

Cardiotonic Agent Milrinone Stimulates Resorption in Rodent Bone Organ Culture

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Abstract

The cardiotonic agent amrinone inhibits bone resorption *in vitro*. Milrinone, an amrinone analog, is a more potent cardiotonic agent with lower toxicity. In contrast to amrinone, milrinone stimulated resorption in cultures of neonatal mouse calvaria and fetal rat limb bones. Threshold doses were 0.1 μ M in calvaria and 0.1 mM in limb bones; maximal stimulation occurred in calvaria at 0.1 mM. Maximal responses to milrinone and parathyroid hormone were comparable. Milrinone concentrations below 0.1 mM did not affect calvarial cyclic AMP. 0.5 μ M indomethacin inhibited milrinone effects in calvaria but usually not in limb bones. 3 nM calcitonin inhibited milrinone-stimulated resorption and there was no escape from this inhibition. Structural homology between milrinone and thyroxine has been reported. We find similarities between milrinone and thyroxine actions on bone, because prostaglandin production was crucial for the effects of both agents in calvaria but not in limb bones, and neither agent exhibited escape from calcitonin inhibition.

Introduction

We have previously shown that the cardiotonic agent amrinone (5-amino-3,4'-bipyridin-6[1H]-one) inhibits stimulated Ca release from neonatal mouse calvaria and fetal rat limb bones in culture (1). The mechanism of this effect is uncertain, although amrinone may be directly affecting a Na-Ca exchange mechanism or some other Ca transport system in bone (2). Amrinone inhibited macromolecular synthesis in bone tissue, which could explain some but not all of the observed effects (2). Milrinone (2-methyl-5-cyano-[3,4'-bipyridine]-6[1H]-one) is an analog of amrinone that differs only in the addition of a methyl group and the replacement of a cyano for an amino group on one pyridine ring of the drug (3). Milrinone has been reported to be a more potent cardiotonic agent with lower toxicity than amrinone. This raised the possibility that milrinone would be a better agent with which to study the mechanism of action of this group of cardiotonic agents on bone. Our results revealed unexpected and striking differences in the action of these two congeners on bone resorption.

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Methods

Organ culture of bone. Neonatal mouse calvaria (4-6 d) were incubated in culture as described previously (4, 5). The medium was Dulbecco's modified Eagle's medium (DMEM;¹ M. A. Bioproducts, Walkersville, MD) supplemented with 15% heat-inactivated (56°C, 60 min) horse serum (Gibco Laboratories, Lawrence, MA). Treatment with hormones and drugs began at time 0 unless otherwise indicated. Medium was changed at least once during each experiment, and fresh treatments were added. Bone resorption was determined by measuring the accumulation of ⁴⁵Ca in the medium at designated intervals.

Fetal rat limb bones were obtained from 19-d fetal Sprague-Dawley rats (Holtzman Co., Madison, WI) after injection of the mothers subcutaneously on the 18th day of gestation with ⁴⁵CaCl₂. Limbs were cultured in BGJ₆ medium (Gibco Laboratories) supplemented with 1 mg/ml bovine serum albumin in stationary culture dishes as described previously (4).

Measurement of Ca. The concentration of total Ca in culture medium from neonatal mouse calvaria was measured by automatic fluorometric titration (Calcette; Precision Systems, Inc., Natick, MA). Limb bones were extracted with 0.5 ml of 0.1 N HCl at the end of each experiment. Aliquots of culture medium and extract were analyzed for ⁴⁵Ca radioactivity by liquid scintillation spectrometry (model 7500; Beckman Instruments, Inc., Fullerton, CA).

β -Glucuronidase activity. Enzyme activity was determined colorimetrically with phenolphthalein glucuronidate as a substrate (Sigma Chemical Co., St. Louis, MO). Medium aliquots were incubated with substrate in a phosphate buffer at 37°C for 72 h. The reaction was stopped by adding cold glycine buffer and the optical density at 540 nm was assayed.

