TRPV-5 Mediates a Receptor Activator of NF-κB (RANK) Ligand-induced Increase in Cytosolic Ca\(^{2+}\) in Human Osteoclasts and Down-regulates Bone Resorption*§

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Most of the signaling effectors located downstream of receptor activator of NF-κB (RANK) activation are calcium-sensitive. However, the early signaling events that lead to the mobilization of intracellular calcium in human osteoclasts are still poorly understood. The Ca\(^{2+}\)-sensitive fluorescent probe Fura2 was used to detect changes in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in a model of human osteoclasts. Stimulating these cells with receptor activator of NF-κB ligand (RANKL) induced a rapid and significant increase in [Ca\(^{2+}\)]\(_i\). Adding extracellular Ca\(^{2+}\) chelators, depleting intracellular stores, and the use of a phospholipase C inhibitor all indicated that the Ca\(^{2+}\) was of extracellular origin, suggesting the involvement of a Ca\(^{2+}\) channel. We showed that none of the classical Ca\(^{2+}\) channels (L-, T-, or R-type) were involved in the RANKL-induced Ca\(^{2+}\) spike. However, the effect of high doses of Gd\(^{3+}\) did suggest that TRP family channels were present in human osteoclasts. The TRPV-5 channel was expressed in osteoclasts and was mainly located in the cellular area in contact with the bone surface. Furthermore, the RNA inactivation of TRPV-5 channel completely inhibited the RANKL-induced increase in [Ca\(^{2+}\)]\(_i\), which was accompanied in the long term by marked activation of bone resorption. Overall, our results show that RANKL induced a significant increase in [Ca\(^{2+}\)]\(_i\), of extracellular origin, probably as a result of the opening of TRPV-5 calcium channels on the surface of human osteoclasts. Our findings suggest that TRPV-5 contributes to maintaining the homeostasis of the human skeleton via a negative feedback loop in RANKL-induced bone resorption.

Transient increases in intracellular calcium are common initiators of signaling cascades, survival signals, and enzyme activation (1). Many extracellular signals modify the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in osteoclasts and, by doing so, modulate their functions (2). For instance, exposing osteoclasts to high doses of extracellular Ca\(^{2+}\) during active bone resorption triggers increases in the [Ca\(^{2+}\)]\(_i\), and inhibits osteoclast function, in a self-modulated loop (3). In addition, osmotic membrane stretches, environmental acidification, or temperature changes all induce increased [Ca\(^{2+}\)]\(_i\), (4, 5). The signaling pathways and metabolic cell functions most commonly reported to be affected by such increases in Ca\(^{2+}\) in the cytosol are perturbations of the resorbing machinery, with a direct inhibition of acid secretion (6–8). In contrast, attachment of osteoclasts to the bone matrix via αβ3 receptors promotes signals that lead to a fall in [Ca\(^{2+}\)]\(_i\), which in turn initiates actin ring formation and triggers resorption (9–11). Moreover, actively resorbing osteoclasts display significantly higher [Ca\(^{2+}\)] than motile cells, but a K\(^{+}\)-induced depolarization of their membranes results in a reduction in [Ca\(^{2+}\)]\(_i\), leading to pseudopodia retraction, actin ring formation, and active resorption (12). This is reversed when the membrane is repolarized and the intracellular Ca\(^{2+}\) increases, leading the osteoclasts to detach from the bone matrix and start to migrate (12). In contrast activation of Ca\(^{2+}\)-dependent intracellular kinases, such as Pyk2, is required for adequate integrin-ligand interaction and osteoclast adhesion to the bone substrate to occur (13, 14). Despite some discrepancies, it is clear that calcium levels in the cytosol are an important factor in controlling osteoclast resorption/migration cycles.

In addition to physico-chemical changes in the cellular microenvironment, hormones and cytokines also alter [Ca\(^{2+}\)]\(_i\), and modify osteoclast function (15–17). The signaling cascades activated by RANKL, the chief regulator of osteoclast differentiation and function, result in activation of the transcription factor NFATc1, which is itself dependent on Ca\(^{2+}\) ions (18). In rodents, RANKL stimulation of osteoclasts triggers a rapid and significant rise in the intracellular Ca\(^{2+}\) concentration, which is required to promote the nuclear translocation of NF-κB and activation of NFATc1 and to stimulate osteoclast survival and bone resorption activity (19, 20). According to these data, RANKL-induced elevation of the [Ca\(^{2+}\)]\(_i\) relies on the recruitment from the intracellular Ca\(^{2+}\) store as a result of phospholipase C activation. However, the mechanism by which RANKL recruits phospholipase C and/or increases cytosolic Ca\(^{2+}\) remains largely obscure and calls for further clarification, espe-

