THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 10, Issue of March 7, pp. 8286–8293, 2003
Printed in U.S.A.

RANK Ligand-induced Elevation of Cytosolic Ca^{2+} Accelerates Nuclear Translocation of Nuclear Factor κB in Osteoclasts*

Received for publication, June 28, 2002, and in revised form, December 9, 2002
Published, JBC Papers in Press, December 20, 2002, DOI 10.1074/jbc.M206421200

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RANK ligand (RANKL) induces activation of NFκB, enhancing the formation, resorptive activity, and survival of osteoclasts. Ca^{2+} transduces many signaling events, however, it is not known whether the actions of RANKL involve Ca^{2+} signaling. We investigated the effects of RANKL on rat osteoclasts using microspectrofluorimetry and patch clamp. RANKL induced transient elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) to maxima 220 nM above basal, resulting in activation of Ca^{2+}-dependent K^+ current. RANKL elevated $[Ca^{2+}]_i$ in Ca^{2+}-containing and Ca^{2+}-free media, and responses were prevented by the phospholipase C inhibitor U73122. Suppression of $[Ca^{2+}]_i$ elevation using the intracellular Ca^{2+} chelator 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) abolished the ability of RANKL to enhance osteoclast survival. Using immunofluorescence, NFκB was found predominantly in the cytosol of untreated osteoclasts. RANKL induced transient translocation of NFκB to the nuclei, which was maximal at 15 min. U73122 or BAPTA delayed nuclear translocation of NFκB. Delays were also observed upon inhibition of calcineurin or protein kinase C. We conclude that RANKL acts through phospholipase C to release Ca^{2+} from intracellular stores, accelerating nuclear translocation of NFκB and promoting osteoclast survival. Such cross-talk between NFκB and Ca^{2+} signaling provides a novel mechanism for the temporal regulation of gene expression in osteoclasts and other cell types.

RANK[1] ligand (RANKL) is a member of the tumor necrosis factor superfamily that plays an essential role in osteoclastogenesis, as well as the activation and survival of mature osteoclasts. This factor is expressed on osteoblasts, stromal cells, B-lymphoid lineage cells, and activated T-cells as a transmembrane ligand and it also exists in a biologically active soluble form (1–3). RANKL acts through its receptor RANK, which is expressed on osteoclast precursors, mature osteoclasts, as well as dendritic cells (4). Osteoprotegerin (OPG) is a soluble decoy receptor, which binds RANKL and blocks its interaction with RANK (4).

Signaling through RANK involves the recruitment of cytosolic tumor necrosis factor receptor-associated factors (TRAFs) 1, 2, 3, 5, and 6, which in turn activate multiple signaling pathways (5–7). For example, the association of RANK with TRAF2 induces activation of c-Jun N-terminal kinase, which leads to phosphorylation of c-Jun and activation of AP-1 (7–9). TRAF6 has been implicated in activation of the nonreceptor tyrosine kinase c-Src and the transcription factor NFκB (10, 11).

NFκB transcription factors are dimers of the five mammalian NFκB proteins: p65 (RelA), RelB, c-Rel, p50 (NFκB1), and p52 (NFκB2). NFκB regulates the expression of a large number of genes involved in cell survival as well as in cellular responses to inflammation and stress (12, 13). Typically, NFκB exists as a heterodimer of p50 and p65 (12). NFκB is retained in the cytoplasm complexed with inhibitory proteins IκBs. RANK signaling involves activation of NFκB-inducing kinase, leading to activation of IκB kinases (IKK) α and β, which in turn phosphorylate serine residues on IκB, targeting it for degradation in the proteasome (5, 11, 14). IκB degradation exposes the NFκB nuclear localization sequence, permitting its nuclear import. Within the nucleus, NFκB acts in concert with other transcription factors to regulate gene expression, with termination of the signal caused by binding of IκB (15). NFκB is essential for osteoclastogenesis, as disruption of both p50 and p52 subunits of NFκB leads to an osteopetrotic phenotype, because of impaired osteoclast differentiation (16).

