Prostaglandin-Independent Stimulation of Bone Resorption in Mouse Calvariae and in Isolated Rat Osteoclasts by Thyroid Hormones (T_4, and T_3) (44217)

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Abstract. **The thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), were found to enhance both neonatal mouse calvarial bone resorption and pit formation on bovine slices by isolated rat osteoclasts. Dosage-dependent release of ^{45}Ca from mouse calvarial bones was observed after 120 hr of culture with 10^{-6}–10^{-8} *M* T_4 and 10^{-6}–10^{-10} *M* T_3. Maximum treatment/control ratios of ^{45}Ca release were recorded for 10^{-7} *M* T_4 and 10^{-8} *M* T_3. Inhibition of ^{45}Ca release stimulated by 10^{-8} *M* T_3 was observed in the presence of 30 n*M* salmon calcitonin at 48 hr and 120 hr of culture with no indication of "escape" by T_3-treated bones. In contrast, stimulation of ^{45}Ca release from mouse calvarial bones by 10^{-7} *M* T_4 and 10^{-8} *M* T_3 was not inhibited by 10^{-6} *M* indomethacin. Formation of PGE_2 and PGI_2 (evaluated by measuring 6-keto-$PGF_{1\alpha}$) by mouse calvariae was also not increased by 10^{-8} *M* T_3 after 120 hr of culture. Furthermore, no increases in cAMP formation were observed in calvarial bone cultures after either 10 min or 24 hr of exposure to 10^{-8} *M* T_3. However, significant inhibition of ^{45}Ca release stimulated by 10^{-8} *M* T_3 was found at 120 hr in the presence of 10^{-3} *M* hydroxyurea. When isolated rat osteoclasts were cultured in the presence of 10^{-7} *M* T_3, a 1.4-fold stimulation of pit number was observed. Pit formation was not affected by addition of 10^{-6} *M* indomethacin to either the control or T_3-treated cultures. These data suggest that the stimulation of bone resorption in neonatal mouse calvariae and activation of isolated rat osteoclasts by the thyroid hormones is not related to either prostaglandin or cAMP formation. In mouse calvariae, the effect on bone resorption of the thyroid hormones is dependent on increased cellular replication, perhaps of osteoclast precursors, or other bone cells involved in the resorptive process.**

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The thyroid hormones are thought to play important regulatory roles in calcium metabolism (1). Normal bone growth and skeletal maturation are deficient in the absence of adequate concentrations of the thyroid hormones (2). The growth promoting effects of the thyroid hormones are believed to be due to both a direct skeletal action of the compounds (3) and an indirect facilitation of the synthesis and secretion of growth hormone (4). In adults, the thyroid hormones are considered to be important regulators of bone turnover (1). In hyperthyroidism, bone turnover is increased, with greater bone resorption occurring than formation (5). This increase in bone resorption can cause a considerable loss of bone mass, and in thyrotoxic patients, an increased osteoporotic fracture rate has been observed (6).

Several studies performed in bone organ cultures have shown that the thyroid hormones can stimulate bone resorption (7–12). However, questions concerning this stimulation, such as the dependency of thyroid hormone action on prostaglandin biosynthesis, remain unanswered. While thyroid hormone–mediated resorption of fetal rat limb bones is

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thought to be independent of prostaglandin production (7, 8), it has been suggested that the resorptive action of the thyroid hormones in neonatal mouse calvariae is prostaglandin-dependent (8, 9). Prostaglandins are believed to be important local regulators of bone resorption (13). In neonatal mouse calvariae, the resorptive effects of agents such as epidermal growth factor (14), platelet-derived growth factor (15), phorbol esters (16), bradykinin (17), and kallidin (18) appear to be completely dependent on increased prostaglandin biosynthesis. In contrast, the bone resorbing actions of compounds such as thrombin (19), interleukin-1 (20), and tumor necrosis factor (21) have been suggested to be mediated by both prostaglandin-independent and prostaglandin-dependent pathways.

In a recent study employing neonatal mouse calvariae, it was suggested that a portion of the resorptive action of the thyroid hormones may be prostaglandin-independent (11). Another recent investigation using isolated osteoclasts has indicated that the action of the thyroid hormones to stimulate bone resorption is indirect, and mediated by osteoblasts (22). The present study testing the effectiveness of thyroxine (T_4) and triiodothyronine (T_3) in neonatal mouse calvarial bones and in isolated rat osteoclasts was initiated to gain a better understanding of the resorptive action of the thyroid hormones and the dependence of this action on prostaglandin biosynthesis.

Materials and Methods

Experimental Design. To investigate skeletal actions of the thyroid hormones, initial experiments were performed to establish the capacity of the thyroid hormones to elicit mineral and matrix breakdown, as well as stimulate lysosomal enzyme release, in neonatal mouse calvariae. In isolated rat osteoclasts, the ability of T_3 to stimulate pit formation on bone slices was evaluated. To determine if bone breakdown stimulated by the thyroid hormones was a prostaglandin-independent or-dependent process, prostanoid formation in neonatal mouse calvariae was evaluated following thyroid hormone treatment. In addition, the resorptive effects of the thyroid hormones in both neonatal mouse calvariae and isolated rat osteoclasts were tested in the presence of the inhibitor of cyclooxygenase enzyme, indomethacin. To further characterize the resorptive effects of the thyroid hormones, the ability of T_3 to stimulate cyclic AMP formation in neonatal mouse calvariae was evaluated. T_3 was also tested in the presence of the hormonal inhibitor of bone resorption, salmon calcitonin (sCT), as well as with the inhibitor of DNA synthesis, hydroxyurea. Lastly, to investigate if interaction between the thyroid hormones and parathyroid hormone (PTH) might occur, T_3 treatment was combined with PTH treatment of mouse calvarial bones.

