

Down-regulation of TRPM6-mediated magnesium influx by cyclosporin A

Akira Ikari · Chiaki Okude · Hayato Sawada ·
Tadanobu Takahashi · Junko Sugatani · Masao Miwa

Received: 24 July 2007 / Accepted: 26 October 2007 / Published online: 17 November 2007
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Abstract Transient receptor potential melastatin 6 (TRPM6) is distributed along the apical membrane of the renal tubular cells and is involved in the reabsorption of magnesium. In this study, we show that TRPM6 expression is suppressed by cyclosporin A (CsA) via a down-regulation of c-Fos expression. TRPM6 was expressed in NRK-52E, but not in Madin-Darby canine kidney cells. In contrast, its homolog, TRPM7, was equally expressed in both cells. In NRK-52E cells, CsA dose-dependently decreased TRPM6 expression without affecting TRPM7 expression. Magnesium load measurements revealed the rise in the intracellular free magnesium concentration ($[Mg^{2+}]_i$) to be inhibited by CsA. The transfection of TRPM6 siRNA decreased TRPM6 expression without affecting TRPM7 expression and inhibited the elevation of $[Mg^{2+}]_i$. CsA did not affect the intracellular distribution of nuclear factor of activated T cells (NFATc). Furthermore, TRPM6 expression was not changed by a NFATc inhibitor. Next, we examined the effect of CsA on the transcription factors c-Fos and c-Jun. CsA decreased c-Fos expression without affecting c-Jun expression. The transfection of c-Fos siRNA suppressed TRPM6 expression without affecting TRPM7

expression. We suggest that CsA decreases TRPM6 expression mediated by inhibition of c-Fos transcription, resulting in a decrease of renal Mg^{2+} reabsorption.

Keywords c-Fos · Cyclosporin A · Transient receptor potential melastatin 6 · Magnesium · Kidney

Abbreviations

CsA cyclosporin A
NFATc nuclear factor of activated T cells
 $[Mg^{2+}]_i$ intracellular free magnesium concentration
TRPM6 transient receptor potential melastatin 6

Introduction

Cyclosporin A (CsA) and tacrolimus (FK506) are used for therapeutic purposes such as organ transplantation, and to treat allergies and autoimmune disease. These drugs are the most potent immunosuppressive agents available today. However, their clinical application can have severe adverse effects including nephrotoxicity, neurotoxicity, and disruptions of mineral homeostasis (Kahan 1989; Bennett 1995; Taque et al. 2004), and so their prolonged administration has been restricted. Two main forms of nephrotoxicity are recognized: an acute impairment of renal function resulting from vasoconstriction and hemodynamic abnormality (Remuzzi and Perico 1995), and a progressive and irreversible impairment of renal function resulting from interstitial fibrosis and tubular atrophy (Myers et al. 1984). The molecular mechanism responsible for the development of CsA-induced nephrotoxicity still remains unclear.

The mechanisms of CsA's actions in T cells are well characterized. Early biological studies revealed that CsA

A. Ikari (✉) · C. Okude · H. Sawada · T. Takahashi ·
J. Sugatani · M. Miwa
Department of Pharmacology-Biochemistry,
School of Pharmaceutical Sciences, University of Shizuoka,
52-1 Yada, Suruga-ku,
Shizuoka 422-8526, Japan
e-mail: ikari@u-shizuoka-ken.ac.jp

J. Sugatani
COE Program in the 21st Century,
School of Pharmaceutical Sciences, University of Shizuoka,
52-1 Yada, Suruga-ku,
Shizuoka 422-8526, Japan

inhibits T-cell activation by blocking the transcription of cytokine genes. CsA binds with high affinity to cyclophilins which are ubiquitous cytosolic proteins with peptidyl-proline-*cis-trans* isomerase activity (Liu et al. 1992). Cyclophilin-CsA complex can associate with calcineurin which belongs to a superfamily of protein serine/threonine phosphatases and inhibit the phosphatase activity. Calcineurin dephosphorylates the nuclear factor of activated T cells (NFATc), translocates it into the nucleus, and activates the gene expression of interleukin-2 and interleukin-4 (Flanagan et al. 1991). CsA inhibits the nuclear translocation of NFAT family members and subsequent gene expression. Furthermore, CsA affects other transcription factor such as AP-1, i.e. c-Fos, and c-Jun (Rincón and Flavell 1994). However, the CsA-targeting factors in nonimmune cells are not well understood.

Hypomagnesemia is frequently encountered in patients treated with CsA (Barton et al. 1987; McDiarmid et al. 1993). Asai et al. (2002) reported that magnesium supplementation prevented CsA-induced nephrotoxicity independently of the rennin-angiotensin system in rats maintained on a low-sodium diet. The disturbance of magnesium homeostasis may be an important component of nephrotoxicity. To prevent or lessen nephrotoxicity, it is important to understand the mechanisms by which CsA causes renal magnesium wasting. Unfortunately, the pathway for the reabsorption of magnesium has remained unclear until recently. Transient receptor potential melastatin 6 (TRPM6) is a cognate protein which constitutes the apical magnesium entry channel in the active transcellular transport of magnesium (Schlingmann et al. 2002; Walder et al. 2002). TRPM7, a close homologue of TRPM6, was characterized as a constitutively active ion channel permeable for divalent cations including magnesium and calcium (Runnels et al. 2002; Schmitz et al. 2003). TRPM6 is expressed predominantly in the intestine and kidney, and may be involved in the absorption and reabsorption of magnesium. In contrast, TRPM7 is ubiquitously expressed in various cells and plays a crucial role in cellular magnesium homeostasis. At present, the effect of CsA on TRPM6 and TRPM7 expression is not fully understood. Therefore, it is necessary to investigate whether the CsA-induced reduction in the reabsorption of magnesium is caused by a decrease in the expression of TRPM6, TRPM7, or both.

In the present study, we examined whether the expression levels of TRPM6 and TRPM7 are decreased by CsA in renal tubular epithelial cells. Our results indicate that CsA decreased TRPM6 expression without affecting TRPM7 expression. Inhibition of NFATc is not involved in the decrease in TRPM6 expression. CsA may decrease TRPM6 expression mediated by c-Fos-dependent transcription, resulting in a decrease of renal Mg^{2+} reabsorption.

Materials and methods

Materials

CsA was obtained from Wako Pure Chemical Industries (Osaka, Japan). Goat anti-TRPM7 (LTRPC7), goat anti-actin, and rabbit anti-NFATc1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-TRPM6 (CHAK2) antibody was from Abgent (San Diego, CA, USA). Mouse anti-E-cadherin antibody was from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit anti-c-Fos antibody was from EMB Biosciences (San Diego, CA, USA). Rabbit anti-c-Jun antibody was from Signal antibody technology (Sunnyvale, CA, USA). 4',6-Diamidino-2-phenylindole (DAPI) was from Dojindo laboratories (Kumamoto, Japan). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). All other reagents were of the highest grade of purity available.

