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High Extracellular Calcium Inhibits Osteoclast-like Cell Formation by Directly Acting on the Calcium-Sensing Receptor Existing in Osteoclast Precursor Cells

Masanori Kanatani, Toshitsugu Sugimoto,[1] Michiko Kanzawa, Shozo Yano, and Kazuo Chihara
Third Division, Department of Medicine, Kobe University School of Medicine, Kobe, Japan

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Although it has recently been suggested that high extracellular calcium ($[Ca^{2+}]_e$) inhibits osteoclast function via a calcium-sensing receptor (CaSR) in mature osteoclasts, the role of CaSR in the regulation of osteoclast formation remains unknown. The present study was performed to investigate whether osteoclast precursor cells possess CaSR and to clarify the possible role of CaSR in the regulation of osteoclast formation. Immunocytochemistry detected CaSR in osteoclast precursor cells derived from spleen cells as well as in osteoblastic MC3T3-E1 cells. The use of reverse-transcription polymerase chain reaction (RT-PCR) with CaSR-specific primers, followed by nucleotide sequencing of the amplified products, also identified CaSR transcripts in osteoclast precursor cells derived from spleen cells as well as in MC3T3-E1 cells. High $[Ca^{2+}]_e$ (3 to 5 mM) concentration dependently inhibited 1,25(OH)2D3- or human parathyroid hormone (hPTH) (1–34)-induced osteoclast-like cell (Ocl) formation from osteoclast precursor cells derived from spleen cells. Further, the CaSR agonist neomycin also concentration dependently inhibited 1,25(OH)2D3- or hPTH(1–34)-induced Ocl formation. Moreover, a calcimimetic which mimics or potentiates the effects of $[Ca^{2+}]_e$ at the CaSR NPS R-467 (1-100 μM) concentration dependently inhibited Ocl formation stimulated by 1,25(OH)2D3 or hPTH(1–34). These findings first demonstrated that osteoclast precursor cells possess CaSR very similar, if not identical, to those in the parathyroid and kidney. Furthermore, the CaSR in osteoclast precursor cells could play a key role in regulating Ocl formation by sensing local changes in $[Ca^{2+}]_e$ at the resorptive sites.

At bone resorption sites, osteoclasts dissolve the mineralized bone matrix and release Ca^{2+} to the extracellular fluid, and the calcium concentration at the resorption sites could rise to as high as 40 mM (1). In the process of bone remodeling, a phase of osteoclastic bone resorption is followed by a period of bone formation (2). This process must include the recruitment of osteoblasts and their precursors to the resorbed bone surface as well as their subsequent proliferation and differentiation into mature functional osteoblasts. Although the coupling of formation to resorption has been considered to be mediated by various kinds of growth factors present in the bone matrix such as transforming growth factor-β (3, 4), our recent study (5, 6) revealed that an increase in extracellular calcium ($[Ca^{2+}]_e$) caused a stimulation of DNA synthesis and a chemotactic response in osteoblastic MC3T3-E1 cells. Thus, we have postulated the possibility that Ca^{2+} released from bone matrix at the resorptive sites might be an important candidate of the coupling factors in bone remodeling. On the other hand, there has been accumulating evidence that an increase in $[Ca^{2+}]_e$ negatively controls osteoclast function (7, 8). Further, our recent study revealed that an increase in $[Ca^{2+}]_e$ inhibited 1,25-dihydroxyvitamin D_3-stimulated osteoclast-like cell formation derived from spleen cells (6). Although recent study (9) suggests that calcium-sensing receptor (CaSR) is present in mature osteoclasts, it remains unknown that osteoclast precursor cells possess CaSR and that CaSR is involved in the inhibitory effect of high $[Ca^{2+}]_e$ on osteoclast formation. In the present study, we first demonstrated clear expression of the CaSR in osteoclast precursor cells as assessed by immunocytochemical staining using a CaSR-specific monoclonal antibody as well as RT-PCR with CaSR-specific primers. We also found that CaSR agonists as well as high $[Ca^{2+}]_e$ inhibit osteoclast-like cell formation induced by bone resorbing factors. These findings suggest that CaSR in osteoclast precursor cells could potentially play a pivotal role in regulating the osteoclast formation within the marrow by sensing local changes in $[Ca^{2+}]_e$ at the resorption sites.

