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Spontaneous and Stimulated Transients in Cytoplasmic Free Ca^{2+} in Normal Human Osteoblast-like Cells: Aspects of Their Regulation

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We characterize two patterns of transients in cytoplasmic free calcium ($[Ca^{2+}]_i$) in normal human osteoblast-like cells (hOB cells). Firstly, spontaneous oscillations in $[Ca^{2+}]_i$ were found to be common. The $[Ca^{2+}]_i$ oscillations were completely inhibited by thapsigargin, indicating that Ca^{2+} fluxes between intracellular Ca^{2+} pools and the cytosol contributed to the generation of the $[Ca^{2+}]_i$ oscillations. Removing extracellular Ca^{2+} either attenuated or completely inhibited spontaneous $[Ca^{2+}]_i$ oscillations. Gadolinium, an inhibitor of stretch activated cation channels (SA-cat channels), reduced the frequency of $[Ca^{2+}]_i$ oscillations. Hence, entry of calcium from the extracellular space, possibly through SA-cat channels also seemed to be of importance in the regulation of these $[Ca^{2+}]_i$ oscillations. The role of the observed spontaneous $[Ca^{2+}]_i$ oscillations in hOB cell function is not clear. Secondly, a decrease in pericellular osmolality, which causes the plasma membrane to stretch, transiently increased $[Ca^{2+}]_i$ in hOB cells. This effect was also observed in a Ca^{2+} free extracellular environment, suggesting that osmotic stimuli release Ca^{2+} from intracellular pools. This finding indicates a possible signaling pathway by which mechanical strain can promote anabolic effects on the human skeleton. © 1999 Academic Press

Key Words: **human osteoblasts; $[Ca^{2+}]_i$ oscillations; SA-cat channels; mechanical strain; osmotic stimuli.**

Observations of spontaneous (1, 2) as well as stimulated oscillations (3–5) in cytoplasmic free calcium ($[Ca^{2+}]_i$) have been reported in many cell types. $[Ca^{2+}]_i$ oscillations may be coupled to numerous cellular functions, e.g. pulsatile insulin secretion (6) and cardiac myocyte contraction (7). Release of calcium from intracellular stores, or influx of Ca^{2+} through calcium channels in the plasma membrane have been demonstrated as constituting underlying mechanisms regulating $[Ca^{2+}]_i$ oscillations (8).

$[Ca^{2+}]_i$ oscillations induced by parathyroid hormone (PTH) in rat osteoblast-like cells (9) have been reported to be mediated by SA-cat (stretch-activated cation) channels. These ion channels are non-selective cation channels (10–12) and are considered to play a role in mediating the anabolic effects of mechanical loading in bone (13), possibly by regulating the release of auto/paracrine regulators of bone cell function. Rawlinson *et al.* have e.g. shown that prostaglandin E_2 release in rat ulnae induced by mechanical loading could be inhibited by gadolinium (14), which is an inhibitor of SA-cat channels (15). Sakai *et al.* have demonstrated that gadolinium also could inhibit fluid shear stress-induced release of transforming growth factor-β from human osteosarcoma cells (16).

Spontaneous $[Ca^{2+}]_i$ oscillations have not previously been reported in hOB cells and little is known about the influence of mechanical stimuli on $[Ca^{2+}]_i$ in these cells. In the present study we demonstrate spontaneous oscillations in $[Ca^{2+}]_i$ in hOB cells and investigate some aspects of their regulation, including the possible role of SA-cat channels. Furthermore, we demonstrate that the $[Ca^{2+}]_i$ response of these cells to osmotic/mechanical stimuli involved mechanical stretch of the plasma membrane.

MATERIALS AND METHODS

Materials. α-MEM medium, fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate buffered saline (PBS), penicillin, streptomycin, *l*-glutamine and trypsin-EDTA were all obtained from Gibco (Grand Island, NE). Fura-2 acetoxymethylester (AM), gadolinium chloride, MgATP, type IV collagenase and alkaline phosphatase staining reagents (naphthol AS-TR and diazonium salt fast-red violet LB) were obtained from Sigma (St. Louis, MO). 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3) was a generous gift from Roche Pharmaceuticals Sweden. Nifedipine and thapsigargin (Sigma) were dissolved in DMSO and 1,25-$(OH)_2$ D_3 in 95% ethanol. Osteocalcin was measured with the ELSA-OST-NAT immunoradiometric kit

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TABLE 1

Statistical Summary Concerning the Presence of Spontaneous $[Ca^{2+}]_i$ Oscillations in Normal Human Osteoblast-like Cells Derived from 11 Patients and Studied at the Single Cell Level

Patient	Gender/age	Oscillating cells*	%
A	Female/70	5/10	50
B	Male/77	7/9	78
C	Female/77	2/8	25
D	Male/51	3/7	43
E	Male/17	3/7	43
F	Male/70	4/12	33
G	Male/70	1/5	20
H	Female/79	3/4	75
I	Female/69	4/8	50
J	Male/67	4/8	50
K	Male/81	2/16	13

* Number of cells displaying oscillations/number of cells studied.