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2 The abbreviations used are: RANKL, receptor activator of NF-κB ligand; TRP, transient receptor potential; TRPV, vanilloid TRP; CTR, calcitonin receptor; DAPI, 4′,6-diamidino-2-phenylindole; siRNA, small interfering RNA; CSF, colony-stimulating factor; CBMs, cord blood monocytes.
cially in human cells, as most work has so far been performed in rodents.

We used a well established model of osteoclast differentiation from umbilical cord blood monocytes (CBMs) to investigate RANKL-induced Ca^{2+} signals in human osteoclasts. We show here that [Ca^{2+}]_i oscillations do indeed occur in human osteoclasts stimulated with RANKL but that they differ considerably from those seen in rodents. In particular, we have identified the vanilloid TRP-5 (TRPV-5) Ca^{2+} channel as a mediator of RANKL-induced increases in the cytosolic level of Ca^{2+}, which may contribute to long term inhibition of bone resorption.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Opti-MEM medium, penicillin, streptomycin, Fungizone, glutamine, and fetal bovine serum were purchased from Invitrogen (Burlington, Ontario, Canada). Ficoll-Paque was purchased from Amersham Biosciences (Montreal, Quebec, Canada). Recombinant human macrophage-CSF and recombinant human granulocyte-macrophage-CSF were purchased from R&D Systems (Minneapolis, MN); goat polyclonal antibodies against calcitonin receptor (CTR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal antibodies against TRPV-5 were from Abcam (Cambridge, MA), and rabbit polyclonal antibodies were from Upstate Biotech Millipore (Billerica, MA); rabbit polyclonal antibodies against L-type and T-type Ca^{2+} channels were purchased from Sigma-Aldrich; those against R-type channels were from Novus Biologicals (Littleton, CO); and rabbit polyclonal antibodies against V-ATPase were purchased from Chemicon (Temecula, CA). Alexa Fluor antibodies and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Soluble human RANKL was produced in our laboratory.

**Human Osteoclast Culture**—Blood was harvested from human umbilical cord at delivery after obtaining informed consent from parturient women, as approved by our institution’s review board. Monocytes were extracted from cord blood by centrifugation on Ficoll and then washed and suspended in Opti-MEM containing 2% fetal bovine serum and antibiotics. They were plated at a density of 3 \times 10^6 cells/ml. After incubating overnight, the cells were washed to remove non-adherent cells. The selected CBMs were cultured for another 3 weeks in the same medium supplemented with granulocyte-macrophage-CSF (100 pg/ml) for the first 3 days and then with macrophage-CSF (25 ng/ml) and RANKL (100 ng/ml). The medium was changed every 3 days. We have previously shown that fully differentiated osteoclasts form under these conditions (21).

**Calcium Measurements**—48 h before the experiment began, the medium was replaced by fresh RANKL-free medium. When the experiment began, the osteoclasts were loaded with Fura2-AM (Invitrogen) in a Tyrode’s buffer (Sigma-Aldrich) equilibrated to pH 7.2 with Heps and bicarbonate and to 298 mosm with sucrose. The cells were then allowed to settle over a heating plate and maintained at 37 °C under a Nikon Eclipse microscope. Using specific filters and Metafluor software, the ratio of Fura2 emissions at 510 nm was recorded after alternating excitation at 340 and 380 nm, corresponding to the bound and unbound fractions of Fura2, respectively. The emission ratios were computed, recorded every 2 s, and then converted into [Ca^{2+}], after calibrating Fura2. After a short equilibration period (1–2 min), the medium was carefully removed and replaced by medium containing 100 ng/ml RANKL. The Fura2-emitted light was recorded every 2 s. Some cells were transfected with TRPV-5 siRNAs (Qiagen, Mississauga, Ontario, Canada) or preincubated with channel blockers before adding the RANKL. Some experiments were performed in a Ca^{2+}-free medium.

