Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:1695-1709
DOI: 10.1159/000495674
Published online: 30 November 2018
Accepted: 22 November 2018

Original Paper

Parathyroid Hormone-Related Protein (1-40) Enhances Calcium Uptake in Rat Enterocytes Through PTHR1 Receptor and Protein Kinase Cα/β Signaling

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Key Words
Parathyroid hormone-related peptide • Intestinal epithelium cells • Calcium transport protein • Calcium uptake

Abstract
Background/Aims: Parathyroid hormone-related protein (PTHrP) is implicated in regulating calcium homeostasis in vertebrates, including sea bream, chick, and mammals. However, the molecular mechanism underlying the function of PTHrP in regulating calcium transport is still not fully investigated. This study aimed to investigate the effect of PTHrP on the calcium uptake and its underlying molecular mechanism in rat enterocytes. ***Methods:*** The rat intestinal epithelial cell line (IEC-6) was used. Calcium uptake was determined by using the fluo-4 acetoxymethyl ester fluorescence method. The expression levels of RNAs and proteins was assessed by RT-PCR and Western-blot, respectively. ***Results:*** PTHrP (1-40) induced rapid calcium uptake in enterocytes in a dose-dependent manner. PTHrP (1-40) up-regulated parathyroid hormone 1 receptor (PTHR1) protein but not 1,25D3-MARRS receptor. Pre-treatment of anti- PTHR1 antibody abolished the PTHrP (1-40)-induced calcium uptake. PTHrP (1-40) significantly up-regulated four transcellular calcium transporter proteins, potential vanilloid member 6 (TRPV6), calbindin-D9k (CaBP-D9k), sodium-calcium exchanger 1 (NCX1) and plasma membrane calcium ATPase 1 (PMCA1), in a dose- and time-dependent manner. Pre-treatment with TRPV6 or CaBP-D9k antibodies or knockout of rat TRPV6 or CaBP-D9k markedly inhibited PTHrP (1-40)-induced calcium uptake, whereas inhibition of NCX or PMCA1 by antibodies or inhibitors had no effect on PTHrP(1-40)-induced calcium uptake. Furthermore, PTHrP (1-40) treatment up-regulated protein levels of protein kinase C (PKC α/β) and protein kinase A (PKA). Pretreatment of PKC α/β inhibitor (but not PKA inhibitor) inhibited

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PTHrP (1-40)-induced calcium uptake. ***Conclusion:*** PTHrP (1-40) stimulates calcium uptake via PTHR1 receptor and PKC α/β signaling pathway in rat enterocytes, and calcium transporters TRPV6 and CaBP-D9k are necessary for this stimulatory effect.

Introduction

Calcium plays a key role in a large number of physiological processes, including muscle contraction, neuromuscular excitability, blood coagulation, enzyme activation, hormone secretion, cell division, and cell membrane stability [1]. In the mammals and birds, extracellular calcium concentrations are regulated by a complex homeostatic system in which three hormones parathyroid hormone [PTH], calcitonin, and 1, 25-dihydroxyvitamin D_3 [1, $25(OH)_2D_3$, the hormonal form of vitamin D] and three target organs (bone, kidneys, and gastrointestinal tract) are involved [2]. In most vertebrates, calcium is mainly absorbed in the small intestine, accounting for approximately 90% of overall calcium absorption under physiological conditions [3]. In mammalian, calcium uptake in the small intestine is through paracellular or transcellular transport. Paracellular calcium transport is passive, non-saturable, and concentration-gradient-dependent, functioning throughout the length of the intestine. By contrast, transcellular transport is active, saturable, energy-dependent, highly regulated, and located mainly in the duodenum and upper jejunum [4, 5].

The process of transcellular calcium transport involves three steps: calcium entry at the apical brush border membranes (BBM) of enterocytes through epithelial calcium channels; calcium association with calcium-binding proteins and moving from the BBM to the basolateral membranes; and calcium extrusion at the basolateral side of the cell membrane into the blood by calcium transporters [4]. Epithelial calcium channels include potential vanilloid member 5 (TRPV5) and member 6 (TRPV6) [6] which are the rate-limiting step of active intestinal calcium absorption [7], and L-type channel Cav1.3 [1]. Cytoplasmic calcium-binding proteins include calbindin-D9k (CaBP-D9k) and calbindin-D28k (CaBP-D28k) [1], while basolateral transporters consist of the sodium-calcium exchanger 1 (NCX1) and plasma membrane calcium ATPase 1 (PMCA1) [8]. The transcellular pathway is regulated by a homeostatic system in which 1, $25(OH)_2D_3$, PTH, parathyroid hormone-related protein (PTHrP), thyroid hormone estrogens, glucocorticoids, and other steroid hormones are involved [4].

PTHrP was first identified as a human tumor-derived hypercalcemic factor in 1987 [9]. Nevertheless, its normal physiologic functions have been revealed in a plenty of subsequent studies. PTHrP ubiquitously expressed in a number of normal cells and tissues, playing versatile physiological functions, such as regulations of smooth muscle contraction [10], tissue/organ development, differentiation, and proliferation [11, 12], and calcium transport [13]. PTHrP has N-terminal homology with parathyroid hormone (PTH), the main hypercalcemic hormone in higher vertebrates. The N-terminal segments (1-34) of two proteins bind with equal affinity to a shared PTH/PTHrP receptor, parathyroid hormone 1 receptor (PTHR1) [14].

In sea bream, PTHrP (1–34) treatment induces an alteration of calcium fluxes in duodenum, hindgut, and rectum of sea bream [15]. In higher vertebrate, both PTH(1-34) and PTHrP(I-34) have a rapid stimulatory effect on calcium uptake in perfused chick duodenum [13]. In the mammal, both PTH(l-34) and PTHrP(1-34) significantly increase the absorption rates of calcium in the reticulorumen of sheep [16]. In humans, both PTHrP (1–34) and PTH (1–34) stimulate calcium transport across the basal the PTH/PTHrP receptor in human syncytiotrophoblast *in vitro* membrane system [17]. Kovacs *et al*. have reported that PTHrP (67-86), but not PTHrP (1-34), regulates fetal–placental calcium transport stimulates calcium transport in human placenta and uterus [18].

Although the stimulatory effect of PTHrP (1-34) on calcium transport has been demonstrated, however, the underlying molecular mechanism is still not fully investigated. In this study, we aimed to investigate the effect of PTHrP on the calcium uptake in rat enterocytes (duodenum cells) and its underlying molecular mechanism.

Materials and Methods

Chemicals

Rat PTHrP(1–40), PTHrP (67-86), and PTHrP (134-143) were purchased from Bachem Americans, Inc (Torrance, USA), and H-89 was obtained from (Cell Signaling Technology, USA), GÖ6983 and GÖ6976 was obtained from Millipore/Merck (USA). Fluo-4am was purchased from Sigma-Aldrich (USA).

Cell culture

IEC-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, USA) supplemented with 8% fetal bovine serum (FBS, Gibco/Life Technologies, USA) and antibiotics (100U/ml penicillin and 100 ug/ml streptomycin) at 37 °C in an atmosphere of 5% CO_2/95% air. Once the cells reached 70% confluence, the culture medium was replaced with serum-free medium for 24 hr. Cells were then exposed to different concentrations of PTHrP in DMEM with 0.5% FBS. For inhibitor studies, serum-deprived cells were treated with 20 μM H89 (a PKA inhibitor, Sigma-Aldrich), 20 μM GÖ6983 (a PKC inhibitor), 20 μM GÖ6976 (a PKCα/β inhibitor), 10 μM nitrendipine (an L-type calcium channel antagonist, Sigma-Aldrich), 20 μM SEA0400 (an NCX inhibitor, Sigma-Aldrich), or 10nM trifluoperazine (a PMCA inhibitor, Sigma-Aldrich) for 30 min, followed by incubation with 6 NM Trip (1-40, Sigma-Aldrich).