[1] To whom correspondence should be addressed at Third Division, Department of Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan. Fax: 078-382-5899 (Japan).

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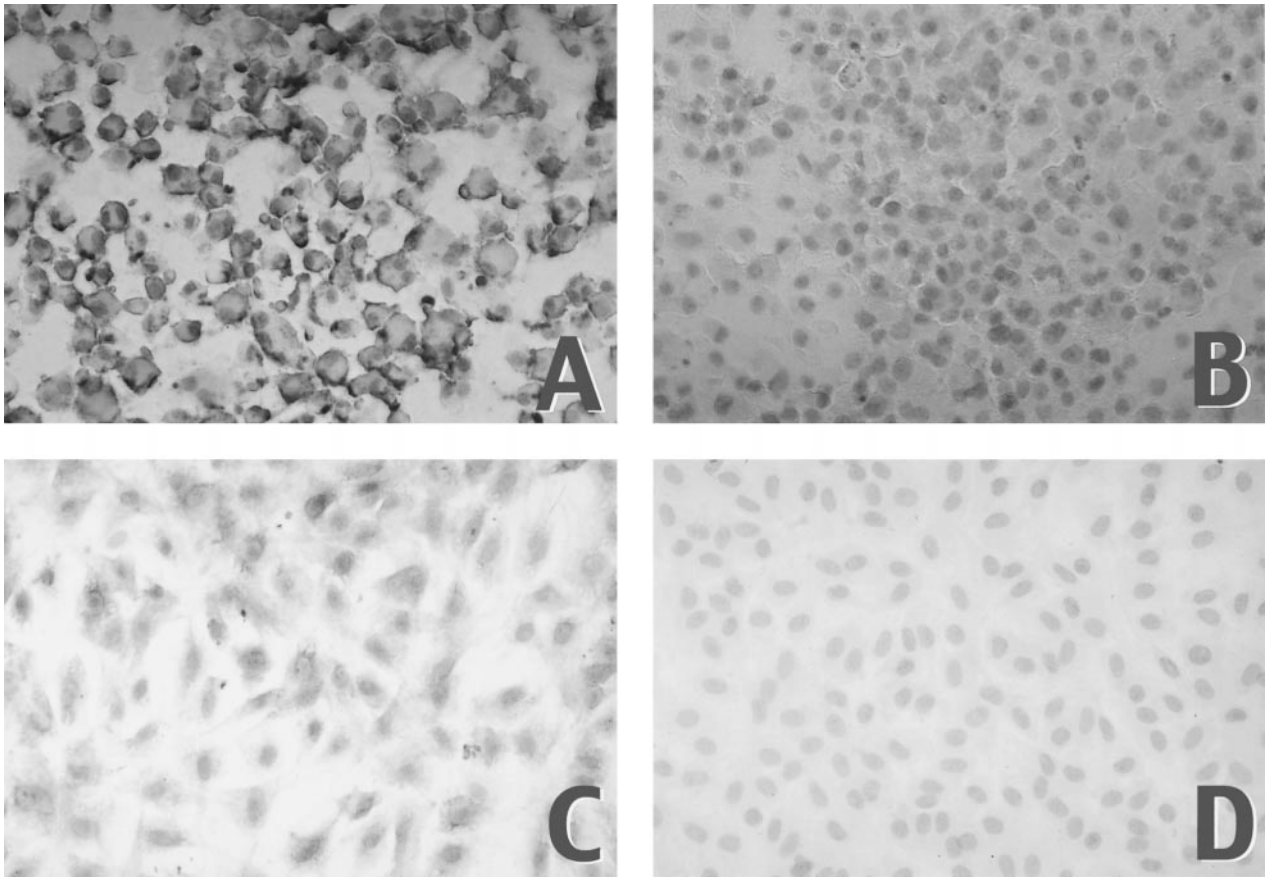

FIG. 1. Immunocytochemistry of osteoclast precursor cells derived from mouse spleen cells and mouse osteoblastic MC3T3-E1 cells carried out as described under Materials and Methods using a CaSR-specific monoclonal antibody (ADD). Immunocytochemistry of osteoclast precursor cells as well as MC3T3-E1 cells revealed strong CaSR staining (A and C, respectively), which were eliminated when the primary antibody was omitted in each control study (B and D, respectively). (Magnification ×400.)

