Journal of Cellular Biochemistry 79:648–661 (2000)

Calcium-Channel Activation and Matrix Protein Upregulation in Bone Cells in Response to Mechanical Strain

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Abstract Femur-derived osteoblasts cultured from rat femora were loaded with Fluo-3 using the AM ester. A quantifiable stretch was applied and $[Ca^{2+}]i$ levels monitored by analysis of fluorescent images obtained using an inverted microscope and laser scanning confocal imaging system. Application of a single pulse of tensile strain via an expandable membrane resulted in immediate increase in $[Ca^{2+}]i$ in a proportion of the cells, followed by a slow and steady decrease to prestimulation levels. Application of parathyroid hormone (10^{-6} M) prior to mechanical stimulation potentiated the load-induced elevation of $[Ca^{2+}]i$. Mechanically stimulating osteoblasts in Ca^{2+}-free media or in the presence of either nifedipine (10 μM; L-type Ca^{2+}-channel blocker) or thapsigargin (1 μM; depletes intracellular Ca^{2+} stores) reduced strain-induced increases in $[Ca^{2+}]i$. Furthermore, strain-induced increases in $[Ca^{2+}]i$ were enhanced in the presence of Bayer K 8644 (500 nm), an agonist of L-type calcium channels. The effects of mechanical strain with and without inhibitors and agonists are described on the total cell population and on single cell responses. Application of strain and strain in the presence of the calcium-channel agonist Bay K 8644 to periosteal-derived osteoblasts increased levels of the extracellular matrix proteins osteopontin and osteocalcin within 24 h postload. This mechanically induced increase in osteopontin and osteocalcin was inhibited by the addition of the calcium-channel antagonist, nifedipine. Our results suggest an important role for L-type calcium channels and a thapsigargin-sensitive component in early mechanical strain transduction pathways in osteoblasts. J. Cell. Biochem. 79:648–661, 2000. © 2000 Wiley-Liss, Inc.

Key words: osteoblast; calcium; mechanical strains; PTH

The skeleton models and remodels its mass and structure in response to the mechanical environment. Application of mechanical strain to bone tissue in vivo and bone-forming cells in vitro has been shown to increase the concentrations of a variety of second messengers, including cAMP, cGMP [Rodan et al., 1975; Binderman et al., 1988] IP_3 [Brighton et al., 1992], and Ca^{2+} [Jones and Bingmann, 1991] to stimulate activity of phospholipases and nitric oxide [Binderman et al., 1988; Pitsillides et al., 1995; Jones et al., 1991], and to enhance release and synthesis of prostaglandins [Somjen et al., 1980; Binderman et al., 1988; Rawlinson et al., 1991; Chow and Chambers, 1994; Thomas and El Haj, 1996; Klein-Nulend et al., 1996, 1997; Zaman et al., 1997]. Strain-activated secondary messenger pathways are associated with a further cascade of responses including G6PD activation [Zaman et al., 1997; El Haj et al., 1990; Dallas et al., 1993; Dodds et al., 1993] and an overall increase in total RNA synthesis [El Haj et al., 1990; Dallas et al., 1993; Raab-Cullen et al., 1994] that includes production of growth factors such as IGFs [Zaman et al., 1997; Raab-Cullen et al., 1994; Rawlinson et al., 1993, Lean et al., 1995] and TGFβ [Klein-Nulend et al., 1996; Neidlinger-Wilke et al., 1995], as well as elevation of specific matrix protein mRNAs [Lean et al., 1995; Zaman et al., 1992; Roelofsen et al., 1995; Sun et al., 1995; Toma et al., 1997] Strain-activated pathways have been linked ultimately to in-

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Received 29 July 1999; Accepted 31 March 2000

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creased bone formation [Sun et al., 1995; Rubin and Lanyon, 1984; Turner et al., 1994].

There is debate over the stages of bone-cell differentiation that are responsive to loading, and whether osteocytes, the terminally differentiated stage, are the key load transducers in bone tissue [El Haj and Thomas, 1994; Lanyon, 1992]. To date, several different bone-cell stages derived from a variety of sources have been shown to be responsive to strain applied both in vivo and in vitro, including osteoblasts [El Haj et al., 1990; Jones and Bingmann, 1991; Jones et al., 1991; Shelton and El Haj, 1992; Dallas et al., 1993; Dodds et al., 1993; Neidlinger-Wilke et al., 1995; Roelofsen et al., 1995; Klein-Nulend et al., 1996, 1997; Zaman et al., 1997], osteocytes [Skerry et al., 1989; El Haj et al., 1990; Dallas et al., 1993; Dodds et al., 1993; Klein-Nulend et al., 1995; Lean et al., 1995], and bone marrow stromal cells [Thomas and El Haj, 1996]. Rawlinson et al. [1995] have suggested that load responses may vary between osteoblasts from different parts of the skeleton.

$[Ca^{2+}]$i plays a critical regulatory role in cellular activities of all eukaryotic cells. Electrophysiologic investigations have highlighted the presence of plasma membrane calcium channels in many bone cell types. In particular, voltage-operated calcium channels (VOCCs) have been detected in primary cultures of rat calvarial, osteoblast-like cells [Chesnoy-Marchais and Fritsch, 1994], chicken osteoclasts [Miyauchi et al., 1990], bone marrow stromal cells [Publicover et al., 1994; Preston et al., 1996], and in virtually all osteoblast-like cell lines that have been studied [Duncan and Misler, 1989; Morain et al., 1992]. Elevation of $[Ca^{2+}]$i is often the first measurable response to a primary stimulus and Ca^{2+} can subsequently elicit both cytoplasmic and nuclear responses and interact with and modulate the activities of other second messengers [Berridge, 1997].