Assay of effects on protein synthesis. Mouse calvaria were cultured as described above. ³H-leucine was added to each tube at a final concentration of 5 μ Ci/ml during the last hour of culture. At the end of the pulse label, cultures were put on ice. Calvaria were removed, washed in ice cold normal saline and then homogenized in 10% TCA + 1 mg/ml unlabeled leucine. Aliquots were removed for total counts and assay of protein content. The remaining homogenate was filtered on glass fiber filters, washed three times with ice cold 10% TCA and once with cold 95% ethanol. Filters were dried overnight and then counted in a liquid scintillation counter. TCA precipitable counts were taken as a measure of ³H-leucine incorporated into protein. The difference between total counts and TCA precipitable counts (soluble counts) provided a measure of the uptake of ³H-leucine. Results were normalized based on the amount of protein per bone. Total protein was assayed by the method of Lowry et al. (6).

Cyclic AMP assay. Freshly dissected calvaria were placed in DMEM (1 bone/0.45 ml) without protein supplementation. After a 20-min preincubation at 37°C, 50 μ l of control or treatment medium was added and the incubation was continued for 20 min. The calvaria were then rapidly removed and each bone was placed in 0.7 ml 90% propanol and extracted as described previously (1). A trace amount of ³H-cyclic AMP (cAMP) was added to each extract to determine recoveries. Medium samples from the original incubation were placed in a boiling water bath

1. Abbreviations used in this paper: CT, calcitonin; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; DMEM, Dulbecco's modified Eagle's medium; PTH, parathyroid hormone.

for 5 min immediately after removal of the calvaria. Samples were stored at -70°C until assayed by radioimmunoassay with a kit purchased from New England Nuclear, Boston, MA.

Statistical method. Analysis of variance was performed on data obtained at each sampling interval (7). A square root transformation of the data for the cAMP assay was done before the analysis of variance.

Materials. Parathyroid hormone (PTH) was used in the form of U. S. Pharmacopeia units (USP) parathyroid injection (100 USP/ml; Eli Lilly & Co., Indianapolis, IN) or synthetic bovine PTH (1-34) (Bachem, Inc., Torrance, CA). Amrinone and milrinone were generously provided by A. E. Soria of Sterling-Winthrop Research Institute, (Rensselaer, NY). Thyroxine (lot #982113) was obtained from Sanabo, Vienna, Austria. Synthetic salmon calcitonin was a gift from Dr. James Bastian of Armour Pharmaceutical Co., Tarrytown, NY). L-4,5- ^3H (N)-leucine, 60 Ci/mmol was obtained from New England Nuclear.

Results

In contrast to our previous findings with amrinone, milrinone did not inhibit PTH-stimulated bone resorption at concentrations up to 1 mM (data not shown). The preparation of PTH (extract or synthetic fragment) used made no difference. As illustrated in Fig. 1, milrinone alone stimulated Ca release from both neonatal mouse calvaria and fetal rat limb bones in culture. The drug was more potent in the calvarial system, in which significant stimulation of resorption was observed at 10^{-7} M milrinone and maximal stimulation occurred at 10^{-4} M. No significant effects of milrinone were observed at concentrations below 10^{-7} M (data not shown). In limb bones significant stimulation was not observed until 10^{-4} M milrinone was used. Even at effective concentrations there was striking variability in the response of individual limb bones to this agent.

Both amrinone and milrinone have been shown to alter cAMP metabolism in cardiac tissue through inhibition of phosphodiesterase activity and this effect has been implicated in the mechanism of their cardiotoxic action (8). In addition, cAMP

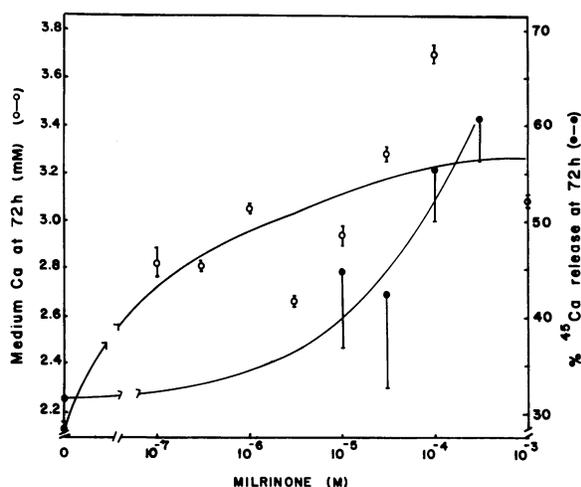


Figure 1. Dose dependence of milrinone-stimulated Ca release in neonatal mouse calvaria and fetal rat limb bones. Bones were cultured with increasing concentrations of milrinone as described in Methods. Medium Ca concentration after 72 h was assayed for neonatal mouse calvaria cultures (open circle) or % total ^{45}Ca release after 72 h was assayed for fetal rat limb bones (solid circle). Data are the mean \pm SE for groups of four bones each. Curve fitting was done with a computer-derived best fit from a nonlinear regression of the data.

has been suggested as a second messenger in the mechanism of action of PTH effects on bone (9). Therefore, we examined the ability of milrinone to stimulate cAMP accumulation in bone. The results indicate that milrinone does significantly increase the acute production of cAMP in the calvaria but only at 10^{-4} M (Table I). This was in contrast to our previous finding that amrinone had no effect on cAMP metabolism in bone (1).

