Activation of Osteocalcin Transcription Involves Interaction of Protein Kinase A- and Protein Kinase C-dependent Pathways*

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Osteocalcin is a major noncollagenous protein component of bone extracellular matrix, synthesized and secreted exclusively by osteoblastic cells in the late stage of maturation, and is considered indicator of osteoblast differentiation. Osteocalcin expression is modulated by parathyroid hormone (PTH) and a variety of other factors. The cAMP-dependent protein kinase pathway has been shown previously to have an essential role in PTH signaling and regulation of osteocalcin expression. To determine the extent to which other pathways may also participate in osteocalcin expression, we used rat and human osteoblast-like cell lines to generate stably transfected clones in which the osteocalcin promoter was fused to a luciferase reporter gene. These clones were examined for their responsiveness to agents known to activate or interfere with protein kinase A (PKA)- and protein kinase C (PKC)-dependent pathways. We have found that forskolin, cAMP, and PTH, as well as insulin-like growth factor I (IGF-I) and basic fibroblast growth factor, all were effective in activating the osteocalcin promoter. Phorbol 12-myristate 13-acetate (PMA) was also a strong inducer of the promoter, indicating that PKC plays a role in expression of osteocalcin. In combination with PTH or forskolin, the effect of PMA was additive to synergistic. Calphostin C, a selective inhibitor of PKC, decreased the PMA-, PTH-, and IGF-I-induced luciferase activity in a dose-dependent manner; a PKA inhibitor, H-89, also blocked the induction by PTH and IGF-I but not by PMA. We conclude that regulation of osteocalcin transcription is mediated by both PKA-dependent and PKC-dependent mechanisms and that the respective kinases reside on a linear or convergent pathway.

Extracellular matrix proteins play an important role in the organization, architecture, and differentiated function of bone (1). A major component of the matrix is osteocalcin, a 5-kDa γ-carboxyglutamic acid-containing noncollagenous protein of uncertain function; one of its possible roles may be to control nucleation of hydroxyapatite crystals (2). Osteocalcin is synthesized and secreted exclusively by mature osteoblasts during the late stage differentiation and mineralization (1, 3) and, thus, may serve both as a marker of bone formation and as an indicator of the maturation stage of osteoblastic cell populations.

Osteocalcin genes have been cloned from several species including rat, mouse, and human (1, 4, 5). The expression of osteocalcin is regulated by a variety of factors, including parathyroid hormone (PTH) (6), 1,25-dihydroxy vitamin D₃ (4, 7), estrogens (8), glucocorticoids (9), growth factors (10, 11), and cAMP (3, 6, 10). The osteocalcin promoter region contains a number of potential regulatory sequences that may be responsive to these factors. Also present are sequences that could confer species and tissue specificity (12, 13).

Parathyroid hormone is a calcium-regulating peptide that plays a significant role in the maintenance of bone function (14). A once-daily administration of PTH-(1–34) results in increased bone mineral density in normal and osteopenic bone in humans and animals (15). This increase in bone density is correlated with enhanced osteoblast activity, leading to increased bone formation (16). While the mechanism of osteoblast activation by PTH is not fully understood, it is well established that the hormone action on target cells is mediated by binding of PTH to a G-protein-coupled receptor (17). Binding is followed by activation of adenyl cyclase, an increase in cAMP level, and activation of cAMP-dependent protein kinase A (PKA). At the same time, phospholipase C (PLC) may also be activated, leading to the release of Ca²⁺ and induction of a protein kinase C (PKC)-associated pathway (18, 19). Mutations causing inactivation or constitutive activation of PTH receptors are associated with profound genetic defects in humans (20, 21).

Our previous studies have shown that PTH activates osteocalcin transcription, at least in part, via the cAMP-dependent PKA pathway (6). These results are consistent with the presence of cAMP regulatory sequences within the osteocalcin gene promoter (6). However, since this promoter contains additional potential response elements, its activation by PTH may involve additional signal transduction pathways. In this report, we present evidence that activation of PTH-mediated osteocalcin transcription involves both PKA- and PKC-dependent pathways.

