Sustained Activation of the Extracellular Signal-regulated Kinase Pathway Is Required for Extracellular Calcium Stimulation of Human Osteoblast Proliferation*

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Elevated levels of $[\text{Ca}^{2+}]_o$ in bone milieu as a result of the resorptive action of osteoclasts are implicated in promoting proliferation and migration of osteoblasts during bone remodeling. However, mitogenic effects of $[\text{Ca}^{2+}]_o$ have only been shown in some, but not all, clonal osteoblast-like cells, and the molecular mechanisms underlying $[\text{Ca}^{2+}]_o$-induced mitogenic signaling are largely unknown. In this study we demonstrated for the first time that $[\text{Ca}^{2+}]_o$ stimulated proliferation of primary human osteoblasts and selectively activated extracellular signal-regulated kinases (ERKs). Neither p38 mitogen-activated protein (MAP) kinase nor stress-activated protein kinase was activated by $[\text{Ca}^{2+}]_o$. Treatment of human osteoblasts with a MAP kinase inhibitor, PD98059, impaired both basal and $[\text{Ca}^{2+}]_o$-stimulated phosphorylation of ERKs and also reduced both basal and $[\text{Ca}^{2+}]_o$-stimulated proliferation. $[\text{Ca}^{2+}]_o$ treatment resulted in two distinctive phases of ERK activation: an acute phase and a sustained phase. An inhibition time course revealed that it was the sustained phase, not the acute phase, that was critical for $[\text{Ca}^{2+}]_o$-stimulated osteoblast proliferation. Our results demonstrate that mitogenic responsiveness to $[\text{Ca}^{2+}]_o$ is present in primary human osteoblasts and is mediated via prolonged activation of the MAP kinase kinase/ERK signal pathway.

Extracellular calcium has been shown to control a variety of cellular functions including secretion, cell growth, differentiation, and motility (1–3). In bone, $[\text{Ca}^{2+}]_o$ has been postulated to play an important role in bone remodeling. Concentrations of $[\text{Ca}^{2+}]_o$ fluctuate dramatically to as high as 40 mM in the local microenvironment as a result of the resorptive action of osteoclasts (4). High concentrations of $[\text{Ca}^{2+}]_o$ have been suggested to stimulate osteoblast proliferation and inhibit osteoclast resorption. Indeed, in vitro studies showed that $[\text{Ca}^{2+}]_o$ and other cations stimulate proliferation in a number of osteoblast-like cell lines (5–8). However, not all osteoblast cell lines are responsive to $[\text{Ca}^{2+}]_o$ (9). For example, the effects of $[\text{Ca}^{2+}]_o$ on proliferation of human osteoblast cell lines are either lacking or marginal (9, 10). Varying degrees of $[\text{Ca}^{2+}]_o$-stimulated proliferation among these clonal osteoblast-like cell lines raise the interesting question of whether calcium responsiveness exists in vivo matured osteoblastic cells.

Studies on $[\text{Ca}^{2+}]_o$ responsive osteoblast-like cell lines have so far provided only limited information regarding the intracellular signaling mechanism underlying $[\text{Ca}^{2+}]_o$ stimulation of osteoblast proliferation (3). Recent studies have shown that multiple intracellular signal pathways in osteoblasts can be activated by $[\text{Ca}^{2+}]_o$ and other cations. High concentrations of $[\text{Ca}^{2+}]_o$ activate phospholipase C, probably through a G-protein-coupled receptor mechanism, which then leads to accumulation of inositol phosphates and diacylglycerol and mobilization of intracellular calcium (8, 11–13). Activation of protein kinase C is also required for mediating the mitogenic effects of $[\text{Ca}^{2+}]_o$ and cations (14). How activation of these signal pathways leads to osteoblast proliferation is not clear.