Western blot

A monolayer of confluent IEC-6 cells was harvested by a modification of the RIPA method (50mM Tris-HCl, pH8.0), followed by Dounce homogenization. Cytosolic supernatants were prepared by centrifuging the extracts at 14, 000 `× g for 30 min. The cytosolic fraction was collected and proteins were quantified by the bicinchoninic acid (BCA) method. All protein samples were size-fractionated in 10% SDS-polyacrylamide gels, followed by electrotransferred to polyvinylidene difluoride (PVDF) membranes at 150 mA for 2 hr at 4 °C. The membrane was blocked with TBS-T (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5% Tween-20 containing 5% non-fat dry milk) for 1 hr at room temperature. The membrane was then incubated with primary antibodies against TRPV6 (1:200, Santa Cruz, USA), Cabp (1:200, Santa Cruz), NCX (1:200, Santa Cruz), PMCA (1:500, Santa Cruz), PTHR1 (1:1000, Abcam, USA), PKC α/β (1:1000, CST, USA), and PKA (1:1000, CST) at 4 °C overnight. Next, the membrane was washed three times with TBS-T, and incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3000 dilution, Invitrogen, USA) for 2 hr at room temperature. The membranes were then visualized using an enhanced chemiluminescent (ECL) technique (Millipore/Merck, USA), according to the manufacturer's instructions. Images were obtained by a model HR55000 Imaging system (GeneGenome, USA). Densitometric analysis for the quantification of the bands was performed using Quantity One software (Bio-Rad Laboratories, USA).

RT-PCR

Total RNA was extracted from IEC-6 cells using TRIzol reagent (Takara, China). Total RNA (2 μg) was reverse-transcribed using first-strand cDNA synthesis kits (Fermentas/Thermo Scientific, USA) according to the manufacturer's instructions. PCR was performed using the reaction mixture (25 μL) contained the following: 2.0 μL reverse transcription (RT) reaction products, 0.5 μmol/l of each primer (sequences were shown in Table 1), 12.5 μL Taq DNA polymerase (Fermentas, USA), and 8.5 μL deionized water. The thermocycling protocol comprised 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55-62°C for 1 min, and extension at 72°C for 80 seconds. The PCR products were then separated on 1.5% agarose gels containing ethidium bromide and visualized using a UV illuminator. Amplification of the expected fragments was confirmed using an automated sequencer.

Table 1. The sequence of primers for RT-PCR

Gene	Primer Sequence
TRPV6	Foreword 5' ATGTGGGCGAGAGTATGGTC-3'; Reverse 5' CCTTCTCCTTGGACTTGCG-3'
calbindin-9k	Foreword 5'GACCTCACCTGTTCCTGTCTG-3'; Reverse 5' GCTCCTTCTTCTGGCTTCATT-3'
NCX	Foreword 5'GGATGTGGTTGAAAATGACCCAGT-3'; Reverse 5' TATGCCATCTTCCGAGACTTCTGA-3'
PMCA	Foreword 5'-CACCGTACTTCACTTGGGCAA T-3'; Reverse 5'-GGCAGGTCATCCAGATACCTGTA-3'
CAV1.3	Foreword 5'- TCAGCGTCAGTGTGTGGAATA-3'; Reverse 5'- CGAAAGGCGAGGAGTTCAC-3'
GAPDH	Foreword 5'-AATGCATCCTGCACCACCAA-3'; Reverse 5'-GTAGCCATATTCATTGTCATA-3'

Table 2. The siRNA sequences for TRPV6 and calbindin-9k

Gene	Primer Sequence
TRPV6	5'-GGUGGAAGAUAGACAAGAUTT-3'; 5'- AUCUUGUCUAUCUUCCACCTT-3'
calbindin-9k	5'-CUCUAGACAAUCUCUUUAATT- 3''; 5'-UUAAAGAGAUUGUCUAGAGTT-3'

Synthesis and transfection of siRNA for TRPV6 and CaBP-D9k

The 21-nucleotide small interfering siRNA sequences specifically targeting rat TRPV6 and CaBP-D9k (sequences were shown in Table 2) were designed and synthesized by GenePharma (China). For siRNA transfection, cells (0.5×10^6 well/m1) were seeded into six-well plates in 2 mL of antibiotic-free DMEM medium 18 hr before transfection. Cells were then transfected with 100 pM siRNA in Opti-MEM® serum and serum-free medium for 6 hr at 37 ℃. After which, the medium was changed to fresh antibiotic-free DMEM medium. All experimental measurements were performed after the confluence reached 70%.

Calcium uptake measurements

For calcium uptake measurements, IEC-6 cells (5×10^5 /ml/well) were seeded onto the 35 mm glass-bottomed dishes. When reaching 60% confluence, cells were starved overnight, followed by incubation with 5μM fluo-4am with 0.01% Pluronic F-127 in Hank's balanced salt solution (HBSS) in the dark at 37 ℃ for 1 hr. Cells were then washed three times with D-Hanks (calcium-free medium) and incubated for a further 30 min. The relevant agonist (diluted in HBSS) was then added to each dish. The fluorescence intensity of the indicator was measured using a confocal laser scanning microscope (FV1000; Olympus, Japan) with the wavelength setting of excitation at 485 nm, emission at 520 nm. For each measurement, 12 cells were randomly selected for analysis before and after adding the stimulators.

Statistical analysis

The data was presented as mean±S. E. M. For multiple group comparison, one-way analysis of variance (ANOVA) with the Fisher's LSD or Student-Newman-Keuls (SNK) post hoc test was used for the data with equal variance, while the Welch's test with Dunnett T3 post hoc test was used for the data with unequal variance. A P value smaller than 0.05 was regarded as statistically significant. All analyses were carried out using SPSS software (v. 13.0; SPSS Inc., Chicago, IL, USA).

Results

PTHrP(1-40) promoted calcium uptake in enterocytes

To identify the bioactive segment of PTHrP responsible for calcium uptake in enterocytes, three segments of PTHrP (1-40, 67-86 and 134-173) were used in the calcium uptake assay. The results showed that among the three segments of PTHrP, only PTHrP(1-40) induced a significant rise in intracellular calcium level ($P<0.05$, Fig. 1A). The reaction was rapid and transient and returned to baseline after 200 seconds (Fig. 2).

PTHrP(1-40) promoted calcium uptake in a dose-dependent manner

To determine if PTHrP(1-40) has a dose-dependent effect on the calcium uptake, different concentrations (0.1, 1, 3, 6 and 10 nM) of PTHrP(1-40) were used in the calcium uptake measurements. As shown in Fig. 1B, 3, 6 and 10 nM of PTHrP(1-40) significantly elevated intracellular calcium level as compared with control (all $P<0.05$), which peaked at 6 nM ($P<0.001$). Therefore, the concentration of 6 nM was selected for the following experiments.

