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Research Article

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Bone Resorption in Organ Culture

INHIBITION BY THE DIVALENT CATION IONOPHORES A23187 AND X-537A

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ABSTRACT The ionophores A23187 and X-537A were used as probes to investigate the possible role of calcium uptake by bone as a mediator for the stimulation of bone resorption induced by parathyroid hormone (PTH) and other agents in cultured mouse calvaria. The ionophores alone at concentrations from 1 nM to 20 μM did not stimulate bone resorption, nor did they potentiate bone resorption stimulated by submaximal concentrations of PTH after either brief (15–60 min) or extended (1–3 day) exposure to the ionophores. Unexpectedly, we found that the ionophores inhibit in a dose-dependent manner bone resorption stimulated by PTH and a wide variety of other compounds (prostaglandin E_2, 1α-hydroxycholecalciferol, 3-isobutyl-1-methylxanthine, and phorbol myristate acetate). This inhibition was not due to irreversible damage to the bones by the ionophores, because the inhibition was reversible even after 24 h of treatment. Inhibition of bone resorption by the ionophores was observed in media of both high and low calcium concentration, indicating that the inhibition was not due to a critical extracellular calcium concentration. Inhibition by the ionophores differs qualitatively in several ways from that produced by calcitonin, a natural inhibitor of bone resorption. Furthermore, A23187 at 1.0 μg/ml had no effect on the accumulation of cyclic AMP in the medium of either control, PTH- or calcitonin-treated calvaria. We conclude that the ionophores A23187 or X-537A do not stimulate bone resorption nor potentiate the effects of stimulators of bone resorption; instead they are inhibitors of bone resorption stimulated by a wide variety of compounds.

INTRODUCTION

It has been proposed (1) that cytosol calcium ion concentration regulates a number of physiological processes. Divalent cation ionophores with relatively high selectivity for calcium ion have been used in several systems to demonstrate the involvement of calcium ion in the action of physiological stimuli (2–5). Because these ionophores either mimic (2–4) or potentiate (5) a physiological stimulus in several systems, we have investigated the effect of the cation ionophores A23187 (6) and X-537A (7) on bone resorption in mouse calvaria in organ culture.

The possibility that calcium ion may be involved in stimulating bone resorption was initially suggested by the report of Parsons et al. (8) that intravenous injection of parathyroid hormone (PTH)[1] in dogs produced a small, but significant, transient decrease in plasma calcium concentration. Both in vivo (9) and in vitro (10, 11) experiments have suggested that this decrease in plasma calcium is due to an accumulation of the ion in bone. These findings led Parsons and his co-workers to hypothesize that calcium uptake by bone is one of the earliest actions of PTH on this target tissue (8, 9). However, our results indicate that instead of mimicking or potentiating the effects of stimulators of bone resorption, these ionophores potently inhibit bone resorption, stimulated by all of the compounds with which they have been tested.

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[1] *Abbreviations used in this paper*: PTH, parathyroid hormone; 1α(OH)-vitamin D_3, 1α-hydroxycholecalciferol; IBMX, 3-isobutyl-1-methylxanthine; PGE_2, prostaglandin E_2; BSA medium and heat-inactivated serum medium denote culture medium supplemented with bovine serum albumin or heat-inactivated serum, respectively.

METHODS

Organ culture of bone. The method used was essentially that described previously (12), based on the procedure of Goldhaber (13).[2] At 4–5 days of age, calvaria (frontal and parietal bones) were removed from the mice under aseptic conditions and fixed to sterile cover slips with a clot formed by mixing chick embryo extract and rooster plasma (1:2, vol/vol). The cover slip and attached calvarium were transferred to a Leighton tube and incubated at 37°C in a rotating drum (0.2 rpm). Each tube contained 2.0 ml of Oxman's modification of Eagle's minimal essential medium, supplemented with 5 mg/ml bovine serum albumin (BSA medium) or with 15% (vol/vol) heat-inactivated (56°C, 60 min) horse serum and 2.5% (vol/vol) heat-inactivated fetal calf serum (heat-inactivated serum medium), 100 μg/ml streptomycin sulfate, 100 U/ml sodium penicillin G, and 10 U/ml sodium heparin. The tubes were gassed with 50% O_2, 5% CO_2, 45% N_2, and sealed with a silicone stopper. In the standard experimental protocol, the bones were incubated for the first 24 h in culture without any treatment, the medium was removed for determination of calcium concentration (the washout sample), 2.0 ml of fresh medium containing the indicated test substances was added, and the tubes were gassed and resealed as described above. Incubation was continued for an additional 48–72 h. A 0.2-ml aliquot of medium was removed for calcium determination, and the tubes were regassed at 24-h intervals.

Measurements of bone resorption. Resorption of bone involves release of the extracellular mineral and degradation of the organic phase of this tissue. Indices used to evaluate bone resorption in vitro include morphological examination of microcinematography (14), morphological scoring of the extent of resorption (12, 15), release from bone of previously incorporated ^{45}Ca (12, 16–18), increase in total medium calcium (^{40}Ca) concentration due to release of calcium from the bone (17, 19), and release of hydroxyproline as an indication of collagen degradation (20). We have found that determination of total medium calcium concentration is more convenient, faster, and considerably less expensive than determination of ^{45}Ca release from bones previously labeled with ^{45}Ca; furthermore, with neonatal mouse calvaria both total calcium and ^{45}Ca release give equivalent results when experiments are assayed by both methods (12, 16, 17, 19, and results described below). In addition, morphological scoring of the extent of bone resorption, which reflects both release of calcium and degradation of matrix, also correlates well with total medium calcium concentration and ^{45}Ca release into the medium (12 and results described below).

Measurement of total medium calcium. Total calcium concentration in the medium was determined with a Corning Model 940 calcium analyzer (Corning Scientific Instruments, Medfield, Mass.). Interference with the determination of medium calcium concentration by this method was observed with unincubated medium containing 10.0 μg/ml A23187. At this concentration the observed calcium concentration was 0.9–1.0 mg/dl lower than the same medium without A23187. No correction for this interference was made, since the amount of A23187 remaining in the medium after incubation with the calvaria was not known. There was no change in observed calcium concentration when medium contained A23187 at concentrations of 1.0 μg/ml or lower.

