

# Parathyroid Hormone Enhances Mechanically Induced Bone Formation, Possibly Involving L-Type Voltage-Sensitive Calcium Channels

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**PTH and mechanical loading might act synergistically on bone formation. We tested the *in vivo* effect of the L-type voltage-sensitive calcium channel (VSCC) blocker, verapamil, on bone formation induced by human PTH-(1–34) (PTH) injection with or without mechanical loading. Adult rats were divided into eight groups: vehicle, verapamil, PTH, or verapamil plus PTH with or without mechanical loading. Verapamil (100 mg/kg) was given orally 90 min before loading. PTH (80 µg/kg) was injected sc 30 min before loading. Loading applied to tibia and ulna for 3 min significantly increased the bone formation rate on both the endocortical surface of tibia and the periosteal surface of ulna ( $P < 0.0001$ ). Treatment with PTH enhanced**

**load-induced bone formation by 53% and 76% ( $P < 0.001$ ) on the endocortical and periosteal surfaces, respectively. Treatment with verapamil suppressed load-induced bone formation rate by 77% and 59% ( $P < 0.01$ ). Furthermore, verapamil suppressed bone formation in rats subjected to PTH plus loading by 74% and 68% ( $P < 0.0001$ ) at the tibia and ulna, respectively. In the groups without loading, neither verapamil nor PTH treatment significantly changed any bone formation parameter. This study indicates that L-type VSCCs mediate load-induced bone formation *in vivo*. Furthermore, PTH enhances load-induced bone adaptation through involvement of L-type VSCCs. (*Endocrinology* 144: 1226–1233, 2003)**

**I**NTERMITTENT administration of the human (h) PTH fragment, hPTH-(1–34) (PTH), stimulates bone formation (1–4). Mechanical loading in combination with PTH treatment has synergistic action on bone formation in rats (5–7). PTH and mechanical loading produce similar responses in osteoblasts, suggesting that one way PTH elicits an anabolic response in bone is by enhancing responsiveness to mechanical loading. An early response to both stimuli is a rapid increase in intracellular calcium ( $[Ca^{2+}]_i$ ) that is dependent on both extracellular  $Ca^{2+}$  entry and  $[Ca^{2+}]_i$  release (8–10). The early increase in  $[Ca^{2+}]_i$  has been linked to increased production of nitric oxide and prostaglandins in osteoblasts (11, 12) and up-regulation of some skeletal growth factors, including IGF-I and TGF $\beta$  (13, 14). Both nitric oxide and prostaglandins have been shown to mediate load-induced bone formation *in vivo* (15–17).

Recent cell culture studies show that PTH enhances the intracellular calcium concentration in osteoblastic cells subjected to fluid shear stress, and that the synergistic effect between PTH and fluid shear is attenuated by L-type calcium channel blockers, such as nifedipine (18, 19). These data suggest that PTH enhances fluid shear-induced calcium signaling in osteoblastic cells through activation of L-type voltage-sensitive calcium channels (VSCCs). We hypothesized that PTH affects load-induced bone formation *in vivo* via L-type VSCCs.

We have previously shown that two L-type calcium chan-

nel blockers, nifedipine and verapamil, suppress load-induced bone formation in rats (20), suggesting that L-type VSCCs play a critical role in mechanically induced bone formation *in vivo*. In the present study we tested the *in vivo* effect of the L-type VSCC blocker, verapamil, on bone formation after PTH injection and/or mechanical loading.

## Materials and Methods

### Experimental animals

A total of 64 adult female Sprague Dawley rats were used for this study. The rats were housed one per cage at the Laboratory Animal Resource Center of Indiana University School of Medicine and were fed standard rat chow and water *ad libitum*. The animals were allowed to acclimate for 2 wk before the experiment began and were approximately 7 months old at the beginning of the study. All procedures performed in this study were in accordance with the Indiana University animal care and use committee guidelines.

### Experimental design

The rats were randomly divided into eight groups ( $n = 8/\text{group}$ ): 1) vehicle treated; 2) verapamil treated; 3) PTH treated; 4) verapamil plus PTH treated; 5) vehicle plus mechanical loading; 6) verapamil plus loading; 7) PTH plus loading; and 8) verapamil, PTH, and loading (Fig. 1). For the groups without loading, one group was given vehicle treatment only. The other three groups were given verapamil orally (100 mg/kg; Sigma-Aldrich, St. Louis, MO) and/or hPTH-(1–34) sc (80 µg/kg; Bachem California, Inc., Torrance, CA) or both verapamil and PTH. The same treatments were given to the remaining four groups with loading. Verapamil and hPTH-(1–34) were given 90 and 30–40 min before loading, respectively. The doses and dosing regimens were selected based on the previous studies (5, 20).

