The role of protein kinase C-δ in PTH stimulation of IGF-binding protein-5 mRNA in UMR-106–01 cells

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Erclik, Mary S., and Jane Mitchell. The role of protein kinase C-δ in PTH stimulation of IGF-binding protein-5 mRNA in UMR-106–01 cells. Am J Physiol Endocrinol Metab 282: E534–E541, 2002.—We have investigated the role of protein kinase C (PKC) signal transduction pathways in parathyroid hormone (PTH) regulation of insulin-like growth factor-binding protein-5 (IGFBP-5) gene expression in the rat osteoblast-like cell line UMR-106–01. Involvement of the PKC pathway was determined by the findings that bisindolylmaleimide I inhibited 40% of the PTH effect, and 1 μM bovine PTH-(3–34) stimulated a 10-fold induction of IGFBP-5 mRNA. PTH-(1–34) and PTH-(3–34) (100 nM) both stimulated PKC-δ translocation from the membrane to the nuclear fraction. Rottlerin, a PKC-δ-specific inhibitor, and a dominant negative mutant of PKC-δ were both able to significantly inhibit PTH-(1–34) and PTH-(3–34) induction of IGFBP-5 mRNA, suggesting a stimulatory role for PKC-δ in the effects of PTH. Phorbol 12-myristate 13-acetate (PMA) stimulated PKC-α translocation from the cytosol to the membrane and inhibited ~50% of the PTH-(1–34), forskolin, and 8-bromoadenosine 3',5'-cyclic monophosphate-stimulated IGFBP-5 mRNA levels, suggesting that PKC-α negatively regulates protein kinase A (PKA)-mediated induction of IGFBP-5 mRNA. These results suggest that the induction of IGFBP-5 by PTH is both PKA and PKC dependent and PKC-δ is the primary mediator of the effects of PTH via the PKC pathway.

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have used the well characterized osteoblastic UMR-106–01 cell line that we and others have used extensively to study osteoblast regulation (22, 33, 34). IGFBP-5 mRNA levels are extremely low in this cell line in the basal state and are increased by stimulation with PTH. We report that PTH selectively regulates the cellular distribution of PKC-δ and that its activation, along with PKA, mediates PTH induction of IGFBP-5 mRNA.

MATERIALS AND METHODS

Cell culture. UMR-106–01 cells (a generous gift from Dr. N. Partridge, St. Louis University, St. Louis, MO) were grown in 50% Dulbecco’s modified Eagle’s medium and 50% Eagle’s medium containing 1 U/ml penicillin, 1 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and supplemented with 5% fetal calf serum (Life Technologies, Burlington, ON, Canada).

Materials. Rat PTH-(1–34), bovine PTH-(1–84), human PTH-(1–31), and bovine PTH-(3–34) were purchased from Bachem Bioscience (King of Prussia, PA). Bisindolylmaleimide I, rottlerin, phorbol 12-myristate 13-acetate (PMA), H-89, forskolin, and 8-bromoadenosine 3’,5’-cyclic monophosphate (8-BrcAMP) were purchased from Biomol (Plymouth Meeting, PA). Isozyme-specific PKC antibodies were purchased from BD Transduction Laboratories (Mississauga, ON, Canada).

Cell treatment and cellular fractionation. After 20–24 h of serum starvation, cells were treated with PTH analogs or PMA (in DMSO) in serum-free media containing 0.1% BSA for 30 min. Cytosol, membrane, and nuclear fractions of cells were prepared by previously described procedures with minor modifications (10). Briefly, cells were washed once with cold PBS and scraped from the plate on ice with 5% fetal calf serum (Life Technologies, Burlington, ON, Canada). Two micrograms of DNA and 8 μl of transfection reagent with the plasmid pSV-β-gal (Promega, Madison, WI), which encodes the reporter gene β-galactosidase, were transiently transfected into each well of a 6-well plate over 48 h and transfected with DNPKC-ε, DNPKC-κ, or vector alone (pcDNA 3.1 +, Invitrogen) using Lipofectamine reagent (Life Technologies, Burlington, ON, Canada). Each well was transfected with 8 μl of DNA and 8 μl of transfection reagent used for each well in serum-free media. After a 24-h incubation, cells were treated with PTH or other agents for an additional 6 h, and then RNA was isolated and hybridized under the same conditions with a 950-bp GAPDH cDNA probe, obtained by digesting GAPDH cDNA (kindly provided by Dr. S. Shimasaki, The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA) with HindIII and SacII (31). The probe was labeled with [α-32P]dCTP by use of the random hexanucleotide-primed method (7). Hybridizations were carried out in the prehybridization solution overnight at 65°C, and washes were performed at 65°C in 30 mM sodium phosphate buffer containing 0.1% SDS. As an internal control, parallel blots were probed and hybridized under the same conditions with a 500-bp GAPDH cDNA probe, obtained by digesting GAPDH cDNA with SfiI and BglII, as previously described (8). Bound RNA was visualized by autoradiography on Kodak X-AR5 film. Signals were quantitated from a phosphorimager using ImageQuant.

