Mutual Up-Regulation of Thyroid Hormone and Parathyroid Hormone Receptors in Rat Osteoblastic Osteosarcoma 17/2.8 Cells*

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ABSTRACT

PTH and thyroid hormone (T₃) stimulate anabolic and catabolic processes in bone predominantly by acting on osteoblasts. Both inadequate and excessive secretion of either hormone can result in clinical bone disorders. In addition, T₃ and PTH related peptide (PTHrP) have multiple effects on a wide number of other tissues modulating both cell differentiation and proliferation. To address the question of whether there might be functional mutual regulation of T₃ receptors (TR) and PTH/PTHrP receptors (PTHR), we studied their expression and receptor-mediated intracellular effects in rat osteoblastic osteosarcoma (ROS) 17/2.8 cells. PTHR were up-regulated by T₃ pretreatment (10⁻¹⁰⁻¹⁰⁻⁶ M) in ROS 17/2.8 cells in a dose-dependent manner. T₃ pretreatment increased both PTH-induced cyclic AMP response element binding protein (CREB) phosphorylation and PTH-induced intracellular calcium transients, and further decreased PTH-induced down-regulation of alkaline phosphatase activity, suggesting that there are functional consequences of the PTHR up-regulation. Pretreatment with PTH (10⁻¹⁰⁻¹⁰⁻⁶ M) or PTHrP (10⁻⁶ M) for 3–4 days resulted in a dose-dependent up-regulation of TR in ROS 17/2.8 cells. CAMP analogues or a calcium ionophore were able to mimic the effect of PTH on TR binding, suggesting that either the cAMP-signaling pathway or Ca²⁺ could be involved in PTH-induced up-regulation of the TR. These observations provide a novel example of mutual interactions between nuclear receptors and membrane receptors and may have significant implications for the regulation of bone remodeling in health and disease. (Endocrinology 142: 157–164, 2001)

BOTH THYROID HORMONE (T₃) and PTH have multiple critical roles in bone. Mice lacking thyroid hormone receptors (TRα¹⁻/⁻, β⁻/⁻) display retarded growth and bone maturation, especially a decrease in longitudinal bone growth (1). In children, hyperthyroidism can cause acceleration of growth and skeletal development (2), whereas hyperthyroidism may accelerate bone loss in adults (3). Conversely, hypothyroidism can be associated with retarded bone maturation and stunted growth. PTH is one of the most important regulators of bone physiology. Hyperparathyroidism can result in bone loss, whereas low-dose and intermittent pulses of PTH stimulate bone formation in animals and humans (4, 5). The fact that both T₃ and PTH stimulate both anabolic and catabolic processes in bone raises interesting questions about their possible interactions and the potential physiologic roles of such coordinate actions. In human subjects, the renal response, as well as the bone response, to administration of PTH is much greater in hyperthyroidism, compared with the responses in hypothyroid or euthyroid subjects (6). These clinical observations suggest that PTH and T₃ have synergistic actions and result in increased bone resorption. It is noteworthy that more than 50 cases of patients with coexistent hyperthyroidism and primary hyperparathyroidism have been reported (7, 8).

Osteoblasts seem to be the major direct cellular target of PTH and T₃ action in bone, because these cells possess both PTH receptors (PTHR) and T₃ receptors (TR). TRα, TRβ, and PTHR have been characterized in cells of the osteoblastic lineage (9, 10).

Other studies documented that T₃ stimulates proliferation of rodent and human osteoblastic cells (11, 12), increases alkaline phosphatase activity (13), and increases production of osteocalcin (14), as well as bone collagen and noncollagen proteins (15). PTH increases DNA synthesis and induces the expression of early-response genes in rat osteoblastic osteosarcoma (ROS) 17/2.8 cells (16, 17). In contrast to these stimulatory effects, PTH decreases alkaline phosphatase activity and collagen synthesis (16, 17). These findings suggest that functional interactions between PTH and T₃ could exist in osteoblasts.