EXPERIMENTAL PROCEDURES

Reagents and Media—Rat PTH-(1–38); human PTH-(1–31), PTH-(1–34), PTH-(13–34), PTH-(53–84), and PTH-(1–84); bovine PTH-(3–34) and PTH-(7–34); and human PTH-related protein-(1–34) were obtained from Bachem (Terrance, CA). Stocks of the polypeptides were prepared in a PTH diluent (1 mM HCl, 0.15 M NaCl containing 1 mg of bovine serum albumin/ml). Except where specified, the rat PTH-(1–38) peptide

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The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; OCN, osteocalcin; OCN-luc, osteocalcin promoter fused to luciferase reporter gene; FSK, forskolin; PMA, phorbol 12-myristate 13-acetate; H-89, N-[2-(p-bromocinnamylaminoethyl)-5-isouquinolinesulfonamide; FBS, fetal bovine serum; PKA, cAMP-dependent protein kinase; PLC, phospholipase C; FGF, human basic fibroblast growth factor; IGF-I, human insulin-like growth factor I; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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was used in the experiments described below. Calphostin C, okadaic acid, forskolin (FSK), 1,9-dideoxy-FSK, dibutyryl-3',5'-cAMP, phorbol 12-myristoyl 13-acetate (PMA), and H-89 were obtained from Alexis Corp. (San Diego, CA). The compounds were dissolved in MeSO and diluted as needed with PTH diluent. Human insulin-like growth factor I (IGF-I), basic fibroblast growth factor, neomycin, and G418 were obtained from Sigma; both were prepared in PTH diluent. Media components, Gene- ticin (G418), and fetal bovine serum (FBS) used for cell cultivation were purchased from Life Technologies, Inc.

**Reporter Vectors, Osteocalcin Probe, RNA Isolation, and RT-PCR Analysis of Osteocalcin Gene Expression**—Plasmids containing the rat or human osteocalcin gene promoter fragments as well as the promoterless pGL-2 basic luciferase gene reporter vector (Promega Corp., Madison, WI) as a backbone (6). The rat and the human promoter sequences (1.54 and 0.84 kilobase pairs, respectively) were generated by PCR on the basis of published information (rat promoter, GenBank™ accession number M23637, nucleotides 1–1154; human promoter, M54013, nucleotides 1–844). The PCR fragments were recovered in pCR®TA cloning vector (Invitrogen Corp., Carlsbad, CA), excised with KpnI and XhoI, and subcloned into pGL-2 digested with KpnI and XhoI. These plasmids (pRaOCN-luc and pHuOCN-luc) were used to establish stable luciferase-expressing clones in ROS17/2.8 and SaOS-2 cell lines, respectively.

**Northern Blot and RT-PCR Analysis of Osteocalcin Gene Expression**—A probe was prepared by “spool labeling” of a full-length osteocalcin DNA obtained by PCR from a rat cDNA library generated previously in this laboratory. The RNA for Northern analysis was extracted from ~80% confluent ROS17/2.8 cells using Ultraspec-IT™ solution from Biotec laboratories (Houston, TX) and enriched for the poly(A)−containing fraction by purification on an Oligotex column (Qiagen, Valencia, CA). Manufacturers' instructions were followed in each case. The RNAs were fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with the osteocalcin-specific probe. A 465-base pair fragment of ubiquitin carrier protein DNA produced from a rat cDNA library by PCR was used to normalize the signal. For the RT-PCR experiments, the ROS17/2.8 cells were grown to confluence, starved in serum-free medium containing 0.1% bovine serum albumin, and treated with various forms of PTH. The RNAs were extracted using Ultraspec-IT™, and 5 μg of total RNA were used for first strand cDNA synthesis (SuperScript™; Life Technologies, Inc.). The product (1–2 μl) was amplified by 10 cycles of PCR using primers specific for osteocalcin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Aliquots of PCR products were fractionated on agarose gels, transferred to a nylon membrane, and probed with radiolabeled osteocalcin and GAPDH DNA. Quantitation was carried out by a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Quantitation of Secreted Osteocalcin**—A slightly modified procedure of van Leeuwen et al. (22) was used to follow osteocalcin production. ROS17/2.8 cells were seeded in six-well culture dishes (4.5 × 10⁵ cells/well) in Ham's F-12 medium containing 10% FBS (HyClone, Logan, UT) and 2 mM glutamine and incubated at 37 °C under a 5% CO₂, 95% air (15). We have previously reported that the medium was replaced and the cells were grown to confluence, starved in serum-free medium containing 0.1% bovine serum albumin, and treated with various forms of PTH. The RNAs were extracted using Ultraspec-IT™, and 5 μg of total RNA were used for first strand cDNA synthesis (SuperScript™; Life Technologies, Inc.). The product (1–2 μl) was amplified by 10 cycles of PCR using primers specific for osteocalcin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Aliquots of PCR products were fractionated on agarose gels, transferred to a nylon membrane, and probed with radiolabeled osteocalcin and GAPDH DNA. Quantitation was carried out by a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Establishment of Stable Clones Expressing OCN-Luciferase Activity**—Rat (ROS17/2.8) and human (SaOS-2) osteoblast-like cell lines were used to generate stable transfectants. Initially, cells were transiently transfected with either the OCN reporter vectors (see below) or the promoterless pGL-2 vector control, which established that FSK or PTH-(1–38) was effective in eliciting OCN-luciferase activation. The cells were maintained in Ham's F-12 or Dulbecco's modified Eagle's medium/ F-12 medium (3:1), respectively, supplemented with 2 mM glutamine and 10% FBS. The cells were grown to ~90% confluence, trypsinized, and inoculated into T25 flasks at a density of 5 × 10⁵ cells/flask. After 24 h, the attached cells were transfected with a mixture (1:1 molar ratio) of the reporter vector (pRaOCN-luc or pHuOCN-luc) and pcDNA3.1 plasmid (Invitrogen), carrying a neomycin resistance gene, a total of DNA produced from a rat cDNA library by PCR was used to normalize the signal. MLX microtiter plate luminometer. The luciferase reaction mix (100 μl) was injected sequentially into the wells. The light signals generated in the reactions were integrated over an interval of 2 s, and the resulting luminescence values were used as a measure of luciferase activity (relative units).