Cell culture

Normal rat kidney (NRK-52E) and Madin-Darby canine kidney (MDCK) cells were obtained from the Japanese Collection of Research Biosciences (Osaka, Japan). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 0.07 mg/ml penicillin-G potassium, and 0.14 mg/ml streptomycin sulfate in a 5% CO₂ atmosphere at 37°C.

Small interfering RNA transfection

TRPM6 and c-Fos small interfering RNAs (siRNA) were produced by Nippon EGT (Toyama, Japan). For TRPM6 siRNA, the oligoribonucleotides were designed as 5'-AACACAAGGGUAUGACUAGTT-3' and 5'-CUAGUCAUACCCUUGUGUUTT-3' (TRPM6 siRNA-1) and 5'-AAGUGAUGAAGCAGGUGUGTT-3' and 5'-CACACUGCUUCAUCACUUTT-3' (TRPM6 siRNA-2). The sequences of c-Fos siRNA were 5'-UUGCCAAUCUACUGAAAGATT-3' and 5'-UCUUUCAGUAGAUGGCAATT-3'. Each pair of oligoribonucleotides was annealed at a concentration of 20 μM. NRK-52E cells were grown to 60% confluence and were transfected with c-Fos or TRPM6 siRNA using Lipofectamine 2000 as recommended by the manufacturer's instructions.

RNA isolation and semiquantitative reverse-transcription-polymerase chain reaction

Total RNA was isolated from cells using ISOGEN (Nippon gene, Tokyo, Japan) and was oligo-dT-primed in the presence of an M-MLV reverse transcriptase (42°C for 30 min and heated at 99°C for 5 min). PCR reactions were

performed in a 20- μ l reaction volume containing 2 μ l of cDNA mixture, 1 \times PCR buffer containing 1.5 mM MgCl₂, 200 μ M of each dNTP, 400 nM of each primer, and 1 U of the TaKaRa rTaq DNA polymerase. The DNA was denatured at 94°C for 2 min before PCR cycling (24–32 cycles) at 94°C for 0.5 min (denaturation), 55°C for 0.5 min (annealing), and 72°C for 1 min (extension). After the last cycle, elongation was extended for an additional 7 min at 72°C before refrigeration. The following primers were used: TRPM6, forward 5'-CTTCTTGGGATACCAAATCAG-3' and reverse 5'-GAAACTTTTCCTAGGTAGCTG-3'; TRPM7, forward 5'-GAATTCATGCTAGAATTGGGCAAGG-3' and reverse 5'-GCGCTTGGTCTTTGGATCATC-3'; c-Fos, forward 5'-GCAGCTATCTCCTGAAGAGGAA-3' and reverse 5'-CTGCTGCA TAGAAGGAACCAG-3'; and c-Jun, forward 5'-ATACGCTGCCAGTGTACACCT-3' and reverse 5'-CCA GCTCGGAGTTTTGCGCTTTC-3'. The PCR products were visualized with ethidium bromide after electrophoretic separation on a 1.5% agarose gel. The TRPM6, TRPM7, c-Fos, and c-Jun primer pairs were designed to amplify fragments of 1,262, 1,011, 515, and 519 bp, respectively.

Preparation of whole cell lysate

Cells were scraped into cold PBS and were precipitated by centrifugation at 3,000 \times g for 2 min. Then, the cells were lysed in a RIPA buffer containing 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), a protease inhibitor cocktail (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride. After sonication for 20 s, the aliquot was used as a whole cell lysate containing plasma membrane, cytoplasm, and nuclear fractions. The nuclear fraction was removed by centrifugation at 6,000 \times g for 5 min and the supernatant was used as a cytoplasmic lysate. The whole cell lysate and the cytoplasmic lysate were solubilized in a sample buffer for SDS-polyacrylamide gel electrophoresis. Protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis was carried out as described previously (Ikari et al. 2001). In brief, the whole cell lysate (60 μ g) or the cytoplasmic extract (60 μ g) was applied to the SDS-polyacrylamide gel. Proteins were blotted onto a PVDF membrane and incubated with each primary antibody followed by a peroxidase-conjugated secondary antibody. Finally, the blots were stained with an ECL Western blotting kit or ECL-plus Western blotting

kit from GE Healthcare Bio-Science. The band density of Western blotting was quantified with Doc-It LS image analysis software (UVP, Upland, CA, USA).

Measurement of intracellular free Mg²⁺ concentration ([Mg²⁺]_i)

[Mg²⁺]_i was determined using a Mg²⁺-sensitive fluorescent dye, mag-fura 2. Cells grown on glass slides were incubated with a Hanks' balanced salt solution (HBSS) containing 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO₃, 3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5 mM Hepes, 0.8 mM CaCl₂, 10 mM glucose supplemented with 2 μ M mag-fura 2 acetoxymethylester (mag-fura 2/AM, Molecular Probes, Eugene, OR, USA) and a detergent, Pluronic F127 (0.025%, w/v, Molecular Probes), at 37°C for 20 min. Then, the mag-fura 2-loaded cells were washed twice with the dye-free HBSS and placed in a glass cuvette. The mag-fura 2 fluorescence was monitored at 1-s intervals using a dual-excitation wavelength spectrofluorometer (Hitachi F-2000, Tokyo, Japan) with excitation at 340 and 380 nm and emission at 495 nm. The ratio of the two fluorescence emission intensities was stored for further analysis. [Mg²⁺]_i was calculated according to the formula of Grynkiewicz et al. (1985), using a dissociation constant (*K_d*) of 1.45 mM for the Mg²⁺-mag-fura 2 complex.

Confocal microscopy

Cells were grown on glass slides. After fixation with 4% paraformaldehyde, the cells were permeabilized with 0.2% Triton X-100. Then the cells were incubated with anti-c-Fos, c-Jun or NFATc1 antibody, followed by incubation with Rhodamine-labeled or fluorescein isothiocyanate (FITC)-labeled secondary antibody containing DAPI. Immunofluorescence microscopy was performed as described previously (Ikari et al. 2004). Immunolabeled cells were visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany) set with a filter appropriate for DAPI (355 nm excitation, 460 nm emission), Texas Red (543 nm excitation, 585–615 nm emission), and FITC (488 nm excitation, 530 nm emission). Images were further processed using Adobe Photoshop (Adobe System, San Jose, CA, USA).

Statistics

Results are presented as means \pm SEM. Differences between groups were analyzed by one-way analysis of variance, and corrections for multiple comparison were made using Tukey's multiple comparison test. Significant differences were assumed at *p*<0.05.

Results

Cell-specific expression of TRPM6

Western blot analysis with TRPM6-specific antibody detected the expression of TRPM6 protein in NRK-52E cells, but not in MDCK cells (Fig. 1). TRPM7 protein was detected in both NRK-52E and MDCK cells. Similarly, reverse-transcription-polymerase chain reaction (RT-PCR) showed that TRPM6 mRNA was endogenously expressed in NRK-52E, but not in MDCK cells (data not shown). In the present study, we used NRK-52E cells to examine whether CsA affects TRPM6 and TRPM7 expression.