Verapamil was dissolved in polyethylene glycol 400 (Sigma-Aldrich) to form a 10-mg/ml solution (21), stirred overnight, wrapped in aluminum foil to protect the solution from light, and stored at room temperature. The vehicle consisted of polyethylene glycol 400. The drug was

Abbreviations: BFR/BS, Bone formation rate/bone surface;  $[Ca^{2+}]_i$ , intracellular calcium; h, human; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; PLSD, protected least significant difference; r, relative; VSCC, voltage-sensitive calcium channel.

Groups	Day 1			Day 5	Day 9	Day 12
	-90 min Verapamil	-30 min PTH	0 min Loading	Calcein	Calcein	Sacrifice
Vehicle				×	×	×
Verapamil	↓			×	×	×
PTH		↓		×	×	×
Verapamil+PTH	↓	↓		×	×	×
Vehicle			×	×	×	×
Verapamil	↓		×	×	×	×
PTH		↓	×	×	×	×
Verapamil+PTH	↓	↓	×	×	×	×

FIG. 1. Overview of the experimental design. Rats were equally divided into eight groups. Half of the animals were treated with vehicle, verapamil, PTH, or verapamil plus PTH without loading. The other half received the various drug treatments and mechanical loading of the ulna and tibia. Verapamil was given 90 min before, and PTH was given 30 min before loading. All animals were labeled with injection of calcein on d 5 and 9, and were killed on d 12.

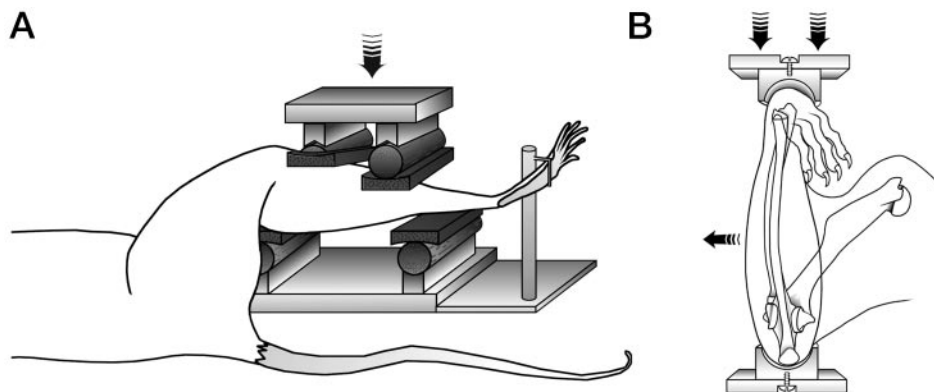


FIG. 2. A, Diagram of the rat tibia four-point bending system. The right tibia is fixed between two upper load points (11 mm apart) and two supports (23 mm apart). When force is applied, a mediolateral bending moment is produced in the central portion of the tibial shaft. This figure is reprinted from the report by Robling *et al.* (54) with permission from the publisher. B, Schematic diagram of the rat ulna loading model. The right forearm is held between upper and lower cups. The force (upper large arrows) is applied through the carpal joint and overlying soft tissues. Axial load is translated into a bending moment (small arrow) in the ulnar diaphysis due to preexisting mediolateral curvature. This figure is reprinted from the report by Robling *et al.* (55) with permission from the publisher.

administered to the rats by gavage. Oral administration of verapamil causes a peak plasma level 90 min later, coincident with loading (22). The hormone vehicle for hPTH-(1–34) was acidified saline containing 2% heat-inactivated rat sera (1). A single sc injection of hPTH-(1–34) leads to peak serum concentration at 30–60 min after administration in rats, also coincident with loading (5, 23). Normal PTH levels are restored within 4 h, and a single sc injection of PTH has no significant effect on plasma calcium levels (22). Rats from the loaded groups were subjected to four-point bending of the right tibia and axial loading of the right ulna.

**Loading protocol**

A single bout of mechanical loading was applied as a haversine wave with a frequency of 2 Hz and a duration of 3 min (360 cycles) to both right ulna and right tibia. For tibial bending, force was applied through

a four-point bending apparatus (Fig. 2A) using a load-controlled, electromagnetic loading device (24). The peak load on the tibia was 63 N. The peak compressive strains at the tibiae midshaft were approximately 3000  $\mu\epsilon$  on the lateral periosteal surface and 1700  $\mu\epsilon$  on the lateral endocortical surface (25). For axial loading on the ulna, force was applied across the flexed carpus and olecanon as described by Torrance *et al.* (26) (Fig. 2B) using a stepper motor-driven spring linkage. The peak load on the ulna was 16.5 N, resulting in a peak compressive strain of 3600  $\mu\epsilon$  on the medial surface of the ulnar midshaft (27). We chose loading protocols that would induce a similar level of new bone formation in the tibia (endocortical surface) and ulna (periosteal surface).

