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Original Article

The β-glucuronidase klotho exclusively activates the epithelial Ca^{2+} channels TRPV5 and TRPV6

Peng Lu, Sandor Boros, Qing Chang, René J. Bindels and Joost G. Hoenderop

Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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Abstract

Background. Active Ca^{2+} reabsorption in the kidney is facilitated by the epithelial transient receptor potential vanilloid Ca^{2+} channel subtype 5 (TRPV5). The complex-glycosylated TRPV5 is expressed at the apical membrane of the renal distal convoluted tubule (DCT) cells where the pro-urine hormone klotho can stimulate its activity by *N*-oligosaccharide hydrolysis. This study investigates whether klotho and its closely related analogue, β-glucuronidase, can activate other renal ion channels than TRPV5 expressed by DCT cells.
Methods. To determine the specificity of this stimulatory effect of klotho and β-glucuronidase, a selection of ion channels and transporters expressed in the kidney (TRPV4, TRPV5, TRPV6 and TRPM6) was screened in transfected HEK293 cells by using Ca^{2+}-influx measurements.
Results. Klotho and β-glucuronidase have been found to significantly increase the activity of TRPV5 and TRPV6, but had no effect on TRPV4 and TRPM6. Furthermore, deglycosylation by endoglycosidase-F also stimulated the activity of TRPV4, TRPV5 and TRPV6, but not of TRPM6.
Conclusions. These results suggest a modulating effect for klotho primarily restricted to the epithelial Ca^{2+} channels TRPV5 and TRPV6.

Keywords: endoF; epithelial calcium channel; β-glucuronidase; klotho; *N*-glycosylation

Introduction

Renal Ca^{2+} reabsorption, intestinal Ca^{2+} absorption and the exchange of Ca^{2+} from bone are the three tightly controlled mechanisms that maintain the delicately balanced Ca^{2+} homeostasis. In kidney, Ca^{2+} can enter the tubular epithelial cells by active reabsorption, which is facilitated by the two epithelial Ca^{2+} channels, transient receptor potential vanilloid type 5 (TRPV5) and transient receptor potential vanilloid type 6 (TRPV6) [1]. Both proteins are members of the transient receptor potential (TRP) channel superfamily and are located at the apical surface of distal convoluted tubule (DCT) and connecting tubule (CNT) cells [1]. In addition to TRPV5 and TRPV6, other TRP family members, such as transient receptor potential vanilloid type 4 (TRPV4) and transient receptor potential melastatin type 6 (TRPM6), are also present in these nephron segments [2]. TRPV4 is abundantly expressed in several segments of the nephron, including the thin and thick ascending limbs as well as in DCT and CNT, and is an osmotically active cation channel with a slight preference for Ca^{2+} [3]. Recent micropuncture studies clearly pointed towards the involvement of TRPV5 in transcellular Ca^{2+} transport across the DCT/CNT tubules, whereas TRPV6 has been speculated to predominantly mediate intestinal Ca^{2+} absorption [4]. The expressions of TRPV5 and TRPV6 channels are tightly controlled by several humoral factors, all known to be involved in regulating the Ca^{2+} homeostasis, such as the parathyroid hormone, 1,25-dihydroxyvitamin D_3 and oestrogens [1]. TRPV5 and TRPV6 harbour a single conserved *N*-glycosylation site, N_{358} and N_{357}, respectively, in the extracellular loop between the first two transmembrane regions [2]. Using different glycobiological tools, it has been shown that native TRPV5 and TRPV6 undergo glycosylation, resulting in high mannose and complex-glycosylated proteins [5]. Additionally, microarray analysis of TRPV5$^{-/-}$ mice has identified klotho, a recently discovered hormone, as a putative regulator of TRPV5 [6].

