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α-Difluoromethylornithine Inhibits Bone Resorption *In Vitro* Without Decreasing β-Glucuronidase Release

PAULA H. STERN, RITA C. LUCAS,[1] and JEROME SEIDENFELD[2]
Department of Pharmacology, Northwestern University, Chicago, Illinois 60611
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SUMMARY

Our previous studies suggested that the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) inhibits bone resorption by mechanisms that are independent of polyamine depletion. To determine whether DFMO prevents calcitriol-stimulated bone resorption by acting at a step before or after osteoclast activation, we compared the effects of DFMO on release of calcium and β-glucuronidase from cultured neonatal mouse calvaria. DFMO, at concentrations of 7.5–20 mM, inhibited release of calcium from calcitriol-stimulated calvaria but failed to inhibit the calcitriol-stimulated increase in β-glucuronidase secretion. In contrast, ornithine, putrescine, spermidine, and spermine, at concentrations with effects on resorption comparable to those of DFMO, inhibited the effects of calcitriol on both calcium and β-glucuronidase release. NaF (0.2 mM), like DFMO, inhibited calcitriol-stimulated calcium release without affecting medium β-glucuronidase activity, whereas elevated phosphate (3 mM) inhibited both activities. The results suggest that DFMO, over the concentration range studied, inhibits calcium release by making the matrix resistant to resorption rather than by acting at a cellular locus.

DFMO is an active site-directed irreversible inhibitor of ODC, the initial enzyme in polyamine biosynthesis (1). The polyamine pathway has been implicated in the biological response of target tissues to PTH and calcitriol. Calcitriol administration increases ODC activity in bone, intestine, and pancreas (2–4). Treatment of osteoblastic cells with PTH likewise increases ODC (5–7). We previously used DFMO to study the role of polyamines, products of the ODC pathway, in hormone-stimulated bone resorption (8). We observed that, whereas DFMO reduces bone putrescine to nondetectable concentrations and inhibits the resorption elicited by PTH or calcitriol in cultured neonatal mouse calvaria, exogenous putrescine does not reverse the inhibition of resorption, despite its circumvention of ODC inactivation. The results suggested that a mechanism other than polyamine depletion might mediate the inhibitory effect of DFMO on bone resorption.

Hormone-stimulated bone resorption is a multistep process, involving specific receptors on osteoblasts, subsequent activation of osteoclasts, and possibly interactions with other cell types (9). Resorption, stimulated by either PTH or calcitriol *in vitro*, is associated with increased osteoclastic activity [i.e., morphological changes and release of lysosomal enzymes (10, 11)]. The release of lysosomal enzymes is, thus, a late step in the processes leading to breakdown of the bone matrix. An agent that inhibits resorption by either directly preventing osteoclast activation or by blocking events before osteoclast activation should inhibit the release of lysosomal enzymes in parallel with its inhibition of bone resorption. As an initial step in determining the site of the inhibitory effect of DFMO on resorption, we compared the effects of the agent on calcitriol-stimulated calcium release and on calcitriol-stimulated β-glucuronidase release.

Materials and Methods

Bone cultures. Calvaria were obtained from 5- to 6-day-old neonatal CD-1 mice of either sex (Charles River, Portage, MI) and were cultured in roller tubes as described previously (12). Bones were cultured in DMEM (M. A. Bioproducts, Walkersville, MD) supplemented with 15% heat-inactivated (56°, 1 hr) horse serum (GIBCO, Grand Island, NY), 2.8 mM L-glutamine (M. A. Bioproducts), 10 units/ml heparin sulfate (Lilly, Indianapolis, IN), 100 units/ml penicillin G potassium (Pfizer, New York, NY), and 0.5 μM indomethacin (Sigma Chemical Co., St. Louis, MO). Calvaria were cultured as individual free-floating bones in roller tubes, which contained 2 ml of medium. Cultures were gassed with a mixture of 50% O_2, 5% CO_2, and 45% N_2 at the start of the experiment and regassed every 24 hr. Media were changed after 48 hr and the incubation was continued for another 48 hr. Bone resorption was determined by measurement of the total

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[1]Recipient of an Advanced Predoctoral Fellowship from the Pharmaceutical Manufacturers Association Foundation. Current address: Department of Medicine, University of Chicago, Chicago, IL 60637.
[2]Current address: Division of Drugs and Toxicology, American Medical Association, 515 N. State St., Chicago, IL 60611.