PTHrP(1-40) promotes calcium uptake through PTHR1

Next, we investigated the effect of PTHrP(1-40) on the expression of PTHrP receptors. As shown in Fig. 3, PTHrP treatment up-regulated the expression of PTHR1 protein in a dose-dependent manner (0.1 to 10 nM, peaked at 3 nM, $P<0.001$, compared with control, Fig. 3A). The time-course study showed that the expression of PTHR1 protein was up-regulated at 6 hr after PTHrP(1-40) treatment ($P<0.05$, Fig. 3B). Neither mRNA nor protein of PTHrP receptor 2 (PTHR2) was detected in IEC-6 cells (data not shown).

The mRNA and protein expressions of 1, 25D3-MARRS (a receptor responsible for 1, 25(OH)2D3-stimulated uptake of calcium in intestinal cells) were up-regulated by 1, $25(OH)_2D_3$ but not by the three PTHrP segments and PTH hormones (Fig. 4).

To confirm if PTHrP(1-40) promoted calcium uptake in enterocytes via PTHR1, anti- PTHR1 antibody was used in the calcium uptake test. After pre-treated with anti- PTHR1 antibody for 5 min, the PTHrP(1-40)-induced calcium uptake in IEC-6 cells was completely blocked (Fig. 5). On the contrary, pre-incubation with antibodies against PTHR2 or 1, 25D3-MARRS (1:200) could not block PTHrP(1-40)-induced calcium uptake (Fig. 5). Taken together, these results suggested that PTHrP(1-40) promotes calcium uptake through PTHR1, but not through PTHR2 or 1, 25D3-MARRS receptor.

We addressed if L-type calcium channels or paracellular pathway participates in the PTHrP(1-40)-induced calcium uptake. As shown in Fig. 6, pre-incubation with nitrendipine (an L-type calcium channel antagonist) did not affect PTHrP(1-40)-induced calcium uptake. On the other hand, Western blot and RT-PCR showed that PTHrP treatment had no influence on the expression of claudin or zo-1 (proteins of the paracellular pathway, data not shown). These results indicated that PTHrP(1-40) promoted calcium uptake through PTHR1 but not through PTHR2, L-type calcium channels or paracellular pathway.

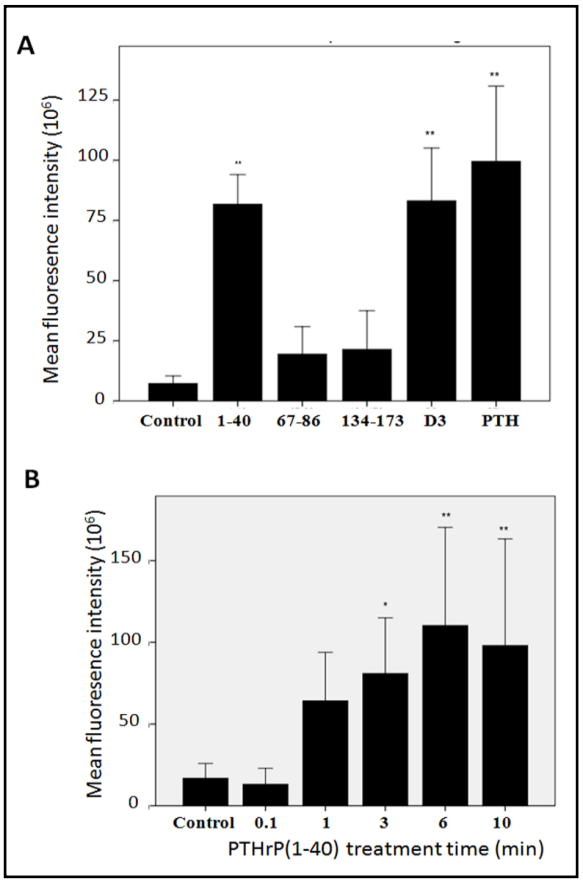

Fig. 1. The effect of PTHrP on the calcium uptake in enterocytes. IEC-6 cells were labeled with fluo-4 am and incubated for 60 min in glass-bottom dishes, followed by treated with different segments of PTHrP (6 nM), D3 and PTH (100 nM). The fluorescence intensity was measured (excitation at 485 nm, emission at 520 nm) by a confocal laser scanning microscope. For each measurement, 12 cells were randomly selected for analysis. (A) The mean fluorescence intensity of each group was presented as a bar chart. 1-40, 67-86, 134-173 were different segments of PTHrP. D3 (1,25(OH)2D3) and PTH (hormones-parathyroid hormone) were used as the positive control. (B) The effect of different concentration of PTHrP(1-40) on the calcium uptake. The error bars represent the SEM (n=3 for each group). $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group.

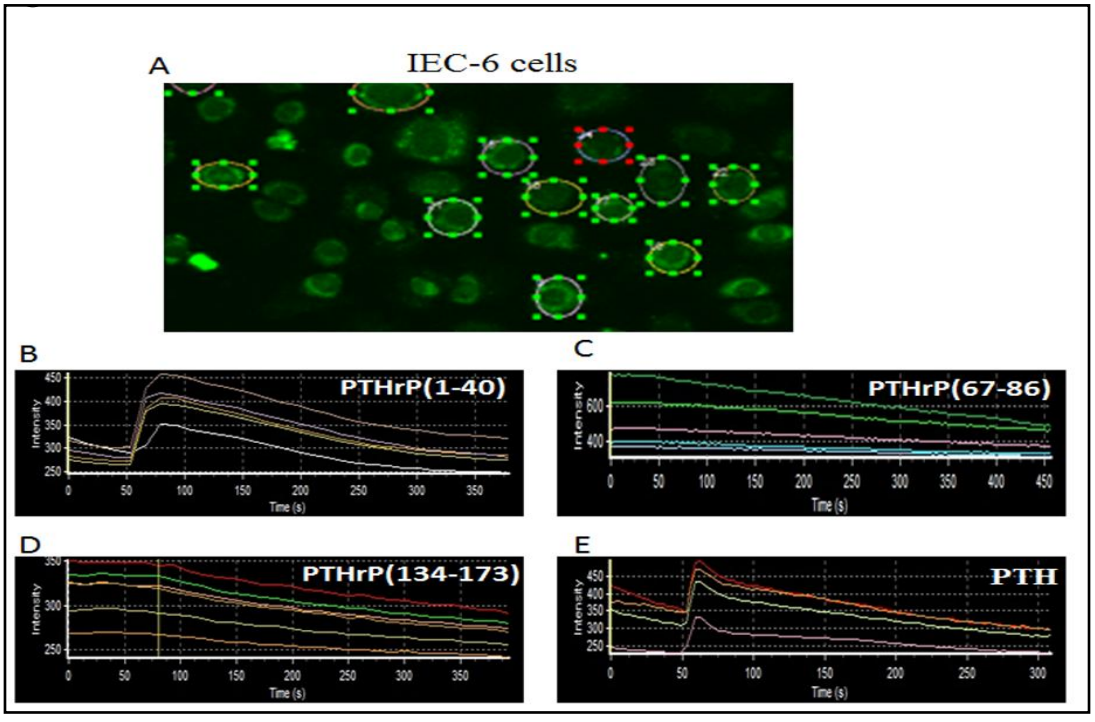

Fig. 2. The effect of PTHrP on the calcium uptake in enterocytes. IEC-6 cells were labeled with fluo-4 am and incubated for 60 min in glass-bottom dishes, followed by treated with different stimulators. The fluorescence intensity was measured (excitation at 485 nm, emission at 520 nm) by a confocal laser scanning microscope. (A) Twelve cells were randomly selected for each analysis. (B-D) After adding the different PTHrP segments, the changes of fluorescence intensity were measured. (E) PTH (hormones-parathyroid hormone) was used as a positive control.