Klotho is a type-I (single-pass) membrane protein, and a member of the β-glycosidase family [6]. The klotho gene is originally identified as a gene mutated in a mouse strain that develops multiple ageing-like phenotypes [7], and it is mainly expressed in the renal DCT cells and the brain choroid plexus, where, following extracellular domain shedding, it is secreted into the extracellular fluid [8]. There is a growing body of evidence that klotho plays a fundamental role in the regulation of Ca^{2+} and phosphate homeostasis [6,9]. Klotho has also been reported to increase the transcellular Ca^{2+} transport in the kidney and choroid plexus cells by augmenting the Na^{+}–K^{+}-ATPase activity

Correspondence and offprint requests to: Joost G. Hoenderop, 286 Physiology, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: +31-24-3610580; Fax: +31-24-3616413; E-mail: j.hoenderop@ncmls.ru.nl

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at the plasma membrane, and stimulating the Na^+/Ca^{2+} exchange through the Na^+/Ca^{2+}-exchanger NCX_1 [9]. A homozygous missense mutation in the deep catalytic cleft of klotho also has been very recently described and found to associate with severe tumoural calcinosis of the dural and carotid arteries in a 13-year-old girl [10]. Additionally, this newly identified calciotropic factor is secreted into the pro-urine where it can hydrolyze the extracellular sugar residues of TRPV5, resulting in increased channel activity [6].

In this present study, we examined whether klotho can activate other renal ion channels than TRPV5 that are also present in DCT cells. TRP channels TRPV4, TRPV6 and TRPM6, with a structural similarity to TRPV5, were selected, expressed in cultured renal cells and subjected to an ion uptake assay, following klotho or β-glucuronidase treatment. Among the selected renal channels, we found that only cells expressing TRPV5 and TRPV6 showed an increased Ca^{2+} influx, suggesting that the stimulatory effect of klotho is exclusively restricted to these epithelial Ca^{2+} channels.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Cambrex Bio Science (Verviers, Belgium). Glutamine and HEPES were purchased from Gibco-BRL (Invitrogen, Breda, The Netherlands) and EndoF was obtained from New England Biolabs (Ipswich, MA, USA). Recombinant mouse klotho was purchased from R&D Systems (Wisconsin, IL, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture and transfection

The human embryonic kidney 293 (HEK293) cells were cultured as described previously [6]. Fifteen micrograms of pCINeo/HA-klotho-IRES-GFP [6] was transfected into cells in an 85-mm petri dish to generate klotho-containing media. For total membrane isolation and $^{45}Ca^{2+}$ uptake assay, cells were transfected with 2 μg pCINeo/IRES-GFP empty vector, pCINeo/HA-TRPV5-IRES-GFP [11], pCINeo/TRPV6-IRES-GFP [11], pCINeo/HA-TRPM6-IRES-GFP [12] or 0.5 μg pCINeo/TRPV4-IRES-GFP [13] in a six-well plate.

Preparation of klotho-containing supernatant

Three days after transfection, the klotho-containing medium was collected and concentrated 10 times in centriprep ultracel YM-30 filtration columns (Millipore, Bedford, MA, USA).

Total cell membrane isolation

Two (TRPV4, TRPV5 and TRPV6) or three days (TRPM6) after transfection, cells were homogenized in a homogenizing buffer [20 mM Tris (pH 7.4/HCl), 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM ethylenediamine tetraacetic acid (pH 8.0/NaOH), 80 mM sucrose, 1 mM phenyl-methylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml pepstatin], and subsequently centrifuged for 15 min at 4000 *g*. Supernatants were transferred to a new tube and centrifuged for an additional 30 min at 16 000 *g*.