Measurement of ^{45}Ca release from calvaria. When ^{45}Ca release from bone was used to determine the extent of bone resorption, the method was that described by Tashjian et al. (16). Mice were injected with 10 μCi of ^{45}Ca at age 1 day. At 4 days of age the calvaria were removed and placed in culture. The bones were incubated in control medium for 24 h, this medium was then replaced with fresh control or treatment medium, and the incubation continued an additional 48 h. Total medium ^{40}Ca was determined as described above. At each sampling period, ^{45}Ca released into the medium was also determined by counting 100-μl aliquots in Bray's scintillation fluid in a liquid scintillation spectrometer.

Morphological scoring of bone resorption. The extent of bone resorption was evaluated morphologically by the basic scoring system described previously (12, 15). The extent of resorption is indicated by a score for the frontal bone and for each of the two parietal bones, reflecting the relative areas of each resorbed. The frontal bone is scored from 0 for no resorption to 6 for complete resorption, and each of the parietal bones is scored from 0 to 4. Because this is a subjective procedure and reliable scoring depends on experience with this technique, the scoring of our experiments was kindly performed by Mr. Steven Isley (Department of Periodontology, Harvard School of Dental Medicine), without knowledge of the treatment groups or the order of samples. The raw scores for each of the three bones were summed to yield a single score (maximum possible 14) for each calvarium.

Measurement of cyclic AMP. Freshly dissected calvaria were placed in Oxman's modification of Eagle's minimal essential medium without protein supplementation (two bones in 0.5 ml). After preincubation for 20 min, 50 μl control or treatment medium was added and the incubation continued for 20 min. The bones were removed and rapidly homogenized in 1.0 ml of ice-cold 5% (wt/vol) trichloroacetic acid. The medium was collected, placed in a boiling water bath for 5 min, and frozen. The bone homogenate was centrifuged to remove precipitated protein, and after adjustment to 0.1 N in HCl, extracted five times with 5 vol of water-saturated diethyl ether. Residual ether was removed by placing in a boiling water bath for 5 min; the solution was then lyophilized and resuspended in distilled water. Cyclic AMP content of bone extracts and unextracted medium were determined by the cyclic AMP protein binding assay described by Gilman (21). The cAMP-dependent protein kinase and protein-kinase inhibitor were prepared from rabbit muscle by the procedures described by Gilman for bovine muscle (21). Digestion of cyclic AMP was performed by incubating 3′:5′-cyclic nucleotide phosphodiesterase from beef heart with unknown samples suspended in 2 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5, at a concentration of 0.04 U/ml for 4 h at 30°C; the incubation tubes were then placed in a boiling water bath for 5 min, cooled, and assayed. Additional control incubations of buffer alone or cyclic AMP standard with and without the cyclic nucleotide phosphodiesterase, or with the phosphodiesterase added immediately before the boiling-water bath, were also performed.

Statistical analysis. Statistical comparisons were performed by separate analysis of variance on the data obtained at each of the sampling intervals and their standard

[2] It should be noted that the report by Goldhaber (13) described two different conditions for culture. When 20% (vol/vol) embryo extract was included in the medium, a remodeling bone culture system was obtained, permitting both bone resorption and formation. When embryo extract was omitted from the medium (as in the present studies), however, no bone formation was observed and the cultures were used exclusively for studying resorption.

TABLE I
Effect of A23187 on Bone Resorption In Vitro

Treatment	Dose	Medium calcium concentration										
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9*a*	Exp. 9*b*	Exp. 9*c*
		mg/dl										
Control		6.5±0.22	7.4±0.47	6.6±0.24	8.0±0.37	6.8±0.45	6.9±0.22	7.2±0.32	7.8±0.36		6.7±0.24	
PTH	0.1 U/ml	9.1±0.22*	12.1±0.47*	9.8±0.24*	—	8.5±0.45‡	11.4±0.22*	13.2±0.32*	11.0±0.36*		9.4±0.24*	
A23187	0.0005 μg/ml	—	—	—	6.9±0.37‡	—	—	—	—	—	—	—
	0.005	—	—	—	6.8±0.37‡	—	—	—	—	—	—	—
	0.00625	—	—	—	—	—	6.5±0.22	—	6.9±0.36	—	—	—
	0.0125	—	—	—	—	—	6.9±0.22	—	7.5±0.36	—	—	—
	0.025	—	—	—	—	—	6.7±0.22	—	6.6±0.36	—	—	—
	0.05	—	—	—	6.8±0.37‡	—	6.5±0.22	—	7.8±0.36	—	—	—
	0.1	6.7±0.22	—	6.7±0.24	—	—	7.1±0.22	7.3±0.32	7.5±0.36	6.2±0.24	7.0±0.24	7.2±0.24
	0.2	—	—	—	—	—	7.4±0.25	—	9.2±0.36§	6.9±0.24	6.2±0.24	7.4±0.24‡
	0.3	—	—	—	—	—	6.6±0.25	7.6±0.32	8.1±0.36	6.6±0.24	7.2±0.24	7.2±0.24
	0.5	—	—	—	6.8±0.43‡	—	6.7±0.25	—	8.1±0.36	—	—	—
	1.0	6.8±0.22	—	—	—	6.4±0.45	—	7.7±0.32	—	—	—	—
	3.0	—	—	—	—	—	—	6.7±0.32	—	—	—	—
	5.0	—	6.3±0.47	—	—	—	—	—	—	—	—	—
	10.0	6.2±0.22	—	—	—	—	—	—	—	—	—	—
S(df)§		0.43 (29)	0.94 (33)	0.48 (26)	0.74 (28)	0.89 (30)	0.43 (26)	0.65 (30)	0.72 (30)		0.47 (33)	

Exps. 1–6 were performed in BSA medium, and exps. 7–9 in heat-inactivated serum medium. A PTH-treated group was not included in exp. 4, and the control group for this experiment was aberrant because even very low doses of A23187 are significantly lower than control and there is no difference in A23187-containing groups over a 1,000-fold concentration range. Exp. 9 used three different lots of A23187: 9*a*, lot 361-V02-248; 9*b*, lot 361-D66-275; and 9*c*, lot 361-D66-282. Most of the other experiments, including exp. 8, were performed with lot 361-V02-248. Treatment means significantly different from the appropriate control values are indicated by: * $P < 0.001$ and ‡ $P < 0.05$. Although the group in exp. 9*c* was significantly different from control, the difference from its own washout mean was not significant. Values are means±SE of groups of three or four bones per treatment group.

§ The standard deviation within groups, s, and the number of degrees of freedom within groups (df) for each experiment.