ABBREVIATIONS: DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; PTH, parathyroid hormone; DMEM, Dulbecco's modified Eagle's medium.

calcium concentration in the culture medium at 24, 48, 72, and 96 hr. Total medium calcium was assayed by automatic fluorometric titration (Calcette calcium analyzer, model 4008; Precision Systems, Inc., Natick, MA).

β-Glucuronidase activity (13). For studies of β-glucuronidase activity released into the bone culture media, 50-μl aliquots of media were taken at the end of culture (generally 96 hr) and mixed with 250 μl of the substrate phenolphthalein glucuronidate (1.5 mM, pH 7.0), 250 μl of phosphate buffer (0.075 M, pH 6.8), and 250 μl of deionized water. Tubes were capped, vortexed, and incubated at 37° for 68–71 hr. The reaction was stopped with 2.5 ml of glycine buffer (0.2 M, adjusted to pH 10.4 with 10% NaOH, and containing 11.7 g/liter NaCl). Absorbance was measured at 540 nm. Previous studies from our laboratory have shown that the assay is linear over this time period. The enzymatic activity was not affected by DFMO, ornithine, putrescine, spermidine, or spermine at the concentrations and under the conditions used in the present experiments.

Chemicals. Calcitriol was generously provided by Dr. M. Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). DFMO was generously provided by Dr. Ekkehard H. W. Bohme (Merrell Dow Research Institute, Cincinnati, OH). Putrescine dihydrochloride, DL-ornithine hydrochloride, and indomethacin were purchased from Sigma (St. Louis, MO). Spermine trihydrochloride and spermine tetrahydrochloride were purchased from Calbiochem (San Diego, CA). Calcitriol and indomethacin were dissolved in absolute ethanol. The concentration of ethanol added to the cultures was less than 0.1%. DFMO, ornithine, and polyamines were dissolved at concentrations of 0.25–2.0 mM in DMEM without bicarbonate and brought to pH 7.0–7.4 with sodium hydroxide.

Statistics. Treatment effects on experimental variables were determined by one-way analysis of variance, followed by the least significant difference test for comparison of means (14). $p < 0.05$ was selected as the level for significance.

Results

Effects of DFMO on calcium and β-glucuronidase release. As shown previously (8), DFMO inhibited the calcitriol-stimulated increase in medium calcium (Fig. 1A). The lowest concentration of DFMO tested, 7.5 mM, produced significant inhibition, and greater effects were seen at 15 and 20 mM DFMO. Although the effects of calcitriol were not completely abolished by DFMO in this study, we have previously achieved complete inhibition with concentrations of 30 mM DFMO (8). In the experiment shown, the higher concentrations of DFMO also inhibited calcium release from unstimulated bones. However, in most studies, DFMO did not lower the calcium in control media, even at DFMO concentrations as high as 40 mM. In contrast to the effects on medium calcium, the increase in medium β-glucuronidase elicited by calcitriol was not affected by DFMO (Fig. 1B).

Effects of ornithine and polyamines on calcium and β-glucuronidase release. Fig. 2 illustrates the effects of ornithine, the substrate for ornithine decarboxylase (Fig. 2A), and the polyamines putrescine, the product of ornithine decarboxylation (Fig. 2B), and spermidine (Fig. 2C) and spermine (Fig. 2D), which are produced by the successive transfer of aminopropyl groups to putrescine and spermidine, respectively. Spermidine, at 0.5 mM, increased medium calcium, whereas spermine at concentrations of 5 mM and greater significantly decreased medium calcium. Calcitriol-stimulated resorption was inhibited in a dose-dependent manner by ornithine at concentrations of ≥30 mM, putrescine at ≥1 mM, spermidine at ≥10 mM, and spermine at ≥5 mM. Spermine, at concentrations of ≥5 mM, inhibited basal calcium release from control bones. Basal calcium release was slightly elevated in this study. In