A variety of extracellular signals have been shown to control various cell functions such as cell proliferation and differentiation through activation of mitogen-activated protein (MAP)1 kinase signal pathways (15, 16). The MAP kinase superfamily has been classified into three subfamilies: extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs), and p38 MAP kinase. ERKs have been best known for their involvement in mediating intracellular signals in cell proliferation, although members of the other two MAP kinase subfamilies have also been reported to be capable of responding to mitogenic stimuli (17–19). In addition, MAP kinase signal cascades have been shown to be involved in other cellular processes such as differentiation, apoptosis, and migration (15, 16). Although $[\text{Ca}^{2+}]_o$ can activate p38 MAP kinase in primary cultures of bovine parathyroid cells and ERK1 in rat 1 fibroblastic cells (20), there is little information on the role and specificity of MAP kinase signal cascades in mediating $[\text{Ca}^{2+}]_o$-stimulated osteoblast proliferation.

In this study, we demonstrate that $[\text{Ca}^{2+}]_o$ exerts a mitogenic response in primary human osteoblasts and MG-63 cells. We provide the first demonstration that $[\text{Ca}^{2+}]_o$-stimulated cell proliferation in osteoblasts is dependent on sustained activation of ERK1 and ERK2 but that other MAP kinase signal pathways, p38 MAP kinase and SAPK/JNK, are not activated by $[\text{Ca}^{2+}]_o$ in osteoblasts.

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1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase, CaR, calcium sensing receptor; MEK, MAP kinase kinase; DMEM, Dulbecco’s modified Eagle’s medium.

2 A. Brown, personal communication.
EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, all chemical reagents were purchased from Sigma. The tissue culture media were purchased either from BioWhittaker, Inc. or from the Tissue Culture Support Center (Washington University). SDS-polyacrylamide gels were purchased from Bio-Rad. The MEKI/MEK2 inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem, Inc. The protease inhibitor mixture was purchased from Roche Molecular Biochemicals.

Cell Culture and Proliferation Assay—Primary human osteoblasts derived from bone chips were prepared as described previously (21). The clonal osteoblastic cell lines (human MG-63, mouse MC3T3-E1, and rat ST2) were routinely maintained in α-minimum essential medium plus 10% fetal bovine serum (Life Technologies, Inc.).

For proliferation assays using [3H]thymidine incorporation, the cells were subcultured into 12-well plates, and the subconfluent cells were maintained in normal culture medium for 48 h. The cells were then incubated in serum-free DMEM/Ham’s F-12 medium (1 mM Ca2+ and 0.9 mM Mg2+) for 24 h. The cells were treated with various agents for time periods as specified in the figure legends. [3H]Thymidine (0.5 μCi; Amersham Pharmacia Biotech) was added to each well and incubated in treatment medium for the last 3 h. The cells were washed once with phosphate-buffered saline and once with 5% trichloroacetic acid before 0.75 ml of 0.1N NaOH was added to each well to lyse the cells. The cell lysates were transferred to scintillation vials along with 10 ml of ScintiVerse (Fisher), and the radioactivity in each vial was measured in a scintillation counter. DNA synthesis measured by [3H]thymidine incorporation was used as an index of cell proliferation.

For assessing cell proliferation by direct cell counting, cells were subcultured into 10-cm dishes that were premarked with 0.5-cm grids. At the end of the treatment period, media were removed immediately, and the cells in 10-cm dishes were lysed by the addition of 0.5 ml of the kinase extraction buffer (20 mM HEPES, 150 mM NaCl, 2 mM Na2VO4, 1 mM NaF, 5 mM EDTA, 10% glycerol, 1% Triton X-100, and protease inhibitor mixture, 1 tablet/10 ml of buffer). The cells were detached from dishes by scraping, and the cell lysates were collected in 1.5-ml microcentrifuge tubes. After a 5-min centrifugation at 12,000 rpm, the supernatants were collected and stored at –80 °C. The protein concentrations of the samples were determined by Bradford protein assay (Bio-Rad).