Many hormones and growth factors likely influence the expression and function of PTH and PTH-related peptide (PTHrP) receptors, and certainly of T₃ receptors. In the case of the PTHR, glucocorticoids and transforming growth factor-β up-regulate PTHR in ROS cells (18–20). 1,25-dihydroxyvitamin D, tumor necrosis factor-α, and retinoic acid down-regulate PTHR and receptor mRNA expression (21, 22). Insulin and hydrocortisone increase TRα transcripts (23, 24), whereas interleukin 1β, interleukin 6, and tumor necrosis
factor-α down-regulate TRα- and TRβ binding capacity in a liver cell line (25).

In addition, both PTH and T3 have been shown to affect the production of a number of growth factors and cytokines in osteoblasts (16, 25, 26). These factors also affect bone remodeling, adding to the potential and complexity of the system. Although it is reasonable to infer that there may be interactions between T3 and PTH in the regulation of these factors, there is only limited information regarding this possibility. The purpose of the current study was to determine whether there are mutual interactions between T3 and PTH at the receptor level in osteoblastic cells and, if so, to examine the mechanisms and consequences of these interactions.

Materials and Methods

Reagents

1,35,3′-[125I]-triiodothyronine ([125I]-T3) was purchased from DuPont NEN Life Science Products (Boston, MA), [125I]-[Nle6,Nle18,Tyr34]-PTH(1–34) ([125I]-PTH) and [α25]-deoxycytidine triphosphate were from Amer-

Sham Pharmacia Biotech (Arlington Heights, IL), Bovine PTH(1–34) amide (PTH) was purchased from Bachem Bioscience Inc. (Torrance, CA), Anti-

CREB (cyclic AMP response element binding protein) and Anti-SEIF3-phos-

phorylated CREB were from Upstate Biotechnology, Inc. (Lake Placid, NY), and AMV reverse transcriptase and Taq polymerase were from Promega Corp. (Madison, WI). All chemicals were obtained commercially and were of the highest purity available.

Cell culture

ROS 17/2.8 cells were a generous gift from the Endocrine Unit, Massachusetts General Hospital (Boston, MA), initially provided by Dr. Gideon Rodan (Merck Sharp & Dohme Laboratories, West Point, PA). Cells were maintained in Ham’s F12 Medium supplemented with 5% FBS (Life Technologies, Inc., Grand Island, NY), with or without resin-

stripping, in a humidified atmosphere of 95% O2-5% CO2 at 37° C. Cells

were plated at a density of 5 × 104 cells/cm2, and experiments were performed with confluent cells in 24-well plates. The medium was changed every other day until the cells reached confluence, and daily thereafter.

PTH receptor binding in ROS cells

Confluent ROS cells were maintained in 5% resin-stripped FBS F12 medium. It is necessary to deplete T3 from the supplemented serum for observation of the action of T3, because bovine serum contains a significant level of T3 (27). The cells were rinsed initially with buffer containing 50 mM Tris (pH 7.7), 100 mM NaCl, 2 mM CaCl2, 2.5 mM KCl, 0.5% heat-

inactivated FBS, and 5% heat-inactivated horse serum, and then incu-

bated in this buffer with 2 × 10⁷ cpm/ml of [125I]-PTH in the presence or absence of unlabeled PTH (10⁻⁶ M) at 16 C for 4 h, as previously described (10). For Scatchard analysis, cells were incubated with [125I]-

PTH in the presence of increasing concentrations of unlabeled PTH. Incubations were terminated by aspirating the supernatant and washing the cells with ice-cold binding buffer. Cell membrane-bound radioac-

tivity was recovered by lysing the cells with 1 mM NaOH, and radioactivity was determined in a γ counter. Specific binding was determined by subtracting binding in the presence of unlabeled PTH (1 μM) from total binding.

