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Stimulation of Neonatal Mouse Calvarial Bone Resorption by the Glucocorticoids Hydrocortisone and Dexamethasone

HERSCHEL H. CONAWAY,[1] DANIEL GRIGORIE,[2,3] and ULF H. LERNER[2]

ABSTRACT

In vitro stimulation of bone resorption was observed with the glucocorticoids hydrocortisone and dexamethasone. Dosage-dependent release of ^{45}Ca from neonatal mouse calvarial bones was found for both steroids, with half-maximal responses for hydrocortisone and dexamethasone of 0.3 and 0.08 μM, respectively. Significant release of stable calcium (Ca^{2+}), inorganic phosphate (P_i), and the lysosomal enzyme β-N-acetylglucosaminidase was noted following treatment of mouse calvariae with either 1 μM hydrocortisone or 1 μM dexamethasone. Additionally, both 1 μM hydrocortisone and 1 μM dexamethasone elicited release of ^{3}H from calvarial bones prelabeled with [^{3}H]proline. The stimulation of bone resorption by the glucocorticoids, as assessed by ^{45}Ca release, was sustained over 120 h of culture. Inhibition of ^{45}Ca release from calvariae treated with either 1 μM hydrocortisone or 0.1 μM dexamethasone was observed with 0.01–30 nM salmon calcitonin (sCT), 0.1 mM acetazolamide, and 0.1 mM of the bisphosphonate AHPrBP. Inhibition of glucocorticoid-induced bone resorption by sCT occurred without "escape from calcitonin-induced inhibition." The ^{45}Ca release stimulated by 1 μM hydrocortisone and 0.1 μM dexamethasone was also inhibited by 10 μM progesterone in a competitive manner and by 1 μM of the antiglucocorticoid RU38486, both of which are modulators of glucocorticoid binding. Prostaglandin E_2 (PGE_2) formation by 10 nM parathyroid hormone (PTH) in neonatal mouse calvarial bones was inhibited by both 1 μM hydrocortisone and 1 μM dexamethasone, but neither compound altered basal PGE_2 formation. Exposure of calvarial bones to the mitotic inhibitors hydroxyurea and mitomycin C inhibited ^{45}Ca release stimulated by 1 μM hydrocortisone and 1 μM dexamethasone. In contrast, addition of 1 ng/ml of recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF) had no effect on ^{45}Ca release elicited by the glucocorticoids. These results suggest that hydrocortisone and dexamethasone stimulate osteoclastic resorption in neonatal mouse calvariae by a receptor-mediated mechanism that is dependent on cellular replication. (J Bone Miner Res 1996;11:1419–1429)

INTRODUCTION

Glucocorticoids were associated with loss of bone and fracture by Cushing in 1932.[1] Prolonged glucocorticoid excess resulting from therapeutic administration of the compounds or due, as in Cushing disease, to increased endogenous production of corticosteroid can cause the development of osteoporosis.[2,3] High levels of glucocorticoids are believed to alter bone remodeling by decreasing bone formation and increasing bone resorption.[2,3] Studies in vitro have suggested a direct inhibitory role for glucocorticoids in bone formation. Glucocorticoids are thought to decrease the proliferation and differentiation of preosteoblasts to osteoblasts.[4–6] In contrast, mechanisms responsible for the osteoclastic resorption of glucocorticoid excess often have been attributed to indirect actions of the compounds. Glucocorticoids decrease intestinal absorption of calcium[7] and increase kidney excretion of the ion.[8] Thus, it has been suggested that glucocorticoids can facilitate, in an indirect manner, the development of secondary hyperparathyroidism.[2,3,9,10]

[1]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas, U.S.A.
[2]Department of Oral Cell Biology, University of Umeå, Umeå, Sweden.
[3]Present address: Institute of Endocrinology, Bucharest, Romania.

Investigations performed in vitro to assess a direct role of glucocorticoids in bone resorption have yielded conflicting results. In studies using fetal rat limb bones, Stern found inhibition with hydrocortisone and dexamethasone of calcium release stimulated by parathyroid hormone (PTH),[11] and Raisz et al.[12] demonstrated inhibition of resorption stimulated by retinol, dibutyryl cyclic adenosine monophosphate (cAMP), prostaglandin E_1 (PGE_1), 25-hydroxyvitamin D, and PTH following cortisol treatment. Tobias and Chambers[13] have also reported the dose-dependent inhibition by hydrocortisone and dexamethasone of bone resorption by osteoclasts disaggregated from neonatal rat limb bones. Recently, Gronowicz et al.[14] observed a transient stimulation of resorption by corticosterone and dexamethasone in fetal rat parietal bones, but this was followed by inhibition of osteoclast function. In contrast, Reid et al.[15] and Lowe et al.[16] have found stimulation of bone resorption by hydrocortisone in cultures of neonatal mouse calvariae. However, in other studies, also using neonatal mouse calvariae, no effect of cortisol or dexamethasone on stable or radioactive calcium release has been observed.[17–19]

More consistent effects of the glucocorticoids have been noted in mouse bone marrow cultures[20] and in cocultures of mouse bone marrow–derived stromal cells and spleen cells.[21] In these studies, dexamethasone has been shown to enhance osteoclast-like cell differentiation induced by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and to increase the capacity of these cells to enhance pit formation on dentine slices. Additionally, in mouse bone marrow cultures, Shuto et al.[20] have suggested that dexamethasone increases osteoclast-like cell generation by inhibiting the endogenous production of granulocyte macrophage-colony stimulating factor (GM-CSF).

The stimulation of mineral mobilization by hydrocortisone treatment observed by Lowe et al.[16] in mouse calvarial bones was sustained, and an additive effect on bone resorption was found by Reid et al.[15] when hydrocortisone and PTH treatments were combined. These data suggest that neonatal mouse calvariae could prove to be a good model for the study of glucocorticoid actions on bone. The present investigation testing the effectiveness of hydrocortisone and dexamethasone in neonatal mouse calvarial bones was initiated in an attempt to gain a better understanding of the direct bone resorptive effects of the glucocorticoids.

