Vitamin D and Dexamethasone Inversely Regulate Parathyroid Hormone-Induced Regulator of G Protein Signaling-2 Expression in Osteoblast-Like Cells

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The PTH/PTHrP receptor stimulates both adenylate cyclase and phospholipase C-dependent signaling pathways via different G proteins. The biological actions of PTH on bone are modified by steroid hormones. PTH induces expression of regulator of G protein signaling (RGS)-2, a putative preferential inhibitor of Gαi-mediated phospholipase C activation. We investigated whether steroid hormones interfere with PTH signaling by modulating PTH-induced RGS-2 expression in osteoblast-like UMR 106-01 cells. PTH (1–34) rapidly and transiently induced expression of RGS-2 mRNA and protein via the cAMP/protein kinase A pathway within 30 min, with maximal protein abundance after 2 h. PTH-induced RGS-2 preferentially bound to Gαi compared with Gαs protein. 1,25-(OH)2D3 pretreatment enhanced PTH-induced RGS-2 mRNA and protein accumulation, whereas dexamethasone preincubation had an attenuating effect. These effects were due to modulation of the RGS-2 gene transcription rate, which increased by 35% with 1,25-(OH)2D3 and decreased by 63% with dexamethasone pretreatment. RGS-2 mRNA half-life was not affected by either steroid. The transcriptional effects of dexamethasone and 1,25-(OH)2D3 were independent of PTH/PTHrP receptor activation and were not explained by effects on cAMP accumulation, cAMP response element-binding protein expression or phosphorylation, or the abundance of the osteoblast-specific transcription factor core-binding factor α (CBFa1/Runx2), a known activator of RGS-2 expression. In conclusion, glucocorticoids and 1,25-(OH)2D3 inversely modulate PTH-induced RGS-2 gene transcription. Regulation of RGS-2 may constitute a novel mechanism by which steroids modulate signaling via the PTH/PTHrP receptor and other G protein-coupled receptors in bone. (Endocrinology 144: 2496–2504, 2003)
PTH-induced inhibition of osteoblast proliferation in vitro, possibly via stimulation of IGF-1 (22). In addition, estrogen and testosterone inhibit PTH-stimulated osteoclast-like cell formation (23, 24). Pretreatment of human osteoblast-like SaOS-2 cells with androgens inhibited PTH-induced cAMP accumulation (25).

In the work presented here, we tested the hypothesis that steroids might also regulate PTH receptor-mediated signaling by modulating PTH-induced RGS-2 expression.

**Materials and Methods**

**Materials**

Recombinant human PTH (1–34) and bovine PTH (3–34) were purchased from Bachem (Heidelberg, Germany). Actinomycin D, cycloheximide, dexamethasone, dihydrotestosterone (DHT), E2, and forskolin were obtained from Sigma–Aldrich Corp. (Munich, Germany) and 1,25-(OH)₂D₃ from Calbiochem (San Diego, CA). RGS-2 antisera was a gift from Kirk M. Druey (NIH, Bethesda, MD), β-actin monoclonal antibody was obtained from Abcam (Cambridge, UK), and cAMP response element-binding protein (CREB), phospho-CREB, and horseradish peroxidase-conjugated (antirabbit, antimouse) antibodies were purchased from Cell Signaling Technology (Frankfurt, Germany). CBF1, Rgs2, Runx2/AML-3 antibodies (Santa Cruz, Heidelberg, Germany) were kindly provided by J. Hess (German Cancer Research Center, Heidelberg, Germany).

**Cell culture**

UMR 106-01 rat osteoblast-like osteosarcoma cells (kindly provided by David Feldman, Stanford University, Palo Alto, CA.) were grown in 75 cm² cell culture flasks at 37 C in humidified 5% CO₂ atmosphere in MEM (with Earle’s salts) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 10 μmol HEPES. Cells were passaged every 3–4 d, and experiments were performed using cells from passage 15–22. Subconfluent cultures were kept in serum-free medium, with or without addition of effector substances, for 24 h before stimulation with PTH, forskolin, or phorbol-12-myristate-13-acetate (PMA).