Transient transfections. The mouse dominant negative PKC-δ K376R (DNPKC-δ), and PKC-ε K376R (DNPKC-ε) constructs were kindly provided by Dr. I. B. Weinstein (Columbia University, New York, NY) (32). Cells were grown to 60–70% confluence in 6-well plates over 48 h and transfected with DNPKC-δ, DNPKC-ε, or vector alone (pcDNA 3.1 +) with Lipofectamine reagent (Life Technologies, Burlington, ON, Canada). Two micrograms of DNA and 8 μl of transfection reagent were used for each well in serum-free media. After a 24-h incubation, cells were treated with PTH or other agents for an additional 6 h, and then RNA was isolated and assessed as outlined. Transfection rates of ~30% were found using this protocol and were assessed as follows. Cells were transfected with the plasmid pSV-β-gal (Promega, Madison, WI), which encodes the reporter gene β-galactosidase. Twenty-four hours after transfection, cells were fixed in 3% formaldehyde (in PBS) for 15 min at room temperature, washed three times with PBS, and then stained with X-gal-containing solution (in PBS: 4 mM K+ ferrocyanide, 4 mM K+ ferricyanide, 4 mM MgCl2, and 0.4 mg/ml X-gal in dimethyl sulfoxide) for 2 h at 37°C. Transfection rates were determined as the percentage of blue-stained cells compared with the total number of cells.

Presentation of data and statistical analysis. The slot blots presented are representative of at least three experiments. The quantitated values of IGFBP-5 mRNA obtained from individual samples were corrected for GAPDH. Statistical analysis was performed by Student’s t-test. The results are expressed as means ± SD.
RESULTS

Mechanisms of PTH-mediated induction of IGFBP-5 mRNA. Previous studies suggested that part of the induction by PTH of IGFBP-5 mRNA in osteoblasts occurs through pathways other than PKA; however, the involvement of the PKC pathway has not been investigated. We utilized PTH fragments that selectively stimulate the PKC or PKA-plus-PKC pathways to determine their relative contribution to PTH effects on IGFBP-5 transcript. As demonstrated in Fig. 1A, under basal conditions, there were very low levels of IGFBP-5 transcript as assessed by phosphorimaging. PTH-(1–34), PTH-(1–84), and PTH-(1–31), each of which can stimulate PKA and PKC, all stimulated a 30- to 45-fold induction of IGFBP-5 mRNA levels. PTH-(3–34), an analog that has been shown to stimulate PKC but not PKA, stimulated a 5- to 20-fold induction of IGFBP-5, suggesting that PKC activation mediates part of PTH stimulation of IGFBP-5 transcript levels.

To further characterize the role of the PKC pathway in PTH induction of IGFBP-5 transcript, cells were treated with either PTH-(1–34) alone or the selective PKA and PKC inhibitors H-89 and bisindolylmaleimide I, respectively. Both inhibitors on their own had no effect on IGFBP-5 transcript levels (data not shown); however, 20 μM H-89 reduced PTH stimulation of IGFBP-5 mRNA from 45-fold to just 17-fold. When cells were cotreated with PTH-(1–34) and 5 μM bisindolylmaleimide I, PTH induction of IGFBP-5 was significantly reduced to just under 30-fold. Figure 1B shows that selective activation of adenylyl cyclase with forskolin resulted in a 25-fold induction of IGFBP-5 transcript levels, and this was inhibited in the presence of 20 μM H-89 to only fourfold (data not shown). Activation of PKC with 1 μM PMA, however, had no effect (Fig. 1B).

Effects of PTH-(1–34), PTH-(3–34), and PMA on PKC isozyme cellular distribution. Our initial findings suggested that IGFBP-5 mRNA is stimulated by PTH partially via the PKC pathway. However, direct activation of PKC by PMA had no effect. Because there are 11 members of the PKC family and only a subset of these is stimulated by PMA, we wished to determine which isozymes were activated by PTH or PMA.

