



Functional Analysis of TRPV6 Polymorphisms

Byung Joo Kim^{1*} and Insuk So²

¹Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan, Korea

²Department of Physiology, Seoul National University College of Medicine, Seoul, Korea

The rate-limiting step of dietary calcium absorption in the intestine requires the brush border calcium entry channel transient receptor potential vanilloid 6 (TRPV6). The putatively-selected TRPV6 haplotype contains three candidate sites for functional differences, namely derived non-synonymous substitutions C157R, M378V and M681T. Functional electrophysiological characteristics between wild-type and mutant (C157R, M378V and M681T) TRPV6 proteins were investigated by cloning the mutant TRPV6 forms, transfecting cell lines, and carrying out electrophysiology experiments via patch clamp analysis. No statistically significant differences in biophysical channel function were found although one property, namely Ca²⁺-dependent inactivation, may show functionally-relevant differences between the wild-type and mutant TRPV6 proteins. This study shows that Ca²⁺-dependent inactivation is one of the good differentiation characteristics in TRPV6, and will be useful in an advancing our knowledge about TRPV6.

Key words: Transient Receptor Potential, single nucleotide polymorphism, TRPV6

Received 15 October 2010; Revised version received 3 November 2010; Accepted 17 November 2010

Advances in DNA sequencing and single nucleotide polymorphism (SNP) genotyping technology are providing the necessary resources to make detailed inferences on the evolutionary and demographic forces that have shaped extant patterns of human genetic variation (Altshuler *et al.*, 2005; Hinds *et al.*, 2005). Stajich and Han (2005) and Akey *et al.* (2004) described a striking signature of positive selection on chromosome 7q34-35 that spans at least 115 kb and encompasses four known genes (KEL, TRPV5, TRPV6 and EPHB6). The rate of transient receptor potential vanilloid (TRPV) 6 protein evolution is significantly accelerated in the human lineage, but only for a haplotype defined by three non-synonymous SNPs (C157R, M378V and M681T) that are nearly fixed for the derived alleles in non-African populations. Interestingly, these three nonsynonymous SNPs have high posterior probabilities for being targets of positive selection, and are therefore strong candidates for mediating the population-specific signatures of selection in this region (Akey *et al.*, 2006). TRPV6 belongs to the mammalian transient

receptor potential superfamily, which consists of 27 genes organized into six subfamilies (Montell *et al.*, 2002; Clapham, 2003). TRPV6 encodes a six transmembrane polypeptide subunit that assembles into tetramers to form cation-permeable pores (Birnbaumer *et al.*, 2003), and is expressed in a wide variety of tissues but is found at the highest levels in the kidney, placenta and intestine (van de Graaf *et al.*, 2004).

TRPV5 and TRPV6 are distinct from other TRP family members as they are the most calcium-selective, and possess strong inwardly rectifying currents (*i.e.*, the inward currents are much larger than outward currents) (Clapham, 2003) and very positive reversal potentials if Ca²⁺ is the charge carrier. Also, both proteins are blocked by Mg²⁺ (Clapham, 2003). Additionally, they have Ca²⁺-dependent inactivation characteristics, and are thought to serve as the rate-limiting step of dietary calcium absorption (Clapham, 2003; Nijenhuis *et al.*, 2003; van de Graaf *et al.*, 2003).

The TRPV6 haplotype was suggested to exist as three non-synonymous polymorphisms, C157R, M378V, and M681T (Akey *et al.*, 2006; Suzuki *et al.*, 2008); and this haplotype produces a gain of function channel. This suggests that the haplotype causes hyperactivation of intestinal Ca²⁺ absorption which in turn leads to absorptive hypercalciuria (Suzuki *et al.*, 2008). However, there are no data about the functional electrophysiological characteristics of these TRPV6 haplotypes.

*Corresponding author: Byung Joo Kim, Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Beomeo-ri, Mulgeum-eup, Yangsan, Gyeongnam 626-870, Korea
Tel: +82-51-510-8469
Fax: +82-51-510-8420
E-mail: vision@pusan.ac.kr

Therefore, in the present study we made seven mutant TRPV6 forms and investigated the functional electrophysiological characteristics of these TRPV6 mutant types.

Materials and Methods

Plasmid constructs and cell culture

GFP-tagged constructs were generated by cloning of TRPV6^{WT}, TRPV6^{C157R}, TRPV6^{M378V}, TRPV6^{M681T}, TRPV6^{C157R and M378V}, TRPV6^{C157R and M681T}, TRPV6^{M378V and M681T}, and TRPV6^{C157R, M378V and M681T}. The constructs were validated by full-length cDNA sequencing. Human embryonic kidney (HEK 293) cells were used for electrophysiological studies. Each construct was transfected with FuGene-6 reagent according to manufacturer's instruction (Roche, Nutley, NJ, USA). HEK293 cells were grown in DMEM containing 10% (v/v) human serum, 2 mM L-glutamine, 2 U/mL penicillin and 2 mg/mL streptomycin at 37°C in a humidity-controlled incubator with 10% CO₂. HEK293 cells were transiently transfected with the vector constructs. Transfected cells were visually identified in the patch clamp apparatus by their GFP fluorescence.

