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Coordinated control of renal Ca^{2+} transport proteins by parathyroid hormone

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Coordinated control of renal Ca^{2+} transport proteins by parathyroid hormone.

Background. The kidney is one of the affected organs involved in the clinical symptoms of parathyroid hormone (PTH)-related disorders, like primary hyperparathyroidism and familial hypocalciuric hypercalcemia. The molecular mechanism(s) underlying alterations in renal Ca^{2+} handling in these disorders is poorly understood.

Methods. Parathyroidectomized and PTH-supplemented rats and mice infused with the calcimimetic compound NPS R-467 were used to study the in vivo effect of PTH on the expression of renal transcellular Ca^{2+} transport proteins, including the epithelial Ca^{2+} channel transient receptor potential, vanilloid, member 5 (TRPV5), calbindins, and the Na^+/Ca^{2+}-exchanger (NCX1). In addition, the effect of PTH on transepithelial Ca^{2+} transport in rabbit connecting tubule/cortical collecting duct (CNT/CCD) primary cultures was determined.

Results. Decreased PTH levels in parathyroidectomized rats or NPS R-467–infused mice, resulted in reduced expression of these proteins, which is consistent with diminished Ca^{2+} reabsorption, causing the development of the observed hypocalcemia. PTH supplementation of parathyroidectomized rats restored the expression of the renal Ca^{2+} transport machinery and serum Ca^{2+} levels, independent of serum 1,25-dihydroxyvitamin D_3 levels and renal vitamin D or Ca^{2+}-sensing receptor mRNA abundance. Inhibition of the PTH-stimulated transepithelial Ca^{2+} transport by the TRPV5-specific inhibitor ruthenium red reduced the PTH-stimulated expression of calbindin-D_{28K} and NCX1 in rabbit CNT/CCD primary cultures.

Conclusion. PTH stimulates renal Ca^{2+} reabsorption through the coordinated expression of renal transcellular Ca^{2+} transport proteins. Moreover, the PTH-induced stimulation is enhanced by the magnitude of the Ca^{2+} influx through the gatekeeper TRPV5, which in turn facilitates the expression of the downstream Ca^{2+} transport proteins. Therefore, the renal transcellular Ca^{2+} transport proteins, including TRPV5, could contribute to the pathogenesis of PTH-related disorders.

Primary hyperparathyroidism is a common endocrine disorder characterized by elevated parathyroid hormone (PTH) levels that are inappropriate to the level of serum Ca^{2+} [1]. This underlines the importance of PTH as an essential component of Ca^{2+} homeostasis. Secretion of PTH from the parathyroid glands is regulated by the ambient Ca^{2+} concentration, sensed by the parathyroid Ca^{2+}-sensing receptor [2]. The Ca^{2+}-sensing receptor is activated by high serum Ca^{2+} concentrations and couples to the inhibition of PTH secretion. Inactivating mutations of the Ca^{2+}-sensing receptor result in familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism [3], whereas autosomal-dominant hypocalcemia is caused by activating mutations [4]. These disorders demonstrate the predominant role of the Ca^{2+}-sensing receptor in controlling parathyroid gland function and, hence, the role of PTH in regulating systemic Ca^{2+} balance. From the clinical symptoms of these PTH-related disorders, like hypo- or hypercalciuria and renal stone formation, it is clear that also renal Ca^{2+} handling is affected.

Regulation of Ca^{2+} reabsorption in the kidney is crucial for the maintenance of normal serum Ca^{2+} levels and occurs via paracellular as well as transcellular routes. Transcellular or active Ca^{2+} reabsorption is the primary target for regulation by calciotropic hormones, including 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and PTH. Active Ca^{2+} reabsorption takes place in the distal convoluted tubule (DCT) and connecting tubule (CNT) of the kidney. Luminal Ca^{2+} enters these tubular cells via the epithelial Ca^{2+} channels, transient receptor potential, vanilloid, members 5 and 6 (TRPV5 and TRPV6), is then transported across the cell in

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[†] Deceased.

Key words: ECaC, TRPV5, TRPV6, PTH, Ca^{2+}-sensing receptor, calcimimetic, NPS R-467, ruthenium red.

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association with Ca^{2+}-carrier proteins (i.e., calbindins) and is finally extruded into the blood stream via the Na^+/Ca^{2+}-exchanger (NCX1) and the plasma membrane Ca^{2+}-ATPase (PMCA1b) [5–8]. PTH receptors have been detected throughout the kidney, as well as in the actively Ca^{2+} transporting tubules DCT and CNT [9, 10]. Therefore, the contribution of these Ca^{2+} transport proteins to the altered Ca^{2+} handling in PTH-related disorders seems likely.

Treatment of primary hyperparathyroidism has been limited to surgical ablation (parathyroidectomy) of the affected glands. A new approach for treating primary hyperthyroidism is to target the mechanisms that regulate the secretion of PTH. The Ca^{2+}-sensing receptor is the first step in this process. In the last decade synthetic compounds, referred to as calcimimetic compounds, have been developed [11, 12]. Type I calcimimetics are full agonists of the Ca^{2+}-sensing receptor, whereas type II calcimimetics are small organic compounds that, upon binding to the Ca^{2+}-sensing receptor, enhance the sensitivity of the Ca^{2+}-sensing receptor to Ca^{2+} in an allosteric fashion, thereby altering PTH secretion by the parathyroid glands. In this way, the type II calcimimetic compounds may provide a novel therapy for treating hyperparathyroidism [11, 12].

The present study aims to gain insight into the molecular mechanism(s) underlying the alterations in renal Ca^{2+} handling in PTH-related disorders. To this end, the in vivo effect of PTH on the expression of proteins involved in renal transcellular Ca^{2+} reabsorption was examined. Here, we demonstrated that PTH affects renal Ca^{2+} handling through the coordinated regulation of the expression of renal transcellular Ca^{2+} transport proteins. Moreover, the PTH-induced increase in Ca^{2+} influx through TRPV5 facilitates the expression of downstream Ca^{2+} transport proteins, thereby further emphasizing the gatekeeper function of TRPV5.

METHODS

Animals

In experiment 1, male Sabra rats, weighing 250 g, had free access to normal rat chow and water. After acclimatization, rats were either sham-operated ($N = 4$) or parathyroidectomized ($N = 8$) by cauterization. After 7 days, Alzet osmotic minipumps were implanted subcutaneously (model 2001) (Durect Corporation, Cupertino, CA, USA). Sham-operated rats received vehicle solution [2% (wt/vol) cysteine HCl] and parathyroidectomized rats received either vehicle solution or bovine PTH 1-34 (Sigma Chemical Co., St. Louis, MO, USA) at a rate of 0.25 U PTH 1-34/hour (parathyroidectomized + PTH) for 7 days. In experiment 2, Male C57BL6 mice, 8 weeks of age, were fed standard chow and given water ad libitum. After acclimatization, mice were divided in four groups of five animals each. Mice were anesthetized and an Alzet model 1007D osmotic minipump (Durect Corporation) was implanted subcutaneously. The pump infused the calcimimetic compound NPS R-467 (NPS Pharmaceuticals, Salt Lake City, UT, USA) at a dose of 10, 30, or 100 μmol per kg body weight per day or vehicle solution [45% (wt/vol) aqueous solution of 2-hydroxypropyl-β-cyclodextrin]. At the end of the treatment periods, animals were sacrificed, and blood and kidney samples were taken. The animal ethics board of the University Medical Center Nijmegen and Hadassah University Hospital Jerusalem approved all animal experimental procedures.