When the interaction between milrinone and amrinone was tested (Table II), milrinone-stimulated Ca release in calvaria was completely inhibited by concurrent treatment with 0.2 mM amrinone, a concentration shown previously to be maximally effective against other known stimulators of bone resorption such as PTH and 1,25-dihydroxyvitamin D_3 , (1,25-[OH] $_2\text{D}_3$) (1). This inhibitory effect of amrinone was observed either after continuous treatment with both drugs (as in Table II) or after a 6-h pretreatment with amrinone alone before subsequent addition of milrinone (Fig. 2). When milrinone was present together with amrinone during the 6-h pretreatment, a significant resorptive response to milrinone was then seen after the amrinone was washed out.

The effect of milrinone on protein synthesis in bone was examined to further compare its effects with those of amrinone. As illustrated in Table III, a maximally effective concentration of milrinone had no significant effect on total protein synthesis in the calvaria after 24-h treatment in culture. This was in contrast to the 54% inhibition of protein synthesis by amrinone under the same conditions. A similar lack of effect of milrinone on protein synthesis has been observed after 72-h treatment of calvaria with the drug (data not shown).

Further characterization of milrinone-stimulated bone resorption is presented in Table IV. Lysosomal enzyme release has been found to be associated with hormonal stimulation of Ca release from bone in vitro (10). Using β -glucuronidase activity as a measure of lysosomal enzyme release, it can be seen that stimulation of Ca release by milrinone was paralleled by a stimulation of β -glucuronidase activity. Calcitonin (CT) completely inhibited both milrinone-stimulated Ca release and β -glucuronidase activity. Interestingly, there was no escape from the inhibitory effect of CT on either parameter. This was in contrast to the expected loss of inhibition by CT of PTH-stimulated resorption and β -glucuronidase activity after an initial inhibitory effect. This difference between the time course of CT-milrinone interaction and PTH plus CT is further illustrated in Fig. 3. The effects of both stimulatory agents were prevented by 24-h treat-

Table I. Acute Effect of Milrinone on Cyclic AMP Production in Calvaria

Treatment	Medium cAMP pmol/bone*	Intracellular cAMP pmol/bone*
Control	2.79 \pm 0.98	11.76 \pm 2.35
Milrinone (10^{-6} M)	2.52 \pm 0.37	14.92 \pm 1.83
Milrinone (10^{-5} M)	3.23 \pm 0.76	13.96 \pm 2.47
Milrinone (10^{-4} M)	4.85 \pm 1.17 [†]	25.28 \pm 7.45 [†]

Calvaria were treated for 20 min at 37°C as indicated. Total medium and intracellular concentrations of cAMP were measured as described in Methods.

* Data are the means \pm SD for groups of four bones.

[†] $P < 0.01$ compared with control.

Table II. Inhibition by Amrinone of Milrinone-stimulated Resorption in Neonatal Mouse Calvaria

Treatment	Medium Ca at 72 h*
	mM
Control	2.47±0.34
Milrinone (10 ⁻⁵ M)	3.62±0.69 [‡]
Amrinone (2 × 10 ⁻⁴ M)	1.59±0.04 [§]
Milrinone + amrinone	1.59±0.03 [§]

Calvaria were treated continuously as indicated for 72 h with a medium change at 24 h.

* Data are the means±SD for groups of four bones each.

[‡] P < 0.001 compared to controls.

[§] P < 0.01.

^{||} P < 0.001 compared to milrinone alone.

ment with CT. By 72 h the rate of PTH-stimulated resorption had recovered despite the continued presence of CT (escape) whereas the effect of milrinone was still completely inhibited.

