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Regulation of Connexin43 Expression and Function by Prostaglandin E_2 (PGE_2) and Parathyroid Hormone (PTH) in Osteoblastic Cells

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Abstract Connexin43 (Cx43) forms gap junctions that mediate intercellular communication between osteoblasts. We have examined the effects of prostaglandin E_2 (PGE_2) and parathyroid hormone (PTH) on gap junctional communication in the rat osteogenic sarcoma cells UMR 106-01. Incubation with either PGE_2 or PTH rapidly (within 30 min) increased transfer of negatively charged dyes between UMR 106-01 cells. This stimulatory effect lasted for at least 4 h. Both PGE_2 and PTH increased steady-state levels of Cx43 mRNA, but only after 2–4 h of incubation. Transfection with a Cx43 gene construct linked to luciferase showed that this effect of PTH was the result of transcriptional upregulation of Cx43 promoter. Stimulation of dye coupling and Cx43 gene transcription were reproduced by forskolin and 8Br-cAMP. Exposure to PGE_2 for 30 min increased Cx43 abundance at appositional membranes in UMR 106-01, whereas total Cx43 protein levels increased only after 4–6 h of incubation with either PGE_2 or PTH. Inhibition of protein synthesis by cycloheximide did not affect this early stimulation of dye coupling, but it significantly inhibited the sustained effect of PTH and forskolin on cell coupling. In summary, both PTH and PGE_2, presumably through cAMP production, enhance gap junctional communication in osteoblastic cell cultures via two mechanisms: initial rapid redistribution of Cx43 to the cell membrane, and later stimulation of Cx43 gene expression. Modulation of intercellular communication represents a novel mechanism by which osteotropic factors regulate the activity of bone forming cells. J. Cell. Biochem. 68:8–21, 1998.

Key words: gap junctions; dye-coupling; connexin43; parathyroid hormone; prostaglandin E_2

Bone remodeling is a dynamic process consisting of repeated sequences of bone resorptive and formative cycles, which require a well coordinated effort of bone cells, osteoblasts, osteoclasts, and osteocytes [Rodan, 1996]. Cells of the osteoblastic lineage play a pivotal role in bone remodeling because they both directly produce and mineralize bone matrix and are the target cells for the major hormones with bone resorptive activity, i.e., parathyroid hormone (PTH), and 1,25-dihydroxycholecalciferol [Narbaitz et al., 1983; Silve et al., 1982]. Osteoblasts are believed to transmit the bone resorptive signals to the osteoclast, possibly by secretion of a soluble factor, although the exact nature of this factor remains undetermined. On the other hand, direct contact between cells of the osteoblastic and osteoclastic lineages is required in certain systems for induction of bone resorption

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[Takahashi et al., 1988], implying a possible role of direct intercellular communication during osteoclastogenesis.

A number of studies have demonstrated that osteoblasts are highly coupled via gap junctions [Donahue et al., 1995; Civitelli et al., 1993; Schirrmacher et al., 1992; Shen et al., 1986; Doty, 1981] which allow signal exchange not only between adjacent osteoblasts, but also between osteoblasts and periosteal fibroblasts, and osteoblasts and osteocytic processes [Palumbo et al., 1990; Doty, 1981; Jeasonne et al., 1979]. In previous work, we have established that osteoblastic cells express connexin43 (Cx43) as the major gap junction protein which mediates intercellular communication in these cells [Steinberg et al., 1994; Civitelli et al., 1993], an observation also reported by other investigators [Donahue et al., 1995; Yamaguchi et al., 1994; Schiller et al., 1992; Schirrmacher et al., 1992]. However, not only do osteoblasts express more than one connexin, but different osteoblastic cells also express different amounts of Cx43 and exhibit different abilities to transfer fluorescent dyes from cell to cell [Steinberg et al., 1994; Civitelli et al., 1993]. The physiologic basis of such a diversity of connexin expression and endogenous communication competence among different osteoblastic cell models is still elusive. Nonetheless, gap junctional communication is a characteristic feature of osteoblastic cells and may be essential for their normal functioning.

Gap junctional communication can be regulated by a variety of physical and chemical factors, including hormonal second messengers, such as cAMP and cytosolic calcium, cytoplasmic pH, and protein phosphorylation [Sáez et al., 1993; Stagg and Fletcher, 1990]. A rapid increase of gap junctional plaques between osteoblasts was reported following incubation of fetal rat calvaria cells with prostaglandin E_2 (PGE_2) [Shen et al., 1986], and stimulation of Cx43 expression and cell coupling by PTH has been reported in rat osteogenic sarcoma [Schiller et al., 1992] and adult rat bone cells [Donahue et al., 1995]. However, the molecular mechanisms by which PGE_2 and PTH regulate gap junctional communication have not been established. Because of the importance of these two factors in bone remodeling and the potential application of PTH as anabolic agent for the treatment of osteoporosis, we have analyzed the action of these two regulators on the expression and function of Cx43 in osteoblasts. The studies described herein demonstrate that both PTH and PGE_2 are able to upregulate gap junctional communication in these cells, via multiple modulatory effects on Cx43 expression and function. Thus, gap junctional communication is an essential regulatory component of osteoblast activity.

MATERIALS AND METHODS

Reagents and Cells

Lucifer yellow CH and calcein-AM were purchased from Molecular Probes (Eugene, OR). Lucifer yellow was dissolved in distilled, deionized water to a 10 mM concentration; calcein-AM was dissolved in DMSO at the concentration of 1 mM. Stock solutions were stored at −20°C in the dark. [α^{32}P]-dCTP was purchased from Amersham (Arlington Heights, IL). Polyclonal rabbit antiserum directed against Cx43 was produced by immunizing rabbits with a synthetic peptide corresponding to amino acids 252–271 of rat Cx43 [Beyer and Steinberg, 1991]. All other chemicals and the tissue culture media were from Sigma Chemicals (St. Louis, MO), unless otherwise indicated.

The osteogenic sarcoma cells, UMR 106-01 were a gift of Dr. Nicola C. Partridge, St. Louis University. These cells have been characterized as having an osteoblastic phenotype [Forrest et al., 1985; Partridge et al., 1983]. UMR 106-01 cells are poorly dye coupled, and express Cx45 in more abundance than Cx43 [Steinberg et al., 1994]. They were maintained in Eagle's MEM, supplemented with 10% FBS. Subcultures until passage #30 were used in these studies. The ROS 17/2.8 cells, also derived from a rat osteosarcoma [Majeska et al., 1980], were also used in some experiments. They were provided by Dr. Gideon Rodan (Merck Research Laboratories, West Point, PA), and were maintained in DMEM/Ham's F-12 (1:1), supplemented with 10% FBS. ROS 17/2.8 cells are well coupled and express abundant Cx43 and no Cx45 [Steinberg et al., 1994].

Dye Coupling

The ability of osteoblastic cells to transfer fluorescent molecules via gap junctions (dye coupling) was assessed using two different techniques, both based on cell-to-cell transfer of fluorescein derivatives. *Intercellular transfer of microinjected Lucifer yellow* was performed as

described [Civitelli et al., 1993]. Briefly, cells adherent to 31 mm glass coverslips were grown to 90% confluence. The coverslips were mounted in a tissue chamber (Biophysica Technology, Baltimore, MD), and placed on the stage of an inverted epifluorescence microscope. Cells from confluent areas of the culture were microinjected with 10 mM Lucifer yellow (1,100–1,200 psi applied for 0.2–0.3 s). Lucifer yellow fluorescence was monitored using a low light silicon intensified target camera (SIT-66, Dage MTI, Michigan City, IN) and the number of adjacent cells containing dye 3–5 min after the injection was recorded, as a measure of dye coupling.