**cAMP generation assay**

Cells were plated in 24-well plates and allowed to grow until 80–90% confluence. After 24 h preincubation with indicated substances or serum-free medium, medium was removed and replaced by serum-free medium containing 1 μm 3-isobutyl-1-methylxanthine with/without PTH. After 10 min of incubation, medium was removed, and reaction was stopped by adding 500 μl of 100% ethanol. After incubation at 4 C for 4 h, ethanol was transferred into 1.5-ml tubes and dried under N₂-atmosphere at 37 C. The pellet was dissolved in cAMP buffer and stored at −20 C. A cAMP-RIA was performed with a commercially available kit (Immundiagnostik, Bensheim, Germany). Briefly, 50 μl of sample were mixed with 20 μl ³H-cAMP (50,000 cpm) and 20 μl cAMP-binding protein, centrifuged for 1 min at 1000 × g, and incubated overnight at 4 C. One hundred microliters charcoal were added, incubated for 2 min at room temperature, and centrifuged (15 min 2500 × g). One hundred microliters of the supernatant were mixed with 250 μl scintillation cocktail and analyzed in a β-counter.

**Multiplex RT-PCR**

After incubation with indicated substances, total RNA was isolated using Rnasy mini columns (QIAGEN, Hilden, Germany), checked for integrity on an agarose gel, and quantified photometrically. One microgram total RNA was reverse transcribed using oligo(dT)/random hexamer primers (10:1). Multiplex PCR amplification was performed by using cDNA template, target primer pairs (RGS-2 forward: GGAAGACCCGTTTGAGGCTAC, RGS-2 reverse: TTTCTCTGCTTTGAGGACCAG), and a mixture of 185 cDNA primers/competimers (Ambion, Inc., Austin, TX). Amplification was performed in a GeneAmp PCR System 2400 (Applied Biosystems, Weiterstadt, Germany) using the following protocol: initial 10 min preheating at 95 C (enzyme activation), followed by 30 cycles of 30 sec at 94 C (denaturation), 45 sec at 57 C (annealing), and 30 sec at 72 C (extension). The amplified products (RGS-2: 281 bp; 188: 488 bp) were separated on a 2% agarose gel and visualized by ethidium bromide staining and UV transillumination. The resulting products were used for the linear logarithmic phase of amplification. Routine control reactions performed by omitting reverse transcriptase or cDNA template showed no reaction product. ODs were analyzed using the Molecular Analyst computer program (Bio-Rad Laboratories, Inc., Ville- bert Lourmat, France). Results were normalized with respect to the density of the multiplexed 185 cDNA product.

**Ribonuclease protection assay**

PCR amplification products were cloned into BP bluscript II KS vectors and in vitro transcribed using ³²P-labeled uridine 5-triphosphate. Twenty micrograms (or 50 ng for detection of 18S cRNA) total RNA were hybridized with the radiolabeled antisense riboprobes at 45 C overnight. The free probe was RNase digested followed by proteinase K treatment. After phenolization and ethanol precipitation, hybrids were diluted in RNA loading buffer and separated on a sequencing gel. The gel was dried and exposed to X-ray film. Bands corresponding to 50% and 100% transcribed products were quantified using a Phosphorlmager (Whatman, England). Cells were grown in 6-well plates (Greiner, Germany) and incubated together with an x-ray film overnight in a cassette with intensifying screens at −80 C. Protected bands were quantified densitometrically and normalized against 18S cRNA bands.

