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Alternate Signaling Pathways Selectively Regulate Binding of Insulin-Like Growth Factor I and II on Fetal Rat Bone Cells

Thomas L. McCarthy,* Changhua Ji, Sandra Casinghino, and Michael Centrella

Yale University School of Medicine, Section of Plastic Surgery, New Haven, Connecticut 06520-8041

Abstract Bone cells synthesize and respond to IGF-I and IGF-II which contribute to bone remodeling and linear growth. In osteoblasts, prostaglandin (PG)E_2 stimulates IGF-I but not IGF-II synthesis through a cAMP-dependent protein kinase A (PKA)-related event. However, protein kinase C (PKC) activation by PGE_2 enhances replication and protein synthesis by less differentiated periosteal cells more so than in osteoblast-enriched cultures from fetal rat bone. Using various PGs and other PKA and PKC pathway activators, the importance of these aspects of PGE_2 activity has now been examined on IGF receptors in these bone cell culture models. PGE_2 and other agents that activate PKA enhanced ^{125}I-IGF-II binding to type 2 IGF receptors on both cell populations. In contrast, agents that activate PKC enhanced ^{125}I-IGF-I binding to type 1 receptors on less differentiated bone cells, and of these, only phorbol myristate acetate (PMA), which activates PKC in a receptor-independent way, was effective in osteoblast-enriched cultures. No stimulator increased total type 1 receptor protein in either cell population. Consequently, ligand binding to type 1 and type 2 IGF receptors is differentially modulated by specific intracellular pathways in bone cells. Importantly, changes in apparent type 1 receptor number occur rapidly and may do so at least in part through post-translational effects. These results may help to predict new ways to manipulate autocrine or paracrine actions by IGFs in skeletal tissue. J. Cell. Biochem. 68:446–456, 1998.

Key words: IGF-I; IGF-II; cAMP; PKA; PKC; prostaglandin; osteoblasts

INTRODUCTION

The insulin-like growth factors (IGF-I and IGF-II) are abundant in the skeleton, and potently effect bone formation. Consistent with the somatomedin hypothesis, administration of IGF-I in vivo stimulates longitudinal bone growth, tibial epiphyseal width, and trabecular bone formation [Russel and Spencer, 1985; Skottner et al., 1987; Guler et al., 1988]. Early studies considered that systemic IGF-I was produced by the liver under the influence of growth hormone (GH). Accordingly, transgenic mice that overexpress GH show accelerated growth following an increase in serum levels of IGF-I [Mathews et al., 1988]. GH also enhances IGF-I synthesis by osteoblasts [McCarthy et al., 1989a], suggesting additional effects on local IGF-I production in bone. Production of IGFs by ostoblasts is also influenced by various hormones that regulate skeletal tissue metabolism [McCarthy et al., 1989a, 1990; Linkhart and Mohan, 1989]. The IGFs therefore function as systemic and local growth and differentiation factors in the skeleton.

In intact fetal rat calvariae in culture, the stimulatory effect of IGF-I on DNA synthesis predominates in the periosteal region, whereas increases in collagen synthesis predominate in the central bone [Hock et al., 1988]. Effects on DNA and collagen synthesis also occur in primary osteoblast-enriched cell cultures prepared from fetal rat bone [Centrella et al., 1989a; McCarthy et al., 1989b; Schmid et al., 1989] that express conventional IGF type 1 receptors [Centrella et al., 1990]. These cells also express IGF-II and abundant type 2 receptors that principally associate with IGF-II [Centrella et al., 1990; McCarthy et al., 1992]. Nonetheless, cross reactivity between IGF-I and IGF-II for binding to type 1 and type 2 IGF

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*Correspondence to: Thomas L. McCarthy, Ph.D., Section of Plastic Surgery, 333 Cedar Street, P.O. Box 208041, New Haven, CT 06520-8041.
E-mail: McCarthyTL@MASPO3.MAS.YALE.EDU

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receptors occurs on various cells, including osteoblasts [Centrella et al., 1990; LeRoith et al., 1995]. Importantly, anabolic effects by both IGFs appear to initiate through type 1 receptors [Mottola and Czech, 1984; Furlanetto et al., 1987; Czech, 1989; Steele-Perkins and Roth, 1990; Ullrich and Schlessinger, 1990; Jones and Clemmons, 1995]. The IGF type 1 receptor is comprised of two ligand binding α subunits and two membrane spanning β subunits [Dahms et al., 1989; Nissely et al., 1991]. It is autophosphorylated by ligand binding, and initiates a series of events for cellular activation [Ullrich and Schlessinger, 1990; Kato et al., 1994; Benito et al., 1996; Blakesley et al., 1996]. The type 2 IGF receptor is a single chain glycoprotein that binds IGF-II, to a lesser extent IGF-I, and mannose-6-phosphate or glycoproteins with mannose-6-phosphate side chains. In this regard, it may shuttle glycosylated proteins, and the IGFs, to intracellular lysosomes, and in so doing limit their activities [Nissely et al., 1991]. Some studies suggest that signaling cascades might also initiate through IGF type 2 receptors, but the prevalence of this and its importance for skeletal cells is not clear [Nissely et al., 1991; Okamoto et al., 1990].

