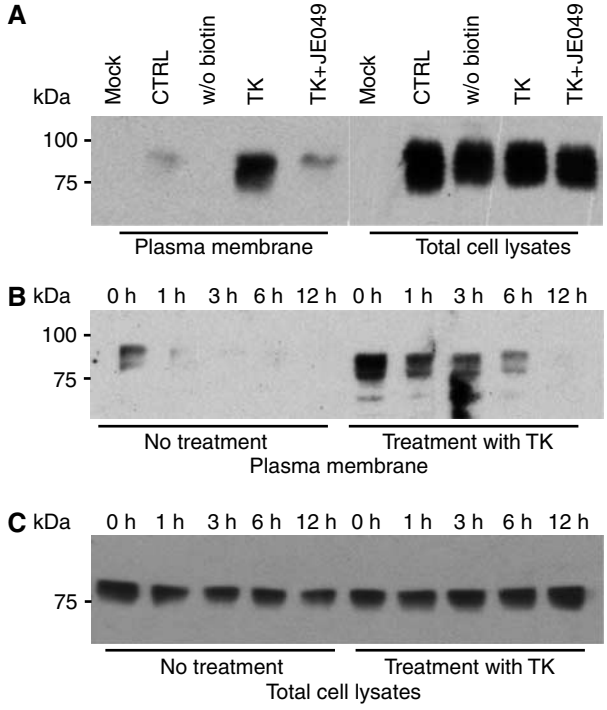

Figure 7 TK affects the plasma membrane expression of TRPV5. (**A**) TRPV5-transfected HEK293 cells were treated with 100 nM TK with or without 1 μM JE049. Cells were subjected to cell surface biotinylation and after lysis precipitated with neutravidin-agarose beads. TRPV5 expression was analyzed by immunoblot for the plasma membrane fraction (left panel) and for the total cell lysates (right panel). As negative controls, mock cells were used and biotin was omitted in the procedure. (**B**, **C**) TK treated or non-treated TRPV5-transfected cells were subjected to biotinylation. Cells were immediately homogenized (time point 0), or further cultured for 1, 3, 6 and 12 h. Subsequently, the cells were lysed, following immunoprecipitation with neutravidin-agarose beads and TRPV5 expression of plasma membrane (B) and total lysates (C) was visualized by immunoblot analysis.

TK effect of delaying channel retrieval from the plasma membrane was abolished in TRPV5 PKC mutants S299A and S654A.

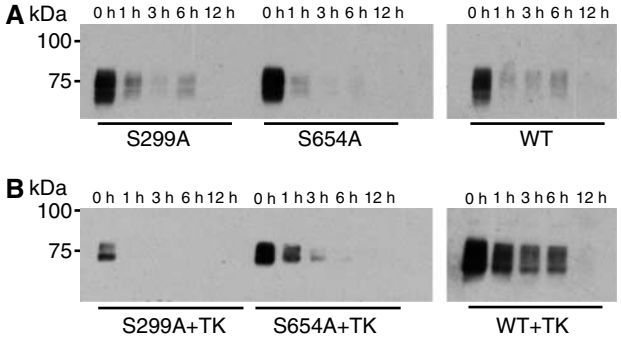

Figure 8 TK does not affect the plasma membrane expression of TRPV5 PKC mutants S299A and S654A. TRPV5 WT, S299A and S654A-transfected HEK293 cells were treated for 1 h with vehicle (**A**) or with (**B**) 100 nM TK. Subsequently, cells were subjected to cell-surface biotinylation and then homogenized at 0, 1, 3, 6 and 12 h after biotinylation. Subsequently, cells were immediately homogenized (time point 0), or further cultured for 1, 3, 6 and 12 h. Then, the cells were lysed, following immunoprecipitation with neutravidin-agarose beads and TRPV5 expression of plasma membrane was visualized by immunoblot analysis. The expression of TRPV5 in the cell lysates was similar in the various conditions (data not shown).