**Immunofluorescence Studies**—Cells were grown in Lab-Tek multichambers and fixed with paraformaldehyde 1% for 10 min. They were then washed, and the autofluorescence was quenched with glycine (0.1 M). Nonspecific binding was blocked by incubating the cells with 5% skimmed milk in phosphate-buffered saline for 1 h. The cells were then incubated with antibodies directed against the various types of Ca^{2+} channels (T-type, L-type, R-type, and TRPV-5) or with the same concentration of polyclonal IgGs from rabbit serum, washed, and incubated with secondary antibodies coupled to Alexa Fluor 546. Finally, DAPI was added to identify multinucleated cells. Pictures were taken with a Nikon Eclipse microscope equipped with specific filters and the Simple PCI software.

**Confocal Microscopy**—Cells were grown and differentiated for 3 weeks on bovine bone slices or on plastic before being fixed with paraformaldehyde 1%. The bone slices were incubated in glycine 0.1 M for 24 h to quench the autofluorescence. The nonspecific epitopes were then blocked using a 5% solution of skimmed milk. Bone or plastic slices were then incubated in a solution of antibodies directed against the TRPV-5 channel, CTR, or V-ATPase diluted in skimmed milk, washed in phosphate-buffered saline, and then further incubated in a solution of secondary antibodies coupled to Alexa Fluor 488. Cells were examined with a scanning confocal microscope (FV1000, Olympus) coupled to an inverted microscope with a \( \times 40 \) oil immersion objective (Olympus). Optical sections were collected at 0.8-\( \mu m \) intervals. Digitized images were computed and processed with Image-Pro+ 6.0 software.

**RNA Inactivation**—Cells were grown as described above and, when required, were transfected with an siRNA that inhibits human TRPV-5 channels (Qiagen). A set of four different siRNAs was used to optimize this inhibition (Table 1). The transfection procedure was performed as indicated by the manufacturer’s instructions using 1 ng/ml siRNA. Briefly, the culture medium was removed and replaced with a mixture containing Hyperfect (Qiagen) and the selected siRNA, diluted in serum and antibiotic-free Opti-MEM. Cells were maintained at 37 °C for 2 h, and fresh culture medium was added again to the dishes. Transfection efficiency was monitored using a non-inhibitory but fluorescent-tagged siRNA as control. The down-

| Name | Catalog number | Target sequence |
|------|----------------|-----------------|
| Hs_TRPV5_3 | S00124369 | TCCCTATGTGGAGTGTTCGA |
| Hs_TRPV5_5 | S01078653 | CCCGCCAGCCCACTCTGAAAA |
| Hs_TRPV5_1 | S00124355 | CCAGAACACCACTCTGAAAA |
| Hs_TRPV5_3 | S00124362 | CGGCTTGAAGACACAATGAT |
regulation of TRPV-5 expression was demonstrated by quantitative PCR and Western blot.

Western Blot Analysis—Mature osteoclasts were washed quickly with cold phosphate-buffered saline and lysed in the presence of protease inhibitors. 50 μg of total proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skimmed milk and incubated overnight at 4 °C with the primary anti-TRPV-5 antibody (1:500). Anti-actin antibodies were used as a loading control. Horseradish peroxidase-conjugated secondary antibodies were used to achieve detection with a chemiluminescent system. Band densities were quantified with a Bio-Rad Versa Doc imaging system (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and normalized for the optical density of the actin bands after stripping and reblotting the membranes.

Quantitative PCR—After siRNA transfection, total RNA was extracted using TRIzol Reagent (Invitrogen) followed by 2-propanol precipitation of total RNA. After being assayed and checked for quality, 1 μg of total RNA was used to perform cDNA synthesis and quantitative PCR amplification. Human primers of TRPV-5 were generated using the RefSeq database and validated by the Rnomics platform of our institution. 100 ng of cDNA, 200 nm of primers, and 5 μl of Power SYBR Green master mix were added to the reaction mix to give a total volume of 10 μl. Amplification and detection of TRPV-5 and of three reference housekeeping genes (HMBS, PSMC4, SDHA) were conducted with a Realplex 2 Master Cycler (Eppendorf, Mississauga, Ontario, Canada). The quantification and normalization of results were based on the computation of target threshold cycle (Ct) values and reference gene Ct values in qBase software. All samples were run in triplicate. Relative expression levels were normalized with respect to a set of reference primer pairs (i.e. the mean of the three housekeeping genes) and to technical and experimental errors. Relative expression quantification analysis relied on the qBase method, as we previously reported (22).