Decrease of TRPM6 expression by immunosuppressants

Administration of FK506 to rats during 7 days enhanced the urinary excretion of magnesium concomitantly with a decrease in TRPM6 expression (Nijenhuis et al. 2004). However, it is unclear whether immunosuppressants affect TRPM7 expression and whether the decrease in TRPM6

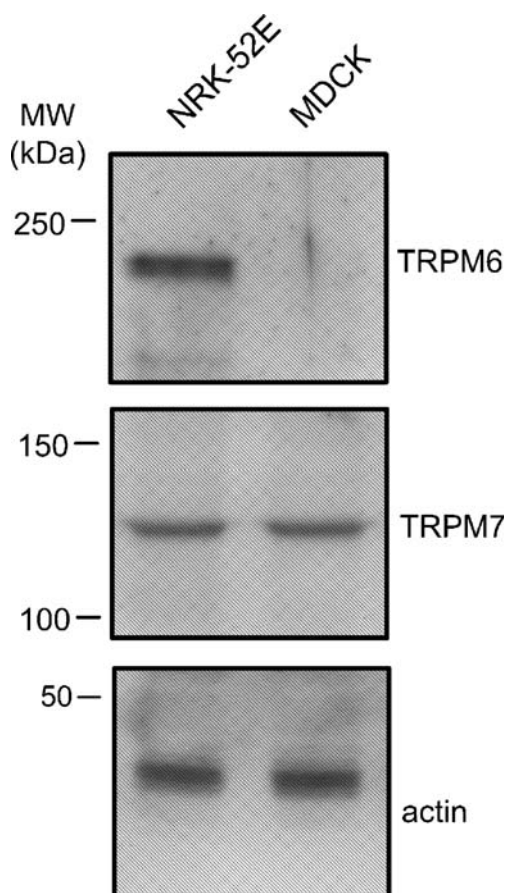


Fig. 1 Expression of TRPM6 and TRPM7 in renal epithelial cells. Cytoplasmic lysates (60 μ g) isolated from NRK-52E and MDCK cells were immunoblotted with anti-TRPM6, TRPM7, or actin antibody. The bands for TRPM6, TRPM7, and actin were detected at about 230, 130, and 40 kDa, respectively. Actin served as an internal control

expression causes the reduction in magnesium transport. NRK-52E cells were incubated with an immunosuppressant such as CsA (0.1–100 μ M) or ascomycin (0.1–100 μ M), a close structural analog of FK506, for 24 h. RT-PCR and Western blot analysis showed that the immunosuppressants dose-dependently decreased TRPM6 expression (Fig. 2). In contrast, TRPM7 expression was not affected by immunosuppressants. These results suggest that immunosuppressants decrease TRPM6 expression and the regulatory mechanism of TRPM6 transcription differs from that of TRPM7.

Inhibition of $[Mg^{2+}]_i$ elevation by CsA

In magnesium-free conditions, the addition of 1 mM $MgCl_2$ in the bath solution caused an elevation of $[Mg^{2+}]_i$ (Fig. 3a,c). The elevation of $[Mg^{2+}]_i$ continued over 5 min. The influx of magnesium mediated via TRPM6 and TRPM7 is inhibited by divalent metal cations (Voets et al. 2004). The elevation of $[Mg^{2+}]_i$ was significantly inhibited by Ni^{2+} and Co^{2+} (Fig. 3a,b), indicating that the elevation of $[Mg^{2+}]_i$ reflects the influx of magnesium from extracellular milieu. CsA significantly inhibited the elevation of $[Mg^{2+}]_i$ (Fig. 3c,d). The inhibitory effect was incomplete even at 100 μ M, suggesting that the remaining increase was caused by TRPM7 or other magnesium transport pathways.

Inhibition of $[Mg^{2+}]_i$ elevation by TRPM6 siRNA

As indicated above, the elevation of $[Mg^{2+}]_i$ was inhibited by divalent metal cations. However, it is doubtful that TRPM6 acts as a magnesium influx channel. Therefore, NRK-52E cells were transfected with TRPM6 siRNA to clarify the involvement of TRPM6 in the influx. Two siRNAs were designed to target different sites of TRPM6. Western blot analysis revealed that both siRNAs decrease TRPM6 expression without affecting TRPM7 expression (Fig. 4a). Both siRNAs significantly inhibited the elevation of $[Mg^{2+}]_i$ caused by the addition of $MgCl_2$ (Fig. 4b). The inhibitory effect of siRNA-2 was more potent than that of siRNA-1. Despite the disappearance of TRPM6, the elevation of $[Mg^{2+}]_i$ was incompletely inhibited by TRPM6 siRNA. These results are similar to those for CsA, supporting the idea that other transport pathways without TRPM6 may be involved in the influx of magnesium.

Effect of CsA on the intracellular distribution and expression of NFATc1

CsA inhibits the immune response-mediated nuclear translocation of NFAT family members and subsequent gene expression (Flanagan et al. 1991). Immunofluorescence analysis showed that NFATc1 is distributed in the nucleus