Primary cultures of rabbit kidney CNT/cortical collecting duct (CCD)

Rabbit kidney CNT/CCD cells were immunodissected from New Zealand white rabbits (5 weeks of age) with monoclonal antibody R2G9 and set in primary culture on permeable filter supports (0.33 cm^2) (Corning-Costar, Cambridge, MA, USA) as described previously in detail [13]. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (Gibco, Paisley, UK) supplemented with 5% (vol/vol) decomplemented fetal calf serum (FCS), 10 μg/mL ciproxin, 10 μg/mL nonessential amino acids (Gibco), 5 μg/mL insulin, 5 μg/mL transferrin, 50 nmol/L hydrocortisone, 70 ng/mL prostaglandin E_1 (PGE_1), 50 nmol/L Na_2SeO_3, 5 pmol/L triiodothyronine, and 5 μmol/L indomethacin, equilibrated with 5% CO_2 95% air at 37°C. Two days after seeding, the cells were incubated for 120 hours with and without 100 nmol/L bovine PTH 1-34 (Sigma Chemical Co.) at the apical (total volume 100 μL) and basolateral (total volume 600 μL) compartment, whereas 10 μmol/L ruthenium red was added only to the apical side. Transport assays were performed with confluent monolayers 7 days after seeding the cells.

Determination of transepithelial Ca^{2+} transport

Confluent monolayers of primary cultures were washed and preincubated in a physiologic salt solution containing (mmol/L) 140 NaCl, 2 KCl, 1 K_2HPO_4, 1 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 5 L-alanine, 10 Hepes/Tris (pH 7.4), and 5 μmol/L indomethacin for 15 minuts at 37°C, adding 10 μmol/L of the TRPV5-mediated Ca^{2+}-influx blocker ruthenium red [14] to the apical side. Subsequently, the monolayers were incubated with 100 nmol/L PTH and 10 μmol/L ruthenium red for a further 90 minutes to measure transepithelial Ca^{2+} transport. At the end of the incubation period, 25 μL samples were collected in duplicate from the apical compartment and assayed for Ca^{2+} concentration using a colorimetric assay kit (Boehringer, Mannheim, Germany). Under the outlined experimental conditions, these polarized renal cells exhibit transcellular Ca^{2+} transport, of which the net apical-to-basolateral Ca^{2+} flux is linear with time for at least 3 hours [13, 15]. Ca^{2+} reabsorption is expressed in $nmol/hour^{-1}/cm^{-2}$.

Analytical procedures

Serum Ca^{2+} concentrations were analyzed using a colorimetric assay kit as described previously [16]. Serum phosphorus levels were measured on a Hitachi autoanalyzer (Hitachi Corp., Tokyo, Japan). PTH levels were determined using either a rat PTH (1-34) immunoradiometric assay or a mouse intact PTH enzyme-linked immunosorbent assay (ELISA) kit (Immunotopics, San Clemente, CA, USA). $1,25(OH)_2D_3$ was measured by immunoextraction followed by quantitation by ^{125}I-radioimmunoassay (RIA) (IDS, Boldon, UK) [17].

RNA isolation and quantitative polymerase chain reaction (PCR)

Total RNA from kidney and primary CNT/CCD cells was isolated using Trizol Reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) according to the manufacturer's protocol. Total DNAse-treated RNA (2 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Gibco BRL) as described previously [18]. Expression of TRPV5, TRPV6, calbindin-D_{28K}, calbindin-D_{9K}, NCX1, PMCA1b, Ca^{2+}-sensing receptor, and vitamin D receptor mRNA, as well as mRNA levels of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT), as an endogenous control, were determined by quantitative real-time PCR on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switserland). The primer and probe sequences of Ca^{2+}-sensing receptor, vitamin D receptor, TRPV5, calbindin-D_{28K}, and NCX1 are depicted in Table 1. Other sequences have been described previously [19, 20].

Immunohistochemistry

Kidney tissue was cut into pieces, placed in 1% (wt/vol) periodate-lysine-paraformaldehyde fixative for 2 hours at room temperature, and incubated overnight at 4°C in phosphate-buffered saline (PBS) containing 15% (wt/vol) sucrose. Subsequently, kidney tissues were frozen in liquid nitrogen and 7 μm sections were cut for the staining procedure. For detection of TRPV5 abundance or colocalization of TRPV5 and Ca^{2+}-sensing receptor, kidney sections were stained with guinea pig anti-TRPV5 antiserum (1:50) as described previously [19] and rabbit antihuman Ca^{2+}-sensing receptor antibody (1:500) (antiserum 4641, a gift from NPS Pharmaceuticals).

Table 1. Sequences of primers and Taqman probes for real-time quantitative polymerase chain reaction (PCR)

Gene		Forward primer	Reverse primer	Probe
CaSR	R	5′-CTTTCCTATCCATTTTGGAGTAGCA-3	5′-GCGAATATCATGGCTTGTAACCA-3′	5′-CGGAAGTTATACCTAATGCACTCCACAGACTCT-3′
	M	5′-CTTTCCTATCCATTTTGGAGTAGCA-3′	5′-GCAAAGATCATGGCTTGTAACCA-3′	5′-CGGAAGTTATACCTGATGCACTCCACAGACTCT-3′
VDR	R	5′-AATGGAGATTGCCGCATCAC-3′	5′-TGTCCACACAGCGTTTGA-3′	5′-AGGACAACCGGCGACACTGCCA-3′
	M	5′-AATGGAGATTGCCGCATCAC-3′	5′-TGTCCACGCAGCGTTTGA-3′	5′-AGGACAACCGGCGACACTGCCA-3′
TRPV5	Rb	5′-CGGGTTGAGAACCATCATGAC-3′	5′-CCTTGTCTGAGCACTTGAATGC-3′	5′-TCCTCTGCGAGTGCTTCGCTATGTGG-3′
CaBP	Rb	5′-GATGGGAAGCTGGAATTAACTGA-3′	5′-CCACACATTTTGATTCCCTGAA-3′	5′-ATGGCCAGGTTACTACCAGTGCAAGAGAATTT-3′
NCX1	Rb	5′-GGGCTGGCAACATTTTAAAGAG-3′	5′-TTTCCGTGTTGACTTCATGCA-3′	5′-AGCTGACCAAGCTAGGAAGGCTGTCAGC-3′

Abbreviations are: CaSR, Ca^{2+}-sensing receptor; VDR, vitamin D receptor; TRPV5, transient receptor potential, vanilloid, member 5; CaBP, calbindin-D_{28K}; NCX1, Na^+/Ca^{2+}-exchanger; R, rat; M, mouse; Rb, rabbit. Sequences of the other target genes used are as described previously [19, 20].

PCR primers and fluorescent probes (5′-FAM to 3-TAMRA) were designed using the computer program Primer Express (Applied Biosystems, Branchburg, NJ, USA) and purchased from Biolegio (Malden, The Netherlands).

Table 2. Effect of parathyroidectomy and parathyroid hormone (PTH) supplementation on serum parameters in Sabra rats[a]

	Sham	Parathyroidectomy	Parathyroidectomy + PTH
PTH *pg/mL*	43.5 ± 14.7	6.1 ± 2.9[b]	14.4 ± 4.6
Ca^{2+} *mmol/L*	2.89 ± 0.06	1.61 ± 0.07[c]	2.51 ± 0.04[d]
Phosphorus *mmol/L*	1.59 ± 0.06	2.15 ± 0.04[c]	1.82 ± 0.10[d]
$1,25(OH)_2D_3$ *pmol/L*	375 ± 9	261 ± 29[b]	234 ± 13[b]

[a] Sham, sham-operated; parathyroidectomy + PTH, parathyroidectomy supplemented with 0.25 U PTH (1-34)/hour. Data are presented as mean ± SE ($N = 4$).
[b] $P < 0.05$ versus sham.
[c] $P < 0.05$ versus sham and parathyroidectomy + PTH.
[d] $P < 0.05$ versus sham and parathyroidectomy.