MATERIALS AND METHODS

Synthetic bovine PTH(1–34) was purchased from Bachem (Bubendorf, Switzerland); recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF) from R&D Systems (Minneapolis, MN, U.S.A.); CMRL 1066 medium from GIBCO (Renfrewshire, Scotland); [^{45}Ca]$CaCl_2$ and the radioimmunoassay kit for PGE_2 from DuPont/New England Nuclear (Dreieich, Germany); L-[5^{3}H]-proline from The Radiochemical Centre (Amersham, U.K.); fetal calf serum (FCS) from Flow Laboratories (Irvine, Scotland); multiwell plastic culture dishes from Costar (Cambridge, MA, U.S.A.); p-nitrophenyl N-acetyl-β-D-glucosaminide, bovine serum albumin (BSA; RIA grade), acetazolamide, hydrocortisone, dexamethasone, progesterone, hydroxyurea, and mitomycin C from Sigma Chemical Company (St. Louis, MO, U.S.A.). Indomethacin was a gift from Merck, Sharp and Dohme (Haarlem, The Netherlands); 3-amino-1-hydroxypropylidene-1, 1-bisphosphonate (AHPrBP) a gift from Henkel KGaA (Düsseldorf, Germany); mifepristone (RU38486) a gift from Roussel-UCLAF (Romanville, France); synthetic salmon calcitonin (sCT) was kindly supplied by Sandoz (Basel, Switzerland); and $1(\alpha),25(OH)_2D_3$ by Hoffmann-LaRoche AG (Basel, Switzerland). Indomethacin, hydrocortisone, dexamethasone, progesterone, $1(\alpha),25(OH)_2D_3$, and RU38486 were dissolved in 99% 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 1.0 mmol HCl containing 1 g/l albumin, rmGM-CSF was dissolved at a concentration of 2 μg/ml in PBS containing 1 g/l albumin, and sCT was dissolved as a stock solution of 30 μmol/l in medium. Each was stored at −80°C. Acetazolamide, hydroxyurea, mitomycin C, and AHPrBP were dissolved directly in culture media.

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: the two parietal bones were divided along the sagittal suture, producing two calvarial halves; the posterior two-thirds of each calvarial half was divided further into anterior and posterior portions, producing four bone fragments.[22] Half calvariae were used 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. The parietal bones were preincubated for 18–24 h in CMRL 1066 medium containing 0.1% BSA and 1 μmol/l indomethacin.[23] Following preincubation, the bones were washed extensively in Tyrode's solution and subsequently cultured for 120 h in multiwell culture dishes (one bone/well) containing 2.0 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 or 10 μCi ^{45}Ca 4 days prior to dissection[24] were dissolved in HCl and aliquots of the media and bones 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 percent of initial radioactivity present in bones.[23]

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,[25] and P_i was measured spectrophotometrically.[26]

Evaluation of prostaglandin synthesis

Prostaglandin production by the bones was assessed by the measurement of prostaglandin E_2 (PGE_2) in culture media at the termination of experiments.[27] The concentration of PGE_2 was determined with a commercially available radioimmunoassay kit. Assay for the prostanoid was performed according to instructions supplied by the manufacturer.

Determination of matrix degradation

Mice were subcutaneously injected with 10 μCi of [^{3}H]proline 4 days before calvarial bone dissection. At the end of experimentation, aliquots of media and hydrolyzed bone samples were analyzed for [^{3}H], and the percentage release of [^{3}H] to the culture medium was determined as described previously.[23]

The release of ^{3}H to culture medium represents release of both [^{3}H]proline and [^{3}H]hydroxyproline. Previous study has shown a good correlation between ^{3}H release and [^{3}H]hydroxyproline release from bone,[28] thus ^{3}H 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-acetylglucosaminidase in culture media and bones. At the end of the cultures, bones were placed in 0.2% (v/v) Triton X-100 in saline for 24 h for removal of enzyme.[29] Culture media and aliquots of supernatants were measured for enzymatic activity. β-N-acetylglucosaminidase was analyzed using p-nitrophenyl N-acetyl-β-D-glucosaminide as substrate.[30] Enzyme release to the media was expressed as percent of total enzymatic activity.

Statistical analysis

Statistical calculations were made with Student's *t*-test for unpaired samples.

RESULTS

In initial experiments, dose response curves of ^{45}Ca release from neonatal mouse calvariae were established for hydrocortisone and dexamethasone. Significantly increased ^{45}Ca release ($p < 0.01$) was recorded for hydrocortisone at concentrations ranging from 10 to 0.1 μM and for dexamethasone at concentrations ranging from 10 to 0.01 μM (Fig. 1). While there was no difference in the maximum percent ^{45}Ca release stimulated by the two agents at 120 h, an approximate 5-fold greater sensitivity to dexamethasone treatment was observed. The half-maximal responses to hydrocortisone and dexamethasone were 0.3 and 0.08 μM, respectively.

Subsequent experiments were performed to compare the effects of hydrocortisone, dexamethasone, and PTH on Ca^{2+}, P_i, and β-N-acetylglucosaminidase release from neonatal mouse calvarial bones. Significantly increased release ($p < 0.01$) of Ca^{2+}, P_i, and the lysosomal enzyme, β-N-acetylglucosaminidase, was observed for 1 μM hydrocortisone, 1 μM dexamethasone, and 10 nM PTH after 120 h of culture (Fig. 2). While greater mineral mobilization and lysosomal enzyme release were noted for dexamethasone in comparison with hydrocortisone, the responses elicited by the glucocorticoids were less than those following treatment with PTH. The Ca^{2+} release stimulated by dexamethasone was 16%, the P_i release 11%, and the β-N-acetylglucosaminidase release 45% of the responses stimulated by PTH.

