Stanniocalcin 2 is positively and negatively controlled by 1,25(OH)₂D₃ and PTH in renal proximal tubular cells

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Abstract

We have previously identified a second mammalian stanniocalcin (STC2) in humans and demonstrated that STC2 inhibits phosphate uptake in an opossum renal proximal tubular cell line (opossum kidney (OK) cells). However, the regulation of *Stc2* gene expression in OK cells is not well understood. In this study, we identified the opossum *Stc2* cDNA sequence. The opossum STC2 amino acid sequence had 78.8% homology with human STC2, and has a conserved putative *N*-linked glycosylation site. Next, we investigated the regulation of *Stc2* gene expression by the classical calcium and phosphate-regulating factors 1,25(OH)₂D₃ and PTH in OK cells. In western blot analysis using affinity-purified anti-STC2 antibody, the secretion of STC2 protein was stimulated by 1,25(OH)₂D₃ in a dose-dependent manner. By contrast, PTH suppressed the induction of STC2 protein secretion by 1,25(OH)₂D₃. Real-time PCR analysis revealed that *Stc2* mRNA expression was increased by 1,25(OH)₂D₃ in a dose- and time-dependent manner. In addition, actinomycin D, an RNA synthesis inhibitor, prevented the effects of 1,25(OH)₂D₃ on *Stc2* gene expression. On the other hand, PTH and phorbol 12,13-myristate acetate, a specific PKC activator, but not 8-bromo-cyclic AMP, a specific PKA activator, reduced the mRNA levels of *Stc2*. In addition, Gö6976, a specific PKC inhibitor, abolished the downregulation of *Stc2* mRNA expression by PTH. Furthermore, we demonstrated that the renal *Stc2* mRNA expression was increased by 1,25(OH)₂D₃ and decreased by PTH *in vivo*. These results suggest that STC2 is positively and negatively controlled by 1,25(OH)₂D₃ and PTH in renal proximal tubular cells.

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Introduction

Inorganic phosphate plays an important role in bone metabolism, energy metabolism, growth, and intracellular signaling (Berner & Shike 1988, Takeda *et al.* 2004, Rigo *et al.* 2007). Phosphate homeostasis is controlled by the absorption of phosphate in the intestine and reabsorption in the kidney. The renal proximal tubule is a particularly important tissue in the regulation of serum phosphate levels (Takeda *et al.* 2004, Nemere 2007).

The serum level of phosphate is regulated by dietary phosphate, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], and PTH, and other factors (Nemere 2007). The type IIa sodium-dependent phosphate cotransporter (NPT2a), which is expressed in the renal proximal tubule, is the major molecule involved in the reabsorption of phosphate (Tenenhouse 2005). 1,25(OH)₂D₃ has been shown to promote the ability of phosphate uptake with the renal brush border membrane and to directly increase the transcriptional activity of the human and mouse NPT2a genes (Taketani *et al.* 1998, Yamamoto *et al.* 2005). By contrast, PTH has been shown to rapidly attenuate NPT2a activity by

stimulating apical endocytosis (Murer *et al.* 1999, Nashiki *et al.* 2005). In addition, it is well known that 1,25(OH)₂D₃ suppresses PTH secretion from the parathyroid gland (Beckerman & Silver 1999) and that PTH increases the serum levels of 1,25(OH)₂D₃ by activating cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1; Brown *et al.* 1999). Because these phosphate-regulating hormones are regulated by each other through a feedback loop, serum phosphate levels are tightly regulated (Barthel *et al.* 2007).

Stanniocalcin is a glycoprotein hormone first identified from the corpuscles of Stannius in bony fish (Wagner *et al.* 1986, Flik *et al.* 1990). Fish stanniocalcin (STC) acts on gill, gut and kidney as a calcium phosphate-regulating hormone (Lafeber *et al.* 1988, Sundell *et al.* 1992, Lu *et al.* 1994). The related mammalian gene, human STC1, was initially isolated based on its sequence homology to fish STC (Chang *et al.* 1995). We have previously identified human STC2, a paralogous gene of STC1, and observed its inhibition of the promoter activity of human NPT2a and phosphate uptake in an opossum renal proximal tubular cell line (opossum kidney (OK) cells). We also observed that *Stc2* mRNA expression was decreased in the kidney and other