Bone clearly contains cells at various stages of osteoblast-like differentiation [Liu et al., 1994] related perhaps in part to individual functions required for skeletal growth or remodeling. Similarly, cell cultures derived from fetal rat parietal bone express, in mass, fewer or higher numbers of features associated with differentiated osteoblasts [McCarthy et al., 1988; Wong and Ng, 1992]. Little is presently known about less differentiated bone cells. They may expand and differentiate in response to specific growth regulators, and when necessary replenish the pool of more differentiated osteoblasts. In membraneous bone, precursor cells may develop from the periosteum. Less differentiated periosteal cells might also support the activities of more differentiated osteoblasts by expressing factors, including IGFs, that influence osteoblast function.

Bone cells are further regulated by agents that activate adenylate cyclase to increase cAMP-dependent protein kinase A (PKA), or by membrane phospholipid turnover and intracellular calcium accumulation to activate protein kinase C (PKC) [Soderling, 1990]. Prostaglandins (PGs) and parathyroid hormone (PTH) activate both PKA and PKC through membrane receptor coupled events. Cross-talk between the two kinase systems is well recognized in bone cells, and these interactions are thought to balance the metabolic effects of endocrine, paracrine, and autocrine agents [Muallem et al., 1989; Hagel-Bradway et al., 1991; Bos et al., 1991; Kozawa et al., 1992; Green and Kleeman, 1992; Freyaldenhoven et al., 1992; Kano et al., 1993; Fukayama et al., 1993; Siddhanti et al., 1995; among others]. We initially reported the effects of various PGs on DNA and collagen synthesis by periosteal and osteoblast-enriched cultures from fetal rat parietal bone. PGE_1, PGE_2, and $PGF_{2\alpha}$ activate less differentiated periosteal cells through PKC mediated events. Of these, PGE_1 and PGE_2, which also potently activate PKA, are inhibitory in osteoblast-enriched cultures, while $PGF_{2\alpha}$ has no significant effect. Nonetheless, phorbol myristate acetate (PMA), a receptor-independent and potent enhancer of PKC, activates both cell populations [Centrella et al., 1994].

Alterations in IGF receptor abundance, affinity, or changes in the ratio of IGF type 1 and type 2 receptors in discrete cell populations may control IGF activity in bone. In this study, we investigated changes in IGF ligand-receptor interactions by activators of PKA and PKC in periosteal and osteoblast-enriched cultures form fetal rat bone. Our results show distinct sensitivities by each receptor type to one or the other kinase system, and confirm the relative importance of each signal transduction cascade in less and more differentiated bone cells.

MATERIALS AND METHODS

Cell Cultures

Primary cell cultures were prepared from the parietal bones of 22-day-old Sprague-Dawley rat fetuses (Charles River Laboratories, Raleigh, NC). Animals were housed and euthanized by methods approved by Yale University Animal Care and Use Committee. Sutures were eliminated during dissection, and bones were digested with collagenase for five sequential 20 min intervals. Cells released during the first 20 min collagenase digestion (periosteal cells) appear less differentiated biochemically. These cultures have a distinctly lower response to transforming growth factor-β1 (TGF-β1) and PTH, and do not make detectable levels of osteocalcin, even when treated with $1,25(OH)_2D_3$. The cell population released during the last three digestions exhibits biochemical character-

istics associated with differentiated osteoblasts, including PTH receptors, a high level of type I collagen synthesis, and a rise in osteocalcin expression in response to $1,25(OH)_2D_3$ [McCarthy et al., 1988; Centrella et al., 1989b]. Histochemical staining demonstrates that approximately 80% of the cells express alkaline phosphatase (T.M., M.C., unpublished data), although this itself is not entirely specific for osteoblasts. Using these criteria, differential sensitivity to TGF-β, bone morphogenetic protein-2, various PGs, and the ability to form mineralized nodules in vitro [Centrella et al., 1994, 1995, 1996], these cells are well distinguished from the less differentiated periosteal cells released during the first collagenase digestion. Cells from the first or the last three digestions were plated at 5,000 or 15,000 per cm^2, respectively, in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES (pH 7.2), 0.1 mg/ml ascorbic acid, penicillin and streptomycin (all from Life Technologies, Gaithersburg, MD), and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). Twenty-four h before treatments, cultures were rinsed twice with serum-free DMEM. All subsequent culturing and treatments were conducted in serum-free DMEM.

Binding Studies

Cultures were pretreated with vehicle or with test agents at the concentrations and for the time intervals indicated. Cultures were then incubated in serum-free medium supplemented with 4 mg/ml BSA and either ^{125}I-IGF-I or ^{125}I-IGF-II (labeled with chloramine T, 2,000 Ci/mmol) for 3 h at 4°C, conditions sufficient to achieve equilibrium binding [Centrella et al., 1989a, 1990]. For Scatchard analysis, cultures were incubated with radioligand without or with increasing amounts of homologous unlabeled IGF to achieve saturation. Nonspecific binding (5–20% of total binding), determined with 1,000-fold molar excess unlabeled IGF-I or IGF-II, was subtracted to calculate specific binding. Binding kinetics were determined by Scatchard plot. Cell surface binding sites for ^{125}I-IGF-I and ^{125}I-IGF-II were visualized as follows: cultures were rinsed with chilled binding medium, cross-linked with 0.1 mM disuccinimidyl suberate, extracted with 1% Triton X-100, and fractionated by electrophoresis on a 7.5% polyacrylamide gel (SDS-PAGE). Bound ^{125}I-labeled complexes were visualized by autoradiography [Centrella et al., 1989a, 1990].