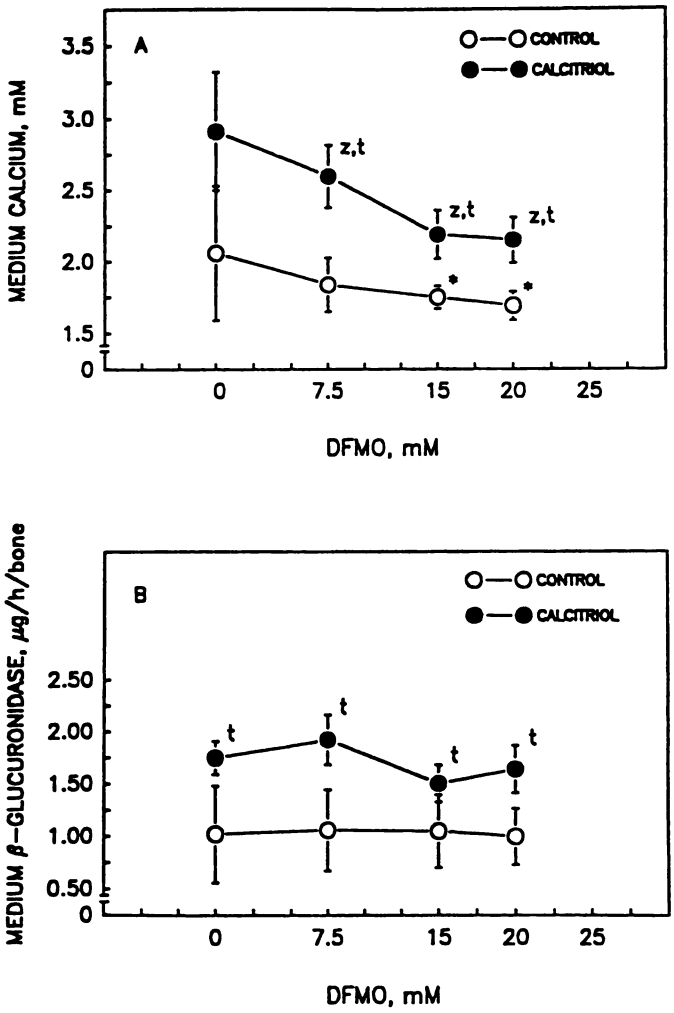

Fig. 1. Dose-dependent effects of DFMO on basal and calcitriol-stimulated resorption (A) and on calcitriol-stimulated β-glucuronidase release (B). Calvaria were cultured for 48 hr in control medium. At 48 hr, media were changed and bones were incubated for an additional 48 hr in control medium or in medium containing DFMO (7.5, 15, or 20 mM), calcitriol (0.5 mM), or calcitriol plus DFMO. Each *point* represents the mean ± standard deviation of responses from 8–10 calvaria. *, $p < 0.05$, compared with untreated control; *t*, $p < 0.05$, compared with the same concentration of DFMO in the absence of calcitriol; *z*, $p < 0.05$, compared with calcitriol alone in the absence of DFMO.

contrast to the results with DFMO, ornithine (30 mM) (Fig. 3), putrescine (7.5 mM) (Fig. 4), and spermidine (7.5 mM) (Fig. 4), added at concentrations that inhibited calcium release, significantly inhibited β-glucuronidase release into the medium as well. Spermine, at 1 mM, significantly inhibited the effect of calcitriol on medium calcium without affecting medium β-glucuronidase significantly. This low concentration of spermine elevated β-glucuronidase activity over control values. However, 5 mM spermine inhibited both activities (Table 1).

Effects of NaF and phosphate on calcium and β-glucuronidase release. Two ions with potential for interactions with the mineral component of the bone matrix were examined for their effects on calcium and β-glucuronidase in the culture system. A full time-course study comparing the effects of DFMO and NaF is shown in Fig. 5. NaF, at 0.2 mM, had effects similar to those of DFMO on medium calcium, although inhibitory effects of NaF on unstimulated bones were seen at earlier times. As was the case with DFMO, NaF inhibited the calcitriol-