tissues of the hypophosphatemic (Hyp) mouse, which is a model of human X-linked hypophosphatemic (vitamin D-resistant) rickets (XLH; Ishibashi *et al.* 1998). Yahata *et al.* (2003) have reported that *Stc2* mRNA expression is increased in the kidneys of precocious aging (klotho mutant) mice. Klotho mutant mice exhibit hypervitaminosis D, hypercalcemia, and hyperphosphatemia (Kuro-o *et al.* 1997, Yoshida *et al.* 2002). However, Honda *et al.* (1999) have documented that renal *Stc2* mRNA expression is reduced by calcitriol, the active form of vitamin D₃, in rats. Thus, the regulatory mechanisms of renal *Stc2* gene expression by $1,25(\text{OH})_2\text{D}_3$ are still unclear.

In the present study, we identified the cDNA sequence of opossum *STC2* and investigated the regulation of *Stc2* gene expression by $1,25(\text{OH})_2\text{D}_3$ and PTH in renal proximal tubular cells.

Materials and methods

Materials

$1,25(\text{OH})_2\text{D}_3$ was obtained from Solvay Pharmaceuticals (Marietta, GA, USA). 22-oxacalcitriol (OCT) and 2β -(3-hydroxypropoxy) calcitriol (ED-71) were kindly provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). Human parathyroid hormone (1–34 fragments) PTH, phorbol 12,13-myristate acetate (PMA), 8-bromo-cyclic AMP (8-bromo-cAMP) and actinomycin D (ActD) were purchased from Sigma-Aldrich. G66976 was purchased from Calbiochem (San Diego, CA, USA).

Cell culture

OK cells were maintained in DMEM (Sigma) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in an incubator with 5% CO₂.

cDNA isolation and DNA sequence analysis

Opossum *Stc2* gene information was obtained from a BLAST search on the UCSC Genome Bioinformatics website (<http://www.genome.ucsc.edu/>) with the amino acid sequence of human *STC2*. On the basis of this information, oligonucleotide primers specific to the *STC2* cDNA sequence were synthesized. The sequences of the upstream and downstream primers were 5'-CCAATGTGTGTGGAGCGGTTTCAGCC-3', and 5'-TCACCTCCGATATCTGAATACTCAGC-3' respectively. Opossum *STC2* cDNA containing an open reading frame was amplified by RT-PCR using total RNA isolated from OK cells. The amplification products were purified and subcloned into a pGEM-T Easy vector (Promega), and manually sequenced using the dye terminator cycle sequencing method (Applied

Biosystems, Foster City, CA, USA). The cDNA sequences of the opossum *Stc2* gene have been deposited in GenBank (<http://www.ncbi.nlm.nih.gov>) under the accession number AB462027.

Antibody purification

Using a 12-amino-acid fragment (residues 291–302: EDEQSEYSDIRR) of the human *STC2* protein deduced from the corresponding gene sequence, we designed an antigenic peptide. This peptide was added to the cysteine residues to permit coupling of the peptides to keyhole limpet hemocyanin (KLH) as a carrier protein for immunization. Rabbits were immunized by repeated s.c. injection of the KLH-coupled peptides emulsified in complete Freund's adjuvant on days 1 and 14 and in incomplete Freund's adjuvant on days 21 and 28. For the affinity purification by a gel column (Cellulofine; Seikagaku-Kogyo, Tokyo, Japan), the antiserum (10 ml) was loaded on the gel coupled with the respective peptide, and the antibodies were eluted with 0.1 mol/l glycine-HCl (pH 2.5), neutralized with 1.0 mol/l Tris, dialyzed into 0.02 mol/l PBS (pH 7.4), and stored at –80 °C.