cAMP Radioimmunoassay

Cultures were pretreated for 5 min with 0.5 mM isobutylmethyl xanthine (IBMX) to inhibit endogenous phosphodiesterase activity. Treatments were in serum-free medium with IBMX for 5 min at room temperature. Treatments were terminated by aspirating the medium, and the cultures were frozen at -75°C. Cells were extracted with 90% n-propanol, and samples were air-dried and dissolved in 50 mM sodium acetate, pH 6.2. An aliquot equivalent to the amount of material present in approximately 20,000 cells was used to measure cAMP using a specific commercial radioimmunoassay kit (Biomedical Technologies, Inc., Stoughton, MA). The amount of cAMP in each sample was determined from the linear portion of a standard curve, and expressed as pmol cAMP per 2 cm^2 culture [McCarthy et al., 1990a; Centrella et al., 1994].

Western Immunoblot

Triton X-100 (1%) extracts were prepared from treated cultures, and nuclei were removed by a 5 min spin at 13,000 xg. Protein content of the extracts was assessed using the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL). An equivalent amount of protein from each sample was loaded onto a reducing 7.5% polyacrylamide gel and electorphoresed overnight at 7 mA constant current. The gel was electroblotted for 45 min at 24 volts, 1 amp (Idea Scientific electroblotter, Minneapolis, MN) onto Immobilon P membrane (Millipore Corp., Bedford, MA) air dried, washed with Tris buffered saline (TBS), blocked with 5% nonfat powdered milk in TBS containing 0.05% Tween 20, and incubated at 4°C for 2 h with chicken anti-type 1 IGF receptor antibody (IgY) (Upstate Biotechnologies Inc., Lake Placid, NY). The blot was washed with TBS containing 5% nonfat powdered milk, and incubated at 4°C for 2 h with donkey anti-chicken IgY antiserum conjugated to horseradish peroxidase (Accurate Chemical Co., Westbury, NY). The filter was again washed with TBS, and material reactive with antibody to type 1 IGF receptor was visualized by chemiluminescence (ECL reagent, Amersham, Arlington Heights, IL).

Statistics

Data were assessed by one way analysis of variance with SigmaStat® software, with the Student-Newan-Keuls method for post hoc comparison. All experiments were performed a minimum of two times.

RESULTS

Treatment for 20 h with PGE_2, PTH, forskolin, or dibutyryl cAMP (db cAMP), which all increase intracelluar cAMP, significantly enhanced ^{125}I-IGF-II binding, but had no effect on ^{125}I-IGF-I binding in primary fetal rat osteoblast-enriched cultures (Fig. 1). When the effect of PGE_2 was examined over a broad range of ligand concentrations, ^{125}I-IGF-I binding was only marginally enhanced at higher ligand concentrations (at or above 400 pmol), while a significant increase in ^{125}I-IGF-II binding occurred over the range of 30–1,000 pmol of ^{125}I-IGF-II (Fig. 2). The effect of PGE_2 was time-dependent, with a small effect at 5 h, and a near two-fold increase in ^{125}I-IGF-II binding at 20 h (Fig. 3). Cycloheximide and tunicamycin each inhibited this effect by PGE_2, indicating a need for ongoing protein synthesis and glycosylation (data not shown). This result is consistent with the slow response time we observed. Scatchard analyses showed curvilinear IGF-I and IGF-II binding. PGE_2 treatment increased the total amount of IGF-II binding consistent with an increase in apparent receptor number, but did not alter the K_a (slope) of high or low affinity binding sites. By this method also, no large or significant changes were noted for IGF-I binding (Fig. 4).

To evaluate effects by PGE_2 on the complexity of the IGF-I and IGF-II binding profiles, cultures were incubated with an intermediate con-

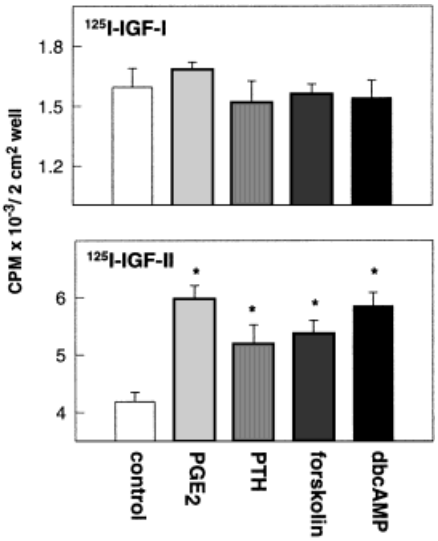

Fig. 1. Effect of various cAMP inducing agents on IGF-I and IGF-II binding in osteoblast enriched cultures from fetal rat bone. Serum-deprived confluent cultures were pretreated for 20 hours in the absence or presence of 1 µM PGE_2, 10 nM PTH, 10 µM forskolin, or 100 µM dibutyryl cAMP. Cultures were labeled with 100 pM ^{125}I-IGF-I or ^{125}I-IGF-II for 3 h at 4°C. Each data point is the average of n = 4 replicate samples per condition.