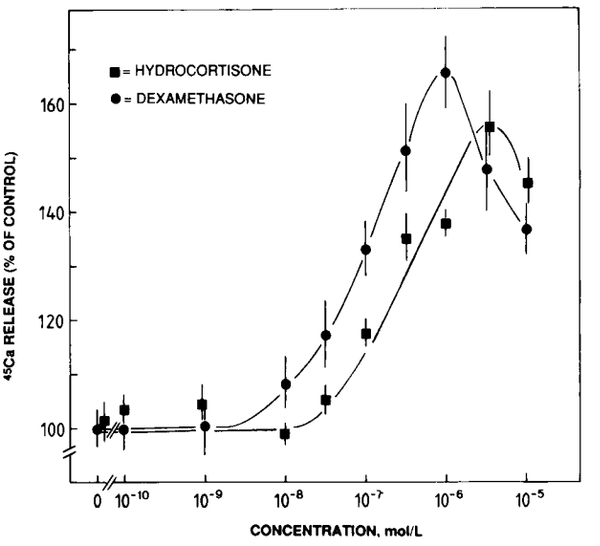

FIG. 1. Dose response curves of ^{45}Ca release stimulated by hydrocortisone and dexamethasone. Mouse calvarial bones were cultured for 120 h as described in Materials and Methods. Values are based on means ≥ 12 bones, and SEM is represented as vertical bars. The percent ^{45}Ca release was significantly different ($p < 0.01$) from control release at 10–0.1 μM hydrocortisone and 10–0.01 μM dexamethasone. The half-maximal responses to hydrocortisone and dexamethasone were 0.3 and 0.08 μM, respectively. The percent ^{45}Ca release in untreated control was 20.3 ± 0.71.

Increased matrix degradation, as assessed by the percent release of ^{3}H, was also found following treatment with the glucocorticoids and PTH (Fig. 3). Exposure of calvarial bones to 1 μM hydrocortisone, 1 μM dexamethasone, and 10 nM PTH for 120 h resulted in significantly enhanced release ($p < 0.01$) of ^{3}H to culture media.

To evaluate the time course of glucocorticoid action, the percent ^{45}Ca release from mouse calvarial bones was determined at 24, 48, 72, 96, and 120 h following treatment with 1 μM hydrocortisone, 1 μM dexamethasone, 0.07 nM PTH, and 10 nM PTH (Fig. 4). Sustained stimulation of ^{45}Ca release was observed for the glucocorticoids and both dosage levels of PTH throughout the experimental period. While the maximum percent of ^{45}Ca release stimulated by hydrocortisone and dexamethasone during the 120 h of culture was less than that observed with either dosage level of PTH, the profile of ^{45}Ca release elicited by each of the steroids resembled the release noted with the lower (0.07 nM) concentration of PTH.

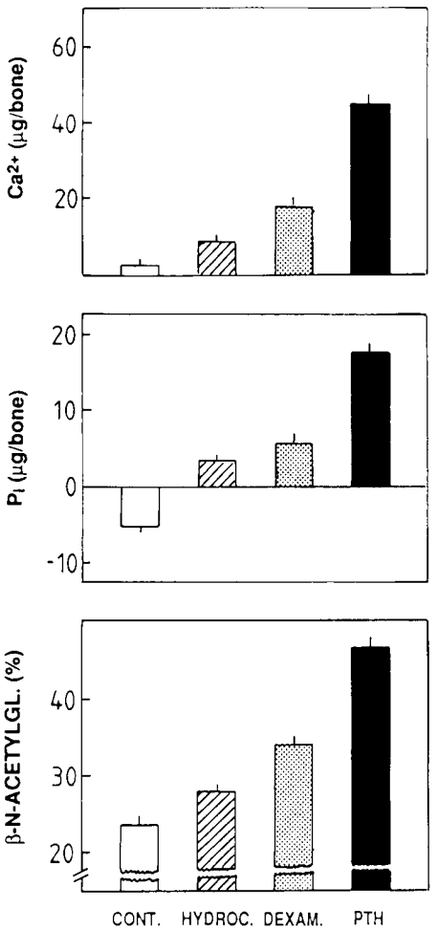

FIG. 2. Stimulation of Ca^{2+}, P_i, and β-N-acetylglucosaminidase release by 1 μM hydrocortisone, 1 μM dexamethasone, and 10 nM PTH. Calvarial bones were cultured for 120 h as described in Materials and Methods. Values are based on means ≥ seven bones, and SEM is represented as vertical bars. Ca^{2+}, P_i, and β-N-acetylglucos-aminidase release in hydrocortisone, dexamethasone, and PTH-treated calvariae was significantly different ($p < 0.01$) from control release of Ca^{2+}, P_i, and β-N-acetylglucosaminidase.

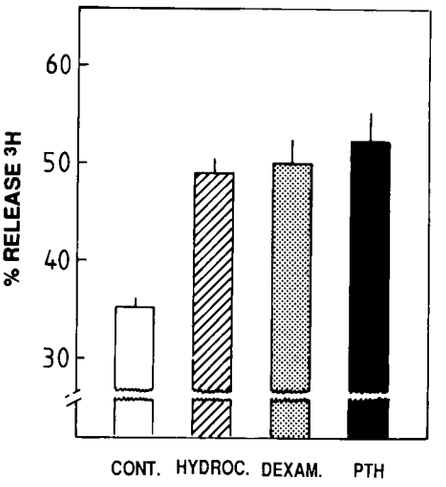

FIG. 3. Stimulation of percent ^3H release by 1 μM hydrocortisone, 1 μM dexamethasone, and 10 nM PTH. Bones were cultured for 120 h as outlined in Materials and Methods. Means were calculated from eight bones and SEM is shown as vertical bars. The percent ^3H release was significantly different ($p < 0.01$) from control release following hydrocortisone, dexamethasone, and PTH treatment.

Experiments were also performed to test the ability of osteoclastic inhibitors of bone resorption to block the resorptive effects of hydrocortisone and dexamethasone. The percent ^{45}Ca release measured following addition of the bisphosphonate, AHPrBP,[31] and the carbonic anhydrase inhibitor, acetazolamide,[32,33] to control, hydrocortisone-treated, and PTH-treated neonatal mouse calvarial bones is shown in Figs. 5a and 5b, respectively. No effect on control resorption was found following exposure of calvariae to either 0.1 mM AHPrBP or 0.1 mM acetazolamide for 120 h (Fig. 5a). However, AHPrBP and acetazolamide proved to be potent inhibitors of ^{45}Ca release stimulated by 1 μM hydrocortisone and 10 nM PTH (Figs. 5a and 5b).

