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Ca^{2+}-ATPase Inhibitors and Ca^{2+}-Ionophore Induce Osteoclast-like Cell Formation in the Cocultures of Mouse Bone Marrow Cells and Calvarial Cells

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Osteoclasts which derive from hemopoietic cells are multinucleated cells responsible for bone resorption. We found that cyclopiazonic acid (CPA), thapsigargin (TG), and 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) induced osteoclast-like cell (OCL) formation in cocultures of mouse calvaria-derived stromal cells and hemopoietic cells such as bone marrow cells and spleen cells. OCLs induced by these compounds showed typical characteristics of osteoclasts such as tartrate-resistant acid phosphatase activity and pit forming activity. These compounds are known as endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitors that increase intracellular Ca^{2+} levels by inhibiting Ca^{2+}-ATPase activity located in the membrane of ER/SR. Ca^{2+}-ionophores such as ionomycin which increase intracellular Ca^{2+} levels also stimulated OCL formation in the cocultures. Differentiation of hemopoietic cells into OCLs induced by these compounds required the presence of calvarial cells. These results indicate that an increase of intracellular Ca^{2+} levels may be a part of signaling pathways to induce osteoclast differentiation in the presence of calvarial cells.

Osteoclasts are multinucleated giant cells that play a critical role in bone resorption. Their precursors are known to derive from mononuclear progenitors existing in hematopoietic tissues. In cocultures of mouse stromal cells and bone marrow cells, osteoclast-like cells (OCLs) were formed in response to several osteotropic hormones and cytokines such as 1α,25-dihydroxy vitamin D_3 [$1\ \alpha$,$25(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), interleukin 1 (IL-1), interleukin 6 (IL-6) together with soluble IL-6 receptors, interleukin 11 (IL-11), and leukemia inhibitory factor (LIF) [1, 2]. Therefore, the differentiation of osteoclast precursors into osteoclasts is believed to be regulated by three independent signal transduction pathways such as cAMP-mediated pathway, gp130-mediated pathway, and 1α,$25(OH)_2D_3$ receptors- mediated pathway [1, 2]. It is also proposed that the target cells for these cytokines and hormones are stromal cells but not osteoclast progenitors, and three independent signaling pathways activate the expression of some osteoclast differentiation factors (ODFs) that can induce osteoclast differentiation [1, 2].

In the last few years, although a large number of studies have been focused on the mechanism of osteoclast differentiation, the mechanism is not fully understood. To gain insights into regulatory mechanisms of osteoclast differentiation, we have attempted to screen low-molecular weight natural products that modulate osteoclast differentiation in a cocultures of mouse calvarial cells and bone marrow cells[3]. In the course of screening, we found that cyclopiazonic acid (CPA), an endoplasmic reticulum (ER)/sarcoplasimic reticulum (SR) Ca^{2+}-ATPase inhibitor, induced OCL formation in the cocultures. Furthermore, Ca^{2+}-ionophore that increase intracellular levels of Ca^{2+} induced OCL formation. In this paper, we report the stimulatory effect of Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore on OCL formation. Our results indicate that the increase of intracellular Ca^{2+} levels may be a part of signaling pathways to induce osteoclast differentiation.

MATERIALS AND METHODS

Animal and chemicals. Five- to eight-week-old male mice and newborn mice of ddY strain were obtained from Sankyo Labo Service Co. (Tokyo, Japan). CPA, 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ), thapsigargin (TG), ionomycin and 1α,$25(OH)_2D_3$ were purchased

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from Sigma Chemical Company (St. Louis, MO). Sodium tartrate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Each compound was dissolved in MeOH, and used for experiments.

In vitro formation of OCL. Five- to eight-week-old male mice were killed by cervical dislocation under light ether anesthesia. Bone marrow cells were collected from femora and tibiae as described previously [3]. Splenic tissues were cut into small pieces in α-minimal essential medium (α-MEM) and pipetted thoroughly. The cell suspension was filtered through 0.1 mm mesh, and the filtrate was collected as spleen cells. To prepare primary calvarial cells, a total of 20 to 30 calvaria collected from newborn mice were subjected to five sequential digestions using 0.1% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.2% dispase (Godo Shusei, Tokyo, Japan) as described [3]. Mouse calvarial cells (2×10^4 cells/well) were cocultured with either bone marrow cells (5×10^5 cells/well) or spleen cells (1×10^6 cells/well) in α-MEM containing 10 % fetal calf serum (CSL Limited, Victoria, Australia) in 48-well plates (CORNING Inc., Corning, NY) (0.4 ml/well). All cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO2 in air. The compounds which induce OCL formation were added to the cocultures just after exchanging the medium on day 3, and the cocultures were maintained for additional 3 days.