The development of fluorescent imaging techniques to observe $[Ca^{2+}]$i has allowed real-time observation of this messenger in bone cells [Ypey et al., 1992]. Such studies have shown that modulation of $[Ca^{2+}]$i is important in activation of osteoblasts by Vitamin D, PGE_2, and parathyroid hormone (PTH), as well as the release of Ca^{2+} from intracellular storage and influx of Ca^{2+} being involved in generation of $[Ca^{2+}]$i transients [Baran et al., 1991; Wiltink et al., 1994; Said Ahmed et al., 2000]. Ca^{2+} also appears to be significant in transducing mechanical stimuli [El Haj et al., 1999]. Jones et al. [1991] reported preliminary data that included an increase in $[Ca^{2+}]$i 100 msec after application of 5,000 μStr to the substratum of cultured osteoblasts. This response was preceded by an increase in IP_3 levels. Vadiakis and Banes [1992] reported that application of cyclic strain to the substratum resulted in increased incorporation of calcium by cultures of ROS 17/2.8 cells, a response that was diminished in the presence of verapamil, a blocker of VOCCs. Shear stress applied in a parallel plate flow chamber produces a $[Ca^{2+}]$i response that is dependent on generation of IP_3 and is attenuated by gadolinium, a blocker of stretch-activated channels, but not by verapamil [Allen et al., 1996; Hung et al., 1996]. Other authors have reported increased $[Ca^{2+}]$i upon osmotic swelling of osteoblastic-like cells [Yamaguchi et al., 1989; Lee and Wong, 1994; Schofield et al., 1994]. These responses are dependent upon extracellular Ca^{2+}, but are also resistant to L-channel blockers. Our work applying force by "optical tweezers" onto single cells has shown how low levels of force (6–8 pN) onto the cell membrane is capable of activating intracellular calcium release [Walker et al., 1999]. Strains applied through the cell substratum, through fluid flow and those induced through osmotic swelling (volume-sensing) may vary in their effects on the cell and therefore in the transduction pathways involved.

In this in vitro study, we have examined the response of $[Ca^{2+}]$i of rat, long-bone-derived osteoblasts to mechanical strain applied to the substratum. In addition, we have measured load-induced up-regulation of the bone matrix proteins osteopontin and osteocalcin. We provide evidence that VOCCs are present in these cells and, using specific modulators, show that they are a significant and necessary component in mechanotransduction, which results in matrix protein up-regulation.

MATERIALS AND METHODS

Femur-Derived Osteoblast Cell Culture

Femur-derived osteoblast cells were prepared and cultured as described by Pitsillides et al. [1995] and Stringa et al. [1995]. Briefly, femora from young male Wistar rats were asceptically removed and stripped of soft tissue. After removal of epiphyses, the marrow was

flushed from the bones and the remaining shafts were dissected into fragments and placed in culture dishes [minimum essential medium (α-MEM) containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1mg/ml)]. After three weeks, the adhered cells were subcultured into membrane-based culture dishes (Petriperm, Bachofer, Germany) and were used after five days. All culture dishes were maintained at 37°C in a 5% CO_2 incubator. Stringa et al. [1995] have carried out extensive characterization of cultures with time, confirming levels of key bone cell markers such as the matrix proteins, alkaline phosphatase, osteopontin, and osteocalcin.

Fluo-3 Loading

Cells were loaded with Fluo-3 by incubation with Fluo-3 AM (10 μM) for 1 h at 37°C in a 5% CO_2 incubator. A nonionic surfactant Pluronic F-127 was used to aid solubilization of the AM ester. A 25% (w/w) stock solution (1 μl) of Pluronic F-127 in dimethyl sulfoxide was mixed with every 10 nmol of AM ester before being dispersed in the media solution. After loading, the cells were washed in phosphate-buffered saline (PBS) and maintained in α-MEM with antibiotics as described above. Experiments were carried out in this medium, supplemented with ethyleneglycol-bis(β-aminoethyl ether) NNN′,N′-tetraacetic acid (EGTA) and drugs as appropriate.

Confocal Microscopy

The 20× objective lens of an inverted microscope with fixed stage was used to stretch the membrane base of the dish, a technique originally developed by Arora et al. [1994] for human fibroblasts. This technique enables realtime measurements to be made on a confocal microscope to monitor levels of Fluo-3 activation. Briefly, the Petriperm culture dish was clamped to the microscope stage and, after obtaining control images over a period of at least 30 s, the microscope objective was raised to deform the base of the culture dish. Following load calibration (see below), a vertical deformation of 1 ± 0.1 mm (calculated as approximately 1,600 ± 50 μStr) was normally applied. After each stretch of 1-s duration, the base of the Petriperm dish was returned to rest to establish optimal focus. Focus was achieved within 1 s of application of stretch. Reproducibility was achieved by a micromanipulator adjustment of the turret movement. Mechanical stimuli were applied under control conditions with added vehicle controls, and in the presence of nifedipine (10 μM; Sigma), Bay K 8644 (500 nm, a gift from Bayer AG, Wuppertal, Germany), EGTA (2 mM; Sigma), thapsigargin (1 μM; Sigma) and Bovine PTH [fragment (1–64); NISB, London].

Transmission and confocal images were obtained with a laser scanning confocal imaging system (MRC-600; Bio-Rad). The cells were excited by laser light (argon/krypton) at 488 nm. The light was attenuated to 3% or less of the maximum to reduce bleaching of the dye. All experiments were carried out at room temperature (20–22°C).

Load Calibration

Calibration of the maximal strains imposed across the expandable membrane dish was carried out. Turret movements of the microscope were applied using the scale on the fine focus, and the vertical distance was measured. In addition, a further procedure described previously in Thomas and El Haj [1996] was used. In particular, five dishes containing cells on Petriperm dishes were visualized at equivalent magnification with video imaging. Measurement by the movement of the lens distance between two focal points can be used to calculate the base deflection. The incremental strains (radial and hoop) have been calculated to be related to the central deflection such that a 10% increase results in a 21% increase in the radial and hoop strains in these dishes [Brodland et al., 1992]. As a result of radial and axial strains, strain levels are not uniform across the circular expandable membrane, with the maximal strain occurring at the outer regions close to the edge of the plate. There was a direct relationship between the deflection of the dish and the maximal calculated strain (0–2,000 μStr) and the movement of the turret (0.2–1.0 mm). Preliminary experiments involved characterizing the cells' fluorescent intensity in the regions around the dish and attempting to make comparisons with calculated strains according to Brodland et al. [1992].

Data Analysis

Image analysis was carried out using customized sequences from the Bio-Rad Comos

software and the confocal image analysis system. Changes in fluorescence were normalized by calculating as a percentage change R from the resting level before stimulation, using the equation R = [(F − F rest)/ F rest] × 100% where F is fluorescence intensity at time t and F rest is the mean of several determinations of F taken before application of stretch. Each experiment was repeated at least three times with separate populations of cells to confirm reproducibility of the results. Significance of responses to specific treatments was tested by use of paired *t*-tests on fluorescence intensity before and after treatment. Differences between responses were tested by comparing mean normalized responses (calculated as above) using Student's *t*-test. Percent responses of cell populations to strain under different conditions were compared using the chi-square test.