Discussion

The present study showed that extracellular TK stimulates TRPV5-mediated Ca^{2+} reabsorption by activating the B2R and subsequently the DAG/PKC pathway resulting in accumulation of TRPV5 channels at the cell surface. This conclusion is based on the following experimental observations: (i) Urinary Ca^{2+} excretion in mice is inversely related to the expression of TK; (ii) TK stimulates Ca^{2+} reabsorption in primary cultures of renal CNT/CCD cells, which can be blocked by the B2R antagonist JE049; (iii) the stimulatory effect of TK is mediated by the PLC/DAG/PKC pathway and requires the two conserved PKC sites S299 and S654 in TRPV5; and (iv) TK enhances Ca^{2+} transport by increasing TRPV5 abundance at the plasma membrane.

TK stimulated TRPV5-mediated Ca^{2+} transport via the B2R receptor. The physiological relevance of this latter

finding is substantiated by the co-localization of B2R with TK (Figueroa *et al*, 1995) and TRPV5 (Hoenderop *et al*, 2003) in DCT and CNT. B2R belongs to the seven-transmembrane domain G protein-coupled receptor superfamily (Hess *et al*, 1992) and signals via $G\alpha_q$ protein with consequent activation of PLC. PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) in inositol trisphosphate (IP_3) and DAG (Blaukat, 2003). Commonly, IP_3 releases Ca^{2+} from the endoplasmic reticulum stores, while DAG activates PKC (Blaukat, 2003). Certainly, our results show that TRPV5 activation requires PIP_2 breakdown following B2R stimulation and relies on DAG as a downstream effector. Subsequently, DAG increases TRPV5 activity via PKC phosphorylation of the channel since potential PKC phosphorylation deficient mutants of TRPV5 at positions S299 and S654 lack the TK effect. This is the first study demonstrating that TRPV5 is activated through its PKC phosphorylation sites. Among the six predicted phosphorylation sites in the TRPV5 sequence, two serines, S299 and S654, are critical for TRPV5 activation by TK. Importantly, TRPV5 closest homolog, TRPV6, did not respond to TK (data not shown). Since the second serine (S654) is not conserved in TRPV6, both serines are apparently critical for stimulation of TRPV5 by TK. In addition, 24-h preincubation with PMA downregulates α, β and ε isoforms of PKC, but not PKC ζ (Frecker *et al*, 2005). This latter PKC isoform is presumably involved in TK increase of TRPV5 currents since the stimulatory effect of PKC was similar in PMA-pretreated and control cells. Interestingly, stimulation of active Ca^{2+} transport in primary cultures of renal CNT/CCD also involves PMA-insensitive PKC isotypes (Hoenderop *et al*, 1999) among which the isotype PKC ζ can be activated by B2R (Christopher *et al*, 2001). However, B2R knockout mice ($B2R^{-/-}$) mice showed no change in urine Ca^{2+} excretion (Picard *et al*, 2005), in contrast with $TK^{-/-}$ mice, suggesting that compensation mechanisms could mask the TK effect on the Ca^{2+} balance in these knockout mice. For instance, it has been shown that in these knockout mice a compensatory induction of the B1 receptor occurs that could theoretically be a new target for TK (Duka *et al*, 2001). Alternatively, TK exerts its *in vivo* effect via a mechanism independent of the B2R.

Previous studies showed that TRP channels can be activated by different signaling molecules of the PLC pathway. For example, the three canonical TRP members TRPC3, 6 and 7 are characterized by their sensitivity to DAG (Hofmann *et al*, 1999). Alternatively, the vanilloid and melastatine members of TRP family, TRPV1 (Chuang *et al*, 2001), TRPV5 (Lee *et al*, 2005), TRPM4 (Nilius *et al*, 2006), TRPM5 (Liu and Liman, 2003), TRPM7 (Runnels *et al*, 2002) and TRPM8 (Liu and Qin, 2005), can be directly regulated by PIP_2. Thus, *in vivo* TRPV5 activation by PIP_2 (Lee *et al*, 2005) or DAG/PKC could adjust TRPV5 activity in response to physiological fluctuations. In addition, Ca^{2+} entering the cell through TRPV5 could prevent the electrostatic interaction between the negatively charged PIP_2 and the channel by screening the negative charge on the lipid head group, as proposed for Mg^{2+} and TRPM7 (Kozak and Cahalan, 2003).

