Parathyroid Hormone-activated Volume-sensitive Calcium Influx Pathways in Mechanically Loaded Osteocytes

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This paper documents for the first time a volume-sensitive Ca\(^{2+}\) influx pathway in osteocytes, which transmits loading-induced signals into bone formation. Stretch loading by swelling rat and chicken osteocytes in hypo-osmotic solution induced a rapid and progressive increase of cytosolic calcium concentration, [Ca\(^{2+}\)]. The influx of extracellular Ca\(^{2+}\) explains the increased [Ca\(^{2+}\)]\(_{i}\) that paralleled the increase in the mean cell volume. Gadolinium chloride (Gd\(^{3+}\)), an inhibitor of stretch-activated cation channels, blocked the [Ca\(^{2+}\)]\(_{i}\) increase caused by hypotonic solutions. Also, the expression of a1C subunit of voltage-operated \(L\)-type Ca\(^{2+}\) channels (a1C) is required for the hypotonicity-induced [Ca\(^{2+}\)]\(_{i}\), increase judging from the effect of a1C antisense oligodeoxynucleotides. Parathyroid hormone (PTH) specifically potentiated the hypotonicity-induced [Ca\(^{2+}\)]\(_{i}\), increase in a dose-dependent manner through the activation of adenyl cyclase. The increases induced by both PTH and hypotonicity were observed primarily in the processes of the osteocytes. In cyclically stretched osteocytes on flexible-bottomed plates, PTH also synergistically elevated the insulin-like growth factor-1 mRNA level. Furthermore, Gd\(^{3+}\) and a1C antisense significantly inhibited the stretch-induced insulin-like growth factor-1 mRNA elevation. The volume-sensitive calcium influx pathways of osteocytes represent a mechanism by which PTH potentiates mechanical responsiveness, an important aspect of bone formation.

Although osteocytes are the most abundant cells in bone, the role of osteocytes in bone remodeling was not clear until recently. Direct inhibition of osteoclastic activity by osteocyte-derived protein through osteoclast-osteocyte attachment was demonstrated (2, 3). Furthermore, intermittent mechanical loading within the physiological range enhances IGF-1 mRNA expression in osteocytes (4, 5), suggesting that these cells transduce signals induced by mechanical stress to osteoblasts. Although various other mediators such as prostaglandins (6–8), cyclooxygenase-2 (encoding prostaglandin G/H synthase), (8, 9), endothelial, constitutive nitric oxide synthase (10), or c-fos (8, 11–13) have been suggested, the exact mechanosensing mechanisms in these cells are not clear. Although the involvement of stretch-activated cation channels (SA-Ca) in the reception of mechanical stress has been reported in many other cell types, none has been functionally demonstrated in osteocytes so far.

The localization of PTH receptors by in situ hybridization (14), as well as the synergistic effects of mechanical stress and PTH (13, 15), on the other hand, indicated the important role of PTH in regulating the signal transduction of mechanical stress in osteocytes. Stretch-activated cation channels and their activation by PTH were demonstrated in UMR 106 osteoblast-like cells by Duncan et al. (16–18) who suggested either the expression of isoforms of the a1C subunit of the voltage-operated Ca\(^{2+}\) channel (VOCC) is required for the activity or that the channel may have homology to the a1C subunit of VOCC. Osteocytes differentiate from osteoblasts along with a dramatic elongation of cytoplasmic processes. This morphological change suggests that their levels of sensitivity to mechanical loading and the mechanisms of response could be distinct.

In this study, we have identified a stretch-activated Ca\(^{2+}\) entry pathway for the first time in both rat and chicken osteocytes by swelling the cells with hypo-osmotic stress. Also, by using single cell [Ca\(^{2+}\)]\(_{i}\) video-image analysis, we demonstrated that osteocyte processes are furnished with volume-sensitive, stretch-activated Ca\(^{2+}\) entry pathways that are ac-

The abbreviations used are: IGF-1, insulin-like growth factor-1; PTH, parathyroid hormone; [Ca\(^{2+}\)], cytotoxic free calcium; SA-Cat, stretch-activated cation channel; Pipes, piperazine-N,N’-bis(2-ethanesulfonic acid); TPA, 12-O-tetradecanoylphorbol-13-acetate; PTHrp, PTH-related protein; VOCC, voltage-operated calcium channel; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ODN, oligodeoxynucleotide.
tivated by PTH. Apparently, the pathways are dependent on the expression of α1C subunit of the VOCC molecule.