Protein Analysis

For the analysis of protein production, the cells were mechanically loaded in a similar manner to the technique used for Fluo-3 measurement using a system previously described in Thomas and El Haj [1996]. Briefly, loading was applied for 30 min at 1Hz frequency and at a magnitude of 1,600 ± 100 μStr to a Petriperm membrane dish as described in the previous section. Cultures were stopped 24 h after loading. The media was stored at −20°C for further analysis and cells were treated as below. In some experiments, immunocytochemstry revealed a uniform staining pattern of osteopontin throughout the cultures. Quantification of immunoctyochemical staining can be difficult. To address the variation in total proteins in response to treatments, Western blotting and radioimmunoassay of the protein levels, osteopontin, and osteocalcin were determined.

For sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cells were lysed using 100μl PBS containing 0.1% Triton X100. Samples of lysate were mixed with equal volumes of dissociation buffer (0.18 M Tris-HCL, pH 6.8; 3% w/v SDS; 30% v/v glycerol; 15% v/v β-mercaptoethanol; and 0.01% w/v bromophenol blue), boiled for 4 min, and subjected to electrophoresis in 12.5% acrylamide gels in a Bio-Rad Mini Protean II electrophoresis apparatus. Proteins were either transferred to Hybond-PVDF membrane using a Bio-Rad Mini Transblot cell overnight at 30V or stained with coomassie blue to determine equal loading. Transfer to Hybond-PVDF was confirmed by staining gels with coomassie blue.

Enhanced chemiluminescence (Pierce, UK Ltd) was used to detect proteins of interest. Antisera against rat osteopontin (mouse monoclonal; Developmental studies hybridoma bank, Johns Hopkins University, Baltimore, MD) and vinculin (mouse monoclonal; Sigma) were used at dilution in PBS 1:2,000. Secondary anti-mouse monoclonal antibodies conjugated with peroxidase were diluted to 1:4,000. Bands were scanned and intensities evaluated using a Black Widow 4800 PP scanner. Western blots were stripped and reprobed. Briefly, the membrane was submerged in stripping buffer (100 mM β-mercaptoethanol; 2% (w/v) SDS; 62.5mM Tris-HCL pH 6.7) and incubated at 60°C for 30 min with occasional agitation. The membrane was then washed for 2 × 10 min in PBS-tween at room temperature.

Media samples (100 μl) were analyzed in duplicate for osteocalcin release 24 h following mechanical stimulation of cells. Osteocalcin levels were measured using a commercial rat osteocalcin EIA kit (Biomedical Technologies Inc. U.K.) Total protein concentrations of each sample were evaluated using the Lowry method and osteocalcin levels were calculated in ng osteocalcin/mg total protein.

Electrophysiological Recording

All recordings of calcium-channel activity were carried out at room temperature (20–22°C) using Ba^+ as the charge carrier. Extracellular saline contained 108 mM $BaCl_2$ and 10 mM HEPES, pH corrected to 7.6 with NaOH (maximum Na^+ content approximately 3 mM [Chesnoy-Marchais and Fritsch, 1988]. Patch electrodes were made from filamented 1.5-mm glass capillaries (Clark Electromedical GC150TF) and fire polished. Electrodes were backfilled with an intracellular saline containing 150 mM CsCl, 5 mM EGTA, 10 mM D-glucose, and 10 mM HEPES, pH was corrected to 7.3 with CsOH [Amagi and Kasia, 1989]. Electrode resistance was 3–7 MΩ.

Cells were uniform in appearance. Isolated cells were selected for recording, to avoid gap junction contacts with neighboring cells. All recordings were made using the whole-cell variant of the patch-clamp technique. Seals of up to 10 GΩ were achieved prior to break-

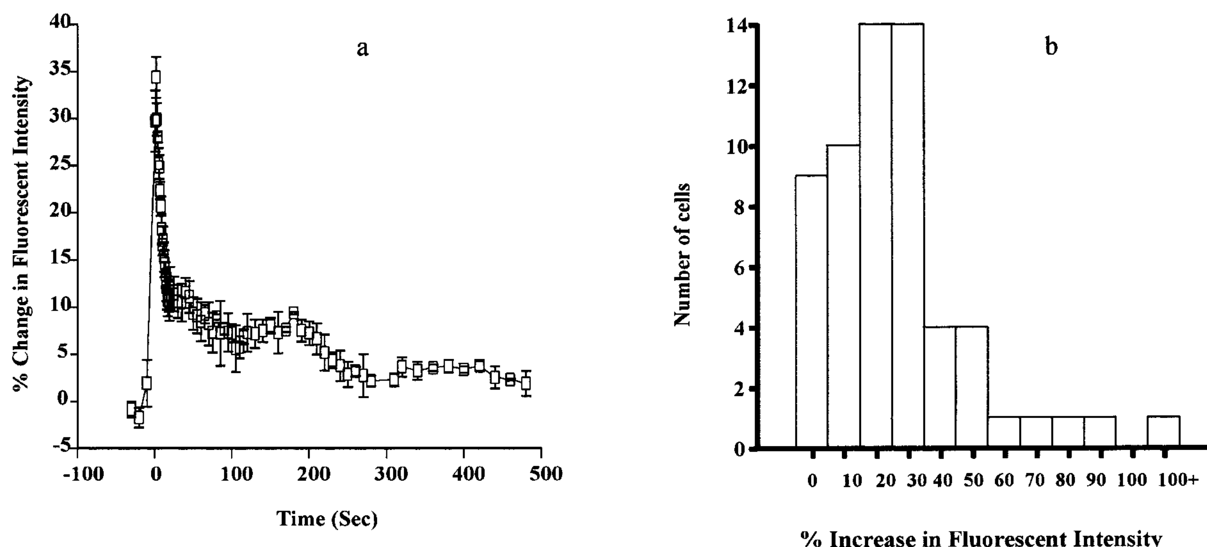

Fig. 1. **a:** Time course of load induced increase in [Ca^{2+}]i-dependent fluorescence of osteoblasts (total population). Post strain increases of fluorescent intensity are expressed as % increases over resting levels. Load applied at 0 sec caused an immediate increase in fluorescent intensity followed by rapid then slower fall to prestimulation levels. Each time point is the mean ± SE from 3 experiments on 60 cells in total. **b:** Levels of percentage change in fluorescence in osteoblasts two seconds after the application of a mechanical strain (1600-2000 μStr). The number of cells in each percentage group is shown on the x-axis. 15% of the cells were found to be unresponsive to strain.

through. Resulting whole-cell input resistances were in the order of 1–5 GΩ. Recordings were commenced within 3–4 min of breakthrough. Cells were held at −60 mV.

Drugs were prepared as stock solutions in ethanol at concentrations of 10 mM and 500 μM for nifedipine and Bay K 8644, respectively. Drugs were added directly to the bath to achieve the final concentration, with ethanol concentration never exceeding 0.001% (v/v).

Statistical Analysis

Data was analyzed for levels of significance using ANOVA or students paired *t*-test. Values are given as mean ± standard error.