The third inhibitor of osteoclastic bone resorption tested was the polypeptide hormone calcitonin. The percent ^{45}Ca release observed at 48 and 120 h following the addition of 30 nM sCT to calvarial bones receiving 1 μM hydrocortisone, 0.1 μM dexamethasone, and 10 nM PTH is shown in Figs. 6a, 6b, and 6c. After 48 h of culture, the ^{45}Ca release recorded following hydrocortisone (Fig. 6a), dexamethasone (Fig. 6b), and PTH (Fig. 6c) treatment was depressed significantly ($p < 0.05$) by sCT addition. However, after 120 h of culture, the ^{45}Ca release measured for PTH + sCT treatment nearly equaled the radioactive calcium release stimulated by PTH alone (Fig. 6c). In sharp contrast, the ^{45}Ca release noted for either hydrocortisone (Fig. 6a) or dexamethasone (Fig. 6b) in the presence of sCT continued to be depressed to control values at 120 h, indicating that "escape from calcitonin-induced inhibition"[34] had not occurred in glucocorticoid-treated calvarial bones. In other experiments, sCT at lower concentrations (0.01–1 nM) were also found to abolish dexamethasone (0.1 μM) stimulated ^{45}Ca release in 120 h cultures (data not shown).

Experiments next were designed to determine if glucocorticoid exposure would affect "escape" of PTH-treated calvarial bones. The percent ^{45}Ca release observed at 48, 96, and 144 h following the addition of 30 nM sCT to calvarial bones treated with 10 nM PTH, 10 nM PTH + 1 μM hydrocortisone, and 10 nM PTH + 1 μM dexamethasone is shown in Figs. 7a, 7b, and 7c. After 48 h of culture, the ^{45}Ca release recorded following PTH (Fig. 7a), PTH + hydrocortisone (Fig. 7b), and PTH + dexamethasone (Fig. 7c) treatment was depressed significantly ($p < 0.01$) by sCT addition. However, after 144 h of culture, the ^{45}Ca release measured for PTH + sCT, PTH + hydrocortisone + sCT, and PTH + dexamethasone + sCT was equivalent to the ^{45}Ca release that had occurred with PTH (Fig. 7a), PTH + hydrocortisone (Fig. 7b), and PTH + dexamethasone (Fig. 7c) treated calvariae, showing that

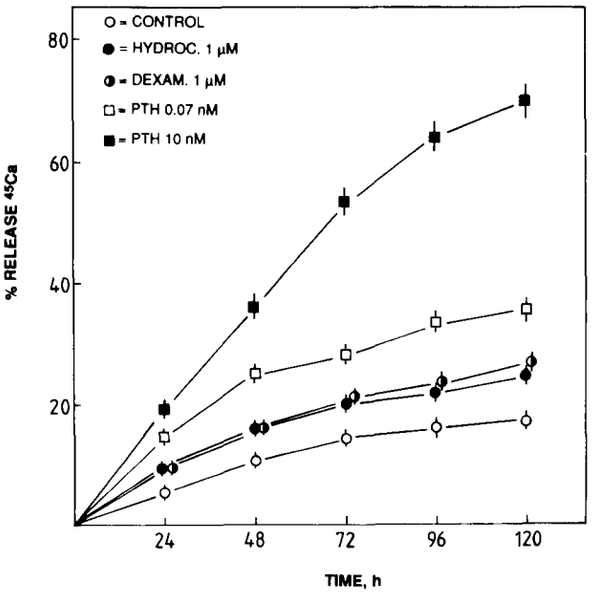

FIG. 4. Time-course for stimulation of percent ^{45}Ca release by 1 μM hydrocortisone, 1 μM dexamethasone, 0.07 nM PTH, and 10 nM PTH. Calvarial bones were cultured for 120 h as described in Materials and Methods with small aliquots of media removed at 24, 48, 72, and 96 h. Values are based on means $\geq$ six bones, and SEM is shown as vertical bars. The percent ^{45}Ca release for hydrocortisone, dexamethasone, 0.07 nM PTH, and 10 nM PTH treatment was significantly different ($p < 0.01$) from corresponding control values at all time intervals.

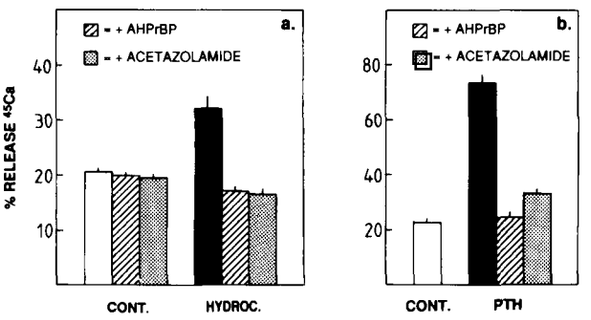

FIG. 5. Inhibition by 0.1 mM AHPrBP and 0.1 mM acetazolamide of percent ^{45}Ca release stimulated by (a) 1 μM hydrocortisone and (b) 10 nM PTH. Mouse calvarial bones were cultured for 120 h as described in Materials and Methods. Means were determined from five bones and SEM is shown as vertical bars. The percent ^{45}Ca release for hydrocortisone and PTH-treated calvariae in the absence of AHPrBP and acetazolamide was significantly different ($p < 0.01$) from control release. In the presence of either AHPrBP or acetazolamide, the percent ^{45}Ca release was significantly different ($p < 0.01$) from release occurring in calvariae receiving only hydrocortisone or PTH treatment.

"escape" had occurred in PTH treated calvarial bones exposed to glucocorticoids.

For determination of the role prostaglandins might play in the calvarial bone resorption observed with the glucocorticoids, the effects of hydrocortisone and dexamethasone on prostaglandin biosynthesis were examined in the presence and absence of PTH (Table 1). While no changes from control values were noted in calvarial bones treated for 120 h with either 1 μM hydrocortisone or 1 μM dexamethasone, both glucocorticoids decreased PGE_2 formation significantly ($p < 0.01$) in calvarial bones exposed to 10 nM PTH.

To investigate if inhibitors of glucocorticoid binding might affect calvarial bone resorption, hydrocortisone and dexamethasone were evaluated in the presence and absence of the competitive antagonist, progesterone,[13,35] and the antiglucocorticoid, RU38486.[35] The percent ^{45}Ca release stimulated by the glucocorticoids in the presence and absence of progesterone is shown in Fig. 8. The ^{45}Ca release elicited by 1 μM hydrocortisone and 0.1 μM dexamethasone treatment for 120 h was decreased significantly ($p < 0.01$) when calvarial bones were exposed to 10 μM progesterone. However, in both instances, the inhibitory effect of progesterone on radioactive calcium mobilization was overcome by an increased concentration of the glucocorticoid. Addition of 10 μM progesterone did not produce a significant depression of 120 h ^{45}Ca release from calvarial bones treated with either 10 μM hydrocortisone or 1 μM dexamethasone. In separate experiments, we have found no effect of 10 μM progesterone on percent ^{45}Ca release stimulated by 10 nM PTH (data not shown).