Activated PKC isozymes have been shown to translocate from one intracellular location to another (26). Therefore, translocation can be used to identify which isozyme(s) could mediate the PTH downstream effects. Cytosol, membrane, and nuclear fractions of UMR-106–01 cells that were untreated (control) or incubated with PTH-(1–34), PTH-(3–34), or PMA were prepared, and the quantity of PKC isozymes present in each fraction was assessed by immunoblotting with isozyme-specific antibodies. Figure 2 demonstrates the distribution of representatives of each of the PKC subfamilies (PKC-α, classical, PKC-ε and PKC-δ, novel, and PKC-γ, atypical). Quantitative analysis of the translocation of each PKC isozyme is shown in Table 1.

PKC-α migrates at an approximate molecular mass of 80 kDa and is predominantly found in the cytosol in resting cells. PTH-(1–34) or PTH-(3–34) failed to stimulate any change in the quantity or distribution of PKC-α. However, incubation with 1 μM PMA for 30 min stimulated the translocation of 54% of PKC-α from the cytosol to the membrane and 17% to the nuclear fractions. This effect was seen as early as 10 min after stimulation and was sustained for almost 1 h (data not shown).

PKC-δ was predominantly present in the membrane fraction and was identified as a doublet migrating at 75 kDa, whereas PKC-ε was localized in the cytosol and
membrane fractions in resting cells and migrated also as a doublet at an approximate molecular mass of 90 kDa (Fig. 2B). The appearance of these two PKC isozymes as doublets has been noted previously and may result from the presence of both phosphorylated and unphosphorylated forms of the proteins (17). PTH-(1–34) stimulated the translocation of 45% of PKC-δ from the membrane to the nuclear fraction, leaving only 13% of the isozyme in the membrane. Likewise, PTH-(3–34) was able to stimulate PKC-δ translocation to the nucleus. PMA did not affect PKC-δ translocation; however, it did reduce the abundance of this isozyme by ~60% after a 30-min incubation. Downregulation of PKC-δ was seen as early as 20 min after addition of PMA and was sustained for at least 1 h (data not shown). PTH-(1–34), PTH-(3–34), and PMA all had no effect on the distribution of the second of the two novel PKC isozymes, PKC-ε; however, PTH-(1–34) did decrease the total amount of PKC-ε by ~40%.

The atypical PKC isozyme PKC-ε was predominantly localized to the cytosol and membrane fractions in resting cells and migrated at an approximate molecular mass of 65 kDa. Neither PTH-(1–34) nor PMA had any effect on the subcellular distribution or amount of this isozyme (Fig. 2C).

Further examination of the time course of PTH-mediated translocation of PKC-δ demonstrated that, within 10 min of incubation with PTH-(1–34), PKC-δ began to translocate to the nucleus (Fig. 3). PKC-δ levels in the nucleus were maximal after 20 min, when 53% of this isozyme was associated with the nuclear fraction, and this was sustained for the next 10 min. After 40–50 min of incubation with PTH, PKC-δ began to move back to the membrane, returning to the basal distribution after 50 min (Fig. 3). Over this 50-min time course of PTH stimulation, the distribution of PKC-α and PKC-ε was unchanged (Fig. 3).

Involvement of PKC-δ in PTH-mediated stimulation of IGFBP-5 mRNA. Having established the ability of PTH to regulate the cellular distribution of PKC-δ, we next investigated whether this isozyme mediated part of the PTH induction of IGFBP-5 transcript. Cells were treated with PTH-(1–34) or PTH-(3–34) alone or in the presence of the PKC-δ specific inhibitor rottlerin. When administered alone, rottlerin had no effect on IGFBP-5 mRNA levels (data not shown). Increasing concentrations of rottlerin progressively suppressed PTH-(1–34) induction of IGFBP-5, such that 5 μM rottlerin inhibited ~45% of the PTH signal (Fig. 4A).

### Table 1. Quantitative Analysis of PKC Isozyme Translocation in Response to PMA, PTH-(1–34), and PTH-(3–34)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Subcellular Fraction</th>
<th>PKC-α</th>
<th>PKC-δ</th>
<th>PKC-ε</th>
<th>PKC-ε</th>
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<tbody>
<tr>
<td>PMA</td>
<td>Membrane</td>
<td>6</td>
<td>114</td>
<td>90</td>
<td>131</td>
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<tr>
<td></td>
<td>Nucleus</td>
<td>7</td>
<td>21</td>
<td>23</td>
<td>91</td>
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<tr>
<td>Control</td>
<td>Cytosol</td>
<td>11</td>
<td>8</td>
<td>13</td>
<td>91</td>
</tr>
<tr>
<td>Treated</td>
<td>Membrane</td>
<td>51</td>
<td>39</td>
<td>83</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Nucleus</td>
<td>21</td>
<td>23</td>
<td>45</td>
<td>68</td>
</tr>
<tr>
<td>PTH-(1–34)</td>
<td>Cytosol</td>
<td>140</td>
<td>39</td>
<td>50</td>
<td>59</td>
</tr>
<tr>
<td>Control</td>
<td>Membrane</td>
<td>2</td>
<td>146</td>
<td>48</td>
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<tr>
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<td>Nucleus</td>
<td>8</td>
<td>16</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>Treated</td>
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<td>PTH-(3–34)</td>
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<td>140</td>
<td>39</td>
<td>50</td>
<td>ND</td>
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Quantitative evaluation of data, expressed in densitometry units, outlined in Fig. 2. PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTH, parathyroid hormone; ND, not determined.
Rottlerin was similarly able to inhibit PTH-(3–34) induction of IGFBP-5 mRNA (Fig. 4B).