Before the loading session, rats were anesthetized with an ip injection of a mixed solution of ketamine hydrochloride (50 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg; The Butler Co.,

Columbus, OH). After loading bouts, rats were allowed normal cage activity.

### Bone labeling, processing, and histomorphometry

All rats were given an ip injection of a fluorochrome bone label (7 mg/kg calcein; Sigma-Aldrich) on d 4 and 9 after loading and were killed 3 d after the second label. The calcein label was administered 4 d after loading because previous experiments have shown that bone formation is initiated 96 h after mechanical loading (28). The right and left tibiae and ulnae were removed, cleaned of soft tissue, and cleaved at the distal and proximal ends to allow proper infiltration of plastic into the marrow cavity. Specimens were immersed in 10% neutral buffered formalin for 48 h to fix the tissues. The specimens were then dehydrated in graded alcohols, cleared in xylene, and embedded in methyl methacrylate. Using a diamond-embedded wire saw (Histo-saw, Delaware Diamond Knives, Wilmington, DE), three transverse thick sections (~70  $\mu$ m) were cut from the tibial diaphyses 4–8 mm proximal to the tibia-fibula junction (this region is under maximal bending during four-point loading) and from ulna diaphysis 2–3 mm distal to the ulnar midpoint, which has been shown to be most responsive to loading (29), and mounted unstained on standard microscope slides.

One slide per limb was read on an Optiphot fluorescence microscope (Nikon, Garden City, NY). Using the Bioquant digitizing system (R&M Biometrics, Nashville, TN), the following primary data were collected, respectively, from the endocortical surface of tibiae and from periosteal surfaces of ulnae at  $\times 150$  magnification: bone perimeter (B.Pm), single label perimeter (sL.Pm), double label perimeter (dL.Pm), and double label area (dL.Ar). From these primary data, the following calculations were performed: mineralizing surface [MS/bone surface (BS) = (1/2 sL.Pm + dL.Pm/B.Pm); percentage], mineral apposition rate (MAR = dL.Ar/dL.Pm $\cdot$ 4 d; microns per day), lamellar bone formation rate (BFR/BS = MAR  $\times$  MS/BS  $\times$  3.65; cubic microns per square microns per year). To examine mechanically induced bone formation, bone formation parameters from the left limb (nonloaded control) were subtracted from right limb values, producing a new set of relative values for each variable: rMS/BS, rMAR, and rBFR/BS.

### Effects of verapamil on blood pressure, serum PTH, and serum calcium

In this study the dose of verapamil administered to rats was much higher than that used in clinical applications. High doses of verapamil may cause hypotension (22) or increase PTH secretion (30, 31), which, in turn, might change the serum calcium level. We looked into these possibilities by conducting a separate experiment to study the effect of a single high dose verapamil treatment (100 mg/kg, orally) on blood pressure, serum PTH, and serum calcium in rats.

Blood pressure was measured in five rats before, 45 min after, and 90 min after a single treatment with verapamil (100 mg/kg). Systolic blood pressure (mm Hg) was measured indirectly using a tail-cuff blood pressure system (model 129, IITC Life Sciences, Woodland Hills, CA). Rats were restrained in holders and placed within a chamber maintained at 27 C. A tail cuff with photoelectric detector was placed at the base of the tail and inflated. As the cuff pressure was slowly released, the photoelectric cell detected the initiation of arterial blood flow through the tail artery, which was recorded as the systolic blood pressure. Several measurements were taken at each time point and averaged.

Blood samples were collected from five untreated rats, five verapamil-treated rats at 45 min after a dose of verapamil, and 90 min after a dose of verapamil (100 mg/kg). Blood samples were allowed to clot at room temperature and then were centrifuged at 2000 rpm for 15 min. Sera were separated from those blood samples for measurements of serum PTH and serum calcium. Serum PTH (picograms per milliliter) was measured using a rat PTH immunoradiometric assay kit (Immunotopics, San Clemente, CA). Serum calcium (milligrams per deciliter) was measured using a Roche Cobas Mira Analyzer (GMI, Inc., Albertville, MN).