**RESULTS**

**PTH Stimulates Osteocalcin mRNA Accumulation and Protein Synthesis**—Initially, we evaluated the time course of OCN induction in response to PTH treatment. This was evaluated by treating ROS17/2.8 cells with PTH-(1–38) and then evaluating either the OCN mRNA (Fig. 1A) or OCN secreted into the medium. Northern blot analysis demonstrates that the level of osteocalcin mRNA began to rise within 1 h of PTH administration and continued to increase for at least 24 h (Fig 1A). The mRNA abundance increased over 4-fold in 24 h as compared with the untreated control.

The effect of PTH on osteocalcin secretion was also confirmed by determination of protein secreted into the medium at various time intervals as well as in response to various concentrations of PTH-(1–38). As shown in Fig. 1 (B and C), osteocalcin levels increased in a time- and dose-dependent manner, with 72 h of PTH treatment showing the best response to PTH-(1–38). Thus, PTH elevates both steady-state osteocalcin mRNA level and the quantity of osteocalcin protein secreted by the cells.

**Effects of Truncated Forms of PTH on OCN Expression**—We next examined the effect of truncated PTH peptides on the level of OCN expression (mRNA abundance and protein secretion). ROS17/2.8 cells were treated with various truncated forms of PTH and the relative abundance of OCN mRNA was measured by RT-PCR, using GAPDH mRNA as an internal control. The results (Fig. 2A) demonstrate that only those PTH fragments (PTH-(1–34), -(1–31), -(1–38), and -(1–84)) that are known to elicit cAMP accumulation (23) stimulated OCN expression. Other peptides (PTH-(3–34), -(13–34), and -(53–84)) that do not bind to PTH receptor and do not elevate cAMP level were ineffective. These mRNA level changes were further confirmed by quantitating the level of OCN secreted into the medium (Fig. 2B).

**Establishment of Stable Geneticin-resistant OCN-Luciferase Transfectant Clones Responsive to PTH**—To determine if PTH elevated its effects at the transcriptional level, we analyzed OCN promoter activity in stable transfecant cell lines generated in ROS17/2.8 and SaOS-2 cells with OCN-luciferase plasmids. Examination of independently and randomly selected geneticin-resistant clones revealed that these clones exhibited a wide variation in both basal luciferase activity and PTH-(1–38) responsiveness (Table I). For subsequent experiments, we chose two rat clones (RG15 and RG24) and two human clones.
(SG12 and SG40) that consistently maintained both basal and PTH-inducible luciferase expression.

