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Receptors for the Carboxyl-Terminal Region of PTH(1–84) Are Highly Expressed in Osteocytic Cells*

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ABSTRACT

PTH is a potent systemic regulator of cellular differentiation and function in bone. It acts upon cells of the osteoblastic lineage via the G protein-coupled type-1 PTH/PTH-related peptide receptor (PTH1R). Carboxyl fragments of intact PTH(1–84) (C-PTH fragments) are cosecreted with it by the parathyroid glands in a calcium-dependent manner and also are generated via proteolysis of the hormone in peripheral tissues. Receptors that recognize C-PTH fragments (CPTHRs) have been described previously in osteoblastic and chondrocytic cells. To directly study CPTHRs in bone cells, we isolated clonal, conditionally transformed cell lines from fetal calvarial bone of mice that are homozygous for targeted ablation of the PTH1R gene and transgenically express a temperature-sensitive mutant SV40 T antigen. Cells with the highest specific binding of the CPTHR radioligand ^{125}I-[Tyr^{34}]hPTH(19–84) exhibited a stellate, dendritic appearance suggestive of an osteocytic phenotype and expressed 6- to 10-fold more CPTHR sites/cell than did osteoblastic cells previously isolated from the same bones. In these osteocytic (OC) cells, expression of mRNAs for CD44, connexin 43, and osteocalcin was high, whereas that for alkaline phosphatase and cbfa-1/osf-2 was negligible. The CPTHR radioligand was displaced completely by hPTH(1–84), hPTH(19–84) and hPTH(24–84) (IC_{50}s = 20–50 nM) and by hPTH(39–84) (IC_{50} = 500 nM) but only minimally (24%) by 10,000 nM hPTH(1–34). CPTHR binding was down-regulated dose dependently by hPTH(1–84), an effect mimicked by ionomycin and active phorbol ester. Human PTH(1–84) and hPTH(39–84) altered connexin 43 expression and increased apoptosis in OC cells. Apoptosis induced by PTH(1–84) was blocked by the caspase inhibitor DEVD. We conclude that osteocytes, the most abundant cells in bone, may be principal target cells for unique actions of intact PTH(1–84) and circulating PTH C-fragments that are mediated by CPTHRs. (*Endocrinology* **142:** 916–925, 2001)

PTH IS THE major physiologic regulator of blood calcium and phosphate, and it exerts potent effects upon cells in bone and cartilage (1). PTH and PTH-related peptide (PTHrP) activate a common G protein-coupled receptor, the type-1 PTH/PTHrP receptor (PTH1R) (2–4). The PTH1R recognizes the highly conserved amino (N)-terminal domain of PTH (and the homologous N terminus of PTHrP) and thus is fully activated by both PTH(1–34) and the intact hormone, PTH(1–84). Carboxyl(C)-fragments of intact PTH(1–84), such as PTH(39–84) or PTH(53–84), neither bind nor activate the PTH1R (5–7). On the other hand, a possible physiologic role for this region of the hormone is suggested by observations that the amino acid sequence of the PTH C-terminal domain is highly homologous across species (8); that PTH C-fragments, arising via both secretion from the parathyroid glands and proteolysis of PTH(1–84) in peripheral tissues, circulate in blood at levels much higher than those of the intact hormone; and that parathyroid secretion of PTH C-fragments is strongly regulated by serum calcium (9–13).

In rat osteoblastic cell lines, fragments from within the sequence PTH(35–84), which cannot activate PTH1Rs, regulate expression of alkaline phosphatase, osteocalcin, collagen α1(I), and IGF binding protein-5 (14–16). Direct effects of PTH C-fragments upon osteoclasts and osteoclast progenitors (17) and upon expression of collagen α1(I) and α1(X) expression in hypertrophic chondrocytes (18) also have been observed. Several such fragments induce cytosolic free calcium transients in human fetal hypertrophic chondrocytes (19). Direct physical evidence of a putative receptor (CPTHR) with binding specificity for C-terminal PTH sequences was obtained by cross-linking of the peptide ^{125}I-[Tyr^{34}]hPTH(19–84) (which does not bind to PTH1Rs) to 40-kDa and 90-kDa proteins in ROS 17/2.8 rat osteoblastic cells (6).

Collectively, these observations suggest that CPTHRs are expressed normally in bone and cartilage and that they may be involved in physiologic control of cell differentiation and function in these tissues. The problem of renal osteodystrophy is of particular interest in this regard, as PTH C-fragments normally are cleared mainly by the kidneys and thus accumulate to very high levels in blood during renal failure (7, 9, 12, 13). Also, hPTH (7–84) potently reduces the calcemic action of intact PTH(1–84) at concentrations much lower than those required for binding to the PTH1R (20).

To establish a model system in which to address the possible functions of CPTHRs in bone, we isolated, from cultures of enzymatically dispersed primary fetal murine calvarial cells, those that expressed CPTHRs in greatest abundance. To eliminate confounding effects of coexpressed PTH1Rs, these cells were derived from fetuses in which most exons encoding the PTH1R had been completely ablated by gene targeting (21, 22). These homozygous mice also were bred to ubiquitously express a transgene encoding a temperature-sensitive mutant SV40 large-T antigen (tsTAg), which

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enabled isolation of conditionally transformed clonal cell lines (21, 22). We report here that the subpopulation of calvarial-derived bone cells that expresses the highest levels of CPTHRs exhibits morphologic and molecular features characteristic of osteocytes, the most terminally differentiated cells of the osteoblast lineage (23, 24). Initial findings indicate that activation of CPTHRs may play a role in regulating osteocytic cell survival and intercellular communication.

Materials and Methods

Materials

Culture media were obtained from the Media Kitchen (Pediatric Surgery, Massachusetts General Hospital, Boston, MA); other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), 1,2-bis (2-aminophenoxy) ethane-tetraacetic acid (BAPTA-AM) and other reagents and chemicals were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Radioactive Na[^{125}I] and ^{32}P-dATP were purchased from NEN Life Science Products (Boston, MA). Recombinant human (h)PTH(1–84), [Tyr^{34}]hPTH(19–84), hPTH(24–84), were gifts of Chugai Pharmaceutical Co. (Shizuoka, Japan) and [Asp^{76}]human PTH(39–84) was purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Cell isolation and culture

Cells were isolated by enzymatic digestion from calvarial bones of 18.5 day-old tsA58(+)/PTH1R(−/−) fetuses, as previously described (21). Animals were maintained in facilities operated by the Massachusetts General Hospital Center for Comparative Resources in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the Institutional Animal Care and Use Committee.

The calvarial bones were dissected aseptically and sequentially digested in 0.5 ml of α-MEM containing 0.1% BSA, 1 mM $CaCl_2$ and 1 mg/ml of collagenase (type I and II; ratio 1:3) (Worthington Biochemical Corp., Freehold, NJ). Bones were sequentially digested six times for 20 min each at 37 C on a rocking platform at 90 oscillations per minute under 5% CO_2 in air. Cells were cultured at 33 C in a humidified atmosphere (95% air/5% CO_2) using growth medium [α-MEM containing 10% FBS (lot no. 1011961 Life Technologies, Inc.) and 1% penicillin-streptomycin (PS)].

HeLa and BHK21 cells were obtained from Dr. Joel Habener (Molecular Endocrine Unit, Massachusetts General Hospital, Boston, MA) and NIH-3T3 cells were provided by Dr. Gino Segre (Endocrine Unit, Massachusetts General Hospital, Boston, MA). MS-1 cells are clonal conditionally immortalized murine bone marrow stromal cells previously isolated in our laboratory (25).

Radioligand binding

The [Tyr^{34}]hPTH(19–84) peptide was radioiodinated with Na[^{125}I] (2000 Ci/mmol) by the chloramine-T method and purified by HPLC, as previously described (6). For binding experiments, cells were plated in 24-well dishes at 100,000 cells/ml and cultured at 33 C for 7–14 days. Confluent monolayers then were washed with 0.5 ml binding buffer [100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.8) plus 5% heat-inactivated horse serum] before incubation with ^{125}I-[Tyr^{34}]hPTH(19–84) (100,000–200,000 cpm/well) in 0.5 ml binding buffer for 4 h at 15 C. Receptor number was ascertained by Scatchard analysis, using [Tyr^{34}]hPTH(19–84) or hPTH(1–84) as competing ligand. Cellular protein was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) and was found to average 0.33 mg/10^6 cells and 0.47 mg/10^6 cells for F cells and C cells, respectively. In some experiments, cells were washed with acidic buffer (50 mM glycine, 150 mM NaCl, pH 4.0) to remove residual bound ligand, before addition of radioligand.

cAMP accumulation

Cells were rinsed twice with assay buffer (135 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2.8 mM glucose, 1.2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4) and then incubated for 15 min at 37 C with the same buffer containing 0.1% heat inactivated BSA, 1 mM isobutylmethylxanthine (IBMX), and agonist, conditions under which cAMP accumulation was found to be linear with time for at least 15 min. The buffer then was rapidly aspirated, the plates were frozen in liquid nitrogen, and the frozen cells subsequently were thawed directly into 0.5 ml of 50 mM HCl. Cell-associated cAMP in the acid extracts was measured using an RIA kit (NEN Life Science Products). Results were expressed as picomoles of cAMP produced per well over 15 min.