EXPERIMENTAL PROCEDURES

Materials

Fetal calf serum was purchased from Life Technologies, Inc. Bacterial collagenase was purchased from Wako Biochemicals (Osaka, Japan). The acetoxyethyl ester of fura-2 (fura-2 AM) was purchased from Dojin (Kumamoto, Japan). Parathyroid hormone and its fragments were from Peptide Institute (Osaka, Japan). All other reagents were of analytical grade from Sigma unless otherwise mentioned.

Osteocytes Culture

Chicken Osteocytes—Chicken osteocytes were isolated from bone marrow-free calvariae of 16-day-old chicken embryos according to the modified method of Van der Plas and co-workers (2, 19). Briefly, calvariae of 16-day-old chicken embryos were minced and digested with 1 mg/ml type I collagenase in the isolation buffer (25 mM Hepes, 10 mM NaHCO₃, 100 mM NaCl, 3 mM K₂HPO₄, 1 mM CaCl₂, 30 mM KCl, 1 mM MgCl₂, 5 mM bovine serum albumin, 5 mg/ml glucose, 7.5 μM α-tosyl-l-lysyl chloromethane, a protease inhibitor) (37 °C, 30 min), washed in PBS, incubated in PBS(−) containing 5 mM EDTA (37 °C, 30 min), and finally digested with collagenase in the isolation buffer (37 °C, 30 min) and cultured on glass coverslips in α-minimal essential medium with 2% chicken serum. Most osteocytes were positively stained with osteocyte-specific monoclonal antibody, OB7.3, kindly provided by Dr. M. J. Alblas and P. Nijweide (University of Leiden, Netherlands).

Rat Osteocytes—Rat osteocytes were prepared from frontal and parietal bones that were dissected aseptically from newborn rats according to the method of Mikuni-Takagaki et al. (5) with some modifications. Briefly, pieces of bone were stripped of periosteal soft tissue and sutured and digested with 25 ml of 0.75 mg/ml collagenase. Cells released after the first 30 min and the second through the fourth 20-min digestion at 37 °C (fractions I–IV) were collected and filtered through a nylon screen with 40-μm pores, and the fraction IV cells cultured as osteoblasts at the original density of 5 × 10⁵ cells/ml on glass coverslips coated with type I collagen. The residual bone pieces were further washed with 4 mM EDTA in Ca²⁺-Mg²⁺-free PBS and then digested with collagenase as before for 20 min each. Isolated 2 × 10⁶ fraction VI cells cultured as young osteocytes on glass coverslips in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and insulin, transferrin, and selenium. Fraction VI cells were cultured on coverslips coated with Matrigel (Collaborative Biomedical Products/Becton Dickinson Labware, Bedford, MA) to stimulate cell differentiation toward the osteocyte phenotype (20).

Measurement of Cell Volume

The mean cell volume of rat osteocytes at various osmolarities was measured using a Coulter counter (CDA-500, Sysmex Co., Kobe, Japan) adapted with a 100-μm diameter aperture as described previously (21, 22). Fraction VI rat young osteocytes (1 × 10⁶) were resuspended in various Ringer’s solutions of different osmolarities, and the cell volume was measured. Cell volume distribution curve was taken, and mean cell volume was calculated by computer software.

Imaging of Cytosolic Calcium, [Ca²⁺]i, and [Ca²⁺]cyt in Hypotonically Stretched Cells

Cytosolic calcium was measured in single cells using the fluorescent calcium indicator, fura-2 as described previously (23–25). Osteocytes cultured for 1–2 days on glass coverslips were washed with PBS and loaded with 5 μM fura 2/AM for 1 h at 25 °C in Ringer’s solution (138 mM NaCl, 5.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM Hepes buffer (pH 7.3 with NaOH)). After two washes with Ringer’s solution, the measuring chamber was mounted to a microfluorometric system and images (F340 and F360) were collected at wavelengths of 340 and 360 nm. The ratio of F340/F360 constructed after background image subtraction disclosed subcellular [Ca²⁺]i localization. The average cell [Ca²⁺]i values were calculated from calibration curve as described (26, 27).

Stretching Procedures on Flexible-bottom Plates

Rat osteocytes cultured on Matrigel were placed 5–7 mm from the edge of the flexible plate wells coated with type I collagen (5, 8). For stretching at the physiological level, the bottom of the plate was deformed by a computer operated vacuum system (Flexercell Strain Unit, Flexercell Corp., McKeesport, PA), with a frequency of 1/2.5 Hz (4 cycles of stretching in 10 s, followed by 50 s of relaxation) for 1 h. The expected strain was between 2,000 με (microstrain) and 4,000 με (0.4% elongation).

