Cell Density and Growth-dependent Down-regulation of Both Intracellular Calcium Responses to Agonist Stimuli and Expression of Smooth-surfaced Endoplasmic Reticulum in MC3T3-E1 Osteoblast-like Cells*

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A two-dimensional intracellular Ca$^{2+}$ imaging system was used to examine the relationship between Ca$^{2+}$ handling and the proliferation of MC3T3-E1 osteoblast-like cells. The resting [Ca$^{2+}$]i level in densely cultured cells was 1.5 times higher than the [Ca$^{2+}$]i level in sparsely cultured cells or in other cell types (mouse fibroblasts, rat smooth muscle cells, and bovine endothelial cells). A high resting [Ca$^{2+}$]i level may be specific for MC3T3-E1 cells. MC3T3-E1 cells were stimulated with ATP (10 μM), caffeine (10 mM), thapsigargin (1 μM), or ionomycin (10 μM), and the effect on the [Ca$^{2+}$]i level of MC3T3-E1 cells was studied. The percentage of responding cells and the degree of [Ca$^{2+}$]i elevation were high in the sparsely cultured cells and low in densely cultured cells. The rank order for the percentage of responding cells and magnitude of the [Ca$^{2+}$]i response to the stimuli was ionomycin > thapsigargin > ATP > caffeine and suggests the existence of differences among the various [Ca$^{2+}$]i channels. All Ca$^{2+}$ responses in the sparsely cultured MC3T3-E1 cells, unlike in other cell types, disappeared after the cells reached confluence. Heptanol treatment of densely cultured cells restored the Ca$^{2+}$ response, suggesting that cell-cell contact is involved with the confluence-dependent disappearance of the Ca$^{2+}$ response. Immunohistochemical analysis of type 1 inositol trisphosphate receptors and electron microscopy showed distinct expression of inositol trisphosphate receptor proteins and smooth-surfaced endoplasmic reticulum in sparsely cultured cells but reduced levels in densely cultured cells. These results indicate that the underlying basis of confluence-dependent [Ca$^{2+}$]i regulation is down-regulation of smooth-surfaced endoplasmic reticulum by cell-cell contacts.

The calcium ion (Ca$^{2+}$) acts as an intracellular messenger and regulates a diverse array of functions in many types of cells (1). Various substances that influence bone remodeling modify the intracellular Ca$^{2+}$ ([Ca$^{2+}$]i) concentration in osteoblasts (2). There are two sources of [Ca$^{2+}$]i in osteoblasts: 1) inflow from the extracellular space and 2) release from intracellular stores, such as the endoplasmic reticulum. The release of Ca$^{2+}$ from the endoplasmic reticulum is a ubiquitous signal in many cells including osteoblasts (3, 4). ATP and caffeine cause inositol trisphosphate (IP3)-induced Ca$^{2+}$ release (IICR) and Ca$^{2+}$-induced Ca$^{2+}$ release (CICR), respectively, from intracellular Ca$^{2+}$ stores.

We previously reported that the response of the Ca$^{2+}$ level in vascular smooth muscle cells (5) to various stimuli is heterogeneous and that it is dependent on the stage of cell growth (6). A heterogeneous Ca$^{2+}$ response has also been reported in an osteosarcoma cell line stimulated by parathyroid hormone (7) and among sub-cell lines of MC3T3-E1 cells treated with bradykinin (8). In the present study, we examined the effects of cell density and growth on [Ca$^{2+}$]i handling mechanisms in MC3T3-E1 cells using a two-dimensional fura-2 imaging system, immunocytochemistry, and electron microscopy, and we identified unique characteristics of these cells. We studied the effect of growth on [Ca$^{2+}$]i handling mechanisms in MC3T3-E1 cells by studying the Ca$^{2+}$ dynamics in cells seeded in the sparse or dense condition.