Immunocytochemical analysis

OK cells grown in plastic cover slips were fixed with 3% (wt/vol) paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 in PBS on ice for 10 min. After blocking with 0.8% BSA in PBS for 1 h, the cells were incubated with anti-*STC2* rabbit antibody and a mouse antibody against the *cis*-Golgi matrix marker protein GM130 (BD Transduction Laboratories, Lexington, KY, USA) for 1 h after which they were washed and labeled with Alexa 488-labeled anti-rabbit IgG (Molecular Probe, Eugene, OR, USA) and Alexa 555-labeled anti-mouse IgG (Molecular Probe). The cells were then washed with Aqua/poly mount and mounted on glass slides (PolyScience, Niles, IL, USA). Slides were analyzed and photographed using a Leica confocal microscope (TCS-SL) (Leica Microsystems GmbH, Mannheim) in the sequential mode.

Western blot analysis

For analysis of the secreted protein, the conditioned medium was centrifuged at 1500 g for 10 min at 4 °C. The supernatant was concentrated by acetone precipitation. Total kidney protein was prepared by standard procedures (Sambrook *et al.* 1989). Briefly, the whole kidney was homogenized in lysis buffer containing 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, and 5 mM

iodoacetamide. Proteins were electrophoresed on 12% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat dried milk in PBS plus 0.05% Tween 20 (PBS-t) at room temperature for 1 h, membranes were incubated with affinity-purified anti-STC2 antibody in 1% nonfat dried milk in PBS-t. The membranes were washed with PBS-t and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibody at room temperature for 1 h. After washing with PBS-t, the membranes were developed using an ECL plus western blotting system (GE Healthcare, Buckinghamshire, UK).

RT-PCR and real-time PCR analysis

Total RNA was extracted with TRIzol reagent (Invitrogen) and then dissolved in RNase-free water. First-strand cDNA was synthesized from 2.5 µg of total RNA

using a first-strand cDNA synthesis kit (Invitrogen). Two microliters of the cDNA were then used for quantitative PCR, with 20–30 cycles of amplification, and the PCR products were then separated by electrophoresis using 2% agarose gels. For PCR amplification, the primer sequences were: opossum STC2, 5'-ATGCCCTGAATGGTAAAGCCCATG-3' and 5'-CACGTAGGGTTCATGCAGTAGCAGATC-3'; opossum and mouse β-actin, 5'-CTGACCCTGAAGTACCCCATGAACA-3' and 5'-CTGGGGTGTGAAGGTCTCAAACATG-3'; mouse STC2, 5'-AGCCCAGGAGAACGTCGGTGTGATTG-3' and 5'-CAGCAGCAGGTTTACAAAGGTCACATAG-3'; mouse CYP27B1, 5'-ATGGTGAAGAA-TGGCAGAGG-3' and 5'-TAGTCGTCGCACAAGGT-CAC-3'; rat STC2, 5'-TACCAGTTGCAGAGGGAATG-3' and 5'-TTACAAGGTCCACGTAGGGT-3'; rat β-actin, 5'-GTCCCAGTATGCCTCTGGTCGTAC-3' and 5'-CCACGCTCGGTCAGGATCTTCATG-3'; and rat CYP27B1, 5'-CAGTTTCGGGAACCCAACTC-3' and 5'-TGCAACTTCGTTTGCCAAAG-3'. RT-PCR was performed with a PCR

A

Opossum	1	MCVERFSQLVTALVLASFDPVLGTEATNPPEGTQDRASPQKGRLSLQNTAEIQHCLVNAGDVGGVFEFCFENN [▲] SCEIRG
		** ** *
Human	1	<u>MCAERLGGFMTLALVLATFDPARG</u> TDATNPPEGPQDRSSQQKGRLSLQNTAEIQHCLVNAGDVGGVFEFCFENN [▲] SCEIRG
Opossum	81	LHDICMTFLHNAGKLDAGKSFIDALKCKAHLRHKFCISRKCPAIKEMVFLQRECYLKHNLCSAAKENIQVMEMI
		** *
Human	81	LHGICMTFLHNAGKFDAQGSFIKDALCKKAHLRHRFGCISRKCPAIREMVSQ [●] LQRECYLKHDLCAAQENTRVIVEMI
Opossum	161	HFKDLLLHEPYVDLVNMLLTCGEEVKEAITRSVQAQCEQNWGSLSILSFCASAAQGEAAAAPERQQQPEGRALRAYQG
		* *
Human	161	HFKDLLLHEPYVDLVNLLLT [●] CGEEVKEAITHSVQVQCEQNWGSLSILSFC [●] TS [●] SAIQKPPTAPPERQPQVDRTKLSRAHHG
Opossum	241	DPGFHLPESGRDASRGAKGEKGGKTHPNAHVRGKAGGQGAQGAQGSNEWEDERA [●] EYS [●] DIRR
		* *
Human	241	<u>EAGHHLPEPSSRETGRGAKGERGSKSPNAHARGRVGGLGAQGPSGSSEW</u> <u>EDEQSEYSDIRR</u>