Microscopy

The fixed cells were imaged using 63X (plan apochromate, 1.4 NA) objective mounted on a Leica SP5 laser scanning microscope (LSM) (Leica, Bensheim, Germany). Cells transfected with GFP constructs were excited at 488 nm and the emission was recorded at 495–530 nm. The images shown were representatives of at least three independent transfections. To determine the localization of TRPV6 from at least three independent transfections were analyzed by LSM and expression of TRPV6 was classified into plasma membrane, endoplasmic reticulum (ER) or mixed (both plasma membrane and ER localization).

Electrophysiology

Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature with three solutions: 1) standard internal solution (145 mM Cs glutamate, 10 mM HEPES, 8 mM NaCl, 1 mM MgCl₂, 2 mM Mg-ATP, and 10 mM EGTA; adjusted to pH 7.2 with CsOH), 2) extracellular solution (145 mM NaCl, 2 mM CaCl₂, 10 mM CsCl, 2.8 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES; adjusted to pH 7.4 with NaOH), and 3) divalent-free (DVF) solution (145 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 10 mM glucose, 10 mM EGTA, 10 mM HEPES; adjusted to pH 7.4 with NaOH).

The ramp protocol consisted of linear voltage ramps changing from –100 mV to +100 mV within 400 msec, applied every

5 sec from a holding potential of +20 mV. The step protocol consisted of a series of 400 msec long voltage steps applied from a holding potential of +20 mV to –100 mV. Current densities, expressed per unit membrane capacitance, were measured from the current at –100 mV during the ramp protocols. Osmotic differences were corrected by adding mannitol to the extracellular solution. Membrane currents, filtered at 5 kHz, were recorded using Axopatch 200B patch clamp amplifier (Axon Instruments, Union City, CA, USA). A gigaseal was achieved by glass pipettes with a resistance of ~2–4 MΩ. Before seal formation, the cells were kept in Ca²⁺-free medium and then exposed to the extracellular solution containing 2 mM Ca²⁺ for up to 5 min. The pClamp V8.0 and Digidata-1200 (Axon Instrument) were used for data acquisition and command pulse application. The sampling rate was 2 kHz.

Statistics

All data are expressed as mean ± SE. Student's *t*-test for unpaired data was used to compare the control and the experimental groups. A *P* value of less than 0.05 was considered to indicate statistically significant differences.

Results

Localization of TRPV6^{WT} in HEK 293 cells

To determine the role of TRPV6 in membrane trafficking, TRPV6 was tagged with GFP fluorescent tags. The intracellular localization of TRPV6-GFP was analyzed by confocal LSM. For this purpose, TRPV6^{WT} constructs were transiently transfected into HEK 293 cells. Figure 1 reveals the localization of TRPV6-GFP in both plasma membrane and ER compartments in HEK 293 cells.

Current–voltage relationships for TRPV6 haplotypes

We made the putatively-selected TRPV6 haplotype that is defined by the three amino acid substitutions: C157R, M378V, and M681T. We also made the double- or triple-mutated TRPV6 forms: C157R and M378V; C157R and M681T; M378V and M681T; C157R, M378V and M681T. No differences in the expression levels or surface expression of each TRPV6 were observed. Figure 1 shows a typical patch clamp experiment in which a voltage ramp protocol was used under different ion conditions. In the absence of any divalent cations (DVF) in the bath solution, large and strongly inward rectifying currents were measured which reversed close to 0 mV (Figure 2A). In this case, the current was mainly carried by Na⁺. This is the typical hallmark of TRPV6 currents (Vennekens *et al.*, 2000; Nilius *et al.*, 2000, 2001a). However,

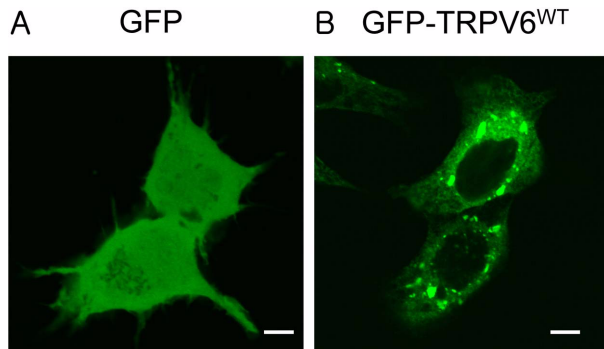


Figure 1. TRPV6^{WT} is localized in endoplasmic reticulum (ER) in HEK 293 cells. HEK 293 cells were transiently transfected with GFP (A) or TRPV6-GFP (B) and fixed 48 hours after transfection. The maximum intensity projections (MIP) taken with the laser scanning microscope (LSM), reveal both plasma membrane and ER localization of TRPV6^{WT} in HEK 293 cells. Bar=5 μ m.

when Ca^{2+} ions was the only charge carrier for inward currents, the reversal potentials were shifted to positive, indicating high selectivity for these divalent cations (Figure 2A). In case of C157R, M378V and M681T, there were not any differences compared to wild-type TRPV6 (Figures 2B-2D). Similarly, in

the cases of double- or triple-mutant types, no differences were observed when compared to wild-type TRPV6 (Figures 3A-3D). This experiment shows that major changes in permeability cannot be expected between the wild-type and the mutant TRPV6 forms.