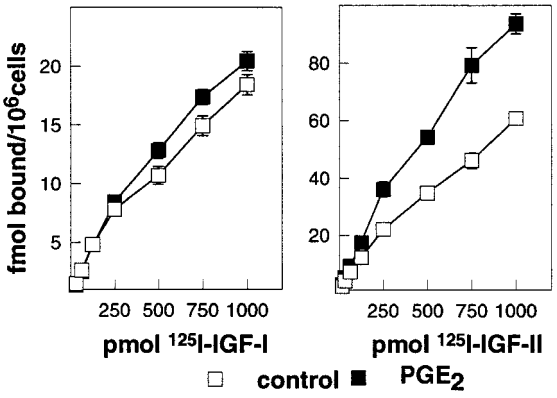

Fig. 2. Effect of PGE_2 on IGF-I and IGF-II binding in osteoblast enriched cultures from fetal rat bone. Serum-deprived confluent cultures were pretreated for 20 h in the absence or presence of 1 µM PGE_2, labeled with 16 to 1,000 pM ^{125}I-IGF-I or ^{125}I-IGF-II (3 h at 4°C). Each data point is the average of n = 4 replicate samples per condition.

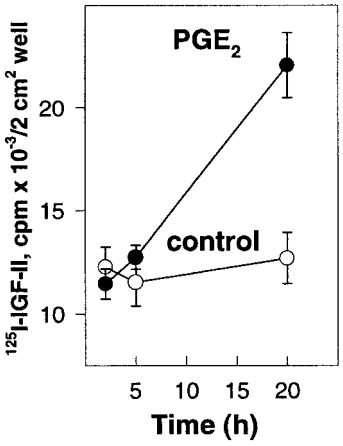

Fig. 3. Time course analysis of IGF-II binding following PGE_2 treatment of fetal rat osteoblast cell cultures. Serum-deprived osteoblast cultures were exposed to serum-free medium with ethanol vehicle or 1 µM PGE_2 for 1, 5, or 20 h. Binding was then carried out using 400 pM ^{125}I-IGF-II for 3 h at 4°C. Cultures were rinsed, extracted with 1% Triton X-100, and the amount of ^{125}I-IGF-II bound determined by gamma spectrometry. Each data point is the average of n = 3 replicate samples per condition.

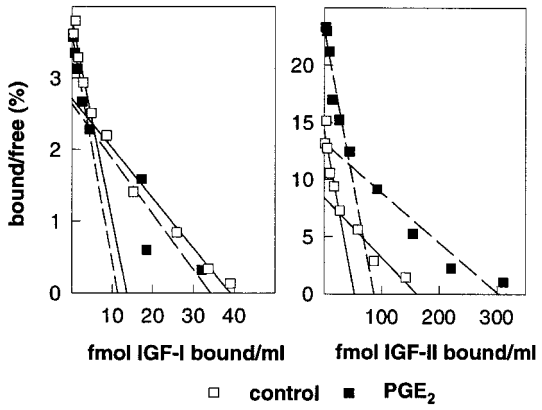

Fig. 4. Effect of PGE_2 on IGF binding in osteoblast enriched cultures from fetal rat bone; Scatchard analysis. Serum-deprived confluent cultures were pretreated for 20 h in the absence or presence of 1 μM PGE_2, labeled with 100 pM ^{125}I-IGF-Ior-II (3 h at 4°C), in the absence or presence of unlabeled homologous ligand (0 to 100,000 pM). Each data point is the average of three replicate samples which differed from each other by 10% or less. Results were analyzed by the method of Scatchard, and the lines shown were determined by linear regression analysis of all data points. Data are representative of n = 4 separate experiments.

centration of ^{125}I-labeled IGF (250 pM), ligand was chemically cross-linked with disuccinimidyl suberate (DSS), and binding sites were assessed by SDS-PAGE and autoradiography. ^{125}I-IGF-I formed binding complexes of 130 and 260 kDa, consistent with monomers and dimers of the α-subunit of the type 1 IGF receptor. A predominant ^{125}I-IGF-II binding complex was observed with an M_r of 250 kDa. Faint binding of ^{125}I-IGF-I at 250 kDa, and ^{125}I-IGF-II at 130 kDa and 260 kDa was also observed (Fig. 5), which were more prominent after longer gel exposure or with higher amounts of radioligand (data not shown). Competition with unlabeled homologous or heterologous ligand was examined to demonstrate ligand specificity. In each case a 10-fold molar excess of unlabeled homologous ligand effectively competed for radioligand binding. A 10-fold excess of unlabeled IGF-II only weakly competed with ^{125}I-IGF-I, while a 10,000-fold excess of insulin was as effective as 10-fold of IGF-I. These amounts of IGF-I and insulin only modestly reduced ^{125}I-IGF-II binding. Binding profiles of this type are consistent with conventional IGF-I and IGF-II receptors in other tissues [Nissley et al., 1991; Werner et al., 1991]. Consistent with total radioligand binding and Scatchard analyses, treatment for 20 h with PGE_2 caused a significant increase in ^{125}I-IGF-II binding to the 250 kDa type 2 IGF receptor band. This effect was preserved at all concentrations of competitive homologous ligand (right panel, Fig. 6). PGE_2 also induced increases in ^{125}I-IGF-II binding within lower M_r complexes approximately 20–40 kDa, possibly reflecting ^{125}I-IGF-II bound to cell surface IGFBPs. Again, similarly large changes in high affinity ^{125}I-IGF-I binding were not found in extracts from PGE_2 treated cultures (left panel, Fig. 6).