In the "*parachute*" *assay*, cells were grown on glass coverslides to confluence. A cell suspension from a parallel culture was prepared and loaded with calcein, a negatively-charged, gap junction permeant dye, using the acetoxymethylester form, calcein-AM. After loading, a small number of loaded cells (~5,000/well) was added to the culture dish just above the cell layer. Within ~15 min, the "parachuted" cells attach to the cell layer, and if gap junctions are present, the dye diffuses from the loaded cell to the cells of the unlabeled culture. Second, third, and higher order cells will acquire the dye, depending on the degree of coupling. Calcein fluorescence was monitored by epifluorescence microscopy (Zeiss Axioscope, Zeiss, Thornwood, NY) using a fluorescein filter set. Dye coupling was quantitated in digitized images by counting the number of cells acquiring dye per parachuted cell. Thus, as for microinjections, dye coupling assessed by the parachute assay depends primarily on cell-to-cell transfer of the fluorescent probe among acceptor cells, which occurs through functional gap junctions. To further prove that the parachute assay reports gap junctional permeability, rather than membrane fusion or other phenomena, we found that heptanol or glycyrrhetinic acid, two gap junction uncouplers, totally inhibited diffusion of calcein among UMR 106-01 and ROS 17/2.8 cells, whereas Dextran Texas red (Molecular Probes, Eugene, OR), a large (~10,000 D) molecular weight dye impermeable to gap junctions, did not pass from parachuted cells to acceptor cells (manuscript in preparation). Parachuted cells are easily distinguishable from acceptor cells because of their very bright fluorescence compared to the surrounding cells, and also because they are in a plane of focus distinct from that of the cell monolayer. Although quantitation is conceptually similar to the microinjection method, in this assay a large number of parachuted cells can be scored in each coverslide, typically more than 50.

RNA Blots

RNA blots were performed as previously described [Civitelli et al., 1993]. Cells were grown to confluence in 100 mm tissue culture dishes, and total cellular RNA was isolated using guanidinium isothiocyanate [Chomczynski and Sacchi, 1987]. Samples (10 µg/lane) were separated on formaldehyde agarose gels and transferred to nylon membranes. The membranes were prehybridized in 0.75 M sodium phosphate, 1.0% SDS, 100 µg/ml salmon sperm DNA, 42°C overnight, followed by overnight hybridization with a cDNA probe for Cx43 [Beyer et al., 1987] at 42°C, and washed under high stringency conditions (30 mM sodium phosphate, 1.0% SDS, 65°C). To verify the integrity and loading uniformity of RNA, the membranes were reprobed with [^{32}P]-dCTP labeled human β-actin cDNA (generous gift of Dr. Bratin Saha, Emory University, Atlanta, GA). The cDNA probes were labeled with 5′-[α^{32}P]-dCTP (3,000 Ci/mmol, aqueous solution) using the Megaprime labeling kit from Amersham (Arlington Heights, IL), according to the manufacturer's recommendations. The intensity of the band hybridizing to the Cx43 probe was quantitated by densitometry, and normalized to the intensity of the βactin band.

Cx43 Promoter Activity

A genomic clone containing exon I and 5′ sequences of the rat Cx43 gene was isolated by hybridization screening of a rat genomic library in lambda DASH (Stratagene, San Diego, CA), using the rat Cx43 cDNA [Beyer et al., 1987] as probe. A DNA fragment extending from 1,339 bases upstream of the transcriptional start site through base +222 was amplified by the polymerase chain reaction. This piece contains transcriptional regulatory sequences and exon I, and exactly corresponds to the rat Cx43 promoter as characterized by Yu et al. [1994]; it also contains all of the regulatory elements characterized by Chen et al. [1994]. This Cx43 promoter was subcloned upstream of the reporter gene luciferase in pGL2 (Promega, Madison, WI). UMR 106-01 cells were cotransfected (in triplicate) in six-well plates with 1 µg of Cx43-pGL2 and 1 µg of RSV-beta galactosidase

(as a transfection control) using the lipofectin reagent (GIBCO BRL, Gaithersburg, MD). Cells were fed with fresh growth medium after 24 h, and 48 h after transfection. Cellular lysates were prepared in 1% Triton X-100, 50 mM Tris-MES ph 7.8, 1 mM DTT, frozen, and thawed twice before assay for luciferase and β-galactosidase activities [Eustice et al., 1991].

Immunoblots

Cells were cultured on 100 mm tissue culture plates to 75–85% confluence, and incubated with the experimental compounds as appropriate. Cells were solubilized in 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.5% BSA, 50 mM TRIS, pH 8, containing a cocktail of protease and phosphatase inhibitors, for whole cell lysates. For enrichment of gap junction plaques [Koval et al., 1995], proteins were extracted in alkaline conditions, using 1 mM $NaHCO_3$, 1 mM phenylmethylsulfonylfluoride, 0.2 µg leupeptin, 0.1 µg pepstatin, pH 8.0, and neutralization in NaOH. Semipurified gap junction proteins were collected by centrifugation at 84,000*g*, and resuspended in the same buffer, before protein electrophoresis. Protein concentration in each sample was determined before electrophoresis using the method of Bradford [1976], and appropriate dilutions were made to ensure that equal amounts of protein were loaded in each lane. Proteins were separated by electrophoresis on 10% polyacrylamide gels with Promega midmolecular weight protein standards, and transferred to PDVF membranes (Millipore Immobilon P, Millipore Corp., Bedford, MA) using a tank transfer apparatus (Trans-blot Cell, Bio-Rad, Richmond, CA). After blocking with 5% nonfat milk in 40 mM TRIS HCl, pH 7.4, the membranes were incubated with the anti-Cx43 antibody at 1:500 dilution in blocking buffer overnight at room temperature, then washed in PBS and incubated for 1 h with an anti-rabbit antibody conjugated to horseradish peroxidase (Tago, Burlingame, CA). The immune reaction was detected by exposing the membranes to autoradiography film (Hyperfilm, Amersham) in the presence of luminol using the ECL detection kit (Amersham), according to the manufacturer's recommendations.

Immunofluorescence

Cells grown on glass coverslips were fixed in methanol/acetone (1:1) for 2 min at room temperature. After washing with PBS, they were incubated with the primary anti-Cx43 antibody (1:200) for 45 min, followed by three washes in PBS and incubation with secondary antibody (rhodamine conjugated goat anti-rabbit IgG (Tago) for further 45 min, as described previously [Civitelli et al., 1993]. The coverslips were washed and mounted on slides for epifluorescence microscopy (Zeiss Axioskop).

RESULTS

Microinjections of Lucifer yellow were first performed on semiconfluent (70–80% confluent) cultures of UMR 106-01 cells, incubated with either PGE_2 or PTH for different times. Approximately 15 min before the end of each incubation time, the coverslips with the cell cultures were placed on the microscope stage and bathed in the same culture medium. At the appropriate time, one or more cells were microinjected with Lucifer yellow. The degree of cell coupling was assessed 5–10 min after the microinjection. Confirming our previous observations [Steinberg et al., 1994], dye transfer occurred only occasionally from the microinjected cell to its neighbors in resting UMR 106-01 cells (Fig. 1A). Incubation with either PGE_2 (10^{-6} M) or PTH (10^{-7} M) rapidly increased the number of coupled cells per microinjected cell within 30 min of incubation with either agonist. Within the first hour of incubation, Lucifer yellow diffusion involved slightly more cells in PGE_2 than in PTH-treated cultures (Table I and Fig. 1B). In the continuous presence of either agonist, dye coupling remained higher than baseline for 4–6 h (Table I and Fig. 1C), and the effect disappeared after 24 h (Table I).