Analyses of the features of TRPV6: current density, Ca^{2+} -dependent inactivation and blocking by Mg^{2+}

To compare these functional electrophysiological data statistically, we measured several parameters. First, current density at -100 mV did not differ significantly between the wild-type and mutant TRPV6 forms under the conditions: DVF and charge carrier Ca^{2+} , respectively (Figures 4A and 4B). Second, the same holds true when data could only be normalized to the DVF current at -100 mV (Figures 4C and 4D).

Next, as an estimate for Ca^{2+} -dependent inactivation, we compared the ratio of the currents at the end of the 400 ms voltage step to -100 mV and the maximal inward current during the step ($I_{400\text{ms}^*-100\text{mV}}/I_{\text{max}}$) for each TRPV6 forms. This ratio reflects the extent of negative feedback inhibition of TRPV6 by Ca^{2+} . No inactivation is present under the DVF

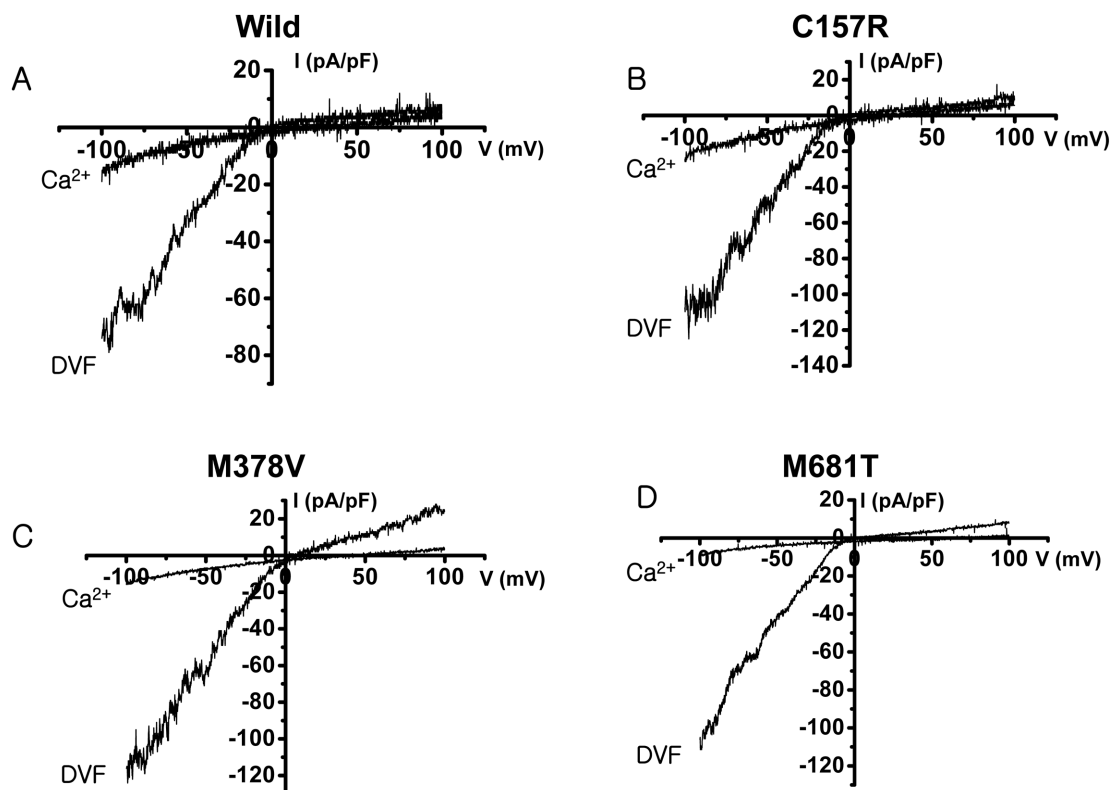


Figure 2. Current-voltage relationships (I-V curves) for the wild and mutant of TRPV6. A) IV curves for wild form obtained from the voltage ramps from -100 mV to $+100$ mV at the holding potential of -60 mV. Note the strong inward rectification in DVF and the shift of the reversal potentials when Ca^{2+} is the charge carrier (DVF: divalent free solution, Ca^{2+} : 30 mM CaCl_2 with NMDG^+ as substitute for Na^+). B) IV curves for C157R form, using the same protocol as in A. C) IV curves for M378V form, using the same protocol as in A. D) IV curves for M681T form, using the same protocol as in A.