Northern blot and RT-PCR analysis

Cells were plated in 10-cm dishes and cultured at the appropriate temperature in growth medium. Total RNA was extracted using the TRIreagent method (Sigma) and quantified by UV absorbance. Fifteen micrograms of total RNA was separated by 1% agarose-gel electrophoresis in the presence of formaldehyde and transferred to a nylon membrane (Bio-Rad Laboratories, Inc. Hercules, CA) using standard procedures. Membranes were hybridized with ^{32}P-labeled cDNA probes labeled by random primer extension, including the α1 subunit of human type I collagen, mouse osteopontin, mouse alkaline phosphatase, rat osteocalcin, mouse connexin 43 (Cx43), cbfa-1/osf-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Rat osteocalcin was a gift of Dr. Marie Demay, mouse Cx43 was a gift of Dr. David Paul and cbfa-1/osf-2 was a gift of Dr. Gerard Karsenty.

RT-PCR was performed on total RNA using the SuperScript Preamplification System from Life Technologies, Inc. with a Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). The following pairs of primers were used for the cDNA amplification: CD44: 5′-CAAGTTTTGGTGGCACACAGC, 3′-GGTTAAGGAAGCTACCTGGC; mouse cbfa-1/osf-2; 5′-TGGAAGGGATGAAAGGCTGC, 3′-CGGTGTTTACCACAGCAGGT and alkaline phosphatase 5′-CCGTTCTTTCTCTATTC, 3′-ACAGATGGGTCCCACCG as previously reported (24, 25). The annealing temperature for CD44 and cbfa1/osf2 was 58 C, and the reaction was repeated for 30 cycles; for alkaline phosphatase the annealing temperature was 54 C, and the reaction was repeated for 35 cycles. PCR products were electrophoresed on 1.5% agarose gels and visualized using ethidium bromide staining.

Von Kossa staining

Cells, plated in six-well dishes, were maintained at 33 C for 3–4 days, until confluent, before refeeding with fresh medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid and transferring to nonpermissive conditions (39 C). Cells were refed with fresh medium twice per week. After 3–4 weeks, the presence of mineralized nodules was assessed by von Kossa staining, as previously described (21). Briefly, after fixation in 95% ethanol for 15 min at 37 C, cells were gradually rehydrated with water and stained with 5% silver-nitrate for 1 h at 37 C. Cells then were exposed to incandescent light (100 W) for 15–30 min.

Western blot analysis and immunocytochemical staining

For Western blot analysis, confluent cell monolayers were washed twice with PBS before lysis by incubation in ice-cold RIPA buffer (50 mM Tris-HCl, pH7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) for 5 min at 4 C. Lysates were centrifuged at 14,000 rpm for 10 min, supernatants were collected and passed ten times through a 22 gauge needle, and proteins were separated by SDS-PAGE (12% acrylamide gel) and transblotted to Hybond nitrocellulose membranes using standard procedures. Membranes were first blocked for 30 min at room temperature in Tris-buffered saline, pH 8.0, plus 0.05% TWEEN-20 (TBS-T) with 5% powdered milk solution, incubated for 60 min with a 1:1000 dilution of first antibody specific for murine Cx43 (Zymed Laboratories, Inc., San Francisco, CA). The membranes were washed several times in TBS-T and then incubated for 60 min with the second antibody/enzyme conjugate (HRP antimouse, IgG diluted 1:5000). Immunoreactive bands were detected by enhanced chemiluminesence assay (ECL, Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions.

For immunocytochemical analysis, cells were plated on glass chamber slides at 72,000 cells/cm^2, cultured for 2 days at 33 C and then incubated for an additional 4–6 days at 39 C. Cells were treated with

vehicle alone (0.1% TFA) or with the appropriate hormone for the time indicated. The immunocytochemical staining was performed as previously described (24). Briefly, cells were fixed in 3% paraformaldehyde/2% sucrose in PBS and incubated for 5 min. in 0.05% Triton-X 100 diluted in PBS. The fixed slides were blocked in 5% BSA in TBS-T buffer for 2 h at room temperature. The cells then were incubated with a 1:125 dilution of anti-Cx43 monoclonal antibody for 30 min at room temperature. The bound antibody was detected using a Vectastain ABC kit, followed by staining with VIP substrate according to manufacturer's instructions (Vector Laboratories, Inc. Burlingame, CA). Counterstaining was performed using 0.5% methyl green. In some cases, anti-Cx43 antibody was omitted to control for nonspecific staining.

Apoptosis

The pyknotic fragmentation of nuclei typical of apoptotic cells was detected using Hoechst-33258 fluorescent dye (Sigma Co., St. Louis, MO). Briefly, cells were cultured on glass coverslips and maintained in culture at nonpermissive conditions for 4–6 days in α-MEM supplemented with 2.5% of FBS and 1% PS. After exposure to the test hormones for 6 h, culture medium was aspirated and cells were fixed in 4% paraformaldehyde in phosphate buffered solution and then stained with Hoechst-33258 (2.5 mg/ml) for 5 min at room temperature. Cells were washed and mounted with glycerol:PBS (9:1, vol/vol). The stained nuclei were visualized by fluorescence microscopy using a DAPI filter. In some experiments, apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction, using the *In situ* Cell Death Detection Kit from Roche Molecular Biochemicals (Indianapolis, IN) following the manufacturer's instructions.

Trypan blue staining was used for routine quantification of cell death, as it previously was shown to correlate well with apoptosis in an osteocytic cell line (26). Briefly, cells were plated at 50,000 cells/well in 24-well dishes at 33 C and shifted to nonpermissive conditions (39 C) after 2 days. Cells were grown at 39 C for an additional 4–6 days in α-MEM supplemented with 2.5% of FBS and 1% PS and then treated with different hormones for an additional 16–18 h. Nonadherent cells were combined with adherent cells that were released from the cultures with trypsin-EDTA, centrifuged, and resuspended in 0.1% trypan blue solution. The percentage of cells exhibiting both nuclear and cytoplasmic staining was determined using a hemocytometer.

Statistical analysis

All results were expressed as the mean ± SD. Each experiment was repeated at least twice. Significance of differences between treatment and control groups was assessed by Student's *t* test using Bonferroni correction.

Results

Cell isolation and C-PTH radioligand binding

Primary mixed calvarial cells, F1, were isolated by sequential collagenase digestion from a PTH1R(−/−)/tsAg(+) animal, as previously described (21). We reported previously that these cells, and osteoblastic subclones derived from them, did not express PTH1Rs, or any other species of Gs-linked PTH receptors, as indicated by the absence of PTH1R DNA by genomic PCR; detectable cAMP response to hPTH(1–34), hPTH(1–84) or hPTHrP(1–36); or specific binding of the ^{125}I-[Nle^{8,18}, Tyr^{34}]bPTH(1–34)NH_2 radioligand. Cells expressing CPTHRs were present in this heterogeneous cell preparation, however, as demonstrated in preliminary experiments by the specific displacement of ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand by intact hPTH(1–84) (data not shown).