### Statistical analysis

The data are expressed as the mean  $\pm$  SEM. Bartlett's test (a test of the homogeneity of variances among groups) indicated that group variances were equal ( $P > 0.5$ ), suggesting that parametric statistical tests were valid. Differences between the loaded (right) and nonloaded (left) limbs were tested using paired  $t$  tests. Differences among group means were tested for significance by ANOVA, followed by Fisher's protected least

**TABLE 1.** Measurements of endocortical bone formation at tibia

Groups	Load	n	MAR ( $\mu$ m/d)		MS/BS (%)		BFR/BS ( $\mu$ m <sup>3</sup> / $\mu$ m <sup>2</sup> ·yr)	
			Mean $\pm$ SEM	$P$ value <sup>a</sup>	Mean $\pm$ SEM	$P$ value <sup>a</sup>	Mean $\pm$ SEM	$P$ value <sup>a</sup>
Vehicle								
Right	N	8	0.63 $\pm$ 0.09	N.S.	26.74 $\pm$ 4.84	N.S.	70.30 $\pm$ 16.15	N.S.
Left		8	0.67 $\pm$ 0.08		24.27 $\pm$ 3.57		65.41 $\pm$ 13.39	
Verapamil								
Right	N	8	0.59 $\pm$ 0.07	N.S.	29.34 $\pm$ 3.89	N.S.	68.32 $\pm$ 12.08	N.S.
Left		8	0.57 $\pm$ 0.06		26.87 $\pm$ 4.60		60.05 $\pm$ 10.94	
PTH								
Right	N	8	0.66 $\pm$ 0.05	N.S.	28.82 $\pm$ 2.92	N.S.	70.96 $\pm$ 10.73	N.S.
Left		8	0.62 $\pm$ 0.05		25.26 $\pm$ 3.37		59.34 $\pm$ 11.36	
Verapamil + PTH								
Right	N	8	0.65 $\pm$ 0.04	N.S.	30.60 $\pm$ 4.13	N.S.	76.70 $\pm$ 14.47	N.S.
Left		8	0.66 $\pm$ 0.05		25.58 $\pm$ 4.27		65.11 $\pm$ 14.56	
Vehicle								
Right	Y	8	0.91 $\pm$ 0.07	<0.01	46.28 $\pm$ 3.88	<0.001	155.98 $\pm$ 18.93	<0.001
Left		8	0.73 $\pm$ 0.07		23.19 $\pm$ 2.90		64.65 $\pm$ 12.51	
Verapamil								
Right	Y	8	0.78 $\pm$ 0.06	<0.05	28.64 $\pm$ 2.82	<0.001	82.00 $\pm$ 10.97	<0.01
Left		8	0.70 $\pm$ 0.08		22.90 $\pm$ 2.93		60.89 $\pm$ 11.21	
PTH								
Right	Y	8	1.00 $\pm$ 0.11	<0.05	55.69 $\pm$ 3.93	<0.001	204.25 $\pm$ 27.69	<0.001
Left		8	0.72 $\pm$ 0.04		24.40 $\pm$ 2.65		64.25 $\pm$ 6.68	
Verapamil + PTH								
Right	Y	8	0.84 $\pm$ 0.07	<0.05	35.00 $\pm$ 5.26	<0.05	110.42 $\pm$ 21.69	<0.01
Left		8	0.70 $\pm$ 0.06		27.52 $\pm$ 3.97		73.56 $\pm$ 15.46	

Values are means  $\pm$  SEM; N, No; Y, yes.

<sup>a</sup> Probability associated with paired  $t$  test between right and left values for each group. N.S. (not significant) indicates that the probability exceeds 0.05.

significant difference test (PLSD) for pairwise comparisons. Statistical significance was assumed if  $P < 0.05$ .

## Results

There were no differences in body weight among the groups at the beginning or end of the experimental period. The treatment with verapamil did not affect the animals' activities throughout the experiment. Compared with the baseline blood pressure ( $118.8 \pm 2.04$  mm Hg), the average blood pressure decreased by 4% and 12% at 45 min ( $113 \pm 2.07$  mm Hg) and 90 min ( $104.6 \pm 6.75$  mm Hg) after treatment with verapamil, respectively, but the changes were not statistically significant. Compared with the normal total serum calcium ( $10.20 \pm 0.07$  mg/dl), serum calcium was significantly increased to  $11.2 \pm 0.22$  mg/dl 45 min after verapamil treatment, but returned to normal levels ( $10.18 \pm 0.17$  mg/dl) 90 min after treatment. There were no significant differences in serum PTH among the normal controls ( $12.38 \pm 2.64$  pg/ml) and animals 45 min after ( $10.13 \pm 2.43$  pg/ml) and 90 min after ( $11.18 \pm 3.92$  pg/ml) a single dose of verapamil.

