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Parathyroid Hormone Activates Phospholipase C (PLC)-Independent Protein Kinase C Signaling Pathway via Protein Kinase A (PKA)-Dependent Mechanism: A New Defined Signaling Route Would Induce Alternative Consideration to Previous Conceptions

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Parathyroid hormone (PTH) is an effective anti-osteoporosis agent, after binding to its receptor PTHR1, several signaling pathways, including cAMP/protein kinase A (PKA) and phospholipase C (PLC)/protein kinase C (PKC), are initiated through G proteins; with the cAMP/PKA pathway as the major pathway. Earlier studies have reported that PTHR1 might also activate PKC via a PLC-independent mechanism, but this pathway remains unclear.


Material/Methods: In HEK293 cells, cAMP accumulation was measured with ELISA and PKC was measured with fluorescence resonance energy transfer (FRET) analysis using CKAR plasmid. In MC3T3-E1 cells, real-time PCR was performed to examine gene expressions. Then assays for cell apoptosis, cell differentiation, alkaline phosphatase activity, and mineralization were performed.

Results: The FRET analysis found that PTH(1–34), [G1,R19]PTH(1–34) (GR(1–34)), and [G1,R19]PTH(1–28) (GR(1–28)) were all activated by PKC. The PKC activation ability of GR(1–28) was blocked by cAMP inhibitor (Rp-cAMP) and rescued with the addition of active PKA- α and PKA- β . The PKC activation ability of GR(1–34) was partially inhibited by Rp-cAMP. In MC3T3-E1 cells, gene expressions of ALP, CITED1, NR4a2, and OSX that was regulated by GR(1–28) were significantly changed by the pan-PKC inhibitor Go6983. After pretreatment with Rp-cAMP, the gene expressions of ALP, CITED1, and OPG were differentially regulated by GR(1–28) or GR(1–34), and the difference was blunted by Go6983. PTH(1–34), GR(1–28), and GR(1–34) significantly decreased early apoptosis and augmented osteoblastic differentiation in accordance with the activities of PKA and PKC.

Conclusions: PLC-independent PKC activation induced by PTH could be divided into two potential mechanisms: one was PKA-dependent and associated with PTH(1–28); the other was PKA-independent and associated with PTH(29–34). We also found that PTH could activate PLC-independent PKC via PKA-dependent mechanisms.

MeSH Keywords: **Cyclic AMP-Dependent Protein Kinase R1alpha Subunit • Parathyroid Hormone • Phosphoinositide Phospholipase C • Protein Kinase C**

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Background

Parathyroid hormone (PTH), the essential regulator that controls calcium ion homeostasis in blood, has a strong anabolic effect on bone formation. Both PTH(1–84), the natural PTH secreted by parathyroid gland, and PTH(1–34), the C-terminus truncated PTH peptide, have been approved to treat osteoporosis. Both PTH(1–84) and PTH(1–34) have similar binding affinity and potent stimulation of type I PTH receptor (PTH1R) [1]. Once stimulated, PTH1R induces multiple signaling cascades including Gs/adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) [2], Gq/11/phospholipase C (PLC)/calcium/protein kinase C (PKC) [2], and G12/13/Ras homolog gene family member A/phospholipase D-signaling pathway [3], as well as G protein-dependent and β -arrestin-dependent activation of extracellular signal-related kinase (ERK)1/2 [4,5]. PTH1R-mediated activation of PKC also occurs through a PLC-independent mechanism [6,7].

PTH augments both bone formation and bone resorption through its effects on both osteoblasts and osteoclasts. The net effect of PTH(1–34) on bone metabolism depends on its administration mode. The application of low and intermittent dose favors bone formation, and the administration of high and/or continuous dose leads to profound bone loss. The mechanism underlying these controversial effects remains unclear. One explanation is that different administration strategies prefer different patterns of signaling cascades and thus cause different biological responses.

Among the second signaling initiated by PTH, cAMP/PKA, has been demonstrated as a central signaling pathway to mediate PTH's biological function [8]. *In vivo*, cAMP/PKA pathway has been identified as the major mediator of bone anabolic actions [9–11]. *In vitro*, via cAMP/PKA signaling, PTH augments BMP's effect [12], activates TRPV5 [13] and receptor activator of NF kappa B ligand [14], inhibits c-Jun N-terminal kinase activity [1] and mitogen-activated protein kinase activation [15], and regulates expressions associate bone metabolism, including osteoprotegerin (OPG) [14,16], Runt-related transcription factor 2 (Runx2), Osterix (OSX) [17], mitogen-activated kinase phosphatase 1 [18], amphiregulin [19], Phex [20], and bone morphogenetic protein 4 [21].

PKC is another important kinase that mediates the function of PTH1R [6,22]. PTH1R activates Ras and MAPK pathway via PKC pathway to stimulate osteogenic proliferation in marrow mesenchymal progenitor cells [23]. PKC is also involved in the mechanism of estrogen inhibition of PTH-stimulated osteoclast formation and attachment [24]. PTH1R can activate PKC via Gq/PLC dependent pathway in which PLC hydrolyzes PIP2 to form DAG and IP3, and stimulates Ca²⁺ release and thereby activates PKCs [25,26]. PLC signaling is not required for PTH's effects on bone metabolism in adult mice [9,27]. PTH1R may

also activate PKC via a PLC-independent mechanism [6], but this signaling pathway still remains unclear.

Signal-selective PTH analogs have been used to study the signaling pathways induced by PTH/PTH1R, based on the specific activation of PTH1R signaling response, which has been linked to key domains of PTH(1–34). The first two amino acids (Ser1-Val2) are crucial for activation of AC/cAMP [28]. The α -amino group of the N-terminal Ser1 is required for PLC activation [6,29]. The analogs of PTH(1–34) with the Ser1 \rightarrow Gly1 mutation lost the ability to activate PLC but still retained AC activity. The Glu19 \rightarrow Arg19 substitution could partially restore the PTH1R binding affinity that would be lowered by the truncation of PTH(29–34) [29]. The C-terminal domain of PTH(1–34) (Gln29-Leu30-Val31-His32-Asn33-Phe34) is the only domain that has been indicated to activate PKCs via a PLC-independent mechanism [6,7]. Recently, using the fluorescence resonance energy transfer (FRET) technique, it was surprisingly shown that cAMP/PKA selective PTH analog [G1,R19]PTH(1–28) [6,29], induced significant PKC activation response. In this paper, we analyzed the role of PKA in the PLC-independent PKC signaling pathway induced by PTH.