RESULTS

Response to Strain

When cultures were bathed in α-MEM (Ca^{2+} = 1.5 mM), application of a load of 1,600 ± 50 μStr resulted in a rapid increase in fluorescence in the body of the cell (Fig. 1A). The greatest response was observed immediately after refocusing the microscope (1 s). Mean fluorescence (whole field of view) was enhanced by 34.4 ± 2.2% (n = 3, $P < 0.05$) at this point, and declined rapidly over the next 50 s (Fig. 1A). Prestimulation levels were not reached within the time duration of monitoring. Application of smaller maximal strains evoked correspondingly smaller increases in fluorescence, revealing a dose dependence of the response in overall total fluorescence of cells (data not shown). The response to loading at ≤1,600 ± 50 μStr was studied in 60 individual cells. The maximal increase in single-cell fluorescent intensity following loading was 110%. The majority of cells showed elevated fluorescence of 10–30%, and 15% of cells produced no measurable response (Fig. 1B). It was not possible to visualize a clear relationship between the axial strain profile, which is applied across an expandable dish, and intracellular calcium flux in individual cells.

Dependence on Ca^{2+} Influx and VOCCs

Addition of 2 mM EGTA to the medium to buffer extracellular Ca^{2+} greatly inhibited the response to application of strain (Fig. 2A). The initial increase in fluorescent intensity observed upon refocusing (1 s) was 4.23 ± 3.31% (whole field of view; n = 3, $P < 0.2$), a response significantly smaller than that seen in cultures stimulated in the absence of EGTA ($P < 0.001$). This rose slowly to 6.68 ± 3.37% at 45 s ($P >$ 0.2). Calcium levels were maintained until

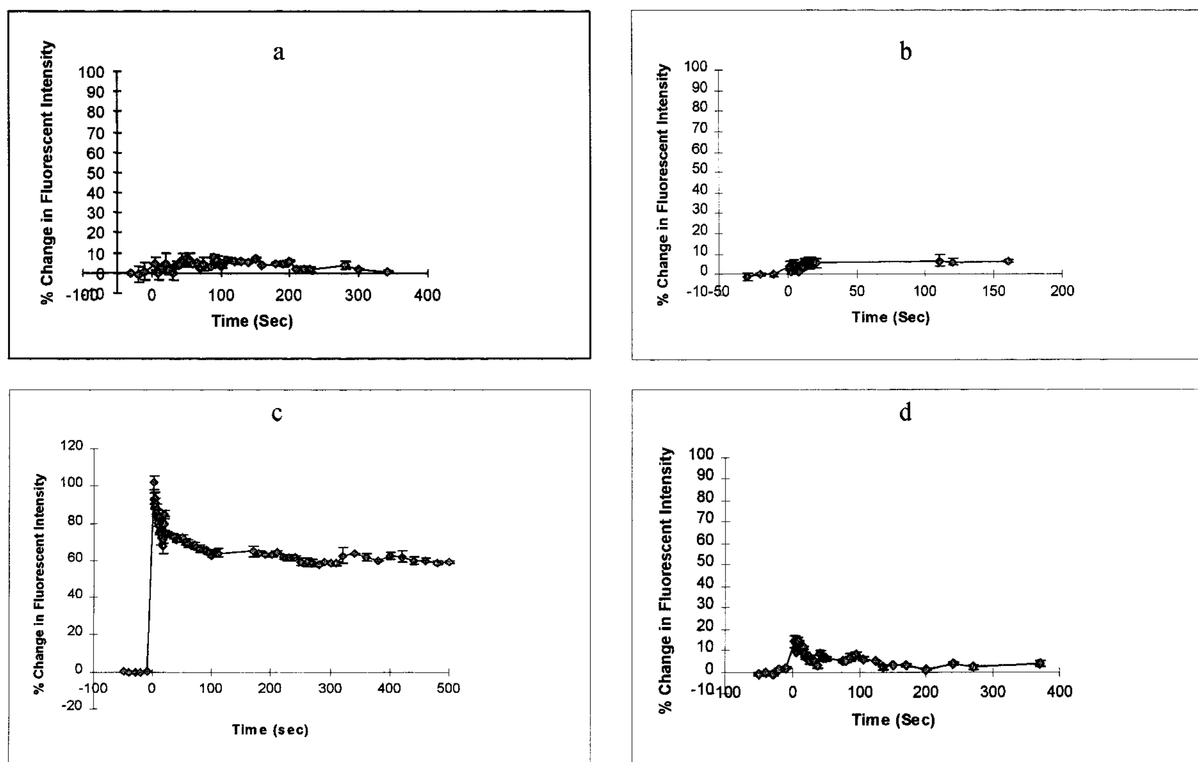

Fig. 2. The effect of pretreatment of osteoblasts with **a:** ethyleneglycol-bis(β-aminoethyl ether) NNN′,N′-tetraacetic acid (EGTA) (2 mM), **b:** nifedipine (10 μM), **c:** Bay K 8644 (500 nM), and **d:** thapsigargin (1 μM) on load-induced changes in $[Ca^{2+}]i$-dependent fluorescence. Pretreatment with EGTA, nifedipine, or thapsigargin all reduced load-induced increases in $[Ca^{2+}]i$-dependent fluorescence. In contrast, load-induced increases were enhanced by the pretreatment of cells with Bay K 8644. In all experiments load was applied at 0 s for duration of 1 s. Each time point shows mean ± SE from three experiments on 40 cells in total.

200 s, when the intensity levels started to decrease towards prestimulation levels. Thirty-four of 40 (85%) individual cells showed measurable increases in fluorescent intensity in response to strain (not significant compared to control).

Stimulating the cells in the presence of L-type calcium blocker, nifedipine, resulted in an increase in fluorescent intensity of 4.3 ± 2.1% after 1 s (whole field of view; n = 3, $P > 0.1$), a response significantly smaller than that seen in the absence of nifedipine ($P < 0.001$; Fig. 2B). This was maintained throughout the monitoring time. Observations of single cells within the population demonstrated that 80% of the cells showed calcium increases of 2–5% in response to strain, 5% gave a greater increase (20–30%). Fifteen percent of cells showed no response to strain in the presence of nifedipine (not significant compared to control).

Pretreatment of cells with Bay K 8644 resulted in an enhancement of the response of fluorescent intensity to application of strain (Fig. 2C). Total fluorescence from all cells in the field of view increased by 101.87 ± 3.47% after 5 s (n = 3; Fig. 2C; $P < 0.05$), a response significantly greater than in control cultures ($P < 0.001$), then decreased before stabilizing at 60% above control levels. Analysis of single cells revealed that 10% did not respond to mechanical strain in the presence of Bay K 8644 (not significant compared to control). The maximal response observed in a single cell was an elevation of 252%, and 80% of the cells showed increases of greater than 100%. No response was measured in vehicle controls or in cells treated with Bay K 8644 alone.

