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Stimulation of Amphiregulin Expression in Osteoblastic Cells by Parathyroid Hormone Requires the Protein Kinase A and cAMP Response Element-Binding Protein Signaling Pathway

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Abstract Parathyroid hormone (PTH), an anabolic agent for bone metabolism, has profound effects on gene expression in the osteoblast. Recently, we identified that amphiregulin (AR), an EGF-like ligand, is an immediate early gene for PTH treatment and has an important role in bone metabolism. In the present report, by using different PTH peptide fragments, protein kinase activators, and inhibitors, we have demonstrated that PTH regulates amphiregulin in a cAMP-protein kinase A (PKA)-dependent manner both in vitro and in vivo. We found that the phosphorylation of cAMP-response element (CRE)-binding protein (CREB) preceded AR transcription after PTH treatment. Moreover, luciferase reporter assays revealed that the binding of phosphorylated CREB to a conserved CRE site in the AR promoter plays an important role in basal, PTH-induced, and prostaglandin E_2 (PGE_2)-induced AR expression in osteoblastic cells. In summary, our data suggest that PTH-induced AR mRNA expression is mediated primarily through cAMP-PKA-CREB signaling.
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Key words: parathyroid hormone; amphiregulin; osteoblastic cells; protein kinase A; cAMP response element-binding protein (CREB)

The adult human skeleton continuously undergoes remodeling, namely, being resorbed and renewed by the actions of osteoclasts and osteoblasts. Parathyroid hormone (PTH), an 84-amino acid polypeptide hormone secreted from the parathyroid glands, plays an important role in this process. Current interest in PTH focuses on its anabolic ability to increase bone mass in humans by intermittent administration (once daily). In bone, the PTH receptor (PTH1R), a seven transmembrane domain receptor coupled to G-proteins, exists in cells of osteoblastic lineage [Juppner et al., 1991]. Binding of PTH to its receptor activates two well-defined signal transduction pathways in osteoblasts. One is the protein kinase A (PKA) pathway [Partridge et al., 1981], in which $G\alpha_s$ stimulates production of cAMP and activation of PKA. The other is the protein kinase C (PKC) pathway [Civitelli et al., 1988] where $G\alpha_q$ activates phospholipase Cβ with subsequent formation of diacylglycerol, PKC activation, and formation of 1,4,5-inositol trisphosphate, thus stimulating an increase in intracellular free Ca^{2+} and related signaling events.

Activation of these pathways by PTH leads to activation of multiple transcription factors, including AP-1, CREB, and Cbfa1 [Swarthout et al., 2002a]. Specifically, PKA activated by PTH is able to phosphorylate CREB at Ser-133 and this phosphorylated CREB is required for the upregulation of the AP-1 family member, c-fos [Tyson et al., 1999], and receptor activator of NFκB-ligand (RANKL), an inducer for osteoclastogenesis [Fu et al., 2002]. A recent computational analysis further reveals that the consensus binding sequence of CREB, CRE,

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has a significantly higher occurrence in the promoter regions of PTH-regulated genes [Qin et al., 2003], suggesting that CREB phosphorylation plays an important role in transducing the PTH signal in osteoblastic cells.

PTH has a profound effect on the gene expression pattern in osteoblastic cells. Our previous microarray experiment revealed that more than 100 genes were differentially expressed after PTH treatment of UMR 106-01 cells, a rat osteoblastic cell line [Qin et al., 2003]. Among those genes, amphiregulin (AR), a member of the epidermal growth factor (EGF) family, has been identified as an immediate early response gene for PTH in osteoblastic cells [Qin et al., 2005]. PTH rapidly and strongly stimulates the expression of amphiregulin not only in several osteoblastic cell lines but also in bone. AR was first isolated from conditioned medium of MCF-7 human breast carcinoma cells exposed to PMA [Shoyab et al., 1988]. It is expressed in vivo by numerous normal human tissues and its expression is regulated by many signals, such as estrogen [Martinez-Lacaci et al., 1995], androgen [Sehgal et al., 1994], other EGF-like ligands (EGF and TGF-α) [Kitadai et al., 1993], vitamin D_3 [Akutsu et al., 2001], and prostaglandin E_2 (PGE_2) [Shao et al., 2003]. Similar to EGF, AR binds and signals through the EGF receptor (EGFR/Erbb1). Overexpression of AR is often observed in several cancers, including breast, colon, stomach, and pancreatic cancers. In bone, AR is a potent growth factor for the preosteoblast but it also strongly inhibits osteoblast differentiation [Qin et al., 2005]. Moreover, AR null mice significantly displayed less tibial trabecular bone than wild-type mice. It is hypothesized that AR is one of the means for PTH to manipulate the bone marrow microenvironment with a possible role in osteoblastogenesis by expanding the pool of preosteoblasts [Qin et al., 2005].