(CIS BioInternational, Gif-Sur-Yvette Cedex, France), detection limit 0.3 ng/ml.

Preparation and characterization of hOB cells. Primary cultures of normal human osteoblast-like cells (hOB cells) were prepared from trabecular bone material obtained during hip- and knee-joint replacement surgery, as previously described (17). None of the bone donors were taking any medication known to affect bone metabolism. The bone material was kept in α-MEM medium supplemented with 10% FBS, 2 mM *l*-glutamine, 50 IU/l penicillin and 50 μg/ml streptomycin at 4°C. Within 12 hours the bone material was scraped with a scalpel and shaken in PBS to remove fat, cartilage and marrow. Thereafter the bone was cut into 2 × 2 mm fragments, which were incubated in protein free α-MEM medium with 1 mg/ml type IV collagenase for 15 minutes at 37°C during continuous shaking. This procedure was repeated four times, with changes of the collagenase solution between each cycle. Finally the fragments were transferred to 75 cm^2 culture flasks containing α-MEM with 10% FBS + *l*-glutamine, penicillin and streptomycin in concentrations as described above to allow osteoblast migration. The cells were grown to confluency (5–8 weeks) with changes of cell medium twice every week. All cell cultures were incubated at 37°C with a humidified atmosphere of 5% CO_2 and 95% air. This study was approved by the Local Ethical Committee at the Karolinska Hospital.

All hOB cell preparations were tested for the presence of alkaline phosphatase activity and osteocalcin production induced by 96 hours of incubation with 1,25-$(OH)_2D_3$ for 96 hours, as previously described (17). All cell cultures used in this study responded with significantly increased osteocalcin production after 1,25$(OH)_2$ D_3 stimulation and displayed positive alkaline phosphatase stainings (data not shown).

Measurement of $[Ca^{2+}]_i$. HOB cells were seeded on glass coverslips, grown to subconfluency, and then incubated in a buffer (125 mM NaCl, 5.9 mM KCl, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 3 mM glucose, 25 mM HEPES and 1mg/ml BSA, pH adjusted to 7.4) with 1 μM fura-2/AM for 20 minutes at 37°C. The coverslips with fura-2 loaded cells were then used as part of the bottom of an open chamber designed for microscopic work, covering a centrally located circular hole in the bottom plate. A rubber ring constituted the chamber wall and was pressed to the coverslip by a circular steel plate, with a central opening and by a thin steel ring. Two cannulas penetrated the top piece of the chamber and were connected to a two channel peristaltic pump (Ismatec), allowing steady superperfusion of the cells. A superperfusion rate of 300 μl/min. was used. The chamber was placed in a holder on the stage of an inverted microscope (Zeiss, Axiovert 35M). The stage itself was thermostatically controlled to maintain a temperature of 37°C in the perfusate inside the chamber. The microscope was equipped with a photon counting photometer and was connected to a SPEX fluorolog-2 CM1T11I system, allowing fluorometry using two excitation wavelengths. The excitation wavelengths (340 and 380 nM) were generated by two monochromators and emitted light was collected through a 510 nm filter inside the microscope. One 340/380 fluorescence ratio was obtained every second. By using a 40×, 0.75 NA, objective and by changing the diameter of a variable diaphragm in the microscope, it was possible to select and measure from a single cell. The figures show representative experiments from a series of at least four performed under identical experimental settings, unless otherwise stated.

Transformation of 340/380 fluorescence ratios into $[Ca^{2+}]_i$ values was done using the formulas in Grynkiewicz *et al.* (18). The K_d for the Ca^{2+}-fura-2 complex was taken as 220 nM (18). Values for maximum and minimum fluorescence ratios were obtained in separate experiments using 1 μl drops of a K^+ rich buffer with an ionic composition similar to the intracellular milieu. The drops contained free fura-2 acid and either a saturating Ca^{2+} concentration or no Ca^{2+} in the presence of EGTA. Such standard curves obtained *in vitro* have been reported to be almost identical to standard curves generated *in situ* (19). In one of the experiments presented (Fig. 5D) values for the background fluorescences were not available, and therefore only the fluorescence ratio is given.