B

Figure 1 (A) Identification of opossum STC2 and its localization in opossum kidney (OK) cells. Opossum *Stc2* cDNA was identified using information obtained from the UCSC genome database website. The opossum STC2 and human STC2 sequences were then aligned. The underlined sequence indicates the predicted signal peptide region. The histidine cluster (HHxxxHH) is double-underlined. The conserved cysteine residues are indicated by dots. The N-glycosylation site is shown as a closed triangle. The box shows the recognition peptide region of the STC2-specific antibody. (B) OK cells were fixed and double immunolabeled with antibodies specific for STC2 (green) and GM130 (red). Yellow signals are indicative of colocalization. Images were taken by confocal laser scanning microscopy. The data presented are from three independent experiments.

Figure 2 Effects of 1,25(OH)₂D₃ and PTH on the secretion of STC2 in OK cells. When OK cells reached 100% confluence, the culture medium was changed to serum-free medium. Then the cells were incubated with the vehicle (final concentration of ethanol, 0.1%) and multiple concentrations of (A) 1,25(OH)₂D₃ for 12 h, or (B) 100 nM 1,25(OH)₂D₃ for different periods of time. The secretory proteins in the medium were collected by acetone precipitation. The protein levels of STC2 were measured by western blot analysis. (C) After treatment with the vehicle (control) or 100 nM 1,25(OH)₂D₃, the vitamin D analogs OCT or ED-71 for 12 h, secreted STC2 was analyzed by western blotting. (D) OK cells were treated with 10 nM 1,25(OH)₂D₃ or 100 nM PTH or both for 12 h.

system (ASTEC, Fukuoka, Japan) using GoTaq Master Mix (Promega). Real-time PCR analysis was performed using the LightCycler (Roche Diagnostics). The prepared first-strand cDNA was PCR amplified using SYBR Premix ExTaq (Takara Bio Inc., Shiga, Japan) in a 20 µl reaction volume, with 4 pmol of each primer. β-actin was used as the internal control. The amplification program was 95 °C for 10 s followed by 50 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. Amplified products were then analyzed using a melting curve, which confirmed the presence of a single PCR product in all reactions (apart from the negative control). The PCR products were quantified by fit point analysis, and results for STC2 and CYP27B1 were normalized to those of β-actin.

Experimental animals

Eight-week-old C57BL/6J male mice and 6-week-old Sprague–Dawley (SD) male rats were purchased from

Japan SLC, Inc. (Shizuoka, Japan). Mice and rats were maintained with 12h light:12h darkness cycles with free access to regular mouse or rat chow and water under pathogen-free conditions. The breeding and handling of all animals in these experiments was approved by the Animal Experimentation Committee of the University of Tokushima. Mice were administered 1,25(OH)₂D₃ (0.2 µg/mouse) orally. After 7 h, the mice were killed. Rats were fed a low-phosphate diet (0.6% calcium, 0.02% Pi) for 3 days (Segawa *et al.* 2002). Then, a bolus of PTH (10 µg/100 g body weight) was injected into the jugular vein. After 0.5 h, the rats were killed.

Statistical analysis

Data are expressed as means ± s.d. Statistical significance between the groups was determined using ANOVA followed by *post hoc* testing using Fisher's protected least significant difference. *P* < 0.05 was considered to be significant.