Bone Resorption—The cells were allowed to settle on bovine bone slices and then cultured for 3 weeks, as described above. On day 17 of culture, they were transfected either with a non-relevant siRNA or with an siRNA that abolishes TRPV-5 expression and allowed to resorb bone until day 22. The bone slices were then removed, washed with sodium hydroxide and distilled water, sonicated to remove cell debris, and stained with 1% toluidine blue containing 1% sodium borate. The resorption pits then showed blue/violet on the slice. Optical light microscopy was used to determine bone resorption. Pictures were taken and analyzed using Simple PCI software (Nikon) to calculate the percentage of the resorbed area on the slice.

Statistical Analysis—Results are expressed as mean ± S.E., and the significance was determined by a paired Student’s t test or analysis of variance with Tukey’s post-test where appropriate. Statistical significance was defined as p < 0.05.

RESULTS

RANKL Induces a Rapid and Sustained Increase in [Ca2+]i—Fully mature human osteoclasts were stimulated with 100 ng/ml RANKL. As shown in Fig. 1A, an immediate increase in the intracellular concentration of free Ca2+ was detected. The average calibrated increase in [Ca2+]i, (calculated from the difference between stabilized ratios before and after stimulation in all the osteoclasts in a field) was more prominent with increasing doses of RANKL (Fig. 1B). More than 80% of the multinucleated cells in the field displayed an increase in [Ca2+]i, of this sort (supplemental Fig. 1).

RANKL-induced Increase in [Ca2+]i, Comes from the Extracellular Medium—To characterize the RANKL-induced increase in the cytosolic Ca2+, we repeated the RANKL stimulation in a Ca2+-free medium. In this experiment, Ca2+ was omitted from the buffer preparation, and the osmolarity was adjusted accordingly with NaCl; EGTA was also added to the stimulation buffer to chelate any remaining Ca2+ ions. Fig. 2A shows that the absence of Ca2+ ions in the culture medium considerably reduced the RANKL-induced increase in [Ca2+]i, which was partially restored when Ca2+ was reintroduced into the extracellular medium. In another set of trials, presented in Fig. 2B, RANKL stimulation was repeated, and the higher levels of Ca2+ were allowed to stabilize for a short period of time. EGTA was then added to the extracellular medium, inducing a drastic fall in [Ca2+]i. As these observations suggested that the Ca2+ ions came from the extracellular medium, we sought to confirm the involvement of intracellular stores in the RANKL-induced Ca2+ response. We therefore preincubated the cells with 0.5 μM thapsigargin overnight to deplete the intracellular Ca2+ stores. After shorter incubations, higher base levels of Ca2+ were still detected in the cytosol, which would have made it difficult to compare these cells with those that had not been preincubated with thapsigargin (supplemental Fig. 2). In addition, all the loading and washing procedures were performed with a medium containing 1 μM thapsigargin to ensure that the stores were completely empty. As shown in Fig. 2C, this treatment did not affect the RANKL-induced Ca2+ response. We also preincubated cells with the phospholipase C inhibitor,
which have been reported to activate extracellular Ca\(^{2+}\) influx via human transient receptor potential (TRP) channels but to inhibit activation of the store-operated TRP channel (23–25). As reported in Fig. 3D, adding 100 \(\mu M\) Gd\(^{3+}\) to the medium induced a significant increase in [Ca\(^{2+}\)]. Moreover, when we added RANKL to the medium, no further increase was detected, suggesting that Gd\(^{3+}\) and RANKL must share a common Ca\(^{2+}\) entry mediator, namely a TRP family channel.

The TRPV-5 Channel Is Expressed in Human Osteoclasts—We wanted to find out whether TRP family channels are present in human osteoclasts. As the TRP family is large, we first targeted the TRPV-5, which has recently been associated with bone resorption and is known to be involved in body Ca\(^{2+}\) homeostasis by regulating the activity of the parathyroid glands (26, 27). Immunofluorescence demonstrated the presence of TRPV-5 in the membrane of human osteoclasts (arrows) (Fig. 4). In comparison, L- and R-type Ca\(^{2+}\) channels displayed a faint or undetectable labeling, whereas T-type channels were detected mainly at the cytoplasmic level (Fig. 4). In addition, using confocal imaging, double labeling of TRPV-5 and of the CTR showed that CTR and TRPV-5 were expressed in osteoclasts with a membrane colocalization (arrow) and a predominant expression of TRPV-5 at the bottom, close to the adhesion surface (Fig. 5A). We wanted to establish the precise location of TRPV-5 in functioning human osteoclasts. We therefore seeded cells onto bone slices, where they were allowed to differentiate before being processed for immunofluorescence labeling. Using a confocal microscope, we observed that TRPV-5 strongly colocalized with the V-ATPase in the bottom slices corresponding to the plasma membrane adjacent to bone and its attachment area (arrows) (Fig. 5B). These findings suggest that TRPV-5 is expressed at the membrane and is predominantly expressed in areas corresponding to the sealing zone and the ruffled membrane.