Electrophysiological studies. Whole-cell Ca^{2+} currents were recorded using the patch-clamp technique (20). Pipettes were pulled from borosilicate glass capillaries (Hilgenfeld, Malsfeld, Germany) on a horizontal programmable puller (DMZ Universal puller, Zeit-Instrumente, Augsburg, Germany). Typical electrode resistance was 3–5 MΩ. Electrodes were filled with a standard internal solution containing (mM): 150 *N*-methyl-D-glycamine, 10 EGTA, 1 $MgCl_2$, 2 $CaCl_2$, 3 Mg-ATP and 5 HEPES (pH 7.2–HCl). The cells were bathed in a solution consisting of (mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 10 $CaCl_2$, 10 TEA and 5 HEPES (pH 7.4–NaOH). Depolarizing voltage pulses (100 ms) to membrane potentials between −60 and +50 mV were given from a holding potential of −70 mV. The resulting currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Acquisition and analysis of current traces were done using the software program pClamp (Axon Instruments). All experiments were performed at room temperature and repeated four times under identical experimental settings.

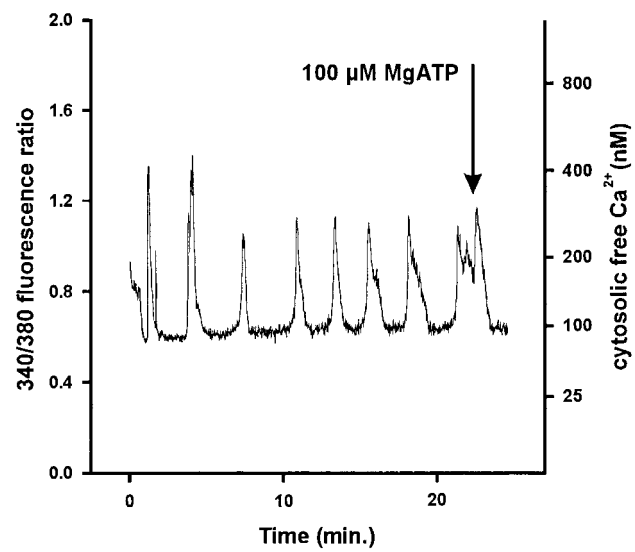

FIG. 1. Spontaneous $[Ca^{2+}]_i$ oscillations in an hOB cell. Addition of 100 μM MgATP (indicated by arrow) results in a superimposed $[Ca^{2+}]_i$ transient.