Interaction of RANKL with RANK is crucial for osteoclast function, however, there are gaps in our understanding of the signaling events leading to activation of NFκB in response to RANKL. Although interaction of RANK with TRAF6 is necessary and sufficient to activate NFκB, dominant negative forms of TRAF molecules are unable to completely block NFκB activation, suggesting that a TRAF-independent pathway is also involved (5, 11). Because Ca^{2+}-sensitive effectors such as calcineurin and protein kinase C (PKC) mediate NFκB activation in T lymphocytes and monocytic cell lines (17, 18), we considered the possible role of Ca^{2+} in the activation of NFκB by RANKL in osteoclasts.

We tested the hypothesis that RANKL signaling in osteoclasts involves elevation of $[Ca^{2+}]_i$, and examined the role of cytosolic Ca^{2+} in cell survival and activation of NFκB. Classical

* This work was supported by The Arthritis Society, Canadian Arthritis Network, and Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RANK, receptor activator of NFκB; AM, acetoxymethyl ester; AP-1, activator protein 1; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; NFκB, nuclear factor κB; IκB, inhibitor of NFκB; IKK, IκB kinase; IK_{Ca}, intermediate conductance Ca^{2+}-dependent K^+ current; OPG, osteoprotegerin; PLC, phospholipase C; PKC, protein kinase C; RANKL, receptor activator of NFκB ligand; TRAF, tumor necrosis factor receptor-associated factor; PBS, phosphate-buffered saline; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

biochemical approaches for studying osteoclasts are limited because of difficulty in isolating cells in sufficient number and purity. Furthermore, osteoclasts are terminally differentiated, and therefore do not proliferate in culture. We overcame these restrictions by studying authentic osteoclasts using single-cell techniques: microspectrofluorimetry and patch clamp to study changes in $[Ca^{2+}]_i$ and membrane currents, and immunofluorescence to assess nuclear translocation of NFκB. We report that RANKL stimulates phospholipase C (PLC) leading to release of Ca^{2+} from intracellular stores, transient elevation of $[Ca^{2+}]_i$, and activation of Ca^{2+}-dependent K^+ current. The effect of RANKL on osteoclast survival was found to be dependent on elevation of $[Ca^{2+}]_i$. Moreover, nuclear translocation of NFκB was slowed when elevation of Ca^{2+} was suppressed or when calcineurin or PKC were inhibited. Thus, phospholipase C and Ca^{2+} signaling are revealed to be important regulators of NFκB activation and osteoclast survival.

EXPERIMENTAL PROCEDURES

Osteoclast Isolation and Culture—Osteoclasts were isolated from the long bones of neonatal Wistar rats or neonatal New Zealand White rabbits as described previously (19). Briefly, long bones were dissected free of soft tissue and cut with a scalpel to release bone fragments into 2–3 ml of osteoclast culture medium that consisted of Medium 199 buffered with 25 mM HEPES and HCO_3^- (Invitrogen, Burlington, Ontario) supplemented with 15% heat-inactivated fetal bovine serum and 1% antibiotic solution (penicillin, 10,000 units/ml; streptomycin, 10,000 μg/ml; amphotericin B, 25 μg/ml). Cells were suspended by repeated passage though a pipette and plated on glass coverslips or 35-mm culture dishes. Rat osteoclasts were incubated at 37 °C in 5% CO_2 for 1 h, then gently washed with phosphate-buffered saline (PBS) to remove nonadherent cells and incubated in fresh medium for at least 1 h before use. To quantify survival, we counted the number of rat osteoclasts in culture dishes at time 0 (time of addition of RANKL or vehicle) and at 24 h. Rabbit osteoclasts were maintained at 37 °C in 5% CO_2 for 2 h after isolation, then fresh culture medium was added and cells were incubated at 37 °C in 5% CO_2 for 2 to 7 days before use. The majority of nonosteoclastic cells were removed from rabbit preparations using Pronase E (0.001% in PBS with 0.5 mM EDTA) for ~5 min at room temperature (22–25 °C) with intermittent agitation (modified from Ref. 20). Osteoclasts were identified by the presence of three or more nuclei, and by their characteristic morphology under phase-contrast microscopy. Rat osteoclast precursors were identified as large (20–40 μm in diameter) mononucleated cells that were generally circular in outline, possessed a region of granular cytoplasm, and often exhibited a broad lamellipod. These mononucleated cells all stained strongly with neutral red, and 92% stained positive for the osteoclast marker tartrate-resistant acid phosphatase. These procedures were approved by the Council on Animal Care of the University of Western Ontario.