To identify cells with high expression of CPTHRs, single colonies were isolated from the mixed F1 population by limiting dilution and then screened for specific binding of ^{125}I-[Tyr^{34}] hPTH(19–84). Among twenty subclones isolated, three clones, initially designated C1, C14, and C59 (*i.e.* C cells)

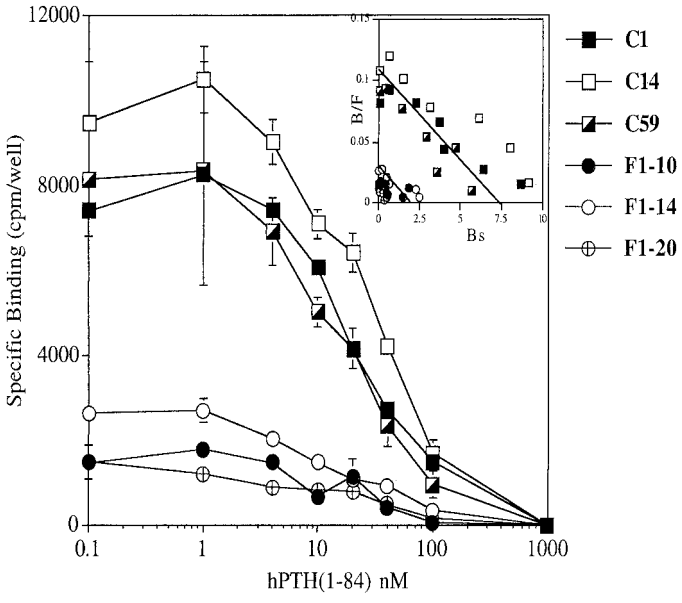

FIG. 1. Competitive ligand binding in C cells (C1, C14, and C59) and F cells (F1–10, F1–14 and F1–20). Different cell lines were tested for expression of CPTHRs by radioligand binding analysis at 15 C using ^{125}I-[Tyr^{34}] hPTH(19–84) as tracer. Cells were plated at 100,000 cells/ml in 24-well plates and maintained in culture at 33 C for 7–10 days. C cells expressed between 1,900,000 and 3,400,000 CPTHRs/cell, whereas the F cells expressed less than 600,000 CPTHRs/cell as determined by Scatchard analysis (insert; ordinate= bound/free, abscissa= specific binding, pmol/mg of protein). Specific binding is expressed as mean ± SD of triplicates in this representative experiment.

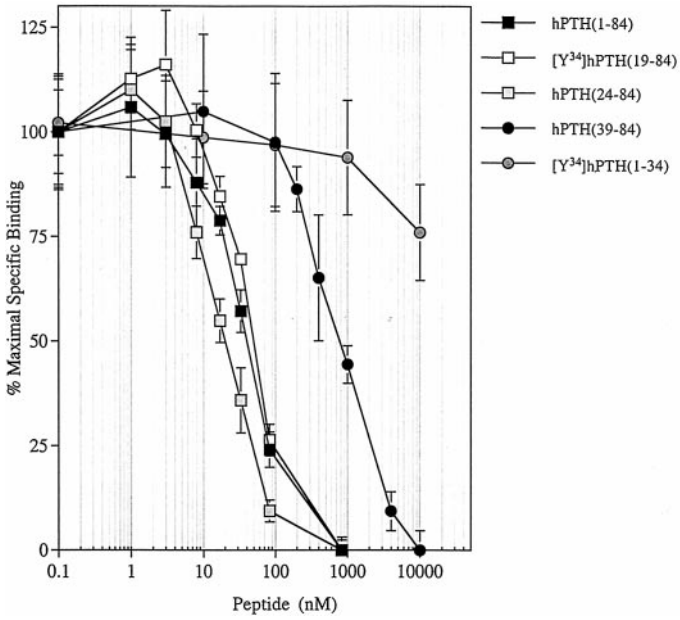

FIG. 2. Binding of N-terminally truncated human PTH fragments to CPTHRs in C59 cells. The human PTH peptides shown were tested for their ability to displace the ^{125}I-[Tyr^{34}] hPTH(19–84) tracer in C59 cells. Experiments were performed as described in Fig. 1. Results are expressed as the percentage of maximal specific binding observed in the absence of competing ligand and are shown as mean ± SD of triplicates in this representative experiment.

were selected for further characterization on the basis of their distinctly high specific binding of the ^{125}I-[Tyr^{34}] hPTH(19–84) radioligand. Like the previously described osteoblastic clonal cell lines (21), these three C cells did not express any other species of Gs-linked PTH receptors, as demonstrated by the absence of a detectable cAMP response to either hPTH(1–34) or hPTH(1–84) (not shown). Comparison of the CPTHR binding of these C cells with those of the previously described osteoblastic (F) cells, which had been selected for high alkaline phosphatase activity rather than for CPTHR binding (21), is shown in Fig. 1. The amount of total radioactivity bound averaged 14.4 ± 3.2% for the three C cell clones, *vs.* 2.98 ± 0.51% for the three previously reported F cell lines. Nonspecific binding, assessed in the presence of 10^{-6} M hPTH(1–84), was 2.8 ± 1.2% and 1.04 ± 0.15% for the C and F cell clones, respectively. As assessed by Scatchard analysis, CPTHR expression by C cells ranged from 1,900,000 to 3,400,000 sites/cell on the C cells but was less than 600,000 sites/cell on the F cells (ranging from 200,000 to 600,000) (Fig. 1, *inset*).

To identify the region(s) of the PTH molecule required for binding to the CPTHR, competitive displacement analysis was performed using various amino-terminally truncated human PTH fragments. As shown in Fig. 2, hPTH(1–84) displaced the ^{125}I-[Tyr^{34}] hPTH(19–84) radioligand as effectively as [Tyr^{34}] hPTH(19–84) (IC_{50} = 20–50 nM for both). Binding by the fragment hPTH(24–84) also was equivalent to that of hPTH(1–84), whereas the shorter peptide hPTH(39–84) was much less potent, with an IC_{50} in the range of 500–700 nM. Human PTH(1–34) displaced the radioligand slightly (24 ± 11.5%; n = 3 experiments) at 10,000 nM but was ineffective at 1000 nM.

Regulation of CPTHR expression

Expression of CPTHRs, as assessed by specific ^{125}I-[Tyr^{34}]hPTH(19–84) binding, was down-regulated in a concentration-dependent manner by pretreatment of C59 cells for 16 h with hPTH(1–84) (Fig. 3A). Binding was reduced 19 ± 5% and 66 ± 7% by preincubation with hPTH(1–84) at concentrations of 200 nM and 1000 nM, respectively. Pretreatment with hPTH(39–84) reduced CPTHR binding by less than 20% when added at 10,000 nM but not at all at 1000 nM (not shown). To assure that this change was due to a reduction in total binding sites and not simply to persistent receptor occupancy by preadministered hPTH(1–84), cells were rinsed, before radioligand addition, with acidic buffer that was found in preliminary experiments to completely and reversibly remove previously bound radioligand. CPTHR binding also was down-regulated following preincubation for 16 h in the presence of calcium ionophore (ionomycin, 1 μM) or active phorbol ester (TPA, 10 nM), which reduced specific binding by 71 ± 3% and 48 ± 12%, respectively. The effects of these drugs were at least partly reversible, as radioligand binding returned to 100% and 63% of controls, respectively, 24 h after their removal (data not shown). No down-regulation occurred when cells were pretreated with inactive phorbol ester (4-α phorbol) at concentrations as high as 100 nM (not shown). Also, the effect of ionomycin (1 μM) was not blocked by prior incubation of the cells with the intracellular calcium chelator BAPTA-AM (25 μM) (not shown). Finally, no down-regulation of CPTHR binding occurred following exposure for 24 h to hPTH(1–34) (1 μM), 1,25(OH)$_2$D$_3$ (10 nM), 8-bromo cAMP (1 mM), forskolin (10 μM), insulin (100 ng/ml), IGF-1 (100 nM) or dexamethasone (100 nM) (data not shown).