Hormones and Chemicals. Synthetic bovine PTH 1-34 was purchased from Bachem, Bubendorf, Switzerland; CMRL 1066 medium from Gibco (Renfrewshire, Scotland), [^{45}Ca] $CaCl_2$, and the radioimmunoassay kits for PGE_2, 6 keto-$PGE_{1\alpha}$, and cyclic AMP (DuPont/New England Nuclear, Dreieich, Germany); L5-[^{3}H] proline (The Radiochemical Centre, Amersham, England); multiwell plastic culture dishes (Costar, Cambridge, MA), choleratoxin (Schwartz/Mann, Orangeburg, NY); p-nitrophenyl *N*-acetyl-β-D-glucosaminide, bovine serum albumin (RIA grade), L-thyroxine (T_4), and L-3, 3′, 5′ triiodothyronine (reverse T_3 [rT_3]) (Sigma Chemical Company, St Louis, MO). L-3,5,3′, triiodothyronine (T_3) was kindly provided by Dr. Klaus Klaushofer, Vienna, Austria; indomethacin a gift from Merch, Sharp and Dohme, Haarlem, The Netherlands; synthetic salmon calcitonin (sCT) supplied by Sandoz, Basel Switzerland; rolipram kindly obtained from Sherring AG, Berlin, Germany. Indomethacin was dissolved in 99% ethanol and thyroxine, T_3 and rT_3 were dissolved in 5% 2 *M* NAOH in ethanol before dilution in medium to the stated concentrations. The final concentrations of ethanol in culture media did not exceed 0.1%. PTH was dissolved at a concentration of 10 μmol/l in 10 mmol/ HCl containing 1 g/l albumin and sCT was dissolved as a stock solution of 30 μmol/l in medium. Both were stored at −80°C. A stock solution of choleratoxin (1 mg/ml) was prepared in Tyrode's solution and stored at −80°C. Rolipram was dissolved directly in culture medium.

Tissue Culture Technique. Parietal bone explants from 6- to 7-day-old CsA mice were used for study. The calvarial bones were dissected in two ways; (1) the two parietal bones were divided along the sagittal suture, producing two calvarial halves; (2) the posterior two-thirds of each calvarial half was divided further into anterior and posterior bone portions, producing four bone fragments (23). Half calvariae were used in experiments evaluating cAMP formation, to assess lysosomal enzyme release and for stable calcium (Ca^{2+}) and inorganic phosphate (P_i) measurements. Microdissected parietal bone fragments (four/ one calvarium) were employed in all other experiments. For the study of cAMP formation, calvarial halves were preincubated for 30 min in CMRL 1066 medium (four calvarial halves/2 ml medium) containing 0.1% bovine serum albumin (BSA) and 30 μmol/l of the phosphodiesterase inhibitor, rolipram (24). Subsequently, bones were incubated for the stated times in the presence of rolipram, with and without addition of test agents. In all additional studies, bones were preincubated for 18 to 24 hr in CMRL 1066 medium containing 0.1% BSA and 1 μmol/l indomethacin (25). Following preincubation, the bones were washed extensively in Tyrode's solution and subsequently cultured for 120 hr in multiwell culture dishes (one bone/well) containing 2 ml CMRL 1066 medium with 0.1% BSA and added test solutions. During both preculture and experimental intervals, the parietal bones were incubated with 5% CO_2 in humidified air at 37°C.

Analysis of Mineral Mobilization. At the end of experiments, bones from mice injected with 1.5 μCi ^{45}Ca 4 days prior to dissection (26) were dissolved in HCl, and aliquots of media and bones were analyzed for radioactivity by liquid scintillation. The extent of bone resorption was

evaluated by the release of ^{45}Ca from bones to the culture medium, expressed as a percentage of the initial radioactivity present in bones (25).

In some experiments, mineral release was determined by measuring stable calcium (Ca^{2+}) and inorganic phosphate (P_i) in media before and after experimentation. Atomic absorption spectrometry was used for Ca^{2+} analysis (27), and P_i was measured spectrophotometrically (28).

Evaluation of Prostaglandin Synthesis. Prostaglandin production by the bones was assessed by measurement of prostaglandins in culture media at the termination of experiments (18). The concentrations of PGE_2 and PGI_2 (quantitated by measurement of the stable metabolite, 6-keto-$PGF_{1\alpha}$) were determined with commercially available radioimmunoassay kits. Assays for both prostanoids were performed according to instructions supplied by the manufacturers.

Determination of Matrix Degradation. Mice were subcutaneously injected with 10 μCi [^{3}H]-proline 4 days before calvarial bone dissection. At the end of experimentation, aliquots of media and hydrolysed bone samples were analyzed for ^3H and the percentage release of ^3H to the culture medium determined as described previously (25). The release of ^3H to culture medium represents release of both [^{3}H]-proline and [^{3}H]-hydroxyproline. Previous study has shown a good correlation between ^3H release and [^{3}H]-hydroxyproline release from bone (29), thus ^3H release is considered to be a good indicator of collagen breakdown.

Measurement of Enzyme Release. The release of lysosomal enzyme from bone was evaluated by measuring β-*N*-acetyl glucosaminidase activity. At the end of the cultures, bones were placed in 0.2% (v/v) Triton X-100 in saline for 24 hr for removal of enzyme (30). Culture media and aliquots of supernatants were analyzed for β-N-acetylglucosaminidase activity using p-nitrophenyl N-acetyl-β-D-glucosaminide as substrate (31). Enzyme release to the media was expressed as a percentage of total enzymatic activity.