The requirement for a 3–5 min lag time to allow the dye to diffuse after microinjection makes it difficult to acquire data from a large number of cells at the same point in time. To corroborate the results obtained with Lucifer yellow microinjections, we applied a new method, the "parachute assay," that allows more precise monitoring of dye diffusion during incubation with an agonist and provides data more suitable for quantitation at distinct time points. The experimental compounds were added directly to the bathing medium after calcein-loaded UMR 106-01 cells were "parachuted" on top of a nonloaded monolayer. To ensure a precise timing of hormone exposure, additions of the experimental compounds were staggered in time so that all incubations ended 2½ h after

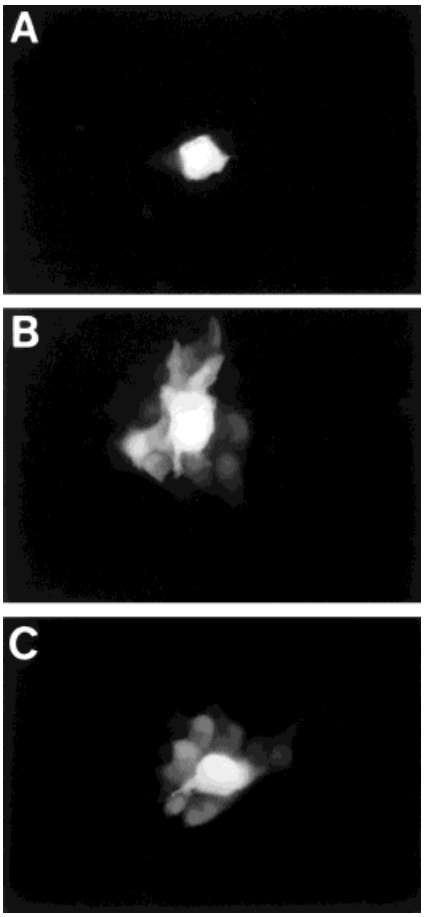

Fig. 1. Stimulation of cell coupling by PGE_2 and PTH in UMR 106-01 osteogenic sarcoma cells (Lucifer yellow microinjection). Cells were grown to subconfluence on glass coverslips. Fluorescence micrographs were taken 5–10 min after microinjection of Lucifer yellow into one cell. Injections were performed before (**A**) or after incubation with either 10^{-6} M PGE_2 for 30 min (**B**), or 10^{-7} M PTH for 210 min (**C**). Objective magnification 100×.

TABLE I. Effect of PGE_2 and PTH on Dye Coupling in UMR 106-01 Cells: Diffusion of Lucifer Yellow From a Microinjected Cell‡

	Agonist	
Incubation time	PGE_2	PTH
Baseline	1.65 ± 2.0 (74)	
20–60 min	6.1 ± 2.7* (12)	3.7 ± 2.6 (41)
4–6 h	4.0 ± 4.3 (26)	5.9 ± 2.7* (45)
24 h	0.6 ± 0.8 (9)	0.7 ± 1.5 (11)

‡UMR 106-01 were cultured to subconfluence and treated with either 10^{-6} M PGE_2 or 10^{-7} M PTH, for the time indicated. After microinjection of Lucifer yellow, dye coupling was expressed as number of coupled cells per microinjected cell. Data represent the average ± SD; in parenthesis is the number of microinjections performed for each condition.
*Significantly different than baseline at the 0.05 level (Kolmogorov-Smirnov test on frequency distributions).

TABLE II. Effect of PGE_2 and PTH on Dye Coupling in UMR 106-01 Cells: Diffusion of Calcein From Donor to Acceptor Cells‡

Incubation time	Agonist		
	Control	PGE_2	PTH
30 min	2.8 (1.9–3.5)	4.0* (3.0–5.2)	3.3 (2.8–3.5)
60 min	2.7 (2.6–3.1)	3.9* (3.8–4.3)	4.6* (3.8–4.8)

‡Calcein-loaded UMR 106-01 cells were added on top of a nonloaded confluent culture. They were then treated with either 10^{-6} M PGE_2 or 10^{-7} M PTH for the time indicated. Dye coupling is expressed as number of coupled cells per cell parachuted. Data represent the median and range (in parenthesis) of three different experiments, with an average of about 30 donor cells scored in each experiment.
*Significantly different than control at the 0.05 level (Fisher's exact test).

parachuting the labeled cell. Preliminary experiments indicated that an apparent steady state of calcein diffusion was rapidly reached within the first 15 min after parachuting. Further propagation of the dye occurred during the following 3 h, but the extent of this slow propagation was minimal (not shown). The experimental design allowed to control for this slight increase in dye diffusion that occurs with time after parachuting. In resting conditions, the average number of acceptor cells for each single calcein-labeled donor cells was higher than the average coupling obtained with the microinjection method (compare Tables I and II). This difference was substantial, and could not be accounted for by the difference in time the fluorescent dyes were allowed to diffuse after injection (15 min) or parachuting (2½ h). Nonetheless, the number of coupled cells per cell was higher in cultures incubated with either PGE_2 or PTH, as compared to dishes in which no addition was made (Fig. 2 and Table 2). Consistent with the microinjection data, at the earliest time-point (30 min) the effect was more pronounced (and statistically significant) in the presence of PGE_2 than in the presence of PTH. At 60 min, the increase in cell coupling was evident for both agonists (Table II). Thus, both PGE_2 and PTH rapidly increase gap junctional permeability to negatively charged dyes in osteoblastic cells.

PGE_2 and PTH activate similar signaling pathways in target cells, including stimulation of cAMP production and activation of phospholipase C [Fujimori et al., 1992; Farndale et al.,

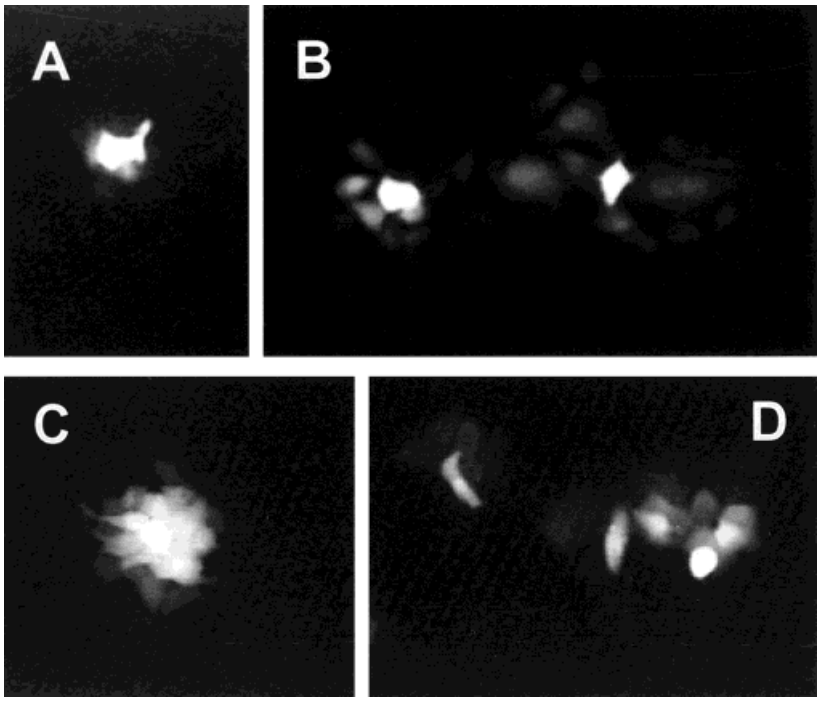

Fig. 2. PTH increases cell-to-cell transfer of calcein in UMR 106-01 cells (parachute assay). A confluent cell monolayer was on coverslips in tissue culture wells. A cell suspension from a parallel culture was loaded with calcein-AM for approximately 1 h. After loading, a small number of calcein-loaded cells was "parachuted" on top of the confluent cell layer. PTH (10^{-7} M) was added to the bathing medium 30 min or 2 h before the end of a 2½ h total incubation after parachuting. Fluorescence micrographs were taken in the absence of PTH (**A**), or after 30 min (**B**) and 2 h (**C, D**) of exposure to the hormone. Objective magnification 20×.

1988; Yamaguchi et al., 1988]. In most cell systems, cAMP stimulates, whereas calcium-dependent signaling inhibits gap junctional communication [Sáez et al., 1993]. Thus, we examined whether generation of cAMP could mimic the action of PGE_2 and PTH on dye coupling in UMR 106-01 cells. Compared to 4.4 ± 0.4 ($n = 5$) coupled cells per donor cell at baseline, cell coupling was increased by forskolin to 6.1 ± 0.5 ($P < 0.05$, $n = 5$) and to 8.2 ± 1.4 coupled cells per cell ($P < 0.01$; $n = 5$) after 30-min and 2-h incubations, respectively. Thus, the time-course of forskolin effect on dye coupling was very similar to those obtained with either PTH or PGE_2, and the magnitude of the effect was commensurate to the two agonists' action. Notably, stimulation of cell coupling was obtained with doses of forskolin (10^{-5} M) that maximally stimulate adenylate cyclase activity.