Stimulation of Ca^{2+} transport through TRPV5 upon TK action could be the result of an increase in either open probability of the channel or in expression of TRPV5 at the plasma membrane. Surface biotinylation analysis showed that TK increases the amount of TRPV5 channels at the plasma membrane. Thus, PKC activation of TRPV5 following the TK application would regulate the balance between constitutive exocytosis and endocytosis in favor of the former leading to the accumulation of TRPV5 at the cell surface. Similarly, the epidermal growth factor prevents the internalization of plasma membrane TRPC3 (Smyth *et al*, 2006). The role of the cytoskeleton in this translocation process is presently unknown. It is possible that PKC-dependent phosphorylation of TRPV5 leads to activation of motor proteins that transport the channels towards the plasma membrane. This process could involve the FKBP52 protein, characterized previously as a TRPV5 regulatory protein (Gkika *et al*, 2006), since FKBP52 is known to interact with the motor protein dynein (Czar *et al*, 1995; Silverstein *et al*, 1999). Interestingly, TRPM7 associates to the actomyosin cytoskeleton upon BK stimulation regulating cell adhesion (Clark *et al*, 2006). Remarkably, the protein synaptotagmin has been proposed to regulate the exocytosis of TRPC5 (Strubing *et al*, 2001). However, the cytoskeletal elements and the motor proteins participating in the incorporation of TRPV5 in the plasma membrane remain to be identified. Furthermore, accumulation of channels at the cell surface can also occur by increased incorporation into the plasma membrane. Indeed, PKC potentiation of TRPV1 promotes the recruitment of a channel vesicular pool to the cell surface (Morenilla-Palao *et al*, 2004). This exocytosis process of TRPV1 is dependent on the soluble *N*-ethylmaleimide-sensitive-factor attachment proteins receptor (Morenilla-Palao *et al*, 2004), known to act as membrane recognition molecules and acceptors for vesicle trafficking, docking and fusion (Duman and Forte, 2003). It would be interesting to investigate the role of similar scaffold proteins in the assembly of TRPV5 and PKC upon TK treatment, considering that such signaling pathways are currently indicated to function in spatially distinct microdomains (Ambudkar, 2006). For instance, the A-kinase-anchoring protein has been described to coordinate the subcellular localization of second messenger-regulated enzymes, such as PKC in order to modulate the activity of K^+ channel, KCNQ/M, upon agonist stimulation via Gq-coupled pathway (Hoshi *et al*, 2005).

The aforementioned data are in support of plasma membrane recycling of TRPV5 protein as mechanism of channel regulation. A tight control of TRPV5 activity is of primordial importance for body Ca^{2+} homeostasis, since TRPV5 constitutes the fine-tuned Ca^{2+} entry step in active Ca^{2+} reabsorption (Hoenderop *et al*, 2005). Besides the hormonal regulation of TRPV5, accessory proteins play a role in modulating channel trafficking and activity (van de Graaf *et al*, 2003; Gkika *et al*, 2004). Together with a recent report on the β-glucuronidase klotho (Chang *et al*, 2005), our study introduces a new mechanism of TRPV5 regulation, which is based on extracellular enzymatic activation. TK and klotho colocalize with TRPV5 in the distal part of the nephron where they activate the channel from the luminal side. A comparable enzymatic regulation is described for the epithelial Na^+ channel, ENaC, present in CNT, by the channel activating proteases, CAP-1, CAP-2 and CAP-3 (Rossier, 2004). Unlike the protease CAP and the β-glucuronidase klotho, TK stimulates TRPV5 indirectly via activation of the B2R that then induces a redistribution of TRPV5 channels towards the plasma membrane. On the contrary, CAPs, which are mem-