EXPERIMENTAL PROCEDURES

Reagents—All reagents used were of analytical grade. Thapsigargin (TG; Sigma Chemical) and ionomycin (IM; Sigma Chemical) were dissolved in dimethyl sulfoxide (Me$_2$SO) and then diluted with phosphate-buffered saline (PBS). The final concentration of Me$_2$SO was less than 0.1% (v/v) and had no action on the handling of [Ca$^{2+}$]i in the MC3T3-E1 cells (data not shown). Caffeine and ATP were purchased from Wako Pure Chemicals (Osaka, Japan). Each reagent was administered by replacing half the volume of extracellular medium with medium containing 2-fold concentrated reagent (5, 6). In a pilot study, we attempted to identify suitable reagents and their appropriate concentrations to analyze the transient [Ca$^{2+}$]i currents. We used 10 mM caffeine, 10 μM ATP, 1 μM TG, and 10 μM ryanodine or IM in the present study.

Cell Culture—MC3T3-E1 mouse clonal osteogenic cells (a generous gift from Prof. S. Yamamoto, Ohu University, Koriyama, Japan) were seeded onto dishes (radius, 1 cm) made of fluorescence-free glass at a density of 1.0 × 10$^4$ cells/dish (sparse condition) or 2.0 × 10$^5$ cells/dish (dense condition). The cells were cultured in α-minimal essential medium (Invitrogen) containing 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma Chemical). The medium was

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1 The abbreviations used are: IP$_3$, inositol trisphosphate; IICR, inositol trisphosphate-induced Ca$^{2+}$ release; CICR, Ca$^{2+}$-induced Ca$^{2+}$ release; F$_{340}$/F$_{380}$, 340- to 380-nm fluorescence ratio; TG, thapsigargin; IM, ionomycin; PBS, phosphate-buffered saline; EC, endothelial cells; IP$_3$-R$_1$, inositol trisphosphate type-1 receptor; eER, smooth-surfaced endoplasmic reticulum.
changed every 48 h after seeding. Caffeine, ATP, TG, or JM was added between 0 and 120 h after seeding, and the peak [Ca\textsuperscript{2+}]\text{,} level upon stimulation was studied. The resting [Ca\textsuperscript{2+}]	ext{,} level in MC3T3-E1 cells seemed to be higher than that in other cells. We compared the [Ca\textsuperscript{2+}]	ext{,} level in MC3T3-E1 cells with that in endothelial cells (ECs) from the bovine aorta, cloned rat vascular smooth muscle cells (A\textsubscript{7r5}, and cloned mouse fibroblasts (NIH3T3), which have been used in studies on [Ca\textsuperscript{2+}]	ext{,} dynamics (5, 6). In single-cell line cultures, each cell line was seeded at a density of 1.0 \times 10^4 cells/dish and cultured for 48 h. In cocultures, EC, A\textsubscript{7r5}, or NIH3T3 cells were seeded together with MC3T3-E1 cells at a density of each cell line of 1.0 \times 10^4 cells/dish and cultured for 48 h.

To examine the effect of cell-cell contact, MC3T3-E1 cells were seeded in dishes at a density of 2.0 \times 10^4 cells/dish (dense condition). After 24 h, 3 \muM heparin, a gap-junctional inhibitor (9, 10), was added to the dishes and the cells were cultured for an additional 48 h. And then, ATP, TG, or JM was added, and the peak [Ca\textsuperscript{2+}]	ext{,} level upon stimulation was studied.