Thyroid hormone receptor binding in ROS cells and calvaria

ROS 17/2.8 cells were lysed in 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), and nuclear proteins subsequently extracted in 0.4 M KCl, 50 mM Tris-HCl, pH 7.5 (extract buffer). For calvaria experiments, calvaria were dissected and cultured with the treatments. The culture medium was changed daily. After 72 h, the calvaria was removed and homogenized with a Polytron. The pellet was resuspended and incubated at 4 C for 60 min in extract buffer. It was then centrifuged for 30 min at 13,000 rpm, and the nuclear protein extract was collected. Equal amounts of nuclear protein extracts were incubated with [35S]-T3, in the presence or absence of unlabeled T3 (4 × 10⁻⁶ M), at 4 C overnight, as previously described (28). Free and bound [35S]-T3 were separated by vacuum filtration through nitrocellulose membranes (Millipore Corporation, Bedford, MA). Radioactivity on membranes was counted with a γ counter. Specific binding was determined by subtracting binding in the presence of unlabeled T3 (4 × 10⁻⁶ M) from total binding.

Western blot analysis

Cytosolic proteins were extracted in a solution (0.9 mM NaCl, 50 mM Na2HPO4, 2.5 mM EDTA, 20 mM NaF, 1 mM phenylmethylsulfonfyl fluoride, and 50 mM HEPES-Tris, pH 7.4) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of cellular protein extracts were resolved by 10% SDS-PAGE and transferred onto nitrocellulose filters. In each experiment, duplicate membranes were prepared. The membranes were incubated with 3% nonfat milk in PBS for 1.5 h and then incubated overnight at 4 C with rabbit polyclonal antibodies against either total CREB or Ser139-phos-

phorylated CREB. Immunoreactive proteins were detected using an antirabbit horseradish peroxidase-conjugated antibody and the en-

hanced chemiluminescence system (Amersham Pharmacia Biotech), as previously described (29). Antibody-antigen complexes were detected with X-Omat film (Eastman Kodak Co., Rochester, NY) and quantified with a densitometer.

RNA isolation, RT-PCR analysis

Cells were extracted in 4 m guanidine isothiocyanate. Total RNA was separated by ultracentrifugation. The pellet was solubilized in 0.1% SDS, precipitated with ethanol, and reconstituted in DEPC-H2O, characterized by agarose gel electrophoresis, and quantitated by spectrophotomot-

eter at A260. The RT-PCR assay methodology has been previously de-

scribed in detail (30). In brief, 1 μg of RNA product was reverse transcribed using random hexamer priming. The complementary DNAs (cDNAs) were then amplified by PCR in the presence of [α32P]deoxy-

cytidine triphosphate using primers for the PTHR receptor (5′GTGCC-

GGCCTGACAGTGCAGCCGCCTAAAGTA3′ and 5′GTGGATGCAGATGACGTATGACATCTAAAGGA3′), as well as cyclinH in an internal control. PCR reactions were separated on acrylamide gels and quanti-

tated by a BAS 1000 phosphoImager (Fuji Photo Film Co., Ltd., Tokyo, Japan). The results are expressed as a ratio of integrated optical density of PTHR to cyclinH.

Assay for alkaline phosphatase activity and protein concentration

For measurement of intracellular alkaline phosphatase activity, cells were first washed with PBS and then ruptured by repeated freeze-thaw cycles and sonication. Alkaline phosphatase activity was measured by the p-nitrophenyl phosphate method (31). Protein concentration was measured by the Bio-Rad Laboratories, Inc. protein assay (Hercules, CA)