**Real-time RT-PCR-based nuclear run-on transcription assay**

Cells from 175-cm² culture flasks were scraped into ice-cold PBS, nuclei were isolated, and assay was performed as described earlier (26). Briefly, cells were lysed in 200 μl buffer A [10 mm HEPES, 1.5 mm MgCl₂, 10 mm KCl, 500 μmol dithiothreitol (DTT), 500 μmol phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin]. Lysate was centrifuged, and the crude nuclear pellet was resuspended in 10 ml ice-cold buffer B (15 mm HEPES, 60 mm KCl, 3 mm/glucose, 100 μm EDTA and EGTA, 1 mm DTT, 50μm spermine, 150 μm spermine, 500 μm PMSF, 2 μg/ml leupeptin, 0.5% Triton X-100). Nuclei were pelleted at 700 × g for 10 min at 4 C, gently resuspended in 10 ml buffer C (15 mm HEPES, 60 mm KCl, 3 mm/ml BSA, 300 μm succrose, 100 μm EDTA and EGTA, 1 mm DTT, 500 μm spermine, 150 μm spermine, 500 μm PMSF, 2 μg/ml leupeptin, and 5 mm MgAc), and repelled again. The final nuclear pellets were resuspended in 230 μl storage buffer (40% glycerol, 75 mm HEPES, 60 mm KCl, 15 mm NaCl, 5 mm MgAc, 100 μm EDTA and EGTA, 1 mm DTT, 500 μm spermine, 150 μm spermine, 2 μg/ml aprotinin and leupeptin), frozen in liquid nitrogen, and stored at −70 C.

To perform nuclear run-on transcription, nuclei were thawed on ice, and 160 μl of the suspension were incubated for 15 min at 22 C with 160 μl of 2× reaction buffer (20% glycerol, 100 mm KCl, 10 mm MgCl₂, 4.5 mm DTT, 1.2 mm ATP, 0.6 mm uridine 5-triphosphate, GTP, CTP, 80 U/ml RNase inhibitor, 500 μm spermidine, and 150 μm spermine). After digestion with Dnase I and proteinase K, total RNA was extracted, quantified, and reverse transcribed as indicated above. To determine the transcription rate of RGS-2, real-time RT-PCR was performed using the Abi Prism 7000 real-time PCR system (Applied Biosystems, Darmstadt, Germany) with specific primers for 18S (forward: ACTTGGTGACGGATTTTG, reverse: CTTTGACCGAGTCTCTTG) and primers specific for nascent, unspliced heterogeneous nuclear RGS-2 RNA (hnRNA) [forward: TTAAGCATCGGTGTTCTGACATGTTA (corresponding to intron 4); reverse: CGAGCCACTTGAGGCTCTTG (corresponding to exon 5)] and Universal Mastermix (PE Applied Biosystems) with SYBR green to detect PCR products at the end of each amplification step. To eliminate interbatch variability, all cDNAs were amplified in a single run. Serial dilutions of an arbitrary cDNA pool were used to establish a standard curve. Relative quantities of RNA levels were determined accounting for amplification efficacy using the software provided with the PCR system. RGS-2 hnRNA levels were normalized to correspond-185 RNA sequences determined within the same run. Reverse transcription controls, in which reverse transcription was omitted, and controls without template did not show a detectable amplification product.
Western immunoblotting

Cells incubated with indicated substances were washed once with cold PBS containing 100 mM Na3VO4, and scraped in 50 μl ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% Triton X) containing a cocktail of proteinase and phosphatase inhibitors (20 mM NaF, 2 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 3 mM benzamidine), vortexed, and centrifuged for 15 min at 15,000 × g. The protein content of the supernatants was measured using the Bradford method (protein assay kit, Bio-Rad Laboratories, Inc., Munich, Germany). Fifty micrograms of protein were separated on polyacrylamide gels at 200 V for 45 min and blotted onto nitrocellulose membranes (105 V, 90 min). After blocking for 1 h in Tris-buffered saline with Tween 20 (10 mM Tris, pH 7.4, 138 mM NaCl, 0.05% Tween-20) containing 5% nonfat dry milk, blots were incubated with primary antibodies at 4°C overnight. Blots were washed three times for 15 min with Tris-buffered saline with Tween 20, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and washed again three times. Immune complexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). Blots were exposed to x-ray films (Kodak, Stuttgart, Germany), and protein bands were quantified densitometrically.

Immunoprecipitation

Cell lysis for immunoprecipitation was performed in 600 μl radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 3 mM benzamidine). Cell lysates were homogenized by passing through a 20-gauge needle and syringe several times and centrifuged at 16,000 × g for 10 min. The supernatant was then incubated with 2 μl of the indicated antibodies on a shaker for 2 h at 4°C. Twenty microliters protein A/G agarose beads were added. After overnight incubation, lysates were centrifuged at 1000 × g for 10 min at 4°C. The pellets were washed four times with PBS, resuspended in electrophoresis buffer, and immunoblotted as described above.