**Two-dimensional (2D) Image Analysis of [Ca\textsuperscript{2+}\text{,} level within individual cells was analyzed as described previously (11-13). Briefly, the cells were washed with PBS and incubated in PBS(+) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 0.5 mM MgCl\textsubscript{2}, and 1 mM Ca\textsuperscript{2+}, pH 7.4. The Ca\textsuperscript{2+}-free solution consisted of PBS in which CaCl\textsubscript{2} was replaced by 1.0 mM EGTA. Cells were loaded with 4 \muM fura-2/acetoxymethyl ester in the same buffer at 37 °C for 30 min, rinsed twice with PBS(+), and then preincubated at 37 °C for an additional 10 min. The recorded fluorescence images were digitized using an on-line image processor (Olympus-Merlin, Tokyo, Japan; Fig. 1A). Fig. 1B shows the change in the relative value of the 340- to 380-nm fluorescence ratio (F\textsubscript{340}/F\textsubscript{380}) in MC3T3-E1 cells upon treatment with ATP. Because of the difficulty in measuring the absolute [Ca\textsuperscript{2+}], concentration caused by problems intrinsic to fura-2 fluoroscopy, the maximum amplitude of the [Ca\textsuperscript{2+}], elevation in response to each stimulant was expressed as the relative value of F\textsubscript{340}/F\textsubscript{380} in relation to the value of F\textsubscript{340}/F\textsubscript{380} in the resting state (5, 6).

Caffeine transiently increased the [Ca\textsuperscript{2+}], level, in both the presence and the absence of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}, L). The degree of [Ca\textsuperscript{2+}], elevation evoked by caffeine in the Ca\textsuperscript{2+}-free solution became progressively smaller as the incubation time increased (data not shown). To avoid fluctuations in the response, we applied caffeine to cells in the presence of 1 mM Ca\textsuperscript{2+}, Ryanodine (10 \muM; Wako Pure Chemicals) was also added to keep the CICR channels open (14) and to prevent the activation of additional CICR. All measurements were made 24 h after seeding to avoid the effects of trypsin, which was used in seeding, on the activation of additional CICR. All measurements were made 24 h after seeding to avoid the effects of trypsin, which was used in seeding, on the activation of additional CICR.

**Immunohistochemistry**—MC3T3-E1 cells incubated in plastic dishes (60 mm in diameter) were fixed with a mixture of equal volumes of ethanol and acetone. The nonspecific binding of antibody was blocked by goat serum, and then the cells were treated with anti-IP3 type-1 receptor (IP\textsubscript{3-R}, polyclonal antibody or non-immunized rabbit IgG at room temperature for 1 h, followed by an LSAB2 kit/ horseradish peroxidase (DAKO Corporation, Carpinteria, CA). The immunopositive was visualized by treatment with amino-9-ethylcarbazol (DAKO) and micrographs were taken at 100× (AX-80; Olympus, Tokyo, Japan).

**Electron Microscopy**—For transmission electron microscopy, the MC3T3-E1 cells were detached from the culture dishes by treatment with trypsin and washed three times with PBS, pH 7.4. After fixing overnight in 2% paraformaldehyde plus 2.5% glutaraldehyde in phosphate buffer, the cells were sedimented at 2000 rpm at room temperature for 5 min, postfixed with 1% osmium tetroxide, dehydrated in graded alcohol, and then embedded in Epox (Epon 812; TAAB Lab, Berkshire, England) overnight at 60 °C. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and observed under an electron microscope (H-7000; Hitachi, Tokyo, Japan).

**Statistical Analysis**—Data were expressed as the mean ± S.E. and were analyzed by one-way analysis of variance. Treatment pairs were compared by Scheffe’s F test. All differences were considered to be statistically significant at p < 0.05.

**RESULTS**

**[Ca\textsuperscript{2+}]: in the Resting State**—The [Ca\textsuperscript{2+}]	ext{,} level in MC3T3-E1 cells in the resting state that had been seeded in the sparse or dense condition was measured at 0, 24, 48, 72, and 120 h after the first medium change (Fig. 2). The [Ca\textsuperscript{2+}]	ext{,} level of the densely seeded cells was 1.5 times higher than that of the sparsely seeded cells at 0 h (1.19 ± 0.01 versus 0.80 ± 0.02, p < 0.01). The [Ca\textsuperscript{2+}]	ext{,} level of the densely seeded cells increased slightly, although significantly, up through 120 h from 1.19 ± 0.01 to 1.33 ± 0.01 (p < 0.01). The [Ca\textsuperscript{2+}]	ext{,} level of the sparsely seeded cells increased significantly from 0.80 ± 0.02 to 1.30 ± 0.01 at 120 h (p < 0.01) and reached the same level as that of the densely seeded cells at 120 h (1.33 ± 0.01, densely seeded, versus 1.30 ± 0.01, sparsely seeded), with no significant change thereafter. These results indicate that the [Ca\textsuperscript{2+}]	ext{,} level of MC3T3-E1 cells increased as proliferation progressed.