Analysis of cAMP Production. At the end of experimental intervals, calvarial bones were placed in 90% n-propanol for extraction of cAMP. The extracts were evaporated, and cAMP in reconstructed samples was measured with a commercially available radioimmunoassay kit using [^{125}I] cyclic AMP as a tracer (24). The assay was performed according to instructions supplied by the manufacturer.

Measurements of Bone Resorbing Activity in Isolated Rat Osteoclasts. The method used was originally described by Boyde *et al.* (32) and Chambers *et al.* (33) and subsequently modified by Arnett & Dempster (34). Long bones were removed from 2- to 5-day-old Sprague-Dawley rats, dissected free from soft tissues, and transferred to prewarmed (37°C) Hanks medium 199 containing 10% fetal bovine serum albumin, antibiotics, and 1-glutamine (200 μg/ml). Cells in the marrow cavity were flushed with a syringe, and aliquots of the cell suspension were transferred to 96-well culture dishes, each containing a single 100-nm thin slice of bovine cortical bone. After allowing the cells to settle for 30 min at 37°C, the bone slices were removed and gently washed in prewarmed Tyrodes solution. The bone slices were then incubated in Hanks medium 199 containing 0.1% albumin with or without test substances. After 48 hr the cells were removed, the slices were dried and stained with toluidine blue, and the number of discrete excavations per slice was determined using bright field microscopy (Nikon Labophot-2, Tokyo, Japan).

Statistical Analysis. Statistical calculations were made using analysis of variance (ANOVA) and the least significant difference (lsd).

Results

Experiments initially were performed in calvariae to establish dose response curves of ^{45}Ca release stimulated by T_4 and T_3. Significant stimulation of bone resorption was observed for T_4 at concentrations ranging from 10^{-6} to 10^{-8} *M*, while mineral mobilization was significantly increased by T_3 at concentrations of 10^{-6} to 10^{-10} *M* (Fig. 1). Although there was no difference in the maximum degree of radioactive calcium release stimulated by T_4 and T_3, an approximate 10-fold greater sensitivity to T_3 treatment was observed. The half-maximal responses to T_4 and T_3 were 1×10^{-9} *M* and 7.5×10^{-10} *M*, respectively. In contrast to the stimulatory effects of T_4 and T_3, significant release of ^{45}Ca did not occur when the inactive analogue rT_3 was tested over a concentration range of 10^{-6}–10^{-11} *M*.

Subsequent experiments were performed to compare the effects of T_3 and PTH on mineral and matrix mobilization in neonatal mouse calvariae. These data are plotted in Figure 2 a and b. Significant release of ^{45}Ca and ^3H was noted for both 10^{-8} *M* T_3 and 10^{-8} *M* PTH. The ^{45}Ca release elicited by T_3 after 120 hr of culture was 12% of the release stimulated by PTH. Collagen breakdown stimulated by T_3

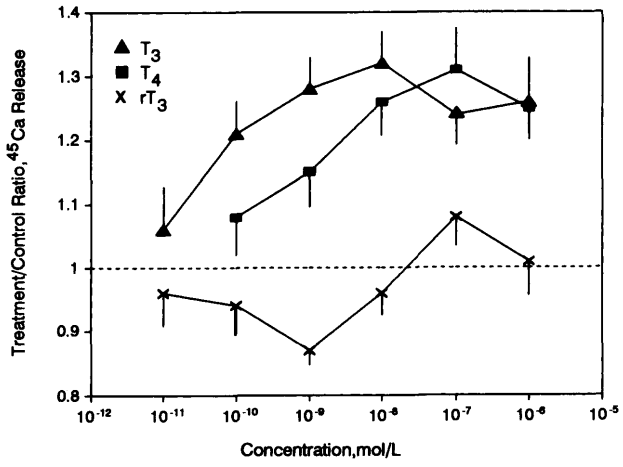

Figure 1. Treatment/control ratios of the percent ^{45}Ca release for T_3, T_4, and rT_3. Calvarial bones were cultured for 120-hr as described in Methods. Values are based on means ≥ 12 bones, and the SEM is shown as vertical bars. The percent ^{45}Ca release versus control was significantly different ($P < 0.01$) for T_4 at 10^{-6}–10^{-8} *M* and T_3 at 10^{-6}–10^{-10} *M*.

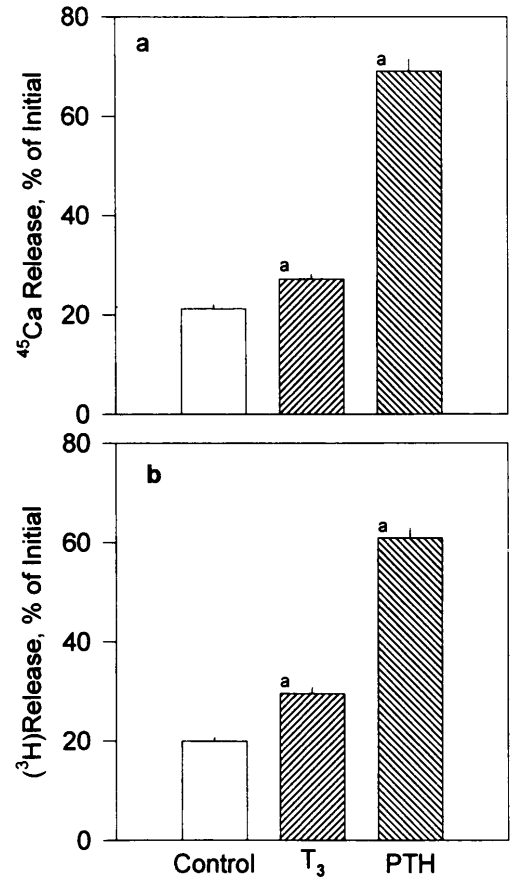

Figure 2. Stimulation of percent ^{45}Ca release (a) and percent [^{3}H] release (b) by 10^{-8} *M* T_3 and 10^{-8} *M* PTH. Calvarial bones were cultured for 120 hr as described in Methods. Values are based on means ≥ 23 bones, and the SEM is shown as vertical bars. The percent ^{45}Ca release and percent [^{3}H] release were significantly different ($P < 0.01$) from control values following T_3 and PTH treatment.