Characterization of OCL. After culture for 6 days, the adherent cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) as described [3]. TRAP-positive multinucleated cells having more than 5 nuclei were counted as OCLs. For pit formation assay, bone marrow cells and calvarial cells were similarly cocultured for 6 days on collagen gel-coated dishes [4]. Cocultures were then treated with 0.1 % collagenase at 37 °C for 20 min to release OCLs from the dishes. OCLs preparations were placed on dentine slices (4 mm diameter), which had been placed in 96-well plates containing 0.2 ml α-MEM supplemented with 10% FCS. After incubation for 24 h, cells were removed from dentine slices, and resorption pits were stained with Mayer's hematoxylin as described previously [4].

RESULTS

When cocultures of mouse calvarial cells and bone marrow cells were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ either for the entire 6 day-culture period or for the last 3 days of the culture period, OCLs were similarly formed in both cocultures (treated with $1\alpha,25(OH)_2D_3$ for 6 days, 445 ± 40 OCLs/well; treated with $1\alpha,25(OH)_2D_3$ for the last 3 days, 439 ± 23 OCLs/well, the mean ± SD of 4 cultures). Therefore, effects on OCL formation of low-molecular weight natural products added on day 3 were examined in the cocultures. As a result of the screening, we found that CPA significantly induced OCL formation. CPA at 5 - 20 μM dose-dependently stimulated OCL formation with a maximal effect at 20 μM and a half maximal effect at 10 μM (Fig. 1A). OCLs formed by CPA showed intense TRAP activity which is known as a marker enzyme of osteoclasts (Fig. 2A), and their morphological appearances were similar to those of OCLs induced by $1\alpha,25(OH)_2D_3$ (Fig. 2C). No TRAP-positive OCLs were formed in the control cocultures (Fig. 2D).

To determine whether the activity of CPA to induce OCL formation is a general feature of ER/SR Ca^{2+}-ATPase inhibitors, the effect of other Ca^{2+}-ATPase inhibitors such as TG and BHQ on OCL formation was examined in the cocultures. BHQ and TG also induced OCL formation in a dose-dependent manner with the maximal effects at 40 nM and 2 μM, respectively (Fig. 1B, C). Although the effective concentrations of these inhibitors in inducing OCL formation were different each other, the dose of each inhibitor required for OCL formation was very close to that affects ER/SR Ca^{2+}-ATPase [10, 11]. Ca^{2+}-ATPase inhibitors are thought to increase intracellular Ca^{2+} levels by inhibiting Ca^{2+}-ATPase activity of the intracellular Ca^{2+} pools such as ER/SR [7, 8, 9]. These results suggest that OCL formation induced by Ca^{2+}-ATPase inhibitors is caused by altering intracellular Ca^{2+} levels.

To confirm that an increase of intracellular Ca^{2+} levels induces OCL formation, the effect of ionomycin, a Ca^{2+}-ionophore, on OCL formation was examined in the cocultures. Ionomycin also significantly induced OCL formation in a dose-dependent manner, and the maximal effects were shown at 1 μM (Fig. 1D and Fig. 2B). These results support the hypothesis that OCL formation induced by Ca^{2+}-ATPase inhibitor is attributed to an increase of intracellular Ca^{2+} levels. In all cases of the present experiments, OCLs were not formed at higher concentrations of the compounds because of the toxicity.