A recent report described structural homology between thyroxine and milrinone that was not shared by amrinone (11). Because thyroxine has been shown to stimulate bone resorption in vitro (12–14), comparisons were made between the effects of thyroxine and milrinone in the calvaria system. The time course of stimulated Ca release was similar for maximally effective concentrations of both agents (Fig. 4). In addition, CT completely inhibited resorption stimulated by both milrinone and thyroxine with no apparent escape from this inhibition over the 72 h of the experiment. In the calvaria, thyroxine-stimulated Ca release appears to be mediated by endogenous prostaglandin production (14). Therefore the ability of indomethacin, a specific prostaglandin synthesis inhibitor, to block the effects of milrinone and thyroxine in this system was examined. Indomethacin completely blocked both thyroxine and milrinone-stimulated Ca release in the calvaria, whereas it had no significant effect on milrinone-stimulated resorption in the limb bones in this experiment (Table

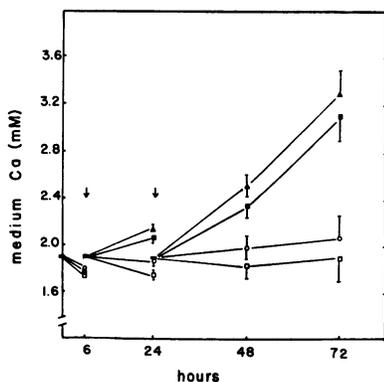


Figure 2. Interaction of milrinone and amrinone in neonatal mouse calvaria. Calvaria were incubated in control medium (open circle) or in the presence of 2 × 10⁻⁴ M amrinone alone (open square) or with amrinone plus 10⁻⁵ M milrinone (solid square). After 6 h, half the control bones (solid triangle) and the bones pretreated with amrinone

or amrinone plus milrinone were washed and the medium was replaced with medium containing 10⁻⁵ M milrinone alone for the remainder of the experiment. At 24 h, medium was changed on all calvaria and fresh treatments were added. (Arrows) indicate the medium changes. (Solid rectangle) indicates the calcium concentration of unin-cubated medium. Data are the mean±SE for groups of four bones.

Table III. Milrinone and Amrinone Effects on Protein Synthesis in Neonatal Mouse Calvaria

Treatment	³ H-Leucine incorporation*	
	TCA precipitable	Soluble
	cpm/mg protein	cpm/mg protein
Control	6820±306	24745±6314
Milrinone (10 ⁻⁴ M)	7024±1371	21643±2065
Amrinone (2 × 10 ⁻⁴ M)	3169±633 [‡]	25574±4126

Calvaria were incubated for 24 h with the appropriate treatment.

* Incorporation of ³H-leucine after a 1-h labeling of the calvaria was assayed as described in Methods. Values are the mean±SD for groups of four bones each.

[‡] P < 0.001 compared with control.

V). Inhibition by indomethacin was seen in only two of eight experiments in limb bones.

Discussion

The results presented here demonstrate that milrinone stimulates Ca release from bone in vitro in a dose-dependent manner. Maximal effects of milrinone were comparable to those of PTH and 1,25-(OH)₂D₃ with respect to the degree of resorption, time course, and effects on lysosomal enzyme release. The stimulatory effect of milrinone was in marked contrast to the predominantly inhibitory effect of amrinone on bone in culture, despite the fact that these two drugs are closely related analogs with comparable cardiotoxic effects. A slight stimulation of basal Ca release from calvaria had been observed occasionally with low concentrations of amrinone (1), but that effect was not highly reproducible. Milrinone was also different from amrinone in that it had no effect on total protein synthesis, while amrinone significantly

Table IV. Calcitonin Interactions with Milrinone on Medium Ca and Effects on β-Glucuronidase Activity

Treatment	24 h Rx		72 h Rx	
	Medium Ca	β-Glucuronidase	Medium Ca	β-Glucuronidase
Control	2.12±0.18	2.11±0.50	2.52±0.73	1.88±0.65
Milrinone (10 ⁻⁴ M)	2.43±0.16*	3.23±0.48*	3.57±0.71 [‡]	3.48±0.81*
CT (3 × 10 ⁻⁹ M)	1.70±0.05*	1.10±0.17 [§]	2.04±0.32	2.03±0.85
Milrinone + CT	1.69±0.05 [‡]	1.55±0.44 [‡]	2.20±0.29 [‡]	2.66±0.70
PTH (0.1 U/ml)	2.37±0.12 [‡]	2.85±0.86 [‡]	4.55±0.74*	4.24±0.67*
PTH + CT	1.67±0.03	1.43±0.48	4.00±0.63*	4.81±0.36*

Calvaria were treated continuously for 72 h with a medium change at 24 h. Medium Ca values are given as mM and are the mean±SD for groups of four bones each. β-Glucuronidase activity was measured as described in Methods on medium aliquots after 24 and 72 h in culture. Data are reported as μg phenolphthalein glucuronidate released per bone per hour and values are the means±SD for groups of four bones each.