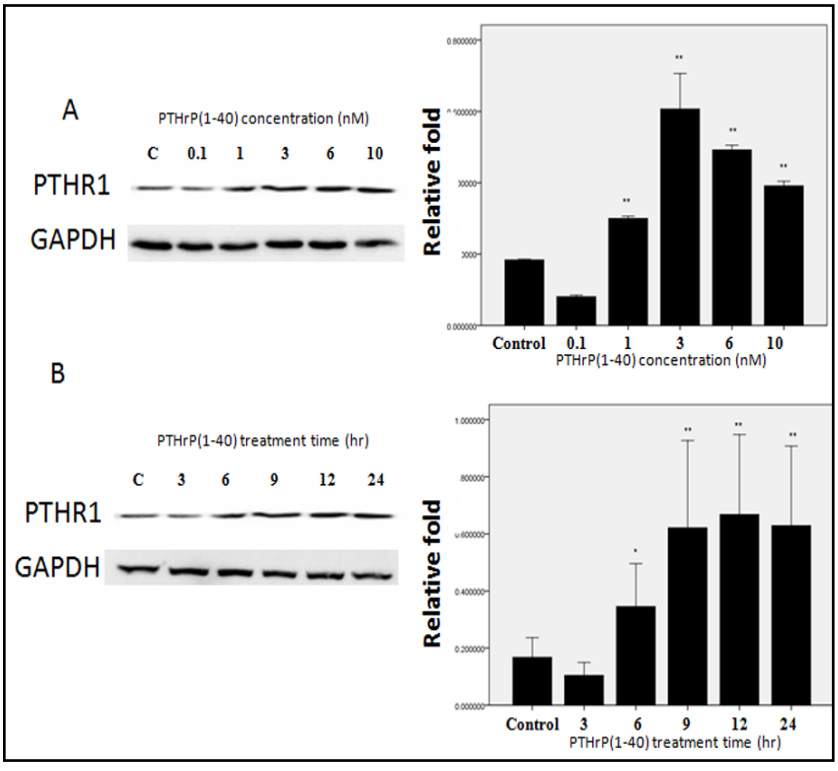

Fig. 3. PTHrP (1-40) up-regulated the PTHR1 expression in a dose-dependent and time-dependent manner. (A) IEC-6 cells were treated with different concentration (0.1 nM to 10 nM) of PTHrP(1-40) for 6 hr, followed by determining the protein level of PTHR1 via Western blot. (B) IEC-6 cells were treated with 6 nM of PTHrP(1-40). All expression levels were normalized to GAPDH. The error bars represent the SEM (n=3 for each group). $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group.

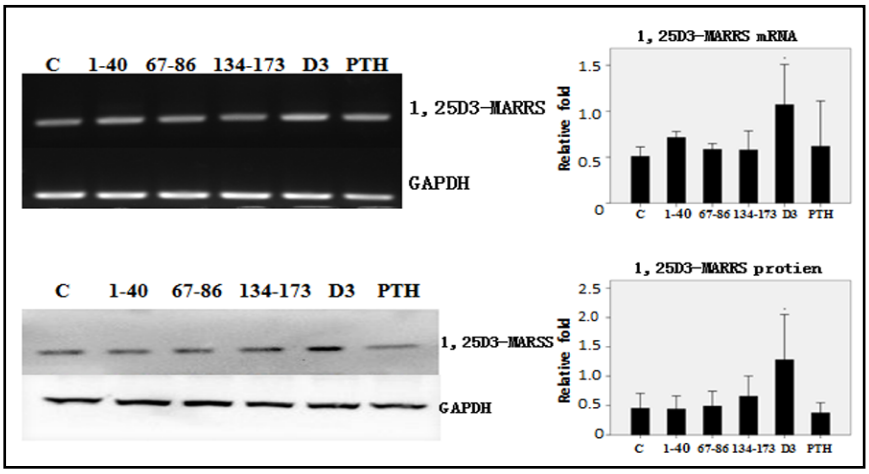

Fig. 4. The effect of PTHrP on the mRNA and protein expression of 1,25D3-MARRS. IEC-6 cells were treated with the three segments of PTHrP for 6 hr, and then RT-PCR and Western blot was performed to assess the expression of 1,25D3-MARRS. All expression values were normalized to GAPDH. The error bars represent the SEM (n=3 for each group). *P<0.05, compared with the control group.

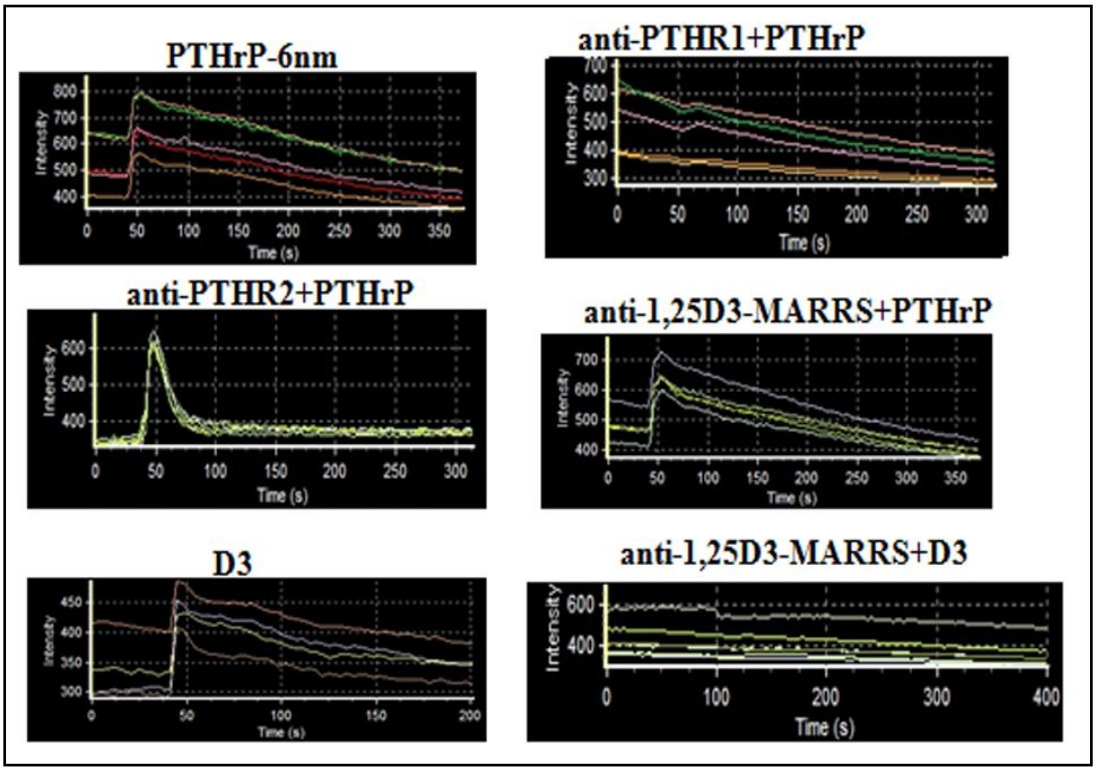

Fig. 5. The role of PTHR1 and 1,25D3-MARRS on calcium uptake induced by PTHrP(1-40). IEC-6 cells were pre-treated with or without the antibody against receptors (anti-PTHR1, anti-PTHR2 or anti-1,25D3-MARRS_antibody, all 1:200 dilution) for 5 min. The calcium uptake was measured before and after adding $1,25(OH)_2D_3$ (130 pM) or PTHrP(1-40) (6 nM).