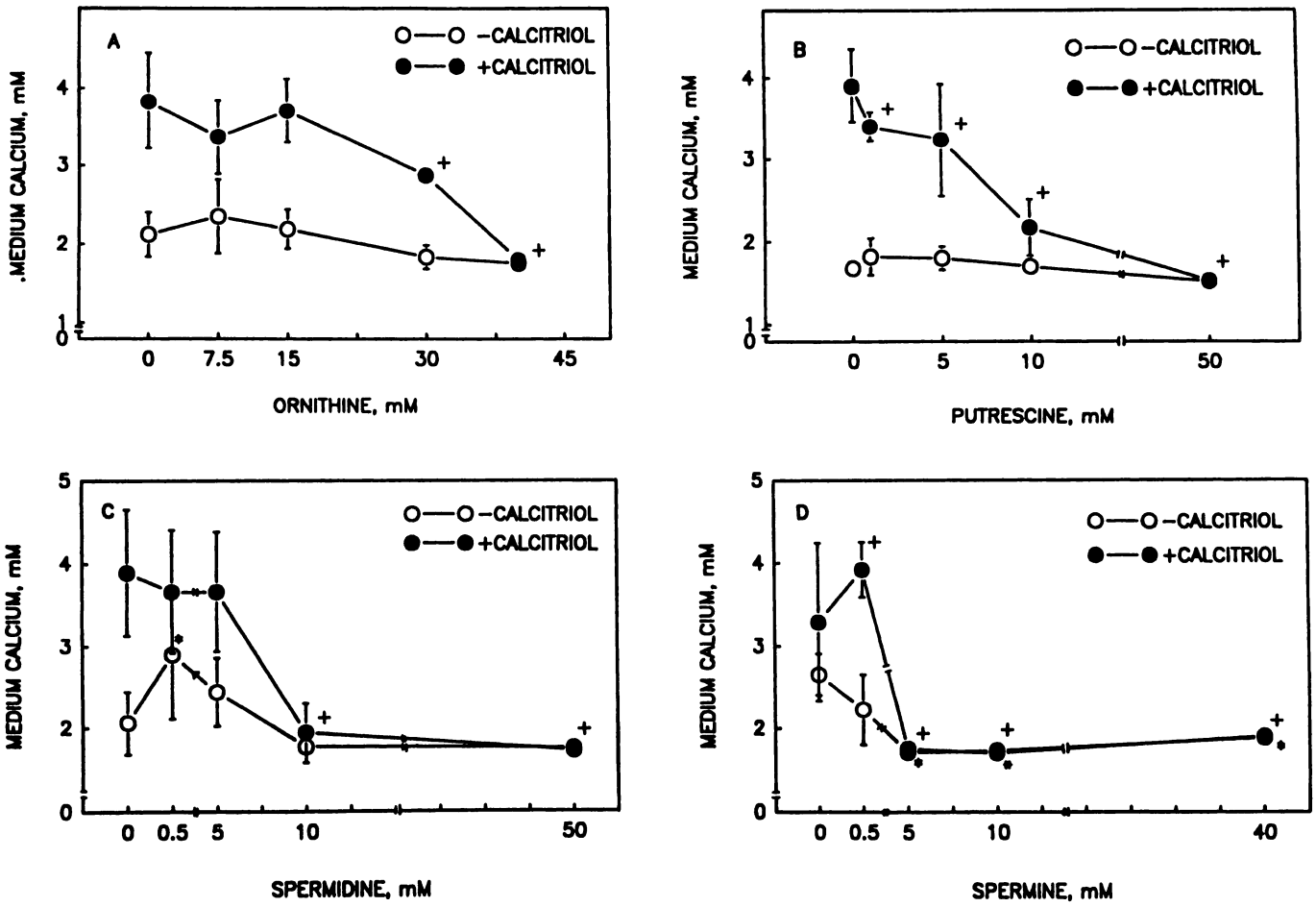

Fig. 2. Effect of ornithine (A), putrescine (B), spermidine (C), and spermine (D) on calcitriol-stimulated resorption. A, Calvaria were precultured for 48 hr with or without ornithine. At 48 hr, media were changed and bones were incubated for an additional 48 hr in control, calcitriol (0.5 mM), ornithine, or ornithine plus calcitriol medium. Each *point* represents the mean ± standard deviation of responses from three or four neonatal mouse calvaria incubated for 96 hr. B–D, Calvaria were precultured in control medium for 48 hr. At 48 hr, media were changed and bones were incubated for an additional 48 hr in control, calcitriol (0.5 mM), polyamine (putrescine, spermidine, or spermine), or polyamine plus calcitriol medium. Each *point* represents the mean ± standard deviation of responses from four neonatal mouse calvaria incubated for 96 hr. *, $p < 0.05$, compared with untreated control; +, $p < 0.05$, compared with calcitriol alone in the absence of polyamine.