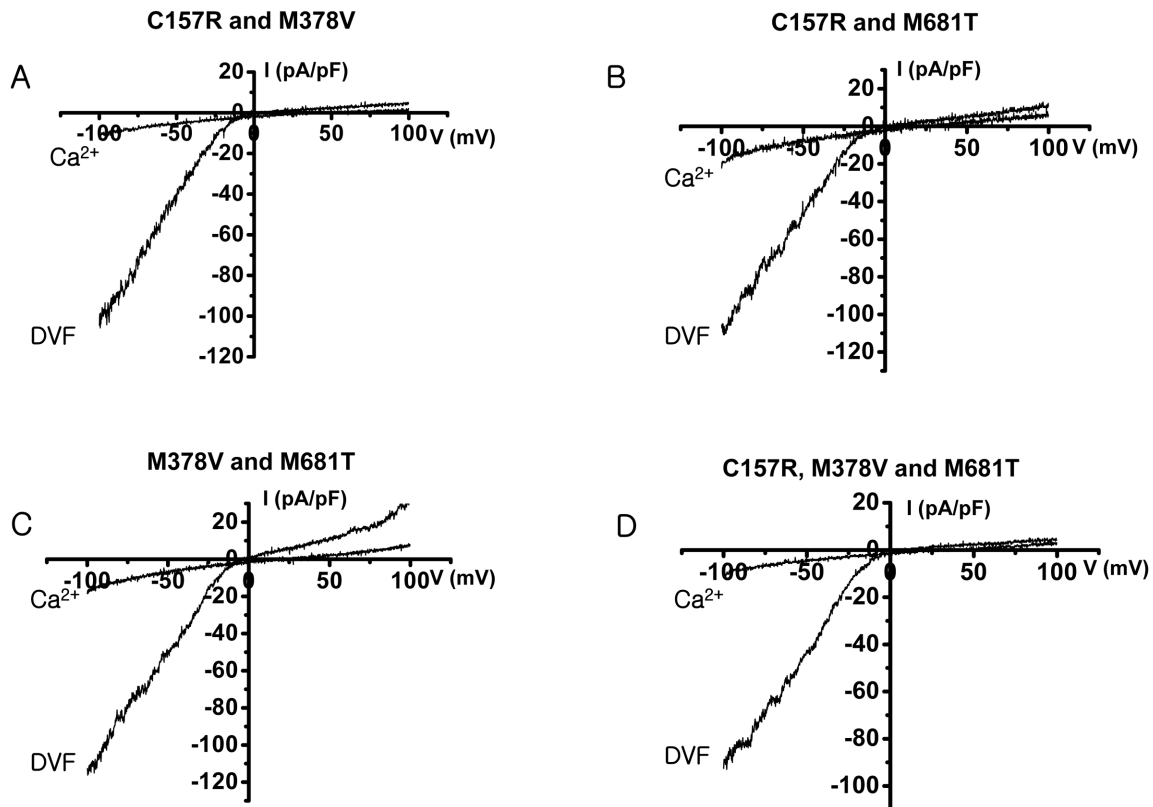


Figure 3. I-V curves for the mutant of TRPV6. A) IV curves for C157R and M378V form obtained from the voltage ramps from -100 mV to +100 mV at the holding potential of -60 mV. Note the strong inward rectification in DVF and the shift of the reversal potentials when Ca^{2+} is the charge carrier (DVF: divalent free solution, Ca^{2+} : 30 mM CaCl_2 with NMDG $^{+}$ as substitute for Na^{+}). B) IV curves for C157R and M681T form, using the same protocol as in A. C) IV curves for M378V and M681T form, using the same protocol as in A. D) IV curves for C157R, M378V and M681T form, using the same protocol as in A.

condition. In the presence of 30 mM Ca^{2+} , inactivation of $34.75 \pm 0.8\%$ in wild-type; $35.64 \pm 1.1\%$ in C157R; $35.75 \pm 1.3\%$ in M378V; $36.81 \pm 1.0\%$ in M681T; $33.72 \pm 1.2\%$ in C157R and M378V; $37.25 \pm 0.6\%$ in C157R and M681T; $34.26 \pm 0.8\%$ in M378V and M681T; and $41.25 \pm 0.8\%$ in C157R, M378V and M681T was observed (Figures 5A and 5B). We additionally measured the current decrease when 1 mM Mg^{2+} was added to the DVF solution in voltage step experiments. Currents were inhibited by $\sim 50\%$ and no significant difference between each TRPV6 form was observed (Figure 5C).

Discussion

The presence of three amino acid substitutions (C157R, M378V, and M681T) on a recently selected haplotype suggests that one (or more) of these substitutions may result in some phenotypic difference (Akey *et al.*, 2006). We therefore investigated possible functional differences between the wild and mutant TRPV6 forms by cloning each form into expression vectors and transfecting the constructs into a HEK 293 cell line. We then used patch clamp analysis to investigate the

electrophysiology of the TRPV6 calcium channel in HEK 293 cells expressing each TRPV6 proteins. The intracellular expression pattern of both constructs was assessed by epifluorescence microscopy and revealed largely plasma membrane-bound GFP fluorescence, compatible with surface expression of the proteins. However, there are no the differences about current-voltage relationship between the wild-type and the mutant TRPV6 forms. This is not surprising because the pore region, which is responsible for this unique permeation pattern (Nilius *et al.*, 2001b; Voets *et al.*, 2003a, 2003b), is the same in each TRPV6 forms. Only Ca^{2+} -dependent inactivation is a good discrimination criteria in TRPV6. Inactivation is a process which is mediated by Ca^{2+} itself entering the cell through the channel (Hoenderop *et al.*, 2001; Nilius *et al.*, 2000, 2002, 2003). It has been shown in detail that extracellular Mg^{2+} blocks both TRPV6 currents (Hoenderop *et al.*, 2001; Nilius *et al.*, 2001a; Vennekens *et al.*, 2001; Hoenderop *et al.*, 2005).