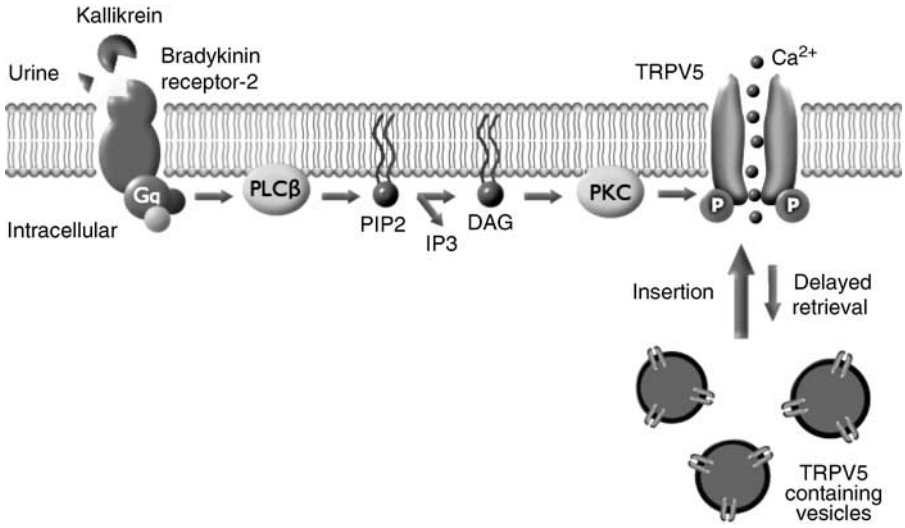

Figure 9 Schematic diagram of TK stimulatory effect on TRPV5. TK activates the B2R and subsequently PLC, leading to the hydrolysis of PIP_2 in IP_3 and DAG. DAG induces subsequently the phosphorylation of TRPV5 through PKC-dependent mechanism with consequent stabilization of TRPV5 channel in the plasma membrane. The increased number of channels at the plasma membrane is the result delayed channel retrieval ensuing enhanced Ca^{2+} transport through TRPV5.

brane-bound serine proteases, act directly on the channel gating by enhancing the open probability of ENaC (Vuagniaux *et al*, 2002) to stimulate epithelial Na^+ absorption. Likewise, klotho directly activates TRPV5 by enzymatic modification of the *N*-glycan to stabilize TRPV5 channels in the plasma membrane (Chang *et al*, 2005).

Interestingly, we showed that an increase in Na^+ supply induced a Ca^{2+} wasting in WT mice. It is well know that a high Na^+ diet increases the plasma volume that triggers the renin–angiotensin–aldosterone system (Nijenhuis *et al*, 2005). These hormones will reduce the reabsorption of Na^+ and consequently Ca^{2+} in the proximal tubules as a negative feedback response. Ultimately, this will increase the urinary excretion of Ca^{2+}. However, the anticipated calciuria was not observed in $TK^{-/-}$ mice, possibly these mice cannot further increase their urinary Ca^{2+} excretion. In addition, the rates of urinary Ca^{2+} excretion in WT and $TK^{-/-}$ mice were similar on a high Na^+ diet, whereas they markedly differed on a normal Na^+ diet. This strongly supports the fact that TK participates in the renal response to a high Na^+ diet and to the attendant decrease in urinary Ca^{2+} reabsorption.

Here, we demonstrated that TK displays autocrine or paracrine stimulation of active Ca^{2+} reabsorption in a dose-dependent manner. As shown in the mice models, reduction in TK expression increases urinary Ca^{2+} excretion due to impaired tubular Ca^{2+} reabsorption. Conversely, genetic ablation of TRPV5 doubled the renal expression of TK possibly due to counterbalance the significant Ca^{2+} wasting observed in the $TRPV5^{-/-}$ mice by maximally stimulating the process of Ca^{2+} reabsorption. Indeed, an increase in dietary Ca^{2+} intake as an alternative maneuver to offset the renal Ca^{2+} loss in $TRPV5^{-/-}$ mice prevented the compensatory increase in TK expression. These results underline the role of TK during Ca^{2+} restriction. It is, therefore, interesting to study the Ca^{2+} excretion in humans with a genetically reduced TK activity. In this respect, a loss-of-function polymorphism of the TK gene in which the active-site arginine at position 53 is changed into a histidine (R53H), reduces by 50–60% TK activity is interesting (Slim *et al*, 2002). Partial genetic deficiency in TK activity of the R53H subjects is associated with a form of arterial dysfunction (Azizi *et al*, 2005). TK is well known as a vasodilator factor of renal cortical blood vessels through BK production, while abnormalities of TK levels has long been documented in the pathogenesis of hypertension (Chao and Chao, 2005). It is therefore tempting to speculate that TK stimulation of TRPV5-mediated Ca^{2+} reabsorption plays a role in TK hypotensive action, since urinary Ca^{2+} leak has been correlated with higher baseline systolic and diastolic blood pressures (McCarron and Reusser, 1999).