Statistics

All experiments were performed at least three times, and samples were usually run in duplicate to account for technical and biological variability within and between experiments. ANOVA or t tests followed by Newman-Keuls testing were used to compare experimental groups as appropriate. Data are given as mean ± sd.

Results

Regulation of RGS-2 expression by PTH in UMR 106-01 cells

Continuous incubation of the UMR 106-01 cells with PTH induced a dose-dependent, rapid, and transient expression of RGS-2. RGS-2 mRNA and protein both became detectable within 30 min (Fig. 1). The abundance of RGS-2 mRNA and protein was maximal after 1 and 2 h, respectively, and decreased markedly during continued incubation with PTH after 4–6 h. RGS-2 expression dose dependently increased, even at excessively high concentrations (between 10⁻⁷ M and 10⁻⁵ M) (Fig. 2). Using the RGS-2 antiserum established by Sullivan et al. (27), we consistently observed two strong independent protein bands induced by PTH, one at a size of approximately 28 kDa and the second band at about 30 kDa. Previous observations detecting RGS-2 protein in HEK293T cells (27) or rat brain homogenates (28) showed only a single band of about 25–30 kDa. Whether the observed second band is an osteoblast-specific splice variant or a phosphorylated RGS-2 molecule remains to be determined.

The PTH-induced stimulation of RGS-2 mRNA expression was independent of de novo protein synthesis as demonstrated by coinoculation with cycloheximide (data not shown).

In agreement with previous mRNA studies (13, 14), we
were able to demonstrate on the protein level that RGS-2 expression is induced exclusively via the cAMP/PKA signaling pathway in osteoblasts and other osteoblast-like cell lines. PTH-induced RGS-2 accumulation was reduced by 70% by cotreatment with H89, a PKA inhibitor (Fig. 3). In contrast, the PKC activator PMA did not induce RGS-2 expression, and coincubation of PTH with the PKC inhibitor bisindolylmaleimide did not reduce PTH-induced RGS-2 protein abundance. Furthermore, PTH (3–34), a fragment that binds to the PTH receptor and activates the PKC/Ca\(^{2+}\) but not the cAMP/PKA pathway, does not induce RGS-2 expression (Fig. 3).

RGS-2 preferentially binds to G\(_{\alpha}\)q subunits in vivo

Biochemical studies have demonstrated a higher affinity of RGS-2 to G\(_{\alpha}\)q than to G\(_{\alpha}\)s subunits (15). To investigate the G protein-binding specificity and kinetics of RGS-2 in vital PTH-treated UMR 106-01 cells, whole-cell lysates were obtained after incubation with PTH for variable time periods, immunoprecipitated with antibodies against G\(_{\alpha}\)q or G\(_{\alpha}\)s, and immunoblotted with anti-RGS-2 antiserum. The comparison between whole-cell lysates and immunoprecipitates showed effective concentration of the G proteins by the immunoprecipitation step. The G\(_{\alpha}\)q antibody had a higher precipitating efficacy (15-fold) than the G\(_{\alpha}\)s antibody (2.5-fold) (Fig. 4 A).

Effects of dexamethasone and steroid hormones on RGS-2 mRNA and protein abundance

The capability of dexamethasone, 1,25-(OH)\(_2\)D\(_3\), DHT, and E2 to induce RGS-2 expression was investigated by RT-PCR. RGS-2 mRNA expression was not induced directly by incubation with any of these compounds alone (data not shown). To evaluate the possibility that the hormones under investigation might exert priming effects on PTH-induced RGS-2 expression, we next preincubated cells with the individual substances in different concentrations for 24 h before stimulating RGS-2 expression with 10\(^{-7}\) M PTH for 2 h. Pretreatment with 1,25-(OH)\(_2\)D\(_3\) increased PTH-induced RGS-2 expression in a dose-dependent fashion. At the maximally effective 1,25-(OH)\(_2\)D\(_3\) dose of 10\(^{-7}\) M, RGS-2 mRNA was increased by 90% (Fig. 5A) and protein abundance by 43% (Fig. 5B). In contrast, dexamethasone preincubation had a dose-dependent attenuating effect on RGS-2 expression, with a maximal mRNA suppression of 40% observed at 10\(^{-7}\) M (Fig. 5A). The observed modulatory effects of the steroid hormones on PTH-induced RGS-2 expression were fully expressed after 6–48 h of preincubation but were not present when cells were preincubated for 1 h. Preincubation
with DHT and E2 had no effects on PTH-induced RGS-2 expression (data not shown).