Suzuki *et al.* (2008) suggested that a haplotype consisting of three polymorphisms (C157R, M378V and M681T) in the TRPV6 gene was found in Ca^{2+} stone patients. The frequency

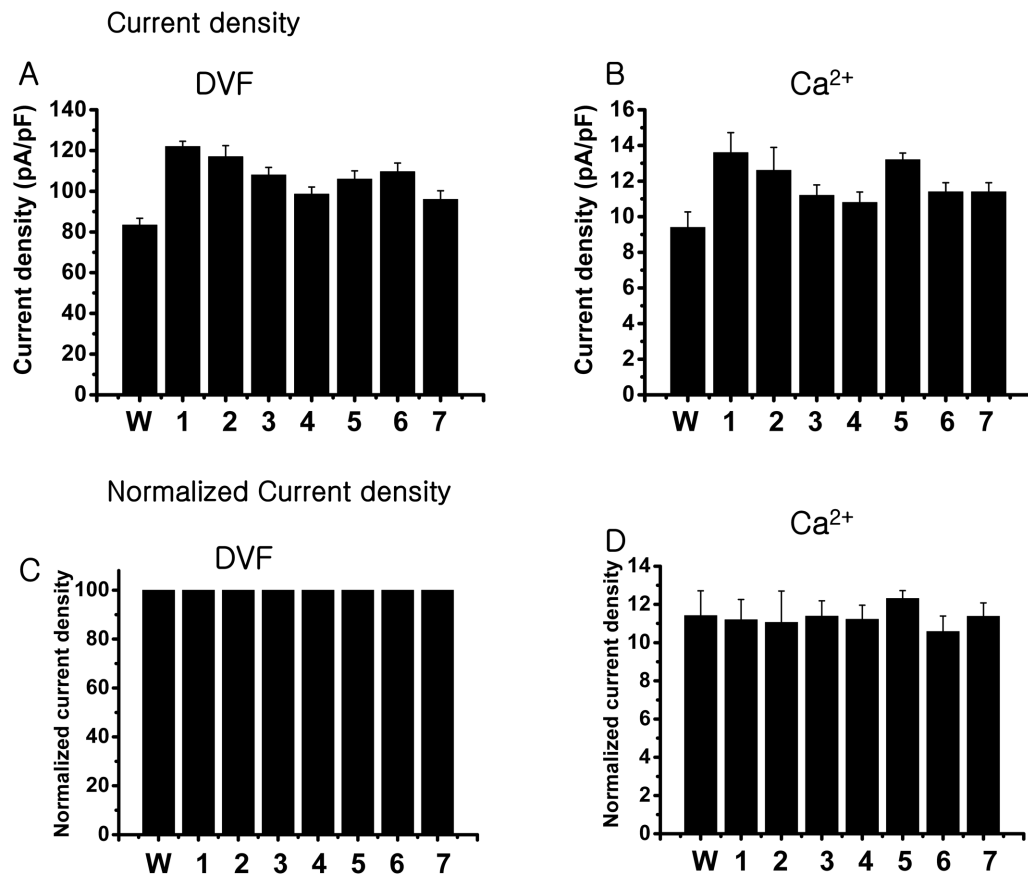


Figure 4. Analyses of key feature of TRPV6: current density. A and B) Current density was measured at -100 mV under DVF and Ca^{2+} ionic conditions for each TRPV6. DVF: divalent free; Ca^{2+} refer to these divalent cations as the only charge carrier for the inward currents. Note that high current densities have been measured indicating high functional expression of the channels. C and D) Current densities were normalized to the current in DVF solution at -100 mV. There are no any differences among each TRPV6 forms. Data from between 4 and 5 cells for all graphs (W: wild; 1: C157R; 2: M378V; 3: M681T; 4: C157R and M378V; 5: C157R and M681T; 6: M378V and M681T; 7: C157R, M378V and M681T).

of the ancestral haplotype (R+V+T) is higher in patients who formed Ca^{2+} stones (8.4 %) when compared to a cohort of individuals who did not (5.4 %). This suggests that the ancestral haplotype is a risk factor for Ca^{2+} stone formation.

One of the amino acid differences of TRPV6, C157R, is located between the third and the fourth ankyrin repeats of TRPV6 which have been shown to be functionally important for multimerization, may initiate a molecular zipper process for protein-protein interaction, and may create an intracellular anchor necessary for functional subunit assembly (Erler *et al.*, 2004). Therefore, a C157R mutation could be functionally significant in protein-protein interactions, and we cannot exclude at this point that protein-protein interactions might differ between each TRPV6 forms. However, channel function *per se* is not significantly changed by the C157R mutation.