PTHrP(1-40) up-regulated the expression of transcellular calcium transporter proteins

Next, we determined the effect of different segments of PTHrP on the expression of transcellular calcium transporter proteins. Both RT-PCR (Fig. 7A) and Western blot (Fig. 7B) showed that PTHrP(1-40) significantly up-regulated the expression of TRPV6, CaBP, NCX1, and PMCA1 (all $P<0.05$), but not L-type calcium channel Cav1.3. The other two segments of PTHrP had no effect on these transport proteins.

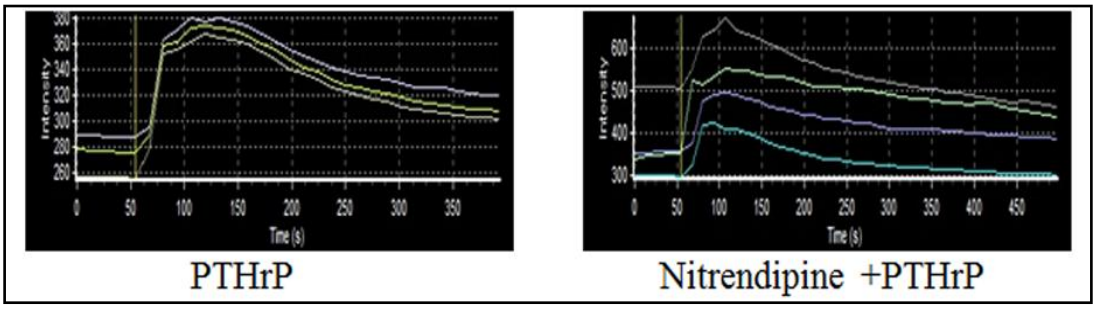

Fig. 6. The role of L-type calcium channel antagonist in the PTHrP(1-40)-induced calcium uptake. IEC-6 cells were pre-incubated with nitrendipine (20nM, an L-type calcium channel antagonist) for 30 min, and the calcium uptake was measured before and after adding $1,25(OH)_2D_3$ (130 pM) or PTHrP(1-40) (6 nM).

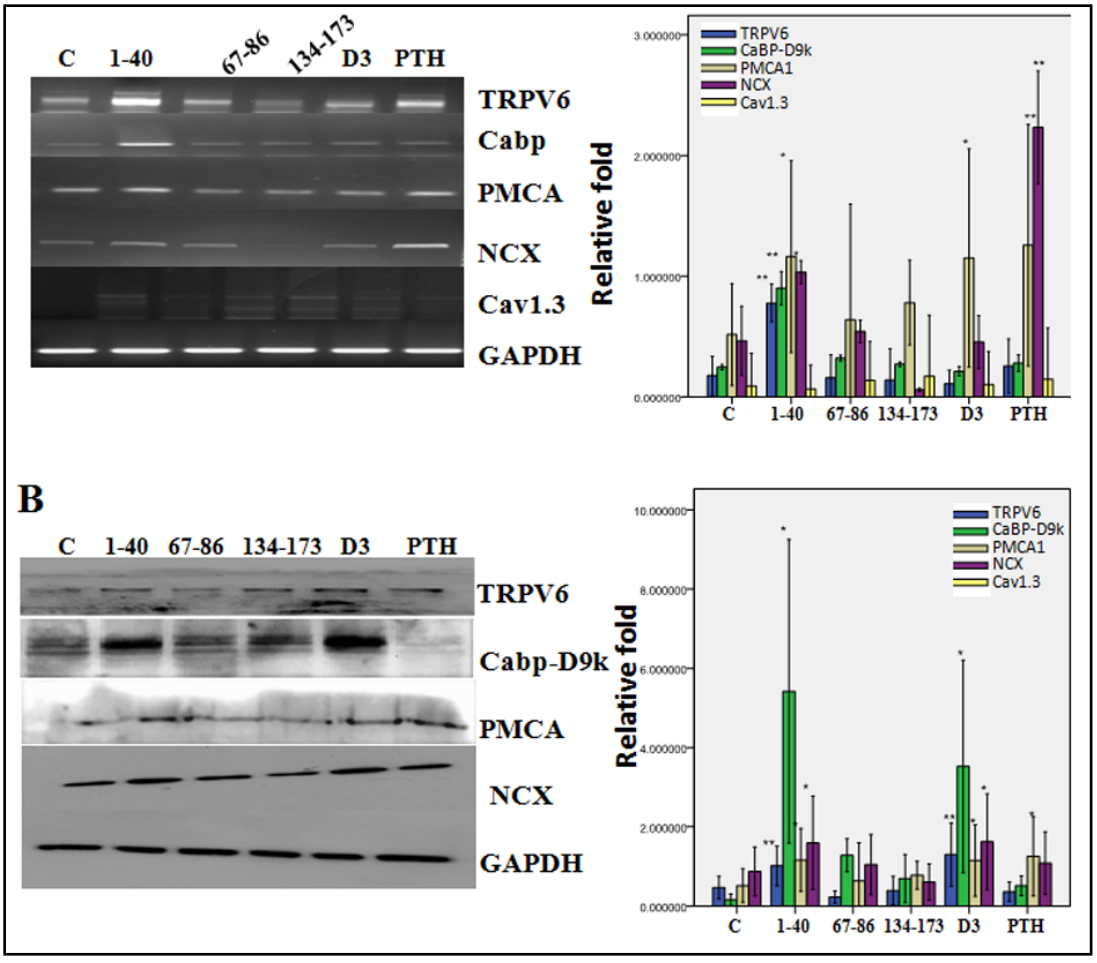

Fig. 7. The effect of PTHrP on the expressions of calcium transport proteins. IEC-6 cells were treated with different stimulators, and then the mRNA (A) and protein levels (B) of TRPV6, CaBP-D9k, PMCA1, NCX and Cav1.3 were determined by RT-PCR and Western blot, respectively. D3 (1,25(OH)2D3) and PTH (hormones-parathyroid hormone) were used as the positive control. All expression values were normalized to GAPDH. The error bars represent the SEM (n=3 for each group). $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group.