* P < 0.001 compared with the respective control group.

[‡] P < 0.001 compared with milrinone alone.

[§] P < 0.05 compared with control.

^{||} P < 0.001 compared with PTH (Lilly extract) alone.

[†] P < 0.01 compared with milrinone alone.

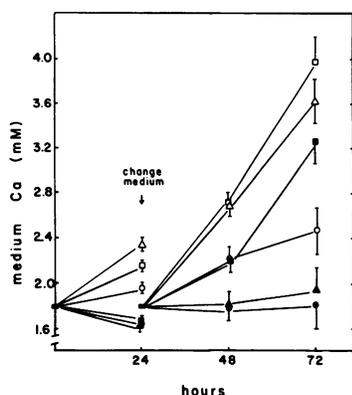


Figure 3. Time course of the effect of CT on milrinone-stimulated and PTH-stimulated resorption. Neonatal mouse calvaria were incubated in control medium (open circle) or in the presence of 0.1 U/ml PTH (Lilly extract) (open square), 10^{-5} M milrinone (open triangle), 10 ng/ml calcitonin (solid circle), PTH + CT (solid square), or milrinone + CT (solid triangle). Medium was

changed at 24 h and fresh treatments added. (Solid rectangle) indicates the calcium concentration of unincubated medium. Data are the mean \pm SE for groups of four bones.

inhibited leucine incorporation into protein in the neonatal mouse calvaria. Although it is unclear what role the effects of amrinone on macromolecular synthesis play in its action on bone, this difference between milrinone and amrinone could partially account for the lower clinical toxicity of oral milrinone compared with oral amrinone in the treatment of congestive heart failure (8).

When the interaction between milrinone and amrinone was tested, amrinone was able to completely block the stimulatory effect of milrinone. We have previously described a unique aspect

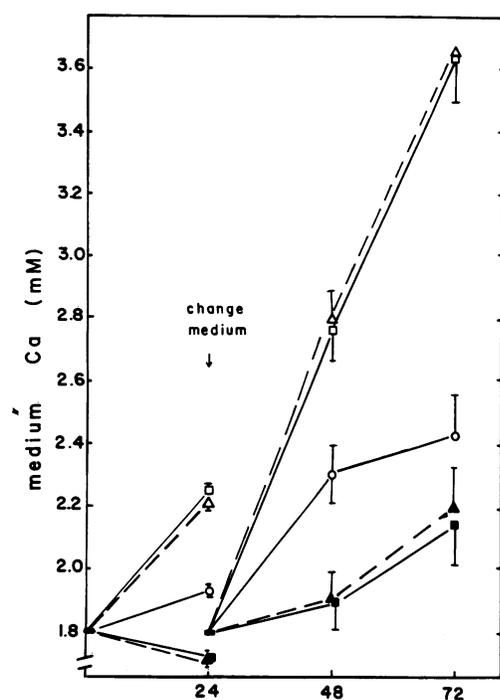


Figure 4. Time course of the effect of CT on milrinone-stimulated and thyroxine-stimulated resorption. Neonatal mouse calvaria were incubated in control medium (open circle) or in the presence of 10^{-5} M milrinone (open triangle), 10^{-6} M thyroxine (open square), milrinone + 10 ng/ml CT (solid triangle) or thyroxine + CT (solid square). (Solid rectangle) indicates the calcium concentration of unincubated medium. Data are the mean \pm SE for groups of four bones.