To visualize TRPV5 and Ca^{2+}-sensing receptor, sections were stained with goat antiguinea Alexa 488–conjugated anti-IgG (1:300) (Sigma Chemical Co.) and goat antirabbit Alexa 594–conjugated anti-IgG (1:300) (Sigma Chemical Co.), respectively. Sections were visualized by confocal laser scanning microscopy (MRC-1024) (Bio-Rad, Richmond, CA, USA). To quantify TRPV5 protein expression, digital images of each kidney section were taken with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY, USA) and the integrated optical density was measured by computer analysis with the Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

Immunoblotting

For protein analysis, frozen kidney tissue and the cultured cells were homogenized in ice-cold solubilization buffer as previously described [21]. Total kidney protein fractions (10 µg) or cell protein fractions (4 µL) were separated on 12% or 16.5% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted to polyvinylidine difluoride (PVDF)-nitrocellulose membranes (Immobilon-P) (Millipore Corporation, Bedford, MA, USA). Blots were incubated with calbindin-D_{28K} antibody (1:10,000) (Sigma Chemical Co.), calbindin-D_{9K} antibody (1:3000) (Swant, Bellinzona, Switzerland), or Na^+/K^+-ATPase antibody (1:10,000) (kindly provided by J.B. Koenderink, Department of Biochemistry, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) [22] and thereafter with peroxidase-conjugated goat antirabbit antibody (1:2000) (Sigma Chemical Co.). Immunoreactive protein was detected using the enhanced chemiluminescence (ECL) method as described by the manufacturer (Amersham, Buckinghamshire, UK). Protein expression was quantified by computer-assisted densitometry with the use of the Image-Pro Plus version 3.0 software (Media Cybernetics).

Statistical analysis

Values are expressed as mean ± SE. Statistical significance of differences between groups was determined by analysis of variance (ANOVA) followed by pair-wise comparisons using the method of least significant difference. An unpaired *t* test was used for comparisons between control and 100 µmol/kg body weight/day groups. Differences in means with $P < 0.05$ were considered statistically significant.

RESULTS

Rescue of parathyroidectomy-induced hypocalcemia by PTH supplementation in rats

Surgical removal of the parathyroid glands resulted in the reduction of serum PTH levels, whereas serum PTH levels tended to increase after infusion with 0.25 U PTH (1-34)/hour (Table 2). Moreover, parathyroidectomy caused a severe hypocalcemia and hyperphosphatemia, which were partially restored by PTH supplementation (Table 2). In addition, serum $1,25(OH)_2D_3$ levels were decreased in parathyroidectomized rats, but no changes were observed after PTH supplementation (Table 2).

Effect of PTH on renal expression of Ca^{2+} transporters

To investigate the effect of PTH on renal Ca^{2+} handling, the expression of genes encoding Ca^{2+} transport proteins involved in transcellular Ca^{2+} reabsorption was examined using quantitative real-time PCR. Surgical removal of the parathyroid glands in rats induced a 70% decrease in TRPV5 mRNA levels, which were completely normalized after PTH infusion (Fig. 1A). In contrast, no significant effects were detected on the expression of TRPV6 mRNA between sham-operated, parathyroidectomy, and parathyroidectomy + PTH rats (Fig. 1B). Similar to TRPV5 expression, mRNA transcripts encoding for calbindin-D_{28K} were down-regulated in parathyroictomized rats and partially restored in the PTH-supplemented animals (Fig. 1C). The expression of calbindin-D_{9K} was not measured, because it is not detectable in rat kidney. In addition, parathyroidectomy reduced the expression of NCX1 (Fig. 1D), whereas PMCA1b mRNA levels were not altered (Fig. 1E). No differences were observed in Ca^{2+}-sensing receptor or vitamin D receptor mRNA levels after parathyroidectomy or subsequent infusion of PTH (Fig. 1F and G).

Subsequently, examination of the expression of renal Ca^{2+} transport proteins by immunohistochemistry revealed a marked decrease in TRPV5 protein

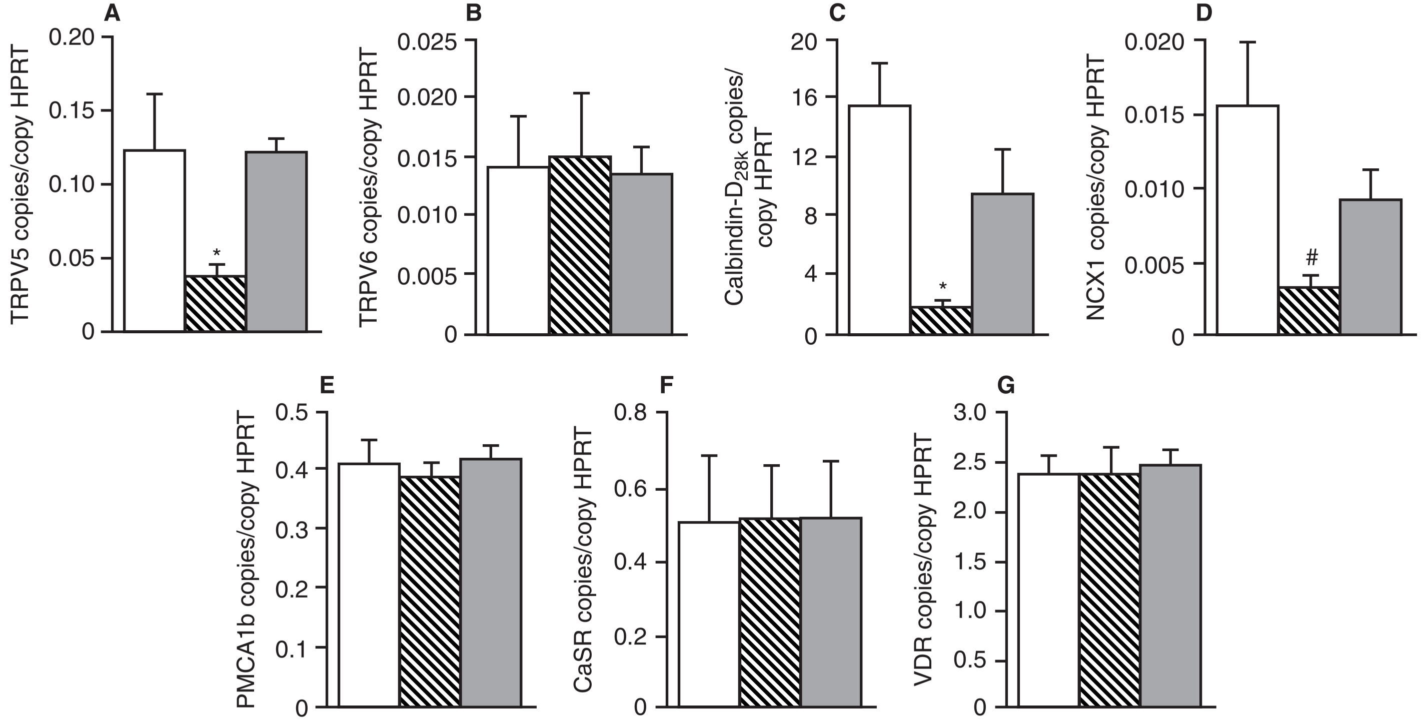

Fig. 1. Effect of parathyroidectomy and supplementation with parathyroid hormone (PTH) (1-34) on mRNA expression levels of genes encoding Ca^{2+} transport proteins in rat kidney. Using real-time quantitative polymerase chain reaction (PCR), renal mRNA expression of transient receptor potential, vanilloid, member 5 (TRPV5) (*A*), TRPV member 6 (TRPV6) (*B*), calbindin-D_{28K} (*C*), Na^+/Ca^{2+}-exchanger (NCX1) (*D*), plasma membrane Ca^{2+}-ATPase (PMCA1b) (*E*), Ca^{2+}-sensing receptor (CaSR) (*F*), and vitamin D receptor (VDR) (*G*) of the different experimental groups were measured and presented as a ratio to hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression. Sham-operated rats (□); parathyroidectomized rats (□); parathyroidectomizied rats supplemented with 0.25 U PTH (1-34)/hour (■). Data are presented as means ± SE ($N = 4$). $^*P < 0.05$ versus all; $^\#P < 0.05$ versus sham.