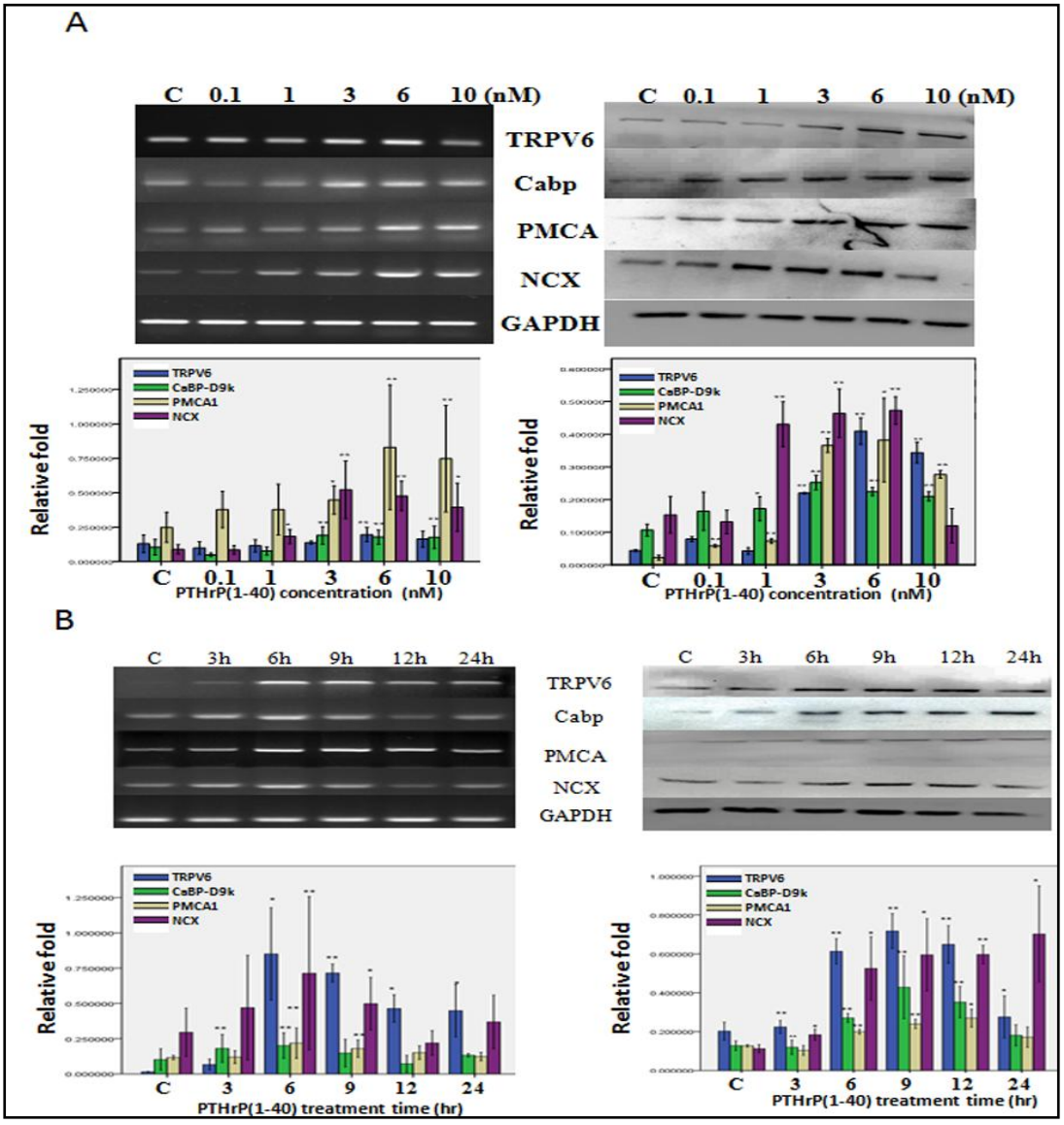

Fig. 8. PTHrP(1-40) up-regulated calcium transport proteins in a dose- and time-dependent manner. IEC-6 cells were treated with different concentration (0.1 nM to 10 nM) of PTHrP(1-40) for 6 hr (A) or treated with 6 nM of PTHrP(1-40) for different time (B). The mRNA and protein levels of TRPV6, CaBP-D9k, PMCA1, NCX and Cav1.3 were determined by RT-PCR and Western blot, respectively. All expression values were normalized to GAPDH. The error bars represent the SEM (n=3 for each group). $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group.

PTHrP(1-40) up-regulated the expression of calcium transporter proteins in a dose- and time-dependent manner

The dose- and time-dependent effects of PTHrP(1-40) on the expression of calcium transporter proteins were also addressed. The results showed that PTHrP(1-40) treatment for 6 hr significantly up-regulated the expressions of TRPV6, CaBP, NCX1 and PMCA1 in both of mRNA and protein levels in a dose-dependent manner (Fig. 8A), with a peak effect at 6 nM of PTHrP(1-40) ($P<0.05$). The time-course study showed that the enhancement effect of PTHrP(1-40) (6 nM) on the mRNA and protein expressions of the calcium transport proteins peaked at 6 hr ($P<0.001$, Fig. 8B), and gradually reduced after 24 hr.

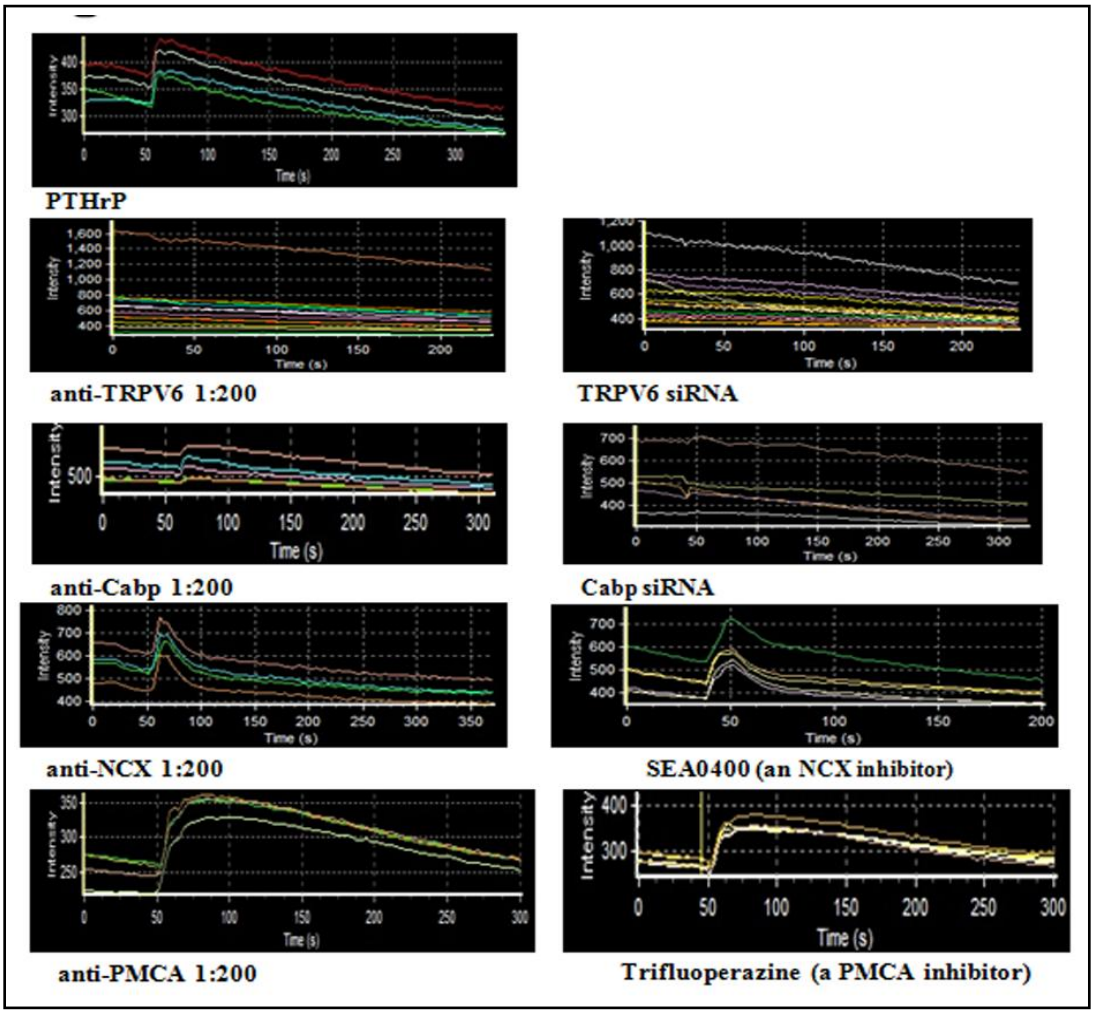

Fig. 9. The role of calcium channel protein on PTHrP(1-40)-induced calcium uptake. IEC-6 cells were pre-treated with the antibody against TRPV6, CaBP-D9k, PMCA1 or NCX (all 1:200 dilution) for 5 min, or pre-treated with SEA04000 (an NCX inhibitor), trifluoperazine (a PMCA inhibitor) for 30 min. IEC-6 cells were transfected with siRNA for rat TRPV6 or CaBP-D9k for gene knockdown. After which, the calcium uptake was measured before and after adding $1,25(OH)_2D_3$ (130 pM) or PTHrP(1-40) (6 nM).