In this report, the detailed mechanisms of how PTH stimulates AR expression in osteoblastic cells were studied. We demonstrated that this regulation requires the cAMP-PKA pathway and CREB phosphorylation. Moreover, the binding of phosphorylated CREB to the conserved CRE site in the AR promoter is important for both basal and signal-stimulated expression of AR in osteoblastic cells.

MATERIALS AND METHODS

Chemicals

Synthetic human PTH(1–31), PTH(13–34), PTH(1–38), and bovine PTH(3–34) were purchased from Bachem (Torrance, CA). Inhibitors H-89 and GF109203X were purchased from Calbiochem (San Diego, CA). Rat PTH(1–34), PGE_2, 8-BrcAMP, and PMA were obtained from Sigma (St. Louis, MO). Antibodies for CREB and phosphorylated CREB (Ser-133) were obtained from Cell Signaling (Beverly, MA).

Cell Culture

UMR 106-01 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 5% (v/v) fetal bovine serum. For experiments, the cells were seeded in 100-mm dishes at a density of 1.2×10^4 cells/cm^2 overnight and then switched to serum-free MEM for 1 day before addition of appropriate agents.

In Vivo Injection of PTH Into Rats

Four-week-old male Sprague–Dawley rats, about 75 g, were purchased from Hilltop (Scottdale, PA). Rats were injected subcutaneously with vehicle (0.9% saline solution) or various PTH peptides (8 μg/100 g) and euthanized using CO_2 after 1 h of injection. The animal protocol was approved by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee. The primary spongiosa samples from distal femur were harvested as described previously [Onyia et al., 1995].

Real-Time RT-PCR

Total RNA was isolated using Tri Reagent (Sigma) followed by an RNeasy kit (Qiagen). TaqMan® Reverse Transcription kit (Applied Biosystems) was used to reverse-transcribe mRNA into cDNA. Following this, PCR was performed on Opticon (MJ Research) using a SYBR® Green PCR Core kit (Applied Biosystems). Each analysis was performed three times with independent sets of cells from hormone treatment to RT-PCR to obtain mean values and SEM shown in the figures. The primers used here were described previously [Qin et al., 2005].

Retrieving AR Promoter Sequences and Multiple Sequences Alignment

AR mRNA sequences (mouse NM_009704, rat NM_017123, and human NM_001657) were

used to do a blast search against an HTGS (high throughput genomics sequence) database to retrieve the corresponding DNA sequences (mouse AC109311.2, rat AC115469.4, and human AC021180.6). The 500 bp sequences upstream of the transcriptional start sites were aligned with each other using ClustalW software available at www.ebi.ac.uk/ClustalW/index.html.

Transient Transfection and Luciferase Assay

Reporter constructs pGL2-A and pGL2-C containing the 5′-flanking region of human AR gene promoter were generous gifts from Dr. Sean B. Lee (NIH) and were described previously [Lee et al., 1999]. pGL2-A mCRE and pGL2-C mCRE, each containing a mutated and non-functional CRE site, were constructed by site-directed mutagenesis (Stratagene) using primers 5′GGGCCGCAGCCCATGGTACCAAGGCCCGGCCGTG3′ and 5′CACGGCCGGGCCTTGGTACCATGGGCTGCGGCCC3′ for pGL2-A mCRE and primers 5′GGGCCGCAGCCCATGGTACCAACTCGAGCTAGCA3′ and 5′TGCTAGCTCGAGTTGGTACCATGGGCTGCGGCCC3′ for pGL2-C mCRE. The dominant-negative KCREB expression vector, pCMV-KCREB, was purchased from BD Biosciences. For transient transfections, UMR 106-01 cells were seeded in six-well plates overnight and then transfected with indicated plasmids using Genejammer (Stratagene) according to the manufacturer's protocol. The next day, the cells were serum-starved and treated with hormones for 4 h. Lysates were analyzed immediately for luciferase activity using the luciferase assay reagent (Promega) and an OptiCompII luminometer (MGM Instruments, Inc., Hamden, CT).