Introduction of Oligodeoxynucleotides

A pair of antisense/sense oligodeoxynucleotides (ODNs) of 24- and a 20-mer antisense ODN were developed from the sequence of α1C cDNA of the α subunit of the calcium channels isolated from the UMR 106/1 cells (28). The sequence of the antisense ODN (24-mer) was 5′-CTCTTGCTG-GCTTGGTCTGGGGCTCA-3′ and the sense ODN was 5′-TGACCCCGACCAACAGCAGGGAGGAG-3′. The sequence of the antisense 20-mer was ACCTTGAGGACACCTCTTCY. Cells were first washed with the permeabilization buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM NaATP, 100 mM Pipes, 0.1% bovine serum albumin (pH 7.4)) and then treated with the buffer containing 2.5 unit/ml streptolyisin O and the appropriate ODN at 100 μM for 5 min at room temperature. The incubation was terminated by replacing the buffer with the normal Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Eighteen hours later, cells were stretched either by swelling them in the hypotonic solution or by stretching them on flexible bottom plates. Ca²⁺ imaging or RT-PCR analysis of IGF-1 mRNA was performed as described under “Experimental Procedures.” Similar procedures were used for application of antisense ODNs to the α1S and α1D isoforms of the α1 subunits of the l-type calcium channels (18, 28).

RT-PCR Analysis of IGF-1

Total RNA was extracted from osteocyte cultures and was reverse-transcribed by random hexamer priming as described previously (8). Each RT reaction mixture contained 1 μg of total RNA, 50 pmol of random 9-mer, and 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.) in a total volume of 20 μl. After denaturation of mRNA at 70 °C for 10 min, the reaction was preincubated for 10 min at 25 °C and incubated at 42 °C for 50 min. Portions were as follows: 0.2–1 μl of the RT product cDNA were amplified using Ready To Go PCR Beads (Amersham Pharmacia Biotech) containing –1.5 units of Taq DNA polymerase. Sense and antisense primers (12.5 pmol) were added, and the reaction was carried out in 27 cycles for IGF-1 and 18 cycles for GAPDH, denaturation at 94 °C for 3 min, followed by set cycles of 30 s at 94 °C for denaturation; 30 s at 55 °C for annealing; and 1 min at 72 °C for polymerization. Primers used are as follows: IGF-1, 5′-GCATCTGCTGATGGTGTGC and 3′-GGCTCTCCTCCTAGTCATTAA; GAPDH, 5′-GCCATACGCCCGCCTTACATTAG and 3′-ACGGAAGGCCATGGCGACTGGCT (8).

For the quantitative real-time PCR analysis of IGF-1 mRNA levels, LightCycler™ System and reagents (Roche Molecular Biochemicals) were used with a double strand DNA binding dye, SYBR Green 1, according to the procedure provided by the manufacturer. Up to 95 °C at 9 s exposure, annealing for 10 s, and extension at 72 °C for 13 s were repeated.

RT-PCR Analysis of the L-type VOCC α1 Subunit in Rat Osteocytes

The PCR primers were oligodeoxynucleotides of 25 and 26 base pairs (bp) designed to amplify 903-bp fragments (29). PCR was carried out for 28 cycles after 2 min denaturation at 94 °C as follows: 30 s at 94 °C for denaturation; 30 s at 55 °C for annealing; and 4 min at 72 °C for polymerization. The amplimers were separated by electrophoresis through 3% agarose gels in Tris borate buffer.

Statistical Analysis

Statistical comparisons were made by one-way analysis of variance. When significant effects were observed, Dunnett’s test was used for multiple comparisons. A p value of less than 0.05 was considered significant.

RESULTS

Hypotonicity-induced Changes in [Ca²⁺]i of Cultured Osteocytes—The basal values of [Ca²⁺]i were not significantly different between young osteocytes (170 ± 7 nM, mean ± S.E., n = 167) and highly purified chicken osteocytes (133 ± 13 nM, mean ± S.E., n = 26). Hypotonic challenge was performed by replacing media by various low Na⁺ Ringer solutions as described previously in UMR 106 cells (17, 18). Cell swelling due to the replacement of the bathing media with hypotonic Ringer’s solution (65 mM NaCl, 182 mosm) caused a rapid and sustained increase of [Ca²⁺]i, in both rat (Fig. 1A) and chicken...
osteocytes (Fig. 1E). The duration of hypotonicity-stimulated
[Ca^{2+}i], increase varied among cells. The net increase in [Ca^{2+}i],
was dependent on the change in osmolarities as shown in Fig.
2. Hypotonicity-induced [Ca^{2+}i], increase was similarly
observed by using mannitol instead of the Ringer’s solution with
different Na+ concentrations to change osmolarities, suggest-
ing that the [Ca^{2+}i], increase observed here is not an artifact
due to Na+ decrease. Hypotonically-induced increases in both
[Ca^{2+}i], and mean cell volumes at different osmolarities are
plotted in Fig. 2. When cells in suspension were exposed to
Ringer’s solutions of lower osmolarity, mean cell volume
increased only 23%, the [Ca^{2+}i], increase of 21 ± 5 nM (n = 10)
was detectable. Thus, the [Ca^{2+}i], increase correlates well with
the changes in cell volume.