It is more difficult to predict any functional changes for the M378V mutant that is located extracellularly in the loop between the first and the second transmembrane helices,

TM1 and TM2. The residue M681T is located in a functionally important region very close to the putative Ca^{2+} /calmodulin binding site between residues 691 and 711 (Nilius *et al.*, 2003). Ca^{2+} /calmodulin binding to this region initiates Ca^{2+} -dependent fast inactivation of TRPV6 which can be reversed by protein kinase C (PKC) dependent phosphorylation (Niemeyer *et al.*, 2001). The inactivation in the Ca^{2+} entry is due to channels closing (*i.e.*, inactivation), a process which is mediated by Ca^{2+} itself entering the cell through the channel and is known as Ca^{2+} -dependent inactivation (Hoenderop *et al.*, 2001; Nilius *et al.*, 2001, 2002, 2003). Ca^{2+} -dependent inactivation acts negative feedback for Ca^{2+} entry through the channel.

Our analysis of several electrophysiological characteristics of TRPV6 demonstrated no significant differences between the mutant types. However, the observed difference in Ca^{2+} -dependent inactivation may nonetheless be biologically relevant. If it were, then the triple-mutant form of TRPV6

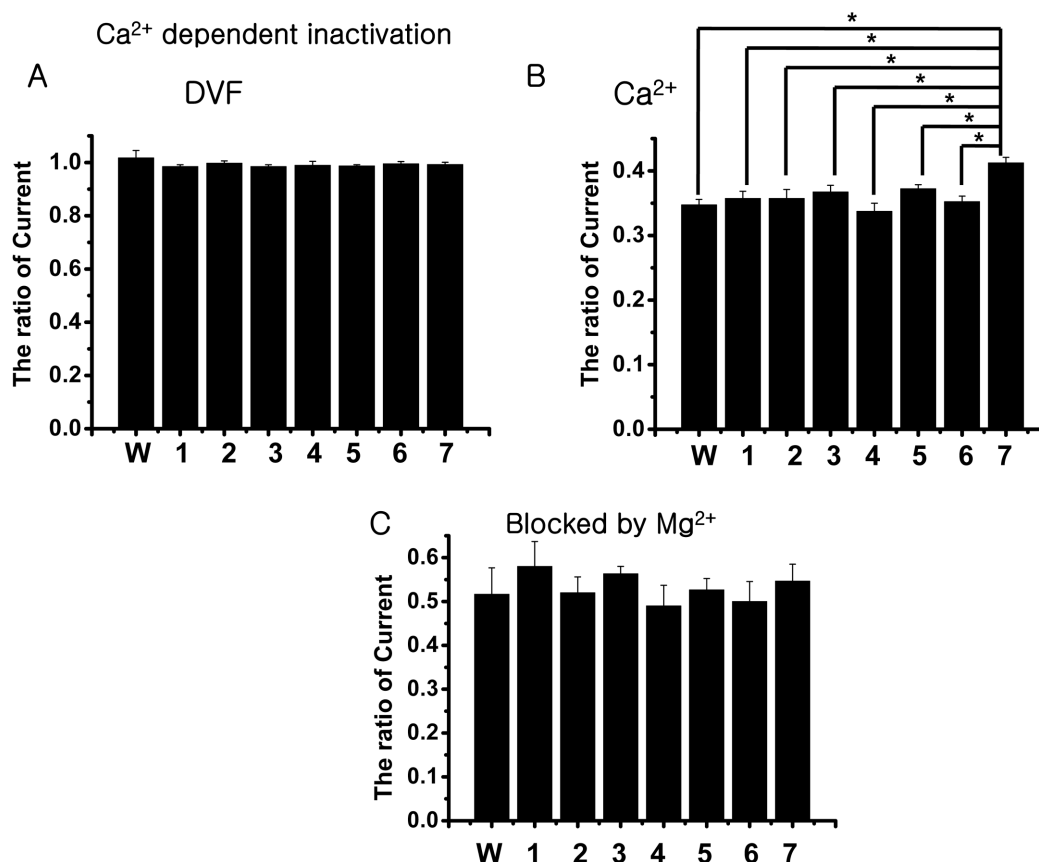


Figure 5. Analyses of key feature of TRPV6: Ca^{2+} dependent inactivation and block by Mg^{2+} . A and B) Ca^{2+} dependent inactivation was estimated by the ratio of the currents at the end of the 400 ms voltage step and the maximal inward current during the step ($I_{400\text{ms}, -100\text{mV}}/I_{\text{max}}$). C) Block of TRPV6 by Mg^{2+} was estimated from step protocols. $I_{1\text{mM Mg}^{2+}, -100\text{mV}}/I_{\text{DVF}, -100\text{mV}}$ is the ratio of the maximal inward current measured from the same cell after changing from DVF to addition of 1 mM Mg^{2+} . (1-ratio) gives the inhibition. Data from between 4 and 5 cells for all graphs (W: wild; 1: C157R; 2: M378V; 3: M681T; 4: C157R and M378V; 5: C157R and M681T; 6: M378V and M681T; 7: C157R, M378V and M681T). * $P < 0.05$.

would be “close” more quickly in response to Ca^{2+} feedback, signifying that it would reabsorb less Ca^{2+} than the other forms. Nonetheless, it is possible that other aspects of TRPV6 could still be affected, such as the putative site of protein-protein interaction in the N-terminus or the site close to a region which is critical for Ca^{2+} dependent inactivation (Nilius *et al.*, 2003). Alternatively, it may be that the amount of TRPV6 channel differs because of expression or stability differences *in vivo*. Despite these extensive electrophysiological experiments, the phenotypic consequences of these different TRPV6 haplotypes remain mysterious. These properties should be explored in the future.