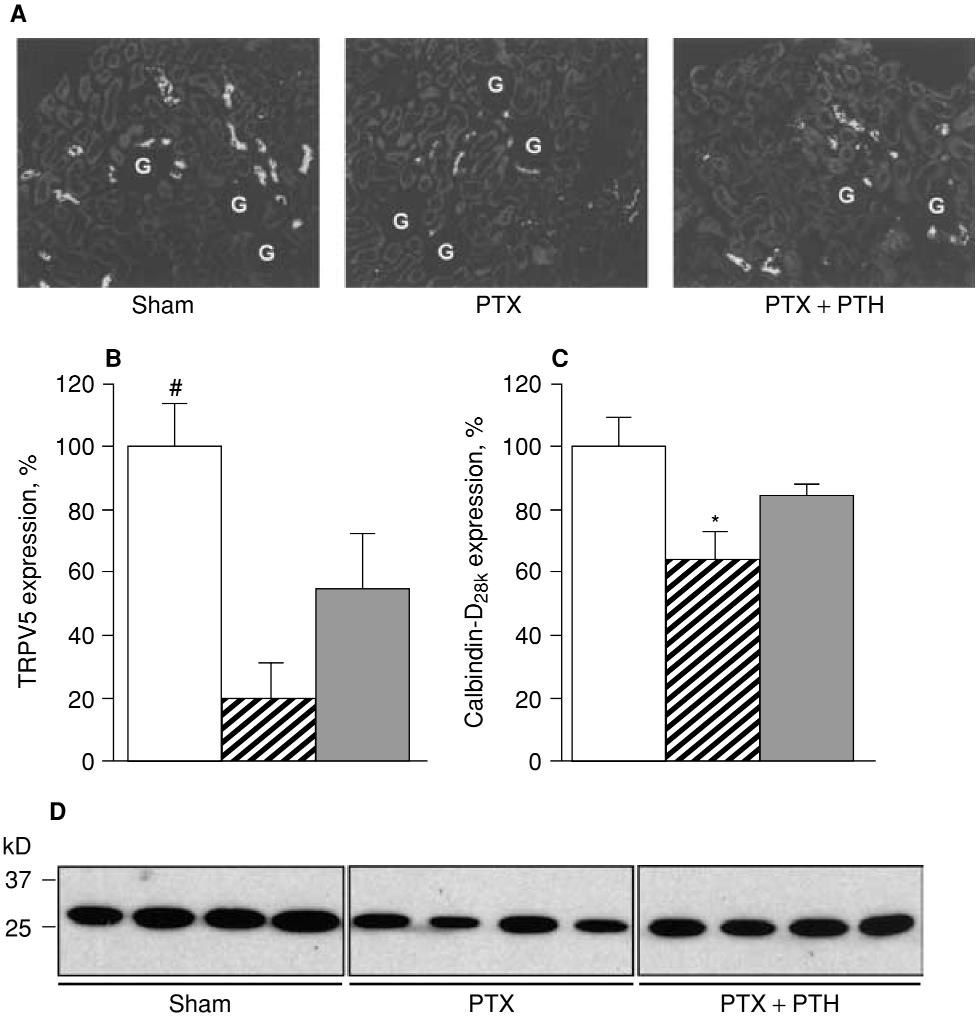

Fig. 2. Effect of parathyoidectomy (PTX) and supplementation with parathyroid hormone (PTH) (1-34) on protein expression levels of transient receptor potential, vanilloid, member 5 (TRPV5) in rat kidney. Immunofluorescence staining of kidney cortex sections of sham, parathyroidectomy, and parathyroidectomy + PTH rats showing the abundance of TRPV5 (*A*), which was quantified by computer analysis of the integrated optical density and expressed in relative percentages (*B*). Glomeruli (G) are depicted in the images. Immunoblots of samples (10 μg protein each) from homogenates of kidney tissue were labeled with antibodies against calbindin-D_{28K} (*D*). Expression of calbindin-D_{28K} protein was quantified by computer-assisted densitometry analysis and expressed in relative percentages (*C*). Sham (□), sham-operated rats; parathryoidectomy (□), parathyroidectomized rats + PTH (■), parathyroidectomized rats supplemented with 0.25 U PTH (1-34)/hour. Data are presented as means ± SE (N= 4). $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus all.

abundance in response to parathyroidectomy, as indicated by the reduced immunopositive staining (Fig. 2A). As observed for TRPV5 mRNA expression, PTH supplementation increased the protein abundance in the parathyroidectomized animals. The corresponding integrated optical density analysis confirmed the significant decline in TRPV5 protein abundance after parathyroidectomy (Fig. 2B). Furthermore, Western blot analysis of calbindin-D_{28K} consistently demonstrated a down-regulation in the protein abundance in parathyroidectomized rats and a subsequent normalization after PTH infusion (Fig. 2D). Densitometric analysis of the intensity of the immunocomplexes confirmed this decrease in calbindin-D_{28K} protein expression after parathyroidectomy (Fig. 2C).

NPS R-467 treatment reduced serum PTH and Ca^{2+} levels in mice

Infusion of the calcimimetic compound NPS R-467 for 7 days reduced serum PTH levels. Furthermore, this reduction was associated with a fall in serum Ca^{2+} concentrations and an increase in serum phosphorus levels. Measurements of circulating $1,25(OH)_2D_3$ levels revealed no effect of NPS R-467 treatment compared with vehicle (Table 3).

Reduction in renal Ca^{2+} transport proteins after NPS R-467 treatment

To address the molecular mechanism responsible for the decreased serum Ca^{2+} levels upon NPS R-467

Table 3. Effect of treatment with NPS R-467 on serum parameters in male C57BL6 mice[a]

	Control	10 μmol/kg	30 μmol/kg	100 μmol/kg
Parthyroid hormone *pg/mL*	20.8 ± 2.5	14.6 ± 1.2[b]	13.0 ± 0.6[b]	10.8 ± 1.2[b]
Ca^{2+} *mmol/L*	2.42 ± 0.02	2.34 ± 0.02	2.26 ± 0.02[b]	2.18 ± 0.06[b,c]
Phosphorus *mmol/L*	2.07 ± 0.19	2.72 ± 0.09[b]	2.72 ± 0.12[b]	2.71 ± 0.06[b]
$1{,}25(OH)_2D_3$ *pmol/L*	309 ± 59	326 ± 14	307 ± 10	227 ± 27

[a] Control, treated with vehicle; 10 μmol/kg, treated with NPS R-467 at 10 μmol/kg body weight/day; 30 μmol/kg, treated with NPS R-467 at 30 μmol/kg body weight/day; 100 μmol/kg, treated with NPS R-467 at 100 μmol/kg body weight/day. Data are presented as means ± SE ($N = 5$).

[b] $P < 0.05$ versus control.

[c] $P < 0.05$ versus 10 μmol/kg.

infusion, the expression of genes encoding Ca^{2+} transport proteins involved in renal transcellular Ca^{2+} reabsorption was examined. TRPV5 mRNA expression was consistently lower in kidneys of NPS R-467–treated mice compared to controls (Fig. 3A), whereas no differences in the expression of TRPV6 were detected (Fig. 3B). Similar to TRPV5 expression, mRNA levels of both calbindin-D_{28K} and calbindin-D_{9K} were down-regulated in response to calcimimetic treatment (Fig. 3C and D). In addition, NPS R-467 treatment inhibited the expression of NCX1 (Fig. 3E), whereas PMCA1b mRNA levels were only down-regulated at 100 μmol/kg body weight/day compared to control animals (Fig. 3F). Infusion with NPS R-467 induced a reduction in the expression of Ca^{2+}-sensing receptor mRNA compared to control mice (Fig. 3G), while no differences were detected in the mRNA expression of vitamin D receptor (Fig. 3H).

Subsequently, the reduced mRNA levels of TRPV5 after NPS R-467 treatment were confirmed by immunohistochemical analysis. As shown in Figure 4A, the staining intensity of the TRPV5 protein was remarkably reduced in kidney cortex sections of NPS R-467–treated mice. Figure 4B depicts the corresponding integrated optical density analysis and demonstrated that TRPV5 protein expression is significantly down-regulated. In addition, the immunoblots shown in Figure 4C and E demonstrated a down-regulation of both calbindin-D_{28K} and calbindin-D_{9K} proteins in the NPS R-467–treated mice compared to controls, which was confirmed by densitometry (Fig. 4D and F).