Intracellular calcium measurements

Cells were harvested, washed, and suspended in a loading buffer consisting of 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, 1 mM CaCl2, and 1% BSA, pH 7.4. Cells were incubated with 2 μM fluo-3 AM (acetoxymethylester) (Molecular Probes, Inc., Eugene, OR), by gentle shaking for 30 min, in loading buffer at room temperature. The cells were centrifuged, washed, and resuspended in loading buffer at a concentration of approximately 10⁶ cells/ml. An LS-5 luminescence spectrophotometer (Perkin-Elmer Corp., Foster City, CA) was used for fluorometric determinations (excitation/emission: 505/530 nm). After a 1-min baseline recording, test solutions were added, and the maximal fluorescence was measured. The intracellular calcium concentrations, in nm quantities, were calculated by the following formula: [Ca²⁺]i = F × (Fmax – F)/F, where Fmax is the dissociation constant for fluo-3 (400 nm), Fmin is the maximal fluorescence measured by addition of digitonin (40 μM), Fmax is the fluorescence of cell suspension without fluo-3 loading, and F is the measured fluorescence of sample in the cuvette.
Statistical analysis, binding parameters

Data are means ± sem. Statistical significance was determined by ANOVA and post test. Differences were considered significant at \( P \leq 0.05 \) (*); \( P < 0.01 \) (**); and \( P < 0.001 \)(***) parameters of binding were determined from the Scatchard plots using the Prism software (GraphPad, San Diego, CA).

Results
Effect of T3 on \([^{125}I\text{-}PTH]\) binding

Pretreatment of ROS 17/2.8 cells with T3 (10^{-10}–10^{-6} M) in F12 medium supplemented with 5% resin-stripped serum, for 3 days, resulted in a biphasic dose-dependent increase in \([^{125}I\text{-}PTH](1–34)\) binding, compared with binding in the vehicle-treated control cells (Fig. 1A). Maximal specific binding (144% of control) was seen with 10^{-7} M T3. The time point for measurement was selected from a time-course experiment (Fig. 1B), which demonstrated that the effect on binding started from day 1 and reached a plateau (134% of control) after 3 days of incubation with T3 (10^{-8} M).

Scatchard analysis showed that T3 increased \([^{125}I\text{-}PTH]\) binding sites by approximately 38%, from 42,963 binding sites/cell (control) to 59,441 binding sites/cell (T3-treated). The binding affinity was changed minimally (Fig. 1C). There was no significant difference in cell numbers after pretreatment of ROS 17/2.8 cells with T3 (10^{-10}–10^{-6} M) for 3 days (Fig. 1D). Therefore, the enhanced binding was attributable principally, if not totally, to an increase in available PTH
receptors, rather than to altered receptor binding affinity or cell proliferation.

**Effect of T₃ on PTH receptor mRNA expression**

RNA was extracted from control ROS 17/2.8 cells and cells treated with T₃ (10⁻⁷ M) for 3 days, respectively. RT-PCR analysis revealed that the level of the PTH receptor transcripts was increased 44% by T₃, as compared with the control (Fig. 2).

**Effect of T₃ on PTH receptor function**

PTH induces intracellular calcium transients in osteoblastic cells (32). The increase reaches a peak within 15 sec. Pretreatment of ROS 17/2.8 cells with T₃ (10⁻⁸ M) for 3 days potentiated the calcium signal elicited by PTH (4 × 10⁻⁹ M) (Fig. 3A). The difference in the [Ca²⁺]i response was 34 nm (48 nm in T₃-treated; 14 nm in control). The increase in PTH-induced intracellular calcium signals is consistent with the up-regulation of the PTH receptor by T₃.

PTH induces phosphorylation of CREB through cAMP-dependent protein kinase A (33). Pretreatment of ROS 17/2.8 cells with T₃ (10⁻⁸ M) for 3 days, followed by a 10-min pulse of PTH (10⁻⁸ M), potentiated PTH-induced CREB phosphorylation, increasing by 2.4-fold, compared with controls that were not treated with T₃ (Fig. 3B). It is likely that the enhanced CREB phosphorylation is a consequence of the T₃ stimulated up-regulation of the PTHR.