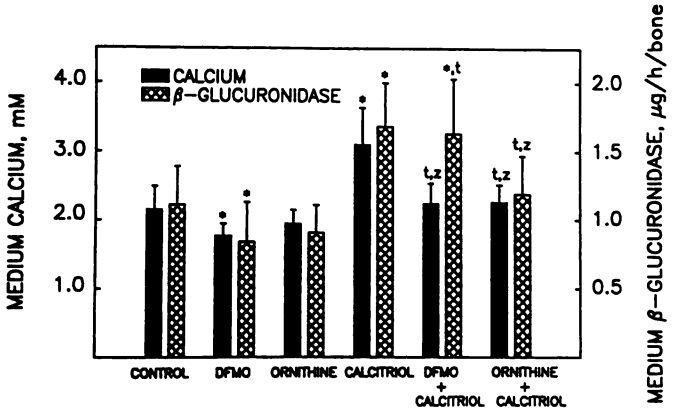

Fig. 3. Effects of DFMO and ornithine on calcitriol-stimulated resorption and release of β-glucuronidase. Calvaria were cultured for 48 hr in the absence or presence of DFMO (15 mM) or ornithine (30 mM). Bones were then incubated for an additional 48 hr in control, DFMO, ornithine, calcitriol, DFMO plus calcitriol, or ornithine plus calcitriol medium. Each *bar* represents the mean ± standard deviation of responses from 14 or 15 calvaria at 96 hr. *, $p < 0.05$, compared with untreated control; *t*, $p < 0.05$, compared with the same treatment in the absence of calcitriol; *z*, $p < 0.05$, compared with calcitriol alone.

stimulated increase in medium calcium without affecting medium β-glucuronidase activity (Table 2). Increasing the phosphate concentration in the DMEM from 0.9 to 3 mM inhibited the increases in medium calcium and in medium β-glucuronidase elicited by calcitriol (Table 3).

Effects of DFMO on calcium and β-glucuronidase release in devitalized bone. When bones were devitalized by five cycles of freezing on solid CO_2 followed by thawing, both medium calcium and medium β-glucuronidase were significantly decreased. Neither calcitriol nor DFMO affected devitalized bones (Table 4). DFMO can inhibit hormone-stimulated resorption, as assessed by release of calcium to the culture medium. A lower concentration of DFMO, 5 mM, was used in the earlier studies, and pretreatment of the bones with DFMO for 48 hr was required to elicit inhibitory effects. The current results indicate that, at DFMO concentrations of ≥7.5 mM (e.g., Fig. 1), the inhibitory effect of DFMO can be elicited without pretreatment.

Discussion

In the present studies, a dissociation of DFMO inhibition of calcium release from the release of β-glucuronidase was ob-

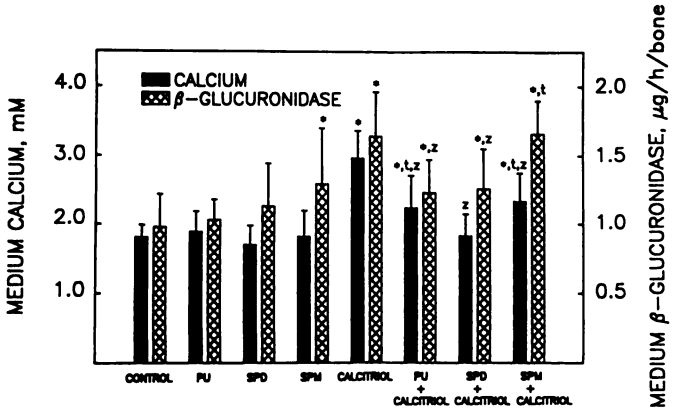

Fig. 4. Effects of putrescine, spermidine, and spermine on calcitriol-stimulated resorption and β-glucuronidase release. Calvaria were cultured for 48 hr in control medium. At 48 hr, media were changed and bones were incubated for an additional 48 hr in control medium or in medium containing putrescine (7.5 mM) (*PU*), spermidine (7.5 mM) (*SPD*), spermine (1 mM) (*SPM*), calcitriol (0.5 nM), or polyamine plus calcitriol. Each *bar* represents the mean ± standard deviation of responses from 14 or 15 calvaria at 96 hr. *, $p < 0.05$, compared with untreated control; *t*, $p < 0.05$, compared with the same treatment in the absence of calcitriol; *z*, $p < 0.05$, compared with calcitriol alone.