MATERIALS AND METHODS

Formation of osteoclast precursor cells and osteoclast-like cells from hemopoietic blast cells derived from mouse spleen cells. Osteoclast-like cell formation from hemopoietic blast cells was measured as previously described in detail (10, 11). In this method, stromal cells were absent in hemopoietic blast cells, as we previously described (12). 5-Fluorouracil (generously provided by Kyowa Hakko Co., Osaka, Japan) was administered to six-week-old female BDF1 mice (Shizuoka Experimental Animal Center, Shizuoka, Japan) at a dosage of 150 mg/kg body wt through a tail vein. Four days after injection, spleen cells were harvested and cell suspensions were prepared. Aliquots of spleen cells (2.6×10^6 cells/ml) were plated onto 35-mm culture dishes in 1 ml of α-minimum essential medium (α-MEM; Ca^{2+}, 1.8 mM) containing 1.2% methylcellulose, 50 U/ml IL-3 (Genzyme Co., Cambridge, MA), 10^{-8} M IL-6 (generously provided by Chugai Pharm. Co. Ltd., Shizuoka, Japan), 10 mg/ml bovine serum albumin and 30% fetal calf serum (FCS). The colonies of hemopoietic blast cells appeared after approximately 7 days and were lifted from the dishes with a 10-μl Eppendorf micropipet. Subsequently, osteoclast precursor cells were prepared from blast cells cultured at a concentration of 10^4/ml in 96-well microplates containing 100 μl of α-MEM supplemented with 5% FCS and 200 U/ml GM-CSF (Genzyme Co., Cambridge, MA) for 7 days. These osteoclast precursor cells were used for immunocytochemistry or reverse transcription-polymerase chain reaction to detect CaR.

To obtain osteoclast-like cells, these precursor cells were cultured in the presence of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] or human parathyroid hormone(1–34) [hPTH(1–34)] for 4 more days. Four days later, the cells were stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts. The number of TRAP-positive MNC with three or more nuclei was measured. These TRAP-positive MNC had various characteristics of osteoclasts, including responsiveness to calcitonin, the existence of calcitonin binding sites (11, 13) and osteoclastic bone resorption, as evidenced by co-culturing with bone rudiments (10).

Immunocytochemistry for CaSR in osteoclast precursor cells. A CaSR antibody, designated ADD was kindly provided by Dr. K. Rogers (NPS Pharmaceuticals, Inc., Salt Lake City, UT). For immunohistochemical detection of CaSR protein, osteoclast precursor cells or MC3T3-E1 cells were cultured in Lab-Tek chamber slides (Nagle Nunc International). After removing media, cells were rinsed with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde (Nacalai Tesque Inc., Kyoto, Japan). After washing three times with PBS, endogenous peroxidase was inactivated with methanol containing 0.3% H_2O_2 for 20 min at room temperature. After washing two times with PBS, nonspecific reaction was blocked with 1.5% skim milk in PBS. The slides were incubated with either ADD (diluted 1:1200 in PBS containing 1% non immunized mouse serum) or control solution (PBS containing 1% nonimmunized mouse serum) overnight at 4°C. After washing thoroughly with PBS, slides were incubated with a biotinylated link antibody (containing anti-

rabbit and anti-mouse immunoglobulins) and peroxidase-labeled streptavidin using LSAB2 kit (DAKO Corp. Carpinteria, CA) (14, 15). Final development was carried out with 3,3-diaminobenzidine (DAB) containing 0.1% H_2O_2 in PBS. Slides were counterstained with hematoxylin and observed by light microscopy.