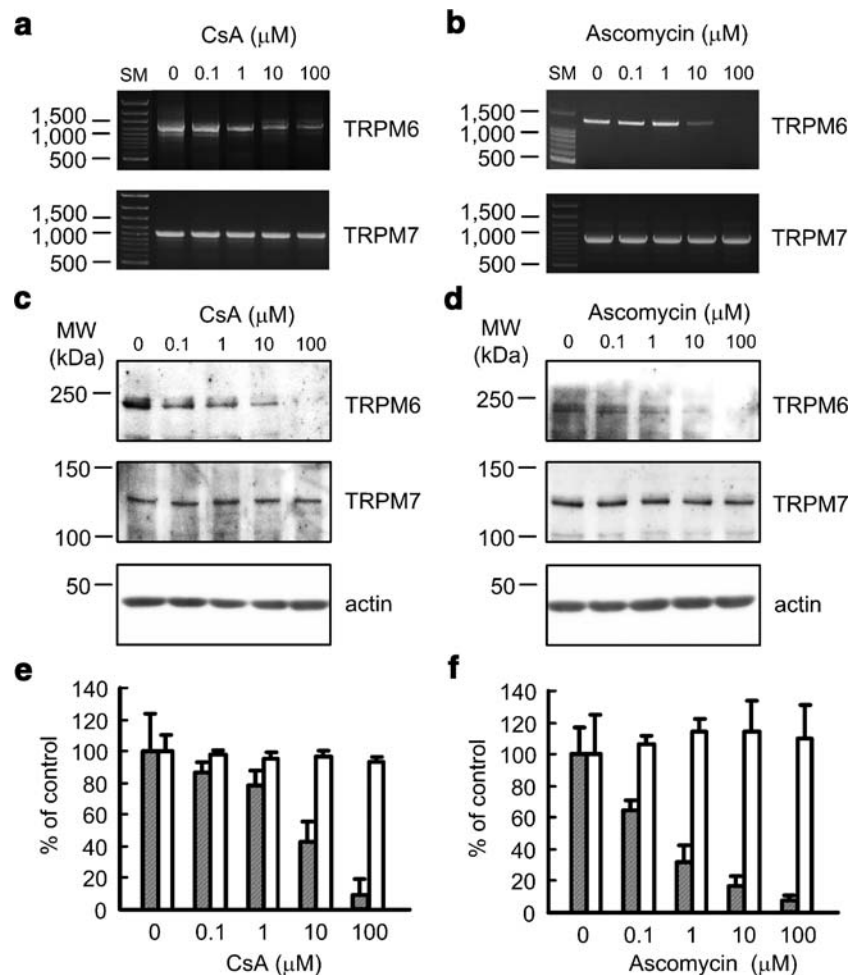


Fig. 2 Decrease in TRPM6 expression by immunosuppressants. NRK-52E cells were incubated with CsA or ascomycin for 24 h at the indicated concentration. **a** and **b** After the isolation of total RNA from the cells, semiquantitative RT-PCR was performed using specific primers for TRPM6 and TRPM7. **c** and **d** Cytoplasmic lysates (60 μg) were immunoblotted with anti-TRPM6, TRPM7, or actin antibody.

e and **f** The band densities of Western blotting were quantified with Doc-It LS image analysis software, and then expressed relative to the value at 0 μM . *Hatched* and *open columns* show TRPM6 and TRPM7 expression, respectively. Actin served as an internal control for normalization purposes. $n=3$

and cytoplasm (Fig. 5a). CsA did not change the intracellular distribution of NFATc1. Next, we checked the amount of NFATc1 protein by Western blotting. CsA decreased TRPM6 expression whereas it did not affect TRPM7 or NFATc1 expression (Fig. 5b). Furthermore, 11R-VIVIT, a NFAT inhibitor (Noguchi et al. 2004) did not decrease TRPM6 expression (Fig. 5c). The activation of NFAT by calcium ionophore and phorbol ester did not change TRPM6 expression (data not shown). These results suggest that NFATc1 distributed in the nucleus of unstimulated cells is not sensitive to CsA and NFATc1 is not involved in the reduction of TRPM6 expression caused by CsA.

Decrease of TRPM6 expression by inhibitor of AP-1

It has been shown that CsA affects the activities of AP-1 in addition to NFAT, implying the presence of another target of CsA as well as the calcineurin/NFAT pathway (Rincón

and Flavell 1994). AP-1 is activated by mitogen-activated protein (MAP) kinase signaling pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (Matsuda et al. 1998; Frigo et al. 2004). We examined the effect of inhibitors of these signaling pathways on TRPM6 and TRPM7 expression by RT-PCR and Western blotting. U0126, an inhibitor of the ERK pathway, decreased TRPM6 expression, whereas SP600125, an inhibitor of the JNK pathway, and SB202190, an inhibitor of the p38 pathway, had no effect (Fig. 6). In the present experimental conditions, Western blotting showed that ERK is phosphorylated and U0126 decreases the level of phosphorylated ERK (data not shown). These inhibitors of MAP kinase signaling pathways did not affect TRPM7 expression. We suggested that TRPM6 expression is regulated by AP-1 transcription factor. Next, we tried to investigate the effect of CsA on the intracellular distribution and the amount of AP-1 protein.

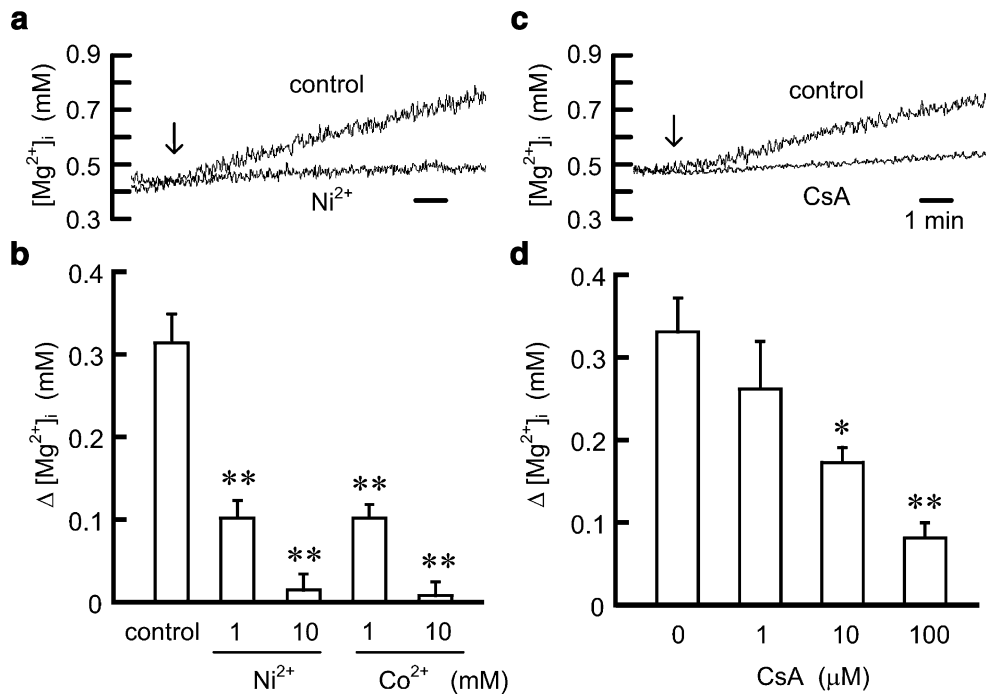


Fig. 3 Inhibition of magnesium influx by divalent cations and CsA. $[Mg^{2+}]_i$ was determined using a magnesium-sensitive fluorescent dye, mag-fura 2. **a** Representative traces of $[Mg^{2+}]_i$ measurements are shown. The cells were placed in a glass cuvette containing 0 or 10 mM Ni^{2+} in the absence of magnesium. Then, 1 mM $MgCl_2$ was added to the bath solution as indicated by the arrow. **b** The cells were placed in a glass cuvette containing 1 or 10 mM divalent cations (Ni^{2+} or Co^{2+}) in the absence of magnesium. After the addition of 1 mM $MgCl_2$, the

increase in $[Mg^{2+}]_i$ for 600 s was calculated. Double asterisks $P < 0.01$ compared with control. $n = 4$. **c** The cells were incubated in the absence and presence of 100 μM CsA for 24 h. Representative traces of $[Mg^{2+}]_i$ measurements are shown. **d** The cells were incubated with CsA for 24 h at the indicated concentration. The increase in $[Mg^{2+}]_i$ for 600 s was calculated. Single asterisk $P < 0.05$ and double asterisks $P < 0.01$ compared with control. $n = 4$