Material and Methods

Peptides

Human PTH(1–34)NH₂ (PTH(1–34)), [G1,R19]PTH(1–28)NH₂ (GR(1–28)) and [G1,R19]PTH(1–34)NH₂ (GR(1–34)) were synthesized by TeraBIO (Guangzhou, China). All the peptides were dissolved in 0.1% trifluoroacetic acid (TFA) to a concentration of 10⁻³ M, aliquoted and stored at –80°C until subsequently diluted into appropriate concentrations with DMEM medium before cell treatment. The peptide concentrations were determined by amino acid analysis. The purity was confirmed to be more than 95%. Compared with GR(1–28), GR(1–34) contains PTH(29–34) domain and has a 10-fold higher binding affinity [6].

Cell culture and transfection

HEK293 cells (GNHu43) and mouse osteoblastic MC3T3-E1 subclone 14 (ATCC CRL-2594) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a DMEM culture medium (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) in a 5% CO₂ humidified atmosphere.

For transient transfection, 150 μ L HEK293 cell suspension (containing 10⁴ cells) was plated onto the cover glass of confocal petri dish (NEST, Hong Kong, China). After the cells were

attached to the cover glass, 1 mL DMEM medium containing 10% FBS was added to the whole dish and the cells were cultured for 24 hours before transfection. The transfection was performed in FBS-free DMEM; 0.9 μ L Fugene 6.0 (Promega, WI, USA) was mixed with 10 μ L DMEM, followed by the addition of 0.3 μ g pcDNA3.1(+)-CKAR plasmid (kindly provided by Dr. Alexandra C. Newton) [30] and DMEM medium to reach the final volume of 20 μ L. Then the mixture was transferred onto the cover glass of a petri dish and cultured for five hours until 1 mL DMEM containing 10% FBS was added. FRET analysis was performed after 72 hours. The process of co-transfection of pHCMV1 plasmid (Gene Therapy Systems, Inc., San Diego, CA, USA) incorporating PTHR1 or DSEL and pcDNA3.1(+)-CKAR was performed as described earlier, except that 6 μ L Fugene combined with 4 μ g plasmid (the mass ratio of PTHR1/DSEL to CKAR was 9 to 1) in the transfection medium.

Stable transfections of HEK293 cells were performed in 6-well plates (Corning Inc., NY, USA) and followed the standard steps for transient transfections. In brief, when the culture reached 60% confluence, transfection mixture containing with 3 μ L Fugene 6.0 and 2 μ g of plasmid (pcDNA3.1(+)-PTH1R or DSEL) was applied to the HEK293 cells. Then 24 hours after the cells grew in medium with 10% FBS, they were trypsinized and plated into 10 cm dishes and cultured for 48 hours. Then the cells were exposed to 500 mg/mL G418 (Sigma-Aldrich, USA) and positive colonies were selected after two weeks.

Cyclic AMP accumulation

At 48 hours after PTHR1 transfection, the cells were washed with assay buffer (135 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 2.8 mM glucose, 1.2 mM CaCl₂, and 20 mM HEPES, pH 7.4) and incubated for 15 minutes at 37°C in the vehicle which was composed of the same buffer containing 0.1% heat inactivated BSA, 1 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich Inc., St. Louis, MO, USA) or 10 nM PTH dissolved in the vehicle. Buffer was then aspirated quickly, plates were frozen immediately in liquid nitrogen, and subsequently, the frozen cells were thawed directly into 50 mM HCl. The cellular cAMP in the acid extracts was measured using a cAMP ELISA Kit (R&D Systems, MN, USA). Data were expressed as nanomoles per well.

FRET measurements

FRET analysis was carried out at room temperature in a dark room with Leica micro system (machine configuration: mode-xyt, speed-400 Hz, zoom-1, objective-HCX PL APO CS 40.0 \times 0.85 UV). Before the analysis, cells were washed with PBS three times and cultured in Hank's solution. FRET spectra were generated by exciting cells at 405 nm. The emissions of CFP and YFP were measured at the wavelength of 475 nm and 535 nm, respectively [30]. The energy transfer from CFP to YFP,

which represents the spatial position of the two molecules that would be changed after the PKC substrate phosphorylated, was presented as the ratio (C/Y ratio) of cyan fluorescence (which increases as FRET decreases) to yellow emission (which decreases as FRET decreases). In each experiment, 10 cells were included in the analysis under 40x magnified field. Results are expressed as mean values \pm SEM of three independent experiments.

Real-time PCR

When 100% confluence was reached, the MC3T3-E1 cells were cultured in DMEM supplement with 1% FBS. PTH peptides were then added in the presence of 10 μ M Rp-cAMP (from Sigma-Aldrich, USA and dissolved in distilled water and added 1.5 hours previously) and/or 1 μ M Go6983 (from Selleck, USA and dissolved in 1% DMSO and added 1 hour previously) or vehicle alone (distilled water or 1% DMSO, respectively). Incubations were continued for a further three hours before extraction of total RNA using an RNeasy Mini Kit (Takara BIO, Dalian, China). Expressions of alkaline phosphatase (ALP), bone sialoprotein (BSP), CBP/P300 interacting transactivator with glutamic acid and aspartic acid rich C-terminal domain 1 (CITED1), osteopontin (OPN), OPG, nuclear receptor subfamily 4 group a member 2 (NR4a2), and OSX were measured by two-step real-time (RT)-PCR. Briefly, the first strand of cDNA was synthesized according to the manufacturer's instructions using a PrimeScript[®] RT reagent Kit (Takara BIO, Dalian, China). For each gene, two specific PCR primers (ALP/fw, 5'-GCTGATATGAGATGCCTT-3', ALP/re, 5'-GCACTGCCACTGCTACT-3'; BSP/fw, 5'-AGGGAAGTACCAGTGTGG-3', BSP/re, 5'-ACTCAACGGTGTGCTTTT-3'; CITED1/fw, 5'-CCACTAGTCTCTGGATCG-3', CITED1/re, 5'-AGCCCCTTGGTACTGGTAT-3'; OPN/fw, 5'-TGACCCAGATCCTATAGCC-3', OPN/re, 5'-CTCCATGTCATCATCATCG-3'; OPG/fw, 5'-ACCTACCCAGAGCAGCTT-3', OPG/re, 5'-TTGTGAAGCTGTGAGGAAC-3'; NR4a2/fw, 5'-CGTTTCAGAAAGTGCCTAGC-3', NR4a2/re, 5'-TTGCCTGGAACCTGGAATAG-3', OSX/fw, 5'-GAGCAAAGTCAGATGGGTAAG-3', OSX/re, 5'-CACCAGTCCAGGCAACA-3'; GAPDH/fw, 5'-TGTCGTGGAGTCTACTGGTG-3'; GAPDH/re, 5'-GCATTGCTGACAATCTTGAG-3') were designed and synthesized by Life Technologies (Shanghai, China). The PCR reactions (94°C, 20 seconds; 60°C, 20 seconds; 72°C, 20 seconds) were performed on an GeneAmp[®] PCR System 9700 (Applied Biosystems, CA, USA) using a SYBR[®] Premix EX Tap[™] (Takara BIO, Dalian, China). Gene expression was normalized to that of GAPDH and then expressed as fold over control.