In conclusion, our data demonstrated that TK reduces urinary Ca^{2+} excretion by an autocrine and paracrine stimulation of TRPV5-mediated Ca^{2+} reabsorption explaining the hypercalciuria in $TK^{-/-}$ mice. Figure 9 illustrates the molecular pathway linking TK to stimulation of TRPV5, as elucidated in the present study. TK activates the B2R and through the PLC derived-messenger DAG initiates the phosphorylation of the TRPV5 PKC sites S299 and S654. Subsequently, Ca^{2+} influx through TRPV5 is enhanced by the accumulation and stabilization of the channel at the plasma membrane. Thus, TK-directed translocation of TRPV5 channels constitutes a mechanism by which renal cells can fine-tune the Ca^{2+} reabsorption.

Materials and methods

Animal experiments

$TK^{-/-}$ mice were produced as previously described (Meneton *et al*, 2001; Picard *et al*, 2005) and fed *ad libitum* two Na^+ diets containing 0.3% w/w or 3% w/w for 14 days. Subsequently, 24 h urine was collected from $TK^{-/-}$ and $TK^{+/+}$ mice housed in metabolic cages and blood was obtained by orbital puncture. Measurements of biological parameters in the animals plasma and urine were performed as previously described (Picard *et al*, 2005). Urinary Na^+, Ca^{2+} and Mg^{2+} excretion was expressed as ratios to urinary creatinine excretion to take into account the variations in urine collection. $TRPV5^{-/-}$ mice were generated as described previously (Hoenderop *et al*, 2003). At the age of 4 weeks, mice were fed *ad libitum* two diets containing 0.02% w/w or 2% w/w Ca^{2+} for 5 weeks and were subsequently placed in metabolic cages.

At 24 h urine samples were collected and subjected to TCA precipitation. The animal ethics board of the Radboud University Nijmegen approved all animal experimental procedures.

Urine protein precipitation by TCA

Of the 24 h urine volume collected of TRPV5$^{-/-}$ mice, 10% v/v was centrifuged at 200 *g* for 10 min at 4°C to remove cell debris. Proteins in the urine were precipitated using ice-cold TCA (Acros organics, NJ, USA). The TCA mixture was centrifuged at 13 000 *g* for 10 min at 4°C, and the pellet was subsequently washed with 300 µl acetone. Next, the pellet was air-dried for approximately 3 min, dissolved in Laemmli buffer containing 0.1 M DTT and 150 mM Tris (pH 8.8), and analyzed by immunoblot analysis for the expression of TK using a rabbit anti-TK antibody (Calbiochem, San Diego, CA, USA). Immunopositive bands were scanned using an imaging densitometer to determine pixel density (Molecular Analyst Software, BioRad Laboratories, Hercules, CA).

DNA constructs and cell culture

The TRPV5 pCINeo/IRES-GFP constructs were generated as described previously (van de Graaf *et al*, 2003). Single and combined PKC mutants were generated by alanine substitution of the six putative phosphorylation sites of TRPV5 (S144A, S299A, S316A, S654A, S664A, S698A) using *in vitro* mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA, USA). The B2R pcDNA3 was a kind gift from Professor G Erdös, MD (Department of Pharmacology, University of Illinois, Chicago, USA). HEK293 cells were transfected at 70% of confluency using polyethylenimine (Polysciences, Inc., Warrington, USA) or Lipofectamin 2000 (Invitrogen Life Technologies, Breda, The Netherlands). After 48–60 h, cells were used for $^{45}Ca^{2+}$ uptake assays, patch-clamp and/or biotinylation experiments. Prior to the assays, cells were incubated for 1 h in serum-free medium containing the particular compound.