To confirm that densely seeded MC3T3-E1 cells in the resting state have a high [Ca\textsuperscript{2+}]	ext{,} level, we measured the [Ca\textsuperscript{2+}]	ext{,} level in both isocultures and cocultures with other cell lines, including mouse fibroblasts (NIH3T3), rat vascular smooth muscle cells (A\textsubscript{7r5}, and ECs from the bovine aorta. The resting state [Ca\textsuperscript{2+}]	ext{,} level of MC3T3-E1 cells was higher than that of the other cell lines both in the isocultures, in which one cell line each had been harvested (Fig. 3A), and in the cocultures at 48 h, in which two cell lines had been cultured in the same dish (Fig. 3B). These results suggest that the high [Ca\textsuperscript{2+}]	ext{,} level in resting state MC3T3-E1 cells may exert a specific function in calcifying tissue.

**Effects of ATP and Caffeine on [Ca\textsuperscript{2+}]: Release from Intracellular Ca\textsuperscript{2+} Stores at Different Cell Densities during Cell Proliferation**—In the following experiments, MC3T3-E1 cells were
incubated in PBS in which CaCl$_2$ had been replaced with EGTA (1.0 mM) to prevent Ca$^{2+}$/H$^{+}$ influx into cells and to selectively evaluate Ca$^{2+}$/H$^{+}$ release from intracellular calcium stores. ATP (10 μM), which stimulates IICR (15, 16), elevated the [Ca$^{2+}$]$_{ij}$ level in 84% of the sparsely seeded cells when it was applied at 24 h after seeding, in 100% of the cells when applied at 48 h, and in 98% of the cells when applied at 72 h (Fig. 4A). In contrast, ATP elevated the [Ca$^{2+}$]$_{ij}$ level in only 18–23% of the densely seeded cells when it was applied between 24 and 120 h.

The relative value of $F_{340}/F_{380}$ upon treatment with ATP at 24 and 48 h in each responder cell in the culture under the sparsely seeded condition was 1.675 ± 0.066 and 1.770 ± 0.068, respectively. Then, it sharply decreased to 1.279 ± 0.023 and 1.038 ± 0.003 when ATP was applied at 72 and 120 h, respectively, when the cell density, because of proliferation, reached that of the densely seeded cells (Fig. 4B).

Caffeine, which stimulates CICR (14), increased the [Ca$^{2+}$]$_{ij}$ level in 61 and 79% of the sparsely seeded cells when it was applied at 24 and 48 h after seeding, respectively. When caffeine was applied at 72 or 120 h, only 0–3% of the sparsely seeded cells had an increased [Ca$^{2+}$]$_{ij}$ level (Fig. 5A). In the densely seeded cells, caffeine had no effect on the [Ca$^{2+}$]$_{ij}$ level when it was applied between 24 and 120 h, respectively, when the cell density, because of proliferation, reached that of the densely seeded cells (Fig. 5B).

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In the sparsely seeded condition, the relative value of $F_{340}/F_{380}$ upon treatment with ATP in caffeine responding cell was larger than that upon treatment with ATP in caffeine non-

responding cell at 24 and 48 h (Fig. 6). These results indicate that cells that responded to caffeine also responded to ATP and suggest that the source of $[\text{Ca}^{2+}]_i$ release was the same smooth-surfaced endoplasmic reticulum (sER) via CICR or IICR, a finding that differs from that in vascular smooth muscle cells under the sparsely seeded condition (5, 6).