Localization of the Ca^{2+}-sensing receptor in mouse kidney cortex

In addition to the presence in the parathyroid glands, the Ca^{2+}-sensing receptor is also expressed in kidney. In Figure 5, the distribution of Ca^{2+}-sensing receptor immunopositive staining in kidney cortex was compared to the TRPV5 localization. The distribution of Ca^{2+}-sensing receptor was most abundant in the basolateral region of the thick ascending limb (Fig. 5A, arrowheads). Importantly, immunopositive staining for Ca^{2+}-sensing receptor (Fig. 5A, asterisks, and C) was also observed in TRPV5 expressing DCT and CNT (Fig. 5B, asterisks, and D). TRPV5 was predominantly localized along the apical domain of these segments, whereas Ca^{2+}-sensing receptor–specific staining revealed a more diffuse pattern.

Inhibition of PTH-stimulated transepithelial Ca^{2+} transport decreased calbindin-D_{28K} and NCX1 expression

To determine the molecular mechanism responsible for the regulation of the Ca^{2+} transport proteins, primary cultures of the rabbit CNT/CCD grown to confluence on permeable supports were employed. Figure 6A shows that in the absence of any stimulus, these monolayers exhibit a net apical-to-basolateral Ca^{2+} flux of 43 ± 2 $nmol/hour^{-1}/cm^{-2}$. Treatment with PTH (100 nmol/L) had a stimulatory effect on transepithelial Ca^{2+} reabsorption (Fig. 6A). Apical addition of the Ca^{2+} channel blocker ruthenium red (10 μmol/L) completely inhibited basal transepithelial Ca^{2+} reabsorption and PTH-stimulated Ca^{2+} transport (Fig. 6A). Furthermore, the stimulatory effect of PTH on Ca^{2+} transport was accompanied by increased mRNA expression of TRPV5, calbindin-D_{28K} and NCX1 (Fig. 6B to D). Importantly, this PTH-induced increase in calbindin-D_{28K} and NCX1 mRNA expression was significantly reduced in monolayers cultured in the presence of ruthenium red, while the mRNA expression of TRPV5 was unaltered after ruthenium red treatment. Immunoblot and densitometric analysis of the intensity of the immunocomplexes of calbindin-D_{28K} confirmed this decrease in calbindin-D_{28K} protein expression after ruthenium red treatment using the Na^+/K^+-ATPase as an internal control for equal loading (Fig. 6E and F).

DISCUSSION

The present study demonstrated that PTH stimulates renal Ca^{2+} reabsorption through the coordinated expression of transcellular Ca^{2+} transport proteins. Moreover, our results indicated that by up-regulating the expression of the TRPV5, the Ca^{2+} influx is increased, which in turn facilitates the regulation of the down-stream Ca^{2+} transport proteins. These findings further emphasize the gatekeeper function of TRPV5.

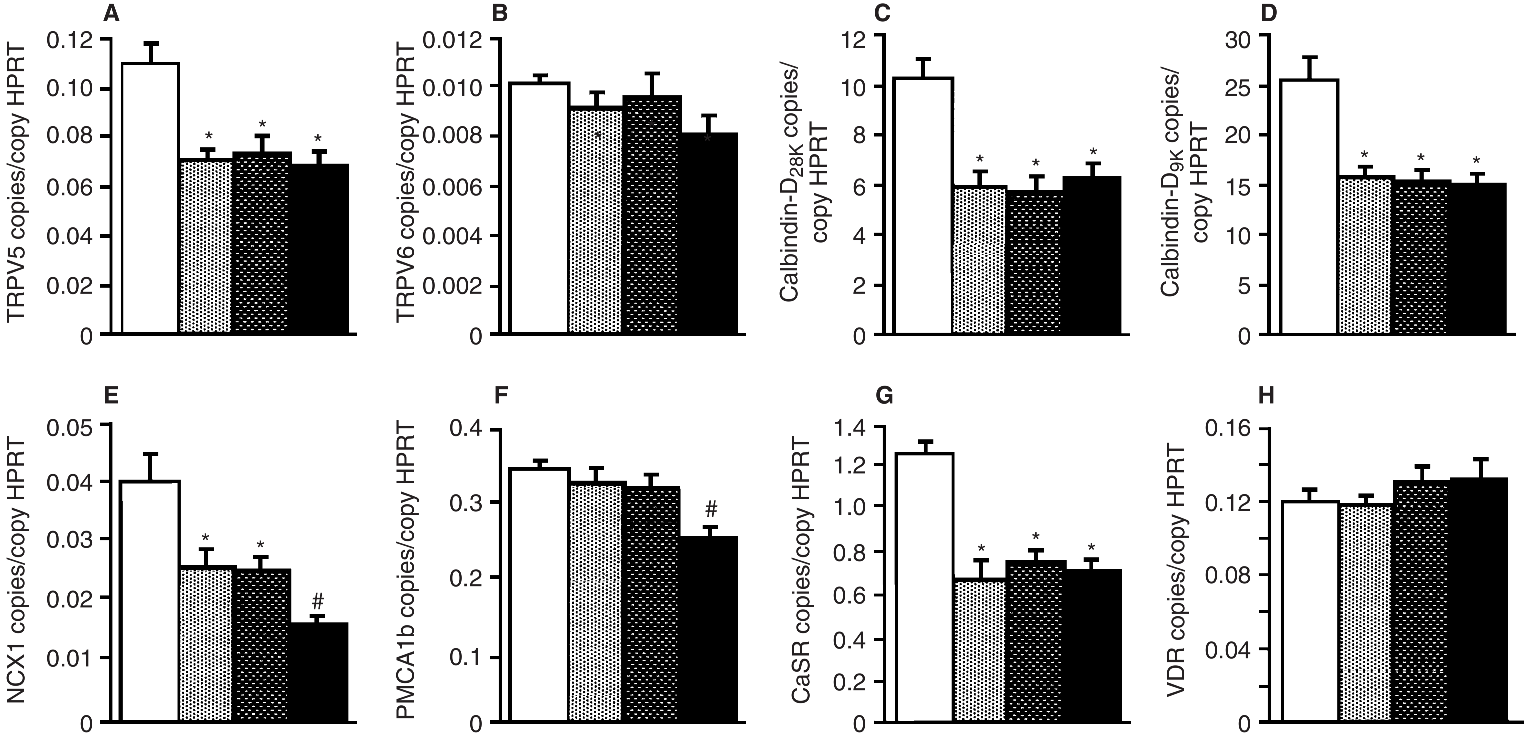

Fig. 3. Effect of NPS R-467 on mRNA expression levels of genes encoding Ca^{2+} transport proteins in the kidney. Using real-time quantitative polymerase chain reaction (PCR), renal mRNA expression of transient receptor potential, vanilloid, member 5 (TRPV5) (*A*), TRPV member 6 (TRPV6) (*B*), calbindin-D_{28K} (*C*), calbindin-D_{9K} (*D*), Na^+/Ca^{2+}-exchanger (NCX1) (*E*), plasma membrane Ca^{2+}-ATPase (PMCA1b) (*F*), Ca^{2+}-sensing receptor (CaSR) (*G*), and vitamin D receptor (VDR) (*H*) of the different experimental groups were measured and presented as a ratio to hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression. Control mice treated with vehicle (□); mice treated with NPS R-467 at 10 μmol/kg body weight/day (□); mice treated with NPS R-467 at 30 μmol/kg body weight/day (□); mice treated with NPS R-467 at 100 μmol/kg body weight/day (■). Data are presented as means ± SE ($N = 5$). $^*P < 0.05$ versus control; $^\#P < 0.05$ versus all.