Effects of Thapsigargin

Application of thapsigargin (1–3 μM) to Fluo-3 labeled cultures induced an increase in

fluorescence within 5–30 s. A peak of fluorescence was reached after 25–100 s (mean increase in fluorescence of whole field of view = 223.3 ± 46.5%, n = 3; $P < 0.1$), after which fluorescence subsided to control levels in 5–10 min of application of the drug (Fig. 2D). A single experiment carried out in EGTA-supplemented medium gave a similar response, a peak increase of fluorescence of 110% (whole field of view) being reached after 80 s. Analysis of single cells showed similar behavior. All cells responded to thapsigargin with increased fluorescence. When cells had been pretreated with thapsigargin for 15–30 min (sufficient for enhanced fluorescence to return to prestimulation levels), application of load induced an increase in total fluorescent intensity (all cells in field of view) of 14.4 ± 2.9% (n = 3; $P < 0.1$) within the first 5 s (Fig. 2D), a response significantly smaller ($P < 0.01$) than that seen in non-pretreated cells. Fluorescent intensity then decreased, but further periods of increased fluorescence were observed at 40 s (8.86 ± 1.1%) and 95 s (8.13 ± 2.28%). Fluorescence then decreased to 3% above control levels and remained so for the duration of the experiment. Analysis of individual cell responses revealed that 20% of the population did not respond to mechanical strain in the presence of thapsigargin (not significant compared to control).

Effect of PTH

A single stretch applied to a population of osteoblasts in the presence of PTH (10^{-6} M) resulted in an increase in fluorescent intensity of 85.0 ± 6.4% (n = 3; $P < 0.05$) within 1 s of the stretch (Fig. 3A), a response significantly greater than in non-pretreated preparations ($P < 0.02$). This response amplitude was calculated using the PTH-enhanced fluorescence as baseline (F rest), the increase with respect to the initial control fluorescence being approximately 135%. The fluorescent intensity then decreased towards the prestrain level. The rise in intracellular calcium was attenuated with the decline occurring over a time period of 800 s compared to <100 s with load alone (Fig. 3A). Analysis of single-cell responses (n = 40) showed that 90% were responsive to strain in the presence of PTH (increased fluorescent intensity levels of 40–252% (not significant compared to control; data not shown).

Figure 3B shows a histogram of the percentage change in fluorescent intensity of the total population of cells in response to the different treatments using the maximal response measured within 1 s of load. Pretreatment of cells with PTH prior to load results in a 40% increase over load alone when compared at 1-s post load (see above). In addition, pretreatment with Bay K 8644 also enhanced load-related elevation by 60–70%. Pretreatment with nifedipine, EGTA, and thapsigargin all reduce the load-related elevation (Fig. 3B).

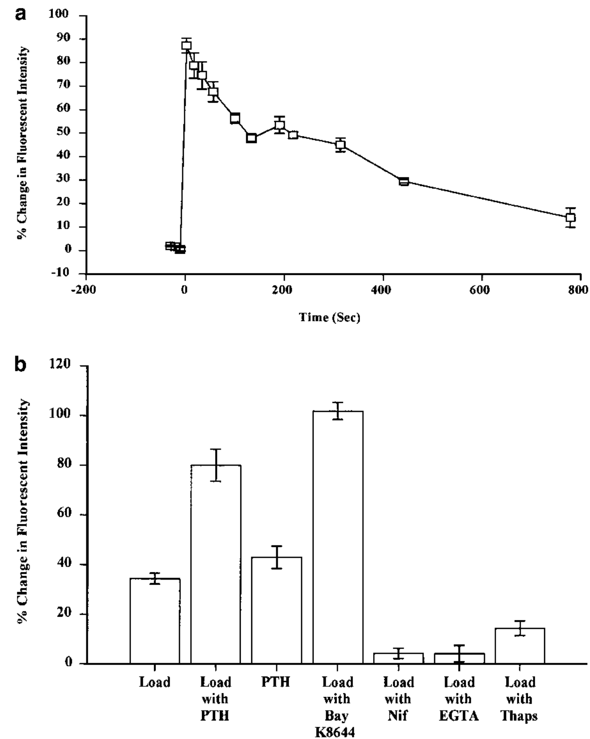

Fig. 3. **a:** The effect of parathyroid hormone (PTH; 2.5 units/ml) on load-related increase in $[Ca^{2+}]i$. Load was applied to the cells 15 min after PTH treatment when fluorescence was stable at a level 30% above the pre-PTH intensity. The time course was monitored for 800 s to enable baseline values to be measured. Values are mean ± SE from three experiments on 40 cells. **b:** Histogram of the effects of various agonists and antagonists on $[Ca^{2+}]i$. Load-related increases in $[Ca^{2+}]i$ were enhanced by PTH (2.5 units/ml) and Bay K 8644 (500 nM), an agonist of L-type calcium channels. Mechanically stimulating osteoblasts in Ca^{2+}-free media (EGTA 2 mM) or in the presence of either nifedipine (L-type channel blocker, 10μM) or thapsigargin (intracellular store calcium releaser, 1μM) reduced load related increase in $[Ca^{2+}]i$. All observations were taken 1 s after load. Values are mean ± SE from three experiments on 40–60 cells.

Synthesis of Matrix Proteins

Osteopontin. Osteopontin was identified on Western blots by immunolabeling, and protein levels were quantified using densitometry.