as assessed by ^{3}H release, equaled 23% of that noted after bones were exposed to PTH. Similar results were obtained when unlabelled calvarial bones were treated with 10^{-8} *M* T_3 and 10^{-8} *M* PTH (Table 1). Enhanced release to media of Ca^{2+}, P_i, and the lysosomal enzyme β-*N*-acetylglucosaminidase was found after 120 hr of treatment with both T_3 and PTH. However, stimulation by T_3 was less than observed following treatment of the calvarial bones with PTH. The Ca^{2+} release was 13%; P_i release was 16%; and β-*N*-acetylglucosaminidase release was 39% of the responses stimulated by PTH.

To evaluate the time course of thyroid hormone action and the ability of the thyroid hormones to "escape from calcitonin-induced inhibition" (35), 10^{-8} *M* T_3 and 10^{-8} *M* PTH were compared both in the absence and the presence of 30 n*M* sCT (Fig. 3 a, b). In the absence of sCT, both T_3 and PTH significantly increased ^{45}Ca release from calvarial bones at 48 and 120 hr. The release of ^{45}Ca by T_3 at 48 hr accounted for 38% of total release, whereas ^{45}Ca release stimulated by PTH at 48 hr was 51% of the 120 hr release. In the presence of sCT, significant inhibition of calcium release stimulated by T_3 and PTH occurred at 48 hr and 120 hr. In the case of T_3, bone resorption was severely blunted by calcitonin treatment with no change over time in the treatment/control ratios of the T_3 + sCT experimental group. In marked contrast to this sustained effect, inhibition by sCT of PTH treated bones was more pronounced at 48 hr of culture then after 120 hr of culture. The inhibition of PTH-mediated bone resorption by sCT was greater than 70% at 48 hr but had decreased to less than 20% by 120 hr.

For determination of the role of prostaglandins in thyroid hormone action, experiments were performed to test the ability of T_4 to stimulate ^{45}Ca release in the presence of the cyclooxygenase inhibitor, indomethacin (Table II). Significant stimulation of bone resorption by 10^{-7} *M* T_4 was observed at 120 hr, not only in bone cultures without indomethacin addition, but also in indomethacin (1 μ*M*)-treated cultures as well. In subsequent experiments, the effect of 1 μ*M* indomethacin on mineral mobilization and the formation of PGE_2 and 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2) in calvarial bones exposed to 10^{-8} *M* T_3 was studied (Table III). As noted in the previous experiments with T_4, significant stimulation of bone resorption by T_3 occurred both in the absence and the presence of indomethacin. Additionally, no changes in PGE_2 and 6-keto $PGF_{1\alpha}$ formation were observed after 120 hr of culture with T_3 although significant production of the prostanoids was found with PTH, which was employed as a positive control. After 120 hr of culture with 10^{-8} *M* PTH in the absence of indomethacin, PGE_2 formation was 919 ± 170 pmol/bone (mean ± SEM; $n = 7$) and 6-keto-$PGF_{1\alpha}$ formation was 661 ± 124 pmol/bone (mean ± SEM; $n = 8$).

Osteoclast cultures settled for 30 min on bone slices and then incubated for 48 hr in medium containing albumin (0.1%) responded to 10^{-8} *M* PTH and 10^{-8} *M* 1,25$(OH)_2$-vitamin D3 with a 1.7-and 2.0-fold stimulation, respec-

Table I. The Effect of T_3 and PTH on the Release of Calcium (Ca^{2+}), Inorganic Phosphate (P_i) and β-*N*-acetylglucosaminidase (β-*N*) from Neonatal Mouse Calvarial Bones

Additions	Amounts (*M*)	Ca^{2+} (μg/bone)	P_i (μg/bone)	β-*N* (% release)
Control	—	30.7 ± 2.6	5.0 ± 0.7	28 ± 1.3
T_3	10^{-8}	40.5 ± 2.7	9.1 ± 0.6[a]	39 ± 1.3[a]
PTH	10^{-8}	107.4 ± 9.4[a]	30.2 ± 1.3[a]	56 ± 2.0[a]

Note. Values are means ± SEM for ≥ 12 bones. T_3 and PTH were added at Time 0 and the bones cultured for 120 hr as described in Methods.
[a] Significantly different from control ($P < 0.01$).