Immunoblotting

Preparation of cell lysates and Western blot analyses were performed as described previously [Swarthout et al., 2002b].

RESULTS

Stimulation of AR Expression by PTH Is PKA-Dependent in Osteoblastic Cells

After binding to its receptor PTH1R, PTH (1–34) stimulates both PKA and PKC pathways in osteoblastic cells [Partridge et al., 1981; Civitelli et al., 1988]. To study which pathway is used to induce AR expression, we took advantage of different peptide fragments of PTH that activate different pathways, and inhibitors and activators of these pathways. As shown in Figure 1A, real-time RT-PCR indicated that rat PTH(1–34, 10^{-8} M) strongly stimulated AR expression 23-fold in UMR 106-01 cells after 1 h of treatment. Human PTH(1–31, 10^{-8} M) (activates PKA, but not PKC) [Jouishomme et al., 1994] retained the ability to induce AR mRNA levels (17-fold). However, the same amount of hPTH(13–34) (activates PKC, but not PKA) [Tyson et al., 1999] had no effect on AR expression. Meanwhile, both the cAMP analog, 8-BrcAMP, and the PKC activator, PMA, by themselves stimulated AR expression to about 19- and 14-fold, respectively, indicating that AR could be regulated by both PKA and PKC pathways. However, in the presence of the PKA inhibitor H-89 [Chijiwa et al., 1990], but not in the presence of the PKC inhibitor GF109203X [Toullec et al., 1991], rPTH(1–34) lost its ability to induce AR expression, suggesting that though AR expression could be activated by PKC pathway, its regulation by PTH is PKA-dependent but not PKC-dependent in osteoblastic cells.

To further investigate this, we injected 4-week old rats with various PTH peptides and then studied AR mRNA levels in the osteoblast-rich femoral metaphyseal primary spongiosa by real-time RT-PCR (Fig. 1B). It is evident that both hPTH(1–38) (activates both PKA and PKC) and hPTH(1–31) have the ability to increase AR mRNA level to 12- and 11-fold, respectively, after 1 h of injection. However, hPTH(3–34) (activates PKC, but not PKA) [Fujimori et al., 1992] had no effect on AR mRNA expression. These data confirm that PTH also regulates AR expression in a PKA-dependent manner in vivo.

AR Promoter Contains a Conserved CRE

The human AR promoter was previously sequenced and a CRE site was identified at −58 to −65, about 30 bp upstream of the TATA box [Plowman et al., 1990]. Note that the numbering of DNA sequence in the present report is different from that previously described because we designate the transcription start site as +1 but the previous publication assigned +1 to the translation start site [Plowman et al., 1990]. Using rat and mouse AR mRNA sequences, we fetched their corresponding promoter sequences from the Genbank database. Alignment of mouse, rat, and human