Transcellular Ca^{2+} transport in renal primary cultures

CNT/CCD tubules were immunodissected from kidney cortex of New Zealand White rabbits (~0.5 kg) using antibody R2G9 and then placed in primary culture on permeable filters (0.33 cm^2; Costar, Cambridge, MA, USA) as described before (Bindels *et al*, 1991). At confluence, monolayers were used for the transepithelial Ca^{2+} transport assay as previously described (Bindels *et al*, 1991). Transepithelial potential difference and resistance were checked before and after transport measurement to confirm the integrity of the monolayer.

$^{45}Ca^{2+}$ uptake assay

HEK293 cells were transfected with TRPV5 pCINeo/IRES-GFP or pCINeo/IRES-GFP cDNA. Ca^{2+} uptake was determined in uptake medium (110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Na-acetate, 2 mM NaH_2PO_4, 20 mM HEPES–Tris, pH 7.4 supplemented with 10 µM felodipine, 10 µM methoxy-verapamil, 1 mM $BaCl_2$ and 1 µCi/ml $^{45}CaCl_2$) for 10 min at room temperature (20–25°C). Each well was washed extensively with stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.5 mM $LaCl_3$, 10 mM Na-acetate, 20 mM HEPES–Tris, pH 7.4) at 4°C, incubated with 0.05% w/v SDS and the lysates were counted for radioactivity using liquid scintillation.

Electrophysiology and solutions

Patch-clamp experiments were performed as described previously (Vennekens *et al*, 2000) in the tight seal whole-cell configuration at room temperature using an EPC-9 patch-clamp amplifier computer controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany). Two voltage protocols were used: a ramp, to establish the *I–V* relation in nominally DVF or in 10 mM Ca^{2+}-containing extracellular solutions, and a hyperpolarizing step protocol to measure the Ca^{2+}-dependent inactivation. Na^+ current densities were calculated from the current at −80 mV during the ramp protocols, while the Ca^{2+} currents were calculated from the current at −80 mV during the ramp protocols or from Ca^{2+} peak values were extracted from the current at −100 mV during the step protocol. The analysis and display of patch-clamp data were performed using Igor Pro software (WaveMetrics, Lake Oswego, USA).

Cell surface labeling with biotin

HEK293 cells were transfected with 15 µg HA-TRPV5, TRPV5-S299A or TRPV5-S654A pCINeo/IRES-GFP or pCINeo/IRES-GFP in poly-L-lysine (Sigma, St Louis, MO, USA) coated 10 cm dishes. At 48 h after transfection, cells were incubated for 1 h with 100 nM TK and 1 µM JE049. The biotinylation assay was performed, cells were homogenized in 1 ml lysis buffer as described previously (Chang *et al*, 2005) using the NHS-LC-LC-biotin (Pierce, Etten-Leur, The Netherlands). Finally, biotinylated proteins were precipitated using neutravidin-agarose beads (Pierce). TRPV5 expression was analyzed by immunoblot for the precipitates (plasma membrane fraction) and for the total cell lysates using the guinea-pig anti-TRPV5 antibody (Hoenderop *et al*, 2000). For the half-life assay, the biotinylation assay was pursued as described above. For time point 0 h, cells were collected from plates and lysed, immediately after biotinylation. Other plates of cells were further cultured after biotinylation for 1, 3, 6 or 12 h, then washed once with ice-cold PBS (pH 7.4), and subsequently homogenized in lysis buffer. All samples were processed as described above.

Compounds

TK, BK, PMA and OAG were purchased from (Sigma, St Louis, MO, USA). U73122 and U73343 were purchased from Upjohn Laboratories (Kalamazoo, MI, USA). JE049, formerly known as icatibant or HOE140, which was a kind gift of Dr J Pünter (Aventis Pharma Deutschland GmbH, Frankfurt, Germany).

Statistical analysis

In all experiments, the data are expressed as mean ± s.e.m. Overall statistical significance was determined by analysis of variance (ANOVA). In case of significance, differences between the means of two groups were analyzed by unpaired *t*-test, while multiple comparisons between groups were performed by Bonferroni *post hoc* tests. $P<0.05$ was considered significant. The statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA).

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