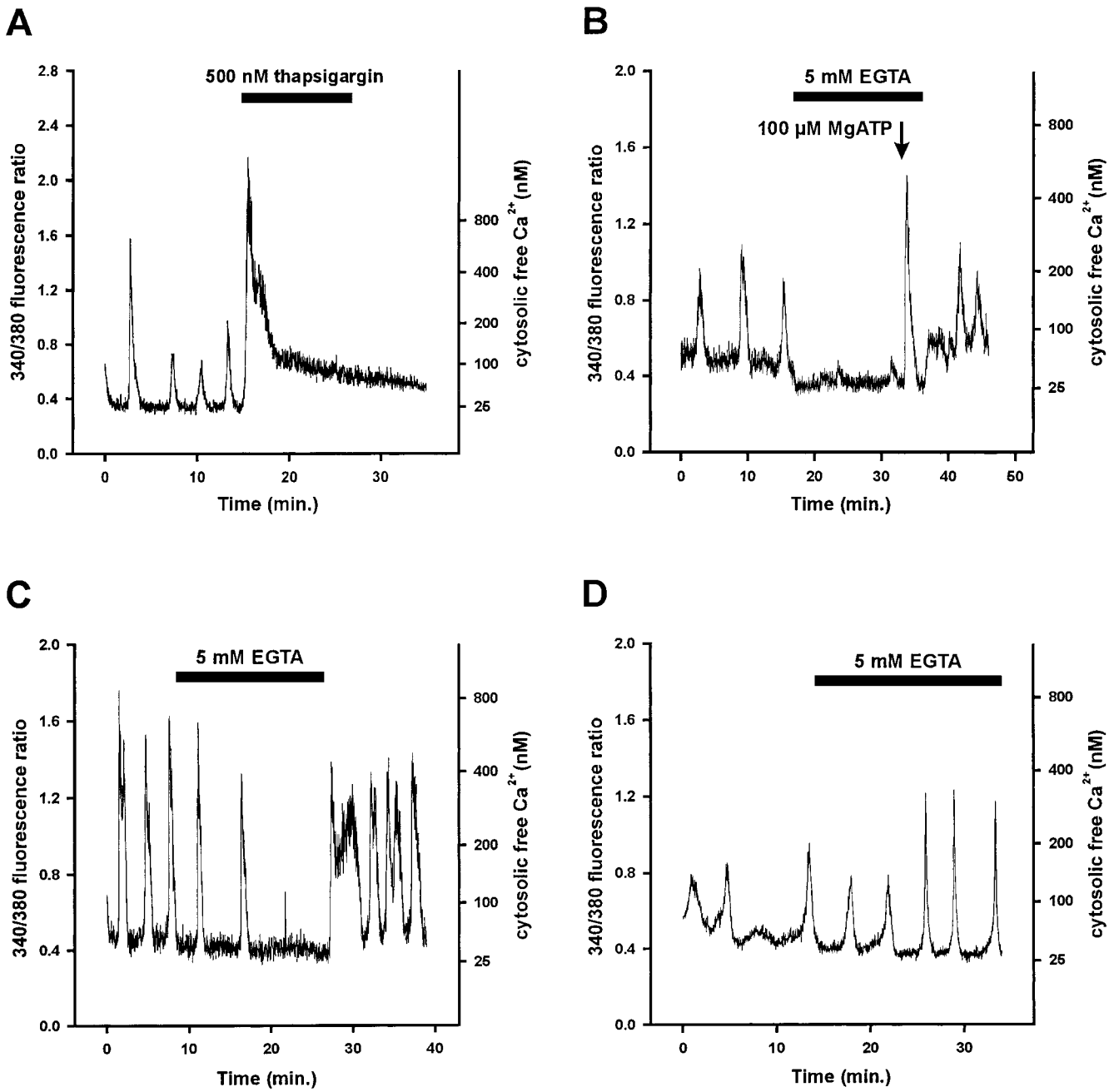

FIG. 2. Cessation of spontaneous $[Ca^{2+}]_i$ oscillations following a $[Ca^{2+}]_i$ increase in an hOB cell exposed to 500 nM thapsigargin (A). Immediate (B), gradual (C), and no (D) inhibition of spontaneous $[Ca^{2+}]_i$ oscillations in hOB cells exposed to 5 mM EGTA. Distinct $[Ca^{2+}]_i$ response in an hOB cell exposed to 100 μM MgATP in the presence of 5 mM EGTA (B). Addition of thapsigargin, EGTA and MgATP indicated by horizontal bars and vertical arrow.

RESULTS

Presence of $[Ca^{2+}]_i$ Oscillations in Single hOB Cells

Spontaneous oscillations were defined as at least 3 transient increases in $[Ca^{2+}]_i$, each having an amplitude of at least 3 times the basal noise amplitude. The percentages of hOB cells displaying spontaneous $[Ca^{2+}]_i$ oscillations in the various cell preparations used in this study are presented in Table 1. The oscillations had a frequency of $\approx$0.25–0.6 $\times$ min^{-1}. The basal $[Ca^{2+}]_i$ level during the oscillations was typically $\approx$25–40 nM and reached peak $[Ca^{2+}]_i$ levels of $\approx$100–700 nM. Cells which did not respond with an increase in $[Ca^{2+}]_i$, either after exposure to 100 μM MgATP (21), or osmotic stimuli (see below), were considered to be anormal, and were disregarded. In cells displaying