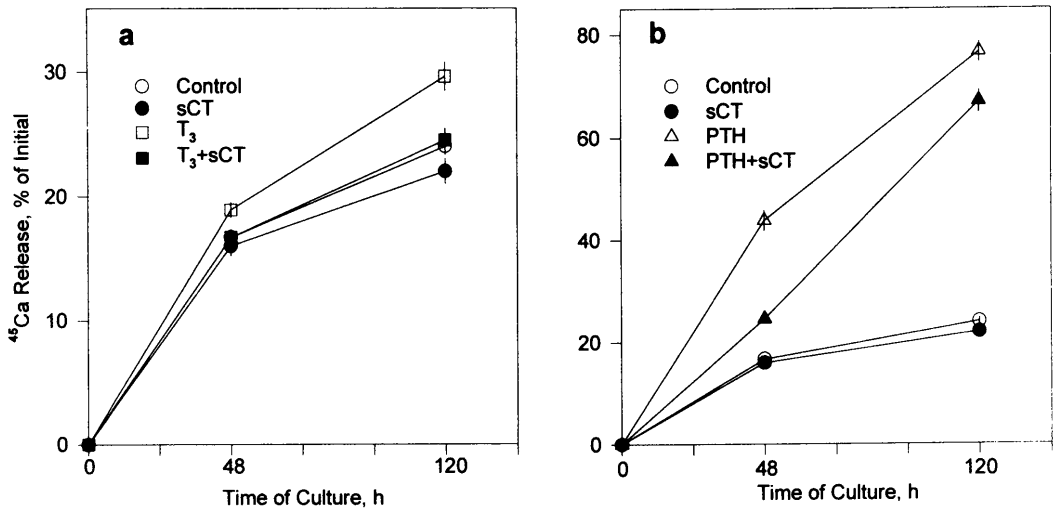

Figure 3. Stimulation of the percent ^{45}Ca release after 48-hr and 120-hr treatment with 10^{-8} *M* T_3 (a) and 10^{-8} *M* PTH (b) in the absence and presence of 3×10^{-8} *M* sCT. Small aliquots of culture media were withdrawn at 48 hr, and the calvarial bones were cultured for an additional 72 hr as described in Methods. Values are based on means ≥ 13 bones, and the SEM is shown as vertical bars when larger than the radius of the symbol. Addition of sCT to control cultures had no significant effect on resorption. In the absence of sCT, the percent ^{45}Ca release caused by T_3 treatment (a) was significantly different from control release after 48 hr ($P < 0.05$) and 120 hr ($P < 0.01$) of culture. Significant differences in ^{45}Ca release were additionally noted at 48 hr ($P < 0.05$) and 120 hr ($P < 0.01$) when T_3-treated calvarial bones were compared to calvarial bones treated with T_3 + sCT, but no differences were observed between control and T_3 + sCT treated calvariae. In the absence of sCT, the percent ^{45}Ca release caused by PTH treatment (b) was also greater than control release after 48 hr ($P < 0.01$) and 120 hr ($P < 0.01$) of culture. There were also significant differences in ^{45}Ca release when PTH treated calvariae were compared to calvariae exposed to PTH + sCT at 48 hr ($P < 0.01$) and 120 hr ($P < 0.01$). However, while the decrease in the degree of inhibition caused by sCT in PTH-treated calvariae between 48 hr and 120 hr was > 50% (b), "escape" was not apparent in T_3 + sCT-treated calvariae (a).

Table II. Stimulation of ^{45}Ca Release from Neonatal Mouse Calvarial Bones by T_4 ± Indomethacin

Additions	Amount (*M*)	^{45}Ca release (% of initial)	
		– indo	+ indo (1 μ*M*)
Control	—	22.2 ± 0.8	24.1 ± 1.0
T_4	10^{-7}	27.0 ± 1.2[a]	31.2 ± 1.1[a]

Note. Values are means ± SEM for ≥ 13 bones. T_4 and indomethacin (indo) were added at Time 0 and the bones cultured for 120 hr as described in Methods. Indomethacin had no significant effect on either control or T_4-treated calvariae.
[a] Significance due to T_4 treatment ($P < 0.01$).

tively, of pit number formation (data not shown). When osteoclasts were cultured in the presence of 10^{-7} *M* T_3, a 1.4-fold stimulation of pit number was obtained (Fig. 4). Addition of 10^{-6} *M* indomethacin to culture media did not affect pit formation in either control cultures or in cultures stimulated by T_3 (Fig. 4). Similarly, the stimulatory effect of 10^{-8} *M* PTH was not significantly affected by indomethacin co-treatment; treatment/control ratios were 1.7 (PTH versus control) in the absence of indomethacin and 1.6 (PTH + indo versus control + indo) in the presence of indomethacin (Fig. 4).

In an effort to gain a better understanding of the mechanisms by which the thyroid hormones stimulate bone resorption, experiments were designed to test the ability of T_3 to stimulate cAMP formation. Following preincubation, calvarial bones cultured with 30 μ*M* rolipram were treated with either 10^{-8} *M* T_3, 0.1 μg/ml choleratoxin (CTX) or 10^{-8} *M* PTH for 10 min and 24 hr (Table IV). No changes in calvarial bone production of cAMP were noted after either 10 min or 24 hr of culture with T_3. In contrast, a significantly increased level of cAMP was found after a 10-min exposure of bones to PTH, and at 24 hr small, but significant, increases in the levels of cAMP were found in CTX-and PTH-treated bones.

Additional experiments were designed to evaluate the dependence of the resorptive effects of the thyroid hormones on DNA synthesis. These data are shown in Fig 5. Significant release of ^{45}Ca was observed following exposure of calvarial bones to 10^{-8} *M* T_3 and 10^{-8} *M* PTH for 120 hr. Although addition of 10^{-3} *M* hydroxyurea had no effect on radioactive calcium release from either the control or PTH-treated bones, inhibition of ^{45}Ca release from bones treated with 10^{-8} *M* T_3 was observed at 120 hr.