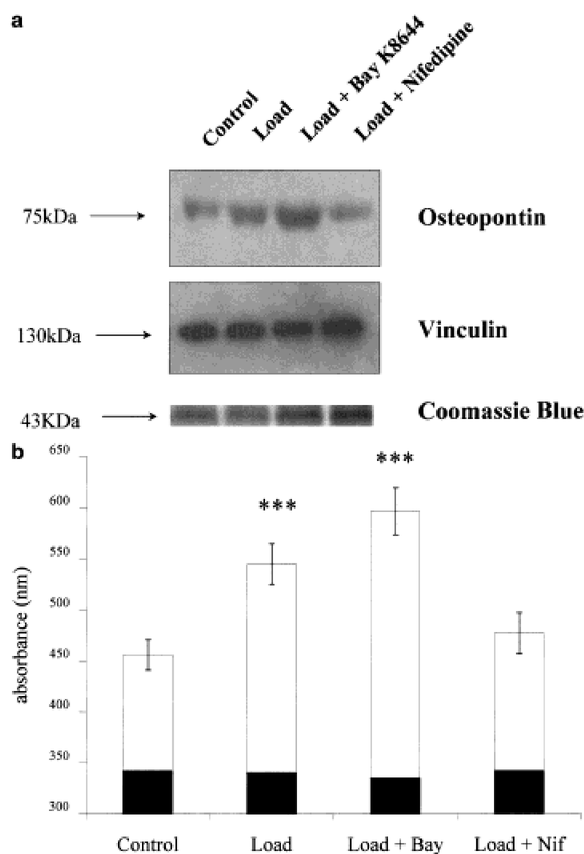

Fig. 4. Western analysis of the effect of L-type calcium channel agonists and antagonists on the synthesis of osteopontin by mechanically loaded osteoblasts. Osteopontin binding to nitrocellulose blot of treated osteoblasts and controls is shown in Fig. 4a. Equal loading was further determined using coomassie staining of proteins on sodium dodecylsulfate-polyacrylamide gel electrophoresis gel (43-KDa protein shown in Fig. 4a). The membrane was stripped and reprobed with an antiserum for vinculin. Following scanning densitometry, levels of each protein band was obtained and shown in Fig. 4b. The black area of each bar within the chart corresponds to vinculin and the upper white area corresponds to osteopontin. Values are mean ± SE from six experiments. Nif, nifedipine; Bay, Bayer K 8644. $^{***}P < 0.001$

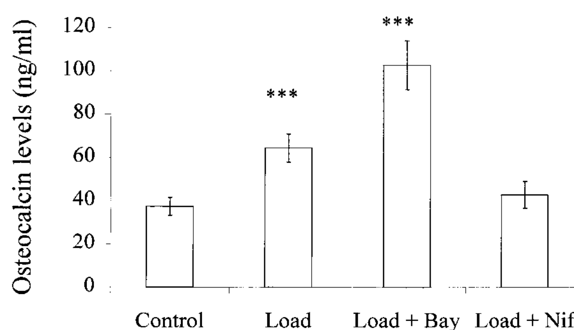

Fig. 5. The effect of load (with and without Bay K 8644 and nifedipine) on osteocalcin release into the media by osteoblasts. Osteocalcin levels were calculated in ng/mg total protein using an EIA kit for osteocalcin levels and Lowry protein method for total protein determination. Values are mean ± SE from six experiments. Nif, nifedipine; Bay, Bayer K 8644. $^{***}P < 0.001$

The antiserum bound predominantly to a band of M_r 75 kDa, which has been shown previously to correspond to the migration of this 44-kDa phosphorylated protein in SDS gels [Sammons et al., 1994] (Fig. 4A). The levels of osteopontin were related to the levels of vinculin, a 130-kDa cytoskeletal protein, which acts as a control. No significant differences in vinculin expression were observed in response to treatments (Fig. 4A, 4B). Coomassie staining of proteins separated on an SDS-PAGE gel further confirmed equal loading of samples (Fig. 4A).

Densitometric levels of osteopontin and vinculin were measured and shown in Fig. 4B. Increased levels of osteopontin were observed in mechanically stimulated cells (23% increase in comparison to control; n = 6) and cells that had been loaded in the presence of Bay K 8644 (31% compared to control; n = 6) in comparison to cells that were not loaded. Load-induced osteopontin elevation was reduced by the addition of the calcium-channel blocker nifedipine (6% compared to control; n = 6).

Osteocalcin. The release of osteocalcin into the media was determined by EIA (Fig. 5). Control media from cells that were not mechanically stimulated produced levels of osteocalcin of 45.7 ± 4.22 ng/mg total protein (n = 9). Similar media levels were observed in samples taken prior to loading or in the presence of bay or nifedipine without mechanical stimulation. Application of mechanical stimulation significantly increased osteocalcin media levels to 76.8 ± 6.61 ng/mg total protein (n = 9; $P < 0.01$). This load-induced osteocalcin response was further significantly elevated by loading in the presence of Bay K 8644 (117 ± 11.28 ng/ml total protein; n = 9; $P < 0.001$), and reduced to control levels when loaded in the presence of nifedipine (50.5 ± 6.34 ng/mg total protein; n = 9; $P < 0.2$).

Presence of VOCCs in Femoral Osteoblasts

The response of $[Ca^{2+}]i$ to strain was inhibited by buffering of extracellular Ca^{2+} with EGTA and was modulated by dihydropyridines in a manner consistent with mediation of Ca^{2+}