Cell apoptosis assay

MC3T3-E1 cells were plated in 6-well plates at 2×10^5 cells/well and incubated at 37°C. After three days, the medium was replaced with α -MEM supplemented with 0.5% FBS. Then 12 hours later, cells were treated with peptides as a 4 hour/24 hour cycle. In brief, after signaling inhibitor pretreatment, peptides were added and remained in culture for four hours, then the medium including the inhibitors and peptides was removed and switched to fresh medium for the next 20 hours. On the third day, soon after the treatment of peptides, cells were suspended with 0.5% trypsin (Sigma, USA) and washed twice with cold PBS, then fixed with cold ethanol for 12 hours. The apoptosis was quantified by using Annexin V-EGFP Apoptosis Detection Kit (GenScript USA Inc., NJ, USA) per the manufacturer's protocol. The apoptotic cells with phosphatidylserines in the outer membrane could be detected with Annexin V-FITC; in addition, propidium iodide (PI), a nucleic dye, could pass the damaged membrane of cells in the middle and late period of apoptosis. Detection of early apoptosis was performed by flow cytometry (Ex=488 nm; Em=530 nm). Flow cytometry was performed on the FACSCanto II Cytometer (BD Biosciences, CA, USA). The BD FACSDiva™ Software v8.0 was used for both acquisition and analysis of apoptosis.

Cell differentiation, alkaline phosphatase activity and mineralization assay

MC3T3-E1 cells were plated in 24-well plates coated with type I collagen at 3×10^4 cells/well, and incubated at 37°C. After three days, the generic medium was replaced with a mineralization medium composed of α -MEM supplemented with 10% FBS, 100 mM β -glycerophosphate, 50 μ g/mL ascorbic acid, and 100 nmol/L dexamethasone. PTH peptides incubated the cells in a 4 hour/48 hour cyclic style as described previously [31]. In brief, the cells were pretreated with 10 μ M Rp-cAMP (1.5 hours before peptides) and/or 1 μ M Go6983 (1 hour before peptides) before each treatment of peptides, then four hours after peptide treatment, cells were switched to fresh medium without peptides or signaling inhibitors for the following 44 hours. ALP staining and ALP activity measurement were taken at the second week; and the investigation of mineralized nodules and the calcium content of the cultures were performed at the fourth week, as previously described [32].

Statistical analysis

All statistical analyses were conducted using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as means \pm standard deviation (SD) (RT-PCR results) or means \pm standard error (SEM) (FRET results). Variables were analyzed using analysis of variance (ANOVA) and Bonferroni's test for post hoc analysis. A *p*-value <0.05 was considered statistically significant.

Results

GR(1–28) activated PKC signaling in HEK293 cells with PTHR1 transfection

In HEK293 cells transfected with CKAR plasmid alone, the PKC activator phorbol ester, (TPA) (Bio-Rad, CA, USA) but not the cAMP analog 8-Br-cAMP, induced FRET response (Figure 1A, 1B). Without PTHR1 transfection, the FRET response could not be detected with PTH(1–34) treatment even at as high a dose as 10 μ M (Figure 1C). When PTHR1 co-transfected into HEK293 cells with CKAR, 100 nM PTH(1–34) induced significant FRET (Figure 1D). So, our experiments verified that FRET signaling generated by CKAR was specific in reporting PKC activation, and with such a system, PTH(1–34) was shown to activate PKC via PTHR1.

With the CKAR reporting system, GR(1–28) induced obvious PKC activation FRET response (Figure 1E). We then repeated FRET analysis in HEK293 cells that were co-transfected CKAR with DSEL, the PLC deficient PTHR1 [24,33], and found that GR(1–28) had a similar effect (Figure 1F).

cAMP specific inhibitor blocked GR(1–28) effect on PLC-independent PKC activation

In order to address the possible role of cAMP/PKA pathway in the PKC activation of GR(1–28), we tested whether the cAMP competitive inhibitor Rp-cAMP could change the effect of GR(1–28) on PKC activation. HEK293 cells stably expressing PTHR1 (HEK293-PTHR1 cells) were established and verified by cAMP generation under the stimulation of hPTH(1–34) (data not shown). CKAR was transfected transiently into the HEK293-PTHR1 (HEK293-PTHR1+CKAR) cells; and 1,000 nM GR(1–28) and 100 nM GR(1–34) both induced FRET response (Figure 2A, 2C). But pretreatment with Rp-cAMP blocked FRET response induced by GR(1–28) and downregulated the effect of GR(1–34) (Figure 2B, 2D).

PKA signaling is required for GR(1–28) ability to activate PLC-independent PKC signaling

After the HEK293-PTHR1+CKAR cells were pretreated by Rp-cAMP, active PKA subunits α and β (5 ng/mL each) (Antibodies-online, Aachen, Germany) were added into the culture, and no FRET was visible within 25 minutes (Figure 3A). When GR(1–28) was added soon after the PKA active subunits were applied, significant FRET response was immediately detected (Figure 3B). This implied that cAMP/PKA signaling could not activate PKC, but it is required for GR(1–28) activating PKC.