To dissect the pathways utilized for this effect and its possible specificity for differentiated osteoblasts, ligand binding was further compared in less differentiated periosteal cells and osteoblast-enriched cultures treated with PGE_2, PGE_1, or $PGF_{2\alpha}$. PGE_2 and PGE_1 rapidly increased intracellular cAMP, while as much as 1 μM $PGF_{2\alpha}$ was ineffective (Fig. 7). Consistent with their effects on cAMP generation, and earlier results in the present studies, 20 h pretreatment with PGE_2 and PGE_1 enhanced IGF-II binding in both cell populations. In contrast, all three PGs enhanced IGF-I binding in the periosteal cell cultures, but did not affect its binding in osteoblast-enriched cultures (Fig. 8). Because PGE_1, PGE_2, and $PGF_{2\alpha}$ share an ability to activate PKC in the presence of specific PG receptors, and we previously found even more potent effects by the receptor-indepen-

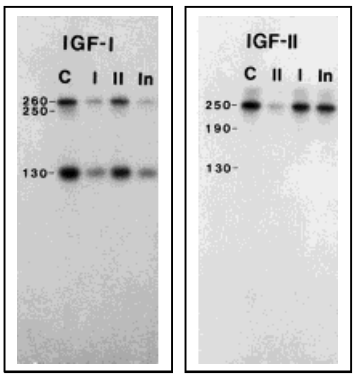

Fig. 5. Characterization of type 1 and type 2 IGF receptors on rat osteoblast cells. Serum-deprived confluent osteoblast cultures were labeled for 3 h at 4°C with 250 pM ^{125}I-IGF-I (**left**) or ^{125}I-IGF-II (**right**). Competition binding was assessed with unlabeled homologous or heterologous ligand, using control medium (C), 2,500 pM IGF-I (I), 2,500 pM IGF-II (II), or 2.5 μM insulin (in), as indicated above each sample lane. The cultures were rinsed, cross-linked with DSS, fractionated on a 7.5% polyacrylamide gel, and ^{125}I-labeled binding complexes were displayed by autoradiography. Numbers on the left (x 10^{-3}) show positions of migrations of IGF receptor complexes relative to molecular weight standards ranging from M_r 14,300 to 200,000.

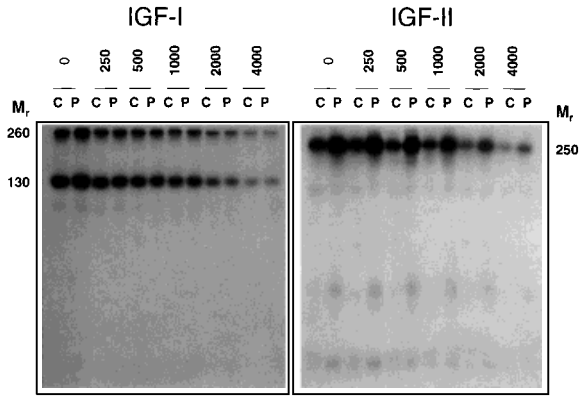

Fig. 6. Effect of PGE_2 on IGF-I and IGF-II binding site distribution in osteoblast cultures from fetal rat bone. Serum-deprived confluent cultures were incubated for 20 h with vehicle or 1 μM PGE_2 and labeled for 3 h at 4°C with 250 pM ^{125}I-IGF-I (**left**) or ^{125}I-IGF-II (**right**). Competition binding was assessed with 250 to 4,000 pM unlabeled homologous ligand (IGF-I or IGF-II) as indicated above each pair of sample lanes. The cultures were rinsed, cross-linked with DSS, fractionated on a 7.5% polyacrylamide gel, and ^{125}I-labeled binding complexes were displayed by autoradiography. Numbers on the left (x 10^{-3}) show positions of migration of IGF receptor complexes relative to molecular weight standards ranging from M_r 14,300-200,000. Data are representative of two or more experiments.

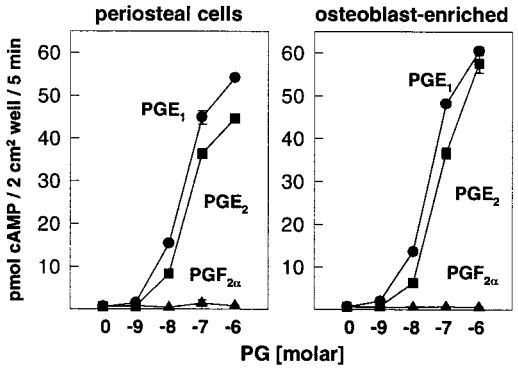

Fig. 7. Effect of various prostaglandins on intracellular cAMP levels in periosteal and osteoblast cell cultures. Serum-deprived confluent cultures were pretreated 5 min with 100 μM IBMX (to inhibit phosphodiesterase activity) then exposed to 1 to 1000 nM PGE_1, PGE_2, or $PGF_{2\alpha}$ for 5 min. Cyclic AMP levels were assessed by radioimmunoassay. Each data point is the average of n=3 replicate samples per condition.

dent PKC activator PMA on cell replication in both bone cell populations [Centrella et al., 1994], we also examined changes in IGF-I and IGF-II binding in PMA treated cultures. Consistent with these results and with the effect of $PGF_{2\alpha}$ on IGF-I binding in Figure 8, PMA potently enhanced IGF-I but not IGF-II binding in both cell cultures (Fig. 9). Using less differentiated periosteal cells to illustrate this effect