To determine if interaction between the thyroid hormones and PTH might affect *in vitro* bone resorption, ^{45}Ca release from mouse calvarial bones was evaluated following 120 hr treatment with 3×10^{-11}, 7×10^{-11}, 10^{-10}, 3×10^{-10}, and 10^{-9} *M* PTH in the absence and presence of 10^{-8} *M* T_3. In these experiments, the mean percentage of ^{45}Ca release ± SEM for control bones receiving no treatment was 24.0 ± 1.0 at 120 hr. Significant stimulation ($P < 0.01$) of ^{45}Ca release was noted at all concentrations of PTH. However, no differences in ^{45}Ca release were observed when bones treated with PTH alone and bones treated with PTH plus T_3 were compared (Fig. 6). Furthermore, in subsequent experiments, no differences in ^{45}Ca release were found for neonatal mouse calvariae pre-treated for 24 hr with 10^{-8} *M* T_3

Table III. Stimulation of ^{45}Ca Release and the Formation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in Neonatal Mouse Calvarial Bones Following Treatment with T_3 ± Indomethacin

Additions	Amount (*M*)	^{45}Ca release (% initial)		PGE_2 formation (pmol/bone)		6-keto-$PGF_{1\alpha}$ formation (pmol/bone)	
		– indo	+ indo (1 μ*M*)	– indo	+ indo (1 μ*M*)	– indo	+ indo (1 μ*M*)
Control	—	21.5 ± 0.6	21.6 ± 0.7	54.4 ± 7.8	12.9 ± 1.3[b]	104.4 ± 22.6	26.9 ± 4.3[b]
T_3	10^{-8}	27.2 ± 0.9[a]	25.5 ± 0.9[a]	69.8 ± 10.2	14.7 ± 1.9[b]	120.0 ± 21.7	39.4 ± 5.2[b]

Note. Values are means ± SEM for ≥ 18 bones. T_3 and indomethacin (indo) were added at Time 0 and the bones cultured for 120 hr as described in Methods.
[a] Significance due to T_3 treatment ($P < 0.01$).
[b] Significance due to indomethacin treatment ($P < 0.01$).

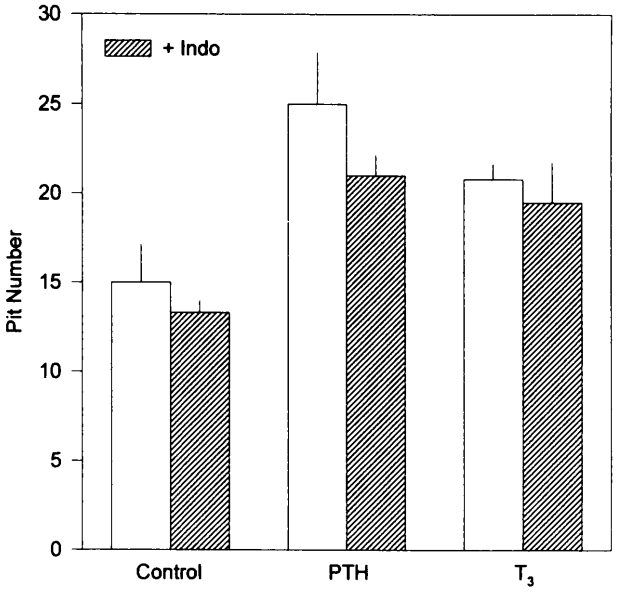

Figure 4. The effect of 10^{-7} *M* T_3 and 10^{-8} *M* PTH on the number of pits excavated by purified rat bone marrow osteoclasts in the absence and presence of 10^{-6} *M* indomethacin (indo). Values shown are means for four bone slices, and the SEM is shown as vertical bars. Indomethacin addition had no effect on pit formation in either control, T_3, or PTH-treated bone slices. In both the absence and presence of indomethacin, pit formation was significantly different from corresponding control values on T_3 ($P < 0.05$) and PTH ($P < 0.01$) treated bone slices.

before treatment for 96 hr with PTH and T_3 (Conaway and Lerner, unpublished observations).

Discussion

The present study provides evidence of the bone resorbing activity of the thyroid hormones. Increased mineral mobilization, matrix degradation, and lysosomal enzyme release were found following thyroid hormone treatment of mouse calvarial bones. These data are in good agreement with previous work by investigators who have studied the resorptive actions of the thyroid hormones in fetal rat limb bones (7, 8), fetal mouse limb bones (12), and neonatal mouse calvariae (8–11). In these studies, increased calcium and lysosomal enzyme release, as well as decreased bone calcium, P_i, and hydroxyproline content were found following thyroid hormone treatment. Furthermore, the slow development of bone resorption observed with T_3 in the current investigation was aligned closely with the delayed time courses of thyroid hormone–stimulated calcium release, which have been reported previously in fetal rat limb bones (7) and in neonatal mouse calvariae (9, 11).

Table IV. Formation of cAMP in Neonatal Mouse Calvarial Bones Following Treatment with T_3, CTX and PTH

Additions	Amount	cAMP (pmol/bone)	
		10 min	24 hr
Control	—	13.2 ± 2.9	3.7 ± 0.9
T_3	10^{-8} *M*	12.1 ± 1.1	3.2 ± 0.6
CTX	0.1 μg/ml	17.4 ± 3.8	39.0 ± 3.7[a]
PTH	10^{-8} *M*	570.0 ± 49.4[a]	11.9 ± 0.7[a]

Note. Values are means ± SEM for 4 bones. T_3, CTX, and PTH were added at Time 0 in the presence of 30 μ*M* rolipram and the bones cultured for 10 min and 24 hr as described in Methods.
[a] Significantly different from control ($P < 0.01$).

Thyroid hormone action is thought to be initiated by the binding of iodothyronines to specific target cell nuclear receptors, and high affinity nuclear receptors for the thyroid hormones have been reported in osteosarcoma cells (36, 37) and in neonatal mouse calvariae (10). The nuclear thyroid hormone receptors, which are referred to as T_3-receptors, bind both T_4 and T_3, but exhibit a 10-fold greater affinity for T_3 (38). The dose response curves of calcium release, recorded following T_4 and T_3 treatment, indicated a greater sensitivity (approximately 10-fold) of the calvarial bones to T_3 exposure. These data favor the idea that nuclear events initiated by T_3-receptor binding were responsible for the resorptive effects of the thyroid hormones.