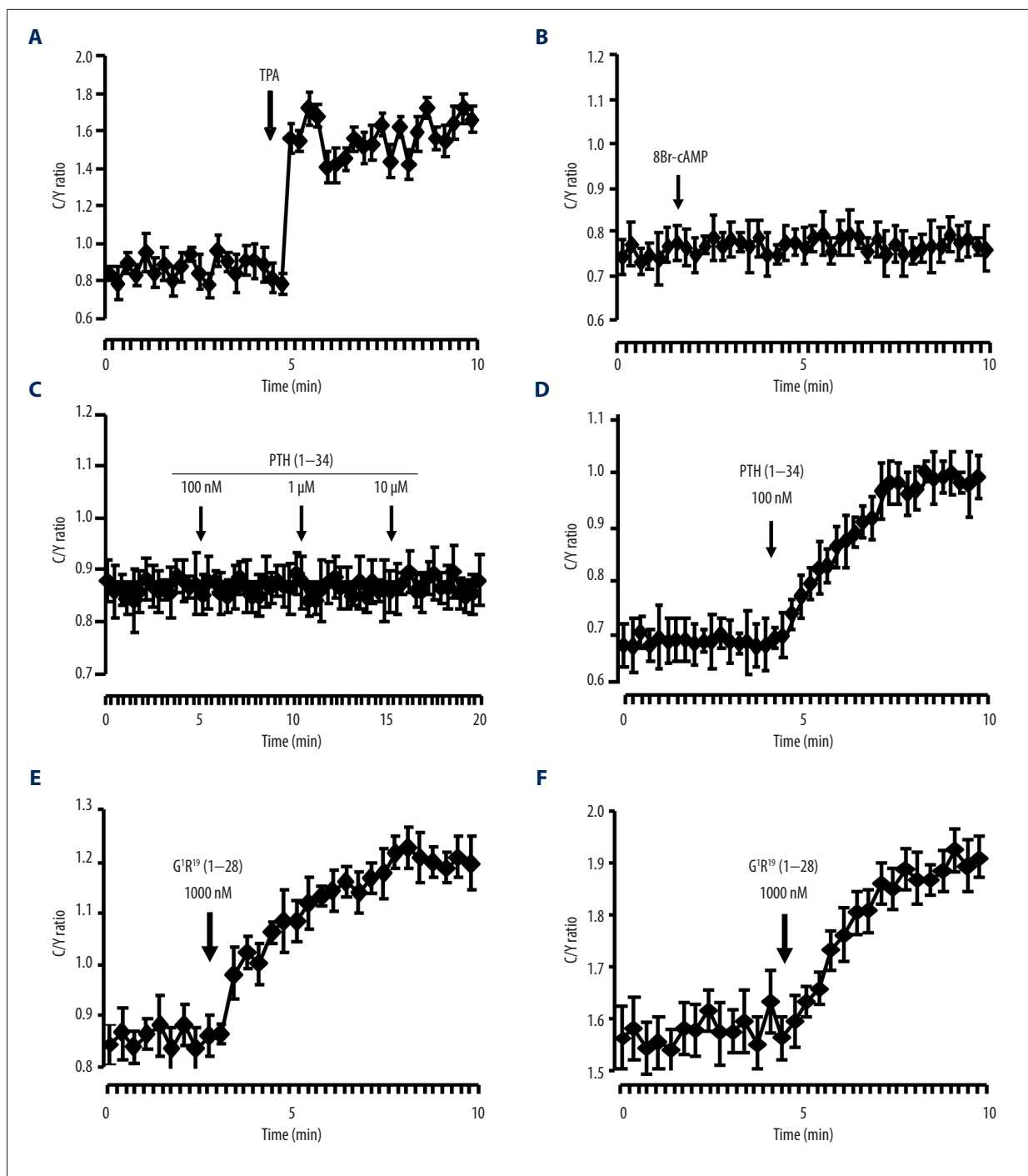


Figure 1. FRET analysis in HEK293 cells. HEK293 were transiently transfected with plasmid of CKAR (A–C) or plasmids combined with CKAR and PTHR1 (D, E) or DESL (F); 72 hours later, FRET was measured after the application of 1 μM TPA (A), 10 μM 8-Br-cAMP (B), 100 nM–10 μM hPTH(1–34) (C), 100 nM hPTH(1–34) (D), and 1,000 nM GR(1–28) (E, F). In each experiment, 10 cells were included in the analysis under 40× magnified field. Results are expressed as mean values ±SEM of three independent experiments.