Under nominally Ca^{2+}-free conditions after replacing the
medium with Ca^{2+}-free Ringer’s solution containing 4 mM
EGTA, hypotonic treatment did not affect [Ca^{2+}i], in either rat
or chicken osteocytes (Fig. 1, C and F). Also pretreatment of the
cells with 2.5 μM thapsigargin, an inhibitor of intracellular
Ca^{2+} store refilling (30), caused a gradual [Ca^{2+}i], increase due
to the release from the internal Ca^{2+} stores but did not affect
the peak level of hypotonicity-induced [Ca^{2+}i], increase (Fig.
1D), suggesting that the influx of extracellular Ca^{2+} is primar-
ily responsible for the hypotonically induced increase in
[Ca^{2+}i]. In contrast, the [Ca^{2+}i], increase was inhibited by 80%
by an inhibitor of the stretch-activated cation channel, Gd3+
(Fig. 1B) (31). Further experiments using antagonists of VOCC,
nitrendipine (10^{-5} M), verapamil (50 μM) showed partial inhibi-
tion in hypotonicity-stimulated Ca^{2+} increases (data not shown
except that with nitrendipine appearing later in Fig. 6).

Micromolar concentrations of these Ca^{2+} channel antagonists
were required for significant inhibition of the volume-sensitive
Ca^{2+} influx pathway.

Involvement of α1C Subunit VOCC—In UMR 106.01 cells, it
was demonstrated by Duncan et al. (18) that antisense oligode-
oxynucleotides, ODNs, derived from the sequence of α1C sub-
unit of voltage-operated calcium channels abolish the whole-
cell conductance (Gm) induced by hypotonic swelling.
Therefore, we first examined the expression of α1C subunit by
RT-PCR according to Mezaros et al. (29). As shown in Fig. 3,
all three sets of primers they reported, PR1/2, PR11/12, and
2514/2759, gave us amplimers of the reported sizes (903, 740,
and 245 bp) in rat osteocytes. Then we tested whether anti-
sense ODNs (24- and 20-mer), directed to IVS6 region of the α1C subunit, have any effects on the hypotonicity-stimulated \([\text{Ca}^{2+}]\), increase observed in rat osteocytes. Antisense ODNs (24- and 20-mer) against the α1C subunit blocked the \([\text{Ca}^{2+}]\), increase by >80% (Fig. 4 and Table I). Neither antisense ODNs against the same IVS6 region in the α1S nor α1D subunit affected the hypotonic stimulation of \([\text{Ca}^{2+}]\). The introduction of these ODNs did not inhibit cell viability as observed by trypan blue exclusion. The volume-sensitive stretch-activated \(\text{Ca}^{2+}\) entry pathways in osteocytes, therefore, are dependent on the α1C subunit of VOCC or a closely related molecule, as was previously shown in UMR 106.01 cells (18).

**Potentiation of the Volume-sensitive Calcium Influx Pathway by PTH Involves Activation of Adenyl Cyclase**—As shown in Figs. 5 and 6, \(10^{-8}\) to \(10^{-6}\) M PTH-(1–34) enhanced the hypotonicity-induced \([\text{Ca}^{2+}]\), increase up to 2.2-fold in a dose-dependent manner. To determine whether activation of adenylyl cyclase is required for stimulation of volume-sensitive calcium influx pathway by PTH, we compared the effects of fully active PTH-(1–34), PTH-(1–31), a selective agonist that activates adenylyl cyclase but not phospholipase C (32), and PTH-(3–34), a selective agonist that activates phospholipase C but not adenylyl cyclase (33). PTH-(1–31) had a comparable effect on hypotonicity-induced \([\text{Ca}^{2+}]\), increase, whereas PTH-(3–34) showed no such effect. Next we studied the diastereoisomers of the phosphorothioate analogue of cAMP, \(\left(S_P\right)_n\)-cAMP, a selective stimulator of protein kinase A, and \(\left(R_P\right)_n\)-cAMP, a selective inhibitor (34, 35). As expected, \(\left(S_P\right)_n\)-cAMP had a stimulatory effect on hypotonicity-induced \([\text{Ca}^{2+}]\), increase similar to PTH-(1–34), whereas \(\left(R_P\right)_n\)-cAMP was inhibitory (Fig. 6). Other substances such as dibutyryl cAMP (10\(^{-3}\) M) and forskolin (10\(^{-5}\) M), which activate adenylyl cyclase signal transduction pathways, also stimulated hypotonicity-induced \([\text{Ca}^{2+}]\), increases. On the contrary, TPA (10\(^{-7}\) M) which activates protein kinase C signaling had no effect on hypotonicity-induced \([\text{Ca}^{2+}]\), increase (Figs. 5 and 6). Adenylyl cyclase signal transduction pathway seems to be required for stimulation of hypotonicity-induced \([\text{Ca}^{2+}]\), increases.