$^{45}Ca^{2+}$-Uptake assay

Two days after transfection,transfected HEK293 cells were reseeded into 24-well plates and incubated with klotho-containing supernatant, 2 μg/ml recombinant mouse klotho, 250 μg/ml β-glucuronidase or 5.5 μg/ml endoglycosidase-F (endoF) in a 300-μl DMEM medium for an additional 16 h, respectively. Subsequent $^{45}Ca^{2+}$ (1 μCi/ml) uptake was performed as described previously [6]. Cells were washed and incubated in a depletion buffer for 10 min at 37°C and incubated in an uptake buffer for 10–30 min at 37°C. The uptake reaction was stopped by washing the cells three times with an ice-cold stop buffer. Cells were lyzed in 500 μl of 0.1% (w/v) SDS and radioactivity was counted in a liquid scintillation counter. The depletion buffer in the case of TRPM6 contained 20 mM HEPES, pH 7.4 adjusted with Tris, 110 mM NaCl, 5 mM KCl, 10 mM Na-acetate, 4 mM L-lactate, 10 mM D-glucose and 1 mM L-alanine. For TRPV4, TRPV5 and TRPV6, this depletion buffer was supplemented with 1.2 mM $MgCl_2$ and 2 mM NaH_2PO_4. For the actual uptake, 1 μCi/ml 45$CaCl_2$ and voltage-gated Ca^{2+} channel inhibitors (10 μM felodipine, 10 μM verapamil) were added to the appropriate depletion buffer. In the case of TRPV4, TRPV5 and TRPV6, 1 mM $BaCl_2$ was also added to the uptake buffer. The uptake reaction was terminated by the addition of a stop buffer, containing 110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 10 mM Na-acetate, 20 mM HEPES, pH 7.4 adjusted with Tris, 0.5 mM $CaCl_2$ and 1.5 mM $LaCl_3$ (TRPV4, TRPV5 and TRPV6), or a depletion buffer with 1.5 mM $LaCl_3$ and 0.5 mM $CaCl_2$ (TRPM6). Data were expressed as mean ± standard error of the mean (SEM). Overall statistical significance was determined by analysis of variance (ANOVA), and P-values <0.05 were considered significant.

Immunoblot analysis

Total membranes were isolated and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblots were incubated overnight at 4°C with primary antibodies including guinea pig anti-TRPV5 (1:4000) [6], rabbit anti-TRPV6 (1:2000) [6], rabbit anti-TRPV4 (1:5000) [14], mouse anti-HA (1:4000, Roche Diagnostics, Almere, The Netherlands) in 1% (w/v) non-fat dried milk in a TBS-T buffer [150 mM NaCl, 10 mM Tris (pH 7.4/HCl), 0.2% (v/v) Tween-20]. After successive washing, immunoblots were incubated at room temperature with the corresponding secondary antibodies, which were goat-anti-guinea pig IgG peroxidase (1:10 000, Sigma), sheep-anti-mouse IgG peroxidase (1:10 000, Sigma) or goat-anti-rabbit IgG peroxidase (1:5 000, Sigma).

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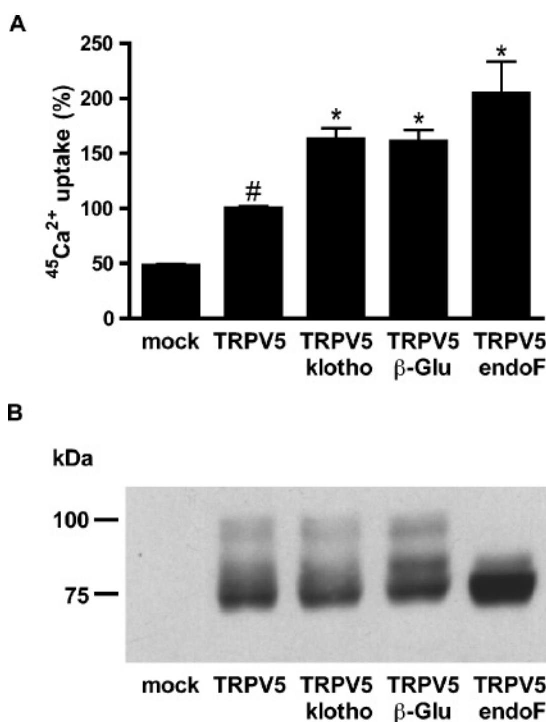

Fig. 1. Stimulation of TRPV5 activity by klotho, β-glucuronidase or endoF. (**A**) HEK293 cells were transfected with TRPV5 or empty vector (mock) cDNA. Then cells were exposed to klotho, β-glucuronidase (β-Glu) or endoF, and subsequently analysed for $^{45}Ca^{2+}$ influx analysis. # indicates a significant difference from mock-transfected HEK293 cells ($P < 0.05$, $n = 6$). * indicates a significant difference from TRPV5-transfected HEK293 cells ($P < 0.05$, $n = 6$). (**B**) Membrane fractions were isolated and treated with klotho, β-glucuronidase or endoF, and subsequently analysed by immunoblot analysis.