Effects of Thapsigargin (TG) and Ionomycin (IM) on the $[\text{Ca}^{2+}]_i$ Level at Different Cell Densities—TG blocks the uptake of $\text{Ca}^{2+}$ from the cytoplasm to the sER (17). When TG was applied at 24, 48, or 72 h, the $[\text{Ca}^{2+}]_i$ level increased in 97.5–100% of the sparsely seeded cells; however, upon application of TG at 120 h, none of the cells had an increased $[\text{Ca}^{2+}]_i$ level (Fig. 7A). The percentage of densely seeded cells with elevated $[\text{Ca}^{2+}]_i$ was only 3–8% when TG was applied between 24 and 120 h. The $F_{340}/F_{380}$ did not change in the densely seeded cells upon treatment with thapsigargin between 24 and 120 h (A). Each point represents the mean ± S.E. of 60 responder cells in three cultures. *, $p < 0.05$, significant difference between MC3T3-E1 cells seeded in the sparse and dense conditions. #, significant difference compared with that at 24 h.

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Effects of Thapsigargin (TG) and Ionomycin (IM) on the $[\text{Ca}^{2+}]_i$ Level at Different Cell Densities—TG blocks the uptake of $\text{Ca}^{2+}$ from the cytoplasm to the sER (17). When TG was applied at 24, 48, or 72 h, the $[\text{Ca}^{2+}]_i$ level increased in 97.5–100% of the sparsely seeded cells; however, upon application of TG at 120 h, none of the cells had an increased $[\text{Ca}^{2+}]_i$ level (Fig. 7A). The percentage of densely seeded cells with elevated $[\text{Ca}^{2+}]_i$ was only 3–8% when TG was applied between 24 and 120 h. The $F_{340}/F_{380}$ did not change in the densely seeded cells upon treatment with thapsigargin between 24 and 120 h (A). Each point represents the mean ± S.E. of 60 responder cells in three cultures. *, $p < 0.05$, significant difference between MC3T3-E1 cells seeded in the sparse and dense conditions. #, significant difference compared with that at 24 h.
All Ca$^{2+}$ ions in internal stores can be non-specifically released by the Ca$^{2+}$ ionophore, IM (2). IM elevated the [Ca$^{2+}$], level in 100% of the sparsely and densely seeded cells. In the sparsely seeded cells, the relative value of $F_{440/380}$ upon treatment with IM at 24, 48, 72, or 120 h after seeding was $2.110 \pm 0.067, 2.058 \pm 0.078, 1.910 \pm 0.042$, and $1.421 \pm 0.021$, respectively (Fig. 8). When densely seeded cells were treated with IM, the $[\text{Ca}^{2+}]$, level increased by a smaller degree. All data on the Ca$^{2+}$ dynamics in the TG and IM experiments supported the dependence of the $[\text{Ca}^{2+}]$, response on cell density. Furthermore, the Ca$^{2+}$ response was smaller upon treatment with TG than upon treatment with IM at each time point. This may indicate that Ca$^{2+}$ was released from other internal stores in addition to the sER.

**Effect of Heptanol on the $[\text{Ca}^{2+}]$, Response**—The relative value of $F_{440/380}$ upon treatment with ATP, caffeine, TG, or IM in the responders was much lower throughout the experiment in the densely seeded cells than in the sparsely seeded cells (Figs. 4, 5, 7, 8). Heptanol, a gap-junctional inhibitor (9, 10), was used to evaluate the effect of cell-cell contact in the densely seeded cells. When densely seeded cells were treated with 3 $\mu$m heptanol, the $[\text{Ca}^{2+}]$, elevation and the relative value of $F_{440/380}$ upon treatment with ATP, TG, or IM recovered (Fig. 9). These results suggest that cell-cell contact may inhibit the Ca$^{2+}$ response of densely seeded cells to ATP, caffeine, TG, and IM.