Analysis of MAP Kinase Activation—Activation of various MAP kinases (ERK1/2, MAP kinase, p38 MAP kinase, and SAPK/JNK) has been shown to occur through dual phosphorylation of threonine and tyrosine residues by upstream MAP kinase kinases. The dual phosphorylation sequences are TEY for ERK1/2, TGY for p38, and TPY for SAPK/JNK. The levels of dual phosphorylation at these positions were measured using at least three grids that were photographed through a CCD camera at 0-, 24-, 48-, and 72-h time points. The cell numbers were measured using PhosphoPlus antibody kits (New England Biolabs, Inc.) following the manufacturer’s instructions. Phosphorylation of specific MAP kinases was detected by affinity-purified antibodies recognizing specific dual phospho-peptide sequences present in these MAP kinases. As a control, antibodies recognizing MAP kinases independent of their phosphorylation status were used to determine the total amount of individual MAP kinases present in the samples. Briefly, protein samples were size-fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad) by electroblotting. The membranes were blocked in 5% nonfat dry milk for 1 h at room temperature followed by an overnight incubation with the primary antibody at 4 °C in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). The membranes were washed three times in TBS-T and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After extensive washing in TBS-T, membranes were subject to a chemiluminescence-based detection assay (New England Biolabs, Inc.).

RESULTS

Proliferative responses to [Ca2+]o, stimulation have been demonstrated in a number of osteoblastic cell lines (6, 9, 10, 22). However, varying degrees of calcium responsiveness were reported among various human and mouse osteoblastic cell lines (5, 9, 10). Thus it raises the interesting question of whether calcium responsiveness is an intrinsic property of osteoblastic cells. To address this question, we assessed the effects of [Ca2+]o, on the proliferation of primary human osteoblasts. Treatment of quiescent primary culture of human osteoblasts with 5 mM Ca2+ resulted in a 5-fold stimulation of DNA synthesis over the control value (1 mM Ca2+) (Fig. 1). To compare the degree of calcium responsiveness of the primary human osteoblast cells with those of clonal osteoblast-like cells, we also determined [Ca2+]o, stimulated cell proliferation of human MG-63 cells, mouse MC3T3-E1 cells, and rat ST2 cells. The proliferative responses to [Ca2+]o, stimulation were found to be similar among all of these osteoblastic cells (Fig. 1).

Demonstration of [Ca2+]o-stimulated proliferation in primary human osteoblastic cells indicates that calcium responsiveness is an intrinsic property of human osteoblast cells and supports the hypothesis that [Ca2+]o, may play a critical role in the bone remodeling process in vivo. The degrees of mitogenic response observed in our studies were very similar among various cell lines across different species, suggesting that the differences in calcium responsiveness reported in the literature may be the results of the different experimental conditions and the specific cell lines established (5, 7, 9, 10). Because the human osteoblastic cell line MG-63 responded to [Ca2+]o, in a manner similar to that of primary human osteoblasts, we therefore chose to use MG-63 cells for all of the subsequent experiments.