Morphology and gene expression

The C cells, which had been selected for high expression of CPTHRs, exhibited a characteristic morphology, including the presence of numerous elongated dendritic processes reminiscent of those seen in mature osteocytes (Fig. 4, A–C). This stellate appearance was seen in all three C cell lines and was distinct from the more cuboidal shape of the osteoblastic (F) cells that previously had been isolated from the same cal-

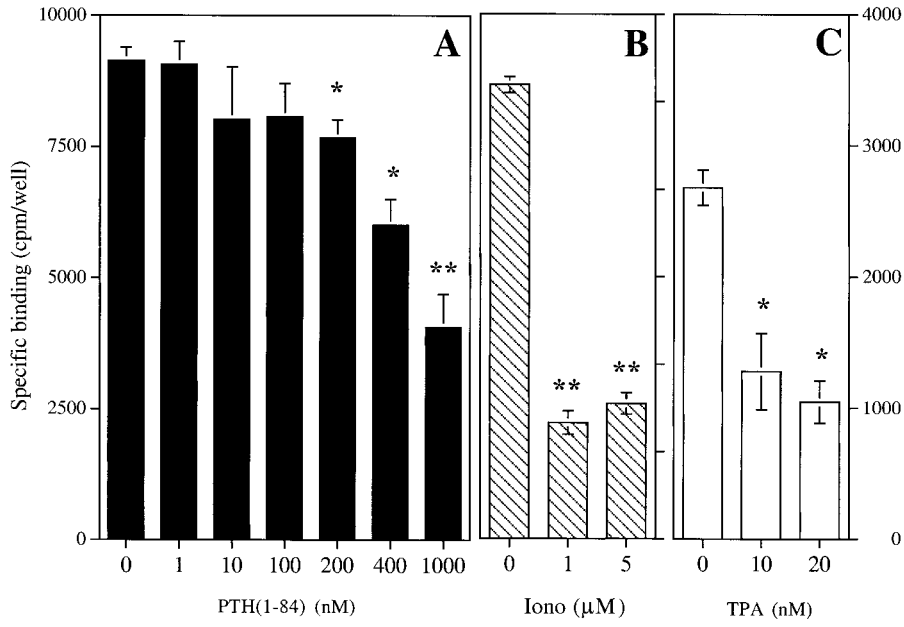

FIG. 3. Homologous down-regulation of CPTHRs in C59 cells. Cells were treated for 16 h with the agent indicated and then rinsed three times with binding buffer and once with an acidic buffer (see *Materials and Methods*), to completely remove any previously added PTH peptide from the receptor. Radioligand binding then was conducted using ^{125}I-[Tyr^{34}]hPTH(19–84) as tracer ± hPTH(1–84) as competing ligand in cells pretreated with (A) hPTH(1–84) at the indicated concentrations (nM), (B) ionomycin (μM), and (C) the active phorbol ester TPA (nM). Specific binding is expressed as mean ± SD of triplicates. * $P < 0.05$, ** $P < 0.01$.

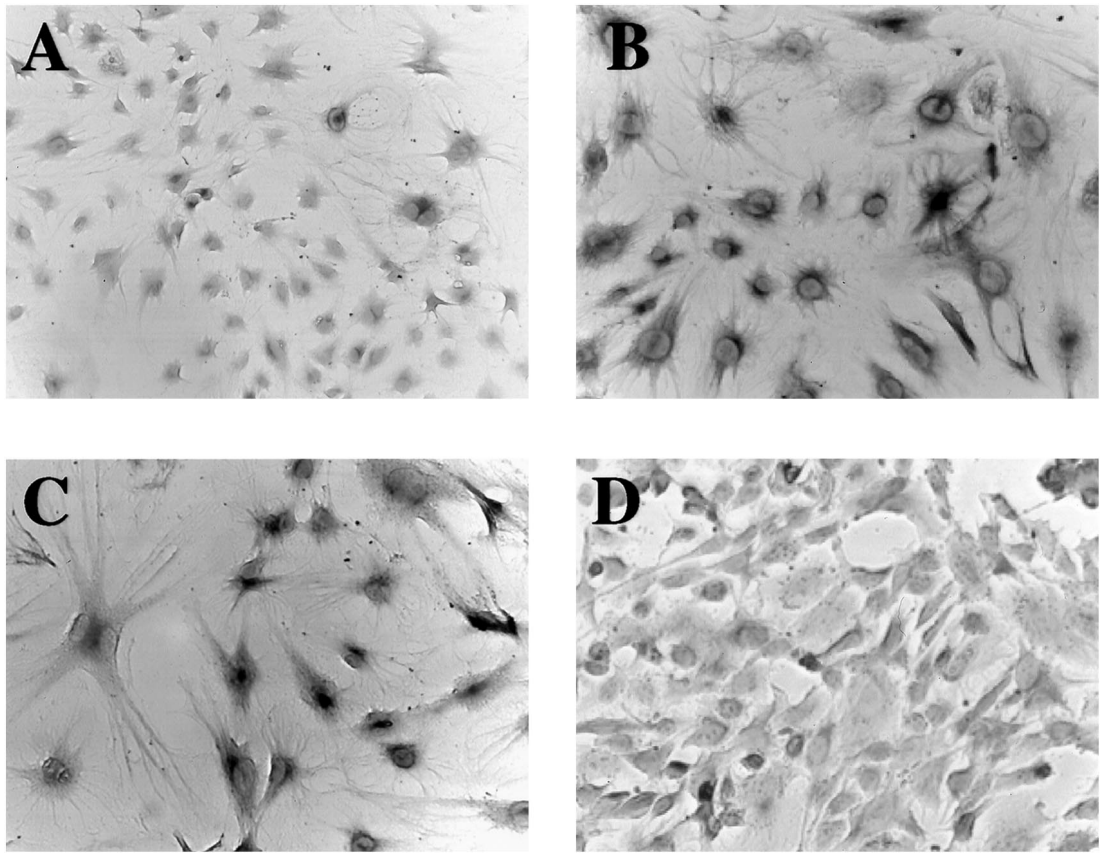

FIG. 4. Connexin 43 expression at permissive and nonpermissive temperature. C59 cells (A and B), C1 (C), and F1–14 cells (D) were plated on glass coverslips at 72,000 cells/cm^2 and cultured at 33 C (A) or at 33 C for 2 days and then at 39 C for 3–5 days (B-D) before staining for Cx43 as described in *Materials and Methods*. Because of their osteocyte-like appearance, C cells were renamed OC cells.

varial digest on the basis of high alkaline phosphatase activity (21) (Fig. 4D). Because of their osteocytic morphology, the three C cell lines were renamed OC cells.

Others have reported that osteocytes, unlike osteoblasts, express little or no alkaline phosphatase, cbfa-1/osf-2, or collagen I but do express high levels of mRNAs for osteocalcin, Cx43, and CD44 (24, 27). To further define the phenotype of the osteocyte-like OC cell lines, expression of specific mRNAs characteristic of osteoblasts or osteocytes was analyzed by Northern blotting and RT-PCR. As shown in Fig. 5A, OC cell expression of osteocalcin and Cx43 mRNAs was higher than in F1–14 osteoblastic cells or in early cultures of normal murine calvarial osteoblasts, whereas osteopontin mRNA was barely detectable in the OC cells. Transcripts for alkaline phosphatase or cbfa-1/osf-2 could not be detected by Northern blot analysis in these cells but were present at low levels, as revealed by RT-PCR (Fig. 5B). Type 1 collagen was well expressed in all of the cell lines. None of the mRNAs measured by Northern blot analysis increased significantly in cells incubated for 5 days at 39 C *vs.* 33 C. Cx43 expression, as detected by direct immunostaining of fixed cells, was observed in cytoplasm and along the dendritic processes (Figs. 4 and 6). After incubation for 2 h with hPTH(1–84) (100 nM) or hPTH(39–84) (1000 nM), Cx43 staining was more prominent, especially in a perinuclear location (Fig. 6). This represented mainly a redistribution of Cx43, as it was not accompanied by an increase in total cellular Cx43 protein, as assessed by Western blotting (Fig. 6D).

The OC cells were capable of mineralization, as detected by von Kossa staining after 4 weeks in the presence of 10 mM β-glycerophosphate and 50 μg/ml of ascorbic acid (Fig. 7). Mineralization (Fig. 7B *vs.* A) was increased when cells were maintained at 39 C, conditions under which the transforming T antigen is inactive.

CPTHR expression in other cells

To determine if abundant CPTHR expression is a specific characteristic of osteocytic cells or a more generalized feature of bone cells, we performed ^{125}I[Tyr^{34}] hPTH(19–84) radioligand binding with several other bone- and non bone-derived cell lines. As shown in Table 1, CPTHR specific binding was much greater in the osteocytic cells than in the unfractionated original calvarial digest (F1) or the previously isolated osteoblastic (F1–14) or bone marrow stromal cells (MS-1) (25). Minimal specific binding was detected on HeLa, BHK1, NIH3T3 cells or, as previously reported, on LLC-PK1 porcine kidney cells (6).