**PTH Time and Dose Response of OCN-luc in Stable Transfectant Lines**—Both ROS17/2.8 and SaOS-2 transfectants exhibited time-dependent induction of reporter gene activity by PTH-(1–38) (Fig. 3), with the highest level of luciferase activity observed at about 8 h (4–30-fold induction, depending on the clone). These results are in agreement with our previous observations on transiently transfected SaOS-2 cells (6). The PTH dose response was also examined in the four stable clones (Fig. 4). The four cell lines show a similar pattern of activation by the hormone, being half-maximally stimulated at PTH concentrations of 7.5 × 10⁻²⁸ to 5 × 10⁻¹⁰ M and reaching a maximum level at 5 × 10⁻⁷ to 5 × 10⁻⁵ M. The activity in clone RG15 showed no leveling off even at the highest dose (5 × 10⁻⁵ M) of PTH used in this experiment.

**Activation of Osteocalcin Promoter-driven Luciferase Gene by Various Truncated Forms of PTH**—We next confirmed that in stable cell lines, distinct domains of PTH evoked specific OCN responses (Refs. 6 and 23; Fig. 2). The PTH analogs PTH-(1–31) and PTH-(1–38), which are known to raise intracellular cAMP, were used for these experiments. The analogs were added at various concentrations to ROS17/2.8 cells, and the OCN promoter activity was measured by luciferase assay. The results showed that PTH-(1–31) was a more potent activator than PTH-(1–38), with a half-maximal activation at 5 × 10⁻²⁸ M and a maximum at 5 × 10⁻⁷ M. In contrast, PTH-(1–38) showed a half-maximal activation at 5 × 10⁻¹⁰ M and a maximum at 5 × 10⁻⁵ M.
concentration and stimulate the PKA pathway (6, 23), were also able to stimulate reporter gene activity in the stable transfectant clones (Fig. 5). A truncated PTH-related protein, PTHrP-(1–34), was nearly as active as PTH-(1–38). In contrast, PTH-(3–34) and PTH-(7–34), the analogues incapable of elevating intracellular cAMP level (23) were unable, at the concentration used, to activate OCN-luc. These results are consistent with the known involvement of PKA in PTH-induced osteocalcin expression. Both rat- and human osteosarcoma-derived stable clones responded similarly in this test.

Treatments That Increase Intracellular Accumulation of cAMP Enhance OCN Transcription—To further evaluate the involvement of the PKA pathway in PTH-induced OCN-luc expression in stably transfected cells, we examined the effect of various concentrations of FSK, a well known activator of adenyl cyclase (6, 24), and dibutyryl cAMP. As shown in Fig. 6A, SaOS-2 cells exposed to dibutyryl cAMP (10⁻⁶ to 10⁻³ M), or FSK (10⁻⁷ to 10⁻⁵ M), demonstrated a concentration-dependent increase in OCN-luc expression. We also evaluated whether treatment with these agents resulted in enhanced OCN protein secretion. The results (Fig. 6B) show that PTH, FSK, and cAMP treatment of ROS 17/2.8 cells resulted in a 5–7-fold stimulation of OCN secretion during the 72-h treatment period. We also evaluated OCN-luc expression in the presence of H-89, a selective inhibitor of the PKA pathway. The results (Fig. 7) of a transient transfection experiment using a single FSK concentration (10⁻⁵ M) and various concentrations of H-89 (0.1–10 μM) indicate a concentration-dependent inhibition of FSK-stimulated OCN-luc expression by H-89. Collectively, these results further confirm that activation of the PKA pathway leads to activation of OCN expression.

Phorbol 12-Myristate 13-Acetate Is a Potent Activator of Osteocalcin Promoter—Because the PTH receptor can signal through phospholipase C and thus, indirectly, through the PKC pathway (18, 19), we asked whether activators of PKC could stimulate OCN transcription. As shown in Fig. 8, when SG12 cells were treated with PMA, the expression of OCN-luc was significantly stimulated (28-fold increase over control level). A combination of PTH and PMA or of FSK and PMA was at least additive, if not synergistic (136- and 166-fold increase, respectively).

TABLE I

| Parent cell lines/ clones | Basal activity | Activity with PTH | Stimulation |
|---------------------------|----------------|-------------------|-------------|
|                           | 5 x 10⁻⁶ μ | 10⁻³ M | |
| ROS17/2.8                 |               |                   |             |
| RG2                       | 1              | 2                 | 2.0         |
| RG4                       | 3              | 13                | 4.3         |
| RG5                       | 0.1            | 1                 | 10.0        |
| RG9                       | 50             | 190               | 3.8         |
| RG10                      | 23             | 34                | 1.5         |
| RG12                      | 332            | 848               | 2.6         |
| RG15                      | 39             | 285               | 7.3         |
| RG23                      | 74             | 362               | 4.9         |
| RG24                      | 1              | 2                 | 22.0        |
| SaOS-2                    |               |                   |             |
| SG5                       | 17             | 32                | 1.9         |
| SG11                      | 3              | 6                 | 2.0         |
| SG12                      | 12             | 158               | 13.2        |
| SG24                      | 0.1            | 2                 | 20.0        |
| SG40                      | 4              | 15                | 3.8         |