Calcitonin is a potent inhibitor of osteoclastic bone resorption, but inhibition by calcitonin of most resorptive agents, such as PTH, 1,25$(OH)_2$-vitamin D_3, and the prostaglandins, is transient (35, 39). Despite the continuous presence of calcitonin, subsequent resorption occurs; it approaches and often equals levels stimulated in the absence of inhibitor. This phenomenon, referred to as "escape from calcitonin-induced inhibition," (35) occurred in PTH-treated calvarial bones in the present study. In contrast, the inhibition of T_3-mediated bone resorption by calcitonin was sustained, with no indication of "escape" by T_3 from the

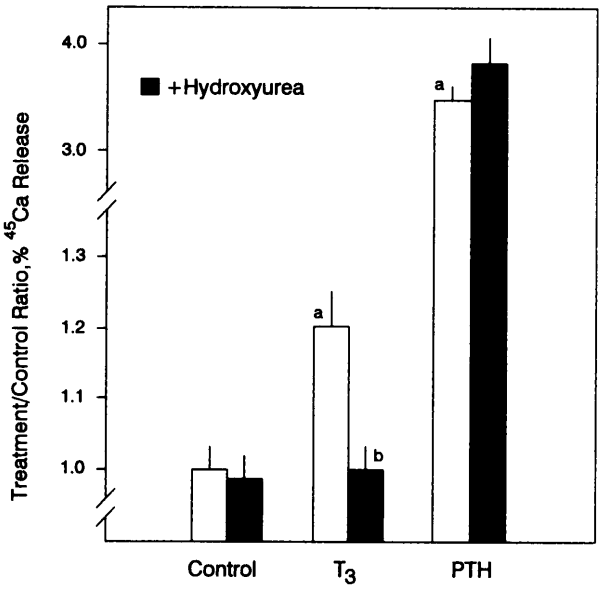

Figure 5. Treatment/control ratios of the percent ^{45}Ca release following treatment with 10^{-8} *M* T_3 and 10^{-8} *M* PTH in the absence and presence of 10^{-3} *M* hydroxyurea. Calvarial bones were cultured for 120 hr as described in Methods. Values are based on means of 16 bones and the SEM is shown as vertical bars. In the absence of hydroxyurea, treatment with either T_3 or PTH caused significant increases [a]($P < 0.01$) in ^{45}Ca release from calvarial bones. Hydroxyurea addition to calvarial cultures had no effect on either control or PTH-stimulated ^{45}Ca release but caused significant inhibition [b]($P < 0.01$) of ^{45}Ca release stimulated by T_3.

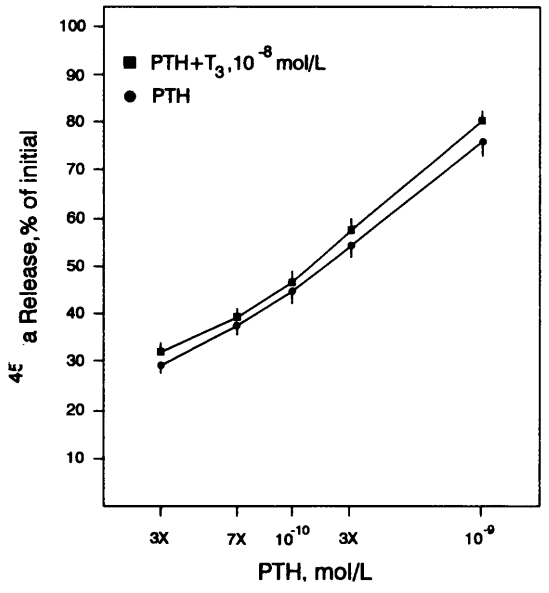

Figure 6. Stimulation of the percent ^{45}Ca release by 3×10^{-11}, 7×10^{-11}, 10^{-10}, 3×10^{-10}, and 10^{-9} *M* PTH in the presence and absence of 10^{-8} *M* T_3. Calvarial bones were cultured for 120 hr as described in Methods. Values are based on means ≥ 16 bones and the SEM is shown as vertical bars. Comparisons of the percent ^{45}Ca release between PTH-treated bones with and without 10^{-8} *M* T_3 treatment revealed no significant differences.

inhibitory influence of calcitonin. This lack of "escape" by the thyroid hormones has been observed previously by other investigators using neonatal mouse calvariae for study (9, 11). However, the reason for this sustained effect of calcitonin is unclear. One recent suggestion is that the inability of the thyroid hormones to "escape" from calcitonin simply may be reflective of the small degree of bone resorption stimulated by these agents (11). Alternatively, recent studies have shown that "escape" from calcitonin inhibition does not occur following stimulation of bone resorption with either phorbol esters (40) or glucocorticoids (41). These data tend to suggest that there may be mechanisms inherent in the actions of certain compounds, such as the thyroid hormones, phorbol esters, and glucocorticoids, which prevent the "escape" process. In testing this latter hypothesis, we have not found (Conaway and Lerner, unpublished observations) that combining T_3 with calcitonin could inhibit "escape" in bones stimulated by a maximum resorptive dosage of PTH. However, we have not tested for an effect of the thyroid hormones on "escape" at lower concentrations of PTH or in the presence of other resorptive agents.