Role of calcium channel proteins on the ability of PTHrP(1-40) to promote calcium uptake

Next, we examined if calcium-transporter proteins are necessary for PTHrP(1-40)-induced calcium uptake. Pre-incubation with antibodies against TRPV6 or CaBP-D9k for 5 min markedly inhibited PTHrP(1-40)-induced calcium uptake (Fig. 9). In addition, knockout of rat TRPV6 or CaBP-D9k consistently abolished PTHrP(1-40)-induced calcium uptake, indicating that TRPV6 or CaBP-D9k are necessary for PTHrP(1-40)-induced calcium uptake. By contrast, pre-incubation with NCX or PMCA antibody (1:200) could not inhibit PTHrP-induced calcium uptake. Consistently, pre-incubation with SEA04000 (an NCX inhibitor) and trifluoperazine (a PMCA inhibitor) for 30 min did not influence PTHrP(1-40)-induced calcium uptake (Fig. 9), indicating that NCX or PMCA was not necessary for PTHrP(1-40)-induced calcium uptake.

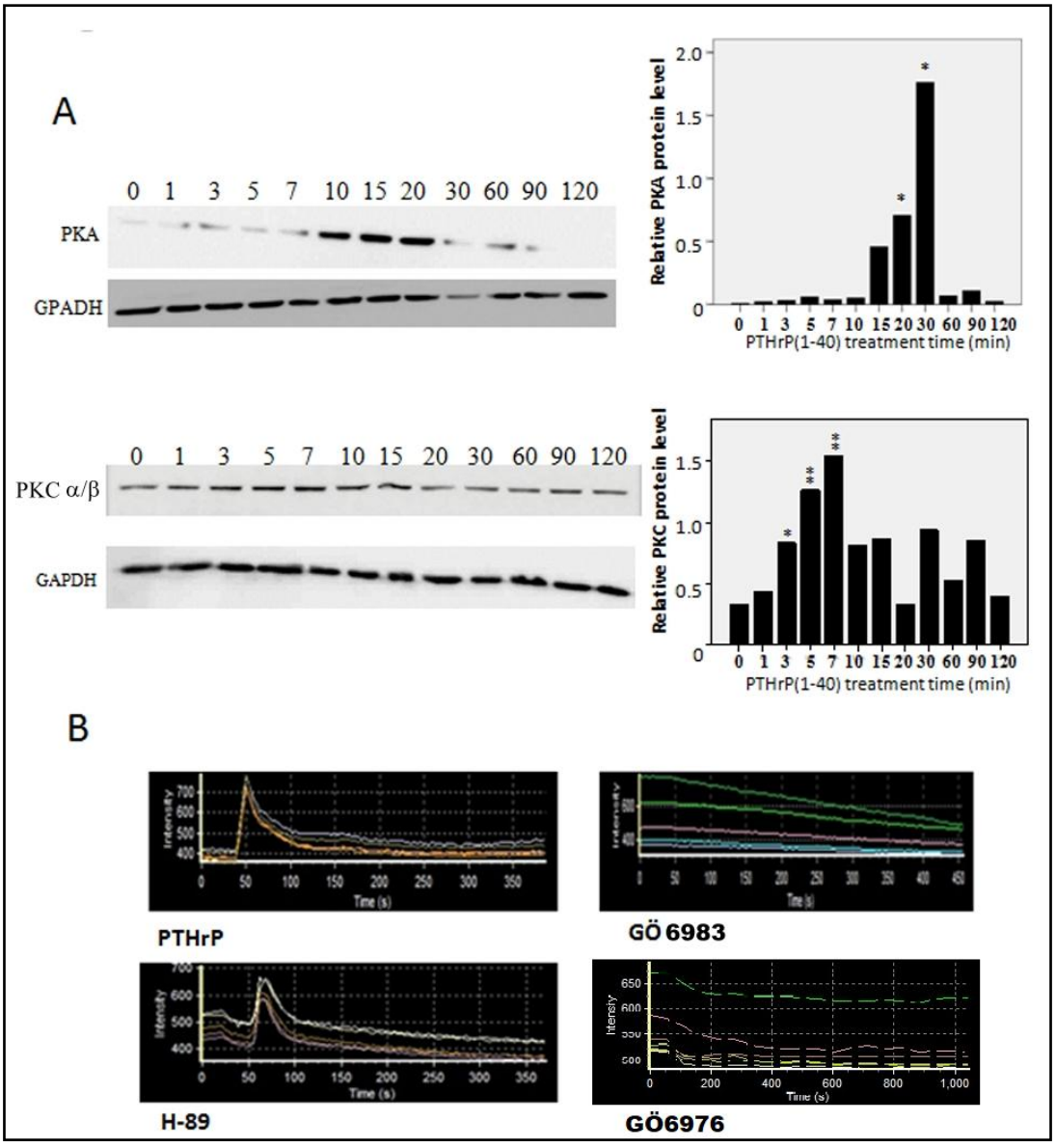

Fig. 10. PTHrP(1-40) induced calcium uptake through PKCα/β but not PKA signaling pathway. (A) The time course study of the effect of PTHrP(1-40) on protein expression of PKCα/β and PKA. IEC-6 cells were stimulated with 6 nM PTHrP(1-40) for different time (0 to 120 min), followed by added with an overdose of cold-PBS to terminate the reaction at the indicated time. The total proteins were collected for determining the protein levels of PKA(c-α PKA type) and PKA(c-α PKA type) by Western blot. All expression values were normalized to GAPDH. $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group (0 min). (B) IEC-6 cells were pre-treated with 20μM H-89 (a PKA inhibitor) or 20μM GÖ6983 (a PKC inhibitor) or 20μM GÖ6976 (a PKC α/β inhibitor), and the calcium uptake was measured before and after adding PTHrP(1-40) (6 nM).

PTHrP(1-40) induced calcium uptake through the PKC α/β but not the PKA signaling pathway

It has been shown that PTHrP-induced calcium uptake is associated with protein kinase C (PKC) [19], and PTHrP can bind PTH3R and activates the protein kinase A (PKA) signaling pathway in enterocytes of sea bream [20]. We then determined if PKC α/β or PKA signaling is involved in PTHrP (1-40) -induced calcium uptake in rat enterocytes. The time-course study showed that PKC α/β protein was significantly up-regulated after 3 min of PTHrP(1-40) stimulation, with the peak effect at 7 min (both $P<0.05$), followed by a gradual

reduction with longer incubation times. Meanwhile, the protein level of PKA (c-αPKA type) was significantly up-regulated by PTHrP(1-40) at 20 min, peaked at 30 min (both $P<0.05$), and gradually reduced to the baseline (Fig. 10A).