The percent ^{45}Ca release determined following the addition of RU38486 to calvarial bones treated with 1 μM hydrocortisone, 0.1 μM dexamethasone, and 10 nM PTH for 120 h is given in Fig. 9. Exposure of calvarial bones to 1 μM RU38486 significantly depressed ($p < 0.01$) mineral mobilization in hydrocortisone- and dexamethasone-treated calvariae but had no effect on ^{45}Ca release from PTH-treated explants.

Additional experiments were designed to evaluate the role DNA synthesis might play in the resorptive action of the glucocorticoids. The percent ^{45}Ca release stimulated by 1 μM hydrocortisone, 1 μM dexamethasone, and 10 nM PTH in the presence and absence of the inhibitor of the DNA synthesis, hydroxyurea, is shown in Table 2. Inhibition of ^{45}Ca release was not observed either in control or PTH-treated calvarial bones exposed to 1 mM hydroxyurea for 120 h. However, hydroxyurea addition produced significant inhibition ($p < 0.01$) of ^{45}Ca release stimulated by hydrocortisone and dexamethasone at 120 h. In agreement with these data, we also found that the ^{45}Ca release response to dexamethasone (1 μM), but not that to PTH (10 nM), was decreased by pretreating the bones with the mitotic inhibitor, mitomycin C (30 μg/ml, 60 minutes; data not shown). Addition of hydroxyurea (1 mM) to bones stimulated by PTH (10 nM) + dexamethasone (1 μM) did not affect the ^{45}Ca release response to the combined treatment with PTH and dexamethasone (data not shown).

Further experiments were performed to determine if addition of rmGM-CSF would alter the resorptive response of the glucocorticoids. The percent ^{45}Ca release stimulated by 0.1 μM dexamethasone, 10 nM PTH, and 10 nM $1,25(OH)_2D_3$

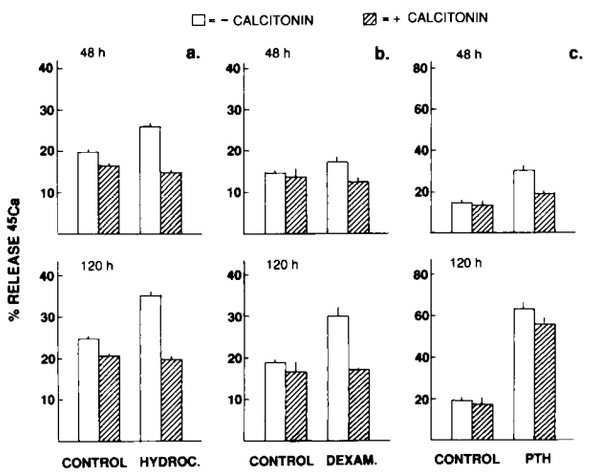

FIG. 6. Stimulation of percent ^{45}Ca release at 48 and 120 h by (a) 1 μM hydrocortisone, (b) 0.1 μM dexamethasone, and (c) 10 nM PTH in the absence and presence of 30 nM sCT. Data are derived from three experiments and based on means ≥ five bones. The SEM is shown as vertical bars. Small aliquots of media were removed at 48 h and the bones cultured for an additional 72 h as described in Materials and Methods. The percent ^{45}Ca release in the absence of sCT was significantly different ($p < 0.05$) from control release in (a) hydrocortisone and (c) PTH treated calvariae at 48 h, and the percent ^{45}Ca release for (a) hydrocortisone, (b) dexamethasone, and (c) PTH treatment in the absence of sCT was significantly different ($p < 0.05$) from control release at 120 h. In the presence of sCT, the percent ^{45}Ca release in (a) control, (a) hydrocortisone, (b) dexamethasone, and (c) PTH-treated calvariae was significantly different ($p < 0.05$) at 48 and 120 h from corresponding calvariae that had not been exposed to sCT. Mean values of percent ^{45}Ca release at 120 h in calvariae receiving only sCT treatment and calvariae exposed to (a) sCT + hydrocortisone or (b) sCT + dexamethasone were almost identical, but more than a 2-fold stimulation of percent ^{45}Ca release had occurred in calvariae treated with (c) sCT + PTH.

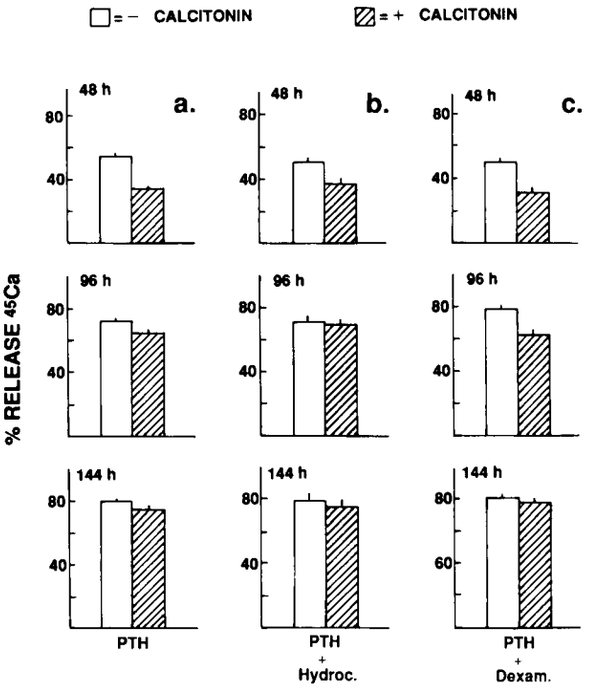

FIG. 7. Stimulation of percent ^{45}Ca release at 48, 96, and 144 h by (a) 10 nM PTH, (b) 10 nM PTH + 1 μM hydrocortisone, and (c) 10 nM PTH + 1 μM dexamethasone in the absence and presence of 30 nM sCT. Values are based on means of eight bones, and SEM is shown as vertical bars. Small aliquots of media were removed at 48 and 96 h, and the bones cultured for an additional 48 h as described in Materials and Methods. The percent ^{45}Ca release in calvariae receiving sCT treatment was significantly different ($p < 0.01$) from calvariae exposed to (a) only PTH, (b) PTH + hydrocortisone, and (c) PTH + dexamethasone at 48 h, as well as calvariae exposed to (a) PTH and (c) PTH + dexamethasone at 96 h, but no differences due to sCT treatment were determined at 144 h in (a) PTH, (b) PTH + hydrocortisone, and (c) PTH + dexamethasone–treated calvariae.