In the UMR 106-01 cells, as in other osteoblastic cell models, intercellular diffusion of negatively charged dyes is mediated primarily by gap junctions formed by Cx43 [Steinberg et al., 1994; Civitelli et al., 1993]. Therefore, we next tested whether PGE_2 or PTH modulate Cx43 expression in these cells. First, RNA blots were performed on total RNA extracts from UMR 106-01 cells incubated with either 10^{-6} M PGE_2 or 10^{-7} M PTH for different times. Both PGE_2 and PTH induced time-dependent increases of steady-state Cx43 mRNA with similar time-courses. An increase of Cx43 mRNA abundance was detectable after approximately 2 h of incubation with either agonist, and the effect lasted for at least 4–6 h (Fig. 3). Importantly, no changes were detected in the level of Cx43 mRNA after 30 min incubation with PGE_2 or PTH, a time at which dye coupling was enhanced (see above). Significant increases of Cx43 mRNA (2.5–1.5-fold of baseline) were obtained in UMR 106-01 cells with PTH concentrations as low as 10^{-9} M. Lower doses were ineffective.

We then studied whether the effect of PTH on Cx43 mRNA was the consequence of gene transcription regulation. Preliminary RNA blot experiments showed that in the presence of actinomycin D, preformed Cx43 mRNA was degraded at the same rate in the presence or in the absence of PTH, although the abundance of mRNA was higher in PTH treated than in un-

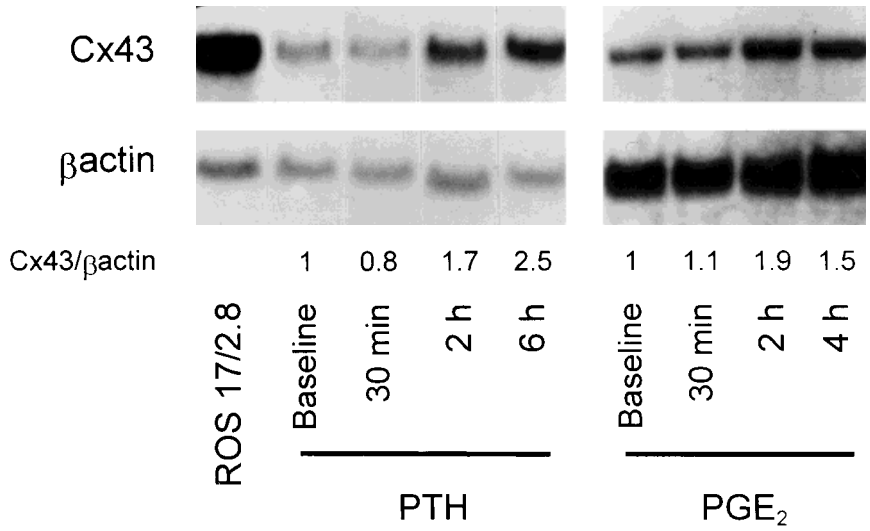

Fig. 3. Effect of PGE_2 and PTH on Cx43 mRNA of UMR 106-01 cells. Confluent cultures were incubated with either 10^{-6} M PGE_2 or 10^{-7} M PTH for the indicated times, and total RNA extracted and separated, as detailed in Material and Methods. Membranes were hybridized with a cDNA probe for Cx43, then washed and rehybridized for human βactin. The intensity of the Cx43 mRNA band relative to that of βactin is indicated. A lane with RNA from calvaria cells in resting conditions is also shown on the left for comparison of relative mRNA abundance.

treated cultures (not shown). These results suggested that the hormone did not alter the half-life of mRNA. UMR 106-01 cells were then transiently transfected with a Cx43-luciferase reporter construct, and exposed to 10^{-7} M PTH for 6 h. The hormone stimulated transcriptional activity of the Cx43 promoter, with a time-course compatible with the steady-state mRNA data, and its action was reproduced by 8Br-cAMP (Fig. 4). Therefore, the stimulatory effect of PTH on Cx43 mRNA is most likely the consequence of transcriptional up-regulation of Cx43 gene promoter.

To verify that changes in Cx43 mRNA abundance resulted in the expected changes in the quantity of Cx43 protein, immunoblots were performed after incubation with either PGE_2 or PTH in whole cell lysates and after alkali extraction, a method that allows enrichment of gap junction proteins. In whole cell lysates, one faint band migrating at around 40 kD was detected in the UMR 106-01 cells, and the intensity of this band increased after treatment with PTH for 4 h or longer without apparent shifts of its electrophoretic mobility (Fig. 5). Based on previous observations [Civitelli et al., 1993; Steinberg et al., 1994], this band corresponds to nonphosphorylated Cx43. Two slower migrating bands of approximate m.w. of 41 kD and 43 kD, corresponding to phosphorylated species of Cx43 [Steinberg et al., 1994] were clearly present in extracts of ROS 17/2.8 cells (Fig. 4). These bands were barely visible in the UMR 106-01 lanes, compatible with the low abundance of Cx43 produced by these cells [Steinberg et al., 1994]. The increase in Cx43 abundance stimulated by PTH was evident after incubations for 4 h or longer, a result consistent with the mRNA data. These findings strengthen the notion that PTH-induced changes of Cx43 protein are delayed compared to the increase in dye coupling. Treatment with PGE_2 had similar effect on Cx43 protein abundance in whole cell lysates of UMR 106-01 cells (Fig. 6). Thus, Cx43-specific bands were more intense, though still faint, after 4-h exposure to PGE_2 compared to baseline. Notably, no changes were detected after 30 min treatment with the prostanoid. In contrast, the intensity of Cx43-specific bands was much stronger in plaque-purified material than in whole cell lysates. In this preparation, PGE_2 treatment increased Cx43 abundance as early as 30 min after cell exposure to the prostanoid, and the effect was maximal after 4 h of incubation (Fig. 6). All Cx43-specific bands were more intense after PGE_2 treatment, but the relative intensity of each band was unchanged. In both whole cell and alkali-extracted preparations, Cx43 protein levels returned to baseline after 24 h.

These results suggest that the initial, rapid phase of PGE_2 and PTH stimulation of cell