Suppression of TRPV-5 Inhibits the RANKL-induced Increase in [Ca\(^{2+}\)]—To find out whether the TRPV-5 channels were functionally involved in the RANKL-induced response, we used RNA interference to inactivate the channel. As shown in Fig. 6A, siRNA generated against human TRPV-5 significantly decreased the expression of TRPV-5 mRNA 18 and 24 h after transfection, a down-regulation reflected by lower protein levels (Fig. 6B). Indeed, Western blot of TRPV-5 and actin allowed

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U73122, to prevent recruitment from intracellular Ca\(^{2+}\) pools as a result of inositol 1,4,5-trisphosphate release after stimulation. This too had no impact on the RANKL-induced increase in [Ca\(^{2+}\)], as compared with control cells (Fig. 2, C and D).

**Classical Ca\(^{2+}\) Channels Are Not Involved in the RANKL-induced Increase in [Ca\(^{2+}\)]—**As the previous results pointed strongly toward the stimulation of the influx of extracellular Ca\(^{2+}\) by RANKL, we wanted to find out whether this involved a Ca\(^{2+}\) channel. We therefore repeated the RANKL stimulations, but this time we added nifedipine, an L-type Ca\(^{2+}\) channel inhibitor, to the extracellular medium after the elevated level of Ca\(^{2+}\) had stabilized. This induced a slight but not significant downward slope, indicating that only a small fraction of Ca\(^{2+}\) transport across the osteoclast membrane is sensitive to dihydropyridines (Fig. 3A). The T-type channel blocker mibebradil was then used under the same conditions and found to have no effect on the elevation of [Ca\(^{2+}\)], after RANKL stimulation (Fig. 3B). To further confirm those observations, we preincubated some cells with both these inhibitors before performing the RANKL stimulation. None showed any change in the increase in [Ca\(^{2+}\)] induced by RANKL (Fig. 3C), thus confirming that neither T-type nor L-type Ca\(^{2+}\) channels were involved in this response. We then tested the effect of high doses of Gd\(^{3+}\),
us to detect one clear band at a position corresponding to 83 kDa, which is the predicted molecular mass of TRPV-5. Only one lower band, described as nonspecific by the antibody supplier, was seen on the membrane. In addition, control undifferentiated cells did not show the band corresponding to TRPV-5, whereas they did display the lower, nonspecific band. Finally, the use of anti-TRPV-5 siRNA selectively decreased the intensity of the 83-kDa band corresponding to TRPV-5 (supplemental Fig. 3). In control osteoclasts, slight oscillations of \([\text{Ca}^{2+}]_i\), which can be attributed to the flickering of the fluorescence, were observed, but no change in this fluorescence pattern was observed between non-treated cells and cells transfected with a non-relevant siRNA or with an siRNA down-regulating TRPV-5 (supplemental Fig. 4). In contrast, the shutdown of TRPV-5 expression almost completely abolished RANKL-induced \([\text{Ca}^{2+}]_i\) responses, with an overall \([\text{Ca}^{2+}]_i\) increase of 3.4 ± 0.9 nM, as compared with 21.8 ± 6.7 nM in cells transfected with a non-relevant siRNA (Fig. 6, C and D).

Suppressing TRPV-5 Expression Increases Bone Resorption—We then attempted to clarify the functional role of TRPV-5 and the associated RANKL-induced influx of \([\text{Ca}^{2+}]_i\). To do this, we cultured osteoclasts on bone slices and transfected them after they had achieved full differentiation and maturation. We then allowed them to resorb for 5 days with TRPV-5 expression suppressed. Under these conditions, we observed that the average area of bone resorbed was much higher in the cells in which TRPV-5 expression had been abolished (33.75 ± 9.7%) than in non-transfected cells or cells transfected with a non-relevant siRNA (12.25 ± 3.97% and 10.75 ± 2.93%, respectively) (Fig. 6E).