Results

Identification of opossum STC2

We obtained the nucleotide sequence of opossum STC2 from the UCSC genome database website. We then identified the opossum *Stc2* cDNA. From the cDNA sequence, we determined both the opossum and human STC2 proteins to be 302 amino acids long. Seventy-eight percent of opossum STC2 was homologous to human STC2 (Fig. 1A). Opossum STC2 had 14 conserved cysteine residues and the same putative N-linked glycosylation site seen in the human gene. However, the C-terminus of the opossum STC2 sequence did not have a cluster of histidines (HHxxxxHH), capable of interacting with divalent metal ions as seen in human STC2 (Moore *et al.* 1999, Ishibashi & Imai 2002). The specificity of the affinity-purified anti-STC2 antibody was confirmed by western blot analysis and a peptide absorption test (Data not shown). Immunofluorescence analysis was performed to localize the STC2 in OK cells. GM130 has been well characterized as the *cis*-Golgi matrix maker protein. When cells were stained with anti-STC2 antibody and anti-GM130 antibody, STC2 expression was partially localized to the *cis*-Golgi matrix, although cells exhibited significant foci of STC2 staining within the cytoplasm (Fig. 1B).

Regulation of STC2 protein secretion by 1,25(OH)₂D₃ and PTH

We next investigated the regulation of STC2 protein secretion by 1,25(OH)₂D₃ and PTH in OK cells. As shown in Fig. 2A, the secretion of STC2 protein was

increased by $1,25(\text{OH})_2\text{D}_3$ in a dose-dependent manner. In a time course study, secreted STC2 protein was detected after 6 h and beyond (Fig. 2B). Because OCT and ED-71 are known as cell-selective or non-calcemic vitamin D analogs (Murayama *et al.* 1986, Miyamoto *et al.* 1993), the possibility that these may uniquely act on the STC2 secretion was examined. In the OK cells, STC2 secretion was also induced by OCT and ED-71, but the magnitude of response was less than that induced by $1,25(\text{OH})_2\text{D}_3$ (Fig. 2C). Interestingly, PTH markedly suppressed the induction of STC2 protein secretion by $1,25(\text{OH})_2\text{D}_3$ (Fig. 2D).

Upregulation of *Stc2* mRNA expression by $1,25(\text{OH})_2\text{D}_3$

We then determined whether the expression of *Stc2* mRNA is affected by $1,25(\text{OH})_2\text{D}_3$ in OK cells. RT-PCR analysis revealed that *Stc2* mRNA levels were increased by $1,25(\text{OH})_2\text{D}_3$ in a dose-dependent manner (Fig. 3A). In a time course study of $1,25(\text{OH})_2\text{D}_3$, upregulation of *Stc2* mRNA expression was evident at 3 h and reached its peak at 12 h, with a 24-fold increase at that time (Fig. 3B). In addition, OCT and ED-71 were observed to stimulate *Stc2* mRNA expression (Fig. 3C). In order to investigate whether upregulation of *Stc2* mRNA expression by $1,25(\text{OH})_2\text{D}_3$ is a result of transcriptional control, we looked at the effects of ActD on STC2 expression. Figure 3D shows that pretreatment with ActD suppressed $1,25(\text{OH})_2\text{D}_3$ -induced *Stc2* mRNA expression.

Downregulation of *Stc2* mRNA expression by PTH

We next investigated the effects of PTH on STC2 expression over multiple doses and times. As shown in Fig. 4A, *Stc2* mRNA expression was decreased by PTH in a dose-dependent fashion. In a time course study, PTH reduced *Stc2* mRNA expression by 50% at 3 h, and expression returned to basal levels at 12 h (Fig. 4B). To determine whether the inhibitory effect of PTH on *Stc2* mRNA expression is mediated by protein kinase A (PKA) and/or protein kinase C (PKC) activation, cells were treated with 8-bromo-cAMP, a specific PKA activator, or PMA, a specific PKC activator. Figure 4C shows that *Stc2* mRNA expression was significantly (<40%) decreased by PMA but not affected by 8-bromo-cAMP. In addition, Gö6976, a specific PKC inhibitor, completely blocked the inhibition of *Stc2* mRNA expression by PTH (Fig. 4D).