Effect of 1,25-(OH)₂D₃ and dexamethasone on forskolin-induced RGS-2 expression

RGS-2 expression was induced not only by PTH but also by forskolin, which stimulates cAMP synthesis independent of PTH receptor activation (Fig. 5B). The possibility that the reciprocal modulation of PTH-induced RGS-2 expression by 1,25-(OH)₂D₃ and dexamethasone might be related to effects at the PTH receptor level was assessed by stimulation with forskolin. Both the stimulating effect of 1,25-(OH)₂D₃ and the attenuating effect of dexamethasone pretreatment on PTH-induced RGS-2 mRNA (data not shown) and protein expres-
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Effect of 1,25-(OH)2D3 and dexamethasone on PTH-induced
RGS-2 mRNA synthesis

RT-PCR-based nuclear run-on assays were performed to investigate the effect of 1,25-(OH)2D3 or dexamethasone pre-treatment on the rate of RGS-2 gene transcription induced by PTH. By using a forward primer corresponding to an intronic sequence of the RGS-2 gene, we measured newly transcribed, unspliced hnRNA. RNA isolated from nuclei not treated with PTH did not show an amplification product of RGS-2 sequence of the RGS-2 gene, we measured newly transcribed, unspliced hnRNA. Preincubation of the cells with 1,25-(OH)2D3 enhanced PTH-induced RGS-2 hnRNA expression by 35%, whereas dexamethasone pretreatment suppressed RGS-2 hnRNA synthesis by 63% (Fig. 6).

Effect of 1,25-(OH)2D3 and dexamethasone on RGS-2
mRNA half-life

RGS-2 mRNA half-life, determined by addition of actino-mycin D after 2 h of PTH incubation (10^{-7} M), was 40 ± 5 min (Fig. 7). Preincubation with 1,25-(OH)2D3 or dexamethasone did not cause significant changes in mRNA half-life [1,25-(OH)2D3: 48 ± 9 min; dexamethasone: 51 ± 12 min].

Effect of 1,25-(OH)2D3 and dexamethasone on PTH-induced
cAMP/PKA/CREB activation

To further elucidate the mechanisms by which dexamethasone and 1,25-(OH)2D3 pretreatment modulate PKA-dependent RGS-2 expression, intermediates of this signaling pathway were assessed. As shown in Fig. 8A, pretreatment with high-dose (10^{-7} M) 1,25-(OH)2D3 for 24 h was associated with a moderate suppression of the cAMP levels induced by 10 min of PTH exposure (85% of control, P < 0.05), whereas high-dose dexamethasone pretreatment had no effect on PTH-induced cAMP accumulation.

Neither the total abundance of CREB nor its PKA-mediated serine phosphorylation on administration of PTH was affected by pretreatment with either compound (Fig. 8B).

Effect of 1,25-(OH)2D3 and dexamethasone on CBFa-1 protein abundance

To further examine the possible mechanism responsible for altered RGS-2 transcription, we investigated the abundance of CBFa-1/Runx2 protein, an osteoblast-specific transcription factor known to bind to the RGS-2 promoter. The abundance of CBFa1 protein in nuclear extracts was not altered by preincubation with either substance (Fig. 9).

Discussion

In this work we further characterized the dynamic induction of RGS-2 by PTH in osteoblast-like cells, demonstrated an inverse modulation of PTH-induced RGS-2 expression by glucocorticoids and vitamin D, and elucidated in part the mechanism of these steroid actions.