FIG. 4. Effect of various concentrations of PTH-(1–38) on OCN-luc activity. Cells in microtiter dishes were treated with indicated amounts of PTH for 8 h, lysed, and assayed for expression of luciferase activity as described above.

FIG. 5. Effect of different analogues of PTH on OCN-luc activity. Each analogue was added at 5 x 10⁻⁶ μ for 8 h, exposed to the peptides for 8 h.
Sensitive, as compared with PTH alone (63-fold) and FSK alone (45-fold)). Very similar results were obtained with SaOS-2 cells transiently transfected with OCN-luc plasmid (not shown).

Independent Activation of OCN Transcription by PTH and PMA—To determine whether PTH and PMA can act through independent pathways to activate osteocalcin transcription, we asked if cells pretreated with one activator are able to respond to a subsequent challenge with the same or the other agent.

SG12 cells were incubated with either PTH or PMA for 12 h and then challenged with either PMA or PTH for additional 8 h. For comparison, the standard 8-h treatment and continuous 20-h exposure were also evaluated. The results (Fig. 9) show that cells exposed to PTH or PMA remained responsive to a subsequent challenge with PMA or PTH, respectively, although the amplitude of response was different. While the long term exposure to PMA significantly diminished response to either agent, the desensitization or down-regulation of the PKC-dependent pathway reduced but, importantly, did not abrogate the PTH-dependent signaling. This indicates that desensitization of one pathway still allows the other pathway to mediate OCN transcription.

Involvement of Protein Kinase C in PTH Activation of OCN Promoter—To obtain further confirmation of the involvement of protein kinases in PTH signaling, we have analyzed the SG12 clone responses to a selective protein kinase C inhibitor, calphostin C (25). As shown in Fig. 10A, calphostin C inhibited the PTH-inducible OCN-luc expression in a dose-dependent fashion. The compound was also effective in abolishing PMA-stimulated OCN-luc expression, indicating a major role for the PKC pathway in regulating osteocalcin transcription. The known PKA inhibitor, H-89 (26), which inhibited the PTH-induced expression (6), had no effect on PMA-induced OCN-luc expression (Fig. 10B).

In order to further define whether or not both the PKA and PKC pathways are activated independently, we evaluated the effects of H-89 or calphostin C on OCN mRNA levels after stimulation with either PTH or cAMP. The results (Fig. 11) show that a 24-h PTH treatment resulted in a 4.9-fold increase in OCN mRNA level (lane 2), while H-89 co-treatment resulted in at least a partial reduction in OCN mRNA levels (2.9- and 2.4-fold, respectively, in comparison with control). A treatment with cAMP resulted in a 3.5-fold increase in OCN mRNA level (lane 5), while H-89 co-treatment...
Insulin-like Growth Factor I Stimulates OCN-luc Transcription—IGF-I, an important effector of bone remodeling, has been shown to up-regulate osteocalcin synthesis in osteosarcoma cells (27). Furthermore, its activity is enhanced by PTH and cAMP and is down-regulated by estrogen (28). Therefore, we determined if IGF-I stimulated OCN-luc expression and if this growth factor acted through PKA- or PKC-dependent pathways, which are primary, if not exclusively, PKA pathway, and both of the pathways act independently of each other.

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mRNA levels (Fig. 11). Finally, forskolin, a non-specific activator of adenyl cyclase, stimulated OCN-luc expression in both ROS17/2.8 and SaOS-2-derived clones (RG15 and SG12, respectively). A forskolin analog, 1,9-dideoxyforskolin, that does not activate adenyl cyclase had no effect on OCN-luciferase expression (results not shown).

In osteoblasts, PTH acts through a cognate G-protein-coupled seven-transmembrane domain receptor (PTHR1) that recognizes both PTH and PTHrP (30). The signal from the receptor is transmitted through the participation of both PKA and PLC pathways. PKA activation has been demonstrated in both normal and osteoblast-like cells, while PLC activation, as measured by calcium release and inositol 1,4,5-trisphosphate production, could only be shown in cells stably transfected to express a high copy number of the PTH receptor gene (31–33). These observations leave uncertain the relative contribution of the PLC and phosphoinositol pathway to PTH signaling, but they do suggest a possible role. Importantly, calcium release could be demonstrated in the absence of cAMP production (31).