### Bone formation on the endocortical surface of tibiae

Histomorphometric measurements show that there was no significant difference in MS/BS, MAR, and BFR/BS between right and left tibiae in the groups without loading, as analyzed by the paired  $t$  tests (Table 1). However, in the loading control group there were significantly higher MS/BS, MAR, and BFR/BS in loaded (right) tibiae ( $P < 0.01$ ) compared with nonloaded (left) tibiae. Significant differences in MS/BS, MAR, and BFR/BS between right and left tibiae were also found for all loading groups regardless of treatment ( $P < 0.05$ ; Table 1). The values for MS/BS, MAR, and BFR/BS of left tibiae were not significantly different among the groups. Treatment with verapamil significantly reduced the mechanical loading effects on bone. Verapamil suppressed the load-induced increase in MS/BS and BFR/BS by 75% ( $P < 0.05$ ) and 77% ( $P < 0.001$ ), respectively (Fig. 3). In contrast, PTH significantly enhanced the load-induced increase in MS/BS and BFR/BS by 36% ( $P < 0.05$ ) and 53% ( $P < 0.01$ ), respectively. Verapamil suppressed rMS/BS and rBFR/BS on the right loaded tibiae in the animals subjected to PTH and loading by 76% ( $P < 0.001$ ) and 74% ( $P < 0.0001$ ), respectively.

### Bone formation on the periosteal surface of ulnae

Histomorphometric measurements showed that there was no significant difference in MS/BS, MAR, and BFR/BS between right and left ulnae in the groups without loading (Table 2). However, there were significantly higher MS/BS, MAR, and BFR/BS in loaded (right) ulnae of the loading control animals ( $P < 0.01$ ) compared with nonloaded (left) ulnae. Significant differences in MS/BS, MAR, and BFR/BS between right and left ulnae were also found for all treatment groups with loading ( $P < 0.05$ ; Table 2). The values for MS/BS, MAR, and BFR/BS of left (nonloaded) ulnae were not significantly different among the groups. Similar to that in tibia, treatment with verapamil significantly inhibited the mechanical loading effects on right loaded ulnae. Verapamil

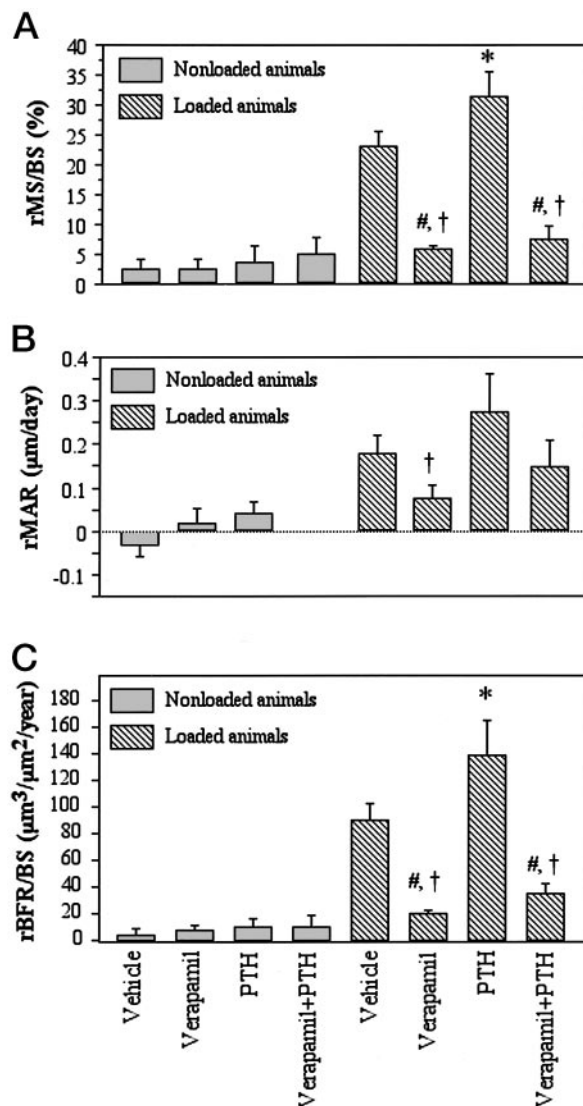


FIG. 3. On the endocortical surface of tibia, the rMS/BS (A) and rBFR/BS (B) were significantly decreased by verapamil. PTH plus loading led to significantly higher rBFR/BS and rMS/BS than the loading alone. Verapamil suppressed the synergistic effect of PTH and loading. There was a significant difference in the rMAR (B) between verapamil treatment and PTH treatment in loaded animals. Results are expressed as the mean  $\pm$  SEM. \*,  $P < 0.05$  vs. vehicle loading group; #,  $P < 0.001$  vs. vehicle loading group; †,  $P < 0.05$  vs. PTH loading group (based on Fisher's PLSD at  $\alpha = 0.05$ ).

suppressed the load-induced increase in MS/BS and BFR/BS in the loading controls by 22% ( $P = 0.056$ ) and 59% ( $P < 0.01$ ), respectively (Fig. 4). PTH significantly enhanced the loading effect on the right loaded ulnae, increasing rMS/BS by 63% ( $P < 0.01$ ) and rBFR/BS by 76% ( $P < 0.01$ ). Furthermore, verapamil suppressed rMS/BS and rBFR/BS on right loaded ulnae in animals subjected to PTH and loading by 39% ( $P < 0.01$ ) and 68% ( $P < 0.001$ ), respectively.