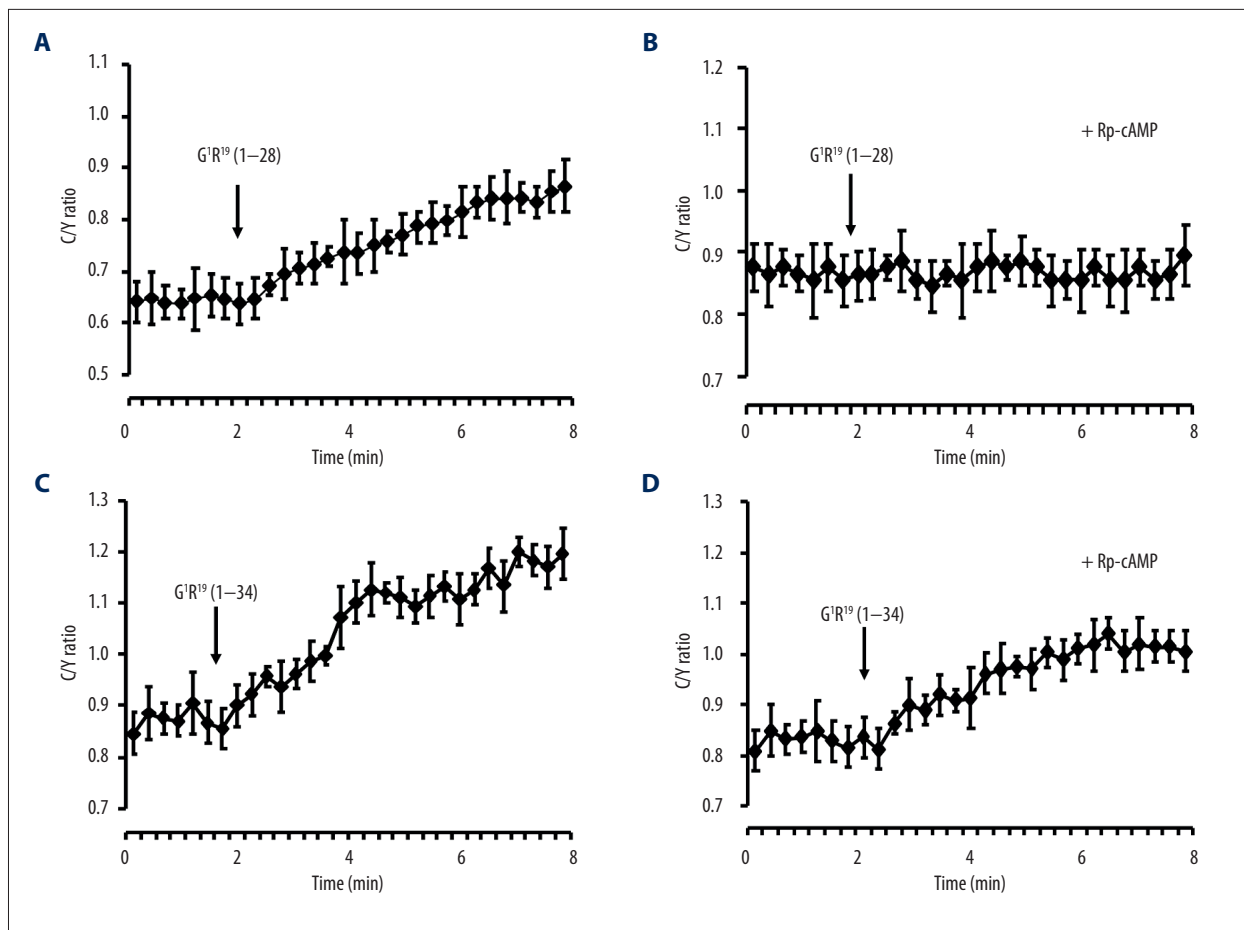


Figure 2. The role of cAMP/PKA signaling in PLC-independent PKC activation. HEK293 cells were stably transfected with PTHR1 and then transiently transfected with CKAR: 1,000 nM GR(1–28) (A) or 100 nM GR(1–34) (C) was applied to the cells and FRET was measured; 1.5 hours after the cells were treated with 10 μ M Rp-cAMP, 1,000 nM GR(1–28) (B), or 100 nM GR(1–34) (D) was then added, followed by FRET measurement. In each experiment, 10 cells were included in the analysis under 40 \times magnified field. Results are expressed as mean values \pm SEM of three independent experiments.

Gene expression regulated by PLC-independent PKC signaling

In MC3T3-E1 cells, the gene expressions of ALP, BSP, CITED1, OPN, OPG, NR4a2, and OSX were measured with or without pretreatment of PKC inhibitor Go6983.

As shown in Figure 4A, the gene expression of ALP (3.72 ± 0.97 fold) and CITED1 (10.84 ± 0.69 fold) increased by GR(1–28) ($p < 0.05$) were then blocked (ALP, 0.97 ± 0.21 fold) or down-regulated (CITED1, 3.10 ± 0.26 fold) ($p < 0.05$) with Go6983 pretreatment. OSX expression was decreased by GR(1–28) (0.31 ± 0.17 fold); but with Go6983 present, it was further decreased (0.10 ± 0.02 fold) ($p < 0.05$). On the contrary, PKC inhibition had a synergistic effect to increased NR4a2 expression (from 1.71 ± 0.16 to 6.44 ± 0.64 fold, $p < 0.05$). BSP and OPN expressions were not changed with Go6983 application. The expression of OPG was decreased more by the combination

of Go6983 and GR(1–28) (0.26 ± 0.02 fold) than by GR(1–28) alone (0.43 ± 1.11 fold), but it could be the additive effect of GR(1–28) and Go6983, without any interactional effect (considering Go6983 itself downregulated OPG expression by 0.52 ± 0.13 fold).

The genes associated with PTH(29–34) were identified by the comparison of the differential regulation of genes by GR(1–28) or GR(1–34) after Rp-cAMP pretreatment. And those genes related to PKC could be sorted out further with PKC inhibition. In the genes we tested (Figure 4B), the expression of ALP was not changed by the combination of GR(1–28) and Rp-cAMP, but increased significantly by the combination of GR(1–34) and Rp-cAMP (3.42 ± 0.31 fold, $p < 0.05$), and the effect was eliminated by Go6983 (0.60 ± 0.01 fold). CITED1 expression was increased by the combination which included both GR(1–28) (5.74 ± 0.98 fold) and GR(1–34) (12.30 ± 0.34 fold), and the effect of GR(1–34) was inhibited by Go6983 (8.12 ± 0.35 fold, $p < 0.05$). After Rp-cAMP

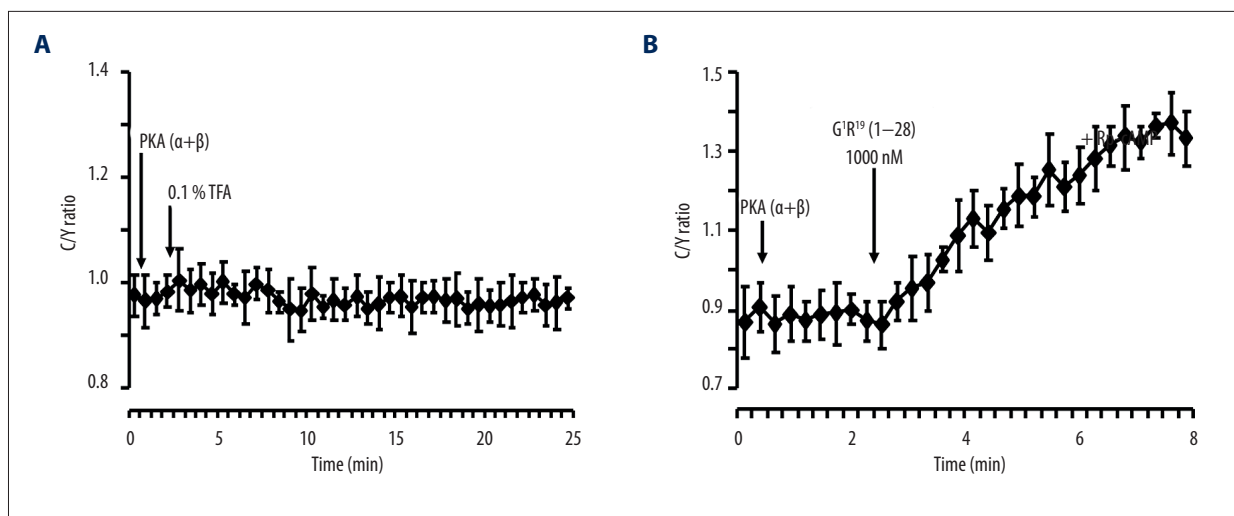


Figure 3. Active PKA subunits rescued the FRET response to GR(1-28) that was blocked by Rp-cAMP. HEK293 cells were stably transfected with PTHR1 and then transiently transfected with CKAR. After the cells were treated with Rp-cAMP for 1.5 hours, active subunits of PKA (including both 5 ng/mL PKA- α and 5 ng/mL PKA- β) were added into the culture and at the same time, FRET was recorded. 0.1% TFA (vehicle) (A) or 1,000 nM GR(1-28) (B) was applied within two minutes after the addition of active PKAs. In each experiment, 10 cells were included in the analysis under 40 \times magnified field. Results are expressed as mean values \pm SEM of three independent experiments.