Regulation of *Stc2* mRNA expression by $1,25(\text{OH})_2\text{D}_3$ and PTH *in vivo*

Finally, we confirmed the effects of $1,25(\text{OH})_2\text{D}_3$ and PTH on renal *Stc2* mRNA expression *in vivo*. We also

Figure 3 Stimulation of *Stc2* mRNA expression by $1,25(\text{OH})_2\text{D}_3$ in OK cells. OK cells were incubated with the vehicle (final concentration of 0.1% ethanol) or multiple concentrations of (A) $1,25(\text{OH})_2\text{D}_3$ for 12 h, or (B) 100 nM $1,25(\text{OH})_2\text{D}_3$ for different periods of time. After treatment with $1,25(\text{OH})_2\text{D}_3$, the cells were harvested and total RNA was extracted. The mRNA levels of *Stc2* were determined by RT-PCR and real-time PCR analysis. (C) OK cells were incubated with the vehicle (control), 100 nM $1,25(\text{OH})_2\text{D}_3$, OCT or ED-71 for 12 h. (D) After pretreatment with 100 nM ActD for 30 min, the cells were exposed to 100 nM $1,25(\text{OH})_2\text{D}_3$. Results were normalized to the mRNA expression level of β -actin. The data are represented as means $\pm$ s.d. ($n=3$). * $P<0.05$, *** $P<0.0001$ compared with vehicle.

Figure 4 Inhibition of *Stc2* mRNA expression by PTH in OK cells. OK cells were treated with the vehicle or different concentrations of (A) PTH for 12 h, or (B) 100 nM PTH for different periods of time. After treatment with PTH, the cells were harvested and total RNA was extracted. The mRNA levels of *Stc2* were determined by RT-PCR and real-time PCR analysis. (C) OK cells were treated with the vehicle (control) or 100 nM PTH, PMA, a specific PKC activator, or 8-bro-cAMP, a specific PKA activator, for 6 h. (D) OK cells were pretreated with 10 μM G66976, followed by incubation with 100 nM PTH for 6 h. The mRNA expression levels of *Stc2* were determined by real-time PCR analysis. Results were normalized to the mRNA expression level of β -actin. The data are represented as means $\pm$ s.d. ($n=3$). * $P<0.05$, ** $P<0.01$ compared with vehicle.

investigated the effects of $1,25(\text{OH})_2\text{D}_3$ and PTH on renal *Cyp27b1* mRNA expression. Figure 5A shows that $1,25(\text{OH})_2\text{D}_3$ administration in mice significantly increased renal mRNA levels of *Stc2* by 160% and decreased renal *Cyp27b1* mRNA levels by 5%, compared with control vehicle mice. It has been reported that serum PTH protein and mRNA levels are decreased by a low-phosphate diet (Kilav *et al.* 1995). Therefore, we fed rats a low-phosphate diet for 3 days, followed by treatment with PTH. After 0.5 h, renal mRNA levels of *Stc2* and *Cyp27b1* were decreased by 50% and increased by 230%, respectively, in PTH-treated rats compared with PTH-untreated vehicle rats (Fig. 5B). In addition, western blot analysis showed that PTH reduced the protein levels of STC2 in kidney (Fig. 5C).

Discussion

Stanniocalcin is a glycoprotein that maintains the intracellular calcium and phosphate homeostasis (Wagner *et al.* 1986, Lafeber *et al.* 1988). In the present study, we have identified opossum STC2 cDNA sequences including the transcriptional start site and coding region. The amino acid sequences of opossum STC2 showed 78% homology to human STC2. The opossum STC2 sequence shows a conserved N-glycosylation site seen in the human sequence. Indeed, secreted opossum STC2 was detected as two bands of 35 and 39 kDa by western blot analysis (Fig. 2). Treatment with N-glycosidase, which removes all N-linked sugar moieties of glycoproteins, showed that these bands were shifted down (data not shown). Moore *et al.* (1999) have reported that a cluster of four to five histidines in the C-terminal region of STC2 was retained not only in humans but also in the mouse, hamster and macaque genes. Interestingly, the opossum STC2 sequence did not include the cluster of histidines, which might associate with a divalent metal ion. Although the function of the C-terminal region of STC2 has not been clarified, this indicates that some functions of the opossum STC2 might be different from those of other mammalian STC2s. McCudden *et al.* (2002) have reported that high-affinity mouse STC1-binding sites and/or receptors are present on both the plasma membrane and mitochondria in nephron epithelial cells and liver hepatocytes, and suggested that STC2 interacts with a different receptor. However, high-affinity STC2 receptor and its signaling pathway have not been established. Because we have observed that STC2 inhibits the NPT2a promoter activity and phosphate uptake in OK cells, the great discovery of STC2-specific receptor and its signaling pathway may be accomplished by the luciferase reporter assay using the NPT2a gene promoter or the phosphate uptake assay.