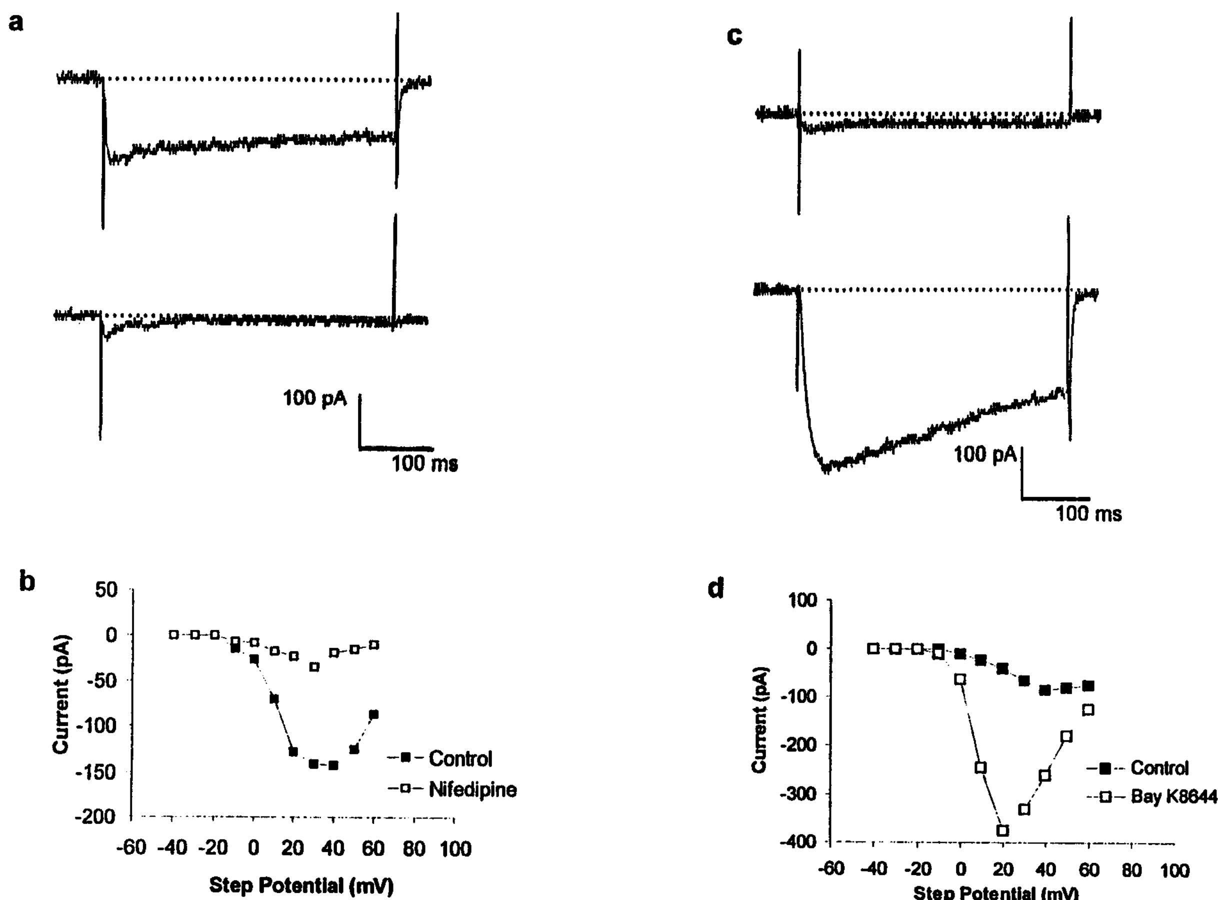

Fig. 6. Modulation of high-voltage activated (L-like) current by dihydropyridines. **a:** Individual currents evoked by steps to ±40mV under control conditions (top) and in the presence of 10 μM nifedipine. Peak current is reduced by almost 90% at this voltage. **b:** Current voltage relationship of an L-like current under control conditions (filled squares) and in the presence of 10 μM nifedipine (open squares). Peak current amplitude is reduced across the voltage range (same cell as a). **c:** Individual currents evoked by steps to ±20mV under control conditions (top) and in the presence of 500 nM Bay K 8644. Peak current is increased almost 10-fold at this voltage. **d:** Current voltage relationship of an L-like current under control conditions (filled squares) and in the presence of 500 nM Bay K 8644 (open squares). Note that in the presence of Bay K 8644, the maximum current amplitude is enhanced and activation occurs at more negative voltages (same cell as c).

influx by L-type VOCCs. We therefore carried out a preliminary electrophysiological investigation to determine whether the cells expressed high-voltage activated (HVA), L-like Ca^{2+} currents and whether the currents could be modulated by dihydropyridines. Recordings from 171 cells, taken between one and five days after subculture of the cells, revealed the presence of both HVA and low-voltage activated (LVA; T-like) currents (Fig. 6). LVA currents were expressed in a high proportion (60%) of day-one cells, but expression fell to only 10% of cells after four days. HVA currents were expressed in 90% of cells on day one (80 of 89) and at a stable level of around 67% (60 of 80) through the following four days.

LVA channels were not affected by dihydropyridine treatment, but the HVA, L-like currents were substantially reduced in the presence of nifedipine and were strikingly enhanced in the presence of Bay K 8644. Figure 6A shows the response of an L-like current to nifedipine (10 μM). The application of nifedipine reduced peak current by approximately 88%, with similar reductions apparent across the entire voltage range (Fig. 6B). Similar reductions were observed in nine other cells. Figure 6C shows the effect of Bay K 8644 (500 nM) on the HVA current (different cell from Fig. 6A). Upon application of Bay K 8644, the maximum current was increased up to fivefold and the voltage necessary to evoke peak current

was shifted by approximately −20 mV, such that at modest depolarizations current amplitude was enhanced by almost 10-fold (Fig. 6D). Traces from Bay K 8644-treated cells also showed current inactivation at more negative voltages and development of marked-tail currents, indicating a slower channel deactivation (Fig. 6C). Similar observations were made in 12 other cells. These responses resemble those of the L-currents seen in primary cultures of rat bone marrow stromal cells [Preston et al., 1996].

DISCUSSION

Application of strain via an expandable membrane to Fluo-3 loaded, cultured femoral osteoblasts resulted in a significant increase in fluorescence. Plots of fluorescence over time suggest that when these first, post-stimulus images were obtained, fluorescent intensity was already falling, having reached a peak during the 1-s period between application of strain and reattaining focus. It is therefore likely that $[Ca^{2+}]$i in femoral osteoblasts is elevated within less than 1 s of application of mechanical load, and that the peak elevation of fluorescence may be significantly greater than that recorded. The percentage increase in fluorescence was found to be directly proportional to the level of strain administered to the cells. The maximum stretch applied (1,600 ± 50 μStr), for a duration of 1 s, resulted in a maximal increase of fluorescent intensity in a whole population of periosteal-derived osteoblasts of 34.4 ± 2.2%. A similar response in fibroblasts subjected to strain by the same methodology was recorded by Arora et al. [1994]. The expandable circular membrane model does not give uniform strain across the dish, as in the case of the four-point bending models. An attempt to correlate strain profiles across the circular membrane as described by Brodland et al. [1992] with fluorescent intensity of cells was not successful.

Analysis of data from single osteoblasts subjected to strain revealed variation in the amplitude response. Of 60 cells analyzed, 85% responded to strain application. Fluorescence in some cells increased by >100% after mechanical strain, the majority of the cells showing increases of between 10% and 30%. These differences may reflect the nonuniformity of the base of the dish, however; they may also reflect the cell cycle as has been found previously using Fluo-3 techniques [Wiltink et al., 1994; Said Ahmed et al., 2000]. There is no comparable, single-cell, study of osteoblasts exposed to strain via stretching of the substrate. However, these data are consistent with observations of responses of $[Ca^{2+}]$i in rabbit long-bone osteoclasts by Xia and Ferrier [1995], who noted that 86% showed increases of fluorescent intensity >10% of the resting levels after stretch of the substrate. As in our data, the response was observed within the first few seconds. In contrast, only 12.5% of fibroblasts were found to exhibit a calcium increase in response to mechanical stimulation using this technique [Arora et al., 1994].