Effect of c-Fos siRNA on TRPM6 expression

Immunofluorescence microscopy showed that c-Fos and c-Jun are distributed in the nucleus (Fig. 7a). CsA did not change the intracellular distribution of c-Fos, but decreased the green fluorescent intensity of c-Fos. In contrast, CsA

did not change the intracellular distribution or green fluorescent intensity of c-Jun. Next, we checked the AP-1 expression by Western blotting. CsA decreased c-Fos expression in a dose-dependent manner, but did not affect c-Jun expression (Fig. 7b,c). To determine the involvement of c-Fos in the regulation of TRPM6 expression, we

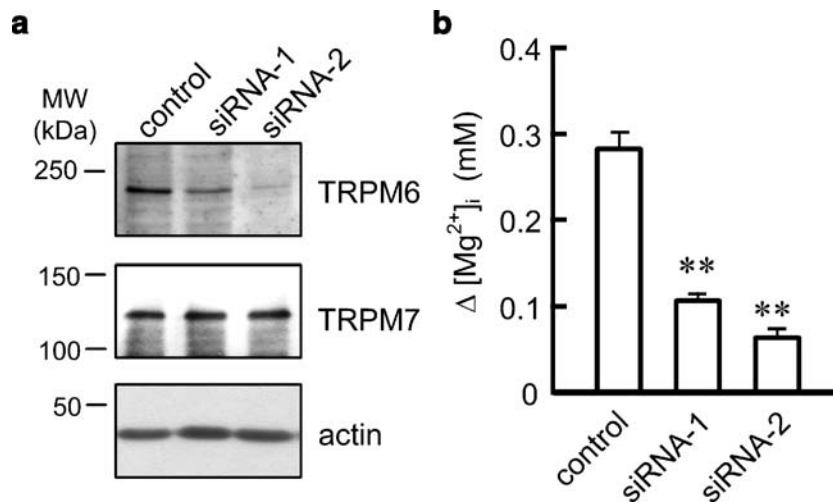


Fig. 4 Inhibition of $[Mg^{2+}]_i$ elevation by TRPM6 siRNA. NRK-52E cells were transfected with TRPM6 siRNAs. **a** Cytoplasmic lysates (60 μg) were immunoblotted with anti-TRPM6, TRPM7, actin antibody. **b** The cells were placed in a glass cuvette in the absence

of magnesium. After the addition of 1 mM $MgCl_2$, the increase in $[Mg^{2+}]_i$ for 600 s was calculated. Double asterisks $P < 0.01$ compared with control. $n = 4$

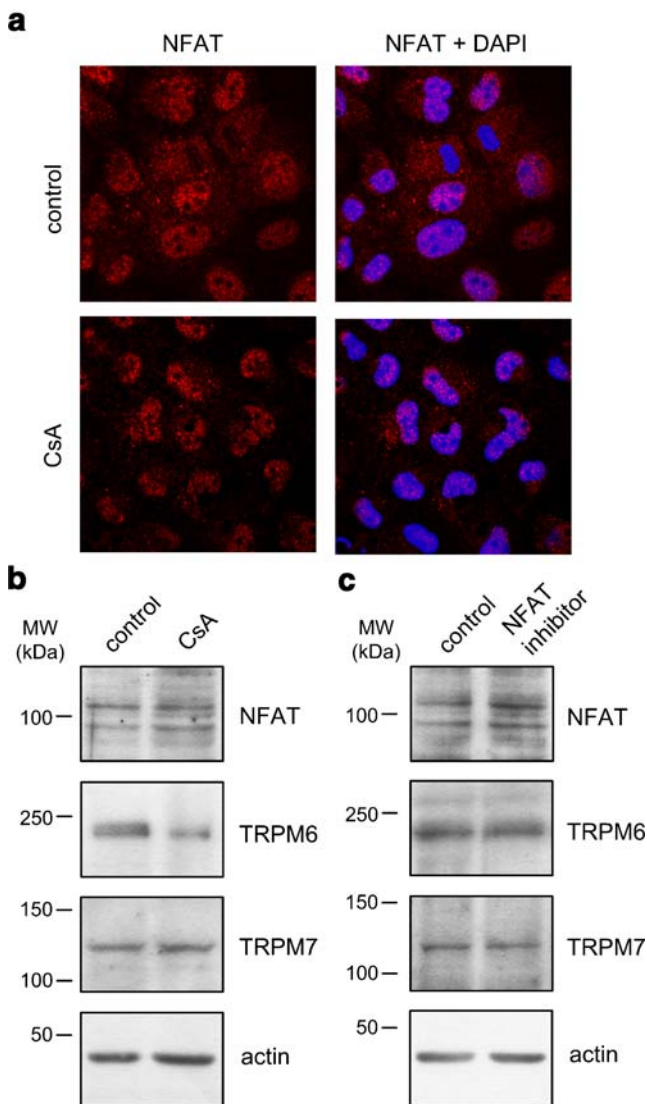


Fig. 5 Effect of CsA on the intracellular distribution and amount of NFATc1 protein. **a** NRK-52E cells were incubated in the absence and presence of 100 μ M CsA for 24 h. After incubation with anti-NFATc1 antibody, the cells were stained with rhodamine-labeled anti-rabbit IgG and DAPI. **b** The cells were incubated in the absence and presence of 100 μ M CsA for 24 h. Whole cell lysates (60 μ g) were immunoblotted with anti-NFATc1. Cytoplasmic lysates (60 μ g) were immunoblotted with anti-TRPM6, TRPM7, or actin antibody. **c** The cells were incubated in the absence and presence of 10 μ M NFAT inhibitor for 24 h. Whole cell lysates (60 μ g) were immunoblotted with anti-NFATc1. Cytoplasmic lysates (60 μ g) were immunoblotted with anti-TRPM6, TRPM7, or actin antibody. Actin was evaluated as an internal control

blocked c-Fos expression using siRNA. As shown in Fig. 8, c-Fos siRNA abrogated the production of c-Fos without affecting c-Jun. Notably, TRPM6 expression was decreased by c-Fos siRNA, but TRPM7 expression was not. Furthermore, c-Fos siRNA significantly inhibited the elevation of $[Mg^{2+}]_i$ (mock 0.36 ± 0.07 mM and c-Fos siRNA 0.14 ± 0.03 mM, $p < 0.05$, $n = 3$). These results indicate that CsA decreases TRPM6 expression via a reduction of c-Fos expression.