In a Ca^{2+} transport assay, while the 157R+681T construct did not significantly increase Ca^{2+} transport activity, the single extracellular polymorphism (378V) significantly increased Ca^{2+} transport activity, suggesting that 378V mainly contributes to the increase in Ca^{2+} transport activity of TRPV6 (Suzuki *et al.*, 2008). However, only a single mutant type was investigated and the double and triple mutant type was not. The

electrophysiological characteristics of TRPV6 mutant type are different from the results of Ca^{2+} transport assay. This results showed that the characteristics of Ca^{2+} measurement is different from electrophysiological characteristics in TRPV6 and biophysical properties of TRPV6 have a complex Ca^{2+} regulatory circuit.

TRPV6 is an epithelial calcium channel which is thought to be the primary protein in the absorption of calcium in the intestine and bone, and hence is a key to absorbing calcium from the diet (Nijenhuis *et al.*, 2003; Hoenderop *et al.*, 2005; Lieben *et al.*, 2010). The TRPV6 triple mutant form may be a crucial regulator of this process and mediator of calcium transfer across the intestinal apical membrane. Some reports suggest that Ca^{2+} -sensing receptor (CaR) polymorphisms are involved in hypercalciuria without kidney stones (Vezzoli *et al.*, 2007). These findings indicate that several genes contribute to hypercalciuria. However, there is no report thus far indicating an association between hypercalciuria with Ca^{2+} stone formation and genetic variations of the genes

responsible for epithelial Ca^{2+} transport, which could directly affect urine Ca^{2+} levels. Therefore, the TRPV6 triple-mutant type may be one of the causes of this disease and a therapeutic target of drugs.

In conclusion, our results show that Ca^{2+} dependent inactivation is a good differentiation characteristic in TRPV6, and this study is useful in advancing our knowledge about TRPV6.

Acknowledgment

This work was supported by Mid-career Researcher Program through NRF grant funded by the MEST (No. 2010-0027710).