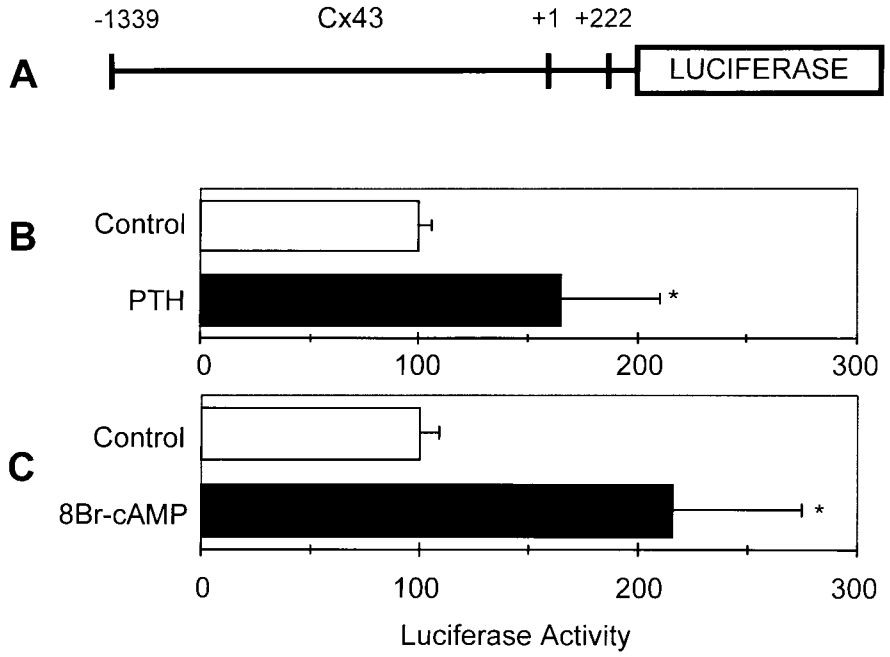

Fig. 4. Transcriptional regulation of Cx43 by PTH and 8Br-cAMP. **A:** Structure of the Cx43 promoter/luciferase construct. A DNA fragment of the rat Cx43 gene including 1,339 bases 5′ from the transcriptional start site (+1) and 222 bases 3′ (including all of exon 1) was subcloned upstream of the reporter gene luciferase and used for co-transfections with an RSV-β-galactosidase control plasmid. **B:** Effect of PTH on Cx43 promoter activity. Triplicate cultures of UMR 106-01 cells were treated with 10^{-7} M PTH or vehicle alone (controls) for the last 6 h of a 48 h transfection. Luciferase activity was determined and normalized to β-galactosidase activity from a cotransfected RSV-β-galactosidase plasmid. Data are presented as percent of control, and represent the mean ± SD from five independent experiments. **C:** Effect of 8-Bromo-cAMP on Cx43 promoter activity. Triplicate cultures of UMR 106-01 cells were treated with 10^{-3} M 8-BrcAMP or vehicle alone (controls) for the last 6 h of a 48 h transfection using the same conditions described for PTH. Data represent the mean ± SD from three independent experiments.

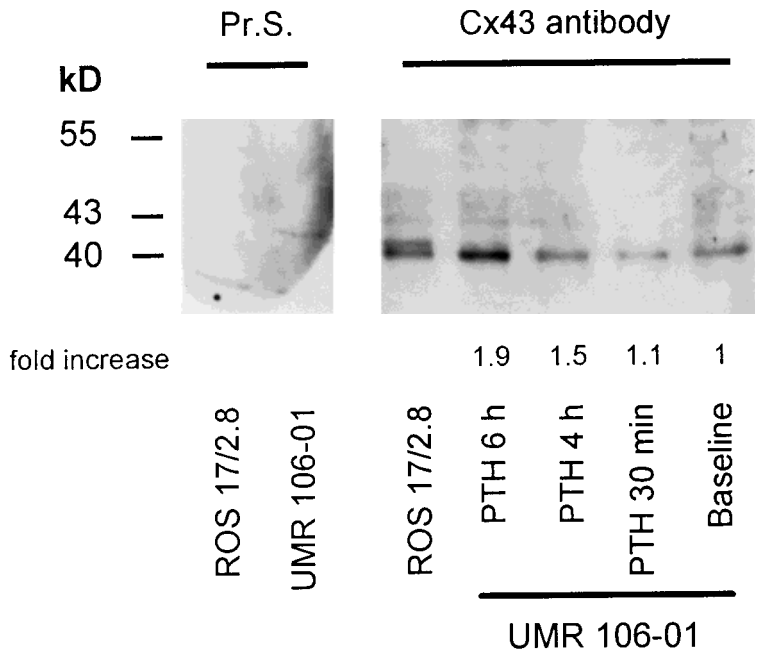

Fig. 5. PTH increases Cx43 protein levels in UMR 106-01 cells. Cells grown to confluence were incubated with PTH (10^{-7} M) for the indicated time, and the protein extract were subjected to PAGE, before blotting with either a Cx43-specific antibody, or preimmune serum (Pr. S.), as indicated. Lanes with extracts from ROS 17/2.8 osteogenic sarcoma cells are also shown for comparison.

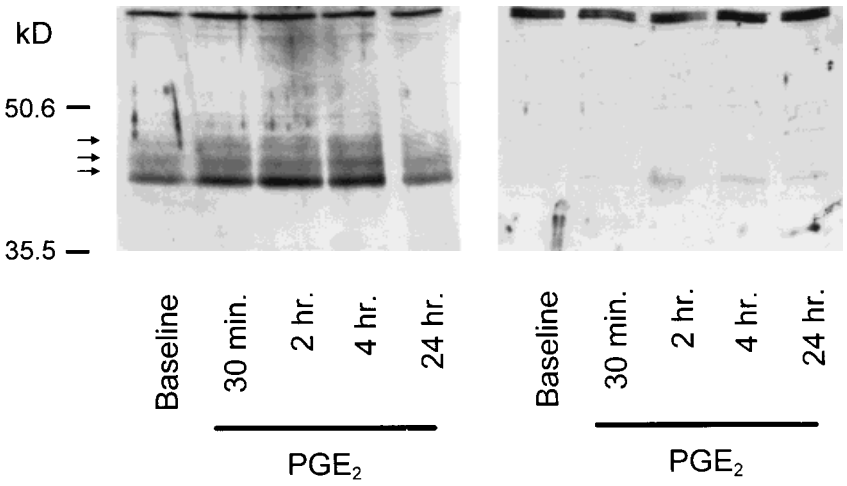

Fig. 6. PGE_2 alters Cx43 protein abundance in UMR 106-01 cells. Cells grown to confluence were incubated with PGE_2 (10^{-6} M) for the indicated time. Alkali-extracted material (left) or either whole cell lysates (right) were blotted with a Cx43-specific antibody.

coupling is independent of *de novo* synthesis of Cx43; whereas for prolonged incubation the stimulatory effect is sustained by increased Cx43 mRNA and protein expression. To further test this hypothesis and to verify that these effects can be reproduced by cAMP stimulation, dye coupling was assessed after treatment with PTH or forskolin in the presence or in the absence of the protein synthesis inhibitor, cycloheximide. As shown in Figure 7, cycloheximide did not significantly affect PTH nor forskolin stimulation of calcein diffusion between UMR 106-01 cells after short, 30 min incubation. Notably, the effect of PTH at this early time point was statistically significant in these experiments, where more than 350 parachuted cells were scored. On the contrary, the effect of PTH was almost completely abolished by cycloheximide, after prolonged, 2-h incubation, whereas forskolin stimulation of calcein diffusion was reduced by approximately 50% (Fig. 7).

The results of the previous experiments are compatible with the hypothesis that the early actions of PGE_2, PTH, and forskolin on gap junctional communication may be related to a redistribution of existing Cx43 to gap junction plaques, as it had been suggested by a previous electron microscopy study on PGE_2-stimulated rat calvaria cells [Shen et al., 1986], and in breast cancer cells after exposure to cAMP analogs [Atkinson et al., 1995]. We thus analyzed the cellular localization of Cx43 by immunofluorescence after incubation with the two agonists. As shown in Figure 8A, very little punctate Cx43 immunostaining was visible in the poorly coupled UMR 106-01 cells in resting conditions. When the cells were exposed to PGE_2 for 30 min, Cx43-specific staining was clearly enhanced at appositional membranes (Fig. 8C). The increased Cx43 immunoreactivity was maintained for at least 3 h of incubation with the prostaglandin (Fig. 8D). PTH incubation also resulted in an increased Cx43-specific immunostaining at areas of cell-cell contact between UMR 106-01 cells, an effect clearly evident after longer (2 h) incubation times (Fig. 8B).

DISCUSSION

By applying a new noninvasive method to assess dye coupling, we herein demonstrate that PGE_2 and PTH, two critical factors involved in systemic and local control of bone remodeling, rapidly increase gap junctional permeability to negatively charged molecules in osteoblastic cells. The data also suggest that this effect is the result of a dual mechanism of gap junction regulation, including both redistribution of preformed connexin proteins, and stimulation of Cx43 gene expression.