TABLE II

Effects of A23187 and IBMX on Submaximal Doses of PTH

Treatment	Medium calcium concentration
	mg/dl
Exp. 1	
None	7.4±0.47
PTH (0.1 U/ml)	12.1±0.47§
PTH (0.02 U/ml)	10.8±0.47§
PTH (0.02 U/ml) + A23187 (5 ng/ml)	10.0±0.47§
PTH (0.02 U/ml) + A23187 (50 ng/ml)	10.6±0.47§
Exp. 2	
None	8.0±0.37
A23187 (5 ng/ml)	6.8±0.37*
A23187 (50 ng/ml)	6.8±0.37*
PTH (0.02 U/ml)	9.9±0.43‡
PTH (0.02 U/ml) + A23187 (50 ng/ml)	9.4±0.37*
PTH (4 mU/ml)	8.1±0.37
PTH (4 mU/ml) + A23187 (5 ng/ml)	7.6±0.37
PTH (4 mU/ml) + A23187 (50 ng/ml)	8.0±0.37
Exp. 3	
None	7.0±0.53
PTH (0.1 U/ml)	11.9±0.46§
A23187 (0.05 μg/ml)	7.1±0.53
A23187 (0.1 μg/ml)	7.0±0.46
A23187 (0.2 μg/ml)	8.1±0.53
A23187 (0.5 μg/ml)	7.8±0.46
PTH (0.01 U/ml)	8.9±0.46‡
PTH (0.01 U/ml) + A23187 (0.05 μg/ml)	8.6±0.46*
PTH (0.01 U/ml) + A23187 (0.1 μg/ml)	8.6±0.46*
PTH (0.01 U/ml) + A23187 (0.2 μg/ml)	9.2±0.46‡
PTH (0.01 U/ml) + A23187 (0.5 μg/ml)	9.1±0.53‡
Exp. 4	
None	6.4±0.53
PTH (0.1 U/ml)	9.7±0.60§
IBMX (4 μM)	6.6±0.53
PTH (0.01 U/ml)	7.4±0.53
PTH (0.01 U/ml) + IBMX (4 μM)	9.2±0.53§

All experiments were performed in BSA medium. Treatment groups received the indicated additions, and the mean calcium concentrations after 48 h of treatment are given. Exp. 2 contains additional data from the experiment listed as exp. 4 in Table I, and the control group in this experiment was aberrant. In exp. 4, PTH (0.01 U/ml) + IBMX (4 μM) is significantly different ($P < 0.05$) from PTH (0.01 U/ml) alone. None of the other additions to submaximal PTH groups produced significant enhancement. Treatment means significantly different from the appropriate control values are indicated by: * ($P < 0.05$); ‡ ($P < 0.01$); and § ($P < 0.001$). Values are means±SE of groups of three to five bones per treatment group.

errors (SE) were calculated with the residual error term. Comparison of treatment means to the mean of the appropriate control group and the standard errors given in the tables and figures were based on these analyses. Statistical significance was determined by a two-tailed Student's *t* test. In addition, a combined analysis of variance for all sampling intervals was also performed on experiments using the standard protocol, and the mean medium calcium concentration after 48 h of treatment for each group was compared to its own washout mean (the value determined after the initial 24-h incubation without treatment). A response was considered a significant increase in bone resorption if it resulted in an increase in mean medium calcium concentration after 48 h of treatment (reflecting a loss of calcium from bone) significantly greater ($P < 0.05$) than both the washout mean for the same group and the 48-h mean for the untreated control group.

Sources of compounds. Ionophores A23187 and X-537A were gifts from Eli Lilly and Company (Indianapolis, Ind.) and Hoffmann-LaRoche, Inc., (Nutley, N. J.), respectively. Prostaglandin E_2 (PGE_2) and 1α-hydroxycholecalciferol (1α(OH)-vitamin D_3) were gifts from The Upjohn Company (Kalamazoo, Mich.). Phorbol myristate acetate (12-O-tetradecanoate-phorbol-13-acetate) (22) was obtained from Consolidated Midland Corp (Chemical Div., Katonah, N. Y.). The source of parathyroid hormone was U.S.P. parathyroid extract (100 U/ml, Eli Lilly and Company). Human calcitonin was synthetic calcitonin-M (310 MRC U/mg, Ciba Pharmaceutical Company, Div. of Ciba/Geigy Corporation, Summit, N. J.), and salmon calcitonin was the synthetic peptide from Armour Pharmaceutical Company (Kankakee, Ill.) (5176 MRC U/mg). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical Co., Inc., (Milwaukee, Wis.). Fresh stock solutions of A23187 and phorbol myristate acetate were prepared immediately before use in dimethyl sulfoxide. X-537A, PGE_2, and 1α(OH)-vitamin D_3 were dissolved in ethanol, and IBMX in 0.1 N NaOH. The final concentration of vehicle in the medium was less than or equal to 0.1% (vol/vol) for both dimethyl sulfoxide and ethanol, concentrations that we find do not affect either basal or stimulated bone resorption.

Oxman's modification of Eagle's minimal essential medium was obtained from Microbiological Associates (Bethesda, Md.). Horse serum and fetal calf serum were obtained from Grand Island Biological Co., (Grand Island, N. Y.), and four-times-recrystallized bovine serum albumin was purchased from ICN Nutritional Biochemicals Div. (International Chemical & Nuclear Corp., Cleveland, Ohio).

3′:5′-cyclic nucleotide phosphodiesterase from beef heart was obtained from Sigma Chemical Company (St. Louis, Mo.), and [^{3}H]adenosine-3′:5′-cyclic monophosphate (G) and $^{45}CaCl_2$ were purchased from New England Nuclear (Boston, Mass.).

RESULTS

Effects of ionophores on bone resorption. To test whether the ionophores could stimulate bone resorption without an added known stimulator, a number of experiments were performed. The results obtained with A23187 are summarized in Table I. In 8 of the 10 experiments performed to test for stimulation of resorption by A23187 alone, none of the A23187 treatment groups had medium calcium concentrations significantly greater than the appropriate control. Although two groups in experiments performed in heat-inactivated serum medium (exps. 8 and 9*c*) had mean medium calcium concentrations significantly above control ($P < 0.05$), the group in exp. 9*c* did not differ significantly from its own washout mean and we therefore do not consider this to be a statistically significant stimulation of resorption. The result

in exp. 8, although yielding a significant stimulation of resorption ($P < 0.05$) by both criteria, was not repeated in exp. 9*a*, in which the same lot of A23187 was used and appears to be an aberrant group. The concentrations of A23187 tested ranged between 1 nM and 20 μM (0.5 ng/ml–10.0 μg/ml). Similar experiments performed with X-537A likewise showed no stimulation of bone resorption over the same concentration range (data not shown).