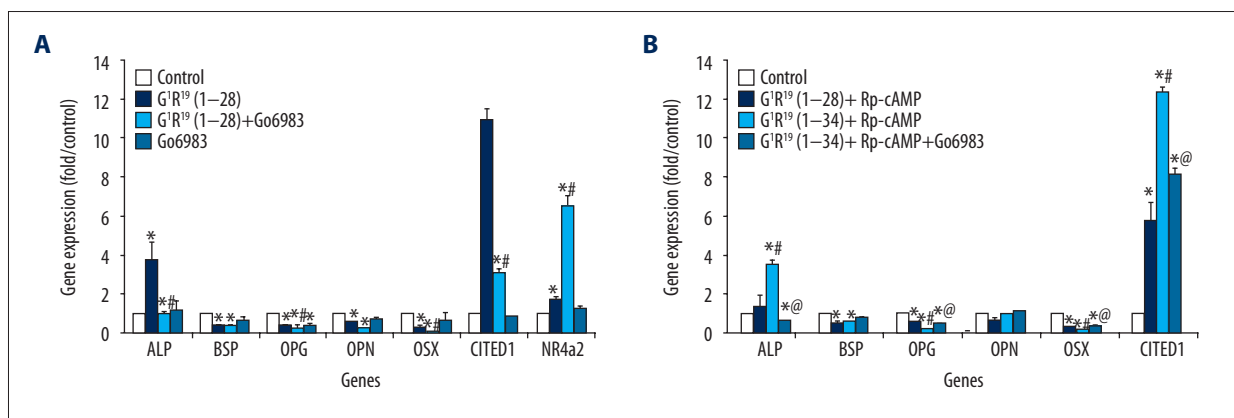


Figure 4. Effect of Go6983 on gene regulation by GR(1-28) or GR(1-34). After MC3T3-E1 cells in culture reached confluence, they were switched into DMEM containing 1% FBS for 12 hours. (A) Go6983 or the vehicle (control) was added into the culture for one hour, followed by treatment of 100 nM GR(1-28) for four hours. Expressions of indicated genes were quantified with real-time PCR. (B) MC3T3-E1 cells were cultured in 1% FBS for 12 hours, then Rp-cAMP (group of GR(1-28)+Rp-cAMP or GR(1-34)+Rp-cAMP) or vehicle (control) was added to the culture. 1.5 hours later, Go6983 (group of GR(1-34)+Rp-cAMP+Go6983) or vehicle were added to the medium before the treatment of 100 nM GR(1-28) or 10 nM GR(1-34) for 4 hours. Expressions of genes were quantified with real-time PCR. Data are expressed as means \pm standard deviation (SD). Variables were analyzed using analysis of variance (ANOVA) and Bonferroni's test for post hoc analysis. * $p < 0.05$ versus control; # $p < 0.05$ versus GR(1-28) (A) or GR(1-28)+Rp-cAMP (B); @ $p < 0.05$ versus GR(1-34)+Rp-cAMP.

treatment, the expression of OPG and OSX were decreased by GR(1-28) (OPG, 0.53 ± 0.05 fold; OSX, 0.27 ± 0.07 fold) and GR(1-34) (OPG, 0.18 ± 0.01 fold; OSX, 0.14 ± 0.01 . $p < 0.05$), but the difference between these two peptides was blunted by Go6983 (OPG, 0.45 ± 0.05 fold; OSX, 0.27 ± 0.12 . $p < 0.05$). The expression levels of BSP and OPN were similarly regulated in the combinations that included GR(1-28) or GR(1-34).

Early apoptosis of MC3T3-E1 cells was inhibited by PTH/PLC-independent signaling via both cAMP/PKA-dependent and -independent mechanism

The apoptosis of MC3T3-E1 cells was induced with low concentrations of FBS. FITC+/PI-cells presented as early apoptotic cells that had contact membrane but abnormal metabolism.

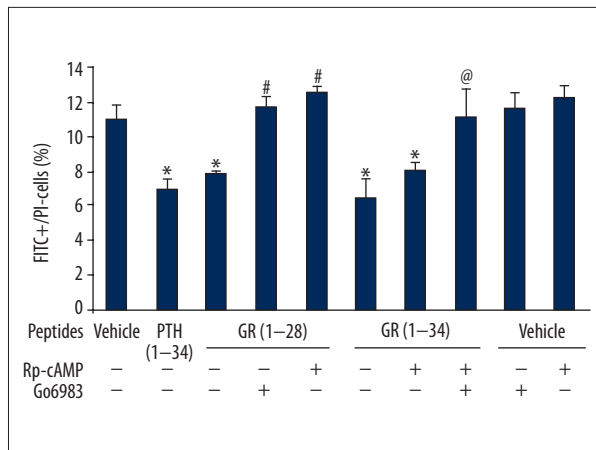


Figure 5. PKC inhibition changed the effects of GR(1-28) and GR(1-34) on early apoptosis of MC3T3-E1 cells. MC3T3-E1 cells were cultured with α -MEM supplemented with 0.5% FBS and treated with peptides as a 4 hour/24 hour cycle. After three rounds of treatment, cells were suspended. The apoptosis was quantified by using Annexin V-EGFP Apoptosis Detection Kit (GenScript USA Inc., NJ, USA) per the manufacturer's protocol. Data are expressed as means \pm standard deviation (SD) of three independent experiments. Variables were analyzed using analysis of variance (ANOVA) and Bonferroni's test for post hoc analysis. * $p < 0.05$ versus control; # $p < 0.05$ versus GR(1-28); @ $p < 0.05$ versus GR(1-34) and GR(1-34)+Rp-cAMP.