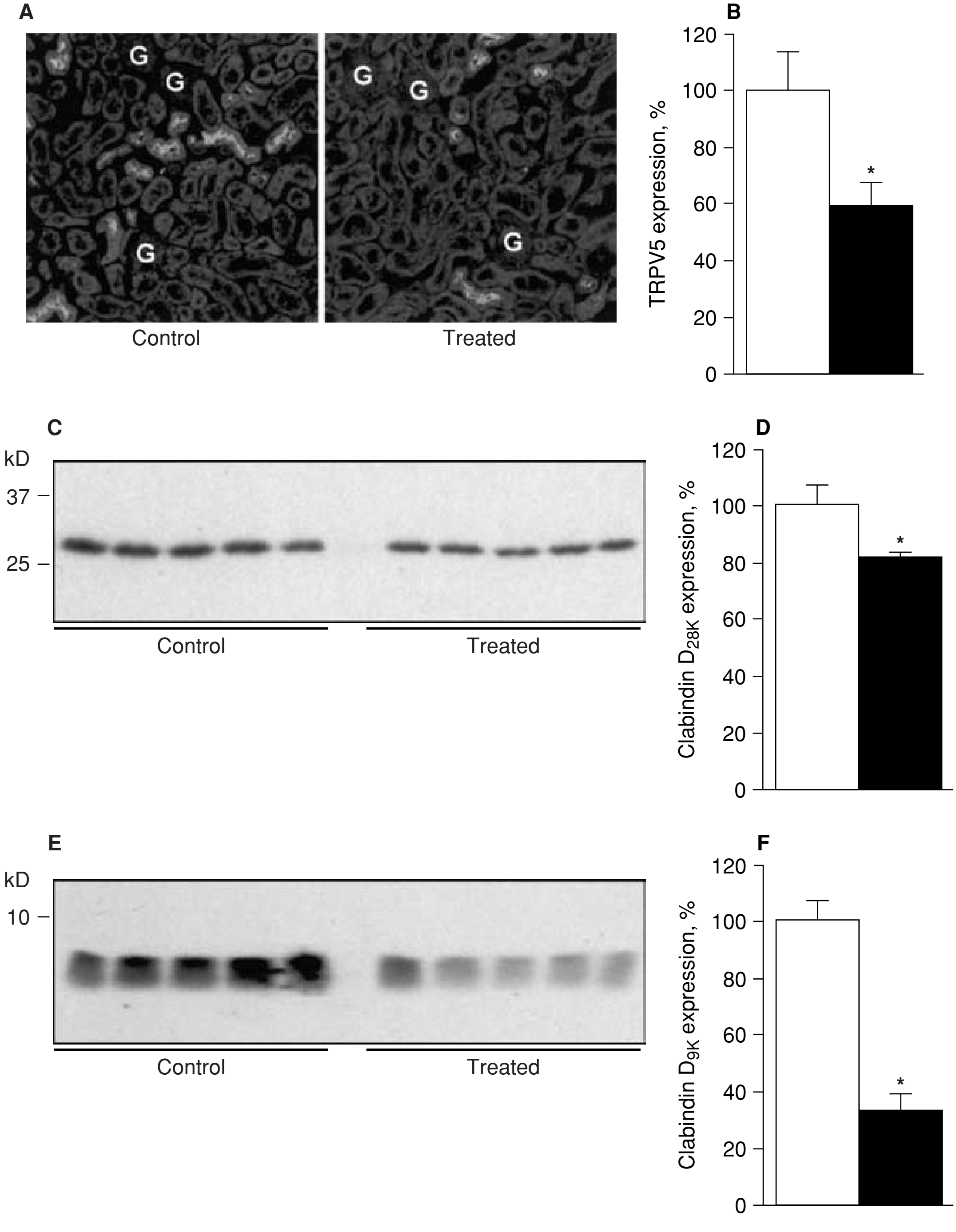

Fig. 4. Effect of NPS R-467 on protein expression levels of transient receptor potential, vanilloid, member 5 (TRPV5) in the kidney. Immunofluorescence staining of kidney cortex sections of control and treated mice showing the abundance of TRPV5 (*A*), which was quantified by computer analysis of the integrated optical density and expressed in relative percentages (*B*). Glomeruli (G) are depicted in the images. Immunoblots of samples (10 μg protein each) from homogenates of kidney tissue were labeled with antibodies against calbindin-D_{28K} (*C*) and calbindin-D_{9K} (*E*). Expression of calbindin-D_{28K} and calbindin-D_{9K} protein was quantified by computer-assisted densitometry analysis and expressed in relative percentages (*D* and *F*). Control (□), mice treated with vehicle; treated (■), mice treated with NPS R-467 at 100 μmol/kg body weight/day. Data are presented as means ± SE ($N = 5$). $^*P < 0.05$ versus control.

The available therapy for treating primary hyperparathyroidism, a disease that so far has resisted pharmacologic intervention, has been limited to surgical removal of the affected glands. Here, we showed that parathyroidectomy in rats results in marked reduction of serum PTH levels and hyperphosphatemia, a well-known symptom in hypoparathyroidism [23–25]. In addition, parathyroidectomy induced the concomitant decrease of renal

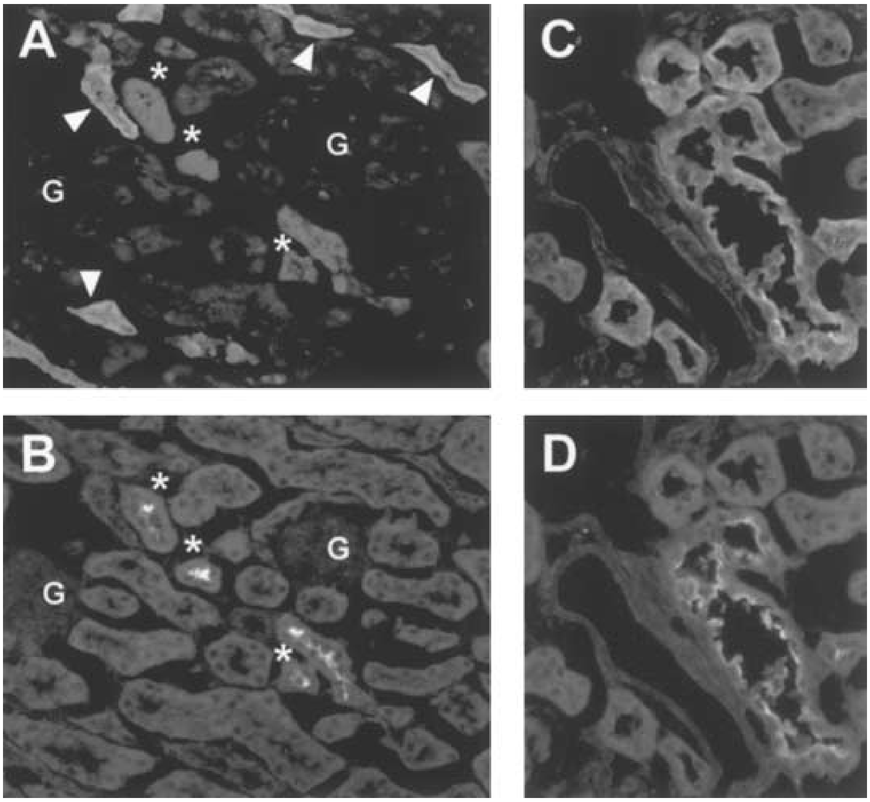

Fig. 5. Immunofluorescence localization of the Ca^{2+}-sensing receptor and transient receptor potential, vanilloid, member 5 (TRPV5). Double staining of a mice kidney cortex section showing staining for Ca^{2+}-sensing receptor (*A*) and TRPV5 (*B*). The asterisks indicate the tubular segments, where Ca^{2+}-sensing receptor and TRPV5 colocalize. The tubules depicted by the arrowheads stained intensely for Ca^{2+}-sensing receptor, but were negative for TRPV5. G is glomerulus. Colocalization of Ca^{2+}-sensing receptor (*C*) and TRPV5 (*D*) in distal convoluted tubule (DCT) and connecting tubule (CNT) of kidney cortex section.

mRNA and protein abundance of TRPV5, calbindin-D_{28K}, and NCX1. As demonstrated by the hypercalciuria present in TRPV5 knockout mice, the decline in renal expression of transcellular Ca^{2+} transport proteins in the present experiment is indicative for a decreased capacity of active Ca^{2+} reabsorption [26] and relates to the development of the observed hypocalcemia. Serum PTH levels tended to increase after PTH supplementation, which was apparently sufficient to restore the expression of the renal Ca^{2+} transport proteins and increase serum Ca^{2+} concentrations. Moreover, these findings imply that PTH, in concert with its well-established effect on Ca^{2+} resorption from bone [27], increases renal Ca^{2+} reabsorption by up-regulating the expression of TRPV5 and other Ca^{2+} transport proteins, thereby maintaining the Ca^{2+} balance. In agreement with this finding, PTH receptors have been detected throughout the kidney, as well as in the actively Ca^{2+} transporting tubules DCT and CNT [9]. Thus, PTH is capable of stimulating active Ca^{2+} transport in kidney through receptor-mediated actions.