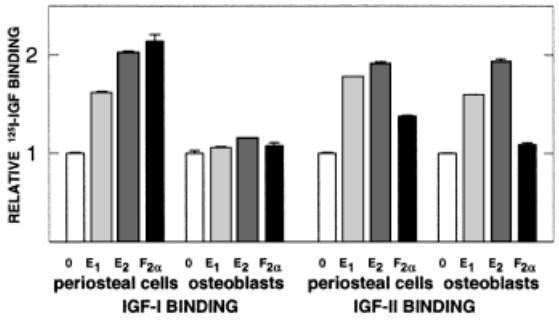

Fig. 8. Effect of various prostaglandins on IGF-I and IGF-II binding in periosteal and osteoblast cell cultures. Serum-deprived confluent cultures were pretreated for 20 h in the absence or presence of 1 μM PGE_2, PGE_1, or $PGF_{2\alpha}$, then incubated with 200 pM ^{125}I-IGF-I or ^{125}I-IGF-II for 3 h at 4°C. Each data point is the average of n = 3 replicate samples per condition.

further, PMA rapidly (within 5–30 min) enhanced IGF-I binding to type 1 receptor. This effect progressed with longer duration of treatment, and was increased by 2.5-fold by 20 h (Fig. 10). By Scatchard analysis, there was an apparent increase in type 1 receptor number with no change in binding affinity (Fig. 11). Co-treatment with hydroxyurea to suppress cell replication did not diminish the increase in IGF-I binding in PMA treated cultures (data not shown). Using the most potent regulators that we had yet detected, PMA for type 1 receptors and PGE_2 for type 2 receptors, the IGF-I and IGF-II binding profiles from periosteal or osteoblast cultures were then directly compared by SDS-PAGE. PMA enhanced IGF-I binding to type 1 complexes of 130 and 260 kDa in both cell populations, while PGE_2 enhanced IGF-II binding to type 2 complexes of 250 kDa. Consistent with these changes and the more potent effects of PGs in periosteal cells [Centrella et al., 1994], IGF-I binding to a 250 kDa complex that co-migrated with the IGF type 2 receptor was also evident with PGE_2 treatment (Fig. 12). Regardless of their stimulatory effects on IGF-I binding, no PKC activator caused significant changes in total type 1 receptor protein by Western blot analysis that could account for this effect in either cell population, even after a 20 h treatment period (Fig. 13). However, a 50% increase in type 1 IGF receptor mRNA was observed in cultures after prolonged treatment (48 h) with PMA (data not shown). Therefore, multiple events may occur in response to PKC activation that alter IGF-I binding. The early effects that we show here may unmask or re-

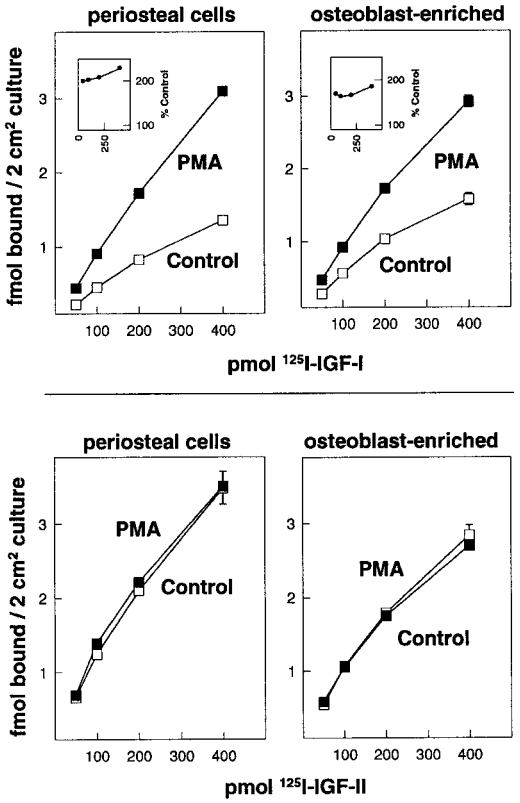

Fig. 9. Effect of phorbol myristate acetate (PMA) on IGF-I and IGF-II binding in periosteal and osteoblast cell cultures. Serum-deprived confluent cultures were pretreated for 20 h in the absence or presence of 1 μM PMA, then incubated with 50 to 400 pM ^{125}I-IGF-I or ^{125}I-IGF-II for 3 h at 4°C. Each data point is the average of three replicates. ^{125}I-IGF-I binding is shown in the upper panel for periosteal and osteoblast cultures, while ^{125}I-IGF-II is shown in the lower panel. Similar results were obtained from two independent binding studies. Inserts indicate % control IGF-I binding following PMA treatment.

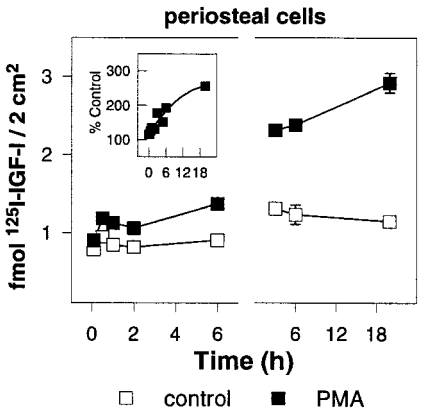

Fig. 10. Time course analysis of IGF-I binding following PMA treatment of periosteal bone cell cultures. Serum-deprived periosteal cultures were exposed to serum-free medium in the absence or presence of 1 μM PMA for 5 min to 5 h (**left**) or 3 to 20 h (**right**). Binding was then carried out using ^{125}I-IGF-I (400 pM) for 3 h at 4°C. The insert shows data from both studies as % control IGF-I binding following PMA treatment. Each data point is the average n = 3 replicate samples per condition.