Calvarial bone resorption stimulated by the thyroid hormones was not dependent on prostaglandin biosynthesis. Prostaglandin's (especially those of the PGE series) are known to be potent stimulators of bone resorption (42), and the compounds are suggested to be important local mediators of bone resorption (13). However, unlike previous studies in neonatal mouse calvariae, which have shown inhibition of calcium release by indomethacin (8, 9, 11) and increased PGE_2 and PGI_2 formation following thyroid hormone treatment (11), calcium release elicited by T_4 and T_3 in the present investigation was not inhibited by indomethacin, and T_3 treatment did not increase PGE_2 and PGI_2 formation. In a recent study by Klaushofer *et al.* (11) using neonatal mouse calvariae, it was reported that the percentage of calcium released following thyroid hormone administration showed greater variation than did release stimulated by other resorptive agents. In experiments where a high level of mineral mobilization occurred following thyroid hormone treatment, the suppression of resorption by indomethacin equaled approximately 50%, leading these investigators to suggest that the resorptive action of the thyroid hormones may be mediated by both prostaglandin-independent and prostaglandin-dependent pathways. The reason for the difference in prostaglandin dependence in the study by Klaushofer *et al.* and the present investigation is unclear, but may be related to variations in culture conditions (preincubation in indomethacin versus no preincubation; albumin versus serum; CMRL 1066 medium versus DMEM, 50% O_2 versus 20% O_2) or different mouse strains. The prostaglandin-independent resorption of bone by the thyroid hormones noted in the present study equalled approximately 15% of the resorption elicited by a maximum dosage of PTH. From the standpoint of prostaglandin dependence, this stimulation of resorption more closely resembles the mobilization effect of the thyroid hormones in fetal rat limb bones. In fetal rat limb bones, resorption of bone by the thyroid hormones also is independent of prostaglandin biosynthesis (7).

In a previous study, Britto *et al.* (22) have shown that

T_3 and T_4 can stimulate pit formation by isolated rat osteoclasts incubated on slices of devitalized bone when cultured with osteoblasts, but not in the absence of osteoblasts or osteoblastic cell lines. These observations suggest that the thyroid hormones, like the resorptive agents PTH and 1,25$(OH)_2$-vitamin D3, act on osteoblasts to stimulate osteoclast activity indirectly. In the current study, T_3 was found to be a good stimulator of isolated rat osteoclasts cultured in the presence of stromal cells/osteoblasts. The number of pits formed on devitalized bone slices following T_3 treatment equalled 82% of the pit formation stimulated by PTH. However, there was no indication that prostaglandins might play a role in the resorptive action of T_3, or that of PTH, for pit formation of neither agent was affected by treatment of cultures with indomethacin, a potent inhibitor of prostaglandin biosynthesis.

In testing other mechanisms by which the thyroid hormones might stimulate bone resorption, the ability of T_3 to increase calvarial bone formation of cAMP was evaluated. Cyclic AMP is an important intracellular messenger that plays a major role in mediating the biological actions of numerous agents, including calcium-regulatory hormones such as PTH and calcitonin (43, 44). Furthermore, previous nonbone-related studies have shown increases in adenylate cyclase activity (45), cAMP content (46), and cAMP-dependent protein kinase activity (47) following thyroid hormone treatment. However, the experiments performed in the current investigation gave no indication that cAMP might mediate the bone resorptive actions of the thyroid hormones. No changes in cAMP content of mouse calvarial bones were observed following exposure to T_3. In contrast, increased cAMP formation was found following both PTH treatment and treatment with a second bone resorbing agent, CTX, whose action, like that of PTH, is believed to be highly dependent on cAMP formation (48). Although use of the phosphodiesterase inhibitor, rolipram, precluded ruling out phosphodiesterase inhibition as a possible mechanism for thyroid hormone action, this event seems unlikely. In an earlier study in fetal rat limb bones where phosphodiesterase inhibition by the thyroid hormones and their analogs was evaluated, inhibition of phosphodiesterase activity was not correlated with bone resorptive activity (7).

In contrast to the lack of prostaglandin and cAMP involvement in the resorptive effects of the thyroid hormones, hydroxyurea was found to be a potent inhibitor of ^{45}Ca release stimulated by T_3. These data suggest that the resorptive effects of the thyroid hormones in neonatal mouse calvariae are dependent on DNA synthesis, perhaps involving replication of osteoclast precursors, or possibly other bone cells involved in the resorptive process. A similar mode of action has been suggested for the stimulation of fetal rat limb bone resorption by T_3 and 3,5,3′-triiodothyroacetic acid (Triac) (49). In these studies, the resorptive effects of T_3 and Triac were blunted by the inhibitor of DNA synthesis, aphidicolin. Furthermore, previous investigations have shown that the resorptive action of PTH is not dependent on cell replication (23, 49, 50), and hydroxyurea was found to have no effect on ^{45}Ca release elicited by PTH in the current study.

There have been suggestions that an important role of the thyroid hormones may be to potentiate the effects of other resorptive agents (51–53). In testing for the possibility of interaction between the thyroid hormones and PTH, we found no enhancement of *in vitro* bone resorption resulting from either combined treatment with PTH and T_3 or following pretreatment of neonatal mouse calvarial bones with T_3. These data also are in good agreement with previous studies in fetal rat limb bones (7). In fetal rat limb bones, the effects of PTH and T_4 on resorption are not synergistic, and the response to PTH is not enhanced by prior T_4 treatment of cultured bones.

In conclusion, we have found that the thyroid hormones can stimulate neonatal mouse calvarial bone resorption in a prostaglandin-independent manner that requires cellular replication. Additionally, we have observed that stimulation of pit formation number by treatment of isolated osteoclasts with thyroid hormone is also a prostaglandin-independent process.

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