We observed a rapid and transient induction of RGS-2 protein expression by PTH evoked by a cAMP/PKA mediated mechanism in UMR 106-01 osteoblast-like cells, confirming and extending to the protein level previous mRNA findings in primary murine and rat osteoblasts and rodent osteoblast-like cell lines (14, 29, 30). These data lend further support to the notion that RGS-2 functions as an immediate-early PTH response gene in bone.

In view of the dual activation of Ga, and Ga,s-mediated signaling pathways by the PTH receptor (3, 31), it was of interest to study whether RGS-2 exhibits binding selectivity to one of these G proteins. In vitro studies have yielded discrepant results: Whereas Heximer et al. (15) observed severalfold higher binding affinity of RGS-2 to Ga,s, Ko et al. (13) observed binding of GST-RGS2 expressed in Escherichia coli bacteria to both G proteins. Thirunavukkarasu et al. (30) found 55% reduction of PTH-induced cAMP accumulation in UMR 106 cells overexpressing RGS-2, but G protein-independent adenylate cyclase stimulation by forskolin had the same effect. The latter finding is compatible with an additional direct effect of RGS-2 on adenylate cyclase (32). Functional effects of RGS-2 on PTH-induced Ga,s-mediated signaling have not been evaluated to date. We used immunoprecipitation to monitor G protein-binding specificity of RGS-2 following stimulation by PTH in vivo. We were able to demonstrate intense binding of RGS-2 protein to Ga,q, which was maximal after 90 min and already decreasing after 2 h of PTH exposure. Binding of RGS-2 to Ga,s appeared much weaker but showed similar temporal dynamics. The observed time course of G protein binding is in keeping with the recent observation that the binding affinity of RGS-2 is highest when G proteins are in the transitional state and diminishes when the G proteins return to their inactive state.
Our results suggest that the interaction of RGS-2 with G proteins is indeed transient and already vanishing when the maximal abundance of RGS-2 protein is reached.

The main aim of the present study was to investigate whether known steroidal effectors of osteoblast metabolism might regulate PTH actions by modulating RGS-2 expression. Whereas RGS-2 expression was not directly induced by dexamethasone or various steroid hormones, pretreatment with 1,25-(OH)2D3 markedly increased and dexamethasone moderately decreased PTH-induced RGS-2 mRNA and protein expression. These effects were specific inasmuch as preincubation with other steroid hormones did not affect PTH-induced RGS-2 expression. 1,25-(OH)2D3 and dexamethasone have been demonstrated to affect the stability of certain mRNA transcripts (19, 33). We ruled out the possibility of posttranscriptional modes of action by actinomycin D chase experiments, which showed no effect of 1,25-(OH)2D3 or dexamethasone on RGS-2 half-life. In subsequent nuclear run-on experiments we were able to demonstrate a clear stimulatory effect of 1,25-(OH)2D3, and an inhibitory effect of dexamethasone, on the rate of RGS-2 gene transcription induced by a given dose of PTH.

The observed reciprocal effects of 1,25-(OH)2D3 and dexamethasone on transcriptional activity of the RGS-2 gene were not mediated by regulation of PTH receptor abundance or function because they were also present when the PTH receptor was bypassed by direct stimulation of adenylate cyclase with forskolin. Moreover, extended exposure to 1,25-(OH)2D3 has previously been shown to suppress, and dexamethasone to stimulate, PTH receptor expression and signaling in osteoblasts (17–20). In concordance with these findings, we observed slightly diminished PTH-induced cAMP levels after 1,25-(OH)2D3 and a slight stimulation with dexamethasone. The consequences of such receptor-related effects, which were of borderline significance with the dose

(13). Our results suggest that the interaction of RGS-2 with G proteins is indeed transient and already vanishing when the maximal abundance of RGS-2 protein is reached.

The main aim of the present study was to investigate whether

FIG. 7. Half-life of RGS-2 mRNA. Cells were incubated with serum-free medium alone (A), 1,25-(OH)2D3 (B), or dexamethasone (C) for 24 h followed by incubation with PTH for 2 h (10−7 M) and treated with actinomycin D (5 mg/liter) for different times. No significant differences were observed between experimental groups.