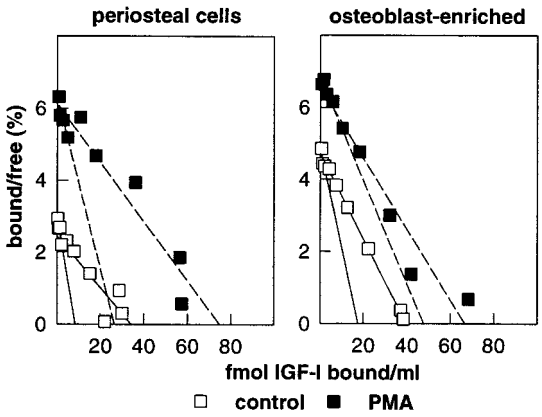

Fig. 11. Effect of PMA on IGF-I binding in periosteal and osteoblast cell cultures; Scatchard analysis. Serum-deprived confluent cultures were treated for 20 h in the absence or presence of 1 μM PMA, and labeled with 12.5 to 100 pM ^{125}I-IGF-I (3 h at 4°C), in the absence or presence of unlabeled IGF-I (0 to 100,000 pM). Each data point is the average of n=3 replicate samples per condition.

veal previously inaccessible type 1 IGF receptors, perhaps by biochemical modifications or changes in cell trafficking. Increases that occur at even later times may depend on new receptor expression.

DISCUSSION

Fetal rat periosteal and osteoblast-enriched cell cultures possess conventional IGF type 1 and type 2 receptors. While preferential ligand binding occurs at each receptor type, cross-receptor competition is also observed, and high levels of insulin effectively reduce ligand binding to type 1 receptors [Centrella et al., 1989a, 1990; Werner et al., 1991]. Using primary fetal rat osteoblast-enriched cultures, we now demonstrate that PGE_2 and other agents that activate PKA increase the apparent number of type 2 IGF receptors, without influencing high affinity ligand binding to type 1 receptors. This effect requires greater than 5 h, ongoing protein synthesis, and active glycosylation and protein translocation to the cell membrane. With high radiologand concentrations, a small increase in ^{125}I-IGF-I binding can also be seen, consistent with limited IGF receptor cross-binding. Radioligand and Scatchard analyses were confirmed by SDS-PAGE binding profiles. The significance of an increase in the amount of IGF type 2 receptor binding in response to PKA activa-

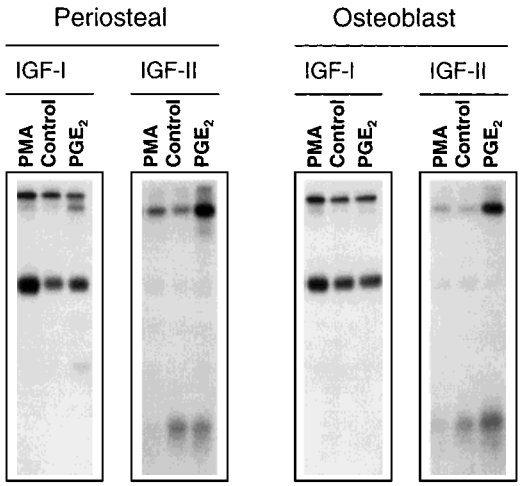

Fig. 12. Comparison of the effect of PMA and PGE_2 on IGF-I and IGF-II binding in periosteal and osteoblast cultures. Serum-deprived confluent cultures were incubated for 20 h in the absence or presence of 1 μM PMA or 1 μM PGE_2, then labeled for 3 h at 4°C with 250 pM ^{125}I-IGF-I or ^{125}I-IGF-II. The cultures were rinsed, cross-linked with DSS, fractionated on a 7.5% polyacrylamide gel under reducing conditions, and the ^{125}I-labeled binding complexes displayed by autoradiography (^{125}I-IGF-I, **left**; ^{125}I-IGF-II, **right**). Lanes 1, 2, and 3 depict treatments with 1 μM PMA (left), control (**center**), and 1 μM PGE_2 (right), respectively; for each panel.

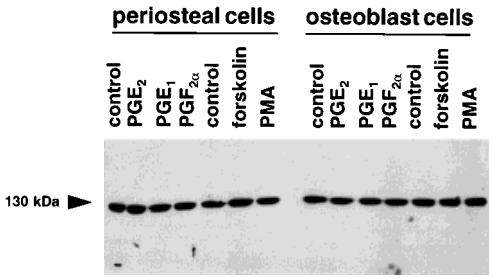

Fig. 13. Western immunoblot analysis of total type 1 IGF receptor protein levels in periosteal and osteoblast cultures treated with various prostaglandins, forskolin or PMA. Serum-deprived confluent cultures were treated for 20 h with control medium, 1 μM PGE_2, 1 μM PGE_1, 1 μM PGF_2, 10 μM forskolin, or 1 μM PMA. A 1% Triton X-100 cell lysate was prepared, protein content determined, and 100 μg of protein from each sample was electrophoresed through a reducing 7.5% SDS-PAGE. Western immunoblot analysis was with chicken anti-type 1 IGF receptor antibody and chemilluminescence after 5 min of film exposure.

tion is hindered by a lack of clear biological effects by type 2 receptor engagement in this and in other cell culture models. However, a change of this type may sequester or divert endogenous IGFs from binding to functional type 1 receptors.