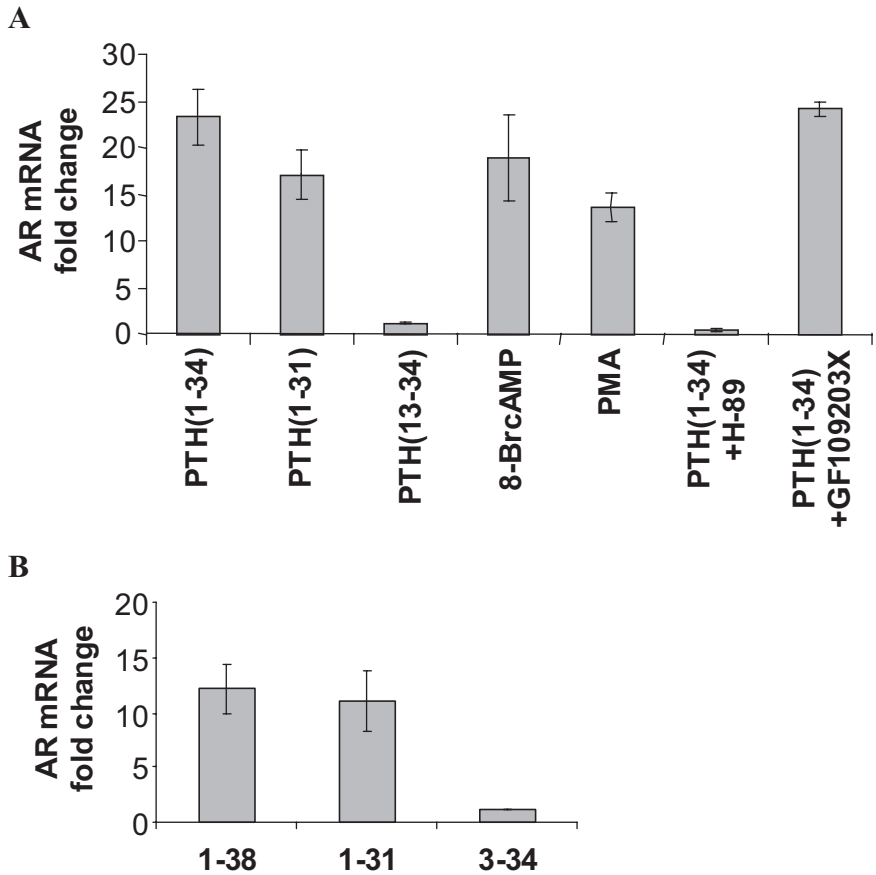

Fig. 1. PTH stimulation of AR expression is PKA-dependent in both an osteoblastic cell line (**A**) and in bone (**B**). A: Serum-starved UMR 106-01 cells were pretreated with the following for 30 min: DMSO (0.1% v/v), 40 μM H-89, or 5 μM GF109203X. Cells were then treated with various 10^{-8} M PTH peptides, 1 mM 8-BrcAMP, or 3 μM PMA as indicated on the X axis, for 1 h before harvesting RNA for real-time RT-PCR analysis to measure the fold change of AR mRNA. B: 4-week old rats were injected subcutaneously with vehicle (0.9% saline solution) or various PTH peptides (8 μg/100 g). The primary spongiosa samples from distal femur were harvested after 1 h and the AR mRNA levels were assayed by real-time RT-PCR.

AR promoters revealed that the CREs are identical in these three species and positioned very similarly (Fig. 2), suggesting that its binding protein, CREB, may play an important role in regulating AR expression.

PTH Regulates AR Expression Through CREB Phosphorylation in Osteoblastic Cells

It is well known that PTH regulates another immediate early gene, *c-fos*, through the PKA and CREB phosphorylation pathway [Tyson et al., 1999]. After PTH treatment, PKA phosphorylates CREB at Ser-133. Phosphorylated CREB binds to the CRE site in the *c-fos* promoter and stimulates its transcription. Our previous studies indicate that PTH regulation of AR expression in osteoblastic cells does not require de novo protein synthesis [Qin et al., 2005]. Moreover, the above data demonstrate that PKA is essential for PTH stimulation of AR and that the AR promoter contains a conserved CRE site. Therefore, we reasoned that the regulation of AR expression by PTH requires CREB phosphorylation.