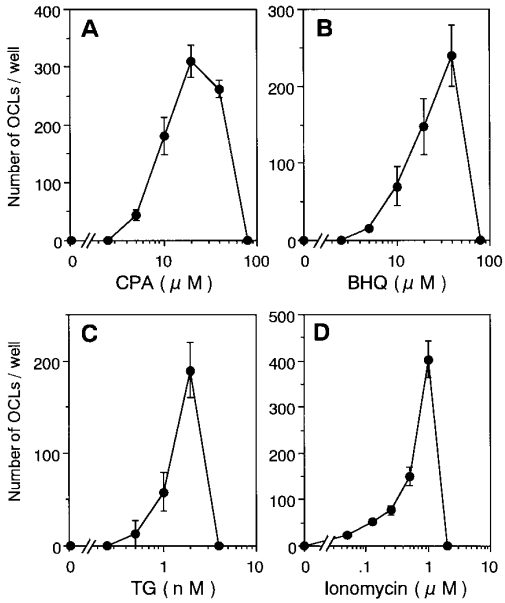

FIG. 1. OCL formation as a function of concentrations of Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore. Mouse bone marrow cells and calvarial cells were cocultured for 3 days. After changing the medium, cells were treated with various concentrations of Ca^{2+}-ATPase inhibitors [CPA (A), BHQ (B), and TG (C)] or Ca^{2+}-ionophore [ionomycin (D)] for an additional 3 days. Then, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than five nuclei were counted as OCLs. The results were expressed as means ± SD of four cultures.