Experiments were then performed to determine whether the ionophores could potentiate bone resorption stimulated by submaximal doses of PTH. The results of experiments with A23187, given in Table II, show no potentiation by A23187 with concentrations between 10 nM and 1 μM (5 ng/ml–0.5 μg/ml). Also shown in Table II are results from exp. 4 with IBMX (a potent inhibitor of cyclic AMP phosphodiesterase activity) and PTH, demonstrating potentiation of the PTH-stimulated resorption by a concentration of IBMX that alone had no effect on bone resorption. These results indicate that although PTH-stimulated bone resorption can be potentiated, A23187 does not enhance or potentiate bone resorption stimulated by PTH.

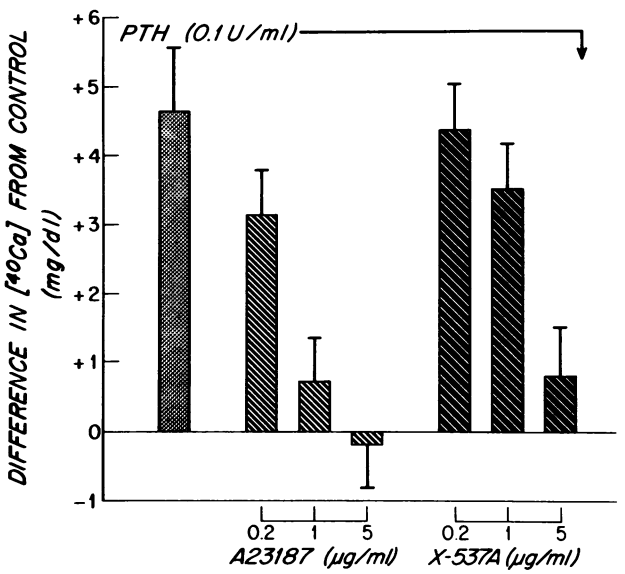

FIGURE 1 Inhibition of PTH-stimulated bone resorption by A23187 and X-537A. Calvaria were maintained in organ culture in BSA medium as described in Methods. At the end of 24 h in culture, fresh medium containing the indicated treatments was added. Total medium calcium was determined after 48 h of treatment. Values given are the mean differences in medium calcium from untreated controls. The brackets give the SE of the mean difference for groups of four bones. All groups except control contained PTH (0.1 U/ml). The first bar to the left gives the extent of bone resorption in the absence of ionophore. Other groups contained A23187 or X-537A at the indicated concentrations.

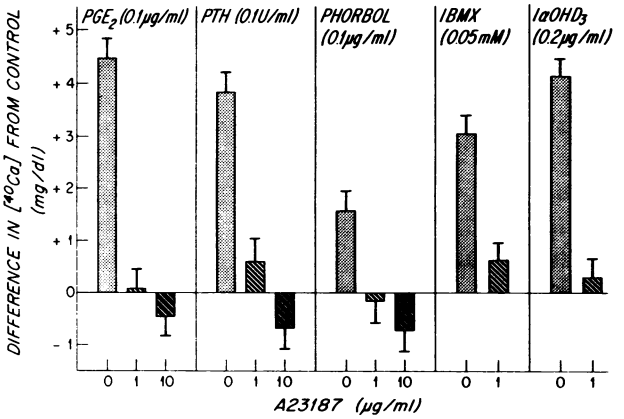

FIGURE 2 Inhibition by A23187 of bone resorption stimulated by a variety of bone resorption-stimulating agents. The compounds noted at the top of each panel were present at the indicated concentrations in the absence or presence (1 or 10 μg/ml) of A23187. Incubations were performed as in Fig. 1. Results are expressed as mean differences from untreated controls; the brackets indicate the SE of the mean difference for groups of four bones.

Inhibition of bone resorption by A23187 and X-537A. As shown above, the ionophore A23187 alone did not stimulate bone resorption, nor did it potentiate PTH-stimulated bone resorption. Similar results were obtained with X-537A. Instead, we observed a dose-dependent inhibition of stimulated bone resorption (Fig. 1). Fig. 1 also shows that A23187 was four to five times more potent than X-537A. The concentrations of ionophores used in this experiment were comparable to those used in other systems (2–5) where the ionophores were shown to mimic the action of physiologic stimuli.

Results presented in Fig. 2 show that A23187 inhibited bone resorption induced not only by PTH, but also by PGE_2, phorbol myristate acetate, IBMX, and 1α(OH)-vitamin D_3. Each of these agents stimulates bone resorption in a dose-dependent manner in this culture system.[3] The concentration of each stimulator used in these experiments was at or near the maximally active dose.

Effects of brief exposure to A23187. Since the inhibitory effects described above were the result of incubations with the ionophores for days, and the uptake of calcium ion by bone observed in vivo or in vitro is a rapid, transient effect (8–11), we have also investigated the effects of brief exposure (15, 30, and 60 min) of the bone to A23187 alone or in the presence of submaximal doses of PTH, followed by incubation in control medium or with the same dose of PTH alone. No stimulation of bone

[3] Ivey, J. L., and A. H. Tashjian, Jr. Manuscript in preparation.

TABLE III
Effect of Brief Exposures to A23187 on Bone Resorption

PTH added	Length of exposure to A23187, min	Medium calcium concentration				
		0	15	30		60
	A23187, μg/ml	0	0.5	0.5	5.0	0.5
U/ml				*mg/dl*		
0		6.8±0.41 (3)	6.6±0.36	6.7±0.36	—	6.2±0.36
0.1		11.0±0.36§				
0.02		9.0±0.50 (2)‡	8.3±0.36*	8.6±0.36‡	9.0±0.36§	9.0±0.41 (3)§

Incubations were performed in BSA medium. Bones were incubated without treatment for 24 h, the medium was removed, and fresh medium containing the indicated concentrations of PTH (0, 0.1, or 0.02 U/ml) or PTH (0 or 0.02 U/ml) + A23187 (0.5 or 5.0 μg/ml) was added. After incubation for the indicated periods of time, medium was removed from the treatment groups receiving A23187, the bones were rinsed three times with 2.0 ml of fresh control medium, and fresh medium containing the indicated concentration of PTH (0 or 0.02 U/ml) was added and the incubation continued for a total treatment period of 48 h. Treatment groups that did not receive A23187 (0 min exposure) were incubated without a second change of medium. Statistical significance from the untreated control group is indicated by: * ($P < 0.05$); ‡ ($P < 0.01$); or § ($P < 0.001$). None of the groups incubated with A23187 differ significantly from the group incubated with the same dose of PTH (0 or 0.02) in the absence of A23187. Values are means±SE of groups of two to four bones per treatment group. Values in parentheses give numbers of bones per group if less than four.