To test this hypothesis, first we studied the time course of CREB phosphorylation and AR mRNA expression in UMR 106-01 cells after PTH treatment. Consistent with our previous report [Tyson et al., 1999], the phosphorylation of CREB at Ser-133 by PTH is a rapid process, with increased CREB phosphorylation detectable at 5 min, peaking between 30 and 45 min and maintained at a substantially high level until 3 h after PTH addition (Fig. 3A). In comparison, AR mRNA levels peaked between 45 and 60 min and were also maintained at a high level until 3 h (Fig. 3B). It seems that CREB phosphorylation precedes AR mRNA

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mouse      G-------GCTCGATCCCCACTTCCTCCCGCGTCAGCTGGGTTTTTGGAGTAGTAACCTC -102
rat        GTAACAGGGCTCGATCCCCACTTCCTCCCGGATCAGCTGAGTTTT-GGGGTAATAACCTC -114
human      CGGGGCGTGTGTCCTCCGCGCGTGGTTTTCGGGTAGC--ACCTTCTGGGGCG----CCGC  -90
                  *     *** * * *  *        ***     **  ** *      ** *

                                      CRE
mouse      CCGCCTCCACCCAGGGCTGGTCCGTGACGTCACGGGCGGTGGCTCCAGC------GGGTC  -48
rat        CCGCCTCCACCCAGGAGAGG--CGTGACGTCACGGGCGGTGGTTCCCGC------GGGTC  -62
human      CTGCCTCCACCCACGGCCGGGCCTTGACGTCATGGGCTGCGGCCCCCTCCCGGCTGAGCC  -30
           * ********** *   **  * ******** **** * **  **  *      * * *
      TATA box
mouse      TATAAAAGCAGCAGGTGCTCGCCGCACCCTGGATCCCGCTTCCTTCGCTCCCTTCTGACC  +13
rat        TATAAA--------GTGCTCACCGCACCCTCCGCCAAGTTCGCTCTGCTCCTTCCTGACT  -10
human      TATAAA-GCGGCAGGTGCGCGCCGCCCTACAGAC---GTTCGCACACCTGGGTGCCAGCG  +27
           ******        **** * **** *         * *  *    **   * *   *

mouse      CGCGGCCACCGCCCGGACATCTCCAGCTGAGCCTGAGGCGCTGGCCGCTCCAGCCGTCCG  +73
rat        CGCGGCCGCTGCCCTGTCATTTTGAGCCGAGCCCTAGGCGCTGGCAGCTCCAGCCTCCTG  +51
human      CCCCAGAGGTCCCGGGACAGCCCGAG--GCGCC----GCGCCCGCCGCCCCGAGCTCCCC  +81
           * *        **  * **     **  * ***    ****  ** ** **   *  *
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Fig. 2. Alignment of mouse, rat, and human AR promoter sequences. The nucleotide sequences are numbered on the right. The transcriptional start site for the human AR promoter is designated as +1 and marked by underlining forward arrow. The conserved CRE and TATA box are indicated by boxes.

expression, indicating that it could be an upstream event of AR transcription.

Next, we studied this hypothesis using a human AR promoter-luciferase reporter system. As shown in Figure 4A, pGL2A and pGL2C contain different lengths of hAR promoter but both plasmids have the conserved CRE site. When UMR 106-01 cells were transiently transfected with pGL2A, PTH treatment strongly induced the transcriptional activity of AR and resulted in a 16-fold increase in luciferase activity (Fig. 4B). This result confirms our previous conclusion that PTH regulates AR expression at the transcriptional level. Mutation of the conserved CRE in pGL2A (pGL2A mCRE) reduced its PTH response to about 7-fold. To confirm the critical role of the CRE in AR transcription, we co-transfected UMR 106-01 cells with pCMV-KCREB. KCREB is a dominant-negative form of CREB. It has an amino acid substitution within the leucine zipper, which results in KCREB-CREB heterodimers with no DNA binding function [Walton et al., 1992]. Co-expression of KCREB but not