**PTH Potentiation of the Volume-sensitive Calcium Influx Pathway, Demonstrated by \([\text{Ca}^{2+}]\) Imaging**—Next the relationship between PTH-induced and hypotonically induced \([\text{Ca}^{2+}]\), changes was investigated by \(\text{Ca}^{2+}\) imaging (Fig. 7). Rat young osteocyte with extended cell processes exhibited relatively homogeneous basal subcellular \([\text{Ca}^{2+}]\), localization (Fig. 7B). Ten to 20 s (Fig. 7, C and D) after adding \(10^{-8}\) M PTH, higher submembranous \(\text{Ca}^{2+}\) concentration (yellow to red) appeared both in the cell processes and cell bodies resulting in dense localization in the cell processes. A very similar pattern in the subcellular \([\text{Ca}^{2+}]\), changes was observed after the hypotonic challenge (within 10–20 s, Fig. 7, F and G), supporting the notion that hypotonically induced cell swelling as well as PTH administration resulted in the \(\text{Ca}^{2+}\) influx into the osteocytes.

The images of the cell in Fig. 7 were further analyzed by averaging the \([\text{Ca}^{2+}]\), values of the pixels (\(2 \times 2\)) within three different areas, as indicated in Fig. 8. The resulting composite time-based plot is shown in Fig. 8 (Fig. 7A, cell process (1), submembranous region of the cell body (2), and perinuclear cytoplasm (3)). PTH-(1–34) (10\(^{-8}\) M) induced a transient in-


Table I

Effect of various ODN on hypotonicity-induced [Ca2+]i, increase in rat osteocytes

Rat osteocytes were incubated at 37 °C for 18 h after the treatment with various oligodeoxynucleotides (Sham: no ODN, streptolysin O treatment only) as described under “Experimental Procedures.” Data are expressed as means ± S.E. Number of experiments is in parentheses.

| DNA        | Effect of hypotonicity-induced [Ca2+]i, increase from basal (nM) |
|------------|---------------------------------------------------------------|
| Sham ODN  | 84.3 ± 11.4 (15)                                               |
| α11 24-mer sense | 112.5 ± 14.2 (15)                               |
| α11 24-mer antisense | 21.5 ± 8.9 (20)*                            |
| α12 24-mer antisense | 16.7 ± 7.3 (9)                 |
| α1S 24-mer antisense | 74.5 ± 6.8 (19)                               |
| α1D 24-mer antisense | 77.8 ± 11.4 (20)                              |

*p < 0.001 versus control (Sham ODN group).



Fig. 5. Effect of various substances on hypotonicity-induced [Ca2+]i, increase in single rat osteocytes. Cell preparations and [Ca2+]i measurements were performed as described under “Experimental Procedures.” A, effect of hypotonic replacement (363 to 182 mosm) on [Ca2+]i, shown as control (same as Fig. 1A). B, effect of PTH-(1–34) (10−7 M). C, PTH-(1–31) (10−7 M). D, dibutyryl cAMP (DibcAMP, 10−5 M). E, TPA (10−7 M). F, PTHrp-(1–34) (10−7 M). Test substance was added to the bathing medium before hypotonic stimulation. Concentration of these substances was kept constant during experiments. Tracings are representative of at least four experiments for each condition (see Fig. 6).