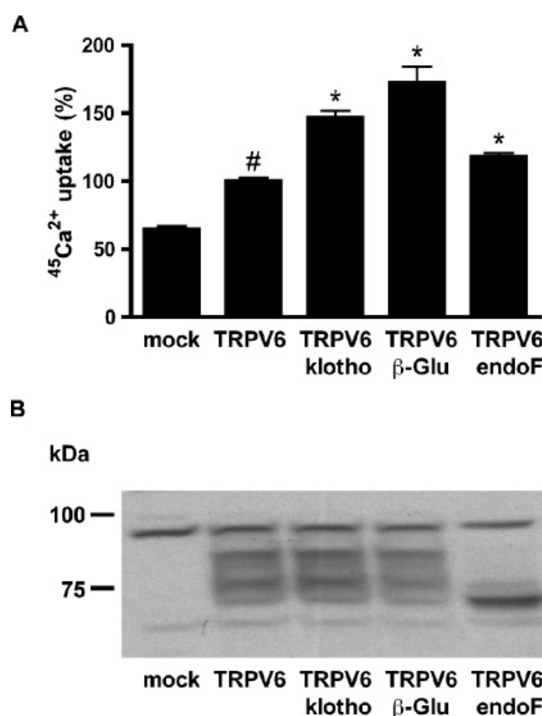

Fig. 2. Stimulation of TRPV6 activity by klotho, β-glucuronidase or endoF. (**A**) HEK293 cells were transfected with TRPV6 or mock cDNA, incubated with klotho, β-glucuronidase (β-Glu) or endoF, and $^{45}Ca^{2+}$ influx was analysed. # indicates a significant difference from mock-transfected HEK293 cells ($P < 0.05$, $n = 6$). * indicates a significant difference from TRPV6-transfected HEK293 cells ($P < 0.05$, $n = 6$). (**B**) Total cell membranes were isolated and treated with klotho, β-glucuronidase or endoF, and immunoblotted.

Results

Klotho, β-glucuronidase and endoF stimulate TRPV5 activity

To determine the effect of different glycosidase enzymes on TRPV5 channel activity, HEK293 cells transiently expressing TRPV5 were subjected to β-glucuronidase, klotho or endoF treatment prior to $^{45}Ca^{2+}$ uptake measurement. The total expression levels of TRPV5 were essentially identical in all cases (data not shown). Cells expressing the TRPV5 channel showed an approximately twofold increase of $^{45}Ca^{2+}$ influx compared to the mock-transfected cells (Figure 1A, first and second bar). Pre-incubation of cells expressing TRPV5 with klotho-containing supernatant resulted in a significant increase in TRPV5 channel activity, compared to untreated cells (Figure 1A, second bar versus third bar). In agreement with previous findings [6], β-glucuronidase-treated TRPV5-expressing cells exhibited similar $^{45}Ca^{2+}$ uptake values to cells incubated in the presence of klotho (Figure 1A, third and fourth bars). Finally, compared to the klotho- or β-glucuronidase-treated cells, we observed an additional ∼40% elevation in the activity of the TRPV5 channel after endoF incubation (Figure 1A, third and fourth bars versus last bar).

Next, we analysed how the three glycosidases affect the glycosylation of TRPV5. First, the total membrane fraction was isolated and subsequently treated with the enzymes. Immunoblot analysis with an anti-TRPV5 antibody showed that two immunoreactive bands with molecular sizes of 75 and 85 kDa, respectively, were detected in the TRPV5-expressing, but not in the mock-transfected cells. Of these, the lower one represents the core protein and the other is the complex-glycosylated form of TRPV5 (Figure 1B, lane 1 versus 2). Treatment with klotho and β-glucuronidase had virtually no effect on the migration of these bands, suggesting that these two enzymes probably cleave off only small parts of the *N*-glycan tree (Figure 1B, lanes 3 and 4). In contrast to klotho and β-glucuronidase, incubation with endoF clearly affected the migration of the TRPV5 reactive bands: the complex-glycosylated band with a mass of ∼85 kDa almost completely disappeared (Figure 1B, lane 5) [5].