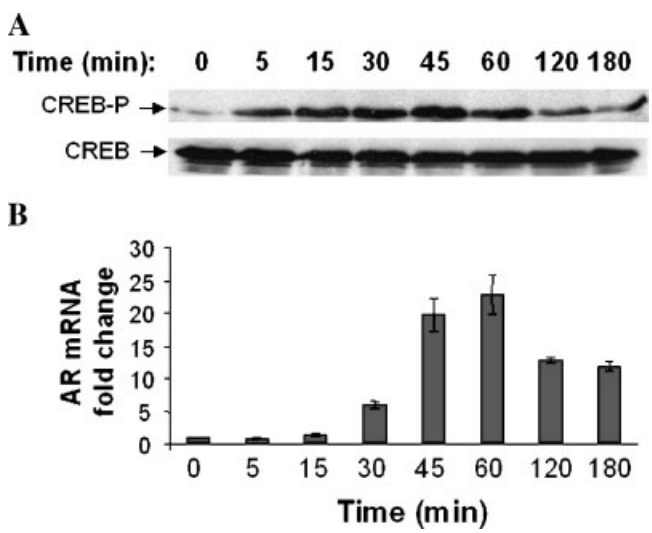

Fig. 3. CREB phosphorylation precedes AR transcription after PTH treatment. Both cell lysates and RNAs were harvested from UMR 106-01 cells at indicated time points after PTH treatment and then subjected to Western blot (**A**) and real-time RT-PCR analysis (**B**).

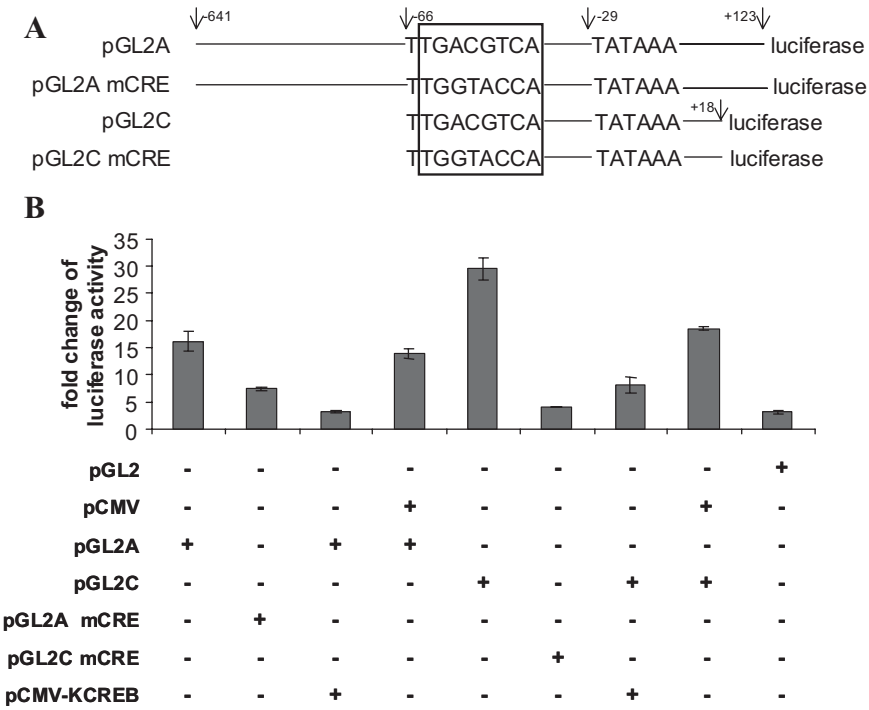

Fig. 4. The CRE in the AR promoter is essential for PTH regulation of AR expression. **A**: A schematic diagram of pGL2 plasmids used in the luciferase assays. **B**: UMR 106-01 cells were transiently transfected with various vectors as indicated and the luciferase activities were measured with or without 4 h of 10^{-8} M rPTH(1–34) treatment. The fold changes in luciferase activity are depicted.

the control vector pCMV greatly reduced the PTH-response of pGL2A, resulting in only a 3-fold increase after PTH treatment. This increase is the same as the PTH-response of pGL2 vector alone (3-fold). It is not known why the pGL2 vector exhibits PTH and PGE_2 responsiveness as shown below. It is interesting to note that pGL2C only contains the CRE site and TATA box of the AR promoter. Similar to pGL2A, it exhibited strong PTH regulation (about 30-fold increase) when transfected into UMR cells (Fig. 4B). Mutation of the CRE site (pGL2C mCRE) almost completely destroyed its PTH-responsiveness, resulting in only a 4-fold increase after PTH treatment. Co-expression of KCREB also reduced the PTH-response of pGL2C to about 8-fold. Taken together, the above data demonstrated that the conserved CRE site plays an important role in PTH regulation of AR expression in UMR 106-01 cells.