## Discussion

We studied the *in vivo* effect of PTH on load-induced bone formation in a system in which the L-type VSCCs were blocked by verapamil. The mechanical loading was carried

**TABLE 2.** Measurements of periosteal bone formation at ulna

Groups	Load	n	MAR ( $\mu\text{m}/\text{d}$ )		MS/BS (%)		BFR/BS ( $\mu\text{m}^3/\mu\text{m}^2\cdot\text{yr}$ )	
			Mean $\pm$ SEM	<i>P</i> value <sup>a</sup>	Mean $\pm$ SEM	<i>P</i> value <sup>a</sup>	Mean $\pm$ SEM	<i>P</i> value <sup>a</sup>
Vehicle								
Right	N	8	0.66 $\pm$ 0.04	N.S.	37.66 $\pm$ 4.04	N.S.	91.86 $\pm$ 12.10	N.S.
Left		8	0.68 $\pm$ 0.05		33.24 $\pm$ 3.26		82.62 $\pm$ 9.65	
Verapamil								
Right	N	8	0.67 $\pm$ 0.06	N.S.	34.38 $\pm$ 1.02	N.S.	84.10 $\pm$ 6.83	N.S.
Left		8	0.68 $\pm$ 0.04		31.76 $\pm$ 1.31		79.83 $\pm$ 8.02	
PTH								
Right	N	8	0.72 $\pm$ 0.06	N.S.	36.19 $\pm$ 3.18	N.S.	98.08 $\pm$ 14.25	N.S.
Left		8	0.70 $\pm$ 0.05		32.42 $\pm$ 3.42		85.48 $\pm$ 12.76	
Verapamil + PTH								
Right	N	8	0.66 $\pm$ 0.04	N.S.	37.02 $\pm$ 2.35	N.S.	90.95 $\pm$ 11.62	N.S.
Left		8	0.64 $\pm$ 0.03		32.10 $\pm$ 1.72		74.62 $\pm$ 3.47	
Vehicle								
Right	Y	8	1.11 $\pm$ 0.10	<0.01	59.73 $\pm$ 2.54	<0.001	239.43 $\pm$ 19.91	<0.001
Left		8	0.71 $\pm$ 0.02		37.70 $\pm$ 1.45		97.92 $\pm$ 5.42	
Verapamil								
Right	Y	8	0.83 $\pm$ 0.06	<0.01	49.34 $\pm$ 3.06	<0.05	149.71 $\pm$ 14.08	<0.01
Left		8	0.57 $\pm$ 0.02		36.95 $\pm$ 2.62		91.22 $\pm$ 11.80	
PTH								
Right	Y	8	1.31 $\pm$ 0.11	<0.01	71.66 $\pm$ 2.49	<0.001	345.88 $\pm$ 37.96	<0.001
Left		8	0.75 $\pm$ 0.02		35.67 $\pm$ 2.81		97.07 $\pm$ 7.51	
Verapamil + PTH								
Right	Y	8	0.80 $\pm$ 0.08	N.S.	55.39 $\pm$ 5.07	<0.01	165.78 $\pm$ 25.13	<0.05
Left		8	0.70 $\pm$ 0.06		33.49 $\pm$ 3.22		85.52 $\pm$ 12.25	

Values are means  $\pm$  SEM; N, No; Y, yes.

<sup>a</sup> Probability associated with paired *t* test between right and left values for each group. N.S. (not significant) indicates that the probability exceeds 0.05.

out using established *in vivo* loading models: rat tibial four-point bending and ulna axial loading (16, 17, 20). Similar to previous studies (16, 17, 20), a single bout of four-point bending was sufficient to significantly increase lamellar bone formation on the endocortical surface of the tibia, and a single bout of axial loading was sufficient to significantly increase lamellar bone formation on the periosteal surface of the ulna. As we have reported previously (20), a single verapamil treatment significantly inhibited load-induced bone formation compared with that in the loading control group. Consistent with a previous report (5), a single PTH treatment significantly enhanced load-induced bone formation compared with loading alone. Moreover, verapamil treatment significantly suppressed bone formation in animals subjected to PTH treatment and loading.