DISCUSSION

Our aim in this study was to investigate \([\text{Ca}^{2+}]_i\) oscillations in human osteoclasts and their physiological implications. Although RANKL effects on \([\text{Ca}^{2+}]_i\) mobilization have already been reported in rodent models (18–20), this is the first time they have been demonstrated in human osteoclasts. We found that RANKL had the immediate
FIGURE 5. Localization of TRPV-5 by confocal imaging. A, at the end of the CBM cultures, immunofluorescence was performed on mature osteoclasts cultured on plastic. The expression of TRPV-5 and of the CTR was evaluated by immunofluorescence double labeling using specific antibodies, and visualized by confocal microscopy using anti-mouse/Alexa Fluor 633 (TRPV-5, red) and anti-goat/Alexa Fluor 488 (CTR, green) secondary antibodies, and counterstaining was performed with DAPI (blue). Cross-sectional (top) and reconstructed images along the z axis (left bottom) are shown. The scale bar represents 10 μm. Fluorograms of two regions are presented: the basolateral membrane (2) and the region close to the plastic surface (1) (green pixels, y axis; red pixels, x axis on fluorogram). B, CBMs were settled on bovine bone slices, and cells were cultured for 3 weeks. At the end of the culture periods, the expression of TRPV-5 and of V-ATPase was evaluated by immunofluorescence using specific antibodies and visualized with anti-mouse/Alexa Fluor 633 (TRPV-5, red) and anti-rabbit/Alexa Fluor 488 (V-ATPase, green) secondary antibodies. Confocal microscopy was performed to determine the localization of TRPV-5 and V-ATPase, and one representative cell is shown. Optical slices were taken every 0.8 μm along the z axis, starting from the top of the cell and ending at the level of the bone slice; three sample slices are shown with each channel (DAPI, V-ATPase, and TRPV-5) followed by a merged reconstitution.
effect of increasing the \([\text{Ca}^{2+}]_i\), and we also demonstrated that the RANKL-induced \([\text{Ca}^{2+}]_i\) response was for the most part attributable to an influx of extracellular \([\text{Ca}^{2+}]_i\) and does not involve any recruitment from intracellular \([\text{Ca}^{2+}]_i\) pools. This differs from what had been shown to occur in rodents, where the RANKL-induced increase in \([\text{Ca}^{2+}]_i\) was abolished by intracellular \([\text{Ca}^{2+}]_i\) chelators and by inhibiting phospholipase C, indicating that recruitment from intracellular \([\text{Ca}^{2+}]_i\) pools was required for the \([\text{Ca}^{2+}]_i\) increases induced by RANKL to occur (19, 20). The mechanism underlying RANKL-induced phospholipase C activation has been studied in rodents, and it has been suggested that two members of the regulator of G protein signaling (RGS) family (RGS 10A and 12) are part of the process (28, 29). These two regulators have been shown to be expressed in human osteoclasts, but their actual involvement in RANKL signaling has not. On the basis of our results, phospholipase C activation does not seem to be involved in RANKL-induced \([\text{Ca}^{2+}]_i\) responses in human osteoclasts. This constitutes a major difference between the species-specific signaling cascades and should be taken into account when exploring osteoclast function in non-human models. This difference could be explained by the involvement of TRP channels as mediator of the RANKL response. It is known that these channels can be activated by various signals, including voltage, extracellular ligands, or intracellular \([\text{Ca}^{2+}]_i\) stores, which display species-specific characteristics (25). For example, murine canonical TRP5 (TRPC5) has been shown to be insensitive to depletion of the \([\text{Ca}^{2+}]_i\) store, in contrast to the human TRPC5, which may display store-operated and/or external ionic activation depending on the cell type (25, 30). If the channels are activated in response to RANKL stimulation, this would reconcile our findings with those of Komarova et al. (20) concerning the recruitment of species-specific mechanisms of activation.

We show here that TRPV-5 channels are highly expressed in human osteoclasts, and this confirms the previous observation that this channel is expressed in human osteoclasts at the
mRNA level (31). We also show that TRPV-5 channels are located on the bone side of the plasma membrane of resorbing human osteoclasts, which is consistent with the subcellular localization already reported in rodents (31). In addition, we show here that the channel displays a subcellular localization consistent with its functions in human cells as the shutdown of TRPV-5 expression not only suppresses the influx of Ca\(^{2+}\) but also induces a major increase in bone resorption. This observation suggests that TRPV-5 down-regulates bone resorption directly from the ruffled border. This is an important observation as both TRPV-5 and TRPV-6 channels are known to be involved in Ca\(^{2+}\) homeostasis, regulating Ca\(^{2+}\) flux through kidney and intestine epithelial cells (32). TRPV-5 knock-out mice have been shown to develop severe hypercalciuria and reduced bone thickness (33), and our observation that TRPV-5 has an inhibitory effect on the process of bone resorption in human osteoclasts also relates TRPV-5 to human bone homeostasis. This is consistent with the close relationships known to exist between kidney, intestine, and bone in calcium and skeletal homeostasis.