T₃ increases alkaline phosphatase activity, and PTH down-regulates alkaline phosphatase activity in ROS 17/2.8 cells (13, 17). After pretreatment of ROS 17/2.8 cells with T₃ (10⁻⁸ M) and/or PTH (10⁻⁸ M) for 3 days, the alkaline phosphatase activity of PTH-treated cells decreased (34.9 ± 0.8% of control). Cotreatment with PTH and T₃ resulted in a further decrease to 15.5 ± 3.6%, compared with the control cells (Fig. 3C).

**Effect of PTH/PTHrP on [¹²⁵I]-T₃ binding**

Pretreatment of ROS 17/2.8 cells with PTH (10⁻¹⁰–10⁻⁶ M) for 4 days resulted in a dose-dependent increase in [¹²⁵I]-T₃ binding, compared with the controls (Fig. 4A).

Maximal specific binding in cells treated with PTH (10⁻⁶ M) was 314% of that seen in controls. The time course of the effect of PTH (10⁻⁸ M) on [¹²⁵I]-T₃ binding showed a maximal effect (245%) on day 3 (Fig. 4B). Scatchard analysis of the binding data indicated that the apparent binding affinity for T₃ was similar in PTH-treated and control cells. Calculation of the number of binding sites per cell revealed that there was an approximately 48% increase in the number of T₃ binding sites in PTH-treated cells, compared with the control cells (7,845 binding sites/cell in the controls; 11,629 binding sites/cell after PTH treatment) (Fig. 4C). Pretreatment of ROS 17/2.8 cells with PTHrP (10⁻⁹ M) for 4 days also increased [¹²⁵I]-T₃ binding to 155%, compared with the controls (Fig. 4B). Because the PTH receptor studied could be activated by both ligands (PTH and PTHrP), it is likely that the relevant receptor is the PTH/PTHrP receptor (see Ref. 35).

**Effect of PTH/PTHrP on [¹²⁵I]-T₃ binding in calvaria**

PTH treatment not only increased [¹²⁵I]-T₃ binding in the osteoblastic cell line but also in mouse calvaria (Fig. 5). This is logical in that the osteoblast is probably the major cell type in the calvaria. For these studies, mouse calvaria were pretreated with PTH (10⁻⁸–10⁻⁶ M) or PTHrP (10⁻⁸ M) for 3 days. [¹²⁵I]-T₃ binding was increased to 353% and 174%, respectively, compared with the controls.

cAMP analogues or calcium ionophore can mimic PTH effects on TR binding

Several signal transduction pathways are activated by ligand binding to the PTH/PTHrP receptors. The predominant responses in these cells are the activation of adenylyl cyclase and increased intracellular calcium. As shown in Fig. 6A, both forskolin (a potent activator of adenylyl cyclase) and 8-Br-cAMP (a cAMP analog) were able to increase [¹²⁵I]-T₃ binding in ROS 17/2.8 cells. Also, (Fig. 6B) ionomycin (a calcium ionophore) increased [¹²⁵I]-T₃ binding in ROS 17/2.8 cells, whereas phorbol-12,13-dibutyrate (10⁻⁷ M) (a protein kinase C stimulator) had no effect. In other studies, concentrations of 10⁻⁸–10⁻⁶ M phorbol-12,13-dibutyrate were also ineffective (data not shown). This suggests that either the cAMP pathway or Ca²⁺ signaling may be involved in the PTH-induced up-regulation of TRs.
The intricate balance of concomitant bone resorption and formation is critical for the maintenance of bone metabolism and structure. Both processes are dependent on the action of osteoblasts and regulated by signals from systemic hormones and locally generated growth factors and cytokines. Among them, PTH and T3 play a particularly important role. The endocrine system offers a number of examples of mutual regulation of peptide and steroid hormone receptors. For example, thyroid hormone treatment has been shown to increase mRNA for cardiac membrane b-adrenergic receptors, thereby potentially augmenting the cardiac responses to catecholamines (34). Glucocorticoids up-regulate, and 1,25-dihydroxyvitamin D down-regulates, PTH receptors in osteoblastic cells (19, 21). Because of the influence of both the T3 and PTH/PTHrP receptor pathway on bone biology, we addressed the question of whether these hormone systems modulate each other in bone cells. This study provides evidence for a positive regulation of the PTH/PTHrP membrane receptors by T3, and of T3 nuclear receptors by PTH. This illustrates that these hormones can act indeed in a synergistic fashion.