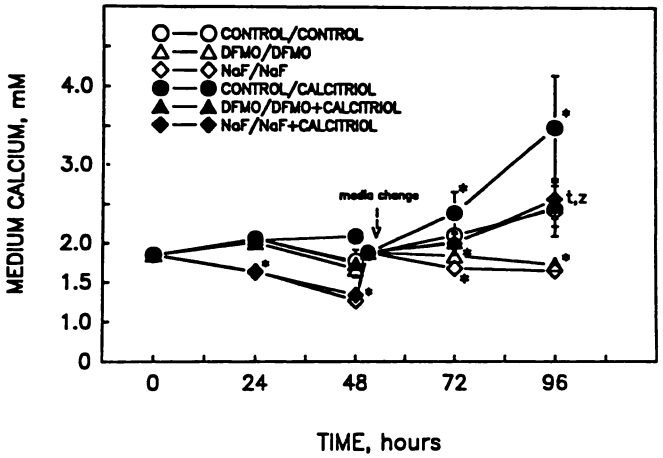

Fig. 5. Time course of effects of NaF or DFMO on basal and calcitriol-stimulated resorption. Calvaria were cultured for 48 hr, with or without DFMO (15 mM) or NaF (0.2 mM). At 48 hr, media were changed and bones were incubated for an additional 48 hr in control medium or in medium containing DFMO or NaF, calcitriol (0.5 nM), calcitriol plus DFMO, or calcitriol plus NaF. Each *point* represents the mean ± standard deviation of responses from five calvaria. *, $p < 0.05$, compared with untreated control at that time point; *t*, $p < 0.05$, compared with DFMO or NaF at that time point; *z*, $p < 0.05$, compared with calcitriol alone at that time point.

TABLE 1

Inhibition by 5 mM spermine of release of both calcium and β-glucuronidase

Calvaria were precultured for 48 hr in control medium. At 48 hr, the medium was changed and bones were incubated for an additional 48 hr with the indicated treatments. Each value represents the mean ± standard deviation of responses from five calvaria incubated for 96 hr.

Treatment	Medium calcium	Medium β-glucuronidase
	mM	*μg of phenolphthalein/hr/bone*
Control	2.62 ± 0.16	1.26 ± 0.17
Spermine, 5 mM	1.65 ± 0.05[a]	1.08 ± 0.09
Calcitriol, 0.5 nM	3.57 ± 0.46[a]	1.73 ± 0.33[a]
Calcitriol + spermine	1.62 ± 0.03[a,b]	1.22 ± 0.16[b]

[a] $p < 0.05$, compared with untreated control.
[b] $p < 0.05$, compared with calcitriol alone.

TABLE 2

Inhibition by NaF of calcitriol-stimulated resorption but not calcitriol-stimulated β-glucuronidase release

Calvaria were precultured in the absence or presence of NaF. At 48 hr, media were changed and bones were incubated for an additional 48 hr with the indicated treatments. Each value represents the mean ± standard deviation of responses from 5–10 neonatal mouse calvaria incubated for 96 hr. Control medium calcium was 2.44 ± 0.35 mM; control β-glucuronidase activity was 1.21 ± 0.26 μg of phenolphthalein/hr/bone.

Treatment	Medium calcium	Medium β-glucuronidase
	% of control	*% of control*
NaF, 0.2 mM	68 ± 4[a]	76 ± 10
Calcitriol	138 ± 20[a]	140 ± 26[a]
Calcitriol + NaF, 0.2 mM	106 ± 10[b,c]	156 ± 25[a,b]

[a] $p < 0.05$, compared with untreated control.
[b] $p < 0.05$, compared with NaF alone.
[c] $p < 0.05$, compared with calcitriol alone.

TABLE 3

Effect of high medium PO_4 on calcium and β-glucuronidase release

Calvaria were precultured for 48 hr in control medium (0.9 mM PO_4) or 3 mM PO_4 medium. At 48 hr, the media were changed and the bones were incubated for an additional 48 hr with the indicated treatment. Each value represents the mean ± standard deviation of responses from five calvaria incubated for 96 hr.