TRPV6 activity is also stimulated by klotho, β-glucuronidase and endoF

We examined whether klotho can also have a stimulatory effect on TRPV6, since this channel is a close homologue of TRPV5 [5]. $^{45}Ca^{2+}$ uptake analysis demonstrated that TRPV6-expressing HEK293 cells displayed a ∼1.5-fold increase of Ca^{2+} influx compared to mock-transfected cells (Figure 2A, first bar versus second bar). After incubation with klotho-containing supernatant, the Ca^{2+} uptake

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of the TRPV6-expressing cells further increased with 40% (Figure 2A, lane 3 versus lane 2), suggesting that the stimulatory effect of klotho on TRPV6 is similar to its effect on TRPV5 as previously shown [6]. The highest TRPV6 activity, however, was observed after incubation with β-glucuronidase as the Ca^{2+} uptake of these cells was almost two times higher compared to the TRPV6-expressing cells without treatment (Figure 2A, fourth bar versus second bar). Furthermore, when endoF was applied to remove the complete *N*-glycan from TRPV6, a mild but significant increase in the TRPV6-mediated Ca^{2+} uptake was also observed (Figure 2A, last bar versus second bar).

Total membrane fractions of TRPV6-expressing HEK293 cells were subjected to klotho-, β-glucuronidase- and endoF-catalyzed glycan hydrolysis. As was the case for TRPV5, incubation with klotho and β-glucuronidase resulted in undetectable changes in the molecular weight of the complex glycosylation of TRPV6 (Figure 2B, lane 2 versus lanes 3 and 4). The complex-glycosylated TRPV6 bands at 85–100 kDa disappeared after endoF treatment, and at the same time, an increase in the amount of the 70-kDa band, representing the core protein, could be observed (Figure 2B, last lane).

TRPV4 activity is enhanced by endoF, but not by klotho or β-glucuronidase

To address the effect of β-glucuronidase and endoF, HEK293 cells transiently transfected with TRPV4 were also subjected to the $^{45}Ca^{2+}$ uptake analysis. TRPV4-transfected cells displayed an approximately twofold increase in Ca^{2+} influx compared to mock-transfected cells (Figure 3A, first bar versus second bar). Incubation with recombinant klotho or β-glucuronidase had no effect on the TRPV4-mediated Ca^{2+} influx, but the addition of endoF to the TRPV4-expressing cells significantly increased their Ca^{2+} uptake (Figure 3A, last bar versus second and third bars). In addition, the total protein expression of TRPV4 was identical under all experimental conditions (data not shown).

As was observed for TRPV5 and TRPV6 (Figures 1B and 2B), klotho or β-glucuronidase treatment had virtually no detectable effect on the glycosylation of TRPV4 (Figure 3B, second lane versus third and fourth lanes), whereas the complex glycans were almost completely removed from TRPV4 by endoF (Figure 3B, last lane versus second lane).

TRPM6 activity does not change upon klotho, β-glucuronidase or endoF treatment

The effect of β-glucuronidase on TRPM6 was also investigated. Functional analysis demonstrated that TRPM6-transfected cells exhibit a ∼1.5-fold increase of Ca^{2+} influx when compared to mock transfectants (Figure 4A, first two bars). However, neither klotho and β-glucuronidase nor endoF could further enhance the TRPM6-mediated Ca^{2+} influx (Figure 4A, last three bars versus second bar).