Table V. Similarity of Indomethacin Effects on Milrinone and Thyroxine-stimulated Resorption in Neonatal Mouse Calvaria and Fetal Rat Limb Bones

Treatment	Measure of resorption
Calvaria	Medium Ca at 72 h (mM)*
Control	2.46 \pm .35
Thyroxine (10^{-6} M)	3.64 \pm .32 [‡]
Milrinone (10^{-5} M)	3.66 \pm .23 [‡]
Indomethacin (5×10^{-7} M)	1.61 \pm .05 [§]
Thyroxine + indomethacin	1.87 \pm .24
Milrinone + indomethacin	2.28 \pm .34 [†]
Limb bones	% ⁴⁵ Ca release at 66 h**
Control	32.2 \pm 3.2
Milrinone (10^{-4} M)	72.3 \pm 9.4 [‡]
Indomethacin (5×10^{-7} M)	30.6 \pm 2.3
Milrinone + indomethacin	72.2 \pm 6.7 [‡]

Calvaria were treated continuously for 72 h with a medium change at 24 h. Limb bones were treated continuously for 66 h.

* Data are the mean \pm SD for groups of four calvaria.

[‡] $P < 0.001$ compared with controls.

[§] $P < 0.01$.

^{||} $P < 0.001$ compared with thyroxine alone.

[†] $P < 0.001$ compared with milrinone alone.

** Data are the mean \pm SD for groups of six limb bones.

of the inhibitory effect of amrinone in bone (1). Inhibition of stimulated bone resorption could be manifested either by continuous treatment with amrinone plus the stimulatory agent or by a relatively short pretreatment with amrinone alone before addition of the stimulator of bone resorption. Inhibition by 6-h pretreatment with amrinone could be prevented if the stimulatory agent was present during the pretreatment period. This pretreatment effect of amrinone was also seen with milrinone-stimulated resorption. This further suggests that the mechanism of the stimulatory effect of milrinone on bone resorption is comparable to that of the more physiologic stimulatory agents.

One other recent report of a divergence of effects of milrinone and amrinone described structural homology between milrinone and thyroxine and demonstrated a thyromimetic effect of milrinone on sarcolemma-enriched rabbit myocardial membrane Ca-ATPase activity (11). Amrinone did not show similar structural homology and had no effect on the Ca-ATPase activity. Thyroid hormones have been shown to stimulate bone resorption in vitro in both fetal rat limb bones (12, 13) and neonatal mouse calvaria (14). Thus comparisons were made between milrinone and thyroid hormone effects in the calvaria system. Our results indicate several similarities between the responses of the bones to these two agents. The time course of maximally effective concentrations of both drugs was similar. The stimulation of Ca release by both milrinone and thyroxine in calvaria was inhibited by a concentration of indomethacin that has been shown previously to completely inhibit endogenous prostaglandin production (15). In limb bones, the effects of both milrinone and thyroxine were not generally inhibited by indomethacin. Another similarity in the responses of the bones to stimulation by milrinone and thyroxine is the lack of escape from inhibition by CT. The complete inhibition by CT over 72 h seems to be unique for these stimulators. All other stimulatory agents that cause

strong resorptive responses (PTH, 1,25-(OH)₂D₃, prostaglandin E₂) exhibit escape after an initial inhibition by CT (16, 17).

The increased potency of milrinone in mouse calvaria compared with the fetal rat limb bones may be explained as a result of the effect being mediated by prostaglandin production in the calvaria. Several agents, including epidermal growth factor (18, 19), transforming growth factor (20, 21), potassium (15), and thrombin (13), whose effect on bone resorption is mediated by endogenous prostaglandin production exhibit different potencies in limb bones and calvaria.

Although milrinone is more potent than amrinone, the pharmacology of these two drugs with respect to their positive inotropic and vasodilatory effects has been quite comparable (3). The mechanism of the cardiotoxic action of these bipyridine compounds is not fully understood, but most current evidence suggests that their effects are due to inhibition of specific phosphodiesterase activity (22, 23). In some systems there are also data to support a more direct effect on Ca transport processes (22, 24). Because amrinone has no effect and only the highest concentration of milrinone tested appears to have effects on cyclic nucleotide metabolism in bone, direct effects on Ca transport may be more important in the actions on bone tissue. Because 10⁻⁴ M milrinone did significantly increase cAMP production in the calvaria, we cannot rule out a contribution of phosphodiesterase inhibition to the mechanism of milrinone-stimulated bone resorption. Alternatively, a mechanism totally unrelated to the cardiotoxic effects of these agents may be operating in our system. This is supported by the observation that changes in prostaglandin metabolism do not appear to be involved in the cardiotoxic effect of milrinone (25). The possibility of a different mechanism seems more likely, given that milrinone and amrinone have opposing effects on bone resorption. Future studies with these agents and other structural analogs could provide further insights into the mechanism of hormonal control of bone resorption.

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