Discussion

Hypomagnesemia is frequently encountered in patients treated with immunosuppressants (Barton et al. 1987; McDiarmid et al. 1993). Magnesium supplementation has been reported to prevent immunosuppressant-induced nephrotoxicity (Asai et al. 2002), suggesting that a disturbance of magnesium homeostasis is involved in the

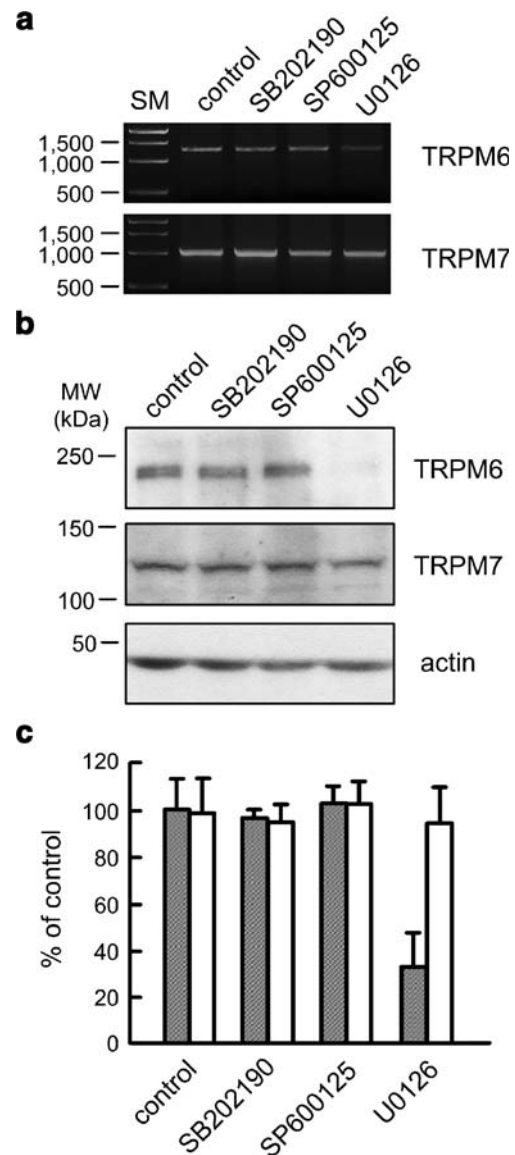


Fig. 6 Decrease of TRPM6 expression by U0126. NRK-52E cells were incubated in the absence and presence of 25 μ M U0126, 10 μ M SP600125, or 1 μ M SB202190 for 24 h. **a** After the isolation of total RNA from the cells, semiquantitative RT-PCR was performed using specific primers for TRPM6 and TRPM7. *Left lane* shows a size marker. **b** Cytoplasmic lysates (60 μ g) were immunoblotted with anti-TRPM6, TRPM7, or actin antibody. **c** The band densities of Western blotting were quantified with Doc-It LS image analysis software, and then expressed relative to the control value. *Hatched and open columns* show TRPM6 and TRPM7 expression, respectively. Actin served as an internal control for normalization purposes. $n = 4-5$

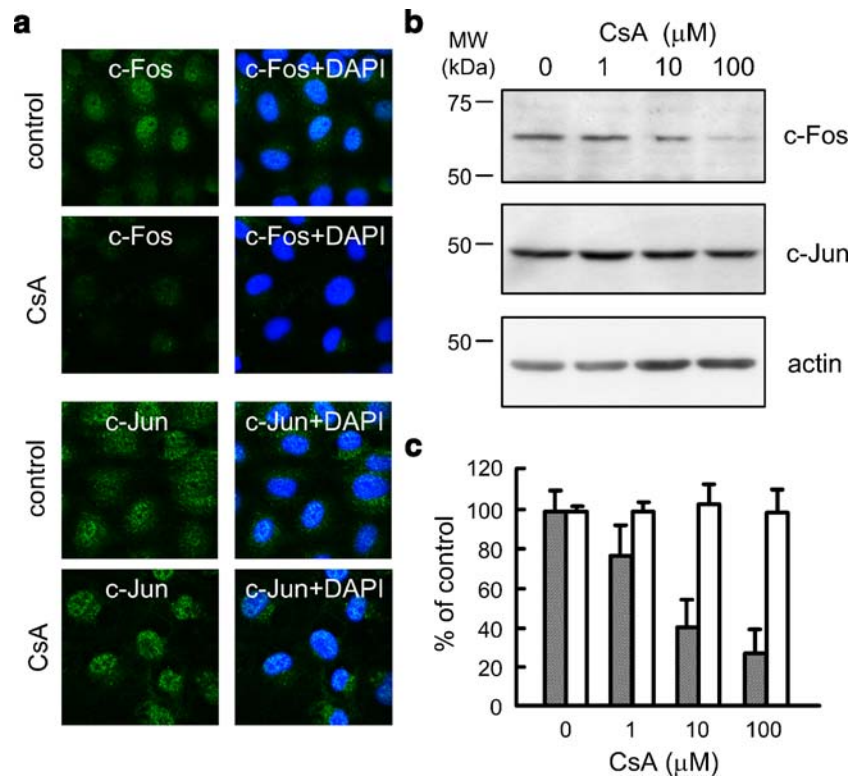


Fig. 7 Decrease of c-Fos expression by CsA. **a** NRK-52E cells were incubated in the absence and presence of 100 μ M CsA for 24 h. After incubation with anti-c-Fos or c-Jun antibody, the cells were stained with FITC-labeled anti-rabbit IgG containing DAPI. **b** The cells were incubated with CsA for 24 h at the indicated concentration. Whole cell lysates (60 μ g) were immunoblotted with anti-c-Fos, c-Jun, or actin

antibody. **c** The band densities of Western blotting were quantified with Doc-It LS image analysis software, and then expressed relative to the value at 0 μ M. *Hatched* and *open columns* show c-Fos and c-Jun expression, respectively. Actin served as an internal control for normalization purposes. $n=3-4$

adverse effects. To prevent or lessen the side effects, we have to fundamentally reveal the mechanisms by which immunosuppressants disturb magnesium homeostasis. In the present study, we found that CsA decreases TRPM6

expression without affecting TRPM7 expression and inhibits magnesium influx in renal tubular epithelial NRK-52E cells.

In the kidney, about 80% of the magnesium in plasma is ultrafiltered through the glomerular membrane and subse-

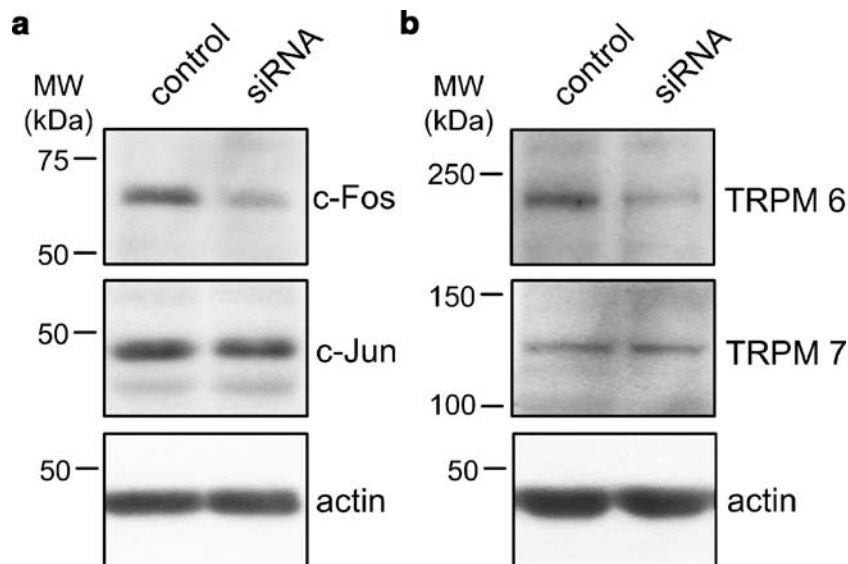


Fig. 8 Decrease of TRPM6 expression by c-Fos siRNA. The cells were transfected with c-Fos siRNA. **a** Whole cell lysates (60 μ g) were immunoblotted with anti-c-Fos, c-Jun, or actin antibody. **b** Cytoplas-