[Ca2+]o-stimulated proliferation in MG-63 cells was dose-dependent (1–10 mM) with maximum stimulation at 5 mM (Fig. 2A). The mitogenic effect of [Ca2+]o, was also time-dependent (3–72 h) with increments observed at 24–72 h in cells treated...
Various extracellular mitogenic signals such as growth factors and cytokines have been shown to stimulate cell proliferation through ERK, p38, or SAPK/JNK MAP kinase signal pathway (17–19). To determine whether \([\text{Ca}^{2+}]_o\)-stimulated osteoblast proliferation was mediated by any of these MAP kinase signal pathways, we first assessed whether any of the MAP kinases were activated by \([\text{Ca}^{2+}]_o\). We took advantage of the fact that activation of all three types of MAP kinases occurs through dual phosphorylation of threonine and tyrosine residues that can be recognized by antibodies specific for the phospho-proteins. The quiescent MG-63 cells were treated with \([\text{Ca}^{2+}]_o\) for various time periods, and activation of ERK1 and ERK2 was measured by Western blotting analysis. At the basal state (1 mM calcium), we detected low but measurable phosphorylation of ERK1 and ERK2 (Fig. 4, top left panel). Thus it indicated that even at 1 mM calcium, low levels of activated ERK1 and ERK2 were present and might contribute to low but measurable basal proliferation. However, a rapid increase (within 5 min) in phosphorylation over basal levels was observed for both ERK1 and ERK2 following 5 mM calcium treatment. The phosphorylation levels of the ERK1 and ERK2 peaked after 5 min of calcium stimulation and gradually declined with time. However, even at 60 min, phosphorylation of ERK1 and ERK2 was still significantly higher than basal levels. We thus examined whether sustained ERK phosphorylation occurred with longer exposure to elevated \([\text{Ca}^{2+}]_o\) (2, 6, 24, 48 h). The increases in phosphorylation of ERK1 and ERK2 were maintained for up to 48 h (Fig. 4, top right panel), indicating that \([\text{Ca}^{2+}]_o\) stimulation resulted in sustained activation of ERK1 and ERK2. This sustained activation was consistent with our finding that \([\text{Ca}^{2+}]_o\)-stimulated proliferation required prolonged treatment of \([\text{Ca}^{2+}]_o\).

The increase in phosphorylation of ERK MAP kinases, especially in the sustained phase, could be due to three mechanisms: 1) an increase in the percentage of phosphorylated proteins in the existing ERK MAP kinase pool; 2) an increase in overall expression levels of ERK MAP kinases without altering the percentage of phosphorylation; or 3) a combination of the two. Western blotting analysis using the antibody recognizing all forms of ERK1 and ERK2 showed that the total amounts of
ERK1 and ERK2 were similar at all time points (Fig. 4, bottom panels), indicating that protein expression was not significantly altered. Thus the increases of phosphorylation in both the acute and the sustained phases were due to the increase in the percentage of phosphorylation in the existing ERK MAP kinases rather than as the consequence of an increase in the overall expression levels of ERK MAP kinases.

The ability of Gd$^{3+}$ to mimic calcium in stimulating osteoblast proliferation raised the question of whether Gd$^{3+}$ can also stimulate ERK phosphorylation. To address this, MG-63 cells were treated with 100 μM Gd$^{3+}$ for various time periods. Gd$^{3+}$ not only stimulates acute phosphorylation of the ERKs but also induces sustained activation of the ERKs up to 24 h (Fig. 5). This pattern of activation is similar to that observed with calcium stimulation, suggesting that two different ions may use a common mechanism for activation of ERKs.

To assess whether other MAP kinases such as p38 MAP kinase and SAPK/JNK were activated by [Ca$^{2+}$]o, we assessed phosphorylation of p38 MAP kinase and SAPK/JNK using phospho-antibodies against phosphorylated peptides derived from p38 MAP kinase and the SAPK/JNK, respectively. We found that p38 MAP kinase was not phosphorylated at either the basal or [Ca$^{2+}$]o-stimulated conditions, whereas sufficient protein expression was detected (Fig. 6, A and B). Similarly, no phosphorylation of SAPK/JNKs was observed when cells were treated with 5 mM [Ca$^{2+}$]o, whereas expression of SAPK/JNKs was readily seen (Fig. 6, C and D). This result demonstrated that [Ca$^{2+}$]o did not activate either p38 MAP kinase or SAPK/JNK in MG-63 cells and that neither p38 MAP kinase nor SAPK/JNK was likely to play any role in [Ca$^{2+}$]o-stimulated osteoblast cell proliferation.