TABLE IV
Effect of Medium Calcium Concentration on Response to PTH and A23187

Treatment	Medium calcium concentration		
	Washout	24-h	48-h
		mg/dl	
Low-calcium medium (2.0 mg/dl)			
Control	4.0±0.18	3.6±0.22	3.9±0.35
PTH (0.1 U/ml)	3.9±0.18	4.2±0.22*	5.8±0.35§
A23187 (1.0 μg/ml)	3.9±0.18	2.9±0.22*	3.0±0.35
PTH + A23187	3.9±0.18	3.0±0.22*	3.2±0.35
Intermediate-calcium medium (5.5 mg/dl)			
Control	7.7±0.18	7.8±0.22	7.6±0.35
PTH (0.1 U/ml)	7.8±0.18	9.9±0.22§	12.8±0.35§
High-calcium medium (10.0 mg/dl)			
Control	11.2±0.18	12.0±0.22	11.8±0.35
PTH (0.1 U/ml)	11.7±0.18*	13.2±0.22§	15.2±0.35§
A23187 (1.0 μg/ml)	11.3±0.18	11.4±0.22*	11.4±0.35
PTH + A23187	11.4±0.18	11.2±0.22‡	11.3±0.35

Incubations were performed as described in Methods in BSA medium used without adjustment (intermediate calcium), or adjusted by the addition of EGTA (low-calcium) or $CaCl_2$ (high-calcium). The concentrations in parentheses indicate the calcium concentrations measured in the unincubated media. The mean calcium concentrations listed as washout were obtained 24 h after the bones had been placed in culture. PTH, A23187, or PTH + A23187 (at the same concentrations used for each compound alone) were added to fresh medium at the end of the 24-h washout period. A23187 and PTH + A23187 treatment groups were not included in the intermediate calcium medium. Statistical significance from the proper control group is indicated as: * $P < 0.05$, ‡ $P < 0.01$, and § $P < 0.001$. Values are means ±SE of groups of four bones per treatment group.

resorption was observed with A23187 alone, nor was the extent of resorption observed with submaximal doses of PTH enhanced by A23187 (Table III). Significant inhibition of the PTH-stimulated resorption was not observed in these short-term exposures to ionophore, due to reversibility of the inhibition as described below.

Medium calcium concentration and inhibition by the ionophores. Since the ionophores were being used to evaluate the role of calcium in the initiation of PTH-stimulated bone resorption, we investigated the effect of medium calcium concentration on the observed inhibition of resorption by the ionophores. Free calcium ion in the medium was complexed with EGTA to decrease the available calcium ion activity, or the available calcium ion activity was increased by the addition of $CaCl_2$. The results of experiments performed with these modified media are shown in Table IV. The inhibitory effect of A23187 on PTH-stimulated bone resorption was observed in both of the modified media, indicating that the observed inhibition was not critically dependent on the medium calcium concentration.

Reversal of inhibition by the ionophores. To rule out the possibility that the observed inhibition of bone resorption was due to irreversible damage to the bone at the ionophore concentrations used, experiments were performed to determine the reversibility of the ionophore effect. Incubations were performed in the presence of PTH or A23187 plus PTH, the medium was then removed, the bones were rinsed thoroughly, medium containing the

second treatment (PTH or A23187 plus PTH) was added, and the incubation was continued. The results show that the inhibitory effect of A23187 was reversible even after 24 h of treatment (Fig. 3). Similar results were also obtained after 5 h of treatment with A23187 and in similar experiments with X-537A (data not shown). These findings indicate that irreversible damage to the bones is not responsible for the effects observed with the ionophores.

Effects of A23187 on bone resorption as determined by three different methods. Because A23187 and X-537A were selected for these studies on the basis of their ability to act as calcium ionophores, it was necessary to demonstrate that the results were not due to an effect on bulk calcium equilibrium between bone and medium, unrelated to bone resorption. Experiments were performed to assess bone resorption by three independent methods. The experiments were performed with calvaria labeled in vivo with ^{45}Ca, as described in Methods. The extent of resorption was determined by: (*a*) measurement of total medium calcium concentration (the method used in the other experiments we have described); (*b*) release of ^{45}Ca into the medium; and (*c*) morphological scoring of the extent of resorption. The results obtained after 48 h of treatment are given in Table V. The spontaneous basal resorption observed in this experiment (and several others performed at the same time) was somewhat greater than is usually observed, and, in this particular experiment, the presence of basal resorption provided additional confirmation of several points made previously. The presence of PTH at 0.1 U/ml produced a significant increase in total medium calcium, ^{45}Ca release, and resorption score above the control values for basal resorption, indicating that the spontaneous resorption was not maximal. Nevertheless, low concentrations of A23187 alone did not enhance basal resorption; instead, significant inhibition of basal resorption was observed at 0.3 and 1.0 μg/ml A23187. Bone resorption stimulated by PTH was also inhibited by A23187 in a dose-dependent manner.