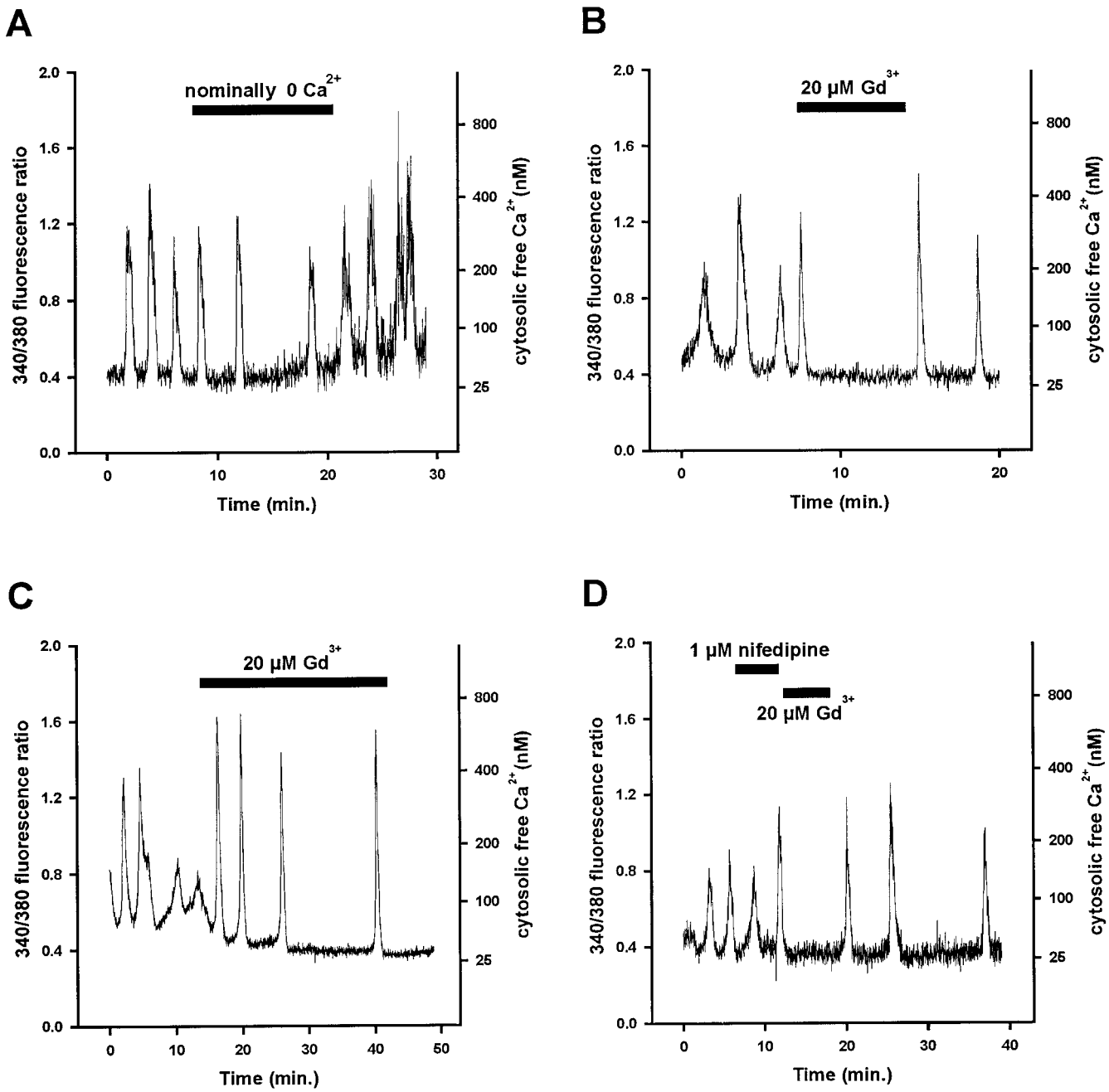

FIG. 3. Reduced frequency of spontaneous $[Ca^{2+}]_i$ oscillations in an hOB cell exposed to a buffer with no added Ca^{2+} (nominally 0 Ca^{2+}) (A). Immediate (B) and gradual (C) inhibition of $[Ca^{2+}]_i$ oscillations in hOB cells exposed to 20 μM Gd^{3+}. An hOB cell first exposed to 1 μM nifedipine before exposure to 20 μM Gd^{3+} (D). Removal of extracellular Ca^{2+} and addition of Gd^{3+} and nifedipine indicated by horizontal bars.

spontaneous $[Ca^{2+}]_i$ oscillations, addition of 100 μM MgATP resulted in an increase in $[Ca^{2+}]_i$ that was superimposed on the oscillations (Fig. 1).