The "parachute assay" reports the same biological phenomenon as the commonly used microinjection methods. However, it greatly simplifies the procedure and allows a far larger number of cells to be examined for dye diffusion. The ability to finely control the experimental conditions (most of the procedural interventions are performed in the tissue culture

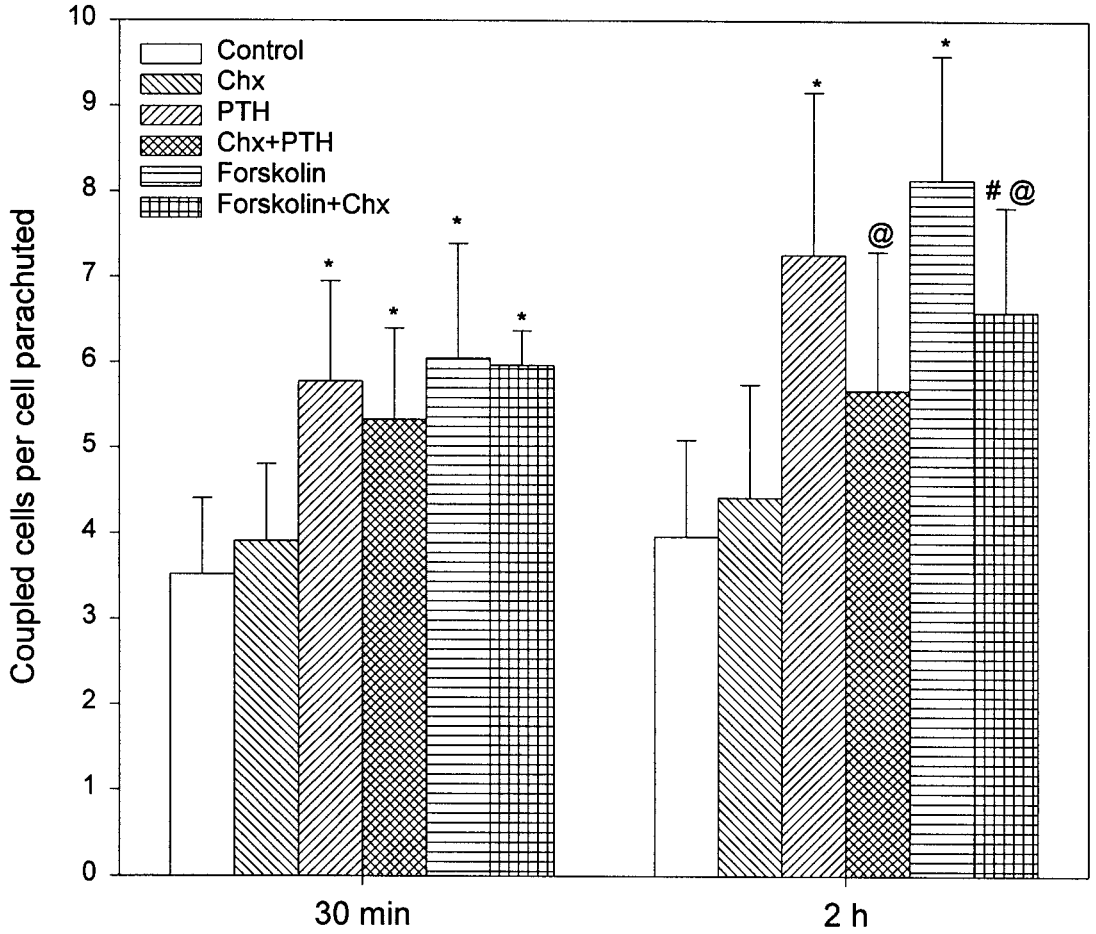

Fig. 7. Effect of cycloheximide (Chx) on PTH and forskolin stimulation of dye coupling in UMR 106-01 cells. Calcein-loaded UMR 106-01 cells were added on top of a nonloaded confluent culture, along with Chx (10^{-5} M) or its vehicle (DMSO). PTH or forskolin were subsequently added 30 min and 2 h before the end of a 2½ h total incubation after parachuting of the donor cells. The number of coupled cells per cell parachuted was then assessed. Data represent the average and SD of seven individual coverslips. Between 50 and 60 donor cells were scored for each condition, in each experiment. Symbols indicate significant difference vs. control (*); vs. Chx (#); and vs. PTH or forskolin (@) at the 0.05 level (Student's *t*-test).

incubator rather than on the stage of a microscope) and incubation times led, in these experiments, to the detection and quantitation of changes in gap junctional communication effected by PGE_2, PTH, and cAMP stimulators as early as 30 min after exposure to the agonist. The use of microinjection methods is problematic and very time consuming for quantitation of dye coupling within short time frames. Application of the parachute assay also disclosed a higher degree of dye coupling between UMR 106-01 cells that it had been previously found using microinjection techniques [Donahue et al., 1995; Steinberg et al., 1994; Schiller et al., 1992]. The reasons for this apparent discrepancy are not immediately obvious, but it is likely that the gap junctions present in UMR 106-01 cells are sensitive to mechanical manipulation, so that disruption of the cell membrane caused by cell impalement results in decreased permeability. In addition to Cx43, UMR 106-01 cells express connexin45 (Cx45) on their surface [Steinberg et al., 1994]. Although gap junctions formed by Cx45 are poorly permeable to negatively charged dyes [Veenstra et al., 1994], Elfgang et al. [1995] have recently found that mouse Cx45 overexpressed in HeLa cells could pass Lucifer yellow injected iontophoretically, although to a lesser degree than Cx43 gap junctions could. Thus, is it possible that the higher dye coupling we observed in UMR 106-01 cells using the parachute assay as compared to Lucifer yellow microinjection may be contributed to by Cx45 channels, whose function is perhaps more sensitive to physical disruption of the cell membrane than Cx43 channels are. The contri-

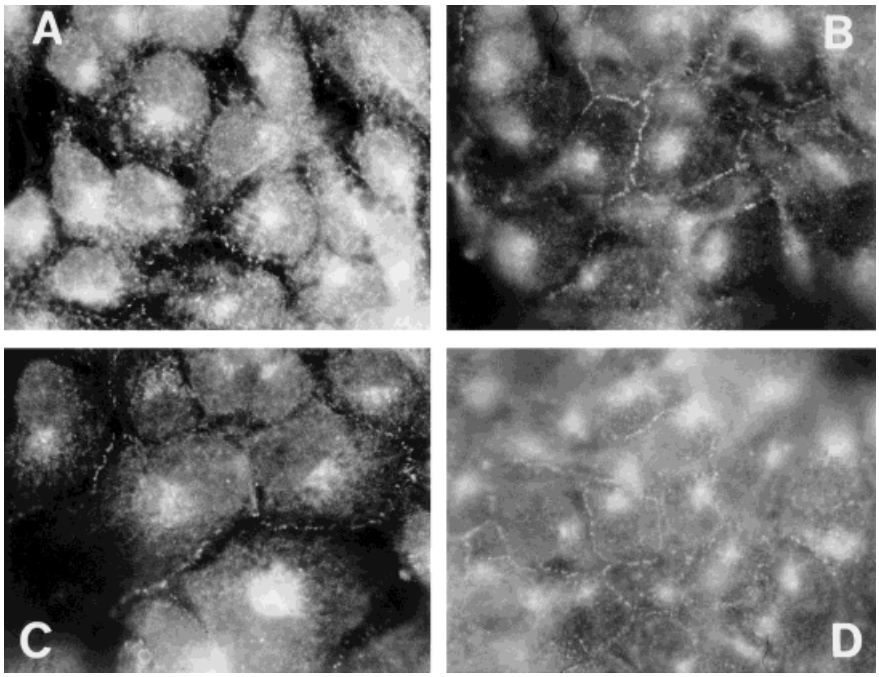

Fig. 8. PGE_2 and PTH increase the abundance of cell-surface associated Cx43. Subconfluent cultures of UMR 106-01 cells grown on glass coverslips were fixed and immunostained with an anti-Cx43 antibody in resting conditions (**A**), or after incubation with either PTH (10^{-7} M) for 4 h (**B**); or PGE_2 (10^{-6} M) for 30 min (**C**), or 4 h (**D**).

bution of Cx45 to PGE_2 and PTH regulation of cell coupling is currently under investigation.