CRE Is Essential for Basal AR Expression

The basal luciferase activities of pGL2A, pGL2C, and their CRE mutant vectors in UMR 106-01 cells were measured and adjusted to total lysate protein amounts. Figure 5 showed that pGL2A exhibited high luciferase activity (2×10^4 U/μg). Loss of the CRE site (pGL2A mCRE) resulted in a 10-fold decrease in basal luciferase activity (2×10^3 U/μg, the same as the pGL2 vector), indicating that the basal expression of AR in osteoblastic cells also requires the

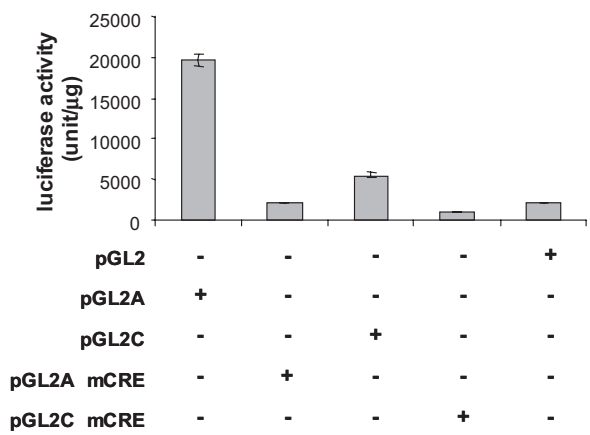

Fig. 5. The CRE in AR promoter is required for AR basal expression. UMR 106-01 cells were transiently transfected with various vectors as indicated and the luciferase activities were measured.

CRE. Studies on pGL2C with the short version of the AR promoter revealed a similar result. The mutated pGL2C mCRE had even lower luciferase activity compared to pGL2C. It is interesting to note that deletion of promoter sequence upstream of the CRE site greatly reduced AR transcriptional activity, as the luciferase activity of pGL2A is about 4-fold higher than that of pGL2C. This result suggests that elements other than the CRE site also contribute to basal AR expression.

Prostaglandin E_2 (PGE_2) Regulation of AR Expression Requires the CRE in Osteoblastic Cells

Our previous study revealed that another osteotropic hormone, PGE_2, quickly and strongly stimulates AR expression in osteoblastic cells [Qin et al., 2005]. Next, we wanted to investigate whether this PGE_2 regulation requires CREB. As shown in Figure 6, both pGL2A and pGL2C in UMR 106-01 cells responded to PGE_2, as their luciferase activities increased about 22- and 27-fold, respectively. Mutation of the conserved CRE site dramatically reduced the fold changes of luciferase activity to 8- and 6-fold, respectively, implying that the CRE site contributes to the PGE_2 regulation of AR. Furthermore, co-transfection with KCREB showed a similar result, as the fold changes of luciferase activities decreased to 4-fold for pGL2A and to 8-fold for pGL2C, providing more evidence that PGE_2 regulates AR expression through the CREB phosphorylation pathway.

DISCUSSION

In this report, we identified the signaling pathways that mediate PTH-induced AR expression in osteoblastic cells. In the osteoblast, PTH activates both PKA and PKC pathways. Using selective pathway agonists and antagonists, we have determined that the stimulation of AR by PTH was cAMP-dependent, since manipulation of the cAMP-PKA signaling pathway alone consistently affected AR expression. Although AR expression could be enhanced by the PKC activator, PMA, manipulation of the PKC pathway had no effect on its mRNA levels after PTH treatment. Furthermore, intermittent injection of PTH peptides confirms that PKA is the main route to regulate AR in vivo. To date, only a few genes, including those encoding IGFBP-5 and TGF-β1, have been found to be regulated by PTH partially or completely through the PKC pathway in osteoblastic cells [Wu and Kumar, 2000; Erclik and Mitchell, 2002]. PTH regulates many more genes, including *c-fos* [Tyson et al., 1999], *collagenase*-3 [Selvamurugan et al., 2000], *TIMP*-2 [Cook et al., 1994], *osteocalcin* [Yu and Chandrasekhar, 1997], *RGS*-2 [Tsingotjidou et al., 2002], *interleukin*-6 [Huang et al., 1998], *RANKL*, *OPG* [Fu et al., 2002], and inducible cAMP early