Treatment	Medium calcium	Medium β-glucuronidase
	mM	*μg of phenolphthalein/hr/bone*
Control	2.37 ± 0.45	1.44 ± 0.33
PO_4, 3 mM	1.37 ± 0.13[a]	1.24 ± 0.18
Calcitriol	3.63 ± 0.40[a]	2.51 ± 0.46[a]
Calcitriol + PO_4, 3 mM	2.01 ± 0.10[a,b,c]	1.83 ± 0.42[b,c]

[a] $p < 0.05$, compared with untreated control.
[b] $p < 0.05$, compared with 3 mM PO_4 without calcitriol.
[c] $P < 0.05$, compared with calcitriol alone.

served. This occurred whether bones were precultured with DFMO (Fig. 3, Table 4) or exposed simultaneously to DFMO and stimulator (Fig. 1). The observed dissociation of the effects of DFMO on medium calcium and medium β-glucuronidase activity is unusual; generally, there is a good correspondence between these indices of bone resorption (10, 13, 15). This finding suggests that the inhibitory effects of DFMO may not be mediated through a cellular pathway but rather through effects on the bone matrix, making it resistant to resorption.

Two ions that can affect the bone matrix, fluoride and phosphate, were compared with DFMO for their effects on resorption and β-glucuronidase activity. The actions of NaF were similar to those of DFMO, i.e., a decrease in medium calcium with no decrease in lysosomal enzyme activity (Table 2). Fluoride can interact with the bone matrix, substituting for hydroxyl ions in the crystalline lattice (16). This modification leads to a less soluble and more stable mineral phase, which could conceivably make it more resistant to resorption. Whether DFMO, which is a fluorinated compound, acts by the same mechanism is unknown. Inactivation of ODC by DFMO results in loss of fluoride (17). However, the quantity of ODC protein in cells is reported to be low (1, 18), and whether sufficient fluoride is generated to reach and react with the critical sites in the bone mineral is unknown. Our results on calcium release from calvaria treated with NaF differed slightly from those reported previously (19), because we were unable to inhibit resorption at 0.1 mM NaF (data not shown) but required 0.2 mM to produce inhibitory effects. Differences between the culture systems, including sources of animals and sera, may explain this disparity.

Elevated phosphate has been shown to inhibit resorption by

TABLE 4

Effects of DFMO and calcitriol on release of calcium and β-glucuronidase in live or devitalized bone

Live or devitalized calvaria were precultured for 48 hr in the absence or presence of DFMO (15 mM). At 48 hr, the media were changed and bones were incubated for an additional 48 hr with the indicated treatments. Each value represents the mean ± standard deviation of medium calcium or β-glucuronidase activity from five calvaria incubated for 96 hr.

Treatment	Medium calcium	Medium β-glucuronidase
	mM	*μg of phenolphthalein/hr/bone*
Live bone		
Control	1.93 ± 0.25	1.09 ± 0.12
DFMO	1.68 ± 0.04[a]	0.82 ± 0.31[a]
Calcitriol	3.13 ± 0.41[a]	1.62 ± 0.18[a]
DFMO + calcitriol	2.28 ± 0.16[a,b,c]	1.64 ± 0.21[a,b]
Devitalized bone		
Control	1.58 ± 0.03[a]	0.24 ± 0.02[a]
DFMO	1.60 ± 0.02[a]	0.25 ± 0.07[a]
Calcitriol	1.56 ± 0.03[a]	0.23 ± 0.15[a]
Calcitriol + DFMO	1.57 ± 0.03[a]	0.34 ± 0.10[a]

[a] $p < 0.05$, compared with live control.
[b] $p < 0.05$, compared with DFMO alone.
[c] $p < 0.05$, compared with calcitriol alone.

a mechanism that is partially dependent upon making the matrix more resistant to resorptive stimuli and is partially cell mediated (20). Therefore, we examined the effects of elevated phosphate in our system. In our studies, 3 mM phosphate inhibited the effects of calcitriol on both calcium and β-glucuronidase release (Table 3).