Shen et al. [1986] observed a rapid (within 20 min) appearance of gap junctions between fetal rat calvaria osteoblasts during incubation with either PGE_2 or PGE_1 by electron microscopy. They proposed that prostanoids promote the assembly of preformed proteins into gap junction channels. Our results confirm and extend these observations, demonstrating that PGE_2 increases gap junctional communication between osteoblastic cells, but without changes in total Cx43 protein levels in whole cell extracts. Earlier work demonstrated that gap junctions may in fact be induced to aggregate without active protein synthesis [Ne'eman et al., 1980; Epstein et al., 1977], a phenomenon that has been recently described in more detail in mammary tumor cells stimulated by cAMP analogs [Atkinson et al., 1995]. This effect of cAMP occurred as early as 30 min of pharmacologic treatment, a time-course comparable to our findings. Because we found substantially greater abundance of Cx43 protein on the surface of UMR 106-01 cells and in plaque-enriched, alkali-extracted material after a short, 30 min exposure to PGE_2, we speculate that PGE_2, via cAMP production, may induce redistribution of preformed Cx43 into functional gap junctions on the plasma membrane of osteoblasts as a rapid response to PGE_2. With time, increased Cx43 protein synthesis is required for sustained enhanced gap junctional communication, as suggested by PGE_2 stimulation of Cx43 mRNA and protein abundance after 2–4 h of incubation.

A similar two-phase mechanism appears to be involved in PTH action. Like PGE_2, PTH also produced an early, rapid effect on gap junctional communication independent of new protein synthesis, whereas new protein synthesis was required to maintain the enhanced dye coupling later in time. Again, a stimulatory action of PTH on Cx43 mRNA and protein was detected at later time-points, most likely the result of transcriptional up-regulation of Cx43 gene expression. In theory, this two-phase regulatory mechanism provides the means of both rapid and sustained responses to hormonal modulators, so that gap junctional intercellular communication can be increased immediately after exposure to the agonist, and remain up-regulated for as long as the hormonal stimulus is applied. In this view, the increased amount of Cx43 required at the cell membrane is initially met by redistribution of preformed protein, and

the intracellular pool is later replenished by an increased rate of Cx43 synthesis.

Stimulation of gap junctional communication and Cx43 mRNA expression by PTH is not restricted to UMR 106-01 cells [Donahue et al., 1995; Schiller et al., 1992]. We and others have obtained similar results in other human osteogenic sarcoma [Schiller et al., 1992; Civitelli, 1997] and rat calvaria cells [Donahue et al., 1995; Schiller et al., 1992], with some cell type-dependent variability. The present work defines the multiple molecular mechanisms that account for this action of PTH on osteoblastic cells. Our data also suggest that stimulation of gap junctional communication is mediated primarily by activation of adenylate cyclase, a signal transduction pathway common to both PTH and PGE_2 [Fujimori et al., 1992; Farndale et al., 1988; Yamaguchi et al., 1988]. The direct demonstration that PTH and cAMP stimulate Cx43 promoter activity provides the molecular mechanisms for the increased steady-state mRNA and Cx43 protein levels observed after prolonged PTH stimulation. These findings extend the results of Schiller et al. [1992], who reported an increased rate of Cx43 mRNA production in the same cell line exposed to PTH. Sequences corresponding to AP-1 and AP-2 sites, as well as a cAMP-binding protein response element have been identified in the proximal 5′ region of the Cx43 promoter [Lefebvre et al., 1995; Yu et al., 1994], and these are possible targets for the regulatory action of PTH. Therefore, while the mechanisms leading to the rapid increase of gap junction function remain unclear, the signals triggered by both PTH and PGE_2 may converge into the cAMP pathway for regulation of Cx43 expression and cellular localization. Analogs of cAMP and phorbol esters can alter the phosphorylation pattern of Cx43 in different cell types [Sáez et al., 1993], and this in turn may affect gap junction function [Sáez et al., 1993; Stagg and Fletcher, 1990]. In our experiments, neither PGE_2 nor PTH produced significant changes in the pattern of Cx43 phosphorylation, despite the increase in Cx43 protein abundance. Thus, Cx43 phosphorylation does not seem to play a significant role in PGE_2, or PTH regulation of gap junctional communication in osteoblasts.

Both PTH and PGE_2 are physiologic regulators of osteoblast function and mineral metabolism. PTH is a potent stimulator of bone resorption, but when administered intermittently it stimulates bone formation [Tam et al., 1982]. Likewise, although prostaglandins had been originally identified as stimulators of bone resorption, under certain conditions PGE_2 can also stimulate production of new trabecular bone [Li et al., 1990; Mori et al., 1992]. The possibility that regulation of intercellular communication between osteoblasts may characterize the "anabolic" action of PTH, and perhaps PGE_2 is intriguing and potentially important for clinical application. *In vivo* studies indicate that the increase in bone formation observed during intermittent treatment with PTH is associated with an increased number of actively secreting osteoblasts [Dobnig and Turner, 1995; Hodsman et al., 1991; Tam et al., 1982]. Conceivably, an increase of intercellular communication within bone remodeling units may enhance the properties of the osteoblastic layer to work as a "functional syncytium," and thus contribute to the anabolic effect of PTH on bone. Consistent with this hypothesis, we have recently found that Cx43 permeability is required for basal expression of genes pivotal to osteoblast maturation and function [Lecanda et al., 1996]. Thus, increased cell-to-cell communication may be part of the stimulatory action of anabolic agent on bone forming cells.

Enhanced communication competence among bone cells may also facilitate the diffusion of locally generated signals, such as mechanical strain, and thus regulate the sensitivity of bone to hormonal and physical factors. The recent demonstration that osteoblast responsiveness to PTH is blunted when gap junctional communication mediated by Cx43 is disrupted [Van der Molen et al., 1996] seems to corroborate this hypothesis. Furthermore, direct intercellular communication may allow transmission of hormonal signals from hormone responsive to unresponsive cells, as demonstrated in experiments with mixed cell populations [Lawrence et al., 1978]. For example, although PTH is antimitogenic in osteogenic sarcoma cells [Reid et al., 1988], in primary cultures the hormone may stimulate proliferation of osteoblasts [Van der Plas et al., 1985; McDonald et al., 1986] and periosteal fibroblasts, which represent osteoprogenitor cells [Van der Plas and Nijweide, 1988]. In the latter system, physical contact between osteoblasts and osteoprogenitor cells was required, since PTH could not directly stimulate cAMP in periosteal fibroblasts [Van der Plas and Nijweide, 1988]. Signal transmission

through direct cell-cell contact and/or gap junctional communication was postulated as a mechanism of the indirect effect of PTH on osteoprogenitor cell proliferation.

In summary, we have established that gap junctional communication between osteoblasts can be regulated at different levels by modulators of bone remodeling. These results provide an additional mechanism which may be involved in the actions of PTH and PGE_2 on osteoblast function and skeletal physiology.

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REFERENCES

Atkinson MM, Lampe PD, Lin HH, Kollander R, Li X, Kiang DT (1995): Cyclic AMP modifies the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. J Cell Sci 108:3079–3090.

Beyer EC, Paul DL, Goodenough DA (1987): Connexin43: A protein from rat heart homologous to a gap junction protein from liver. J Cell Biol 105:2621–2629.

Beyer EC, Steinberg TH (1991): Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. J Biol Chem 266:7971–7974.

Bradford M (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254.

Chen Z-Q, Lefebvre D, Bai X, Reaume AG, Rossant J, Lye SJ (1994): Identification of two regulatory elements within the promoter region of the mouse connexin43 gene. J Biol Chem 270:3863–3868.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocianate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Civitelli R, Beyer EC, Warlow PM, Robertson AJ, Geist ST, Steinberg TH (1993): Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. J Clin Invest 91:1888–1896.

Civitelli R (in press): Cell-cell communication in bone. In Zaidi M (ed): "Molecular and Cellular Biology of Bone." Greenwich, CT: JAI Press, Inc.