**Immunodetection of IP$_3$-R$_1$—Ca$^{2+}$** was released from CICR channels in response to caffeine only in sparsely seeded cells up to between 48 and 72 h. The Ca$^{2+}$ efflux from IICR channels was also closely related to the Ca$^{2+}$ released from the sER. These results suggest that IICR may play a more significant role than CICR in calcium handling in osteoblasts. To date, three isoforms of the IP$_3$ receptor have been cloned, and the type-1 receptor is believed to be ubiquitous. Using a specific antibody to IP$_3$-R$_1$, we investigated the expression of this receptor in sparsely and densely seeded cells to determine whether it is involved in the Ca$^{2+}$ response. A high level of IP$_3$-R$_1$ protein was detected in sparsely seeded cells upon culture for 24 h (Fig. 10B), and a low level was detected upon culture for 120 h (Fig. 10C). The level of immunostaining was low in densely seeded cells at both 24 and 120 h (Fig. 10, D and E). The level of IP$_3$-R$_1$ protein expression was associated with the amount of Ca$^{2+}$ released from IICR channels.

**Electron Microscopy of sER**—Transmission electron microscopy was used to ascertain the relationship between the [Ca$^{2+}$], level and the amount of sER in osteoblasts. Many organelles, including sER, were observed in the sparsely seeded cells at 24 h (Fig. 11, A and B). However, at 120 h of culture, the sER had been reduced, and many rough-surfaced endoplasmic reticula and mitochondria were detected after cell proliferation (Fig. 11, C and D). Many rough-surfaced endoplasmic reticula and mitochondria were observed and reduced number of sER were seen in densely seeded cells that had been cultured for 24 h (Fig. 11, E and F) or 120 h (Fig. 11, G and H). Thus, the reduced Ca$^{2+}$ response of densely seeded cells to ATP, caffeine, TG, and IM can be explained by the reduced number of sER. These results suggest that the expression of sER is regulated by cell proliferation.

**DISCUSSION**

Heterogeneity of the [Ca$^{2+}$], level among MC3T3-E1 cells was observed even before stimulation and was dependent on cell density (sparsely seeded or densely seeded). The [Ca$^{2+}$], level in cells in the resting state ranges from 50 to 400 nM and varies according to cell type and function (14). Because estimation of the absolute [Ca$^{2+}$], level by fura-2 fluorescence is inac-
Curate because of several problems intrinsic to fura-2 fluorescence (11–13), the [Ca\(^{2+}\)] level in the resting state obtained in previous reports cannot be simply compared with that obtained in the present study. The relative value of \( F_{340}/F_{380} \) in reference to \( F_{340}/F_{380} \) in the resting state (11) was used to evaluate the Ca\(^{2+}\) response. To compare the [Ca\(^{2+}\)] levels of various cells at the resting state, we used \( F_{340}/F_{380} \) in iso- and cocultures of MC3T3-E1 cells and other established cell lines (EC, A549, and NIH3T3).

It is unclear why the [Ca\(^{2+}\)] level was higher in the densely seeded MC3T3-E1 cells than in the sparsely seeded cells or other cell lines. We consider that this characteristic is specific for bone-forming MC3T3-E1 osteoblast-like cells. In the densely seeded MC3T3-E1 cells, the number of sER was reduced, indicating a reduced amount of stored Ca\(^{2+}\). Consequently, it is suggested that in the resting state, the [Ca\(^{2+}\)] level increases. However, the [Ca\(^{2+}\)] level in densely seeded cells was about 1.5 times higher than that in the sparse condition or other cell lines, and the degree of the increase in [Ca\(^{2+}\)] level was not necessarily so high that it would be toxic for cells. The presence of an increased [Ca\(^{2+}\)] level in the resting state of MC3T3-E1 cells, especially in densely seeded cells, seems to be informative for estimating the physiological significance of bone-forming cells. We believe that the increased [Ca\(^{2+}\)] level may be advantageous for the release of Ca\(^{2+}\) during calcification from the point of view of ionic strength.