ERK1 and ERK2 are known to be activated by the upstream enzyme MEK1 and MEK2, and such activation can be blocked by the MEK1/MEK2 inhibitor PD98059 (26–28). To test whether PD98059 could block activation of ERK1 and ERK2 by [Ca$^{2+}$]o, we treated MG-63 cells with 50 μM PD98059 for 1 h before calcium stimulation. PD98059 treatment not only markedly reduced basal phosphorylation of ERK1 and ERK2 but also completely eliminated [Ca$^{2+}$]o-stimulated phosphorylation (Fig. 7).

To address the question of whether activation of the MEK/ERK signal cascade (phosphorylation of ERK1 and ERK2) was functionally important for [Ca$^{2+}$]o-stimulated phosphorylation, we measured [Ca$^{2+}$]o-stimulated [3H]thymidine incorporation in the presence and absence of PD98059 (Fig. 8). PD98059 treatment not only severely impaired [Ca$^{2+}$]o-stimulated proliferation but also markedly reduced basal cell proliferation (Fig. 8). This result showed that ERK MAP kinases were important in both [Ca$^{2+}$]o-stimulated and basal cell proliferation. It was consistent with the effects of PD98059 on phosphorylation of ERK1 and ERK2 (Fig. 7), where both basal and [Ca$^{2+}$]o-stimulated phosphorylation of ERK1 and ERK2 were blocked by PD98059. This result indicated that there might be a direct association of ERK1 and ERK2 activation with cell proliferation in MG-63 cells.

To rule out the possibility that the decrease in cell proliferation was due to cell death as a result of the potential cytotoxicity of PD98059, we carried out a recovery experiment, in which MG-63 cells were first treated with PD98059 for 24 h and then washed three times for 1 h each with normal DMEM/Ham’s F-12 medium containing 1 mM Ca$^{2+}$ to remove PD98059. The cells were then treated with 1 or 5 mM Ca$^{2+}$ for an additional 24 h (Fig. 8). As shown in Fig. 8, 5 mM calcium treatment of MG-63 cells following the removal of PD98059 resulted in a
A 3-fold increase in cell proliferation over that of 1 mM calcium (recovery group). The fold induction was similar to the fold induction in the control group. Regaining calcium responsiveness after removal of PD98059 indicated that MG-63 cells were still functionally viable after 24 h of PD98059 treatment. Thus blocking phosphorylation of ERK1 and ERK2 with PD98059 suppressed both basal and [Ca$^{2+}$]$_o$-stimulated osteoblast cell proliferation, indicating that the phosphorylation of ERK1 and ERK2 was critical for these two processes.

To directly assess whether p38 MAP kinase activity was required for calcium-stimulated proliferation, we incubated MG-63 cells with a potent p38 MAP kinase inhibitor, SB203580, which has an in vitro IC$_{50}$ of $-0.6$ μM (29, 30). Both 1 and 10 μM concentrations of SB203580 failed to inhibit calcium-stimulated osteoblast proliferation (Fig. 9). This result further confirmed that p38 MAP kinase was not critical for calcium-stimulated osteoblast proliferation.

A number of recent studies suggested that sustained MAP kinase activation was required for cellular functions such as cell proliferation and differentiation (31–34). To assess whether the acute or the prolonged activation of ERK1/2 was responsible for mediating mitogenic effects of [Ca$^{2+}$]$_o$, we first tested whether transient exposure to high calcium was sufficient to cause sustained activation of ERK1/2 and increase cell proliferation. MG-63 cells were transiently exposed to 5 mM [Ca$^{2+}$]$_o$ for 1 h and then incubated in normal growth medium (1 mM [Ca$^{2+}$]$_o$) for an additional 1 or 23 h. The cells were then either harvested for assessing ERK activation or treated with...
[\textsuperscript{3}H]thymidine to measure cell proliferation. One hour after removal of high calcium medium, ERK phosphorylation levels were reduced back to the basal level, and subsequent incubation with 1 mM calcium up to 24 h did not result in reactivation of the ERKs (Fig. 10). Transient exposure to 5 mM \([\text{Ca}^{2+}]_o\) (1 or 2 h) did not result in increase in \([\text{H}]\)thymidine incorporation at 24 h either (Fig. 11). These results indicate that transient exposure to high calcium causes acute activation of ERKs but does not lead to sustained activation of ERKs. Acute activation of ERK1/2 by transient calcium treatment was not sufficient to stimulate osteoblast proliferation. This result suggested that the continuing presence of high calcium is required for sustained activation of ERKs and stimulation of osteoblast proliferation.