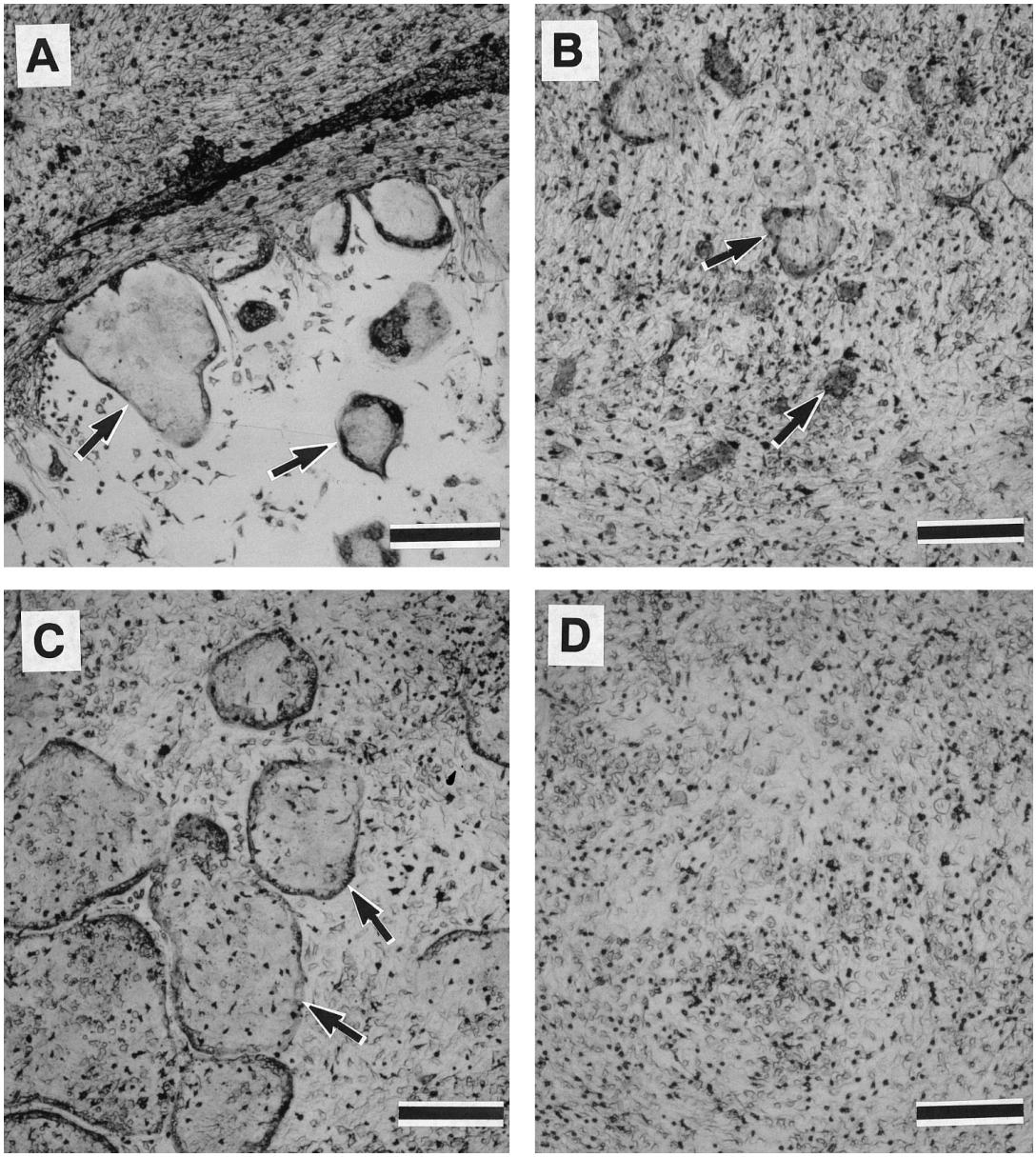

FIG. 2. OCL formation induced by CPA and ionomycin. Mouse bone marrow cells and calvarial cells were cocultured for 3 days. Then, the cells were treated with 20 μM CPA (A) or 1 μM ionomycin (B) for an additional 3 days. 10^{-8} M $1,25(OH)_2D_3$ (C) and MeOH (D) were used as the positive and negative controls, respectively. After coculture for 6 days, cells were fixed and stained for TRAP. Arrows indicate TRAP-positive OCLs. All bars indicate 250 μm.

In order to examine the bone resorbing abilities of OCLs induced by CPA, OCLs formed on the collagen gel in the presence of 20 μM CPA were recovered by collagenase digestion and cultured on dentine slices for 24 h. CPA-induced OCLs formed resortpion pits on dentine slices similarly as $1\alpha,25(OH)_2D_3$- induced OCLs did (Fig. 3). OCLs induced by other Ca^{2+}-ATPase inhibitors (TG and BHQ) and Ca^{2+}-ionophore (ionomycin) also formed similar resorption pits on dentine slices (data not shown).

It has been suggested that stromal cells play an essential role in differentiation of osteoclast progenitors into osteoclasts induced by osteotropic hormones and cytokines [1, 2]. Therefore, we examined whether OCL formation induced by Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore similarly requires the presence of calvarial cells or not. The spleen cell preparation which contained OCL progenitors but not OCL formation-supporting stromal cells was cultured with or without calvarial cells. Ca^{2+}-ATPase inhibitors and Ca^{2+}-iono-

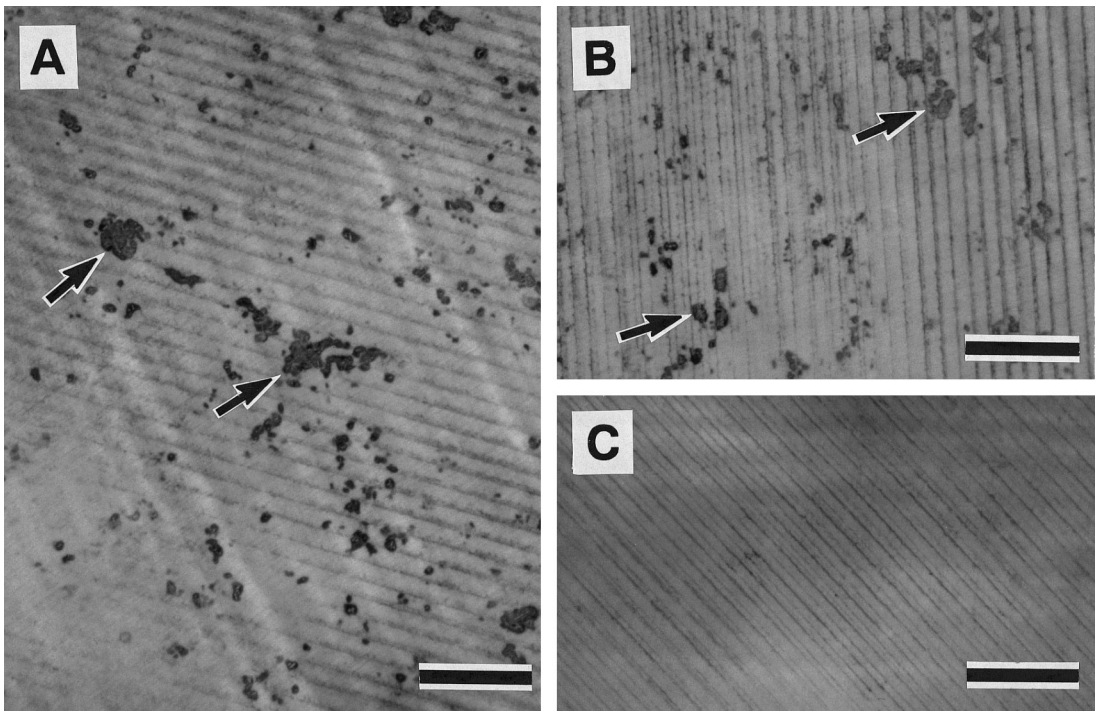

FIG. 3. Pit formation by OCLs induced by CPA. Mouse bone marrow cells and calvarial cells were cocultured for 3 days on collagen gel-coated dishes and treated with 20 μM CPA (A), 10^{-8} M 1α,25$(OH)_2D_3$ (B), or vehicle (MeOH) (C) for an additional 3 days. Cells were then recovered from the dish by collagenase treatment, and aliquots of the cell preparation were placed on dentine slices for 24 h. After removal of cells, the slices were stained with Mayer's hematoxylin solution to visualize resorption pits (arrows). All bars indicate 250 μm.

phore induced OCL formation only in the presence of calvarial cells (Table 1).