To further substantiate the involvement of PKC-δ in mediating PTH effects on IGFBP-5 transcript levels, PKC-δ activity was inhibited by a dominant negative mutant. The K376R mutation has been shown to eliminate the function of PKC-δ and is able to function as a dominant negative when transfected into cultured cells (19). DNPKC-δ or vector alone (pcDNA3.1) was transfected into UMR-106–01 cells, and 24 h later, cells were treated with PTH-(1–34) or PTH-(3–34). As shown in Fig. 5A, DNPKC-δ was able to inhibit the induction of IGFBP-5 stimulated by 10 nM PTH-(1–34) by ~30% compared with cells transfected with vector alone. When cells were transfected with DNPKC-ε, which also contained the K376R mutation and has previously been shown to act as a potent dominant negative mutant (32), the PTH induction of IGFBP-5 transcript was unaffected (Fig. 5A). DNPKC-δ was also able to partially block the PTH-(3–34) induction of IGFBP-5 mRNA by 25% compared with cells transfected with vector alone (Fig. 5B). Western blots of extracts from control cells or those transfected with DNPKC-δ or DNPKC-ε are shown in Fig. 5C and demonstrate the increased expression of these PKC isozymes in the transfected cells.

Effect of PMA on PKA-induced stimulation of IGFBP-5 mRNA. Our PKC isozyme translocation studies indicated that PMA stimulated the translocation of PKC-α and downregulated PKC-δ. The lack of effect of PMA alone on IGFBP-5 mRNA levels suggested that PKC-α does not stimulate IGFBP-5 transcription (Fig. 1). We also investigated whether PMA could affect IGFBP-5 gene expression stimulated by PKA. UMR-106–01 cells were treated with 5 μM forskolin, 1 mM 8-BrcAMP, or 10 nM PTH-(1–34) for 6 h in the presence or absence of 1 μM PMA. PMA blocked 50% of PTH-, forskolin-, and 8-BrcAMP-induced stimulation of IGFBP-5 transcript (Fig. 6).

**DISCUSSION**

In the present report, we have demonstrated that both the PKA and PKC pathways mediate PTH stimulation of IGFBP-5 mRNA levels in UMR-106–01 osteoblastic cells. The functional involvement of PKA in PTH induction of IGFBP-5 is demonstrated in the present study by partial inhibition in the presence of H-89. However, this PKA inhibitor could not suppress 40% of PTH activation of IGFBP-5. Evidence that the PKC pathway mediated this additional PTH-stimulated IGFBP-5 came from several observations. First, the selective PKC inhibitor bisindolylmaleimide I partially blocked PTH stimulation of IGFBP-5. Second, PTH-(3–34), which is not able to stimulate adenylyl cyclase in UMR cells, was able to significantly stimulate IGFBP-5.

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Because there was very little evidence for PKC regulation of IGFBP-5 transcription in the literature, we proceeded to investigate the regulation of the activity of PKC isozymes in UMR-106–01 cells. It has been reported by Sanders and Stern (29) that the related UMR-106 cell line expresses eight PKC isoforms, including PKC-α, -βI, -βII, -δ, -ε, -η, -ι, and -ζ. We were unable to detect expression of either the β or η isoforms in the 106–01 cells, likely due to differences in the cell lines or the antibodies used in the two studies. We found that PTH-(1–34) and PTH-(3–34) had no effect on the distribution of PKC-α, -ε, and -ι but were able to stimulate translocation of PKC-δ to the nucleus, with a corresponding decrease in the membrane fraction over a 10- to 40-min time course. Others have previously reported that PKC-δ is translocated to the nucleus, in a number of different cell lines. For example, Wang et al. (35) demonstrated by means of fluorescence microscopy that bryostatin stimulated the rapid translocation of PKC-δ to the nucleus in Chinese hamster ovary cells. Translocation occurs after PKC activation, and peptide inhibitors of PKC isozyme translocation act as antagonists of PKC activity (15).