in the absence and presence of 1 ng/ml rmGM-CSF is shown in Table 3. Addition of 1 ng/ml rmGM-CSF was found to have no effect on 120 h ^{45}Ca release from control, dexamethasone, PTH, or $1,25(OH)_2D_3$-treated calvarial bones. In subsequent experiments, an increased concentration of rmGM-CSF (10 ng/ml) was employed. Addition of the growth factor again was found to have no effect on 120 h ^{45}Ca release from control, dexamethasone, or $1,25(OH)_2D_3$-treated calvariae (data not shown).

DISCUSSION

The present study provides evidence for the stimulation of bone resorption in neonatal mouse calvarial bones by the glucocorticoids, hydrocortisone, and dexamethasone. In previous investigations, Reid et al.[15] and Lowe et al.[16] observed increased ^{45}Ca release from neonatal mouse calvarial bones treated with hydrocortisone. We have found stimulation of ^{45}Ca release with hydrocortisone, as well as with a second glucocorticoid, dexamethasone. In addition, increased lysosomal enzyme release, matrix degradation, and bone Ca^{2+} and P_i release were observed following exposure of calvarial bones to both glucocorticoids. A sustained stimulation of mineral mobilization from neonatal mouse calvarial bones following exposure to glucocorticoid also had been reported previously by Lowe et al.[16] In the current study, calcium release stimulated by both hydrocortisone and dexamethasone was sustained over 120 h of culture. This observation of sustained mineral release together with the evidence of increased matrix degradation and lysosomal enzyme release suggest that neonatal mouse calvariae can provide a good model for the study of the direct resorptive effects of glucocorticoids on bone.

The reason for the stimulation of bone resorption by glucocorticoids in neonatal mouse calvariae when inhibitory

TABLE 1. EFFECT OF HYDROCORTISONE AND DEXAMETHASONE, IN THE ABSENCE AND PRESENCE OF PARATHYROID HORMONE, ON PROSTAGLANDIN E_2 BIOSYNTHESIS IN NEONATAL MOUSE CALVARIAL BONES

Additions	Amount (μM)	PGE_2 (pg/ml/bone) −PTH	PGE_2 (pg/ml/bone) +PTH*
Experiment 1			
—	—	23 ± 5	175 ± 49†
Hydrocortisone	1	24 ± 4	17 ± 2‡
Experiment 2			
—	—	12 ± 1	385 ± 159†
Dexamethasone	1	14 ± 3	19 ± 3‡

* Parathyroid hormone was added to a final concentration of 10 nmol/l.

† Significantly different from untreated control ($p < 0.05$).

‡ Significantly different from PTH alone ($p < 0.01$).

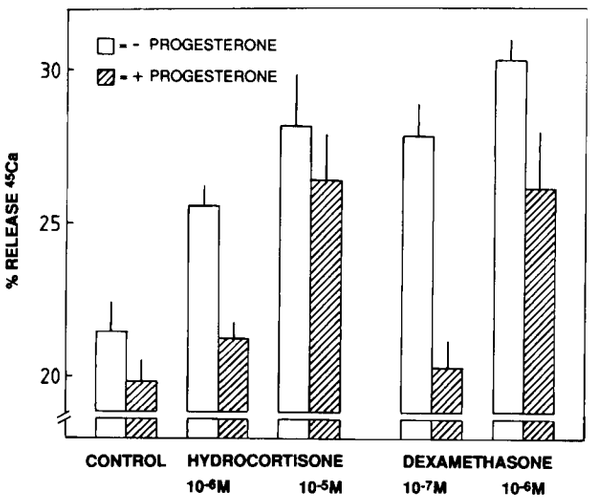

FIG. 8. Stimulation of percent ^{45}Ca release by 1 μM hydrocortisone, 10 μM hydrocortisone, 0.1 μM dexamethasone, and 1 μM dexamethasone in the absence and presence of 10 μM progesterone. Neonatal mouse calvarial bones were cultured for 120 h as described in Materials and Methods. Values are based on means ≥ five bones, and SEM is shown as vertical bars. The percent ^{45}Ca release in the absence of progesterone was significantly different ($p < 0.05$) from control release following 1 μM hydrocortisone, 10 μM hydrocortisone, 0.1 μM dexamethasone, and 1 μM dexamethasone treatment. In the presence of 10 μM progesterone, the percent ^{45}Ca release was significantly different ($p < 0.01$) from release that occurred in calvariae treated with only 1 μM hydrocortisone or 0.1 μM dexamethasone.

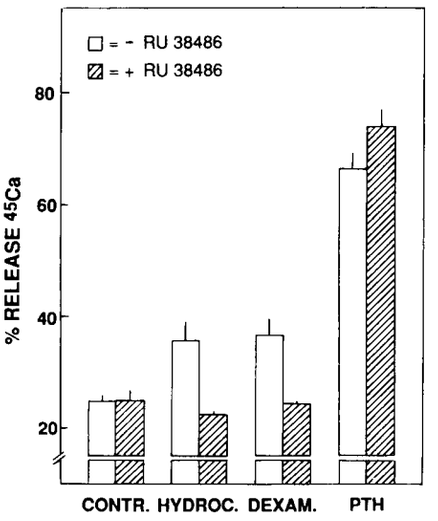

FIG. 9. Stimulation of percent ^{45}Ca release by 1 μM hydrocortisone, 0.1 μM dexamethasone, and 10 nM PTH in the absence and presence of 1 μM RU38486. Calvarial bones were cultured for 120 h as described in Materials and Methods. Values are based on means for five bones, and SEM is represented as vertical bars. The percent ^{45}Ca release in the absence of RU38486 was significantly different ($p < 0.01$) from control release following hydrocortisone, dexamethasone, and PTH treatment. In the presence of RU38486, the percent ^{45}Ca release was significantly different ($p < 0.01$) from release which occurred in calvariae receiving only hydrocortisone or dexamethasone treatment.