TRPV-5 might have a direct influence on the bone resorption function of osteoclasts by activating a stop signal in the resorbing osteoclasts mediated by Ca\(^{2+}\) entry. Previous studies performed with TRPV-5\(^{-/-}\) mice indicated that the number of osteoclasts was greater when TRPV-5 expression was abolished (31), thus suggesting that this channel might also down-regulate osteoclast formation. However, the same study also detected a concomitant inhibition of resorption (both in vivo and in vitro) in osteoclasts generated from TRPV-5\(^{-/-}\) mice despite the increase in osteoclastogenesis. This paradoxical observation could be related to the fact that deficient TRPV-5 osteoclast precursors generate malfunctioning osteoclasts. Our model was different as TRPV-5 expression was inhibited in osteoclasts that had already differentiated. It is also conceivable that there are differences between human and murine TRPV-5 function.

Recent data have highlighted the role of TRPV-4 channels in RANKL-induced [Ca\(^{2+}\)], oscillations in osteoclasts (34). Interestingly, the kinetics of RANKL-induced TRPV-5 activation differs from that of TRPV-4. Indeed, although TRPV-4 induces transient oscillatory [Ca\(^{2+}\)], elevations in rodent osteoclasts, we show in our study that TRPV-5-mediated [Ca\(^{2+}\)], elevations in human osteoclasts remain stable for more than 10 min after stimulation. This was illustrated even more clearly by the fact that no [Ca\(^{2+}\)], elevation was observed when the culture medium was depleted of Ca\(^{2+}\) but that Ca\(^{2+}\) entry was detected as soon as Ca\(^{2+}\) was reintroduced into the medium, even if this occurred more than 3 min after the RANKL stimulation. In addition, TRPV-4 channels are expressed exclusively on the basolateral side of osteoclast membrane in mice, and a TRPV-4-mediated influx of Ca\(^{2+}\) is required for the osteoclast terminal differentiation and bone resorbing activity to occur as a result of triggering NFATc1 activation (34). Our study suggests that TRPV-5 is also crucial in RANKL-induced osteoclast activity and that the presence of TRPV-5 in the ruffled membrane of human osteoclast is necessary for the RANKL-induced increase in intracellular Ca\(^{2+}\) and down-regulation of bone resorption to occur. If this is so, it would imply that this channel acts as a negative feedback and controls the ending of RANKL-induced bone resorption.