DISCUSSION

Present experiments have shown that both Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore induce OCL formation in the cocultures of bone marrow and calvarial cells. The OCLs induced by these compounds showed typical characteristics of osteoclasts such as high TRAP activity and pit forming activity. The Ca^{2+} level in cytosol of eucaryotic cells is regulated by both the release of Ca^{2+} from intracellular Ca^{2+} pools such as ER/SR and the entry of Ca^{2+} across the cytoplasmic membrane. These two events are closely coupled to each other, the entry of Ca^{2+} into cytoplasm being triggered by emptying the pools in response to inositol triphosphate [5, 6]. Ca^{2+}-ATPase inhibitors (CPA, TG and BHQ) deplete Ca^{2+} in the ER/SR store by inhibiting Ca^{2+}-ATPase located in the membrane of ER/SR and, as a result, an influx of extracellular calcium described as "capacitative entry" takes place [7, 8, 9]. The concentrations of CPA, TG and BHQ sufficient to induce OCL formation were comparable to those required to inhibit ER/SR Ca^{2+}-ATPase [10, 11]. Ca^{2+}-ionophore, ionomycin, also induced OCL formation. It has been reported that a Ca^{2+}-ATPase inhibitor increases IL-6 mRNA expression and IL-6 secretion in murine peritoneal macrophages via the increase of intracellular Ca^{2+} levels [12]. Furthermore, Tanapat et. al. have reported that ER/SR Ca^{2+}-ATPase inhibitors induce IL-4 pro-

TABLE 1

Requirement of Calvarial Cells for OCL Formation Induced by Ca^{2+}-ATPase Inhibitors and Ca^{2+}-Ionophore

		Number of OCLs/well	
Treatment	Concentration	Spleen cells/ calvarial cells	Spleen cells
CPA	20 μM	150 ± 25	0 ± 0
BHQ	40 μM	122 ± 18	0 ± 0
TG	2 nM	131 ± 36	0 ± 0
Ionomycin	1 μM	184 ± 36	0 ± 0
Vehicle		0 ± 0	0 ± 0

Note. Mouse spleen cells were cocultured with calvarial cells for 3 days. After changing the medium, cells were treated with vehicle, Ca^{2+}-ATPase inhibitors (CPA, BHQ, and TG), or Ca^{2+}-ionophore (ionomycin) for an additional 3 days. Cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than five nuclei were counted as OCLs. The results were expressed as means ± SD of four cultures.

duction and secretion in IL-3-dependent cell lines [13]. These results suggest that the increased intracellular Ca^{2+} level is involved in production of cytokines which modulate osteoclast differentiation. This hypothesis is also supported by a finding that high levels of extracellular Ca^{2+} stimulated OCL formation in mouse bone cell cultures [14].

The differentiation of osteoclast precursors into osteoclast is proposed to be regulated by three independent signal transduction pathways such as cAMP-mediated, gp130-mediated and $1\alpha,25(OH)_2D_3$ receptors-mediated pathways [1, 2]. The present study suggests that the increase of intracellular Ca^{2+} levels is another signaling pathway to induce osteoclast differentiation. Alternatively the increase of intracellular Ca^{2+} level may be accompanied with three independent signal transductions.

Previous studies have indicated that stromal cells play an indispensable role in osteoclast differentiation from progenitor cells [1,2]. It is also proposed that the target cells of osteotropic factors and hormones in inducing OCL formation are stromal cells but not osteoclast progenitors in the cocultures. The present study showed that OCL formation induced by Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore also required the presence of calvarial cells, suggesting that the intracellular Ca^{2+}-mediated signals are also functioning in stromal cells. Further studies are required to identify the target cells of Ca^{2+}-ATPase inhibitors and Ca^{2+}-ionophore and to clarify how an increase of intracellular Ca^{2+} levels in the target cells induces osteoclast differentiation.

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