Calcimimetic compounds provide a novel non-surgical therapy for hyperparathyroidism by enhancing the sensitivity of the Ca^{2+}-sensing receptor to extracellular Ca^{2+}, thereby suppressing the secretion of PTH and normalizing serum Ca^{2+} levels [11]. Studies of inherited disorders caused by mutations in Ca^{2+}-sensing receptor clearly suggested that this receptor is the mechanism for Ca^{2+}-mediated regulation of PTH secretion [28]. To determine whether NPS R-467 could indeed effectively lower PTH levels and affect renal Ca^{2+} handling, mice were treated for 7 days with the calcimimetic compound NPS R-467. In agreement with previous findings, NPS R-467 successfully decreased serum PTH levels [12, 29]. In addition to the suppression of PTH, serum Ca^{2+} concentrations were reduced after NPS R-467 treatment, whereas serum phosphate levels were increased. Interestingly, a similar concurrent down-regulation of the expression of the renal Ca^{2+} transport proteins (i.e., TRPV5, calbindin-D_{28K} and calbindin-D_{9K}, NCX1, and PMCA1b) was observed in kidneys of NPS R-467–treated mice as for the parathyroidectomized rats, and indicative for a decreased capacity of active Ca^{2+} reabsorption. This finding is consistent with complementary findings in other studies. A short-term use of the type II calcimimetic compound NPS R-568 was previously associated with an increase in urinary Ca^{2+} excretion in hypercalcemic patients with primary hyperparathyroidism [30]. Recently, Fox et al [abstract; Fox J et al, *J Am Soc Nephrol* SA-FC179, 2003] showed that injections with NPS R-467 in rats increased the fractional excretion of Ca^{2+}. Taken together, our findings suggested that PTH stimulates the coordinated expression of transcellular Ca^{2+} transport proteins in kidney, which could contribute to increased Ca^{2+} reabsorption and positively affect Ca^{2+} balance.

Active Ca^{2+} reabsorption is a primary target for the regulation by calciotropic hormones. We have previously

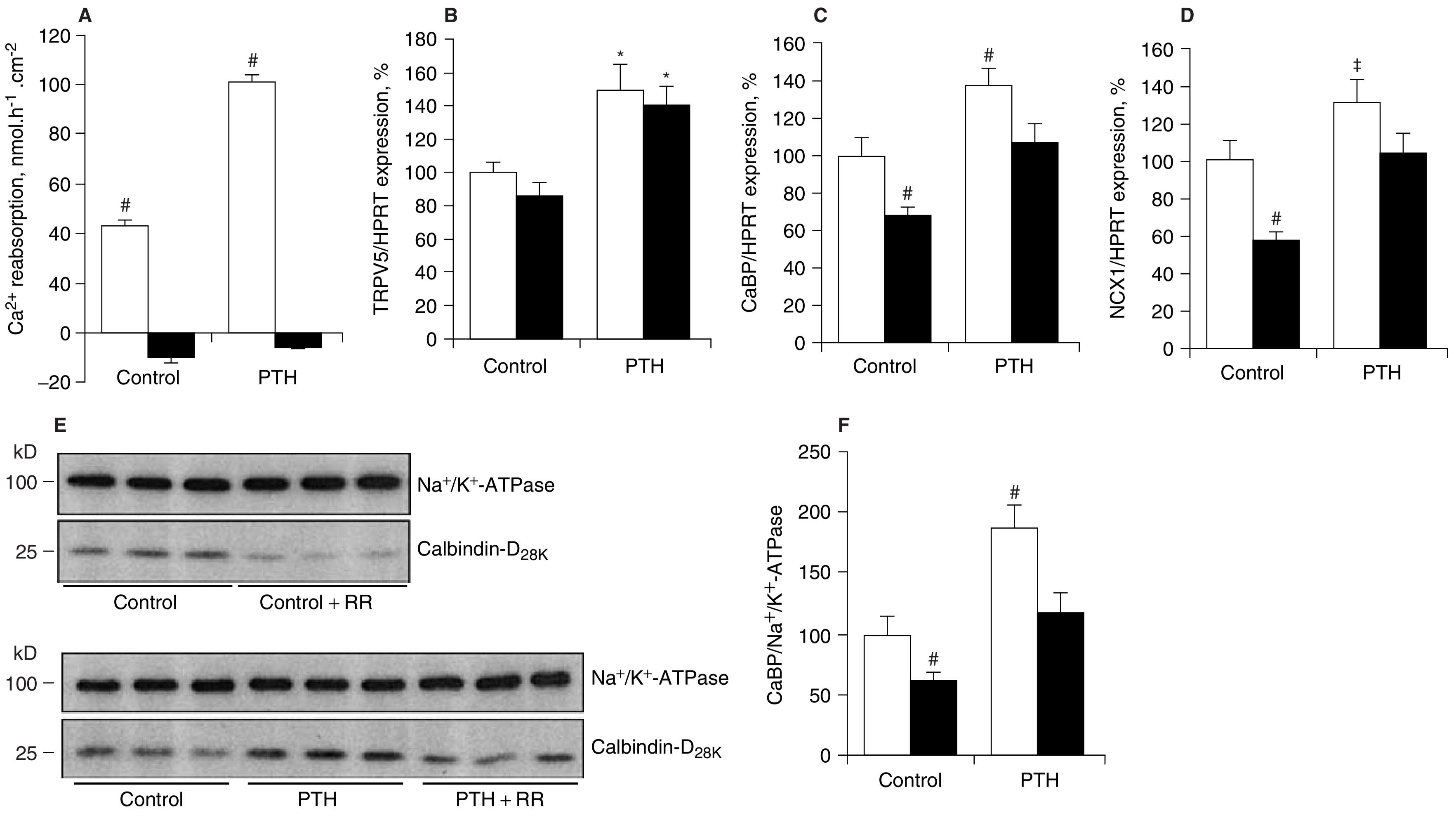

Fig. 6. Effect of parathyroid hormone (PTH) and ruthenium red on Ca^{2+} transport and expression levels of genes encoding Ca^{2+} transport proteins in primary cultures of rabbit kidney connecting tubule/cortical collecting duct (CNT/CCD). (*A*) Transepithelial Ca^{2+} transport across confluent monolayers was measured in the absence or presence of PTH and ruthenium red (RR). At the end of the incubation period apical medium was collected to determine the amount of Ca^{2+} transported across the monolayer. Using real-time quantitative polymerase chain reaction (PCR), mRNA expression of transient receptor potential, vanilloid, member 5 (TRPV5) (*B*), calbindin-D_{28K} (*C*), and Na^+/Ca^{2+}-exchanger (NCX1) (*D*), of the different experimental groups were measured and presented as a ratio to hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression. (*E*) Western blot analysis of homogenates from rabbit CNT/CCD cultured in the absence or presence of PTH and ruthenium red, labeled with antibodies against calbindin-D_{28K} and Na^+-K^+-ATPase, as an internal control for equal loading. (*F*) Densitometric analysis of the corresponding blots. Calbindin-D_{28K} expression is presented as a ratio to Na^+/K^+-ATPase expression in relative percentages. Control, unstimulated cells; PTH, cells stimulated with 100 nmol/L PTH for 120 hours; cultured in the absence (■) or presence (□) of 10 μmol/L ruthenium red. Data are presented as means ± SE ($N = 9$). $^{\#}P < 0.05$ versus all; $^{*}P < 0.05$ versus control in the presence and absence of ruthenium red; $^{\ddagger}P < 0.05$ versus control in the absence of ruthenium red.