Our results indicate that PTHrRs were up-regulated by T3 pretreatment (10^{-10}-10^{-6} M) in osteoblastic ROS 17/2.8 cells (Fig. 1A, 2). The minimal effective dose of T3 was 10^{-10} M. Because 99.5% of T3 is normally bound to serum protein, after equilibrium the free T3 concentration in resin-stripped serum medium would be less than 10^{-11} M, which is in the human physiological range. Moreover, T3 pretreatment increased both PTH-induced intracellular calcium transients and PTH-induced CREB phosphorylation (Fig. 3, A and B), possibly through up-regulation of PTHrR by T3 treatment. In ROS 17/2.8 cells, T3 enhances (and PTH decreases) alkaline phosphatase activity, and cotreatment of PTH/T3 results in augmented decrease of enzyme activity (Fig. 3C). This suggested that the effect of PTH on alkaline phosphatase activity is predominantly receptor-mediated, and that T3-induced up-regulation of PTHrRs results in potentiation of the suppressive effect of PTH on alkaline phosphatase. Because T3 can stimulate cell proliferation in certain circumstances, the number of cell membrane receptors could be increased by this mechanism. Although [3H]-thymidine incorporation was slightly increased in T3-treated cells (data not shown), there were no significant changes in cell number when cells were cultured in medium containing resin-stripped serum (Fig. 1D). Therefore, the enhanced [125I]-PTH binding was attributable principally, if not totally, to an increase in available PTH receptors, rather than to altered receptor binding affinity or cell proliferation.

T3 receptors were up-regulated in ROS 17/2.8 cells, after pretreatment with PTH (10^{-10}-10^{-6} M) for 3–4 days, in a dose-dependent manner (Fig. 4). This up-regulation of TR binding was also seen after PTHrP (10^{-9} M) treatment. This suggests that the effects are mediated through a PTH/PTHrP...
receptor, i.e. a type 1 receptor. As of now, three subtypes of PTHRs have been characterized: type 1 is activated by both PTH and PTHrP; type 2 is activated by PTH only; and type 3 is activated by PTHrP only (35).

Pretreatment with either PTH or PTHrP likewise increases [125I]-T3-specific binding in mouse calvaria (Fig 5) and osteoblastic UMR106 cells (data not shown). This demonstrates that PTH/T3 interactions were not restricted to ROS 17/2.8 cells but also occur in other cells of the osteoblast lineage. Both cAMP analogues and the calcium ionophore, ionomycin, were able to mimic PTH effect on TR binding (Fig. 6, A and B), suggesting that either the cAMP-signaling pathway or increases in intracellular calcium could mediate PTH-induced up-regulation of TR.

Scatchard analysis showed that T3 (10⁻² to 10⁻⁶ M) increased the binding sites for the other hormone by approximately 38% and 48% per cell, respectively. This increase could be at the level of transcriptional regulation of the receptors. Regulation of PTH/PTHrP and TR receptor binding and mRNA expression by other hormones have been reported. PTH/PTHrP receptor availability was up-regulated (87%) by hydrocortisone (2×10⁻¹⁷ M); and simultaneously, the level of receptor transcripts was also increased in ROS 17/2.8 cells (18, 19). In the case of the TR, insulin up-regulated nuclear thyroid hormone binding (60%) and increased TRα mRNA expression in bovine aortic endothelial cells (23).