Characterization of Spontaneous $[Ca^{2+}]_i$ Oscillations in hOB Cells: Electrophysiological Studies

Application of thapsigargin, a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, caused a strong increase in $[Ca^{2+}]_i$ followed by a complete and irreversible inhibition of $[Ca^{2+}]_i$ oscillations (Fig. 2A). When Ca^{2+} was omitted from the perfusion buffer and 5 mM EGTA was added, a reversible inhibition of $[Ca^{2+}]_i$ oscillations was observed (n = 14). The inihibitory effect was either immediate (Fig. 2B) or gradual (Fig. 2C), but in two of the cells which were studied, there was no effect even after ≈15 minutes exposure to 5 mM EGTA (Fig. 2D). There was a distinct increase in $[Ca^{2+}]_i$ in cells which were exposed to 100 μM MgATP

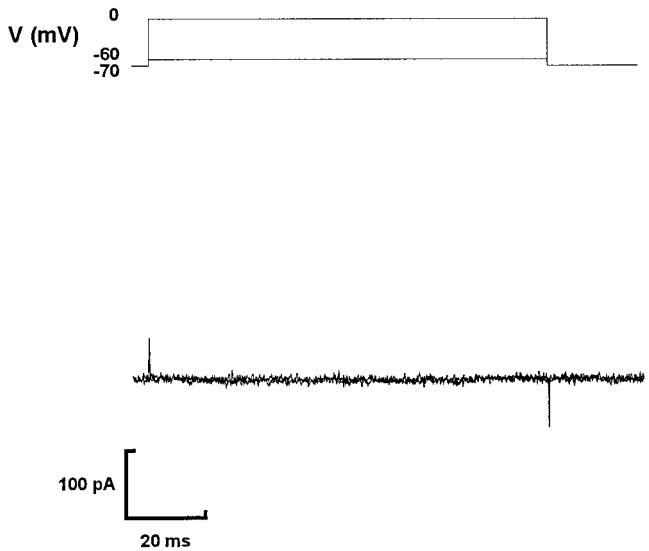

FIG. 4. Recording of Ca^{2+} currents from hOB cells using the whole-cell configuration of the patch-clamp technique. The upper part of the figure illustrates depolarizations to −60 and 0 mV from a holding potential of −70 mV. As can be seen from the two corresponding superimposed current traces (nearly indistinguishable) in the lower part of the figure, no voltage-activated Ca^{2+} currents could be observed.

in the presence of 5 mM EGTA (Fig. 2B). This shows that the inhibitory effect of EGTA on the oscillations was not due to total depletion of intracellular Ca^{2+} pools. A buffer containing no added Ca^{2+} and no EGTA, reduced the frequency of the oscillations (−44 ± 8%, mean ± S.E.M., n = 5), but did not completely inhibit the oscillations (Fig. 3A). The frequency of the oscillations was also reduced by 20 μM gadolinium (Gd^{3+}) (−62 ± 3%, mean ± S.E.M., n = 5), and this effect was also either immediate or gradual (Figs. 3B, 3C, 3D). The amplitude of the $[Ca^{2+}]_i$ oscillations did not appear to be influenced by Gd^{3+}. There was no effect on the oscillations of 1 μM nifedipine, an inhibitor of plasma membrane L-type Ca^{2+} channels (Fig. 3D), or of stopping the peristaltic perfusion system (data not shown).

Recordings of Ca^{2+} currents from hOB cells using the whole-cell configuration of the patch-clamp technique were performed to study the possible presence of voltage-gated calcium channels. As shown in Fig. 4, no voltage activated Ca^{2+} currents could be observed in our hOB cells.

$[Ca^{2+}]_i$ Responses to Osmotic Stimuli in hOB Cells

Depolarization of the plasma membrane potential of the hOB cells with 50 mM KCl (100 milliosmoles) did not elicit any change in $[Ca^{2+}]_i$. However, when the basal perifusion buffer was reinstated, there was a transient $[Ca^{2+}]_i$ increase (Fig. 5A). A similar effect could be produced by first increasing the osmolality with 100 milliosmoles of sucrose, and then reinstating the basal perifusion buffer (Fig. 5B). Thus, a decrease in osmolality from a hyperosmolar environment to a physiological osmolality resulted in a transient $[Ca^{2+}]_i$ increase. Repeating this type of stimulus in a single cell caused repeated $[Ca^{2+}]_i$ transients to occur (Figs. 5A, 5B). A similar effect could be produced by decreasing the osmolality of the basal experimental buffer by 100 milliosmoles (Fig. 5C). An increase in $[Ca^{2+}]_i$ caused by a relative decrease in osmolality could also be observed in the presence of 5 mM EGTA (Fig. 5D), or 20 μM Gd^{3+} (data not shown). This shows that the $[Ca^{2+}]_i$ increase in response to osmotic stimuli was caused by release of Ca^{2+} from intracellular stores.