To identify the source of the rise in $[Ca^{2+}]$i, effects of modified media and channel modulators were investigated. Buffering of extracellular Ca^{2+} with EGTA significantly reduced the amplitude of the response, and similar inhibition was achieved by pretreatment with the specific, L-type VOCC blocker nifedipine. In contrast, pretreatment with the L-channel agonist Bay K 8644 produced a threefold enhancement of the response to strain compared to non-pretreated preparations. Analysis of single-cell responses in nifedipine and EGTA experiments showed that 15% of the cells appeared to be unresponsive to strain (a proportion similar to that seen under control conditions), but the majority of the remaining cells showed calcium increases of only 2–5%. These data suggest that nifedipine and EGTA attenuated the strain-induced $[Ca^{2+}]$i response in the whole population of strain-responsive cells, rather than completely inhibiting the response in a subpopulation of cells. Similarly, 10% of cells remained unresponsive in the presence of Bay K 8644, but the response of the strain-sensitive cells was greatly enhanced. Electrophysiological recordings from femoral osteoblasts demonstrated the presence of HVA and LVA VOCCs. Furthermore dihydropyridines, at the concentrations used in the Fluo-3 experiments, modulate HVA, L-like currents in a manner consistent with their effects upon load-induced $[Ca^{2+}]$i response. From these data it can be concluded that VOCCs are present in the cells and that they are an important component of the load-induced Ca^{2+} response transduction pathway in osteoblasts derived from long bones. The finding that modulation of VOCCs by dihydropyridines modifies the amplitude of the cellular response, but not the

proportion of responsive cells, is in accord with a role in primary transduction rather than in secondary, intercellular communication. The response of fibroblasts to load may be similar. Load-related responses in these cells have been shown to be inhibited by Gd^{3+}, which is known to block stretch-activated and L-type Ca^{2+} channels.

Incubation with nifedipine or EGTA did not completely abolish the response of the cells to strain, leaving a residual 4% increase in fluorescence. It is therefore possible that intracellular calcium stores also play a part in mechanotransduction. Exposure of cells to thapsigargin, which has been shown to deplete calcium from internal stores, caused an increase in fluorescence that returned to control levels in 5–10 min. These data suggest that femoral osteoblasts contain Ca^{2+} stores that are emptied in the presence of thapsigargin, after which $[Ca^{2+}]i$ returns to normal levels, despite the continued presence of the drug. Subsequent loading produced an increase in fluorescent intensity (whole field of view) of only 14.4 $\pm$ 2.9%, less than half that of non-pretreated preparations. From this it can be concluded that a thapsigargin-sensitive store contributes to the strain-induced elevation of $[Ca^{2+}]i$. The thapsigargin-sensitive component of the response to loading appears to be much greater than is the residual response seen in cultures strained in the presence of EGTA or nifedipine. We therefore suggest that the thapsigargin-sensitive response may be, at least in part, mediated by calcium-induced calcium release [Fasolata et al., 1994; Berridge, 1997] in response to L-channel mediated Ca^{2+} influx. In fibroblasts, the response of $[Ca^{2+}]i$ was also dependent on contributions from both extracellular and intracellular sources of $[Ca^{2+}]i$ [Amagi and Kasai, 1989]. Futhermore, Xia and Ferrier [1995] observed that both magnitude and duration of the strain-induced $[Ca^{2+}]i$ responses of cultured osteoclasts were greater in cells that were bathed in thapsigargin than those without the drug. It has been reported in various cell types that thapsigargin-induced depletion of calcium stores results in a further calcium influx across the cell membrane. This effect, known as capacitative Ca^{2+} entry [Duncan et al., 1992], may explain the observation of enhanced responsiveness of thapsigargin-treated osteoclasts. There is no evidence for involvement of capacitative entry in the present study.

Second-messenger pathways stimulated by mechanical strain may induce responses similar to the anabolic effects of PTH, which has been shown to increase bone mass [Reinholt et al., 1990]. Addition of PTH to osteoblasts induce and result in elevated cAMP levels [Schofl et al., 1991; Wiltink et al., 1994; Said Ahmed et al., 2000]. Data from these studies indicate that the response of osteoblasts to PTH is dependent on both extracellular and intracellular sources of Ca^{2+}. Our data suggest that mechanically stretching osteoblasts similarly produces an increase in $[Ca^{2+}]i$ from extra/intra cellular sources. It is therefore possible that the effect of PTH and mechanical strain on osteoblasts may be via similar, perhaps convergent, transduction pathways. It has been suggested previously that stretch-activated Ca^{2+} ion channels might be such a point of convergence [Duncan et al., 1992]. Were such a convergence to occur, a combination of treatment with PTH and application of strain might be expected to act synergistically, producing a greater than additive response. This was observed in our experiments in that mechanically stimulating cells resulted in an increase in total fluorescence (whole field of view) of 34.4 $\pm$ 2.2%, whereas stimulation after pretreatment with PTH resulted in increases of 80 $\pm$ 6.4% above the PTH-enhanced baseline [Said Ahmed et al., 2000]. It may also be that the additive effects are mediated through the PTH-mediated cAMP messenger pathway.

Our data also show that VOCCs are important in enabling the activation of further stages of the pathway (up-regulation of the bone matrix proteins osteopontin and osteocalcin). Osteopontin and osteocalcin are noncollagenous matrix proteins located in the extracellular bone matrix and have been suggested to be important in both bone formation and resorption [Weinreb et al., 1990; Duncan et al., 1992]. Furthermore, osteopontin has previously been shown to be a mechanoresponsive gene [Raab-Cullen et al., 1994] in rat calvarial cells and chick osteoblasts and our Western analysis confirms these findings in rat femoral osteoblasts. Immunocytochemical analysis was not able to correlate any individual variation in calcium response with levels of osteopontin. We therefore cannot exclude the possibility that cell/cell communication may play a role in

this response following initial channel activation. Blocking the response with the calcium-channel antagonists does indicate, however, that the initial step of calcium flux in a proportion of cells is a critical one. Our data show that strain-related increases in osteopontin and osteocalcin were reduced by the treatment of nifedipine and enhanced by Bay K 8644, further suggesting the involvement of L-type calcium channels in mechanotransduction. These data also indicate that strain-induced, VOCC-dependent $[Ca^{2+}]i$ responses are likely to be functionally significant during the early phase in strain transduction, potentially resulting in bone formation and ultimately bone modeling. The model used in this study allows us to monitor strain effects on cells using confocal microscopy. Four-point bending models have been developed that apply uniform strain across the base of the substrates [Jones et al., 1991] and it is necessary to address whether these results obtained using a circular membrane model translate to research conducted in vivo and using other load models.

In this article we have demonstrated using this methodology that mechanical stimulation of osteoblasts activates the Ca^{2+} message system both through activation of L-type Ca^{2+} channels and through Ca^{2+} release from a thapsigargin-sensitive component within 1 s post-stimulation. It is becoming increasingly clear that these responses are involved in early mechanotransduction. Our work suggests that calcium mediation may be a factor in strain-induced up-regulation of osteopontin and osteocalcin proteins. These pathways may be convergent with the action of PTH and potentially with other hormones on the cells.

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