Calcium uptake test showed that GÖ6983 (a PKC inhibitor, 20μM) and GÖ6976 (a PKC α/β inhibitor, 20 μM) pretreatment inhibited PTHrP(1-40)-induced calcium uptake. However, H-89 (a PKA inhibitor, 20μM) pretreatment did not affect PTHrP(1-40)-induced calcium uptake, indicating that PTHrP promotes calcium uptake through the PKC α/β but not PKA signaling (Fig. 10B).

Discussion

In this study, we investigated the effect of PTHrP on the calcium uptake in rat enterocytes and its underlying molecular mechanism. The results showed that among the three segments of PTHrP, only PTHrP(1-40) induced rapid and significant calcium uptake in enterocytes in a dose-dependent manner. PTHrP(1-40) up-regulated PTHR1 protein but not 1, 25D3-MARRS receptor. Pre-treatment of anti- PTHR1 antibody for 5 min completely blocked the PTHrP(1-40)-induced calcium uptake. In addition, PTHrP(1-40) significantly up-regulated transcellular calcium transporter proteins, TRPV6, CaBP, NCX1 and PMCA1 (but not L-type calcium channel Cav1.3) in a dose- (peaked at 6 nM) and time- (peaked at 6 hr) dependent manner. Meanwhile, pre-treatment with antibodies against TRPV6 or CaBP-D9k for 5 min or knockout of rat TRPV6 or CaBP-D9k markedly inhibited PTHrP(1-40)-induced calcium uptake, whereas inhibition of NCX or PMCA1 by antibodies or inhibitors had no effect on PTHrP(1-40)-induced calcium uptake. Furthermore, PTHrP(1-40) treatment up-regulated protein levels of PKCα/β and PKA. Pretreatment of PKC α/β inhibitor inhibited PTHrP(1-40)-induced calcium uptake. Taken together, these results suggested that PTHrP(1-40) stimulated calcium uptake via PTHR1 receptor and PKCα/β signaling in rat enterocytes, and calcium transporters TRPV6 and CaBP are necessary for this effect.

Studies have shown that the three bioactive subdomains (the N-terminal, mid-segment, C-terminal sequences) of PTHrP have different physiological functions. For example, the N-terminal segment (PTHrP(1-34)) can stimulate calcium uptake in chick duodenum [13] and stimulate cortisol release in teleost interregnal cells [20]. The mid-segment of the PTHrP can promote maternal to fetal calcium transfer across the placenta [18], while the carboxy-terminal fragment inhibits bone resorption [21], stimulates osteoblast growth [22], and is involved in calcium signaling in hippocampal neurons [23]. In our study, we also found that only the N-terminal segment (PTHrP(1-40)) can provoke calcium uptake and up-regulate the transcellular calcium proteins in rat enterocytes, which is in line with the findings of previous studies on intestinal calcium uptake in sea bream [15], chick [13] and sheep [16].

PTHR1 is a member of the family 2 B1 G-protein coupled receptors with seven transmembrane domains [24]. Immunohistochemical analysis shows that PTHR1 expresses in several human normal tissues, including intestine, kidney, and bone [25]. Binding of ligands (PTH or PTHrP) to PTHR1 triggers different intracellular signaling pathways, including the PKA signaling activation, PKC signaling activation, cAMP accumulation and intracellular calcium release [26]. In this study, PTHR1 was detectable in rat enterocytes and could be up-regulated by PTHrP in a dose- and time-dependent manner. Pre-incubation with PTHR1 antibody was able to significantly inhibit PTHrP(1-40)-induced calcium uptake. Meanwhile, PTHrP(1-40) treatment also up-regulated PKCα/β protein level in rat enterocytes and PKC inhibitor pre-treatment completely abolished PTHrP(1-40)-induced calcium uptake. These data suggested that PTHrP(1-40) may bind to PTHR1 on the cell membrane of enterocytes, which subsequently activated PKCα/β signaling pathway and eventually induced calcium uptake. It is worth to further investigate the how PTHrP(1-40)-induced PKCα/β signaling activation induces calcium uptake in enterocytes. PTHR2 is a second G protein-coupled PTH receptor with more than 50% amino acid homology with PTHR1 [27]. However, the PTHR2 receptor only responds to PTH, but not PTHrP [28], and has a restricted distribution in

humans. In this study, PTHR2 was undetectable in rat enterocytes and anti-PTHR2 antibody pre-treatment had no effect on the PTHrP(1-40)-induced calcium uptake. Similar results were observed in the 1, 25D3-MARRS receptor. These results suggest that the stimulatory effect of PTHrP(1-40) on calcium uptake in rat enterocytes is mainly through PTHR1 but not PTHR2 or 1, 25D3-MARRS receptors.

Transcellular calcium uptake is predominant in the duodenum [29] and is associated with several calcium-transporter proteins. Our data showed that PTHrP(1-40) can up-regulate TRPV6, CaBP-D9k, PMCA1, NCX but not L-type calcium channel and the paracellular pathway proteins. Inhibition of TRPV6 or CaBP-D9k significantly inhibited the PTHrP(1-40)-induced calcium uptake, whereas blocking of NCX or PMCA1 had no effect. Since TRPV6 channel is the rate-limit-step for calcium entry [6], and CaBP-D9k facilitates calcium movement across the cytosol [1], these data indicated that TRPV6 and CaBP-D9k are necessary for PTHrP(1-40)-induced calcium uptake. NCX1 and PMCA1 are responsible for calcium extrusion from the basolateral membrane [8], which are not required for calcium uptake procedures.

There are still some limitations of this study. First, this is an *in vitro* study, and an *in vivo* model is necessary to validate the findings of the current study. We did not investigate the detailed mechanism between PTHrP(1-40)-induced PKCα/β signaling and the calcium uptake. In addition, there are 15 isozymes in the PKC family in human [30]. It should be further investigated if PTHrP(1-40) has an effect on the other PKC isoforms and PKC signaling pathways. Furthermore, we did not investigate if PTHrP has an effect on calcium transport, and whether the calcium transport proteins NCX and PMCA1 participate in this process. Fluo-4am is a fluorescence probe reflecting the changes of intracellular calcium level, which cannot be used to study calcium transport. Isotopes are required for the calcium transport test. All these limitations should be addressed in the following study.

Conclusion

In summary, our study demonstrated that PTHrP(1-40) can stimulate rat calcium uptake in rat intestinal cells via the PTHR1 receptor and PKCα/β signaling pathway. PTHrP(1-40) can up-regulate transcellular calcium proteins, of which TRPV6 and CaBP-D9k are necessary for calcium uptake in rat enterocytes. Our findings are helpful for better understanding the mechanisms of calcium uptake in mammal enterocytes.

Acknowledgements

This work was supported by the grants from National Natural Science Foundation (81170354), Guangdong Provincial Science and Technology Plan Fund (2011B031800195) and Natural Science Foundation of Guangdong Province (S2012010009343). We also wish to acknowledge the following institutions for their support of this work: Department of Neurobiology and the Clinical Research Center of Nanfang Hospital affiliated with Southern Medical University. We also thank Doctor Chudi Chen and our technicians Jianjun Li and Yong Zeng.

Disclosure Statement

The authors declare no conflicts of interest.

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