PLC activation leads to generation of inositol 1,4,5-trisphosphate and diacylglycerol, in turn leading to Ca²⁺ mobilization and activation of PKC. We evaluated activators of PKC (PMA, IGF-I, and FGF) for their ability to modulate OCN-luciferase expression in osteosarcoma cells. The data indicate that PMA was able to activate OCN independently of the PKA-mediated mechanism. Several pieces of evidence point to the importance of PKC in effecting osteocalcin expression. First, PMA actions were at least additive or synergistic with those of PTH or FSK (Fig. 8). Second, H-89 treatment did not abolish stimulation by PMA (Fig. 10B), showing that activation of PKC can activate osteocalcin promoter activity independently of the PKA pathway. Third, cells desensitized to PTH-(1–38) by an extended treatment with the hormone were still able to respond robustly to PMA stimulation and vice versa (Fig. 8). Finally, the growth factors (IGF-I and FGF), known for their actions through both PKA- and PKC-dependent pathways, strongly stimulated OCN-luc expression in a manner additive with PTH (Fig. 12 and data not shown).

The experiments with protein kinase inhibitors (H-89 and calphostin C) revealed that each of the inhibitors could block activation of the OCN promoter. Thus, calphostin C, a highly selective inhibitor of protein kinase C (34), was able to abolish OCN-luc transcription elicited by either PTH or PMA (Figs. 10 and 11) or IGF-I (Fig. 12) but was ineffective in blocking cAMP-stimulated OCN expression (Fig. 11). On the other hand, a PKA inhibitor, H-89, while strongly diminishing OCN-luc expression when PTH, cAMP, FSK, or IGF-I (Figs. 7, 11, and 12) was the inducing agent, had no effect on PMA-induced OCN-luc expression (Fig. 9). Collectively, these results suggest that both PKA and PKC activation pathways are involved in PTH signaling. However, it is not clear whether these activation steps are independent of or interdependent on each other. Most of the evidence suggests that they act independently. First, H-89 blocks cAMP- or PTH-stimulated, but not PMA-stimulated, OCN transcription. Second, calphostin C blocks PMA- or PTH-
stimulated, but not cAMP-stimulated, OCN transcription. Thus, the simplest interpretation of these data is that activation of either of the pathways leads to OCN transcription, and PTH activation of OCN utilizes both of the pathways. However, an additional possibility is that although the initial signals generated by PTH and PMA differ, the PTH-dependent and PKC-dependent transduction pathways converge on an element common to both (e.g. adenyl cyclase) that serves to transmit the signals necessary to promote expression of the osteocalcin gene. This notion is consistent with the well-documented ability of G-proteins, PKA, and PKC to activate differentially various isoforms of adenyl cyclase (35). The synergy between PTH and PMA and between PMA and FSK (Fig. 8) is also consistent with such an interpretation.

The extent to which PTH signals via PKC remains an open question. However, it is quite possible that factors that elicit PKC signaling (such as IGF-1 and FGF-2) may work in concert with PTH to enhance OCN transcription. Taken together, our data point to the existence of multiple regulatory circuits involved in transcriptional control of osteocalcin expression. Which of these circuits is also responsible for the temporal and tissue-specific pattern of osteocalcin biosynthesis remains to be elucidated.

The role of OCN in bone mineralization is not clear. OCN is produced by osteoblasts during a late stage of maturation. Recent study suggests that OCN knockout animals may develop higher bone density, suggesting that it may serve to inhibit mineralization (36). PTH promotes osteoblast differentiation in vitro and also causes an increase in OCN mRNA levels (16). Bone formation and maturation are complex processes and clearly involve several steps in tandem (12). They could involve an initial expansion of osteoblasts, followed by formation of early osteoid matrix. Once an appropriate amount of matrix is laid down, it is certainly possible that mineralization could proceed utilizing enzymes such as alkaline phosphatase or matrix proteins such as bone sialoproteins. If OCN is indeed a true inhibitor of mineralization, it may serve as a termination signal for bone formation once the bone matrix is fully matured (37). PTH may play a role in all aspects of the process, including OCN production.

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