Fig. 6. Effect of various agents on hypotonicity-induced [Ca2+]i, increase in rat osteocytes. Experiments were performed as described in Figs. 1 and 5. Rat osteocytes were challenged with hypotonic replacement of buffer (363 to 182 mosm) after incubations with various agents or control vehicle shown here for 3 to 5 min if not specified. [Ca2+]i was measured as described under “Experimental Procedures.” Data are expressed as the amplitude of the effect of hypotonic replacement (363 to 182 mosm) on [Ca2+]i, in the presence of various substances, normalized to the response obtained in the presence of vehicle only. Means ± S.E. Number of experiments is in parentheses. *p < 0.05 versus control (vehicle only treated); **p < 0.01 versus control, ***p < 0.001 versus control. The control values in vehicle only treated groups are 100 ± 15 nM (n = 16) (vehicle H2O, 0.1%) and 108 ± 12 nM (n = 27) (vehicle EtOH, 0.1%).

Dependently up-regulated IGF-1 mRNA levels regardless of the presence (5th and 6th lanes) or absence (2nd and 3rd lanes) of stretching.

Elevation of the IGF-1 mRNA Level in Stretched Osteocytes Involves Activation of the Volume-sensitive Calcium Influx Pathway—Fig. 10 shows semi-quantitative analysis of stretch-induced IGF-1 mRNA up-regulation in the presence of various substances. Whereas PTH-(1–34) dose-dependently up-regulated IGF-1 mRNA levels regardless of the presence or absence of stretching, PTH-(3–34) had no such effect. PTH-(1–31) and dibutyryl cAMP (10−5 M) had an effect comparable to PTH-(1–34) on stretching-induced IGF-1 mRNA expression. We have also tested whether the volume-sensitive stretch-activated Ca2+ entry pathways of rat osteocytes described here is involved in stretch-induced IGF-1 mRNA up-regulation. Gd3+ (10−5 to 2 × 10−6 M, data not shown except that with 10−5 M Gd3+) and nitrendipine (10−6 M), which inhibited Ca2+ increases by hypotonic solution, inhibited stretch-induced IGF-1 mRNA elevation by 33–60%. Similarly, antisense ODN (24-mer) against the α1C subunit blocked the stretch-induced IGF-1 mRNA elevation by 51% (Fig. 10).
common type of cation entry in various cells, which are exposed to mechanical stress. SA-Cat was reported to be activated by cell swelling during the cell volume increase (31). In this study, we have characterized the stretch-activated Ca\(^{2+}\) entry pathways, which may represent SA-Cat activities in both rat young osteocytes and in highly purified chicken osteocytes. By swelling the cells in hypo-osmotic solution, single cell [Ca\(^{2+}\)]\(_i\) video-image analysis (25) visualized the hypotonicity-induced [Ca\(^{2+}\)]\(_i\) increase, a common feature of osteocytes in both species. Moreover, Ca\(^{2+}\) influx from the extracellular space explains the increased [Ca\(^{2+}\)]\(_i\) by hypotonicity in most part, since the increase was not observed in a Ca\(^{2+}\)-free medium containing EGTA. The Ca\(^{2+}\) influx was visualized in a single cell as shown in Fig. 7. This is the first presentation of subcellular Ca\(^{2+}\) distribution in osteocytes. Interestingly, Ca\(^{2+}\) increase by hypotonic swelling was predominantly seen in submembranous regions along the cell processes. A major event of osteocytic differentiation from osteoblasts is the development of extensive processes, which connect cell bodies between many osteocytes. Tanaka-Kamioka et al. (36) elegantly demonstrated that processes of chicken osteocytes were organized primarily by actin filaments. It has been suggested that cell processes connected through gap junctions sense mechanical strain and/or fluid shear stress resulting in the transmission of signals to neighboring osteocytes and osteoblasts (37). Our subcellular Ca\(^{2+}\) imaging clearly demonstrates a rapid Ca\(^{2+}\) influx, mainly along the cell processes. When bathed in hypotonic solution, osteocytes rapidly swell due to the influx of water through plasma membrane. Typically, mean cell volume rapidly reaches a peak within 3 min and gradually decreases. Such a time course of the regulatory volume changes is similar to that of the [Ca\(^{2+}\)]\(_i\)-increase that was measured separately in single cells. In addition, the peak [Ca\(^{2+}\)]\(_i\) increase caused by the hypotonic exposure and measured in single cells on a glass coverslip paralleled the increase in mean cell volume, which was measured in the cells suspended in the same hypotonic solution. All these data indicate that the Ca\(^{2+}\) influx pathway observed here is cell volume-dependent and is possibly membrane stretch-activated. The Ca\(^{2+}\) influx in turn, may activate Ca\(^{2+}\)-activated Cl\(^-\) channels as well as Ca\(^{2+}\)-activated K\(^+\) channels leading to the decreased cell volume as reported in other cell types (31, 38). Under the conditions employed, the [Ca\(^{2+}\)]\(_i\)-increase was detectable when the mean cell volume increased by 23% from the basal volume. This result indicates that osteocytes are more sensitive than endothelial cells, in which 20% elongation on silicon membrane caused little change in [Ca\(^{2+}\)]\(_i\) (39). Osteocytes appear to be extremely sensitive to mechanical strain since our previous study of anabolic reaction showed that young osteocytes respond to low levels of mechanical strain (up to 4,000 μE) to which osteoblasts do not respond (5). It is quite likely that the development of extensive cell processes, which is accompanied by differentiation of osteocytes, allows these cells to acquire sensitivity to stretching by the SA-Cat concentrated along the processes, as we presented in this article.