mic lysates (60 μ g) were immunoblotted with anti-TRPM6, TRPM7, or actin antibody. Actin was evaluated as an internal control

quently reabsorbed in consecutive segments of the nephron (Cole and Quamme 2000; Konrad et al. 2004). Active reabsorption of magnesium occurs in the distal convoluted tubule (Quamme 1997). In patients having hypomagnesemia with secondary hypocalcemia, a disorder characterized by a combined defect of intestinal and renal magnesium transport, mutations of TRPM6 have been identified (Schlingmann et al. 2002; Walder et al. 2002). Mutations lead mostly to a truncated TRPM6 protein. The biophysical characterization of TRPM6 is controversial. Chubanov et al. (2004) reported that TRPM6 specifically interacts with TRPM7 to form a functional ion channel complex in *xenopus* oocytes and HEK293 cells, while Li et al. (2006) reported that TRPM6 forms functional homomeric channels as well as heteromeric TRPM6/7 complexes. TRPM6 functions as a magnesium channel in the presence of TRPM7. TRPM6 expression is upregulated by 17β -estradiol in the mouse kidney without TRPM7 expression being affected (Groenestege et al. 2006) and is downregulated by FK506 in the cortex of the rat kidney (Nijenhuis et al. 2004). The expression of TRPM7, but not TRPM6, is upregulated by angiotensin II in vascular smooth muscle cells from Wistar Kyoto rats (Touyz et al. 2006). These reports predict that TRPM6 and TRPM7 expression levels are regulated in a different manner. Unfortunately, it has not been determined whether TRPM7 expression is affected by immunosuppressants. Furthermore, the molecular mechanisms involved in the regulation of TRPM6 and TRPM7 expression are unknown. We found that the mRNAs and proteins of TRPM6 and TRPM7 are expressed in the unstimulated NRK-52E cells. It is worth noting that TRPM6 expression was decreased by CsA, but TRPM7 expression was not. Mutations in the gene encoding TRPM6 cause hereditary hypomagnesemia (Schlingmann et al. 2002; Walder et al. 2002), indicating that TRPM6 is necessary to reabsorb magnesium in the kidney. We suggest that immunosuppressants decrease renal TRPM6 expression without affecting TRPM7 expression, resulting in hypomagnesemia.

The addition of $MgCl_2$ caused an elevation of $[Mg^{2+}]_i$ in NRK-52E cells under the magnesium-free conditions. The elevation was inhibited by Ni^{2+} and Co^{2+} , indicating that TRPM6 and/or TRPM7 function as magnesium influx channels. CsA significantly, but not completely, inhibited the rise in $[Mg^{2+}]_i$. The remaining increase may be caused by TRPM7. Recently, it has been reported that SLC41A1 and SLC41A2 function as a magnesium transporter (Goytain and Quamme 2005a; Goytain and Quamme 2005b). RT-PCR revealed that both SLC41A1 and SLC41A2 are expressed in NRK-52E cells (data not shown). CsA changed neither SLC41A1 nor SLC41A2 expression. There is a possibility that a magnesium transporter other than TRPM7 is involved in the influx of

magnesium in the CsA-treated cells. Anyway, CsA decreased TRPM6 expression and the magnesium influx.

NFAT regulates the inducible expression of many cytokines as well as cell surface receptors, which is critical for an immune response. CsA inhibited the immune response mediated by inhibiting the phosphatase activity of calcineurin and the nuclear translocation of NFAT family members in activated T cells. The intracellular distribution and amount of NFATc1 protein were not affected by CsA. Furthermore, an inhibitor of the NFAT pathway did not affect TRPM6 expression (Fig. 5). TRPM6 expression was not inducible but was constitutive in NRK-52E cells. We suggest that the inhibition of the NFAT pathway by CsA is not related to the regulation of constitutive TRPM6 expression. Andoh et al. (1996) reported that the mechanism of hypomagnesemia and tubular injury induced by immunosuppressants is unclear but may be independent of calcineurin. Other transcriptional regulatory factors may be involved in the reduction of TRPM6 expression caused by CsA.

The transcriptional activity of AP-1 is regulated by ERK1/2, JNK, and p38 pathways. CsA activated the ERK1/2 pathway in the renal epithelial MDCK cells (Kiely et al. 2003) whereas it inhibited the phosphorylation of ERK1/2 induced by parathyroid hormone in the mouse distal convoluted tubule cells (Kim et al. 2006). At present, we do not know why CsA has opposite effects in these cells. AP-1 activity is determined by the amount of protein and its intracellular distribution. Immunofluorescence studies showed that c-Fos and c-Jun are mainly distributed in the nucleus in control cells (Fig. 7). CsA decreased c-Fos expression in a dose-dependent manner. In contrast, the distribution and amount of c-Jun protein were not affected by CsA. These results indicate that c-Fos, but not c-Jun, is needed to regulate TRPM6 expression. U0126, an inhibitor of the ERK1/2 pathway, decreased TRPM6 expression similar to CsA (Fig. 6) whereas inhibitors of the JNK and p38 pathways did not affect TRPM6 expression. The endogenous expression of TRPM6 may be regulated by the ERK1/2 pathway and c-Fos. It is worth noting that c-Fos siRNA decreases TRPM6 expression without affecting the expression of c-Jun or TRPM7 (Fig. 8). We demonstrate that c-Fos plays a critical role in the regulation of TRPM6 expression. These results reveal, for the first time, that CsA decreases TRPM6 expression mediated by a reduction of c-Fos expression.

Taken together, TRPM6 and TRPM7 were expressed in rat renal epithelial NRK-52E cells. CsA decreased c-Fos and TRPM6 expressions without affecting TRPM7 expression. U0126 decreased TRPM6 expression whereas SP600125 and SB202190 did not. Transfection of c-Fos siRNA decreased the expression of TRPM6 and elevation of $[Mg^{2+}]_i$ similar to CsA. These results indicate that the

decrease of c-Fos expression contributes to the reduction of TRPM6 expression caused by CsA. The development of a novel immunosuppressant, which is free of inhibitory effects on c-Fos expression, may lead to a decrease in the occurrence of hypomagnesemia.

Acknowledgements This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan, a grant-in-aid for Encouragement of Young Scientists (to A.I.), and by grants from the Ichiro Kanehara Foundation, the Salt Science Research Foundation, no. 0633, and the SRI academic Research Grant (to A.I.). Akira Ikari and Chiaki Okude contributed equally to this work.