Reverse-transcribed polymerase chain reaction amplification of mouse CaSR in osteoclast precursor cells and MC3T3-E1 cells. Total RNA was extracted from osteoclast precursor cells or MC3T3-E1 cells by the acid guanidinium thiocyanate-phenol–chloroform extraction methods (16). Each first-stranded cDNA was synthesized from 1 μg of total RNA using Molony murine leukemia virus reverse transcriptase (GIBCO BRL). Polymerase chain reaction (PCR) was performed at a final concentration of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of forward primer, 0.4 μM of reverse primer, and 1 μl of ELONGASE enzyme mix (GIBCO BRL). Two different sets of primer pairs were used: 5′-ATGGTTTGGCTACTGTTTGG-3′, sense; 5′-CAGAGCCTTGGAGACGGTGT-3′, antisense, designed from the partially cloned extracellular domain of the mouse AtT-20 cell CaSR (17); 5′-AGAAGTTCCGAGAGGAAGCC-3′, sense; 5′-ACCTGTTGCC-ACCTTCTTCG-3′, antisense, designed from the extracellular domain of the rat kidney CaSR (18). The first primer pair was designed to span one intron of the CaSR gene, to distinguish products amplified from cDNA and genomic DNA. To perform "hot-start" PCR, the enzyme was added during the initial 3-minute denaturation and was followed by 35 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 47°C, and 1 min extension at 72°C). The reaction was completed with an additional 10-min incubation at 72°C to allow completion of extension. PCR products were fractionated on 1.2% agarose gels. The presence of 319 and 480 nucleotide-long amplified products, respectively, were indicative of positive PCRs. The PCR products in the reaction mixture were purified using the QIAEX II gel extraction kit (Qiagen GmbH, Hilden, Germany) and subjected to direct, bidirectional sequencing employing the same primer pairs used for PCR by means of an automated sequencer (ABI PRISM 377; Applied Biosystems, Foster City, CA), using a dye-terminator cycle sequencing, FS Ready Reaction kit.

Statistical analysis. Data are expressed as means ± SEM. The data shown in the figures and the significance within each set of data were representative of at least three separate cell preparations. Similar results were obtained from other cell preparations. The significance of a difference between comparable group was determined by Student's *t* test or Duncan's multiple-range test.

RESULTS AND DISCUSSION

To clarify whether the CaSR is expressed in osteoclast precursor cells, we first investigated the presence of the receptor in osteoclast precursor cells derived from mouse hemopoietic blast cells by immunocytochemistry. We employed MC3T3-E1 cells as a positive control for detecting CaSR because the expression of CaSR in MC3T3-E1 cells was shown by immunocytochemistry and RT-PCR (19). Immunocytochemistry of osteoclast precursor cells as well as osteoblastic MC3T3-E1 cells, performed with a CaSR-specific monoclonal Antibody (mAb), revealed positive CaSR staining (Figs. 1A and 1C, respectively). On the other hand, no immunoreactivity was observed when the primary antibody was omitted in each control study (Figs. 1B and 1D, respectively). The immunoreactivity was observed in osteoclast precursor cells as well as in MC3T3-E1 cells, showing that osteoclast precursor cells derived from mouse hemopoietic blast cells express CaSR protein.

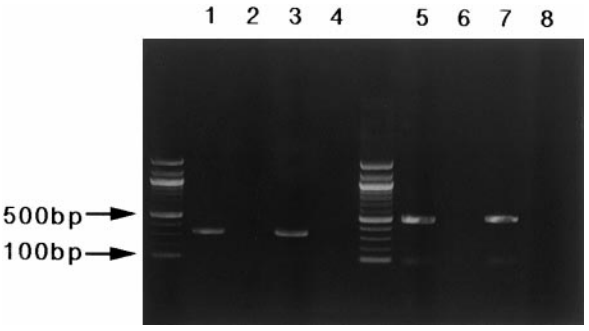

FIG. 2. Identification of CaSR transcripts in osteoclast precursor cells derived from mouse spleen cells and mouse osteoblastic MC3T3-E1 cells using RT-PCR with two sets of CaSR-specific primers, performed as described under Materials and Methods. Two products were amplified from reverse transcribed RNA isolated from osteoclast precursor cells as well as from MC3T3-E1 cells, which were of the expected sizes, 319 bp (lane 1 and lane 3, respectively) and 480 bp (lane 5 and lane 7, respectively), for CaSR-derived products. The PCRs without RT showed no products (lanes 2, 4, 6, and 8, respectively) in osteoclast precursor cells as well as in MC3T3-E1 cells. Lanes 1, 2, 5, and 6; osteoclast precursor cells. Lanes 3, 4, 7, and 8, MC3T3-E-1 cells.