As shown in Figure 5, the percentage of early apoptotic cells treated by PTH(1-34) (6.93 ± 0.69), GR(1-28) (7.84 ± 0.06) and GR(1-34) (6.48 ± 0.86) were significantly reduced compared with vehicle (11.25 ± 0.79). The effect of GR(1-28) was blocked by either Go6983 (11.74 ± 0.42) or Rp-cAMP (12.51 ± 0.42). And the effect of GR(1-34) was partially reduced by Rp-cAMP (8.02 ± 0.45) then eliminated with additional Go6983 (11.08 ± 1.51).

Cellular differentiation

At week two, ALP activity in MC3T3-E1 cell lysate was measured. In the vehicle treatment, the basic ALP activity was 2.59 ± 0.18 U/gprot. After the cells were intermittently treated with PTH(1-34), GR(1-28), and GR(1-34), ALP activity increased to 5.03 ± 0.18 , 3.59 ± 0.31 , and 4.21 ± 0.39 U/gprot, respectively. The effect of GR(1-28) was abolished with Go6983 (2.84 ± 0.15 U/gprot) and Rp-cAMP (2.40 ± 0.36 U/gprot). And the effect of GR(1-34) was attenuated by Rp-cAMP (3.52 ± 0.04 U/gprot); while combination of Go6983 and Rp-cAMP could ablate the effect (Figure 6B). The quantified ALP activity was coincident with ALP stain experiments (Figure 6A).

At week four, the accumulated calcium in cell cultures was extracted and the concentration of calcium in the cell lysate

was measured. As compare with vehicle treatment (0.26 ± 0.07 mmol/gprot), PTH(1-34), GR(1-28), and GR(1-34) increased calcium concentration to 0.47 ± 0.02 , 0.34 ± 0.00 , and 0.44 ± 0.02 mmol/gprot, respectively. Just like ALP analysis aforementioned, Go6983 (0.25 ± 0.1 mmol/gprot) and Rp-cAMP (0.28 ± 0.01 mmol/gprot) could remove the effect of GR(1-28). Rp-cAMP (3.52 ± 0.04 mmol/gprot) was partially eliminated and the combination of Go6983 and Rp-cAMP eliminated the effect of GR(1-34) (Figure 6C). Formation of mineralized nodules, stained by Alizarin red, was another index to show the calcification ability of cells *in vitro*. More calcified nodules were seen with the treatment of PTH(1-34), GR(1-28), and GR(1-34). The inhibitions of Go6983 and Rp-cAMP on GR(1-28) or GR(1-34) on mineralized nodules were the same as the situation on calcium measurements described earlier (Figure 6A).

Discussion

PTH has a strong anabolic effect on bone formation, and PTH could induce bone marrow mesenchymal stem cell chondrogenic differentiation [34]. In this paper, two mechanisms were found to be involved in the PLC-independent PKC signaling pathway that was initiated by PTH/PTHr1. One was PKA signaling dependent and related to the domain of PTH(1-28). The other was PKA-independent and related to the domain of PTH(29-34). Both PKA-dependent and PKA-independent PLC-independent PKC signaling pathway were found to play important roles in mediating PTH's effect on bone metabolism according to our data analysis of gene expression, apoptosis, and *in vitro* differentiation. As far as we know, PKA-dependent PKC activation has not been previously reported; this finding might lead us to reconsider PKA's role associated with N-terminus of PTH peptide.

The CKAR system has been identified only as a response to PKC activator (phorbol dibutyrate) but not the strong PKA simulator forskolin [30,35]. The PKC activation specificity to the CKAR system was again confirmed in HEK293 cells, in that TPA induced strong FRET response and 8-Br-cAMP did not. In our experiments, GR(1-28) was shown to activate PKC, both in wildtype PTHR1 and in its PLC-inefficient mutant DSEL. The results in our FRET analysis were coincident with gene expression measurements in which PKC inhibitor changed the effects of GR(1-28) on individual genes expressing in MC3T3-E1 cells.

The ability of GR(1-28) to activate PKC was linked with cAMP/PKA because the FRET response induced by GR(1-28) was totally blocked by Rp-cAMP; and then PKA active subunits (the factor downstream of cAMP in the signaling cascades) reversed the effect of Rp-cAMP. But active PKA subunits alone did not activate PKC. These results implied that cAMP/PKA itself could not activate PKC, but was required for GR(1-28) or PTH(1-28)