References

- Andoh TF, Burdmann EA, Fransechini N, Houghton DC, Bennett WM (1996) Comparison of acute rapamycin nephrotoxicity with cyclosporine and FK506. *Kidney Int* 50:1110–1117
- Asai T, Nakatani T, Yamanaka S, Tamada S, Kishimoto T, Tashiro K, Nakao T, Okamura M, Kim S, Iwao H, Miura K (2002) Magnesium supplementation prevents experimental chronic cyclosporine a nephrotoxicity via renin-angiotensin system independent mechanism. *Transplantation* 74:784–791
- Barton CH, Vaziri ND, Martin DC, Choi S, Alikhani S (1987) Hypomagnesemia and renal magnesium wasting in renal transplant recipients receiving cyclosporine. *Am J Med* 83:693–699
- Bennett WM (1995) The nephrotoxicity of immunosuppressive drugs. *Clin Nephrol* 43(Suppl 1):S3–S7
- Chubanov V, Waldegger S, Mederos y Schnitzler M, Vitzthum H, Sassen MC, Seyberth HW, Konrad M, Gudermann T (2004) Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. *Proc Natl Acad Sci USA* 101:2894–2899
- Cole DE, Quamme GA (2000) Inherited disorders of renal magnesium handling. *J Am Soc Nephrol* 11:1937–1947
- Flanagan WM, Corthésy B, Bram RJ, Crabtree GR (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* 352:803–807
- Frigo DE, Tang Y, Beckman BS, Scandurro AB, Alam J, Burow ME, McLachlan JA (2004) Mechanism of AP-1-mediated gene expression by select organochlorines through the p38 MAPK pathway. *Carcinogenesis* 25:249–261
- Goytain A, Quamme GA (2005a) Functional characterization of the human solute carrier, SLC41A2. *Biochem Biophys Res Commun* 330:701–705
- Goytain A, Quamme GA (2005b) Functional characterization of human SLC41A1, a Mg²⁺ transporter with similarity to prokaryotic MgtE Mg²⁺ transporters. *Physiol Genomics* 21:337–342
- Groenestege WM, Hoenderop JG, van den Heuvel L, Knoers N, Bindels RJ (2006) The epithelial Mg²⁺ channel transient receptor potential melastatin 6 is regulated by dietary Mg²⁺ content and estrogens. *J Am Soc Nephrol* 17:1035–1043
- Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450
- Ikari A, Nakajima K, Kawano K, Suketa Y (2001) Polyvalent cation-sensing mechanism increased Na⁺-independent Mg²⁺ transport in renal epithelial cells. *Biochem Biophys Res Commun* 287:671–674
- Ikari A, Hirai N, Shiroma M, Harada H, Sakai H, Hayashi H, Suzuki Y, Degawa M, Takagi K (2004) Association of paracellin-1 with ZO-1 augments the reabsorption of divalent cations in renal epithelial cells. *J Biol Chem* 279:54826–54832
- Kahan BD (1989) Cyclosporine. *N Engl J Med* 321:1725–1738
- Kiely B, Feldman G, Ryan MP (2003) Modulation of renal epithelial barrier function by mitogen-activated protein kinases (MAPKs): mechanism of cyclosporine A-induced increase in transepithelial resistance. *Kidney Int* 63:908–916
- Kim SJ, Kang HS, Jeong CW, Park SY, Kim IS, Kim NS, Kim SZ, Kwak YG, Kim JS, Quamme GA (2006) Immunosuppressants inhibit hormone-stimulated Mg²⁺ uptake in mouse distal convoluted tubule cells. *Biochem Biophys Res Commun* 341:742–748
- Konrad M, Schlingmann KP, Gudermann T (2004) Insights into the molecular nature of magnesium homeostasis. *Am J Physiol Renal Physiol* 286:F599–F605
- Li M, Jiang J, Yue L (2006) Functional characterization of homo- and heteromeric channel kinases TRPM6 and TRPM7. *J Gen Physiol* 127:525–537
- Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL (1992) Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry* 31:3896–3901
- Matsuda S, Moriguchi T, Koyasu S, Nishida E (1998) T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J Biol Chem* 273:12378–12382
- McDiarmid SV, Colonna JO, Shaked A, Ament ME, Busutil RW (1993) A comparison of renal function in cyclosporine- and FK-506-treated patients after primary orthotopic liver transplantation. *Transplantation* 56:847–853
- Myers BD, Ross J, Newton L, Luetscher J, Perlroth M (1984) Cyclosporine-associated chronic nephropathy. *N Engl J Med* 311:699–705
- Nijenhuis T, Hoenderop JG, Bindels RJ (2004) Downregulation of Ca²⁺ and Mg²⁺ transport proteins in the kidney explains tacrolimus (FK506)-induced hypercalciuria and hypomagnesemia. *J Am Soc Nephrol* 15:549–557
- Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, Tanaka K, Tanaka N, Matsui H (2004) A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* 10:305–309
- Quamme GA (1997) Renal magnesium handling: new insights in understanding old problems. *Kidney Int* 52:1180–1195
- Remuzzi G, Perico N (1995) Cyclosporine-induced renal dysfunction in experimental animals and humans. *Kidney Int Suppl* 52: S70–S74
- Rincón M, Flavell RA (1994) AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J* 13:4370–4381
- Runnels LW, Yue L, Clapham DE (2002) The TRPM7 channel is inactivated by PIP(2) hydrolysis. *Nat Cell Biol* 4:329–336
- Schlingmann KP, Weber S, Peters M, Niemann Nejsum L, Vitzthum H, Klingel K, Kratz M, Haddad E, Ristoff E, Dinour D, Syrrou M, Nielsen S, Sassen M, Waldegger S, Seyberth HW, Konrad M (2002) Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. *Nat Genet* 31:166–170

- Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. *Cell* 114:191–200
- Taque S, Peudener S, Gie S, Rambeau M, Gandemer V, Bridoux L, Bétrémieux P, De Parscau L, Le Gall E (2004) Central neurotoxicity of cyclosporine in two children with nephrotic syndrome. *Pediatr Nephrol* 19:276–280
- Touyz RM, He Y, Montezano AC, Yao G, Chubanov V, Gudermann T, Callera GE (2006) Differential regulation of transient receptor potential melastatin 6 and 7 cation channels by ANG II in vascular smooth muscle cells from spontaneously hypertensive rats. *Am J Physiol, Regul Integr Comp Physiol* 290:R73–R78
- Voets T, Nilius B, Hoefs S, van der Kemp AW, Droogmans G, Bindels RJ, Hoenderop JG (2004) TRPM6 forms the Mg^{2+} influx channel involved in intestinal and renal Mg^{2+} absorption. *J Biol Chem* 279:19–25
- Walder RY, Landau D, Meyer P, Shalev H, Tsolia M, Borochowitz Z, Boettger MB, Beck GE, Englehardt RK, Carmi R, Sheffield VC (2002) Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. *Nat Genet* 31:171–174