We next performed RT-PCR analysis of CaSR expression in osteoclast precursor cells. RT-PCR with two sets of CaSR-specific primers (Fig. 2), one of which was intron-spanning to preclude amplification of a similar sized product from any contaminating genomic DNA, amplified two products of the expected sizes, 319 bp (Fig. 2, lanes 1 and 3) and 480 bp (Fig. 2, lanes 5 and 7), for cDNA synthesized from CaSR transcripts in osteoclast precursor cells as well as in MC3T3-E1 cells. No products were observed when the RT was omitted during synthesis of cDNA (Fig. 2, lanes 2, 4, 6, and 8, respectively) in osteoclast precursor cells as well as in MC3T3-E1 cells. DNA sequence analysis of the two PCR products from osteoclast precursor cells as well as from MC3T3-E1 cells revealed 100% and 95% sequence identities with the mouse AtT-20 cell CaSR sequence (17) and the rat kidney CaSR sequence (18), respectively (data not shown). The 480-bp PCR product showed one amino acid difference from the rat sequence at position 132. In MC3T3-E1 cells, our findings of DNA sequence analysis of PCR products are compatible with the previous report (19). In this study, the possibility of amplification of CaSR from genomic DNA was totally eliminated because the primer pair was designed to span one intron of the CaSR gene, the primer pair amplified a product of the size expected for a CaSR-derived product, and no PCR product was amplified without RT. These results showed that the PCR products corresponded to CaSR sequences, indicating the presence of bona fide CaSR transcripts in osteoclast precursor cells.

Thus, the present study shows that osteoclast precursor cells express both CaR protein and mRNA. Then, We next examined the effect of high concentra-

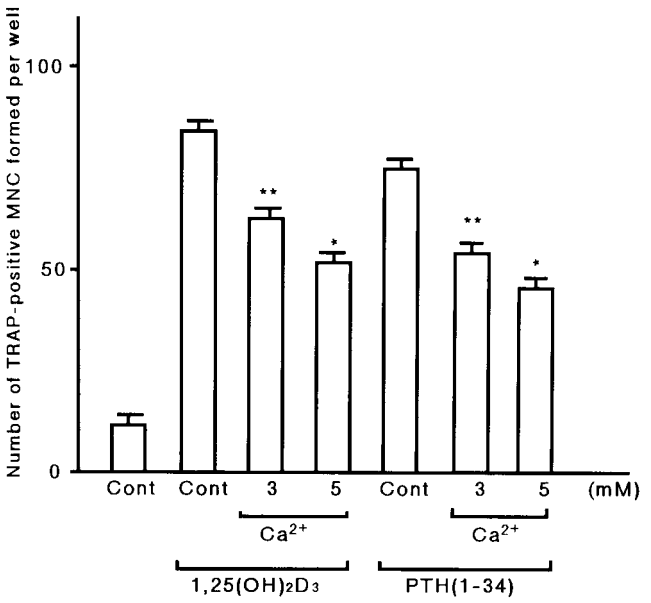

FIG. 3. Effect of high concentrations of $[Ca^{2+}]_e$ on $1,25(OH)_2D_3$- or hPTH(1-34)-induced osteoclast-like cell formation from osteoclast precursor cells. TRAP-positive MNC were counted 4 days after treatment with high concentrations of $[Ca^{2+}]_e$ in the presence of 10^{-8} M $1,25(OH)_2D_3$ or 10^{-8} M hPTH(1-34), as described under Materials and Methods. Each bar represents the mean ± SEM of four determinations. The concentration of $[Ca^{2+}]_e$ of the control was 1.8 mM. $*P < 0.01$, compared with $1,25(OH)_2D_3$- or hPTH(1-34)-treated control. $**P < 0.05$, compared with $1,25(OH)_2D_3$- or hPTH(1-34)-treated control.

tions of $[Ca^{2+}]_e$ on $1,25(OH)_2D_3$- or hPTH(1–34)-induced osteoclast-like cell formation from osteoclast precursor cells. As we previously reported (6, 11), both 10^{-8} M $1,25(OH)_2D_3$ and 10^{-8} M hPTH(1–34) significantly stimulated TRAP-positive MNC formation from osteoclast precursor cells (Fig. 3). The concentration of $[Ca^{2+}]_e$ of the control medium was 1.8 mM. High $[Ca^{2+}]_e$ (3 to 5 mM) concentration dependently inhibited $1,25(OH)_2D_3$- or hPTH(1–34)-induced TRAP-positive MNC formation from these osteoclast precursor cells (Fig. 3). Furthermore, neomycin (100 and 500 μM), a CaSR agonist, and NPS R467 (1–100 μM), a calcimimetic, which mimics or potentiates the effect of $[Ca^{2+}]_e$ on the CaSR, concentration-dependently inhibited TRAP-positive MNC formation stimulated by $1,25(OH)_2D_3$ or hPTH(1–34) (Fig. 4). These results strongly suggest that the inhibitory effect of high $[Ca^{2+}]_e$ on osteoclast-like cell formation stimulated by $1,25(OH)_2D_3$ or hPTH(1–34) would be mediated through CaSR in osteoclast precursor cells. Thus, it is possible that the CaSR in osteoclast precursor cells could sense high levels of $[Ca^{2+}]_e$ released from mineralized bone matrix during osteoclastic resorption of bone, thereby providing a signal for osteoclast precursor cells that ceases further differentiation at the end of resorption phase in the bone remodeling.