Test Substances—Soluble RANKL (murine recombinant 158–316) and OPG (human recombinant 21–194 fused at the N terminus to the Fc domain of human IgG1) were kindly provided by Amgen (Thousand Oaks, CA) and RANKL (human recombinant 151–316 fused at the N terminus to a linker peptide and a FLAG tag) was purchased from Alexis Corp. (San Diego, CA). U73122 and U73343 were obtained from Calbiochem (La Jolla, CA), dissolved in chloroform, aliquoted, evaporated under N_2, and stored at −80 °C. On the day of each experiment, U73122 and U73343 were reconstituted in dimethyl sulfoxide and added to the physiological buffer or medium bathing the cells. BAPTA-acetoxymethyl ester (AM) and calcein blue-AM were obtained from Molecular Probes (Eugene, OR) and stock solutions were prepared in dimethyl sulfoxide. Osteoclasts were loaded with the BAPTA or calcein blue by incubation in loading medium (HCO_3^--free Medium 199 buffered with HEPES supplemented with 15% fetal bovine serum and 1% antibiotic solution) containing 50 μM BAPTA-AM or calcein blue-AM for 10 min at room temperature. Cyclosporin A, FK506, bisindolylmaleimide I and V were obtained from Calbiochem and stock solutions were prepared in dimethyl sulfoxide.

Fluorescence Measurement of Cytosolic Free Ca^{2+} Concentration—$[Ca^{2+}]_i$ of single rat osteoclasts and osteoclast precursors was monitored using microfluorimetric techniques. Cells on glass coverslips were loaded with 1.5 μM fura-2-AM (Molecular Probes) for 40 min at room temperature in loading medium. Coverslips were then placed in a chamber mounted on the stage of a Nikon Diaphot inverted phase-contrast microscope, and superfused at room temperature with physiological buffer containing (in mM): NaCl, 130; KCl, 5; glucose, 10; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 20; adjusted to pH 7.4 with NaOH; 280–290 mOsmol/liter. The ratio of fluorescence emission at 510 nm with alternate excitation wavelengths of 345 and 380 nm was measured using a Deltascan illumination system (Photon Technology International, London, ON, Canada) as described previously (21). RANKL was applied locally to cells by pressure ejection from a micropipette. In some studies, cells were superfused with a Ca^{2+}-free physiological buffer, supplemented with 0.5 mM EGTA.

Electrophysiology—The whole cell patch clamp configuration was used to record membrane currents as described previously (22). Electrode solution contained (in mM) KCl, 140; HEPES, 20; $MgCl_2$, 1; EGTA, 0.1; adjusted to pH 7.2 with KOH; 280–290 mOsmol/liter. Pipette resistance before seal formation was 3–5 MΩ. Cells were superfused in physiological buffer at room temperature. Currents were recorded with Axopatch-1D amplifier, filtered, and digitized at 2–5 kHz using pClamp 6.0 (Axon Instruments, Union City, CA).

NFκB Localization by Immunofluorescence—Osteoclasts on glass coverslips were incubated with or without RANKL in osteoclast culture medium at 37 °C and at the indicated times fixed with 4% paraformaldehyde (10 min), washed in PBS (3× 5 min); permeabilized with 0.1% Triton X-100 in PBS (10 min), washed in PBS (3× 5 min); and blocked with 1% normal goat serum in PBS (NGS) for 1–2 h at room temperature. Monoclonal antibody to p65 (catalog number sc-8008, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:100 in NGS and applied overnight at 4 °C, followed by washing in PBS and incubation for 2 h at room temperature with biotinylated goat anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:200 in NGS. After incubation (2 h, room temperature) with fluorescein-conjugated streptavidin (Vector Laboratories Inc.) (1:100 in NGS), coverslips were washed, mounted on slides with Vecta-Shield (Vector Laboratories Inc.), and examined using a Zeiss LSM 510 laser-scanning confocal microscope. We assessed localization of fluorescent label in all osteoclasts on each coverslip (usually 40–70 cells/coverslip). Osteoclasts were rated positive for nuclear localization if fluorescence intensity of one or more nuclei exceeded that of the cytoplasm.