HEK293 cells transfected with TRPM6 were subjected to isolation of total cell membranes and to subsequent treatment with klotho, β-glucuronidase or endoF. Treatment of

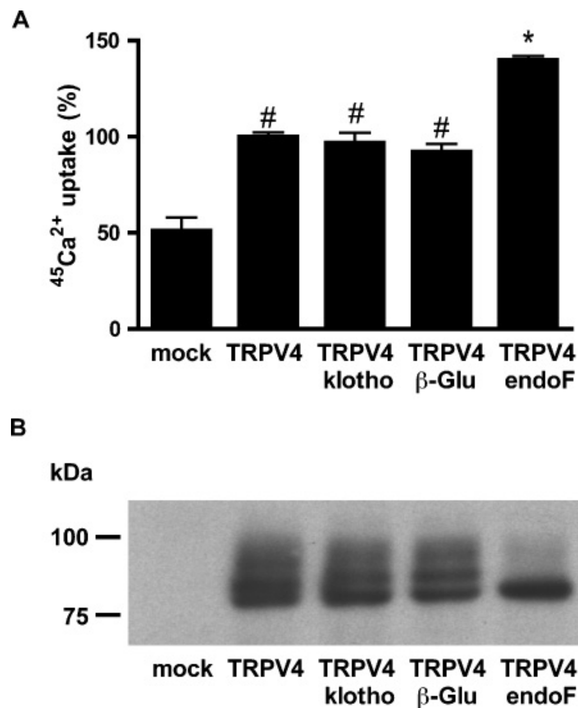

Fig. 3. EndoF-mediated deglycosylation increases the activity of TRPV4. (**A**) HEK293 cells transfected with TRPV4 and incubated with klotho, β-glucuronidase (β-Glu) or endoF were subjected to the $^{45}Ca^{2+}$ uptake assay. # indicates a significant difference from mock-transfected HEK293 cells ($P < 0.05$, $n = 6$). * indicates a significant difference from TRPV4-transfectants ($P < 0.05$, $n = 3$–6). (**B**) Isolated total membranes were treated with klotho, β-glucuronidase or endoF, and subsequently analysed by immunoblotting.

TRPM6 with these enzymes had no detectable effect on the molecular size of TRPM6 (Figure 4B, last four lanes), suggesting that the *N*-glycosylation of this protein may be different from those of TRPV5 or TRPV6.

Discussion

Klotho, a novel hormone synthesized in the kidney and secreted in the pro-urine, has recently been suggested as a new regulator of Ca^{2+} homeostasis [9]. In the kidney, secreted klotho increases the activity of TRPV5 channels in renal DCT cells [6]. Here, we investigated whether klotho or β-glucuronidase has an extended stimulatory effect among the TRP protein family members, such as TRPV4, TRPV6 and TRPM6, all known to be localized at the apical surface of DCT cells. Our most important finding is that next to TRPV5, klotho and β-glucuronidase also stimulate the homologous epithelial Ca^{2+} channel TRPV6 (Figures 1A and 2A), but do not affect TRPV4 or TRPM6 (Figures 3A and 4A). Ca^{2+} uptake experiments suggest that in the case of TRPV5, klotho and β-glucuronidase result in equally high channel activities, which was further increased by the endoF-mediated complete deglycosylation (Figure 1A). For TRPV6, the highest channel activity was observed after β-glucuronidase treatment (Figure 2A). TRPV5 and TRPV6 channels exhibit 75% homology at the amino acid level [15]. Both TRPV5 and TRPV6 have

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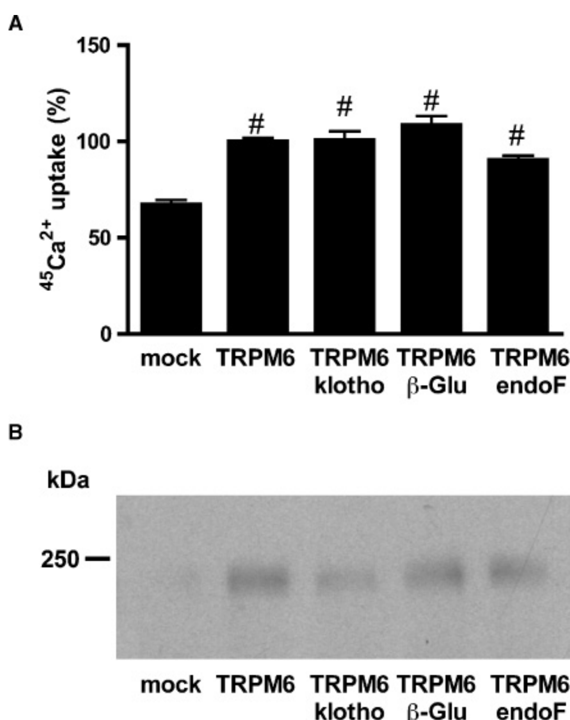