Apoptosis

It was shown recently that PTH(1–34), presumably acting via PTH1Rs, can reduce the rate at which osteoblasts and

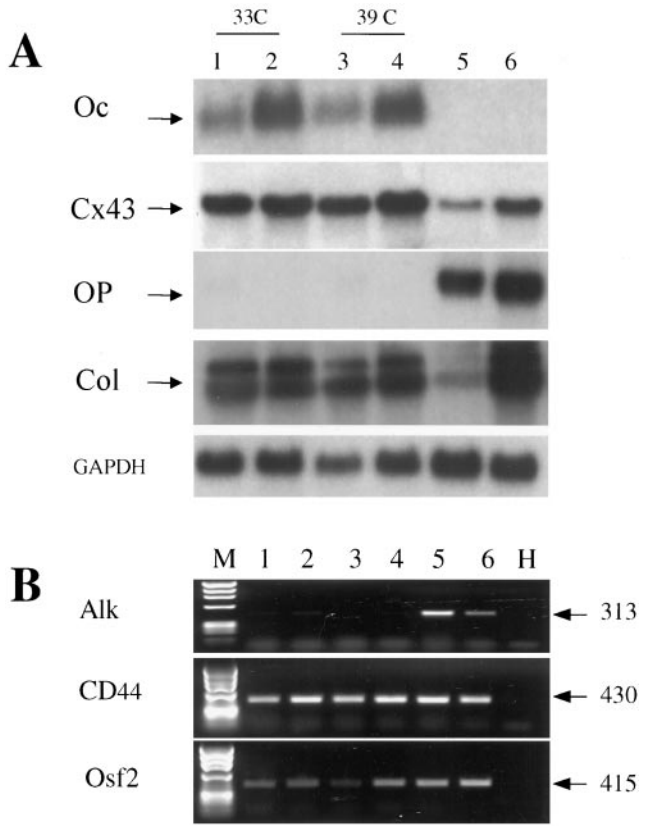

FIG. 5. mRNA expression in OC cells. OC14 lanes (1 and 3) and OC59 (lanes 2 and 4) cells were cultured at 33 C for 7 days (lanes 1 and 2) or at 33 C for 2 days and at 39 C for 5 additional days (lanes 3 and 4) before RNA extraction. F1–14 cells (lane 5) also were cultured at 33 C 2 days and at 39 C for 5 additional days whereas primary calvarial osteoblasts (lane 6) were cultured at 37 C. Expression of specific mRNAs was assessed by Northern blotting or by RT-PCR as described in *Materials and Methods*. A, Northern blot analysis of mRNAs for osteocalcin (Oc), connexin 43 (Cx43), osteopontin (OP), and collagen α1 (I) (Col) and GAPDH. B, RT-PCR for alkaline phosphatase (Alk), CD44 and cbfa-1/osf-2 (Osf2). M, Molecular weight. H, Reaction with primers in the absence of cDNAs. The expected size for each PCR product is as indicated.

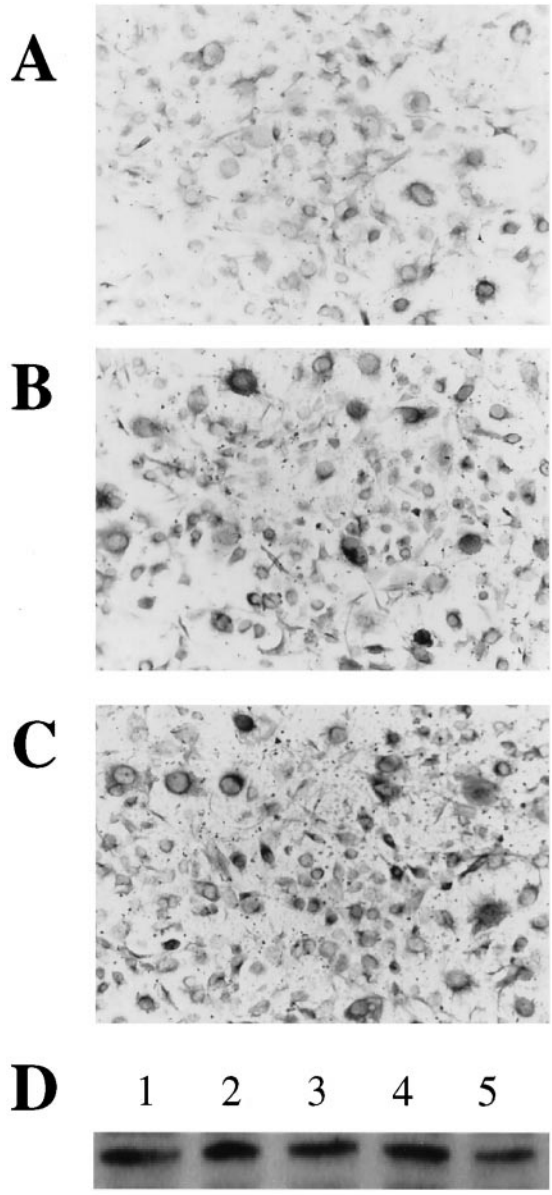

FIG. 6. Connexin 43 regulation by CPTHR ligands. OC14 cells were plated on glass coverslips at 72,000 cells/cm^2, cultured at 33 C for 2 days and then shifted at 39 C for 3–5 days before stimulation with CPTHR ligands. OC14 cells were treated for 2 h with (A) vehicle alone, (B) 100 nM hPTH(1–84), or (C) 1000 nM hPTH(39–84). Cells were fixed and stained for Cx43 expression as described in *Materials and Methods*. D, Western blot for Cx43 in OC14 cells cultured at 33 C (lane 1) or at 39 C (lanes 2–5), as described in *Materials and Methods* and treated for 2 h with vehicle alone (lanes1–3) or with 100 nM hPTH(1–84) (lanes 4 and 5).

osteocytes undergo apoptosis *in vivo* and *in vitro* (26, 28). As osteocytes are terminally differentiated osteoblasts, it was of interest to determine if CPTHR activation might play a role in regulating apoptosis in the OC cells. As shown in Fig. 8 (A and B) when OC cells, which lack functional PTH1R genes, were incubated for 6 h with 100 nM hPTH(1–84), increased nuclear pyknosis and chromatin condensation appeared, as revealed by DNA staining with Hoechst dye 33258. Increased apoptosis also was observed using a TUNEL immunocytochemical assay (Fig. 8, C and D). Similarly, when cell death was monitored using trypan blue staining of combined adherent and nonadherent cells from these cultures (26), hPTH(1–84) induced a doubling in trypan blue-stained cells after 16 h (Fig. 9A). This effect of hPTH(1–84) upon cell death was concentration dependent and maximal at 10 nM, with an EC_{50} in the range of 0.1 nM (Fig. 9B). The effect was mimicked by 100 nM hPTH (24–84) and 1000 nM hPTH(39–84), which produced, respectively, 1.8- and 1.5-fold increases over basal. (Fig. 9, C and D). As expected, hPTH(1–34) (1000 nM) was inactive, consistent with its inability to effectively bind to CPTHR sites on these cells (Fig. 2). In these experiments, the percentage of trypan blue-stained cells in controls was substantial (20–30%), perhaps because of prolonged incubation in reduced serum at the nonpermissive temperature. A similar proapoptotic effect of hPTH(1–84) was observed in analogous experiments performed at 37 C, however, where basal rates of cell death were lower (Fig. 9A). Cell death triggered by hPTH(1–84) was blocked completely by prior addition of the caspase-3 inhibitor DEVD (50 μg/ml) (Fig. 9E).

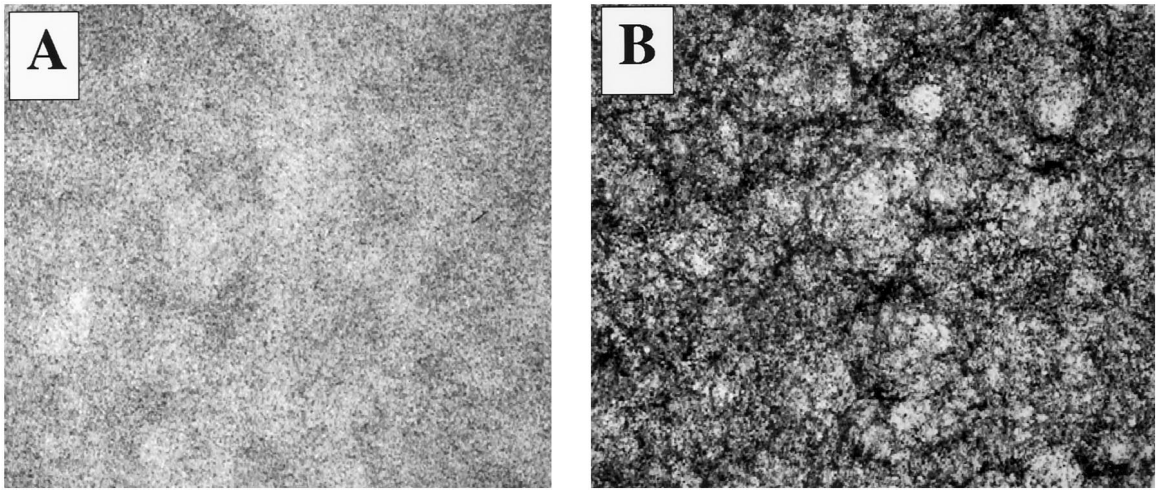

FIG. 7. Mineralization by OC 14. OC 14 cells were grown to confluence at 33 C on 6 well-dishes and then maintained for an additional 4 weeks at 33 C (A) or 39 C (B) in the presence of 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid before visualizing calcium phosphate deposition by von Kossa staining. Magnification, 40×.