In conclusion, the present study first demonstrated that osteoclast precursor cells possess CaSR very similar, if not identical, to those in parathyroid and kidney and that CaSR is at least in part involved in the regulation of osteoclast formation by $[Ca^{2+}]_e$. Taking the evidence that high $[Ca^{2+}]_e$ inhibits osteoclast activity via CaSR existing in mature osteoclast (9) into account, the high $[Ca^{2+}]_e$ at the resorptive site would directly inhibit not only osteoclast function but also differenti-

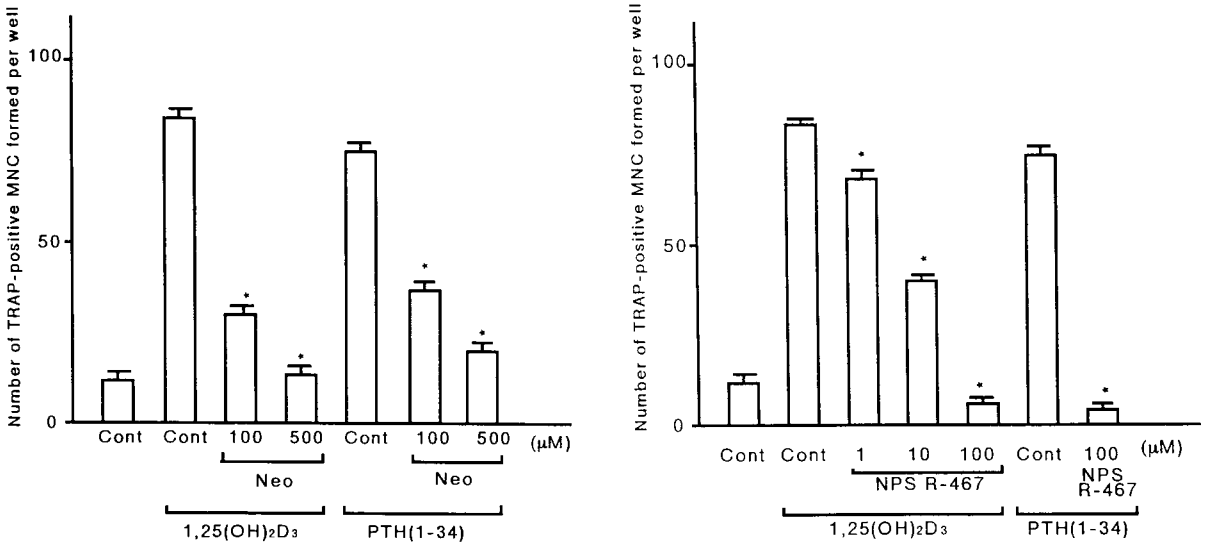

FIG. 4. Effect of a CaSR agonist and a calcimimetic on $1,25(OH)_2D_3$- or hPTH(1-34)-induced osteoclast-like cell formation from osteoclast precursor cells. TRAP-positive MNC were counted 4 days after treatment with the indicated concentrations of a CaSR agonist, neomycin or a calcimimetic, NPS R-467 in the presence of 10^{-8} M $1,25(OH)_2D_3$ or 10^{-8} M hPTH(1-34), as described under Materials and Methods. Each bar represents the mean ± SEM of four determinations. The concentration of $[Ca^{2+}]_e$ of the control was 1.8 mM. $*P < 0.01$, compared with $1,25(OH)_2D_3$- or hPTH(1-34)-treated control.

ation presumably via CaSR. The present data provide evidence about an important role of CaSR in the regulation of bone remodeling.

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