Pharmacological studies to characterize the osteocyte SA-Cat using different ion channel blockers revealed the following. Hypotonicity-induced Ca\(^{2+}\) influx in osteocytes, in most part, is Gd\(^{3+}\)-inhibitable but relatively insensitive to blockers of VOCC, such as a dihydropyridine derivative, nitrendipine, or verapamil, which are only effective at micromolar concentrations. The overall pharmacological characteristics of this Ca\(^{2+}\) influx described here may be similar to that of UMR 106 cells (18, 40). Since the molecular identity of the SA-Cat is uncertain, we can only speculate on the nature of the protein from the properties of the osteocyte Ca\(^{2+}\) influx pathways. Antisense

DISCUSSION

Several in vivo and in vitro studies of osteocytes demonstrated that mechanical stress, either directly applied on vital bone, by exposing to pulsating fluid flow, or by deformation of cells plated on silicon membrane, induced rapid responses in mRNA expression of various genes as described in the Introduction. This accumulating evidence as well as biomechanical studies suggest that osteocytes respond to mechanical stress and transmit signals to other cells in bone. Stretch-activated cation channels (SA-Cat), or mechanosensitive channels are a

![Fig. 7. Digital image analysis of [Ca\(^{2+}\)]\(_i\), in single fura-2 loaded rat osteocytes during exposure to PTH-(1-34) (10^{-8} m) and hypotonic Ringer's solution (182 mosm). A, phase contrast image. An isolated rat osteocyte with long processes is visible. B, basal 340/360 nm ratio image, converted to [Ca\(^{2+}\)]\(_i\), C–E, sequence of images taken 10, 20, and 120 s after the addition of 10^{-6} m PTH. F and G, sequence images taken 10 and 20 s after the replacement of hypotonic Ringer's solution (182 mosm) in the presence of PTH. The ratio color scale, converted to [Ca\(^{2+}\)]\(_i\), as described under “Experimental Procedures”, is shown on the right of the figure.](http://www.jbc.org/)

![Fig. 8. Time-based plot of the experiment illustrated in Fig. 7. [Ca\(^{2+}\)]\(_i\), was averaged within three different subcellular areas (2 x 2 pixels) indicated in Fig. 7A at different time points during stimulation with PTH and hypotonic Ringer's solution (182 mosm).](http://www.jbc.org/)
The value of antisense ODN-treated group is significantly (p, stretched rat osteocytes. Cells were stretched in the presence of sense or antisense ODNs 18 h prior to the experiments. Data are expressed as the amplitude of the response in antisense ODN-treated group, normalized to the sense ODN-treated group; mean ± S.E., n = 3. The ratio of IGF-1/GAPDH amplimers was calculated from PCR results with LightCyclerTM System and SYBR Green I dye. Data in the presence of various substances are normalized to the value of vehicle experiment. Mean ± S.E., n = 6. **, p < 0.01 versus stretched control (S); *, p < 0.05 versus stretched control (S). For the experiments of α1C antisense ODN (24-mer), cells were loaded with sense or antisense ODNs 18 h prior to the experiments. Data are expressed as the amplitude of the response in antisense ODN-treated group, normalized to the sense ODN-treated group; mean ± S.E., n = 3. The value of antisense ODN-treated group is significantly (p < 0.05) different from sense ODN-treated group although the value of sense-treated group was variable depending on the experiment.