DISCUSSION

The present study shows that spontaneous $[Ca^{2+}]_i$ oscillations are a common phenomenon in normal human osteoblast-like cells (hOB cells). To our knowledge such spontaneous oscillations have not been shown previously. Bidwell *et al.* demonstrated PTH-induced $[Ca^{2+}]_i$ oscillations in ROS 17-2.8 rat osteosarcoma cells (9). However, these $[Ca^{2+}]_i$ oscillations were not spontaneous and the possible involvement of stretch activated cation (SA-cat) channels was not studied. The $[Ca^{2+}]_i$ oscillations were also of a higher frequency and displayed a lower amplitude than those observed in the present study. The spontaneous oscillations in the present study seem to be dependent on Ca^{2+} fluxes between intracellular pools and the cytosol, as they were completely inhibited by thapsigargin. However, influx of extracellular Ca^{2+} also seems to be of significance as the oscillations in most experiments could be inhibited by withdrawal of Ca^{2+} from the extracellular environment. The entry of Ca^{2+} through the plasma membrane might be mediated by SA-cat channels, since the frequency of the oscillations also was inhibited by gadolinium.

In two cases the $[Ca^{2+}]_i$ oscillations appeared to be independent of extracellular Ca^{2+}. Surprisingly, the amplitude of the oscillations in these particular cells seemed to increase when the cells were exposed to a Ca^{2+} free environment. Even if the experiments in this study were performed on primary cultures testing positive in osteoblast marker assays, it is possible that the two cells which displayed oscillations independent of extracellular Ca^{2+}, may have had a non-typical phenotype.

Since SA-cat channels seem to play a role in the generation of spontaneous $[Ca^{2+}]_i$ oscillations, we wanted to study the effects of mechanical stretch of the plasma membrane of hOB cells. A relative decrease in pericellular osmolality causes cellular swelling, and consequently a mechanical stretch of the plasma membrane. This procedure of osmolality decrease has previously been used to mimic mechanical stress on cells (12). We found that a relative decrease in pericellular