To directly demonstrate the involvement of PKC-δ in PTH-(1–34) stimulation of IGFBP-5, we blocked the kinase activity of PKC-δ with the selective inhibitor rottlerin at concentrations between 0.5 and 5 μM. Rottlerin is a natural toxin that has been shown to inhibit PKC-δ with an IC₅₀ of 3–6 μM by interaction with the ATP-binding domain (36). Other PKC isozymes and PKA are inhibited at higher rottlerin concentrations.

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concentrations of 30–100 µM, but the concentrations of rottlerin used in our assays were too low to affect these kinases. The only other kinase reported to be significantly inhibited by this compound in the low micromolar range is calmodulin kinase III (rottlerin IC50 of 5.3 µM). Therefore, we used a dominant negative PKC-δ mutant, DNPKC-δ (K367R), to discriminate further the role of PKC-δ in PTH effects on IGFBP-5. DNPKC-δ significantly blocked the PTH regulation of IGFBP-5, whereas DNPKC-ε had no effect on PTH (1–34) induction of IGFBP-5. PTH (3–34) stimulation of IGFBP-5 transcript was also partially inhibited by both rottlerin and DNPKC-δ. The failure of these agents to completely inhibit the PTH (3–34) effect suggests that a fraction of the PTH (3–34) regulation of IGFBP-5 transcript is mediated by a pathway that is both PKA and PKC-δ independent. There are a number of signaling pathways that could be stimulated by Gβγ subunits released after PTH (3–34) stimulation of Gαq/11 through the PTH receptor (3). The IGFBP-5 gene has been shown to be under the transcriptional control of a plethora of transactivating factors and, subsequently, a large number of signaling pathways (28). Thus Gβγ-subunits released by PTH (3–34) could potentially mediate the stimulation of a pathway(s) whose activity also regulates IGFBP-5.

Previous attempts to investigate whether the PKC pathway was involved in PTH regulation of IGFBP-5 were performed by attempting to mimic the PTH effect with PMA. Nasu et al. (25) found that PMA was unable to mimic PTH stimulation of IGFBP-5 transcript. We also found that PMA could not induce IGFBP-5 mRNA; furthermore, it inhibited the stimulation of IGFBP-5 by PTH (1–34). Because PMA selectively activated PKC-α in our cells, as evidenced by its translocation to the cell membrane, PKC-α appears to have an inhibitory effect on IGFBP-5 transcription. Our demonstration that PMA is also able to partially abrogate the stimulation of IGFBP-5 mRNA induced by forskolin or 8-BrcAMP indicates that the inhibitory effects of PMA occur through cross-regulation of the PKA pathway downstream of adenylyl cyclase. The inhibitory effects of PMA were also directly demonstrated in osteoblast-enriched cultures from fetal rat calvaria, where PMA was shown to inhibit basal expression of IGFBP-5 mRNA (28). PMA could also be regulating IGFBP-5 through the downregulation of PKC-δ; however, this seems unlikely, given that induction of IGFBP-5 by forskolin and 8-BrcAMP likely occurs by PKA activation independently of PKC-δ regulation.

IGF-I has been shown to act through phosphatidylinositol 3-kinase to regulate IGFBP-5 gene expression in vascular smooth muscle cells (6). Osteoblasts also produce IGF-I, and PTH stimulates its synthesis through a PKA-dependent mechanism. We found that, after 6 h of treatment with exogenously added IGF-I, IGFBP-5 transcript levels were not changed in our cells (M. S. Erçlik, unpublished observations), a finding consistent with previous reports by others (4). Thus it is unlikely that there is an IGF-I component to the PTH-induced accumulation of IGFBP-5 mRNA under our assay conditions.

Because there were very low amounts of the transcript in unstimulated cells, it is likely that PTH induces IGFBP-5 mRNA through transcriptional mechanisms. The IGFBP-5 promoter has been cloned and well characterized. A number of AP-2 consensus binding sites have been identified throughout the length of the promoter, and studies in human fibroblasts have demonstrated AP-2 regulation of IGFBP-5 gene expression (5). Because AP-2 has been shown to mediate transcription events in response to both PKA and PKC activation (13), it is an attractive hypothesis to suggest that PTH-stimulated PKC activation works through AP-2 to stimulate IGFBP-5 gene expression. Further studies to characterize the molecular mechanisms of regulation of the IGFBP-5 promoter by PTH are necessary to lend insight into the complex regulation by both PKA and PKC of IGFBP-5 gene expression.

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REFERENCES