FIG. 8. Effects of dexamethasone and 1,25-(OH)2D3 preincubation on PTH-induced cAMP accumulation (A) and CREB phosphorylation (B). Cells were grown until subconfluency and preincubated for 24 h with serum-free medium or indicated substances. A. After incubation with PTH for 10 min (10−7 M) in medium containing 3-isobutyl-1-methylxanthine, cAMP was measured as indicated in Materials and Methods. B. Cells were incubated with 10−7 M PTH for 20 and 60 min, and phosphorylation of CREB was determined by Western immunoblot using a phosphorylation site-specific antibody.

FIG. 9. Effects of dexamethasone and 1,25-(OH)2D3 pretreatment on CBFa1/Runx2 protein abundance. Cells were incubated for 24 h with serum-free medium or indicated substances. CBFa1 protein was detected in nuclear extracts by Western immunoblotting.
and exposure time used in our experiments, would, if anything, have resulted in modifications of PTH-dependent gene expression opposite to those observed.

Further downstream the activation pathway, the abundance and PTH-induced phosphorylation of CREB, the major nuclear transcription factor activated by PKA, was found unaltered. This leaves the possibility that transcription factors other than CREB are inversely regulated by either of the two steroids. The recently characterized promoter region of the RGS-2 gene apparently does not contain consensus vitamin D3 or glucocorticoid-responsive elements (30). However, three osteoblast-specific (OSE) elements have been identified that serve as consensus binding sites for CBFA1/Runx2, an essential factor for osteoblast differentiation and function (34, 35). Dexamethasone suppresses CBFA1 protein abundance and DNA-binding activity by a posttranscriptional mechanism in primary rat bone, but not human or mouse, osteoblasts (36, 37). In the UMR 106-01 osteoblast-like cells used here, we observed no change in nuclear CBFA1 protein concentration following dexamethasone incubation. Furthermore, CBFA1 gene and protein expression was found either positively or negatively regulated by 1,25(OH)2D3 in primary human osteoblasts and different rodent osteoblast cell lines, respectively, depending on duration of exposure and the state of differentiation (38, 39). We observed no effect of 1,25-(OH)2D3 exposure for 24 h on nuclear CBFA1 protein abundance in UMR 106–01 cells, in accordance with findings in primary rat osteoblasts (37).

The apparently lacking regulation of CBFA1 on the protein level does not entirely rule out modulating actions of dexamethasone or 1,25-(OH)2D3 because the DNA-binding and transactivating capacity of CBFA1 is also affected by post-translational mechanisms such as protein phosphorylation (40, 41). Besides the OSE2 elements, a CAAT box was identified in the murine, rat, and human RGS-2 promoter sequence. The β and δ isoforms of the CAAT/enhancer-binding protein (C/EBP) family have recently been shown to synergize with CBFA1 in stimulating bone-specific protein expression (42). 1,25-(OH)2D3 induces C/EBP-β and -δ expression in primary rat osteoblasts (42), and glucocorticoids either up- or down-regulate C/EBP isoforms in different cell types (43). In preliminary experiments we observed constitutive, unregulated C/EBP-β and -δ mRNA expression in UMR 106 cells (data not shown) but cannot exclude post-transcriptional regulation of these factors by dexamethasone or 1,25-(OH)2D3. Modulation of translational efficiency, protein–protein interactions, cellular localization, and phosphorylation-mediated changes in DNA-binding activity, activation potential, and nuclear localization have recently been described for several C/EBPs (43, 44). Finally, other as-yet-unidentified nuclear proteins binding to the RGS-2 promoter may be involved in the reciprocal regulation of RGS-2 expression by glucocorticoids and vitamin D.

In summary, we have demonstrated that glucocorticoids and vitamin D inversely regulate PTH-induced RGS-2 expression via a transcriptional mechanism. Taken together with the observed preferential binding of RGS-2 to the Gαq subunit, regulation of RGS-2 constitutes a novel mechanism by which steroids can modulate cellular responses to signals mediated via G protein-coupled receptors.

Acknowledgments

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