oligodeoxynucleotides (24- and 20-mer) against the α1C subunit of UMR106 Ca2+ channel significantly inhibited hypotonic increase of [Ca2+]i, by 75–80% (Table 1). In contrast, antisense oligodeoxynucleotide (24-mer) against the α1D and α1S subunits of UMR106 Ca2+ channel had no significant effects. These results suggest the common properties of SA-Cat to that in UMR 106 osteoblast-like cells (18). Considering the fact that UMR 106 is a transformed cell line of heterogenous phenotype containing cells at various stages of differentiation in the osteoblast lineage, it is not surprising that they express SA-Cat of similar characteristics. We have used the antisense oligodeoxynucleotides against the region S’ of the S6 domain IV of α1C subunit of VOCC, and we have clarified that the expression of α1C subunit of Ca2+ channel is required for the function of stretch-activated Ca2+ influx pathways in osteocytes. The Ca2+ influx in osteocytes is sensitive to micromolar concentration of dihydropyridine and that the depolarization of cell membrane by high potassium solution (KCl 141 mM, NaCl 20 mM Ringer’s solution) induced the [Ca2+]i increase (70 ± 8 (S.E.) (n = 6) in 54.5% (6/11) cells examined. These observations suggest either the heterogeneity of the population or that the number of the molecules of α1C subunit of VOCC in osteocytes is not large enough to create persistent [Ca2+]i increases in response to membrane depolarization. In rat smooth muscle cells, Langton (41) reported that a VOCC itself is activated by membrane stretch. It is clear that α1C subunit of Ca2+ channel observed here has a pivotal role in the Ca2+ influx in stretched osteocytes. Whether these α1C channels act as an SA-Cat or that SA-Cat is an independent entity which is somehow regulated by α1C subunit of VOCC, however, remains to be answered.

We have found that PTH enhanced hypotonically stimulated Ca2+ influx in osteocytes. The relationship between the effects of PTH and mechanical loading has already been the subject of many studies. Synergistic effects of mechanical stress and PTH on bone formation were reported in in vivo studies (13, 15). In primary rat osteoblasts, Carvalho et al. (42) reported that mechanical strain induced cAMP and IP3 increases and that PTH augmented these effects. As described in the Introduction, PTH potentiated stretch-activated cation channels in UMR 106 cells (16). Recently, it was demonstrated by in situ hybridization that PTH primed loading-induced c-fos mRNA expression in osteocytes (13). They also demonstrated in rat bones that PTH injection augmented osteogenic responses to mechanical loading. Moreover, no osteogenic response was seen in thyrroparathyrectomized rats. Under the conditions they tested, c-fos expression was detected only in the osteocytes of those thyroparathyrectomized rats that were mechanically stimulated together with PTH administration. These results suggest that PTH either sensitizes strain-sensing mechanism or is involved in the downstream signaling. Our results showed that PTH up-regulated strain sensing of osteocyte by enhancing Ca2+ influx resulting in up-regulation in the IGF-1 mRNA level. The presence of PTH receptors in osteocytes has been demonstrated.
recently by in situ hybridization (14). In rat osteocytes, hypotonicity-induced Ca\textsuperscript{2+} influx was enhanced by pretreating cells with PTH in a dose-dependent manner (Figs. 5 and 6). Subcellular Ca\textsuperscript{2+} imaging (Figs. 7 and 8) suggested that PTH and hypotonic treatment stimulated [Ca\textsuperscript{2+}]\textsubscript{i}, increase predominately along the cell processes. PTH receptors and stretch-activated Ca\textsuperscript{2+} channels are likely to be co-localized in the processes acting as a mechanosensor. The PTH receptor is known to stimulate G protein-coupled cAMP production as well as phospholipase C activation (43). As shown in Fig. 6, enhancement of hypotonicity-induced Ca\textsuperscript{2+} influx by PTH was mimicked by dibutyryl cAMP, forskolin, (S)\textsubscript{p}-cAMP, a specific activator of protein kinase A, and PTH-(1–31), a specific agonist that activates adenyl cyclase but not phospholipase C (32). Neither PTH-(3–34), another specific agonist that activates only the phospholipase C (33), nor TPA which activates protein kinase C have stimulatory effect on the [Ca\textsuperscript{2+}]\textsubscript{i} increase. Moreover, (R)\textsubscript{p}-cAMP, a specific inhibitor of protein kinase A, is inhibitory on the hypotonicity-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase. Thus, activation of adenylate cyclase by PTH is required in its potentiation of hypotonicity-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase. Besides PTH-(1–34), PTHrp-(1–34) acted similarly, whereas C-terminal fragment of PTH-(35–84) was not effective. The rapid increase in cAMP concentration in stretched rat osteocytes (5) is likely representative of mechanosensing machinery that PTH synergistically up-regulates IGF-1 mRNA expression is mediated through the activation of adenylate cyclase (Figs. 9 and 10). It should be noted that PTH up-regulates IGF-1 mRNA levels in many helpful discussions.

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