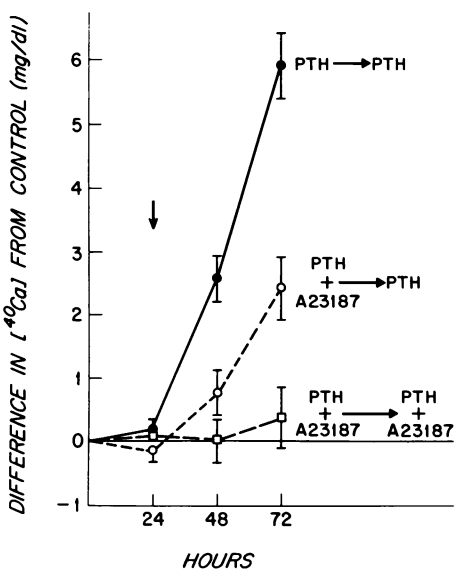

FIGURE 3 Reversal of the inhibition of bone resorption by A23187 after removal. Calvaria were placed in organ culture as described in Methods; however, the BSA medium added to the treatment groups at zero time contained either PTH (0.1 U/ml) (●); or PTH (0.1 U/ml) + A23187 (1.0 μg/ml) (○, □). After 24 h of incubation, indicated by the vertical arrow, medium was removed from all groups, and the bones were rinsed three times each with 2.0 ml of fresh control medium for a total of 5 min. Fresh medium containing either 0.1 U/ml PTH (●, ○), or 0.1 U/ml PTH + 1.0 μg/ml A23187 (□) was then added to the treatment groups and incubation continued an additional 48 h. Data are presented as mean differences in medium calcium concentration from the untreated control group. The vertical bars give the SE of the mean differences of four bones per group. Mean calcium concentrations (in mg/dl) for the control group were: 24 h, 7.3±0.12; 48 h, 7.0±0.24; and 72 h, 6.8±0.34.

TABLE V
A23187 and Bone Resorption Determined by Three Methods

Treatment	Total medium [^{40}Ca]	Morphological score	^{45}Ca released
	mg/dl	*out of 14*	*cpm/100 μl* $\times 10^{-3}$
Control	10.8±0.47	4.6±0.63	5.1±0.37
A23187 (0.1 μg/ml)	11.4±0.47	4.8±0.63	5.6±0.37
A23187 (0.3 μg/ml)	9.4±0.47*	1.3±0.63‡	3.9±0.37*
A23187 (1.0 μg/ml)	8.3±0.47§	1.2±0.63‡	3.2±0.37§
PTH (0.1 U/ml)	14.5±0.47§	7.0±0.63*	7.3±0.37§
PTH + A23187 (0.1 μg/ml)	13.8±0.47§	6.5±0.63*	7.3±0.37§
PTH + A23187 (0.3 μg/ml)	10.5±0.47	3.0±0.63	4.4±0.37
PTH + A23187 (1.0 μg/ml)	8.4±0.47‡	1.3±0.63‡	3.4±0.37‡

Calvaria were labeled with ^{45}Ca in vivo and incubations were performed in BSA medium. All bones were incubated for 24 h in control medium and the medium was then replaced with fresh medium containing the indicated treatments. Incubations were continued for an additional 48 h. Morphological scores are given as means of raw scores, with a maximum possible score of 14 indicating total resorption. Treatment means significantly different from the untreated control group are indicated by: * $P < 0.05$; ‡ $P < 0.01$; and § $P < 0.001$. Values are mean±SE for groups of four bones.

The responses observed with each of the three methods for determining the extent of bone resorption were in excellent agreement. The correlation coefficients obtained when the mean values for total medium calcium were compared to the mean values for ^{45}Ca release and morphological scoring were 0.99 and 0.97, respectively, both values indicating a high degree of correlation not expected by chance alone ($P < 10^{-5}$). The extent of correlation among these three methods of assessing bone resorption observed in this experiment agrees well with previous results obtained in experiments not involving A23187 and X-537A (unpublished observations). Thus, we

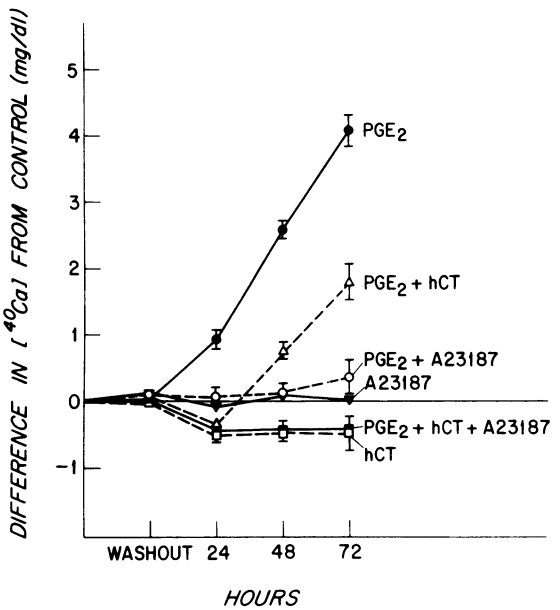

FIGURE 4 Comparison of the effects of calcitonin and A23187 on bone resorption stimulated by PGE_2. Data are plotted as mean differences from control, and the vertical bars are SE of the mean difference for groups of four bones. All groups were incubated with control heat-inactivated serum medium for the first 24 h (indicated as washout), and the medium was replaced with fresh medium containing the indicated treatments and incubated for an additional 72 h. Aliquots of medium (0.2 ml) were removed at 24-h intervals for determination of medium calcium concentration. The treatment groups contained: PGE_2 (0.1 μg/ml) (●); A23187 (1.0 μg/ml) (▼); human calcitonin (hCT) (0.1 μg/ml) (□); PGE_2 (0.1 μg/ml) + A23187 (1.0 μg/ml) (○); PGE_2 (0.1 μg/ml) + hCT (0.1 μg/ml) (△); and PGE_2 (0.1 μg/ml) + A23187 (1.0 μg/ml) + hCT (0.1 μg/ml) (■). Medium calcium concentrations for controls were: washout, 7.2±0.09 mg/dl; 24 h, 7.3±0.09 mg/dl; 48 h, 7.2±0.12 mg/dl; and 72 h, 7.0±0.17 mg/dl.

conclude that the inhibition of stimulated bone resorption observed with A23187 and X-537A is not due to an effect on bulk calcium equilibrium between bone and medium unrelated to bone resorption, but rather is fundamentally related to the resorption process itself.

Comparison of inhibition of bone resorption by A23187 and calcitonin. The experiment shown in Fig. 4 compares the bone resorption-inhibitory action of A23187 with that of calcitonin, a physiologic inhibitor of bone resorption. It can be seen that the inhibition produced by A23187 persisted as long as the ionophore was present. However, calcitonin prevented bone resorption for approximately 24 h, after which resorption resumed, despite the continued presence of the hormone, and proceeded at a rate similar to that of bones exposed to PGE_2 alone. This phenomenon has been observed previously by Wener et al. and has been termed "escape" (23). In other experiments (data not shown), the inhibitory effect of A23187 persisted for as long as 96 h in the continued presence of the ionophore. Furthermore, calcitonin consistently produced a small but significant reduction in medium calcium concentration, due to net calcium uptake by the bones, whereas this effect was not seen with the ionophores. Lastly, when calcitonin and A23187 were tested together, the ionophore did not prevent the uptake of calcium due to calcitonin but did prevent escape.