We conclude that the synergism between PTH and mechanical loading in their effect on bone formation is mediated in part by  $\text{Ca}^{2+}$  entry into the osteoblast through L-type VSCCs. This conclusion is supported by *in vitro* experiments showing that PTH pretreatment of osteoblasts before mechanical loading produces a greater peak  $[\text{Ca}^{2+}]_i$  response than loading alone (18, 32, 33) and the observation that PTH can modulate VSCC-mediated  $\text{Ca}^{2+}$  influx and at least partially amplify L-type VSCC currents in various cell models (19, 34–38). In addition, PTH can increase the activity and single channel conductance of the mechanically sensitive, cation-selective channel in UMR106.01 osteoblast-like cells (39). Changes in the activation kinetics of the mechanically sensitive channel produce a membrane depolarization that could activate L-type VSCCs.

The earliest response of osteoblasts to fluid shear or mechanical strain is a rapid increase in  $[\text{Ca}^{2+}]_i$  (8), which can be

suppressed by L-type VSCC blockers (33). On the other hand, PTH can enhance shear flow-induced increase in  $[\text{Ca}^{2+}]_i$  osteoblastic cells, which can also be suppressed by the L-type VSCC blocker nifedipine (19). Furthermore, in osteoblasts, blockade of L-type VSCCs using either verapamil or nifedipine inhibits  $[\text{Ca}^{2+}]_i$  signals and consequent cellular responses induced by PTH (18, 40–42). The L-type VSCC is the best characterized channel among the voltage-sensitive and voltage-insensitive currents that have been measured in osteoblasts, stromal precursor cells, osteocytes, and osteoblast-like clonal cell lines (for review, see Ref. 43). These channels may play a role in normal bone physiology. Low doses of nifedipine given to growing rabbits over 10 wk significantly reduce cancellous and cortical bone volume, MAR, and the length of the epiphyseal growth plate (44).

Intermittent administration of PTH increases bone mass and improves bone structure *in vivo* (1–3). Osteoblasts are the primary target cells for the anabolic effects of PTH on bone tissue. PTH may stimulate differentiation of osteoprogenitors (1, 45, 46) and possibly prolong cell longevity (47). Most actions of PTH-(1–34) on osteoblasts are mediated by the PTH-1 receptor, a G protein-coupled receptor with seven membrane-spanning domains (48). PTH can activate the cAMP/protein kinase A pathway, the inositol lipid/ $\text{Ca}^{2+}$  (release of intracellular  $\text{Ca}^{2+}$ )/protein kinase C pathway, or both in osteoblast-like cells from rat or human (see review in Ref. 49). Like mechanical loading, PTH can also increase  $[\text{Ca}^{2+}]_i$  in bone cells by increasing both extracellular  $\text{Ca}^{2+}$  entry and intracellular  $\text{Ca}^{2+}$  release. PTH could alter calcium channel kinetics via cAMP or PKC (40, 50), possibly through phosphorylation of sites on the various subunits of the channel protein. We have recently demonstrated that the PTH-