To directly test whether sustained activation of ERK1/2 as the result of prolonged treatment of high calcium is required for calcium-stimulated osteoblast proliferation, we first treated MG-63 cells with 5 mM \([\text{Ca}^{2+}]_o\) for 1 h and then added PD98059 to the medium (Fig. 12). The delayed treatment of cells with PD98059 suppressed the proliferative effect of \([\text{Ca}^{2+}]_o\). The degree of suppression was similar to that obtained by pretreatment with PD98059. (PD98059 was added 1 h prior to addition of 5 mM \([\text{Ca}^{2+}]_o\).) Thus it confirmed that acute activation of ERK1 and ERK2 was not sufficient for \([\text{Ca}^{2+}]_o\)-stimulated proliferation. It also demonstrated that prolonged treatment with high calcium in the absence of sustained activation of ERKs was not sufficient for stimulation of osteoblast proliferation, which indicated that prolonged activation of ERK1 and ERK2 was critical for \([\text{Ca}^{2+}]_o\)-stimulated osteoblast proliferation.

**DISCUSSION**

Successful bone remodeling requires balanced rates of bone formation and bone resorption. The rates of bone formation are dependent on the number of functionally active osteoblasts. It is thus of prime importance to identify the extracellular factors in the bone milieu that regulate the proliferation of osteoblasts. In this study, we demonstrated that \([\text{Ca}^{2+}]_o\) is indeed such an extracellular factor that stimulated proliferation of primary human osteoblast cells and clonal osteoblast cell lines across a number of species. We also provided clear evidence for the first time that \([\text{Ca}^{2+}]_o\) stimulation of human osteoblasts resulted in both an acute phase and a sustained phase of ERK1 and ERK2 activation, which were through dual phosphorylation of critical threonine and tyrosine residues. The sustained activation was required for proliferative responses, whereas the acute activation was not sufficient. We found that although p38 MAP kinase and SAPK/JNK were present in MG-63 cells, they were not activated by \([\text{Ca}^{2+}]_o\) stimulation. Thus our studies suggest that the ability to sense \([\text{Ca}^{2+}]_o\) is present in in vivo matured human osteoblasts, supporting the hypothesis that \([\text{Ca}^{2+}]_o\) plays an important role in the bone remodeling process. Our findings also establish a molecular and cellular mechanism for \([\text{Ca}^{2+}]_o\)-stimulated osteoblast proliferation. The mitogenic effects of \([\text{Ca}^{2+}]_o\) are mediated through sustained activation of
specific ERK1 and ERK2 MAP kinases. Our results do not support the possibility that other MAP kinases such as p38 MAP kinase and SAPK/JNK are involved in \( \left[ Ca^{2+}\right]_o \)-stimulated osteoblast proliferation.

The discovery that ERK1 and ERK2 MAP kinases play a key role in \( \left[ Ca^{2+}\right]_o \)-stimulated osteoblast proliferation raises an interesting question regarding the molecular nature of the upstream signaling cascades leading toward activation of ERK1 and ERK2. Growth factor receptors and G-protein-coupled receptors have all been shown to activate ERK MAP kinase signal cascades through a number of mechanisms (15, 35, 36). Several studies provided molecular evidence that the cloned G-protein-coupled CaR was present in osteoblastic cells, suggesting that the changes in \( \left[ Ca^{2+}\right]_o \), may be sensed by the CaR, which then activates intracellular signal cascades (5, 6, 37). A very recent study demonstrated unambiguously that the mRNA and protein of the CaR were present in MG-63 cells and that the CaR was actively coupled to a K\(^+\) channel in MG-63 cells (13). Thus this evidence clearly resolved the controversial issue of the presence of the CaR in osteoblast cells and supported the hypothesis that the CaR is a possible candidate for mediating \( \left[ Ca^{2+}\right]_o \)-stimulated osteoblast proliferation.