shown that the expression of TRPV5, as well as the other proteins involved in active Ca^{2+} transport, is positively regulated by $1,25(OH)_2D_3$ and other hormones, like estrogens [19, 31, 32]. $1,25(OH)_2D_3$ is synthesized primarily as a result of the conversion of 25-hydroxyvitamin D_3 by the renal enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase). PTH is the major regulator of renal 1α-OHase [33]. In turn, $1,25(OH)_2D_3$ controls parathyroid gland growth and suppresses the synthesis and secretion of PTH. Moreover, $1,25(OH)_2D_3$ and its analogues are frequently used as treatment for secondary hyperparathyroidism [34, 35]. Therefore, $1,25(OH)_2D_3$ could be responsible for the observed effect on the expression of Ca^{2+} transport proteins. However, the decline in TRPV5, calbindin-D_{28K} and calbindin-D_{9K} and NCX1 expression after NPS R-467 treatment was not due to decreased levels of $1,25(OH)_2D_3$. In contrast, the decrease in serum PTH levels in the parathyroidectomized rats did result in reduced $1,25(OH)_2D_3$ levels. However, PTH supplementation did not alter serum $1,25(OH)_2D_3$ levels, but was sufficient to concomitantly elevate the expression of TRPV5, calbindin-D_{28K}, and NCX1. This supports a regulatory mechanism primarily controlled by PTH. However, a possible mechanism by which PTH could affect expression is an increased sensitivity for $1,25(OH)_2D_3$ via elevated vitamin D receptor expression. $1,25(OH)_2D_3$ exerts its biologic actions by binding to the vitamin D receptor and the response in the target tissues to $1,25(OH)_2D_3$ is directly related to the vitamin D receptor abundance [33]. Parathyroidectomy, PTH supplementation, or NPS R-467 infusions did, however, not change vitamin D receptor mRNA levels, suggesting that the alterations in renal Ca^{2+} transport protein expression were not associated with changes in renal vitamin D receptor expression. Together, these findings imply that PTH stimulates renal Ca^{2+} reabsorption by up-regulation of the expression of TRPV5 and other Ca^{2+} transport proteins, independently of $1,25(OH)_2D_3$.

In addition to the presence in parathyroid glands, the Ca^{2+}-sensing receptor is also expressed in other cells involved in systemic Ca^{2+} homeostasis. Notable among these are certain epithelial cells in kidney cortex, particularly those in the cortical thick ascending limb (TAL) and DCT [36]. Therefore, the observed effect on serum Ca^{2+} levels after NPS R-467 infusion could also be explained by a direct action of the Ca^{2+}-sensing receptor on the Ca^{2+} transport mechanisms in kidney. Immunohistochemical analysis of kidney cortex sections confirmed the intense basolateral expression of Ca^{2+}-sensing receptor in TAL. Moreover, we demonstrated that the Ca^{2+}-sensing receptor is present in DCT and CNT, confirming previous observations [10, 36]. However, the acute hypocalcaemic response to the type II calcimimetic compound NPS R-568 was abolished in (thyro) parathyroidectomized rats infused with Ca^{2+} or PTH, suggesting that the effects of the calcimimetic compounds are totally dependent on the parathyroid Ca^{2+}-sensing receptor–mediated inhibition of PTH secretion [29, 37]. In addition, pharmacologic evidence from a study using NPS R-467 in rats suggested that renal Ca^{2+}-sensing receptor contributes to the regulation of Ca^{2+} reabsorption by modulating the response to PTH rather than by affecting transport mechanisms directly [abtstract; Fox, et al, *J Am Soc Nephrol* SA-FC179, 2003]. Nevertheless, in the present study, the calcimimetic compound NPS R-467 could directly affect serum Ca^{2+} levels via inhibitory actions on the renal Ca^{2+}-sensing receptor. Remarkably, after NPS R-467 treatment Ca^{2+}-sensing receptor mRNA levels were down-regulated, which could indeed mediate the decreased expression of Ca^{2+} transport proteins by directly affecting expression or by modulating the effect of PTH [38, 39]. However, parathyroidectomy in rats did not change the Ca^{2+}-sensing receptor mRNA levels, suggesting that differences in Ca^{2+}-sensing receptor expression are not entirely responsible for the observed effect on renal Ca^{2+} transport proteins. In addition to abundance, the activity of the Ca^{2+}-sensing receptor could play a role in directly affecting Ca^{2+} transporter expression. It is unlikely that Ca^{2+}-sensing receptors on parathyroid cells and kidney cells are exposed to different levels of NPS R-467. However, the cellular environment in which the receptor is expressed could profoundly influence its response to ligands. This has already been established for G protein–coupled receptors as well as for the different effects of calcimimetics on PTH and calcitonin secretion [37, 40]. Studies of inherited disorders caused by mutations in Ca^{2+}-sensing receptor clearly suggest that this receptor is the mechanism for Ca^{2+}-mediated regulation of both PTH secretion and Ca^{2+} excretion by the kidney [28]. However, no data are present about the physiologic function of the Ca^{2+}-sensing receptor in DCT/CNT and its possible effect on Ca^{2+} reabsorption. Our findings suggest that there are additional or alternative mechanisms to account for the altered renal Ca^{2+} handling, involving PTH-mediated regulation of the expression of renal Ca^{2+} transport proteins.

In various studies exploring the regulatory role of $1,25(OH)_2D_3$, estrogens, PTH, and dietary Ca^{2+}, we observed the concomitant up-regulation of Ca^{2+} transport proteins in kidney [19, 32]. Likewise, genetic ablation of TRPV5 in mouse resulted in a decreased expression of calbindin-D_{28K} and NCX1. As transcellular Ca^{2+} reabsorption in DCT/CNT was abolished in these TRPV5 knockout mice, the concomitant decrease in Ca^{2+} transport proteins, even in the presence of elevated $1,25(OH)_2D_3$ levels, suggested a regulatory mechanism controlled primarily by TRPV5 [26]. We, therefore, hypothesized that the Ca^{2+} influx through TRPV5 predominantly controls the expression of the downstream Ca^{2+} transport proteins. To further substantiate the effect of PTH on Ca^{2+} influx, rabbit primary cell cultures were used. These cultures retain many characteristics of the original epithelium, including a net apical-to-basolateral Ca^{2+} transport and hormone responsiveness [13]. In rabbit, the CNT and CCD have been implicated in active Ca^{2+} reabsorption and it has been shown that the transcellular Ca^{2+} transport proteins are all present in these cells [5, 18, 21, 41]. Using polarized monolayers of the rabbit CNT/CCD, we demonstrated that PTH stimulates transepithelial Ca^{2+} transport, which was accompanied by an increased expression of TRPV5, calbindin-D_{28K}, and NCX1. Subsequently, the entry of Ca^{2+} through TRPV5 was blocked with ruthenium red, which is a potent inhibitor of TRPV5 with an inhibition constant (IC_{50}) of around 110 nmol/L [14]. Blockage of TRPV5 by ruthenium red eliminated PTH-stimulated transepithelial Ca^{2+} transport and simultaneously decreased the expression of calbindin-D_{28K} and NCX1, whereas TRPV5 expression remained unaffected. Apparently, there are two mechanisms that are responsible for the effect on calbindin-D_{28K} and NCX1 expression in the primary CNT and CCD cell monolayers. The magnitude of the Ca^{2+} influx through TRPV5 predominantly controls the expression of calbindin-D_{28K} and NCX1. In addition, direct stimulation by PTH results in increased expression of TRPV5, calbindin-D_{28K}, and NCX1.

Taken together, our findings imply that the magnitude of the Ca^{2+} influx through TRPV5 controls the expression of the Ca^{2+} transport proteins. In addition, these results confirm the direct effect of PTH on the expression of these renal proteins. Moreover, they explain the coordinated regulation of the renal Ca^{2+} transport proteins and further emphasize the gatekeeper function of TRPV5.

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