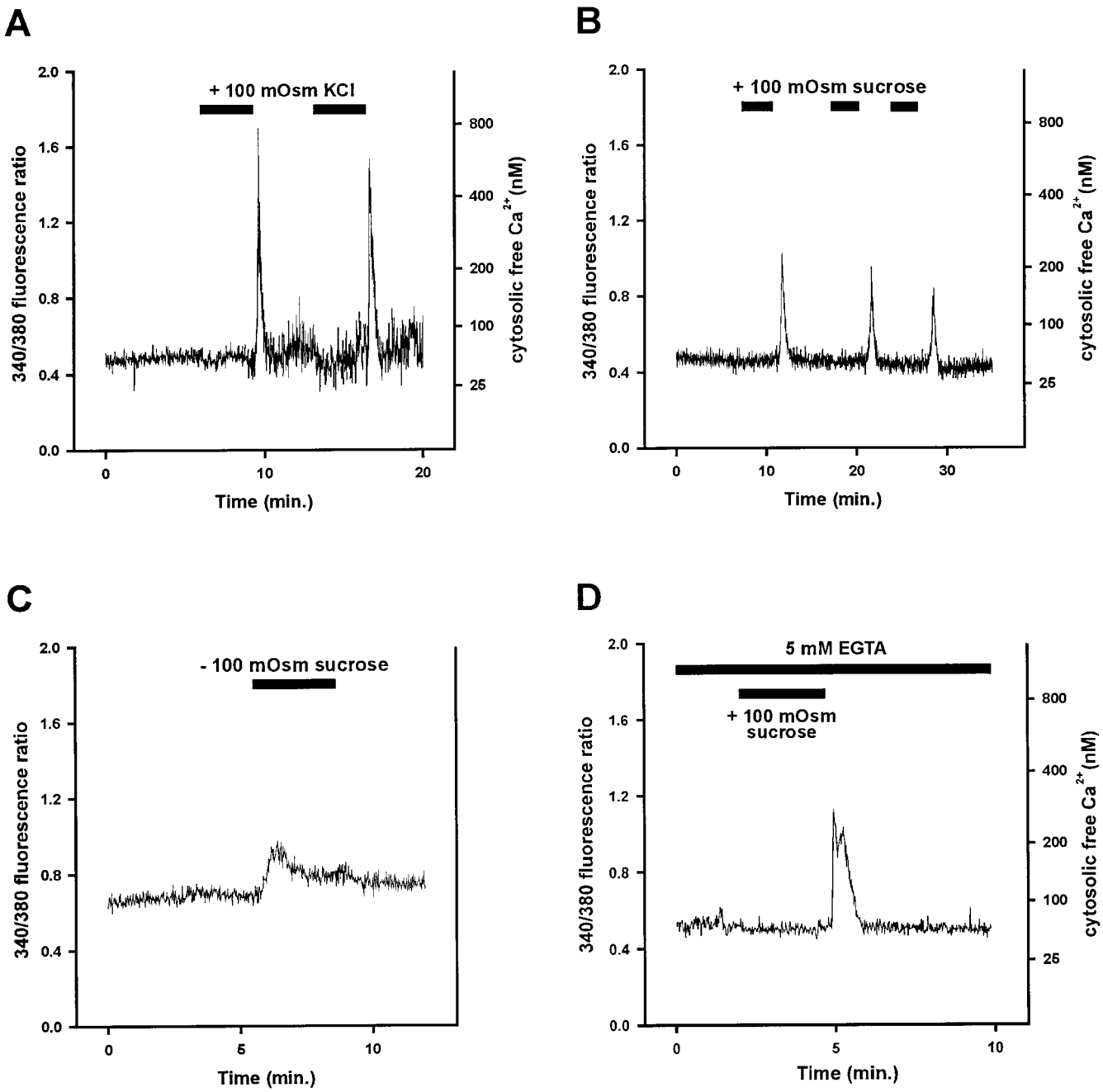

FIG. 5. $[Ca^{2+}]_i$ in hOB cells exposed to relative decreases in osmolality, either after a primary increase by 50 mM KCl (100 milliosmoles) (A) or 100 mM sucrose (100 milliosmoles) in the presence of extracellular calcium (B), or by a primary decrease in osmolality (C), or 100 mM sucrose in the presence of 5 mM EGTA (no added calcium) (D). In (C) the cells were first perfused with a buffer containing 100 mM sucrose and 25 mM NaCl (instead of 125 mM NaCl). The osmolality was decreased by withdrawing sucrose. This procedure allowed a decrease in osmolality to be imposed without changing extracellular ion concentrations. Buffer changes indicated by horizontal bars.

osmolality increases $[Ca^{2+}]_i$ in hOB cells. This effect could also be demonstrated in the absence of extracellular Ca^{2+}, and could not be inhibited by Gd^{3+}. The $[Ca^{2+}]_i$ increases in response to osmotic stimuli in hOB cells was thus caused by Ca^{2+} release from intracellular stores. In contrast to the present study, Yamaguchi *et al.* (12) found that $[Ca^{2+}]_i$ increases in response to hypoosmotic stimuli in a rat osteoblast-like cell line (UMR 106-01) were totally dependent on extracellular Ca^{2+}. Furthermore, Dascalu *et al.* (22), using another type of rat osteoblast-like cell line (RCJ 1.20), found that a relative *increase* in osmolality elevated $[Ca^{2+}]_i$. In these respects, human and rat osteoblast-like cells seem to be different.

Voltage-gated Ca^{2+} channels have previously been reported in various osteosarcoma cell lines (11, 23, 24) and in rat calvarial osteoblasts (25), and have been suggested to mediate hormonal effects (26). The presence of voltage-gated Ca^{2+} channels in hOB cells has not been investigated previously. We have excluded the presence of voltage-gated Ca^{2+} channels in our hOB cells using several experimental methods. First, the $[Ca^{2+}]_i$ oscillations which we here report, were not dependent on influx of Ca^{2+} through plasma membrane voltage-gated calcium channels, since nifedipine (a blocker of L-type voltage-gated calcium channels) had no effect on the oscillations. Second, we were unable to detect $[Ca^{2+}]_i$ increases in response to plasma membrane depolarization with 50 mM K^+, or Ca^{2+} currents using electrophysiological methods. Instead, $[Ca^{2+}]_i$ increased when the elevated K^+ concentration returned to normal, possibly because of a corresponding reduction in osmolality (12).

To our knowledge, there has been no previous report of spontaneous $[Ca^{2+}]_i$ oscillations in osteoblast-like cells. Future studies are needed to clarify the possible role of this phenomenon in bone metabolism. It would be of interest to investigate the possible presence or absence of spontaneous $[Ca^{2+}]_i$ oscillations in osteoblasts derived from patients with osteoporosis or osteomalacia. Studies of possible correlations between the presence of spontaneous $[Ca^{2+}]_i$ oscillations and the magnitude of bone matrix protein and skeletal growth factor secretion are also warranted. Our results suggest that $[Ca^{2+}]_i$ elevation in normal human osteoblast-like cells in response to osmotic-mechanical stimuli may be an early event in the chain of reactions which mediate the anabolic effects of mechanical loading on the human skeleton.

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