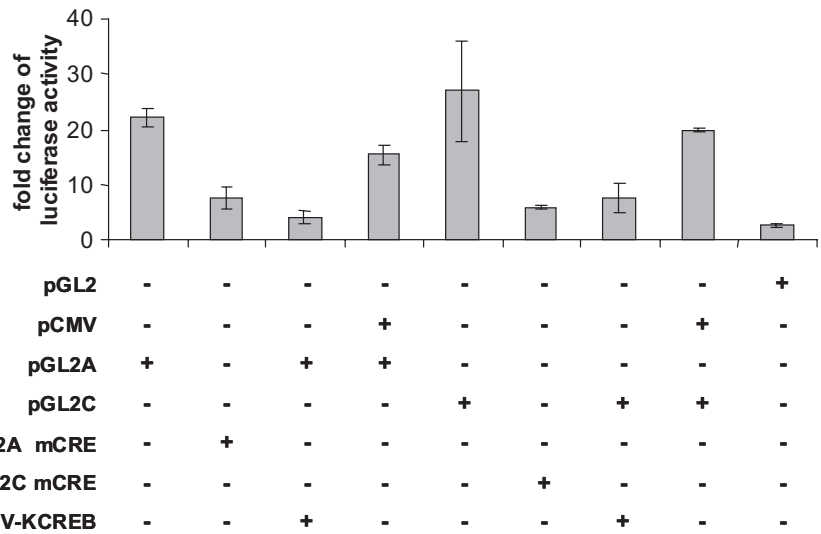

Fig. 6. The CRE in AR promoter is essential for PGE_2 regulation of AR expression. UMR 106-01 cells were transiently transfected with various vectors as indicated and the luciferase activities were measured with or without 4 h of 10^{-6} M PGE_2 treatment. The fold changes in luciferase activity are depicted.

repressor (*ICER*) [Nervina et al., 2003] etc., through the PKA pathway. Our current result provides another example that the main signal transduction pathway utilized by PTH in bone is cAMP-PKA.

CREB belongs to a family of cAMP-responsive activators which also includes cAMP response element modulator (CREM) and activating transcription factor 1 (ATF-1). It was originally identified as a target of the cAMP signaling pathway, yet later studies revealed that it is also a target for other signaling pathways activated by a diverse array of stimuli. All signaling pathways that activate CREB lead to phosphorylation of Ser-133, which is required for CREB-induced gene transcription [review in Shaywitz and Greenberg, 1999]. In PTH signaling, phosphorylation of CREB by PKA is an essential step for *c-fos* stimulation [Tyson et al., 1999]. Interestingly, PTH also strongly stimulates the expression of ICER, a truncated CREM, and a dominant negative repressor of CREB, through the cAMP-PKA pathway in osteoblastic cells [Nervina et al., 2003], providing a feedback mechanism to down-regulate PTH-stimulated signals. Moreover, a computational promoter analysis reveals that the CRE site has a significantly higher occurrence in the promoters of PTH-regulated genes compared to all possible promoters [Qin et al., 2003]. All these data strongly suggest that CREB plays an important role in PTH function. Our current data identify another CREB target in PTH signaling and hence further support the notion that the cAMP-PKA-CREB pathway is one of the major pathways for PTH regulation of immediate early genes in the osteoblast. It would be interesting to study how bone-specific CREB-deficient mice respond to PTH intermittent injections to determine whether it is essential for regulating the genes required for PTH's anabolic functions.

In addition to PTH, we found another osteotropic hormone, PGE_2, which also strongly stimulates AR expression through the CREB phosphorylation pathway. This result is expected as the main signal pathway activated by PGE_2 in osteoblastic cells is the PKA pathway [Kawaguchi et al., 1995]. Recently, another report demonstrated that PGE_2 regulates AR through CREB phosphorylation in colon cancer cells [Shao et al., 2003]. This result is consistent with our findings and further suggests that binding of phosporylated CREB to the conserved CRE site in the promoter is one of the major routes to manipulate AR expression.

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