TABLE 1. CPTHR binding in various cell lines

Cell line	Total binding (%TC)	Specific binding (%TC)
OC 59	11.9 ± 1.2	10.2 ± 2.8
F1	4.1 ± 1.4	2.8 ± 2.5
F1–14	3.2 ± 0.3	2.4 ± 1.7
MS-1	2.9 ± 0.1	2.1 ± 0.1
NIH-3T3	1.6 ± 0.2	0.6 ± 0.2
HeLa	0.9 ± 0.1	0.5 ± 0.1
BHK21	0.6 ± 0.1	0.4 ± 0.1

Data shown were obtained from experiments in which total cpm of ^{125}I[Tyr^{34}]hPTH(19–84) added ("TC") ranged between 66,000 and 140,000 cpm/well. Values shown are means ± SD for triplicates. Specific binding was obtained after subtraction of radioligand bound in the presence of 1000 nM hPTH(1–84).

Discussion

Osteocytes comprise over 90% of bone cells, yet their functions, and the involvement of systemic hormones in regulating them, are incompletely understood. Located in interconnecting lacunae deep within the mineralized matrix of bone, osteocytes are believed to fulfill an important mechanosensory function, whereby they transduce shear and strain forces into chemical signals that communicate this information to other osteocytes, osteoblasts and, ultimately, osteoclasts (28–30). Osteocytes express PTH1Rs, and their response to mechanical forces *in vivo* is enhanced by, and may even require, circulating PTH (31).

We report here that clonal murine calvarial-derived cell lines with many characteristics of osteocytes also express unusually large numbers of receptors that specifically recognize the C-terminal portion of intact PTH. In fact, these cells express considerably higher levels of CPTHRs (up to 10-fold more per cell) than do other bone cell subtypes, including mature osteoblasts (21). It is difficult to identify the osteocytic phenotype with complete confidence *in vitro*, absent the normal surrounding lacunar environment, but the three conditionally immortalized, clonal cell lines that we selected exclusively on the basis of high CPTHR expression share many morphological features and a distinctive profile of gene expression with normal osteocytes (23) and with a previously characterized osteocytic cell line (24). These include a stellate shape, with numerous dendritic processes, and abundant expression of mRNAs for osteocalcin, Cx43, and CD44 but not for alkaline phosphatase or cbfa-1/osf-2, which are more characteristic of early or mature osteoblasts. The osteocytic phenotype of these cells is most intense when they are maintained at a temperature (*i.e.* 39 C) that is nonpermissive for expression of functional transforming tsTAg. For example, mineralization (Fig. 7) was much more intense in cells cultured at 39 C than at 33 C, as we previously observed in other clonal osteoblastic cells isolated from these mice (21).

The OC cells were isolated intentionally from animals that genetically lack functional PTH1Rs to eliminate possible confounding effects of coexpressed PTH1Rs and to allow us to focus directly upon CPTHR-dependent actions of intact PTH. The results of radioreceptor binding assays in OC cells, using the ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand, were remarkably similar to those reported previously with ROS17/2.8 cells (6, 16), which suggests that the CPTHRs on these different cell types probably are similar or identical. Thus, like Inomata *et al.*, we found that hPTH(1–84) and the hPTH(19–84) peptide bound equivalently to CPTHRs and that hPTH(39–84) bound more weakly, with an IC_{50} roughly 15-fold higher. We have further narrowed the ligand domain responsible for this higher affinity interaction, moreover, by studying hPTH (24–84), which was found to bind at least as well as hPTH(1–84). This indicates that CPTHRs require sequences within the region hPTH(24–38) for high affinity binding, although other, more C-terminal domains also must be involved to account for the residual affinity of hPTH(39–84). The small amount of binding observed at high concentrations of hPTH(1–34) is consistent with the presence of a binding determinant within hPTH(24–38), most of which is contained within hPTH(1–34). It seems that this domain probably cannot function well independently of the cooperative effects of more C-terminal sequences, however. The manner in which of these independent domains contribute to the

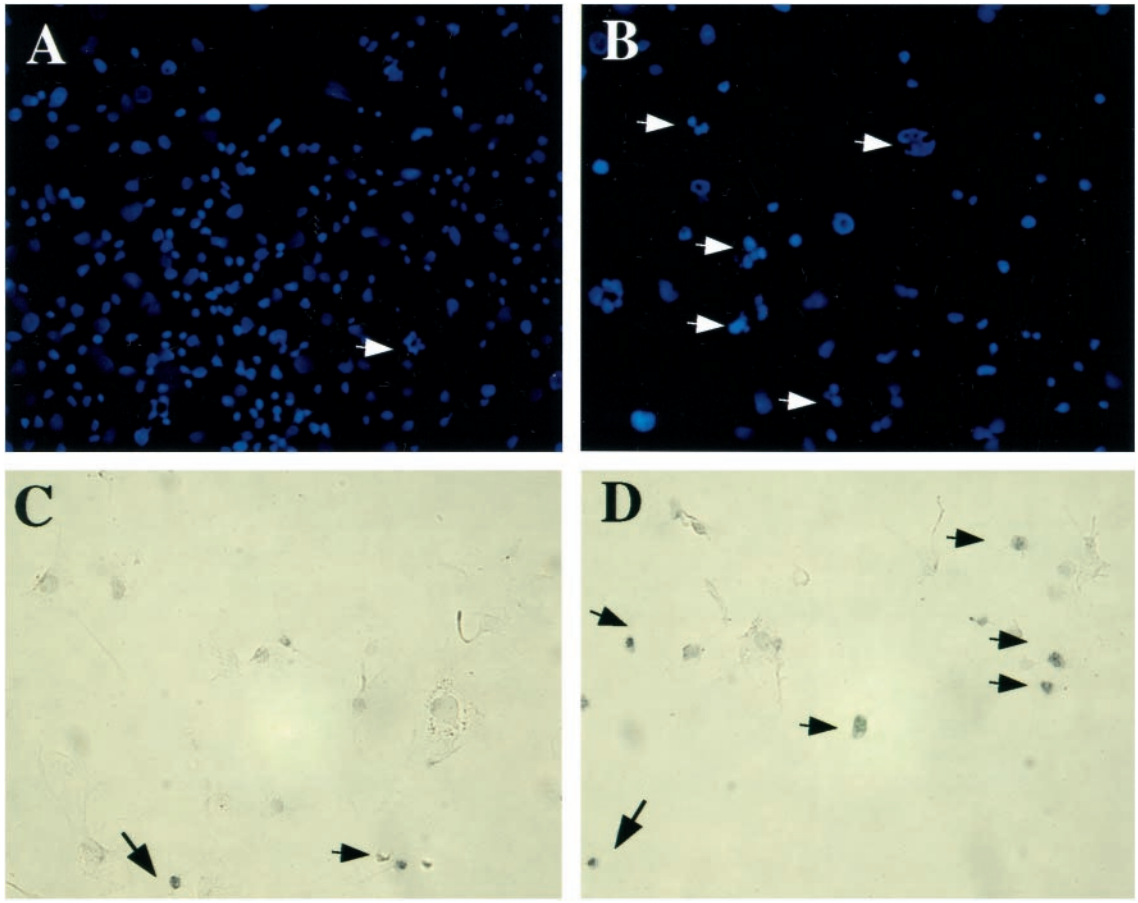

FIG. 8. Induction of apoptosis by PTH(1–84) in OC14 cells. Cells plated on glass coverslips and maintained at 33 C for 3 days were shifted to 39 C for 5–7 days before addition of 100 nM hPTH(1–84) (B, D) or vehicle alone (A, C) for 6 h. At the end of the incubations, cells were fixed and stained with Hoechst 33258 (A and B) or for TUNEL (C and D), as described in *Materials and Methods*. *Arrows* indicate apoptotic cells. Of the total Hoechst 33258 stained cells counted (>200), 20 ± 2.1% and 29 ± 0.7% of the cells were apoptotic in the control and in PTH-treated, respectively. Similarly, in TUNEL assay, there were 5.5 ± 0.4 and 8 ± 0.3 TUNEL positive cells/field in the control and in the PTH-treated, respectively.; 200× magnification.

formation of an active conformation of the ligand remains obscure, especially as available spectroscopic studies do not suggest a highly ordered secondary structure for the C-terminal sequence of PTH (32–34).