Effect of late addition of the ionophores. Further experiments were performed to determine whether the ionophores inhibited PTH-stimulated resorption only when added together with the hormone, which might be the case if an early event in the stimulation of resorption, such as PTH-induced RNA or protein synthesis, was inhibited by the ionophores, or whether the ionophores could also inhibit after stimulation of resorption had been initiated. The data shown in Fig. 5A were obtained with heat-inactivated serum medium, and indicate that although calcitonin produced a complete, but transient, inhibition of bone resorption, the ionophores, at concentrations producing complete inhibition when added at the same time as PTH (Figs. 1 and 2), or PGE_2 (Fig. 4) reduced the rate of resorption only slightly when added 24 h after PTH. When this same experiment was performed in BSA medium, however, both calcitonin and the ionophores completely inhibited the resorption (Fig. 5B). This difference in the effects of the ionophores and calcitonin in the two media was repeated with identical results. It was also observed in experiments using different lots of the two media. At the present time the basis for this differential effect of the media with respect to the action of the ionophores is not known. Unfortunately, the finding of both of the possible results complicates the interpretation of the experiment. Since complete inhibition of previously stimulated resorption is obtained in the BSA medium, it appears that the ionophores may act after the initial events involved in the stimulation of bone resorption. This interpretation must be considered tentative, however, until the differential effects of the two media are understood.

Effects on cyclic AMP accumulation. Another possible site of action for the inhibitory effect of the ionophores was the adenylate cyclase-cyclic AMP system, which is stimulated by PTH (24) and inhibited in membrane preparations by elevated calcium ion concentrations (25). Measurements of cyclic AMP extracted from calvaria appeared to overestimate the amount of cyclic AMP present, for after phosphodiesterase treatment of the samples, significant inhibition of labeled cyclic AMP binding, which would be measured as cyclic AMP, persisted. Parallel

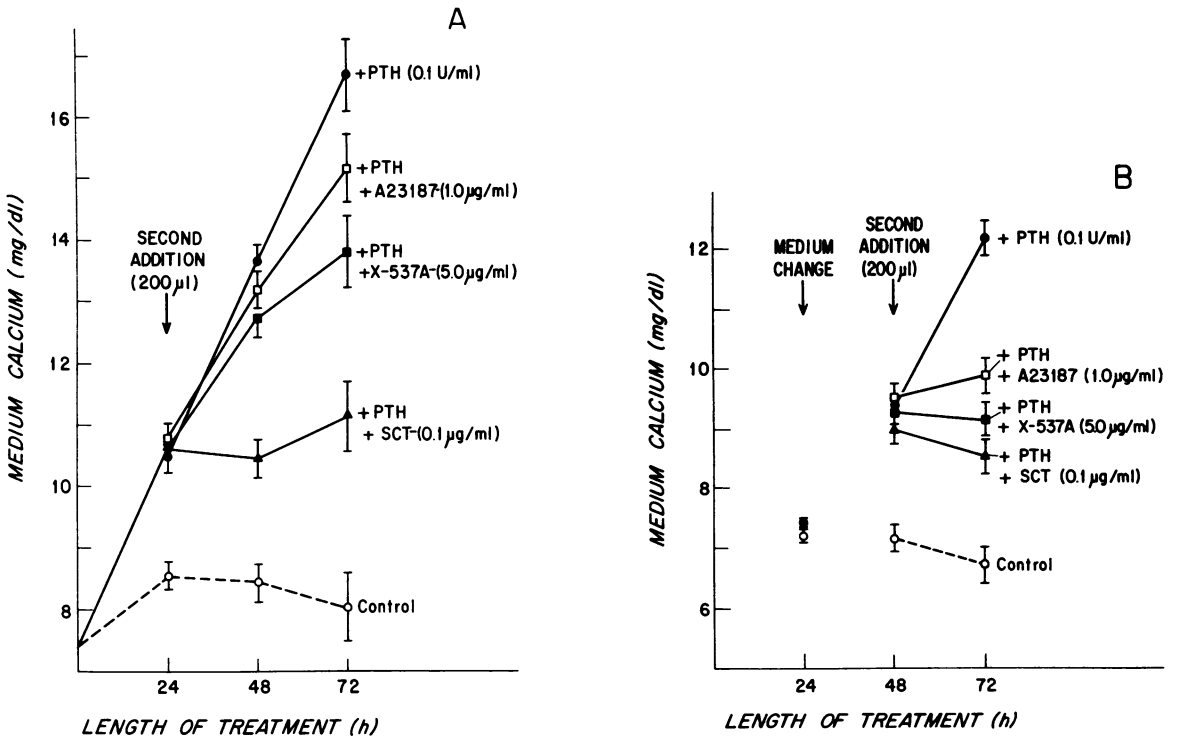

FIGURE 5 Effects of calcitonin and the ionophores after initiation of PTH-stimulated bone resorption. Calvaria were placed in culture in heat-inactivated serum medium (A) or BSA medium (B) as described in Methods. All groups, except control, received medium containing PTH (0.1 U/ml) immediately. In experiment A, after 24 h of incubation, a 200-μl aliquot of medium was removed for calcium determination, and 200 μl of control medium was added to the control group (○), and the group labeled + PTH (●); to the other groups were added 200 μl of medium containing the indicated additions at 10 times their final concentrations, so that the final concentrations were those indicated in the figure: A23187 (1.0 μg/ml) (□); X-537A (5.0 μg/ml) (■); and salmon calcitonin (SCT) (0.1 μg/ml (▲). Incubation was continued for an additional 48 h. In exp. B, the medium was replaced after 24 h with fresh control medium (control) or fresh medium containing PTH (0.1 U/ml). After incubation for an additional 24 h, a 200-μl aliquot was removed and 200 μl additional BSA medium was added as in experiment A. Incubation was continued for an additional 24 h. The additional 24-h incubation with PTH alone in exp. B was used to insure a high rate of resorption at the time the inhibitors were added. Data are plotted as mean medium calcium concentrations (not mean differences), the vertical bars give the SE for groups of four bones. The intercept on the ordinate in A indicates the calcium concentration of the unincubated medium; this value was not determined for the experiment in B.

incubations of authentic cyclic AMP with phosphodiesterase demonstrated that the nucleotide was completely destroyed by this treatment. A similar observation has been reported by Nagata et al. (26). We have found, however, that during incubation cyclic AMP accumulates in the medium and reflects indirectly amounts of cyclic AMP in the calvaria,[4] in a manner similar to that described by Clark et al. (27) with astrocytoma cells in culture. Thus, values obtained by determining accumulated cyclic AMP in the medium after a 20-min incubation in the presence and absence of hormones and/or A23187 are shown in Fig. 6. Similar data have also been obtained after 10-min incubations. In addition, the qualitative changes in "apparent" cyclic AMP extracted from the bone are similar when increases from control are compared. These results show no effect of A23187 on basal control cyclic AMP levels or on the levels obtained on stimulation with PTH or calcitonin.