Fig. 4. TRPM6 is insensitive to klotho, β-glucuronidase or endoF treatment. (**A**) HEK293 cells expressing TRPM6 were exposed to klotho, β-glucuronidase (β-Glu) or endoF, and subsequently assayed for $^{45}Ca^{2+}$ uptake. # indicates a significant difference from mock-transfected HEK293 cells ($P < 0.05$, $n = 6$). (**B**) Total cell membranes were treated with klotho, β-glucuronidase or endoF, and subsequently analysed by immunoblot analysis.

been identified as high-mannose and complex-glycosylated proteins [5], which can take place on a conserved *N*-glycosylation site, N_{358} or N_{357}, respectively in the first extracellular loop [2]. In fact, the *N*-glycosylation-deficient mutant of TRPV5 was not activated by klotho or β-glucuronidase, although it exhibited proper membrane trafficking [6]. These findings together with the present observations suggest that the *N*-glycosylation is crucial for the klotho-mediated stimulation of TRPV5 and TRPV6.

We next examined the effect of β-glucuronidase on other members of the TRP protein family. Since deglycosylation increases TRPV4 channel activity [16], endoF was applied to remove the entire *N*-glycan of TRPV4. This resulted in an enhanced TRPV4-mediated Ca^{2+} influx (Figure 3A). However, these experiments also clearly indicated that klotho and β-glucuronidase have no effect on TRPV4 channel activity (Figure 3A). Presumably, the *N*-oligosaccharides essential for klotho or β-glucuronidase-mediated activation of TRPV5 and TRPV6 are not present in TRPV4. TRPV4 is also a Ca^{2+}-permeable cation channel, and it plays a key role in cell volume regulation [17]. In the case of TRPV4, the consensus *N*-glycosylation site N_{651} has been identified within the pore-forming loop between the fifth and sixth transmembrane segments of TRPV4 [18], whereas the *N*-glycosylated asparagines of TRPV5 and TRPV6 are located in the first extracellular loop [6]. The glycosylation-deficient mutant TRPV4-N_{651}Q displays an increased Ca^{2+} uptake resulting from the enhanced expression on the plasma membrane [18]. Our findings are in agreement with the recent study of Imura *et al.* [9], suggesting that *N*-glycosylation of TRPV4 is important in the regulation of channel activity, independent from klotho.

None of the three glycosidases (klotho, β-glucuronidase or endoF) simulated the activity of TRPM6. In the kidney, TRPM6 is predominantly present in the apical surface of DCT cells [19], where it plays a role in the maintenance of Mg^{2+} balance [20]. However, TRPM6 is also permeable for Ca^{2+} [21]. Up-to-date, detailed information regarding TRPM6 *N*-glycosylation remains elusive. Although there is a potential *N*-glycosylation site at the asparagine residue N_{787} between the first and second transmembrane segments of human TRPM6, treatment with endoF had no significant effect on the Ca^{2+} influx in cells transiently expressing TRPM6 (Figure 4A). Moreover, no difference could be observed between the untreated and the endoF-treated TRPM6 containing cell lysates after immunoblot analysis (Figure 4B).

In summary, our data indicate that klotho specifically activates the epithelial Ca^{2+} channels TRPV5 and TRPV6. In the absence of circulating klotho, TRPV5 will not be well expressed at the luminal membrane [6], resulting in Ca^{2+} wasting as it was recently demonstrated by an increased fractional excretion of Ca^{2+} in klotho-deficient mice [22]. These results altogether further substantiate the importance of klotho in maintaining the Ca^{2+} balance.

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Conflict of interest statement. None declared.

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