Figure 5 Control of the *Stc2* mRNA expression by 1,25(OH)₂D₃ and PTH *in vivo*. C57BL/6J male mice were administered 1,25(OH)₂D₃ orally (0.208 µg/mouse). After 7 h, the mice were killed. (A) The mRNA expression levels of *Stc2* and *Cyp27b1* in mouse kidney were determined by RT-PCR and real-time PCR analysis. After SD male rats were fed a low-phosphate diet (0.02% Pi, 0.6% Ca) for 3 days, PTH (10 µg/100 g body weight) was injected into the jugular vein. (B) After treatment for 30 min, the mRNA expression levels of *Stc2* and *Cyp27b1* in rat kidney were determined by RT-PCR and real-time PCR analysis. Results were normalized to the mRNA expression level of β-actin. The data are represented as means ± s.d. (*n* = 3–5). **P* < 0.05, ***P* < 0.01 compared with vehicle. (C) The renal STC2 protein levels were measured by western blot analysis.

1,25(OH)₂D₃ and PTH play a critical role in calcium and phosphate homeostasis in the kidney (Beckerman & Silver 1999, Brown *et al.* 1999). We first examined the effects of 1,25(OH)₂D₃ and PTH on the secretion of STC2 protein in OK cells. Western blot analysis indicated that secretion of STC2 protein was increased by 1,25(OH)₂D₃; however, its secretion was strongly suppressed by PTH. In accordance with our findings regarding STC2 protein secretion, *Stc2* mRNA expression was dose- and time-dependently increased by 1,25(OH)₂D₃. Furthermore, the increase in *Stc2* mRNA in response to 1,25(OH)₂D₃ was completely blocked by actinomycin D. These data suggest that the induction of STC2 protein secretion by 1,25(OH)₂D₃ is mediated by transcriptional control. Contrary to our findings with 1,25(OH)₂D₃, PTH significantly suppressed *Stc2* mRNA expression. To determine the inhibitory mechanism of PTH on STC2 expression, we examined the signaling pathways of PKA and PKC. Using PMA, 8-bromo-cAMP, and G66976, we revealed that activation of the PKC pathway by PTH is an important negative regulator of STC2 expression. These data suggest that PTH suppresses the 1,25(OH)₂D₃-induced STC2 secretion by PKC-mediated downregulation of its mRNA expression. Finally, we confirmed that *Stc2* mRNA expression was regulated by 1,25(OH)₂D₃ and PTH *in vivo*. In mice, which were orally administered 1,25(OH)₂D₃, renal *Stc2* mRNA expression was significantly increased. Furthermore, in the kidneys of rats injected with PTH, *Stc2* mRNA and protein expression was rapidly reduced (Fig. 5). In addition, the plasma levels of phosphate, not calcium, were increased by 1,25(OH)₂D₃, but PTH did not change plasma calcium and phosphate levels (data not shown). Because previous studies using *klotho* mutant mice have suggested that STC2 expression is positively regulated by blood phosphate concentration (Yahata *et al.* 2003), the renal *Stc2* gene expression might be directly and indirectly regulated by 1,25(OH)₂D₃. Thus, renal *Stc2* mRNA expression was strictly controlled by not only 1,25(OH)₂D₃ but also PTH. Therefore, our data might explain the discrepancy between the previous reports showing that vitamin D enhanced (Yahata *et al.* 2003) or suppressed renal *Stc2* gene expression (Honda *et al.* 1999).

In conclusion, we have identified a novel STC2 amino acid sequence in opossum and demonstrated that STC2 secretion is positively and negatively regulated by 1,25(OH)₂D₃ and PTH in OK cells. Furthermore, we have shown that *Stc2* mRNA expression is controlled by 1,25(OH)₂D₃ and PTH *in vitro* and *in vivo*.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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