The mechanism of T3/PTH interactions is still unclear. The TRα1 promoter contains three putative CRE and one putative TPA site (36), and TRβ1 promoter contains one putative TRE (37). All of these elements could be potentially regulated by PTH through phosphorylation of CREB, TR, AP1, or other thyroid receptor auxiliary proteins and coregulators, including calmodulin. TR not only can bind to TRE but also can interact with Jun and Fos (38), as well as with RXR, which are

Fig. 4. Effect of PTH on [¹²⁵I]-T₃-specific binding in ROS 17/2.8 cells. Confluent cells were incubated in F12 medium and treated with a different dose of PTH or vehicle (C) for 4 days, respectively. Media were renewed daily. T₃ receptor binding was then assayed as described in Materials and Methods. Statistical significance was determined by ANOVA and post test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, Dose-response curve of effect of PTH (10⁻¹⁰–10⁻⁶ M) and vehicle (C) on [¹²⁵I]-T₃-specific binding. Data are means and SEM of 3 determinations. B, Time course of effect of PTH (10⁻⁸ M), PTHrP (10⁻⁸ M), and vehicle (C) on [¹²⁵I]-T₃-specific binding. Data are means and SEM of 2–4 determinations. Panel C, Scatchard plot of effect of PTH (10⁻⁸ M) on [¹²⁵I]-T₃-specific binding. Cells were incubated with [¹²⁵I]-T₃ in the presence of increasing concentrations of unlabeled T₃, overnight at 4 C. T₃ receptor binding was then assayed as described in Materials and Methods. B/F, Bound-to-free ratio.

Fig. 5. Effect of PTH/PTHrP on [¹²⁵I]-T₃-specific binding in mouse calvaria. Eight-day-old mice calvaria were incubated in DMEM containing 10% heat-inactivated horse serum and treated with different doses of PTH (10⁻⁷–10⁻⁶ M), PTHrP (10⁻⁸ M), or vehicle (C) for 3 days, respectively. Media were renewed daily. Bone was homogenized, and cytosolic proteins were extracted. T₃ receptor binding was then assayed as described in Materials and Methods. Data are means and SEM of 3 determinations and are representative of two experiments. Statistical significance was determined by ANOVA and post test. **, P < 0.01; ***, P < 0.001.
potential targets for TR activation of PTHR expression, because the PTHR promoter contains putative AP-1 and RXRβ sites (39).

In conclusion, the present experiments demonstrate that PTH treatment increases the number of thyroid hormone receptors in osteoblastic ROS 17/2.8 cells in a time- and dose-dependent manner. Conversely, T3 treatment increases the number of PTH receptors and receptor transcripts in ROS 17/2.8 cells as well. This is a novel example of mutual regulation between nuclear receptors and membrane receptors, an observation that has significant implications for the regulation of bone remodeling. Further characterization of the involved TR isoforms, the signaling pathways, and the regulation of the promoter regions of both the PTH/PTHrP receptor and TRs may shed further light on these interactions.

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Fig. 6. Effect of activation of signaling pathways on [125I]-T3-specific binding in ROS 17/2.8 cells. A, Effect of forskolin (FSK) or Br-cAMP on [125I]-T3-specific binding in ROS 17/2.8 cells. Confluent cells were incubated in F12 medium containing either 10 μM FSK, 100 μM Br-cAMP, or 10−9 M PTH for 3 days. Media were renewed daily. T3 receptor binding was then assayed as described in Materials and Methods. Data are means and SEM of 3 determinations and are representative of two experiments. Statistical significance was determined by ANOVA and post test. ***, P < 0.01; **, P < 0.05; *, P < 0.001. B, Effect of phorbol-12,13-dibutyrate and ionomycin on [125I]-T3-specific binding in ROS 17/2.8 cells. Confluent cells were incubated in F12 medium containing either 10−9 M phorbol-12,13-dibutyrate (PDBu) or 10−9 M ionomycin (Iono) for 4 days. Media were renewed daily. T3 receptor binding was then assayed as described in Materials and Methods. Data are means and SEM of 3 determinations and are representative of two experiments. Statistical significance was determined by ANOVA and post test. **, P < 0.05; ***, P < 0.01.

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