Enumeration of CPTHR sites on the OC cells led to estimates of 1,900,000 to 3,400,000 per cell, which was 6- to 10-fold higher than on the osteoblastic (F) cells obtained from the same bones and at least 5-fold higher than on ROS 17/2.8 cells (6). A survey of other available cell lines indicated that, with the exception of a bone marrow stromal cell line (MS-1) (25), little or no binding was detectable on NIH-3T3, HeLa or BHK21 fibroblast cells, as was reported previously for OK and LLC-PK1 kidney cells, YCC cells, and SaOS-2, MG63 and UMR106–01 osteosarcoma cells (6). Thus, the very high abundance of expressed CPTHRs on osteocytic OC cells is unique, even among other bone cells tested, and suggests that these receptors may play an important role in osteocyte regulation. The possibility that bone is an important C-PTH target tissue *in vivo* is further supported by our recent observation that ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand binds specifically to frozen sections of 18.5-day normal mouse fetal calvarial bone (unpublished results) and that hPTH (7–84) reduces the calcemic effects of intact PTH(1–84) *in vivo* at concentrations much lower than those required for effective antagonism of the PTH1R (20).

We observed homologous down-regulation of CPTHR binding following preincubation with hPTH(1–84). This response required relatively high concentrations of ligand (>100 nM), in contrast to the effects upon apoptosis, and was mimicked by addition of active phorbol ester or ionomycin. The failure of BAPTA to block the ionomycin effect suggests mediation by a sustained influx of calcium, presumably from the extracellular compartment, as opposed to a rapid burst of calcium released from intracellular stores. This is consistent also with our previous report of CPTH-stimulated calcium uptake in SaOS-2 human osteoarcoma cells (35). Also, in C-PTH-treated human fetal chondrocytes, others have reported increased cytosolic free calcium due to activation of calcium influx via nickel-insensitive channels (18). Our down-regulation data are most compatible with involvement of a calcium-dependent PKC, although further study will be needed to pursue this possibility and to clarify the role and source of the calcium signal implicated in this response.

Functional studies of CPTHRs on OC cells have pointed so far to involvement in at least two general types of cellular processes: cell survival and intercellular communication. We

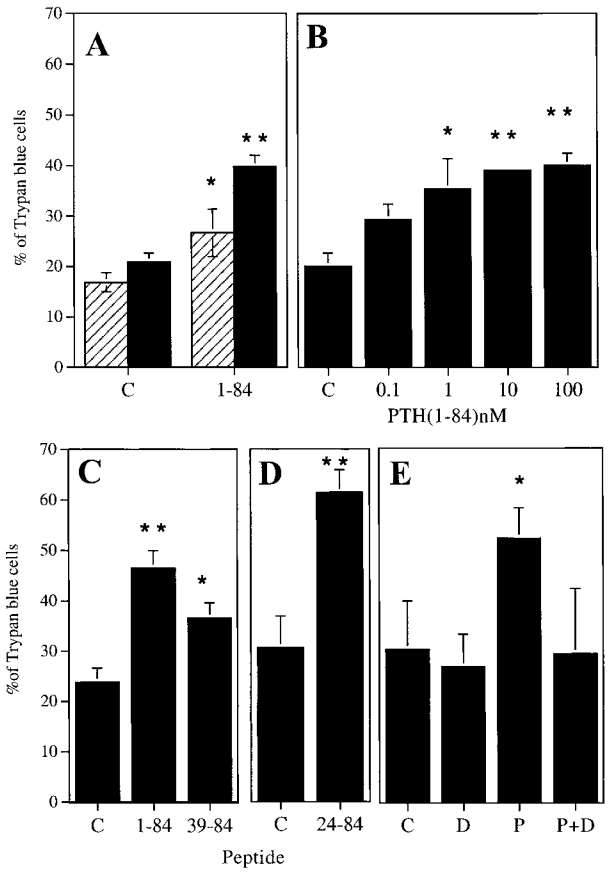

FIG. 9. Cell death induced by in PTH in OC14 cells. OC14 cells were plated, cultured at 33 C for 3 days and then incubated at 37 C (*hatched bars* in A) or 39 C (*solid bars* in A; B–E) for 5–7 days before addition of PTH peptides for a further 16 h, followed by trypan blue staining (see *Materials and Methods*). A, Response to hPTH(1–84), 100 nM; B, Human PTH 1–84 dose response; C, Response to hPTH(1–84), 100 nM and hPTH(39–84),1000 nM; D, Response to hPTH(24–84), 100 nM; E, Caspase dependence (C = controls) The Caspase-3 inhibitor DEVD (D) was added 1 h before addition of 100 nM hPTH(1–84) (P). Results are expressed as mean ± SD of quadruplicate determinations of the percentage of nonvital trypan blue-stained cells. * $P < 0.05$, ** $P < 0.01$.

observed a rapid CPTHR-dependent change in cellular expression of Cx43, a gap-junction protein important in mediating intercellular communication and previously shown to be expressed by both osteoblasts and osteocytes (36, 37). The possible physiologic significance of this action is not yet clear. The response of osteocytes to mechanical stress *in vivo* is promoted by PTH(1–34) and blocked by parathyroidectomy (38). It is interesting to speculate that activation of CPTHRs by C-PTH fragments, preferentially secreted by the parathyroids during hypercalcemia (9), might trigger communication from osteocytes to other bone cells so as to limit osteoclast formation or activity and thereby exert negative feedback on bone resorption. Further understanding of CPTHR function and ligand activity, facilitated by availability of these osteocytic cell lines, will be needed to pursue such hypotheses.

We also observed that CPTHR-interacting ligands increased the rate of osteocyte cell death *in vitro*. As this action was accompanied by evidence of increased nuclear pyknosis, chromatin condensation and TUNEL staining and was completely blocked by the caspase-3 inhibitor DEVD, we conclude that apoptosis, mediated by activation of the caspase cascade, is the likely mechanism. Importantly, this proapoptotic effect was observed also with the C-terminal fragment hPTH(39–84) (at an appropriately higher concentration), which indicates that the ligand domain required for CPTHR activation of this response likely is located C-terminal to the above-mentioned hPTH(24–38) region needed for high-affinity binding. In contrast, others (26) have observed an opposite, anti-apoptotic action of hPTH(1–34), via the PTH1R activation, in dexamethasone-stimulated osteoblastic and osteocytic cells. In OC cells, which lack endogenous PTH1Rs, hPTH(1–84) exerts only a pro-apoptotic effect (via CPTHRs), as does hPTH(39–84). This suggests the possibility that PTH1Rs and CPTHRs may exert opposite effects upon cell survival in osteocytes and that these cells may be able to integrate signals arising from these two types of PTH receptors in response to discoordinate changes in circulating intact hormone and C-PTH fragments. In renal insufficiency, for example, excessive CPTHR activation by high levels of circulating CPTH fragments might lead to exaggerated, pathological loss of osteocytes and contribute to some forms of renal osteodystrophy (39).

Expression of the PTH1R by osteocytes has been documented *in vivo* (40), and it is of interest to consider the potential for interactions between PTH1Rs and CPTHRs in these cells. It is possible that the genetic absence of PTH1R in our cells may have modified in some way the expression or function of CPTHRs. Almost certainly, the response of these PTH1R-null cells to hPTH(1–84) *in vitro* differs from that of authentic osteocytes *in vivo*, in which both receptor types presumably can be activated concurrently by the intact hormone. Preliminary evidence, in ROS 17/2.8 cells, indicates that C-PTH fragments may modify the function of hPTH(1–84) [but not hPTH(1–34)] in cells that express both types of PTH receptors (41). The possible involvement of PTH1Rs in modulating CPTHR function, or vice versa, now can be addressed directly in our OC cells by reconstituting them through transfection with cDNA encoding PTH1Rs; efforts in this direction are underway in our laboratory.