REFERENCES

1. Supanchart, C., and Kornak, U. (2008) Arch. Biochem. Biophys. 473, 161–165
2. Blair, H. C., Robinson, L. J., and Zaidi, M. (2005) Biochem. Biophys. Res. Commun. 328, 728–738
3. Bennett, B. D., Alvarez, U., and Hruska, K. A. (2001) Endocrinology 142, 1968–1974
4. Micelli, S., Meledeo, L., Picciarelli, V., and Gallucci, E. (2006) Front. Biosci. 11, 2035–2044
5. Tsuzuki, T., Okabe, K., Kajiya, H., and Habu, T. (2000) Jpn. J. Physiol. 50, 67–76
6. Radding, W., Williams, J. P., Hardy, R. W., McDonald, J. M., Whitaker, C. H., Turbat-Herrera, E. A., and Blair, H. C. (1994) J. Cell Physiol. 160, 17–28
7. Teti, A., Colucci, S., Grano, M., Barattolo, R., Argentino, L., and Zambonin Zallone, A. (1990) Boll. Soc. Ital. Biol. Sper. 66, 1–4
8. Van Epps-Fung, C., Williams, J. P., Cornwell, T. L., Lincoln, T. M., McDonald, J. M., Radding, W., and Blair, H. C. (1994) Biochem. Biophys. Res. Commun. 204, 565–571
9. Kajiya, H., Okabe, K., Okamoto, F., Suzuki, T., and Soeda, H. (2000) J. Cell. Physiol. 183, 83–90
10. Miyauchi, A., Alvarez, J., Greenfield, E. M., Teti, A., Grano, M., Colucci, S., Zambonin-Zallone, A., Ross, F. P., Teitelbaum, S. L., Cheres, D., et al. (1993) Osteopetros. Int. 3, Suppl. 1, 132–135
11. Miyauchi, A., Hruska, K. A., Greenfield, E. M., Duncan, R., Alvarez, J., Barattolo, R., Colucci, S., Zambonin-Zallone, A., Teitelbaum, S. L., and Teti, A. (1990) J. Cell Biol. 111, 2543–2552
12. Kajiya, H., Okamoto, F., Fukushima, H., Takada, K., and Okabe, K. (2003) Am. J. Physiol. Cell Physiol. 285, C457–C466
13. Destaing, O., Sanjay, A., Itzstein, C., Horne, W. C., Toome, D., De Camilli, P., and Baran, R. (2008) Mol. Biol. Cell 19, 394–404
14. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Baradley, C., Antoine, E., Levy, J., Gailit, J., Bowett, D., Horne, W. C., and Baran, R. (2001) J. Cell Biol. 152, 181–195
15. Evans, K. E., and Fox, S. W. (2007) BMC Cell Biol. 8, 4
16. Komarova, S. V., Shum, J. B., Paige, L. A., Sims, S. M., and Dixon, S. J. (2003) Calcif Tissue Int. 73, 265–273
17. Mangashetti, L. S., Khatip, S. M., and Wani, M. R. (2005) J. Immunol. 175, 917–925
18. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Iisikhi, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) Dev. Cell 3, 889–901
19. Komarova, S. V., Pereverzev, A., Shum, J. B., Sims, S. M., and Dixon, S. J. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 2643–2648
20. Komarova, S. V., Pilkington, M. F., Weidema, A. F., Dixon, S. J., and Sims, S. M. (2003) J. Biol. Chem. 278, 8286–8293
21. Roux, S., Lambert-Comeau, P., Saint-Pierre, C., Lépine, M., Sawan, B., and Parent, J. L. (2005) Biochem. Biophys. Res. Commun. 333, 42–50
22. Mchou, L., Chamoux, F., Couture, J., Morisette, J., Brown, F. P., and Roux, S. (2010) Bone 46, 598–603
23. Touskova, K., Vyklicky, L., Susankova, K., Benedikt, J., and Vlachova, V. (2005) Mol. Cell Neurosci. 30, 207–217
24. Trebak, M., Bird, G. S., McKay, R. R., and Putney, J. W., Jr. (2002) J. Biol. Chem. 277, 21617–21623
25. Zeng, F., Xu, S. Z., Jackson, P. K., McHugh, D., Kumar, B., Fountain, S. J., and Beech, D. J. (2004) J. Physiol. 559, 739–750
26. Minke, B. (2006) Cell Calcium 40, 261–275
27. Vennekens, R., Owsianik, G., and Nilius, B. (2008) Curr. Pharm. Des. 14, 18–31
28. Yang, S., and Li, Y. P. (2007) J. Bone Miner. Res. 22, 45–54
29. Yang, S., and Li, Y. P. (2007) Genes Dev. 21, 1803–1816
30. Okada, T., Shimizu, S., Wakamori, M., Maeda, A., Kurosaki, T., Takada, N., Imoto, K., and Mori, Y. (1998) J. Biol. Chem. 273, 10279–10287
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31. van der Eerden, B. C., Hoenderop, J. G., de Vries, T. J., Schoenmaker, T., Buurman, C. J., Uitterlinden, A. G., Pols, H. A., Bindels, R. J., and van Leeuwen, J. P. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 17507–17512
32. Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2005) Physiol. Rev. 85, 373–422
33. Hoenderop, J. G., van Leeuwen, J. P., van der Eerden, B. C., Kersten, F. F., van der Kemp, A. W., Mérialat, A. M., Waarsing, J. H., Rossier, B. C., Vallon, V., Hummler, E., and Bindels, R. J. (2003) J. Clin. Invest. 112, 1906–1914
34. Masuyama, R., Vriens, J., Voets, T., Karashima, Y., Owsianik, G., Vennekens, R., Lieben, L., Torrekens, S., Moermans, K., Vanden Bosch, A., Bouillon, R., Nilius, B., and Carmeliet, G. (2008) Cell Metab. 8, 257–265