The inhibitory effects of DFMO appear to be similar to those of (*N*-[3-*N*-(benzyloxycarbonyl)-amino-1-(*R*)-carboxypropyl]-L-leucyl-*o*-methyl-L-tyrosine *N*-methylamide), an inhibitor of mammalian collagenase (21). Both agents inhibit the stimulated release of calcium, whereas neither inhibits stimulated β-glucuronidase release. Electrostatic interactions between DFMO and the polar side chains of collagen could occur and result in inhibition of collagen degradation.

In contrast to the effects of DFMO, concentrations of ornithine, putrescine, and spermidine that inhibited calcium release also decreased medium β-glucuronidase activity. Although spermine at low concentrations inhibited calcium release without affecting medium β-glucuronidase, slightly higher concentrations prevented both effects of calcitriol. These observations suggest that the inhibitory effects of ornithine and the polyamines are exerted at a cellular step before secretion of β-glucuronidase by osteoclasts. However, there could be additional effects of these compounds on the bone matrix.

The inhibitory effects of ornithine and the polyamines on resorption are not surprising, because another positively charged molecule, protamine, has been shown to inhibit resorption (22, 23). The mechanism by which highly protonated molecules inhibit resorption is not known. Possible explanations for inhibition at a cellular level include osmolarity and/or ionic strength effects and changes in surface charge. The respective mono-, di-, tri-, and tetrahydrochloride salts of ornithine, putrescine, spermidine, and spermine were used. Therefore, the increases in both medium osmolarity and ionic strength were greater than would be expected on the basis of polyamine concentrations alone. We previously found that addition of 60 mM sucrose fails to inhibit resorption in the calvarial culture system (24). Thus, the increases in osmolarity produced by the polyamines are not likely to be responsible for the inhibitory effects. Additional experiments with salts of polycationic metals and nonbiological organic polycations are needed to evaluate the role of increased ionic strength in mediating these effects. Calculations by the simplified Gouy-Chapman equation (25) provide evidence that membrane surface charge effects of the polyamines are consistent with the polyamine concentrations required to inhibit resorption. Polyamines may hyperpolarize cells, thereby interfering with membrane depolarization. Although calcitriol has been shown to elicit rapid changes in intracellular calcium in osteoblasts (26, 27), it is not known whether membrane depolarization is essential to the effect of calcitriol to stimulate bone resorption.

Oxidation of polyamines by diamine oxidase results in the production of acrolein, a toxic metabolite (28). Diamine oxidase is present in some tissues (28), and it is possible that calvaria have significant diamine oxidase activity. However, this is an unlikely explanation for the effects of putrescine, because aminoguanidine (a diamine oxidase inhibitor) does not reverse the toxic effects of putrescine on fibroblast proliferation, although it does reverse the effects of spermidine and spermine (29). In addition, the use of heat-inactivated horse serum decreases the likelihood of substantial metabolism of polyamines in the medium by the diamine oxidase pathway (30, 31).

We had previously found that addition of exogenous putrescine did not reverse the inhibitory effects of DFMO on resorption. This observation, in fact, suggested that inhibitory effects of DFMO on bone resorption may have been due to mechanisms other than inhibition of the ODC pathway. One possible explanation for the failure of putrescine to restore bone-resorbing activity was that treatment with DFMO killed the bones. Several of the current observations make this unlikely. First, bones killed by repeated freezing and thawing had significantly lower levels of medium calcium (decreased by 25%) and medium β-glucuronidase activity (decreased by 75%) than control bones (Table 4). Although we sometimes observed small decreases (<20%) in medium calcium and β-glucuronidase release from control bones with DFMO pretreatment, in most experiments these indices of bone resorption were unchanged in the presence of DFMO. In addition, the failure of DFMO to decrease β-glucuronidase activity in hormone-stimulated bones provides further evidence against cell death.

Our original question, concerning a requirement for polyamines in stimulated bone resorption, remains unanswered. However, the observation that DFMO inhibits hormone-stimulated resorption without affecting lysosomal enzyme activity suggests that the agent has a noncellular mechanism of action in the cultured calvaria. Bone is distinguished from other tissues by its calcified matrix. Our results suggest that agents that inhibit resorption selectively by interactions with the matrix could have potential for the treatment of abnormal bone turnover.

Acknowledgments

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Send reprint requests to: Dr. Paula H. Stern, Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611.