![Figure 10](image1.png)

**Fig. 10.** Transient calcium stimulation induces acute activation but not sustained activation of ERK 1/2 MAP kinases. Western blot analysis was performed with extracts from cells treated with 1 or 5 mM calcium for specified time points using an antibody against the active form (phosphorylated form) of ERK 1/2 MAP kinases (A) or an antibody recognizing both active and inactive forms of ERK 1/2 MAP kinases (B). Lanes 1–8, cell extracts from cells that were treated with 1 or 5 mM calcium for 5 min, 1 h, 2 h, and 24 h. Lanes 9–12, cell extracts from cells that were treated with 1 or 5 mM calcium for 1 h and then switched to 1 or 5 mM calcium for 1 or 23 h.

![Figure 11](image2.png)

**Fig. 11.** Transient calcium stimulation does not induce proliferation of MG-63 cells. MG-63 cells were incubated in 1 mM (open bars) or 5 mM (filled bars) calcium at 37 °C for 24 h (24 hr). For transient calcium treatment, cells were treated with 1 or 5 mM calcium for 1 h or 2 h and then switched to 1 mM calcium for 24 h (1 hr and 2 hr). \[^{3}H\]\textsuperscript{[H]}Thymidine incorporation was used as an index of cell proliferation.

![Figure 12](image3.png)

**Fig. 12.** Delayed inhibition of MEK by PD98059 suppresses \( \left[ Ca^{2+}\right]_o \)-stimulated proliferation of MG-63 cells. MG-63 cells were incubated in 1 mM (open bars) or 5 mM (filled bars) calcium at 37 °C for 24 h. For the pretreatment group, cells were treated with 50 \( \mu \)M PD98059 in DMEM/Ham’s F-12 medium (1 mM calcium) for 1 h before the calcium concentration in the medium was adjusted to 1 or 5 mM. For the post-treatment group, PD98059 (50 \( \mu \)M) was added to DMEM/Ham’s F-12 medium after cells were treated with 1 or 5 mM calcium for 1 h. \[^{3}H\]\textsuperscript{[H]}Thymidine incorporation was used as an index of cell proliferation.
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specificity displayed by MG-63 cells in this study is functionally consistent with cation specificity of the cloned G-protein-coupled CaR (1, 38). However, the critical issues of whether the cloned CaR is the only calcium sensor in MG-63 cells and whether it plays any functional role in mediating [Ca\(^{2+}\)]\(_o\)-stimulated osteoblast proliferation remain unsolved (3, 9). More direct functional studies are needed to firmly establish a role for the CaR as an upstream component in [Ca\(^{2+}\)]\(_o\)-stimulated osteoblast proliferation.

Although acute activation of MAP kinase signal cascades by extracellular signals have been established in many cell systems, sustained activation of MAP kinases has only been reported in a limited number of studies, all of which indicate that the sustained activation, but not the acute activation, plays a more critical role in cell proliferation, differentiation, and migration as well as gene expression (31, 33, 39). This study establishes an important role for sustained activation of ERK1 and ERK2 in mediating the mitogenic effects of [Ca\(^{2+}\)]\(_o\), on osteoblast cell proliferation. Further studies examining the molecular mechanism underlying sustained activation will provide insight regarding how [Ca\(^{2+}\)]\(_o\) regulates osteoblast proliferation and the bone remodeling process.

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