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References

1. **Heersche JNM, Bellows CG, Aubin JE** 1994 Cellular actions of parathyroid hormone on osteoblasts and osteoclasts differentiation. In: Bilezikian JP, Marcus R, Levine MA (eds) The Parathyroids. Basic and Clinical Concepts. Raven Press, New York, pp 83–91
2. **Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV, Potts Jr JT, Kronenberg HM, Segre GV** 1992 Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc Natl Acad Sci USA 89:2732–2736
3. **Jüppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski Jr LF, Hock J, Potts Jr JT, Kronenberg HM, Segre GV** 1991 A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science 254:1024–1026
4. **Jüppner H** 1999 Receptors for parathyroid hormone and parathyroid hor-

mone-related peptide: exploration of their biological importance. Bone 25:87–90
5. **Pines M, Adams AE, Stueckle S, Bessalle R, Rashti-Behar V, Chorev M, Rosenblatt M, Suva LJ** 1994 Generation and characterization of human kidney cell lines stably expressing recombinant human PTH/PTHrP receptor: lack of interaction with a C-terminal human PTH peptide. Endocrinology 135:1713–1716
6. **Inomata N, Akiyama M, Kubota N, Jüppner H** 1995 Characterization of a novel parathyroid hormone (PTH) receptor with specificity for the carboxyl-terminal region of PTH-(19–84) [see comments]. Endocrinology 136:4732–4740
7. **Bringhurst FR, Stern AM, Yotts M, Mizrahi N, Segre GV, Potts Jr JT** 1988 Peripheral metabolism of PTH: fate of biologically active amino terminus *in vivo*. Am J Physiol 255:E886–E893
8. **Khosla S, Demay M, Pines M, Hurwitz S, Potts Jr JT, Kronenberg HM** 1988 Nucleotide sequence of cloned cDNAs encoding chicken preproparathyroid hormone. J Bone Miner Res 3:689–698
9. **D'Amour P, Labelle F, Lecavalier L, Plourde V, Harvey D** 1986 Influence of serum Ca concentration on circulating molecular forms of PTH in three species. Am J Physiol 251:E680–E687
10. **Bringhurst FR, Stern AM, Yotts M, Mizrahi N, Segre GV, Potts Jr JT** 1989 Peripheral metabolism of [35S]parathyroid hormone *in vivo*: influence of alterations in calcium availability and parathyroid status. J Endocrinol 122:237–245
11. **Hanley DA, Takatsuki K, Sultan JM, Schneider AB, Sherwood LM** 1978 Direct release of parathyroid hormone fragments from functioning bovine parathyroid glands in vitro. J Clin Invest 62:1247–1254
12. **Martin K, Hruska K, Greenwalt A, Klahr S, Slatopolsky E** 1976 Selective uptake of intact parathyroid hormone by the liver: differences between hepatic and renal uptake. J Clin Invest 58:781–788
13. **Dambacher MA, Fischer JA, Hunziker WH, Born W, Moran J, Roth HR, Delvin EE, Glorieux FH** 1979 Distribution of circulating immunoreactive components of parathyroid hormone in normal subjects and in patients with primary and secondary hyperparathyroidism: the role of the kidney and of the serum calcium concentration. Clin Sci 57:435–443
14. **Murray TM, Rao LG, Muzaffar SA** 1991 Dexamethasone-treated ROS 17/2.8 rat osteosarcoma cells are responsive to human carboxylterminal parathyroid hormone peptide hPTH (53–84): stimulation of alkaline phosphatase. Calcif Tissue Int 49:120–123
15. **Nasu M, Sugimoto T, Kaji H, Kano J, Chihara K** 1998 Carboxyl-terminal parathyroid hormone fragments stimulate type-1 procollagen and insulin-like growth factor-binding protein-5 mRNA expression in osteoblastic UMR-106 cells. Endocr J 45:229–234
16. **Takasu H, Baba H, Inomata N, Uchiyama Y, Kubota N, Kumaki K, Matsumoto A, Nakajima K, Kimura T, Sakakibara S, Fujita T, Chihara K, Nagai I** 1996 The 69–84 amino acid region of the parathyroid hormone molecule is essential for the interaction of the hormone with the binding sites with carboxyl-terminal specificity. Endocrinology 137:5537–5543
17. **Kaji H, Sugimoto T, Kanatani M, Fukase M, Chihara K** 1995 Carboxyl-terminal peptides from parathyroid hormone-related protein stimulate osteoclast-like cell formation. Endocrinology 136:842–848
18. **Erdmann S, Muller W, Bahrami S, Vornehm SI, Mayer H, Bruckner P, von der Mark K, Burkhardt H** 1996 Differential effects of parathyroid hormone fragments on collagen gene expression in chondrocytes. J Cell Biol 135:1179–1191
19. **Erdmann S, Burkhardt H, von der Mark K, Muller W** 1998 Mapping of a carboxyl-terminal active site of parathyroid hormone by calcium-imaging. Cell Calcium 23:413–421
20. **Slatopolsky E, Finch J, Clay P, Martin D, Sicard G, Singer G, Gao P, Cantor T, Dusso A** 2000 A novel mechanism for skeletal resistance in uremia. Kidney Int 58:753–761
21. **Divieti P, Lanske B, Kronenberg HM, Bringhurst FR** 1998 Conditionally immortalized murine osteoblasts lacking the type 1 PTH/PTHrP receptor. J Bone Miner Res 13:1835–1845
22. **Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM** 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663–666
23. **Nijweide PJ, Burger EH, Klein-Nulend J, Van der Plas A** 1996 The osteocyte. In: Bilezikian JP, Raisz LG, Rodan G (eds) Principle of Bone Biology. Academic Press, New York, vol 1:115–126
24. **Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF** 1997 Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res 12:2014–2023
25. **Liu BY, Guo J, Lanske B, Divieti P, Kronenberg HM, Bringhurst FR** 1998 Conditionally immortalized murine bone marrow stromal cells mediate parathyroid hormone-dependent osteoclastogenesis in vitro. Endocrinology 139:1952–1964
26. **Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt M, Manolagas S** 1999 Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J Clin Invest 104:439–446
27. **Hughes DE, Salter DM, Simpson R** 1994 CD44 expression in human bone: a novel marker of osteocytic differentiation. J Bone Miner Res 9:39–44
28. **Manolagas SC** 2000 Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 21:115–137
29. **Mullender MG, Huiskes R** 1997 Osteocytes and bone lining cells: which are the best candidates for mechano-sensors in cancellous bone? Bone 20:527–532
30. **Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH** 1995 Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts—correlation with prostaglandin upregulation. Biochem Biophys Res Commun 217:640–648
31. **van der Plas A, Aarden EM, Feijen JH, de Boer AH, Wiltink A, Alblas MJ, de Leij L, Nijweide PJ** 1994 Characteristics and properties of osteocytes in culture. J Bone Miner Res 9:1697–1704
32. **Fiskin AM, Cohn DV, Peterson GS** 1977 A model for the structure of bovine parathormone derived by dark field electron microscopy. J Biol Chem 252:8261–8268
33. **Gronwald W, Schomburg D, Harder MPF, Mayer H, Paulsen J, Wingender E, Wray V** 1996 Structure of recombinant human parathyroid hormone in solution using multidimensional NMR spectroscopy. Biol Chem Hoppe-Seyler 377:175–186
34. **Wray V, Federeau T, Gronwald W, Mayer H, Schomburg D, Tegge W, Wingender E** 1994 The structure of human parathyroid hormone from a study of fragments in solution using 1H NMR spectroscopy and its biological implications. Biochemistry 33:1684–1693
35. **Fukayama S, Schipani E, Juppner H, Lanske B, Kronenberg HM, Abou-Samra AB, Bringhurst FR** 1994 Role of protein kinase-A in homologous down-regulation of parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid in human osteoblast-like SaOS-2 cells. Endocrinology 134:1851–1858
36. **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
37. **Ziambaras K, Lecanda F, Steinberg TH, Civitelli R** 1998 Cyclic stretch enhances gap junctional communication between osteoblastic cells. J Bone Miner Res 13:218–228
38. **Chow JW, Fox S, Jagger CJ, Chambers TJ** 1998 Role for parathyroid hormone in mechanical responsiveness of rat bone. Am J Physiol 274:E146–E154
39. **Bonucci E, Gherardi G** 1977 Osteocyte ultrastructure in renal osteodystrophy. Virchows Arch 373:213–231
40. **Lee K, Deeds JD, Bond AT, Jüppner H, Abou-Samra AB, Segre GV** 1993 In situ localization of PTH/PTHrP receptor mRNA in the bone of fetal and young rats. Bone 14:341–345
41. **D'Amour P, Dorais C, Rousseau L, Roy L, Brossard JH** 1997 Synthetic hPTH(7–84) enhance the cAMP repsonse to hPTH(19–84) in dexamethasone treated ROS 17/2.8 clonal cells. J Bone Miner Res [Suppl] 12:S318