effects occur in other bone resorption assays is not known. Lowe et al.[16] have reported that the addition of 5% serum to culture medium inhibited the resorptive action of hydrocortisone in neonatal mouse calvarial bones. We have found that the addition of 1% and 3% FCS causes a slight decrease in treatment/control ratios of hydrocortisone and dexamethasone as well as PTH-treated calvarial bones because of increased control resorption, but that serum has no effect on the maximum percent ^{45}Ca release elicited by the glucocorticoids and PTH (Conaway and Lerner, unpublished observations). The use of tissue derived from the mouse rather than the rat also may play a role in determining the response to glucocorticoids. In osteoblast-like cells derived from rats and mice, species differences for cell proliferation[36] and stimulation of $1,25(OH)_2D_3$ receptors[37,38] have been found following glucocorticoid treatment. The use of fetal versus neonatal tissue for study may be another variable. In an investigation using bone particles that were implanted in rats, De Franco et al.[39] suggested that glucocorticoids inhibit the recruitment and/or differentiation of osteoclast progenitor cells while stimulating the activity of multinucleated osteoclasts. If the numbers of osteoclasts in particular stages of differentiation in different bone tissues are not the same, the responses to glucocorticoid treatment may differ as well. In the mouse calvarial system used in the present study, stimulatory effects on bone resorption are due mainly to stimulation of the number of multinucleated osteoclasts. In another study, however, we have prestimulated the bones with PTH to enhance osteoclastic numbers before the addition of glucocorticoids, and still no inhibition was found. In other experiments, we have simultaneously added PTH and glucocorticoids so that

TABLE 2. EFFECT OF HYDROCORTISONE, DEXAMETHASONE AND PARATHYROID HORMONE, IN THE ABSENCE AND PRESENCE OF 1 mM HYDROXYUREA, ON ^{45}Ca RELEASE FROM NEONATAL MOUSE CALVARIAL BONES

Stimulator	*Amount (μM)*	*Percent ^{45}Ca release*	
		−HU	*+HU*
—	—	24.41 ± 1.55	28.57 ± 3.52
Hydrocortisone	1	34.80 ± 2.49*	27.76 ± 1.60‡
Dexamethasone	1	41.55 ± 2.45*	28.15 ± 2.20‡
PTH	0.01	64.88 ± 2.46*	71.59 ± 4.21†

Values are means ± SEM for six bones. Hydrocortisone, dexamethasone, and PTH were added at time 0 and the bones cultured for 120 h, as described in Materials and Methods.

* Significantly different from control −HU ($p < 0.01$).

† Significantly different from control +HU ($p < 0.01$).

‡ Significantly different due to HU treatment ($p < 0.01$).

TABLE 3. EFFECT OF DEXAMETHASONE, PARATHYROID HORMONE, AND $1,25(OH)_2D_3$, IN THE ABSENCE AND PRESENCE OF 1 ng/ml rmGM-CSF, ON ^{45}Ca RELEASE FROM NEONATAL MOUSE CALVARIAL BONES

Stimulator	*Amount (μM)*	*Percent ^{45}Ca release*	
		−rmGM-CSF	*+rmGM-CSF*
—	—	26.7 ± 1.4	29.3 ± 2.0
Dexamethasone	0.1	46.8 ± 3.8*	45.5 ± 3.1†
PTH	0.01	69.2 ± 4.1*	64.3 ± 2.5†
$1,25(OH)_2D_3$	0.01	73.2 ± 5.3*	62.3 ± 4.4†

Values are means ± SEM for six bones. Dexamethasone, PTH, and $1,25(OH)_2D_3$ were added at time 0 and the bones cultured for 120 h as described in Materials and Methods.

* Significantly different from control −rmGM-CSF ($p < 0.01$).

† Significantly different from control +rmGM-CSF ($p < 0.01$).

glucocorticoids would be present during PTH-induced osteoclast recruitment. But again, no inhibition of ^{45}Ca release was seen (Conaway et al., unpublished observations).

The first step in the series of events leading to stimulation of nuclear activity by glucocorticoids is binding of steroid to receptor protein in target cells, and glucocorticoid receptors have been found in osteoblasts.[40] In the present study, the greater potency of dexamethasone relative to hydrocortisone for stimulation of bone resorption (approximately 5-fold) suggests that receptor binding and subsequent nuclear activity were responsible for the resorptive actions of the glucocorticoids. Progesterone and RU38486 are modulators of glucocorticoid binding to receptor protein. Progesterone is a physiological antagonist of cortisol[13,35] and RU38486 (mifepristone; 17β-hydroxy-11β-(4-dimethylaminophenyl) 17α-(1-propynyl)-estra-4, 9 dien-3-one) is a derivative of 19-nortestosterone and a potent antiglucocorticoid,[35] which has been used as an effective drug in the treatment of Cushing's syndrome.[41] Specific inhibition of resorption by progesterone in a competitive manner and by RU38486 provides additional evidence favoring an action of the glucocorticoids which was mediated by nuclear transcriptional events.

Calcium release stimulated by hydrocortisone and dexamethasone in neonatal mouse calvarial bones was inhibited by the bisphosphonate AHPrBP,[31] the carbonic anhydrase inhibitor acetazolamide,[32,33] and the polypeptide hormone calcitonin. Inhibition of bone resorption by AHPrBP is believed to be due, in large measure, to a direct inhibitory effect of the bisphosphonate on osteoclast function and the compound has been used for treatment of Paget's disease[42] and in osteolytic lesions associated with neoplastic bone disease.[43] The carbonic anhydrase inhibitor, acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), is another well known inhibitor of bone resorption, both in vitro[32] and in vivo.[33] The enzyme carbonic anhydrase is present in osteoclasts and is thought to facilitate osteoclastic bone resorption through the generation of hydrogen ions from carbon dioxide by the CO_2 hydration reaction. The third agent that blocked mineral mobilization stimulated by hydrocortisone and dexamethasone was calcitonin. Calcitonin is a potent hormonal inhibitor of bone resorption which stimulates production of the second messenger, cAMP, in osteoclasts.[44] Inhibition of calcium release by AHPrBP, acetazolamide, and calcitonin suggests that the resorptive response of the glucocorticoids in neonatal mouse calvarial bones was mediated by osteoclasts. Additional evidence indicating osteoclast-mediated bone resorption by the glucocorticoids was the observation that both hydrocortisone

and dexamethasone stimulated release of the lysosomal enzyme, β-N-acetylglucosaminidase. Other bone-related cells, such as monocytes and macrophages, contain specific binding protein for glucocorticoids,[45] and Teitelbaum et al.[46] have shown that glucocorticoids can enhance in vitro resorption of devitalized bone by rat peritoneal macrophages. However, unlike bone resorption stimulated by osteoclasts, macrophage dissolution of bone particles is not associated with the release of the lysosomal enzyme, β-N-acetylglucosaminidase.[47]