DISCUSSION

The studies reported were initiated to investigate the effects of the divalent cation ionophores A23187 and X-537A on bone resorption. The results presented indicate that the two ionophores used do not

[4] Tashjian, A. H., Jr., and J. E. Tice. Unpublished observations.

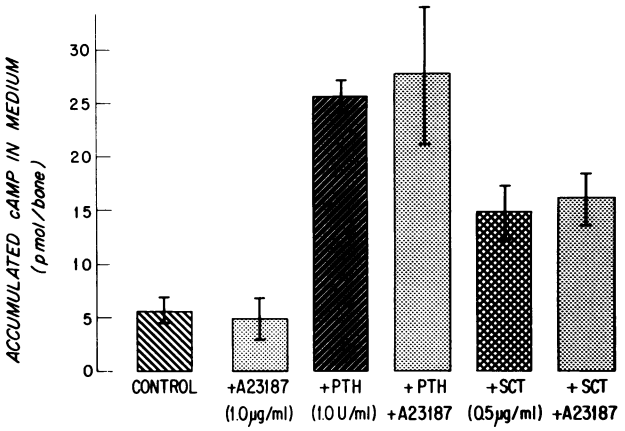

FIGURE 6 Lack of effect of A23187 on basal and stimulated cyclic AMP accumulation in bone culture medium. Incubations were performed as described in Methods for 20 min after addition of the indicated treatment and accumulated cyclic AMP in the medium was measured. SCT denotes salmon calcitonin, and combinations contained both compounds at the same concentrations used individually. The vertical bars indicate ranges for two or three separate incubations.

stimulate bone resorption, nor do they potentiate the action of PTH. Unlike many studies in which A23187 or X-537A were used to study the role of calcium in physiological processes, the results obtained do not agree with those expected if calcium played a major role in the stimulation of bone resorption. If A23187 and X-537A increase intracellular calcium concentration by enhancing calcium transport across membranes, as is generally believed (2–7), calcium uptake alone appears insufficient to initiate bone resorption, since exposure to either A23187 or X-537A alone for brief (15–60 min) or extended (1–3 day) periods did not stimulate resorption. Our results also suggest that the magnitude of the calcium uptake does not determine the magnitude of the stimulation of bone resorption, since even brief exposures (15–60 min) to A23187, comparable to the length of the calcium uptake observed in vivo (8, 9) and in vitro (10), did not increase the bone resorption obtained with submaximal concentrations of PTH. At the present time it is not known whether the initial uptake of calcium by bone is an absolute requirement for the stimulation of bone resorption by PTH.

Unexpectedly, we have found that these ionophores are potent inhibitors of bone resorption stimulated by a wide variety of compounds. The results presented suggest that the ionophores inhibit bone resorption by acting at a locus distal to the initial site of hormone binding to receptor and the stimulation of cyclic AMP accumulation, and common to all of the agents tested. In several respects the inhibition of bone resorption by the ionophores is similar to the effect of diphenylhydantoin, reported to inhibit PTH- and 25-hydroxycholecalciferol-stimulated bone resorption without affecting the action of PTH on cyclic AMP accumulation (28).

The inhibition of bone resorption by the ionophores differs from that of calcitonin: (*a*) by not exhibiting the escape from inhibition characteristic of calcitonin; (*b*) by not stimulating cyclic AMP accumulation in bone; (*c*) by not causing a measurable net movement of calcium into bone; and (*d*) by failing to inhibit completely bone resorption previously stimulated by PTH in heat-inactivated serum medium (although inhibition was complete in BSA medium).

The data presented in Table V show that the effects of the ionophores are not due to actions on bulk calcium equilibrium between bone and medium unrelated to bone resorption, since three independent methods for assessing the extent of bone resorption all showed inhibition of bone resorption by A23187. Other studies (2–5) that employed divalent cation ionophores for investigating the role of calcium ion in various physiological events have used incubations from minutes up to 2 h, while the inhibitory effects observed in the present investigation occur over 1–3 days. However, the lack of significant stimulation or potentiation of bone resorption by the ionophores was not due solely to the length of incubation with the ionophores, since brief (15–60 min) exposure to the ionophores either with or without submaximal doses of PTH also had no effect on the extent of resorption observed.

A23187 and X-537A were used in these studies because of their ability to bind and transport calcium. However, because both ionophores have been shown to transport monovalent cations as well, it is possible that the effect observed is due to the movement of ions other than calcium (7, 29–33). Further studies will be required to investigate this possibility.

Contrary to the results presented here, Dziak and Stern (34) have reported that A23187 at concentrations of 0.1, 0.3, and 1.0 μg/ml increased release of ^{45}Ca from prelabeled fetal rat long bones in culture, which they interpret as evidence for stimulation of bone resorption. Higher concentrations (3.0 and 10.0 μg/ml) had no effect. Because of this report we have retested the same lot of A23187 used in their studies at the same concentrations and have observed consistent, dose-related inhibition of bone resorption by all three measurement techniques. Thus at present we have no explanation for the discrepancy in results.

From the data presented in this communication, we conclude that the ionophores A23187 and X-537A do not stimulate bone resorption or potentiate the effects

of PTH or other stimulators of bone resorption in cultured mouse calvaria. At no concentration of the ionophores have we observed a consistent stimulation of bone resorption under a variety of experimental conditions. We have consistently found inhibition of bone resorption stimulated by a wide variety of compounds. The mechanism by which the ionophores inhibit bone resorption in a reversible, dose-dependent manner is not known at present, but we believe that these compounds may be useful tools for investigating bone metabolism.

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