We focused on the cell density- and growth-dependent changes in the [Ca\(^{2+}\)], response of osteoblast-like cells to various stimuli. There is a study that focused on the downstream signaling mechanism via Ca\(^{2+}\), not only from internal stores but also from extracellular Ca\(^{2+}\) in osteoblasts (18). Whether the [Ca\(^{2+}\)], response of osteoblast-like cells to various stimuli creates cross-talk with extracellular Ca\(^{2+}\)-sensing pathways is of much interest and requires further investigation (19).

Among the agonists employed in the present study, ATP, which stimulates IICR, caused [Ca\(^{2+}\)], elevation when applied between 24 and 120 h to both sparsely and densely seeded cells. In contrast, caffeine, which opens CICR channels, induced [Ca\(^{2+}\)], elevation in only the sparsely seeded condition. CICR may be inhibited more strongly than IICR in densely seeded cells. On the other hand, cells showing CICR channel function reacted to ATP, and the other cells that did not reveal an IICR channel response did not respond to caffeine either. Therefore, IICR channels mainly handle the calcium response in MC3T3-E1 cells. We previously showed that the expression of CICR and IICR channels, in addition to voltage-dependent Ca\(^{2+}\) channels, is influenced by cell growth and enhanced by the differentiation of vascular smooth muscle cells and that they are independently regulated from each other (5, 6). The percentage of responding cells and the magnitude of the Ca\(^{2+}\) response were much lower in sparsely seeded vascular smooth muscle cells than in densely seeded cells. Thus, [Ca\(^{2+}\)] handling in MC3T3-E1 cells differs from that in vascular smooth muscle cells.
TG depletes Ca\textsuperscript{2+} from the sER and induces [Ca\textsuperscript{2+}]\textsubscript{i} elevation via capacitative Ca\textsuperscript{2+} entry. A sufficient dose of IM, an ionophore that forms artificial [Ca\textsuperscript{2+}] channels in membranes and releases all Ca\textsuperscript{2+} from the sER, also increased the [Ca\textsuperscript{2+}]\textsubscript{i} level even when Ca\textsuperscript{2+} entry did not occur in PBS(−). The rank order of the degree of [Ca\textsuperscript{2+}]\textsubscript{i} increase was IM > TG = ATP > caffeine. These results indicate that there is a large amount of Ca\textsuperscript{2+} in the sER of sparsely seeded MC3T3-E1 cells and that IICR channels of the sER mainly control [Ca\textsuperscript{2+}]\textsubscript{i} elevation in MC3T3-E1 cells. These findings are fundamentally compatible with the results of the study of Zach et al. (19). In sparsely seeded MC3T3-E1 cells, the presence of a high level of IP\textsubscript{3}-R\textsubscript{1} and a large number of sER was morphologically confirmed by immunohistology and electron microscopy, respectively.

The [Ca\textsuperscript{2+}]\textsubscript{i} level in osteoblasts has been reported to be correlated with the seeded cell density, the cell line, morphology, and receptor distribution (7, 8, 12). Various factors influence the [Ca\textsuperscript{2+}]\textsubscript{i} level in individual cells at rest and after agonist administration. A previous study suggested that gap junctions and gap-junctional intercellular communication play pivotal mechanotransduction mechanisms in bone (20). In this study, the results of heptanol treatment revealed that gap-junctional cell-cell contact also contributes to the cell density-dependent, heterogeneous [Ca\textsuperscript{2+}]\textsubscript{i} response to agonists. In the proliferating phase of osteoblasts, cells without cell-cell contact have both CICR and IICR channels or abundant sER. We previously showed that IP\textsubscript{3}-R\textsubscript{1} may regulate the proliferation of vascular smooth muscle cells (21). During bone formation in physiological growth or pathological repair after a bone fracture, enhanced IP\textsubscript{3}-R\textsubscript{1} expression may be required for the proliferation of osteoblasts. To elucidate the precise significance of ICR modulation, more specific and detailed studies should be conducted in the future.

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