References

- Altshuler, D., Brooks, L.D., Chakravarti, A., Collins, F.S., Daly, M.J. and Donnelly, P. (2005) International HapMap Consortium A haplotype map of the human genome. *Nature* 437(7063), 1299-1320.
- Akey, J.M., Eberle, M.A., Rieder, M.J., Carlson, C.S., Shriver, M.D., Nickerson, D.A. and Kruglyak, L. (2004) Population history and natural selection shape patterns of genetic variation in 132 Genes. *PLoS Biol.* 2, e286.
- Akey, J.M., Swanson, W.J., Madeoy, J., Eberle, M. and Shriver, M.D. (2006) TRPV6 exhibits unusual patterns of polymorphism and divergence in worldwide populations. *Hum. Mol. Genet.* 15(13), 2106-2113.
- Birnbaumer, L., Yildirim, E., Abramowitz, J. and Yidirim, E. (2003) A comparison of the genes coding for canonical TRP channels and their M, V and P relatives. *Cell Calcium* 33(5-6), 419-432.
- Clapham, D.E. (2003) TRP channels as cellular sensors. *Nature* 426(6966), 517-524.
- Erler, I., Hirnet, D., Wissenbach, U., Flockerzi, V. and Niemeyer, B.A. (2004) Ca^{2+} -selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. *J. Biol. Chem.* 279(33), 34456-34463.
- Hinds, D.A., Stuve, L.L., Nilsen, G.B., Halperin, E., Eskin, E., Ballinger, D.G., Frazer, K.A. and Cox, D.R. (2005) Whole-genome patterns of common DNA variation in three human populations. *Science* 307(5712), 1072-1079.
- Hoenderop, J.G., Nilius, B. and Bindels, R.J. (2005) Calcium absorption across epithelia. *Physiol. Rev.* 85(1), 373-422.
- Hoenderop, J.G., Vennekens, R., Muller, D., Prenen, J. and Droogmans, G. (2001) Function and expression of the epithelial Ca^{2+} channel family: comparison of the epithelial Ca^{2+} channel 1 and 2. *J. Physiol. (Lond.)* 537(3), 747-761.
- Hughes, D.A., Tang, K., Strotmann, R., Schöneberg, T., Prenen, J., Nilius, B. and Stoneking, M. (2008) Parallel selection on TRPV6 in human populations. *PLoS ONE* 3(2), e1686.
- Lieben, L., Benn, B.S., Ajibade, D., Stockmans, I., Moermans, K., Hediger, M.A., Peng, J.B., Christakos, S., Bouillon, R. and Carmeliet, G. (2010) Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. *Bone* 47(2), 301-308.
- Montell, C., Birnbaumer, L. and Flockerzi, V. (2002) The TRP channels, a remarkably functional family. *Cell* 108(5), 595-598.
- Niemeyer, B.A., Bergs, C., Wissenbach, U., Flockerzi, V. and Trost, C. (2001) Competitive regulation of CaT-like-mediated Ca^{2+} entry by protein kinase C and calmodulin. *Proc. Natl. Acad. Sci. USA* 98(6), 3600-3605.
- Nijenhuis, T., Hoenderop, J.G., Nilius, B. and Bindels, R.J. (2003) Pathophysiological implications of the novel epithelial Ca^{2+} channels TRPV5 and TRPV6. *Pflügers Arch.* 446(4), 401-409.
- Nilius, B., Prenen, J., Hoenderop, J.G., Vennekens, R., Hoefs, S., Weidema, A.F., Droogmans, G. and Bindels, R.J. (2002) Fast and slow inactivation kinetics of the Ca^{2+} channels ECaC1 and ECaC2 (TRPV5 and TRPV6). Role of the intracellular loop located between transmembrane segments 2 and 3. *J. Biol. Chem.* 277(34), 30852-30858.
- Nilius, B., Prenen, J., Vennekens, R., Hoenderop, J.G., Bindels, R.J., Droogmans, G. and Nilius, B. (2001a) Modulation of the epithelial calcium channel, ECaC, by intracellular Ca^{2+} . *Cell Calcium* 29(6), 417-428.
- Nilius, B., Vennekens, R., Prenen, J., Hoenderop, J.G., Bindels, R.J. and Droogmans, G. (2000) Whole-cell and single channel monovalent cation currents through the novel rabbit epithelial Ca^{2+} channel ECaC. *J. Physiol. Lond.* 527(2), 239-248.
- Nilius, B., Vennekens, R., Prenen, J., Hoenderop, J.G., Droogmans, G. and Bindels, R.J. (2001b) The single pore residue Asp542 determines Ca^{2+} permeation and Mg^{2+} block of the epithelial Ca^{2+} channel. *J. Biol. Chem.* 276(2), 1020-1025.
- Nilius, B., Weidema, F., Prenen, J., Hoenderop, J.G., Vennekens, R., Hoefs, S., Droogmans, G. and Bindels, R.J. (2003) The carboxyl terminus of the epithelial Ca^{2+} channel ECaC1 is involved in Ca^{2+} -dependent inactivation. *Pflügers Arch.* 445(5), 584-588.
- Stajich, J.E. and Hahn, M.W. (2005) Disentangling the effects of demography and selection in human history. *Mol. Biol. Evol.* 22(1), 63-73.
- Suzuki, Y., Pasch, A., Bonny, O., Mohaupt, M.G., Hediger, M.A. and Frey, F.J. (2008) Gain-of-function haplotype in the epithelial calcium channel TRPV6 is a risk factor for renal calcium stone formation. *Hum. Mol. Genet.* 17(11), 1613-1618.
- van de Graaf, S.F., Boullart, I., Hoenderop, J.G. and Bindels, R.J. (2004) Regulation of the epithelial Ca^{2+} channels TRPV5 and TRPV6 by 1- α ,25-dihydroxy vitamin D3 and dietary Ca^{2+} . *J. Steroid Biochem. Mol. Biol.* 89-90(1-5), 303-308.
- van de Graaf, S.F., Hoenderop, J.G., Gkika, D., Lamers, D., Prenen, J., Rescher, U., Gerke, V., Staub, O., Nilius, B. and Bindels, R.J. (2003) Functional expression of the epithelial Ca^{2+} channels TRPV5 and TRPV6 requires association of the S100A10-annexin 2 complex. *EMBO J.* 22(7), 1478-1487.
- Vennekens, R., Hoenderop, J.G., Prenen, J., Stuijver, M., Willems, P.H., Droogmans, G., Nilius, B. and Bindels, R.J. (2000) Permeation and gating properties of the novel epithelial Ca^{2+} channel. *J. Biol. Chem.* 275(6), 3963-3969.
- Vennekens, R., Prenen, J., Hoenderop, J.G., Bindels, R.J., Droogmans, G. and Nilius, B. (2001) Pore properties and ionic block of the rabbit epithelial calcium channel expressed in HEK 293 cells. *J. Physiol.* 530(2), 183-191.
- Vezzoli, G., Terranegra, A., Arcidiacono, T., Biasion, R., Coviello, D., Syren, M.L., Paloschi, V., Giannini, S., Mignogna, G., Rubinacci, A., Ferraretto, A., Cusi, D., Bianchi, G. and Soldati, L. (2007) R990G polymorphism of calcium-sensing receptor does produce a gain-of-function and predispose to primary hypercalciuria. *Kidney Int.* 71(11), 1155-1162.
- Voets, T., Janssens, A., Droogmans, G. and Nilius, B. (2003a) A single pore residue determines calcium selectivity, inward rectification and pore size of TRPV6 (CaT1/EcaC2). *Biophys. J. (Annual Meeting Abstracts)* 2724-Pos.
- Voets, T., Janssens, A., Prenen, J., Droogmans, D., Nilius, G. (2003b) Mg^{2+} -dependent gating and strong inward rectification of the cation channel TRPV6. *J. Gen. Physiol.* 121(3), 245-260.