Although calcitonin, like AHPrBP and acetazolamide, is an inhibitor of osteoclastic bone resorption, inhibition by calcitonin of most resorptive agents is transient.[34] Despite the continuous presence of calcitonin, the initial inhibition of bone resorption is followed by increased resorption which approaches and often equals that stimulated in the absence of inhibitor. This phenomenon is referred to as "escape from calcitonin-induced inhibition"[34] and has been observed with many well known stimulators of bone resorption, such as PTH, $1,25(OH)_2D_3$, and the prostaglandins. In the present study, "escape" occurred with PTH treatment and when PTH was combined with glucocorticoids. However, "escape" was not noted when calvarial bones were exposed to calcitonin and either hydrocortisone or dexamethasone in the absence of PTH. This sustained inhibition by calcitonin of bone resorption stimulated by hydrocortisone and dexamethasone has not been observed previously and was seen with supraoptimal as well as suboptimal concentrations of the hormone.

An additive effect of cortisol on the inhibition of fetal rat limb bone resorption by calcitonin has been shown by Raisz et al.,[12] and in hypercalcemic patients, the combination of glucocorticoids and calcitonin is reported to produce a greater and more sustained hypocalcemia than treatment with either agent alone.[48,49] Down-regulation of calcitonin receptors is suggested to be related to the "escape" phenomenon,[50] and glucocorticoid exposure has been shown to enhance the numbers of calcitonin receptors in cancer cell lines[51,52] and in osteoclast-like cells formed in cocultures of mouse osteoblastic cells and marrow cells in the presence of $1,25(OH)_2D_3$.[53] Up-regulation of calcitonin receptors in neonatal mouse calvarial osteoclasts could play an important role in the lack of "escape from calcitonin-induced inhibition" which occurred in the present study following hydrocortisone and dexamethasone treatment. However, the observation of "escape" in the presence of PTH + glucocorticoids, but sustained inhibition when only glucocorticoids were present with calcitonin, suggests a further dependence of the "escape" phenomenon on mechanisms that can modify or overcome up-regulation of calcitonin receptors by glucocorticoids.

Glucocorticoids are believed to function as inhibitors of prostaglandin formation in numerous tissues, including bone, by decreasing arachidonic acid release from membrane phospholipids[54] and/or by inhibiting arachidonic acid metabolism via interaction with cyclo-oxygenase.[55] The absence of an effect of hydrocortisone and dexamethasone on basal PGE_2 formation, while both steroids proved to be potent inhibitors of PGE_2 formation stimulated by PTH, suggests that resorption stimulated by the glucocorticoids in neonatal mouse calvariae is not dependent on prostaglandin biosynthesis, but is mediated by a prostaglandin independent pathway.

Depending on experimental conditions, glucocorticoids have been reported to either inhibit or stimulate bone cell growth.[36] Inhibition of ^{45}Ca release in mouse calvarial bones stimulated by hydrocortisone and dexamethasone was observed with the mitotic inhibitors hydroxyurea and mytomycin C. The finding that hydroxyurea did not affect basal or PTH-stimulated resorption, together with the observation that bone resorption stimulated by cotreatment with PTH and dexamethasone was unaffected by hydroxyurea, indicate that this mitotic inhibitor, neither in the absence nor in the presence of dexamethasone, did not decrease glucocorticoid-induced ^{45}Ca release by nonspecific cell toxicity. These data indicate that the resorptive effects of the glucocorticoids in neonatal mouse calvariae are dependent on cellular replication, perhaps of preosteoclasts or other bone cells involved in the resorptive process. In contrast, the resorptive effect of PTH in neonatal mouse calvariae (present study) and in fetal rat long bones[56] cannot be reduced by mitotic inhibitors, suggesting that PTH enhances the number of osteoclasts in these bone organ cultures by increasing the differentiation and/or fusion of mononuclear progenitor cells at a postmitotic level. Glucocorticoids also have been implicated in other mechanisms that could play a role in the stimulation of neonatal mouse calvarial bone resorption observed with hydrocortisone and dexamethasone. In addition to the evidence in rat bone implants presented by DeFranco et al.[39] for stimulation of multinucleated osteoclasts by glucocorticoids, enhanced differentiation by glucocorticoids of osteoclast-like cells induced by $1,25(OH)_2D_3$ in mouse bone marrow cultures and in cocultures of spleen cells and the bone marrow-derived stromal cell lines, MC3T3-G2/PA6 and ST2, also has been reported.[20,21] However, unlike the dependence of osteoclast-like cell formation stimulated by dexamethasone on inhibition of GM-CSF production in mouse bone marrow cultures,[20] no effect of exogenous rmGM-CSF addition on the resorptive response to glucocorticoids in neonatal mouse calvariae was observed. Thus, in addition to the requirement for $1,25(OH)_2D_3$ in bone marrow cultures, the presence of GM-CSF may represent a second fundamental difference in osteoclast enhancement by glucocorticoids in the two systems. Another possible mechanism suggested for the glucocorticoids comes from a study by Bar-Shavit et al.[57] In this investigation, it was found that glucocorticoids modulate cell surface oligosaccharides, increasing the binding of cells to the surface of bone, and this might provide a means not only for aiding stimulation of osteoclastic bone resorption by glucocorticoids but also for potentiation of the effects of other osteolytic agents.

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Address reprint requests to:
Dr. Ulf H. Lerner
Department of Oral Cell Biology
University of Umeå
S-901 87 Umeå, Sweden

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