In contrast to effects by PKA activation, PGE_2 and other agents that activate PKC enhanced binding to type 1 IGF receptors on less differentiated bone cells, suggesting additional cAMP-independent effects of PGE_2 in this cell population. Based on our earlier studies with various PGs and other PKA and PKC activators in these cells [Centrella et al., 1994], PMA replicated this effect in osteoblast-enriched cultures. Therefore, while both pathways appear active in both bone cell populations, osteoblast-enriched cultures are differentially sensitive to PG receptor coupled PKA activation, which selectively increases the levels of IGF type 2 receptors.

We observed subtle differences in total IGF binding in response to individual PGs by comparison to receptor-independent activators of PKA (forskolin) or PKC (PMA). They may in part relate to the various PKC isoforms previously noted in osteoblasts [Sanders and Stern, 1996]. Unless different substrates for these enzymes can be identified with certainty, however, this alone cannot explain differences between PGs and PMA in osteoblast-enriched cultures. They may also result from variations in specific PG receptors [Muallem et al., 1989; Suda et al., 1996] or their abilities to couple separate but interacting signaling pathways during bone cell differentiation. Furthermore, $PGF_{2\alpha}$ modestly enhanced total IGF-II binding in periosteal cell cultures without increasing cAMP. This seemingly inconsistent effect may result from cross-binding among IGFs and IGF receptors [Centrella et al., 1990], from PKC activation by $PGF_{2\alpha}$ [Centrella et al., 1994], and from increased ligand binding to IGF type 1 receptor in response to PKC activation (these studies).

Rapid increases in IGF-I binding in response to PKC activation occurred through an increase in apparent type 1 receptor number with no change in binding affinity or total cellular type 1 receptor protein levels. Because type 1 receptor mRNA levels rise slightly after 48 h of treatment [unpublished observation], multiple events may regulate short vs. long term stimulation of PKC activity. Early effects may uncover previously inaccessible receptors, whereas later effects result from new receptor expression. Even though our studies reveal the need for new protein synthesis and presumably an increase in type 2 receptor protein to observe stimulatory effects by PKA on IGF-II binding, lack of commercial antibody for rat IGF type 2

receptor prevents us from examining changes in this molecule by immunoblot analysis.

Only few earlier studies examined the influence of protein kinase activation on IGF binding or receptor expression. In contrast to our results, a subpopulation from transformed human osteosarcoma SaOS-2 cells, expressing low levels of alkaline phosphatase and presumably pre-osteoblast-like, exhibit an increase in IGF type 1 receptor mRNA in response to dbcAMP, but no change in type 2 receptor mRNA. In those studies, no effect occurred in more differentiated SaOS-2 cultures expressing high alkaline phosphatase activity [Mohan et al., 1993]. However, consistent with our results, in the continously cultured murine osteoblast-like MC3T3-E1 cell line, activation of PKC modestly elevated ^{125}I-IGF-I binding, while db-cAMP had no effect [Hakeda et al., 1994].

Whereas PKC activators can increase ligand binding to IGF type 1 receptor, and may do so without inducing new IGF receptor synthesis by bone cells, little is still known about their effects on IGFBP expression in skeletal tissue [Conover et al., 1993; Hakeda et al., 1994]. In contrast, PKA activators rapidly induce the synthesis of IGF-I and select IGFBPs in cultured bone cells [McCarthy et al., 1990, 1992, 1994; Bichell et al., 1993; Torring et al., 1991; Schmid et al., 1991, 1992]. The many actions by cAMP on components of the IGF axis may further influence the kinetics of IGF receptor binding as well as IGF actions in skeletal tissue. Because the IGF type 1 receptor transduces intracellular signals for both IGF-I and IGF-II, a redistribution of IGF binding between type 1 and type 2 receptors by select protein kinase activation may modulate the activity of IGFs on bone cell metabolism. Although we found a greater level of type 1 receptor with PKC activation, it is not certain if this engenders an increase in IGF-I activity. We have been unable to assess this directly because of the similar biological effects by IGF-I and PKC activators on the parameters that we now measure in bone cell cultures [Centrella et al., 1994, and unpublished observations]. However, Chinese hamster ovary cells stably transfected to overexpress PKCα and human IGF type 1 receptor exhibit a reduced capacity for IGF-I-dependent phosphorylation of IRS-1 and GAP-associated p60, components of the IGF-I signal transduction cascade [Danielson et al., 1995], suggesting a reduction in IGF-I activity in some PKC activated cells. Other parameters, or exposure to IGF-I at times when primary PKC effects have diminished, may allow us to address this in future studies.

In summary, we find discrete changes in IGF binding on bone cells in response to activators of PKA and PKC, limited in part to cells with different levels of committment to the osteoblast phenotype. These complex changes may result from an increase in type 2 receptor availability due to new receptor synthesis, from biochemical changes that unmask type 1 receptors, or from changes in receptor cycling (down-regulation) due to fluctuations in the levels of IGF-I, IGF-II, or IGFBPs. Consequently, new information that further defines the agents or the events that modulate the IGF axis in bone will assist the development of strategies to take best advantage of the anabolic potential of IGFs as autocrine and paracrine skeletal growth factors.

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