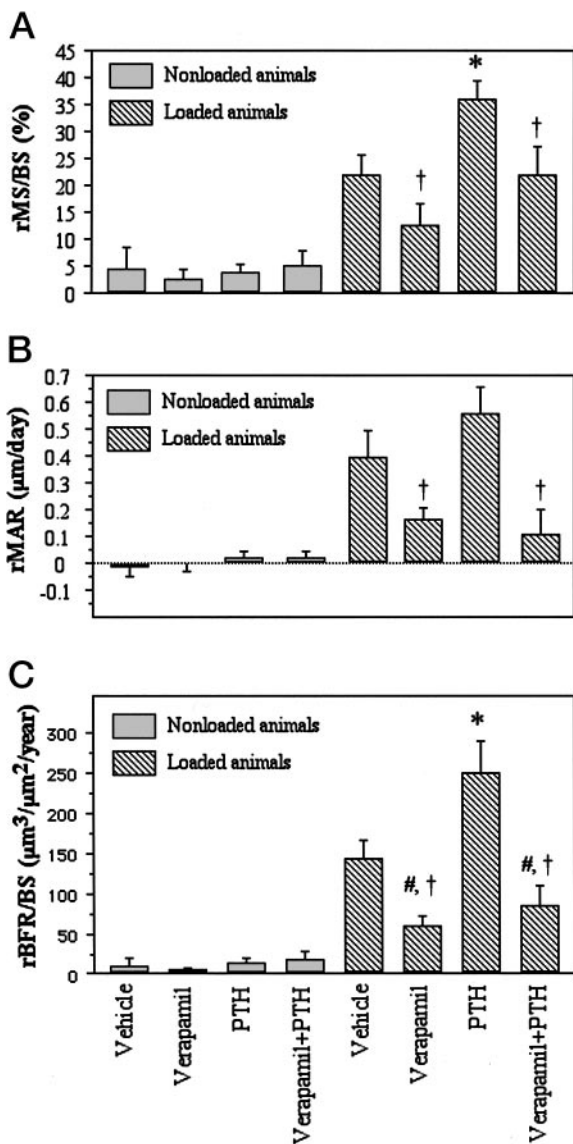


FIG. 4. On the periosteal surface of the ulna, the rMS/BS (A) and rBFR/BS (C) were significantly decreased by verapamil. PTH combined with loading led to significantly higher rBFR/BS and rMS/BS than loading alone. As in the tibia, verapamil suppressed the synergistic effect of PTH and loading. There was a significant difference in the rMAR (B) between verapamil treatment and PTH treatment in loaded animals. The rMAR (B) in animals receiving verapamil, PTH, plus loading was also significantly lower than that in PTH plus loading animals. Results are expressed as the mean  $\pm$  SEM. \*,  $P < 0.05$  vs. vehicle loading group; #,  $P < 0.001$  vs. vehicle loading group; †,  $P < 0.05$  vs. PTH loading group (based on Fisher's PLSD at  $\alpha = 0.05$ ).

enhanced peak  $[Ca^{2+}]_i$  response to mechanical stimulation can be blocked by the L-type VSCC inhibitor, nifedipine. Furthermore, this enhanced response could be mimicked through activation of the protein kinase A, but not the protein kinase C, pathway (18).

The dose of verapamil used in the present study was higher than the typical dose (4 mg/kg) used in humans. The goal of clinical use is to partially block L-type VSCCs, reducing their activity in the heart and thus treating arrhythmias. To completely block the L-type VSCC, as we intended

in this study, a higher dose was necessary. Verapamil at 100 mg/kg was chosen because of its previously demonstrated lack of toxicity on normal bone formation (20). A high dose of verapamil, 100 mg/kg, did not change blood pressure or serum PTH level. Treatment with 100 mg/kg verapamil also did not significantly affect bone formation in the left (control) bones of the rats in the present study, nor was bone formation affected in the vehicle controls. Our data strongly suggest that 100 mg/kg verapamil given as a single dose does not substantially affect the rats' overall health or skeletal biology.

Even with the high dose of verapamil used in this study, bone formation induced by mechanical loading and by PTH plus mechanical loading was not abolished completely. Administration of verapamil suppressed BFR by 59–77%, which is substantial, yet mechanical loading significantly increased bone formation even in the presence of verapamil. Considering the high dose of verapamil used, it is reasonable to assume that the majority of L-type VSCCs in bone cells were blocked. Consequently, there must be another signaling pathway independent of the L-type VSCC that contributes about 25–40% to the bone formation resulting from mechanical loading or PTH plus loading.

At the 100-mg/kg dose, verapamil might have effects not specific to the L-type VSCC. Previous studies have shown a possible effect of verapamil on PTH secretion (30, 31, 51, 52). In our study, serum PTH did not change after a dose of verapamil, nor was the serum calcium level different from normal at the time of loading. These findings suggest that verapamil treatment did not have substantial side-effects, and the observed effects of treatment were probably due to the effect of verapamil on calcium channels. Verapamil has also been reported to modulate *p*-glycoprotein, a multidrug transporter on cell membrane (53). We cannot rule out this pathway as a mechanism for the verapamil response in the present study. However, we know of no evidence relating *p*-glycoprotein to mechanically induced bone formation. In our previous study we showed that 20 mg/kg of both verapamil and another L-type VSCC blocker, nifedipine, suppressed mechanically induced bone formation. The primary effect of both drugs was on L-type VSCCs, and at the 20-mg/kg dose, few nonspecific effects should be expected (22). Verapamil administered at 100 mg/kg had very similar effects on load-induced bone formation as the 20-mg/kg dose (20), suggesting that the higher dose of verapamil does not activate pathways in addition to the primary effect on L-type VSCCs. Other considerations include the possibility that verapamil alters the vasculature by inducing vasodilation. Our data demonstrate that a single high dose of verapamil lowers blood pressure somewhat, but not significantly. It is possible that altered hemodynamics might affect mechanotransduction in bone tissue by affecting extracellular fluid flow near bone cells. However, as noted above, bone cell mechanotransduction is suppressed by lower doses of verapamil that have much less effect on vascular tone. It seems most likely that the effect of verapamil on bone mechanotransduction was not due to changes in hemodynamics.

In conclusion, the present study suggests that blockade of L-type calcium channels by verapamil suppresses load-induced bone formation on the endocortical surface of tibia and the periosteal surface of ulna. PTH improved load-

induced bone formation in the absence of verapamil, but the improvement was nullified by verapamil. This suggests that PTH enhances mechanically induced bone formation through involvement of L-type calcium channels *in vivo*.

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