Dobnig H, Turner RT (1995): Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 136:3632–3638.

Donahue HJ, McLeod KJ, Rubin CT, Andersen J, Grine EA, Hertzberg EL, Brink PR (1995): Cell-to-cell communication in osteoblastic networks: Cell line-dependent hormonal regulation of gap junction function. J Bone Miner Res 10:881–889.

Doty SB (1981): Morphological evidence of gap junctions between bone cells. Calcif Tissue Int 33:509–511.

Elfgang C, Eckert R, Lichtenberg-Fraté H, Butterwerk A, Traub O, Klein RA, Hülser DF, Willecke K (1995): Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. J Cell Biol 129:805–817.

Epstein ML, Sheridan JD, Johnson RG (1977): Formation of low-resistance junctions *in vitro* in the absence of protein synthesis and ATP production. Exp Cell Res 104:25–30.

Eustice DC, Feldman PA, Colberg-Poley AM, Buckery RM, Neubauer RH (1991): Sensitive method for the detection of β-galactosidase in transfected mammalian cells. Biotechniques 11:739–742.

Farndale RW, Sandy JR, Atkinson SJ, Pennington SR, Meghi S, Meikle MC (1988): Parathyroid hormone and prostaglandin E_2 stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblast cultures. Biochem J 252:263–268.

Forrest SM, Ng KW, Findlay DM, Michelangeli VP, Livesey SA, Partridge NC, Zajac JD, Martin TJ (1985): Characterization of an osteoblast-like clonal cell line which responds to both parathyroid hormone and calcitonin. Calcif Tissue Int 37:51–56.

Fujimori A, Cheng S, Avioli LV, Civitelli R (1992): Structure-function relationship of parathyroid hormone: Activation of phospholipase C, protein kinase A and C in osteosarcoma cells. Endocrinology 130:29–36.

Hodsman AB, Steer BM, Fraher LJ, Drost DJ (1991): Bone densitometric and histomorphometric responses to sequential human parathyroid hormone (1–38) and salmon calcitonin in osteoporotic patients. Bone Min 14:67–83.

Jeasonne BG, Faegin FA, McMinn RW, Shoemaker RL, Rehm WS (1979): Cell-to-cell communication of osteoblasts. J Dent Res 58:1415–1419.

Koval M, Geist ST, Westphale EM, Kemendy AE, Civitelli R, Beyer EC, Steinberg TH (1995): Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43. J Cell Biol 130:987–995.

Lawrence TS, Beers WH, Gilula NB (1978): Transmission of hormonal stimulation by cell-to-cell communication. Nature (Lond) 272:501–506.

Lecanda F, Towler DA, Cheng S, Steinberg TH, Civitelli R (1996): Osteocalcin (OC) and bone sialoprotein (BSP) gene expression is transcriptionally regulated by gap junctional permeability. J Bone Miner Res 11:S122.

Lefebvre D, Piersanti M, Bai X, Chen Z, Lye SJ (1995): Myometrial transcriptional regulation of the gap junction gene, connexin43. Reprod Fertil Dev 7:603–611.

Li XJ, Jee WSS, Patterson-Buckendahl P (1990): Transient effects of subcutaneously administered prostaglandin E_2 on cancellous and cortical bone in young adult dogs. Bone 11:353–364.

Majeska RJ, Rodan SB, Rodan GA (1980): Parathyroid-hormone responsive clonal cell lines from rat osteosarcoma. Endocrinology 107:1494–1503.

McDonald BR, Gallagher JA, Russell RGG (1986): Parathyroid hormone stimulates the proliferation of cells derived from human bone. Endocrinology 118:2445–2449.

Mori S, Jee WSS, Li XJ (1992): Production of new trabecular bone in osteopenic ovariectomized rats by prostaglandin E_2. Calcif Tissue Int 50:80–87.

Narbaitz R, Stumpf WE, Sar M, Huang S, DeLuca HF (1983): Autoradiographic localization of target cells for 1,25-dihydroxyvitamin D_3 in bones from fetal rats. Calcif TIssue Int 35:177–182.

Ne'eman Z, Spira ME, Bennett MVL (1980): Formation of gap and tight junctions between reaggregated blastomeres of the killifish. Am J Anat 158:251–262.

Palumbo C, Palazzini S, Marotti G (1990): Morphological study of intercellular junctions during osteocyte differentiation. Bone 11:401–406.

Partridge NC, Alcorn D, Michelangeli VP, Ryan G, Martin TJ (1983): Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. Cancer Res 43:4308–4314.

Reid LR, Civitelli R, Avioli LV, Hruska KA (1988): Parathyroid hormone depresses cytosolic pH and DNA synthesis in osteoblast-like cells. Am J Physiol 255:E9–E15.

Rodan GA (1996): Coupling of bone resorption and formation during bone remodeling. In Marcus R, Feldman D, Kelsey J (eds): "Osteoporosis." San Diego, CA: Academic Press, 289–299.

Sáez JC, Berthoud VM, Moreno AP, Spray DC (1993): Gap junctions: Multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. Adv Second Messenger Phosphoprotein Res 27:163–198.

Schiller PC, Mehta PP, Roos BA, Howard GA (1992): Hormonal regulation of intercellular communication: Parathyroid hormone increases connexin43 gene expression and gap-junctional communication in osteoblastic cells. Mol Endocrinol 6:1433–1440.

Schirrmacher K, Schmitz I, Winterhager E, Traub O, Brummer F, Jones D, Bingmann D (1992): Characterization of gap junctions between osteoblast-like cells in culture. Calcif Tissue Int 51:285–290.

Shen V, Rifas L, Kohler G, Peck WA (1986): Prostaglandins change cell shape and increase intercellular gap junctions in osteoblasts cultured from rat fetal calvaria. J Bone Miner Res 1:243–249.

Silve CM, Hradek GT, Jones AL, Arnaud CD (1982): Parathyroid hormone receptor in intact embryonic chicken bones: characterization and cellular localization. J Cell Biol 94:379–386.

Stagg RB, Fletcher WH (1990): The hormone-induced regulation of contact-dependent cell-cell communication by phosphorylation. Endocr Rev 11:302–325.

Steinberg TH, Civitelli R, Geist ST, Robertson AJ, Hick E, Veenstra RD, Wang H, Warlow PM, Westphale EM, Laing JG, Beyer EC (1994): Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. EMBO J 13:744–750.

Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Mosley JM, Martin TJ, Suda T (1988): Osteoblastic cells are involved in osteoclast formation. Endocrinology 123:2600–2603.

Tam CS, Heersche JNM, Murray TM, Parsons JA (1982): Paratyhroid hormone stimulates the bone apposition rate independently of its resorptive action: Differential effects of intermittent and continuous administration. Endocrinology 110:506–512.

Van der Molen MA, Rubin CT, McLeod KJ, McCauley LK, Donahue HJ (1996): Gap junctional intercellular communication contributes to hormonal responsiveness in osteoblastic networks. J Biol Chem 271:12165–12171.

Van der Plas A, Feyen JHM, Nijweide PJ (1985): Direct effect of parathyroid hormone on the proliferation of osteoblast-like cells; A possible involvement of cyclic AMP. Biochem Biophys Res Commun 129:918–925.

Van der Plas A, Nijweide PJ (1988): Cell-cell interactions in the osteogenic compartment of bone. Bone 9:107–111.

Veenstra RD, Wang H, Beyer EC, Brink PR (1994): Selective dye and ionic permeability of gap junction channels formed by connexin45. Circ Res 75:483–490.

Yamaguchi DT, Hahn TJ, Beeker TG, Kleeman CR, Muallem S (1988): Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR 106-01 cells. J Biol Chem 263:10745–10753.

Yamaguchi DT, Ma D, Lee A, Huang JT, Gruber HE (1994): Isolation and characterization of gap junctions in the osteoblastic MC3T3-E1 cell line. J Bone Miner Res 9:791–803.

Yu W, Dahl G, Werner R (1994): The connexin43 gene is responsive to estrogen. Proc Roy Soc London 255:125–132.