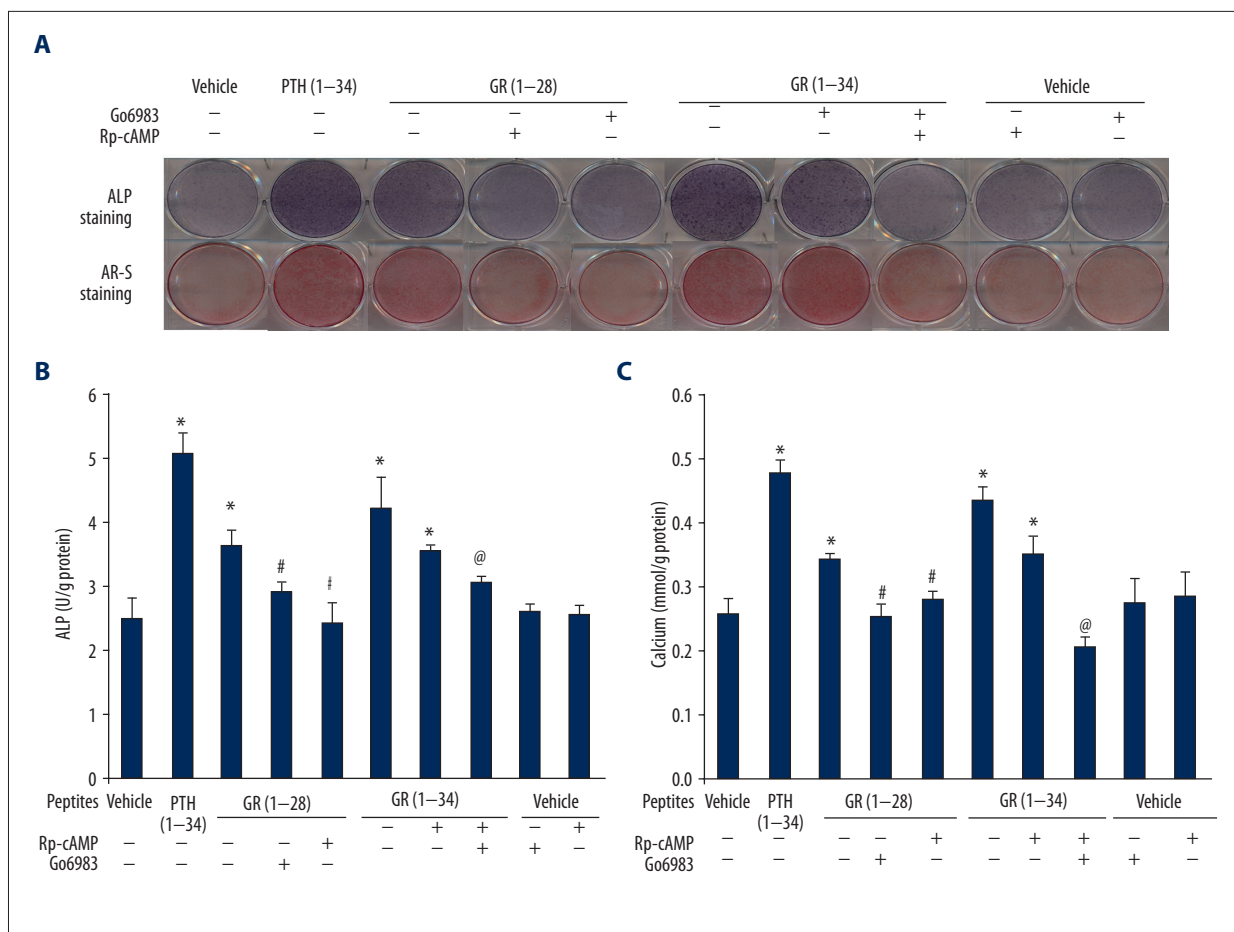


Figure 6. PKC inhibition altered the effects of GR(1-28) and GR(1-34) on osteoblastic differentiation of MC3T3-E1 cells. MC3T3-E1 cells were plated in 24-well plates coated with type I collagen in mineralization medium. PTH peptides incubated the cells in a 4 hour/48 hour cyclic style. Alkaline phosphatase (ALP) staining and ALP activity measurement at the second week, as well as the investigation of mineralized nodules and the calcium content of the cultures at the fourth week were performed. Data are expressed as means±standard deviation (SD). Variables were analyzed using analysis of variance (ANOVA) and Bonferroni's test for post hoc analysis. * $p < 0.05$ versus control; # $p < 0.05$ versus GR(1-28) or GR(1-28)+Rp-cAMP; @ $p < 0.05$ versus GR(1-34) and GR(1-34)+Rp-cAMP.

to activate PKC. This newly discovered PLC-independent PKC signaling pathway was cAMP/PKA-dependent and associated with PTH(1-28).

The connection between PKA and PKC signaling pathway has been indicated previously in the literature. It has been shown to synergistically stimulate calcium uptake in distal convoluted cells [36]. In human periodontal ligament cells, PTH(1-34), via PKC-dependent and PKA-independent pathways, has been shown to mediate proliferative and apoptotic signaling [37]. But the crosstalk between PKA and PKC remains poorly understood. Our results provide new information that suggests the relationship between PKA and PKC is not only in parallel but also in cascade.

We also examined whether PKC inhibition could change the effect of GR(1-28) on gene expressions in MC3T3-E1 cells. Out of seven genes tested, the expressions of four genes regulated by GR(1-28) were significantly changed by the pan-PKC inhibitor Go6983. And the genes altered by PKC inhibition included transcription factors (NR4a2 and CITED1) and bone marker genes (ALP and OSX). Especially, GR(1-28) regulated ALP gene expression was mediated totally by PKC. These results strongly support that GR(1-28) has the ability to activate PKC, and implies that PKA-dependent PLC-independent PKC signaling is involved in PTH's function in multiple aspects. It is known that cAMP/PKA is the dominant signaling that mediates PTH's biological function [8], and the finding of PKA-dependent, PLC-independent PKC signaling suggests the additional contributions of PKC. This definitely provides a new outlook to study the signaling pathway of PTH.

The activation of PKC induced by GR(1–28) was blocked by pre-treatment of the cells with cAMP inhibitor. On the contrary, the effect of GR(1–34) was partially inhibited. This, once again, supports that the domain of PTH(29–34) is associated with PLC-independent PKC signaling shown in previous studies [6,7], and that this pathway is independent of cAMP/PKA signaling. Based on our findings that GR(1–28) activated PKC and was dependent on cAMP/PKA signaling, we treated MC3T3-E1 cells to inhibit the effect of cAMP/PKA as well as PKA-dependent PKC signaling and then compared the gene expressions. The gene expressions of ALP, CITED1, and OPG were found to be differentially regulated by GR(1–28) or GR(1–34). And the difference in gene regulation between GR(1–28) and GR(1–34) was significantly blunted by PKC inhibition. These results provided essential evidence to define the signaling pathway of PKA-independent PLC-independent PKC pathway, which could be mapped to PTH(29–34).

In order to probe the functions of PLC-independent PKC pathway, we further performed the analysis of apoptosis and *in vitro* differentiation. PTH(1–34), GR(1–28), and GR(1–34) significantly, decreased early apoptosis and augmented osteoblastic differentiation. The effects of GR(1–28) were ablated by either PKC inhibition or PKA inhibition. Considering PKA's role to activate PKC signaling, this suggested that GR(1–28)'s functions on apoptosis and osteoblastic differentiation depended on PKC signaling. However, GR(1–34)'s effects on apoptosis and

osteoblastic differentiation could only partially be inhibited by PKA inhibition. Remaining effects associated with PTH(29–34) were finally eliminated by PLC inhibition. This once again highlighted PKC's role in osteoblastic function.

Conclusions

Our study demonstrated that PLC-independent PKC pathway could be activated via two mechanisms. One is PKA-dependent and associated with PTH(1–28); the other is PKA-independent and associated the domain of PTH(29–34). Both PKA-dependent and PKA-independent of PLC-independent PKC pathway had the capacity to inhibit apoptosis, and augment osteoblastic differentiation. Whether these two mechanisms are redundant or performed different functions needs further study.

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Conflicts of interest

None.

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