Statistical Analyses—Data are presented as representative traces, as percentages of total cells tested, or as mean ± S.E. with sample size (n) indicating the number of osteoclasts for Ca^{2+} fluorescence or electrophysiology studies, or the number of separate cell preparations for immunofluorescence studies. Differences were assessed by one-way analysis of variance for correlated samples, followed by a Tukey or Bonferroni test and accepted as statistically significant at $p < 0.05$. Sigmoid curves were fit by nonlinear regression using Prism (GraphPad Software, Inc., San Diego, CA). Error bars were omitted where they were smaller than the symbol.

RESULTS

RANKL Induces Elevation of Cytosolic Free Ca^{2+}—Rat osteoclasts were loaded with fura-2, and Ca^{2+} was monitored by microspectrofluorimetry. Osteoclasts had basal $[Ca^{2+}]_i$ of 154 ± 4 nM (n = 118, mean ± S.E.). Osteoclasts responded to soluble RANKL with elevation of $[Ca^{2+}]_i$, which typically peaked and then declined slowly, even in the continued presence of RANKL. Upon washout of RANKL, $[Ca^{2+}]_i$ returned promptly to basal levels (Fig. 1*A*). Multiple $[Ca^{2+}]_i$ transients could be elicited by successive applications of RANKL (Fig. 1*B*), although the subsequent responses were slightly decreased in amplitude. No responses were observed when osteoclasts were stimulated with vehicle (n = 25). Moreover, OPG blocked the ability of RANKL to induce $[Ca^{2+}]_i$ elevations in osteoclasts that were responsive to multiple applications of RANKL alone (n = 3).

The proportion of osteoclasts responding to RANKL with elevation of $[Ca^{2+}]_i$ was dependent on the concentration of RANKL (Fig. 1*C*). $[Ca^{2+}]_i$ elevations were elicited by concentrations of RANKL as low as 10 pg/ml. The maximum proportion of osteoclasts (~60%) responded to RANKL at 10–100 ng/ml, with half-maximal effects at ~0.1 ng/ml. When the amplitudes of the RANKL-induced Ca^{2+} transients were quantified, similar concentration dependence was observed (Fig. 1*D*). At concentrations of 10–100 ng/ml, RANKL elevated $[Ca^{2+}]_i$ to peaks of 220 ± 30 nM above basal (based on 15

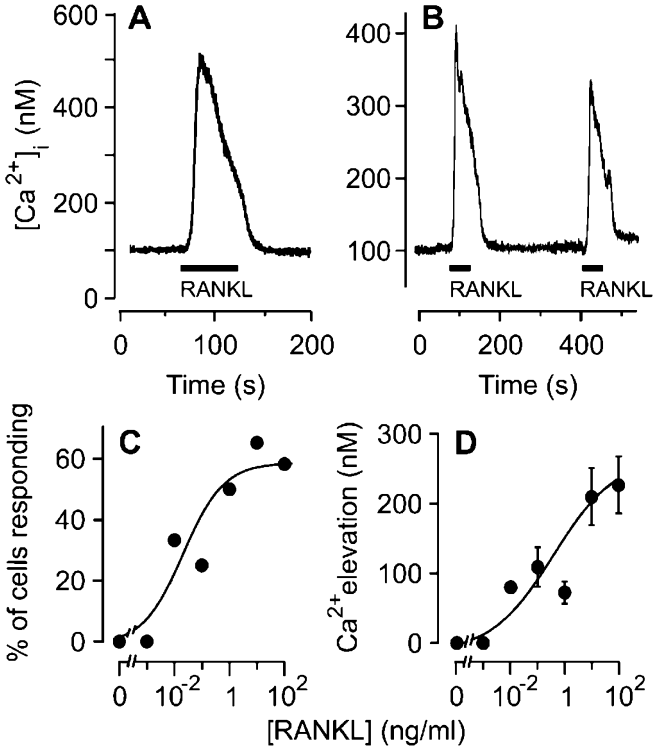

FIG. 1. **RANKL elicits $[Ca^{2+}]_i$ elevations in rat osteoclasts.** Single rat osteoclasts were loaded with fura-2, bathed in physiological buffer, and $[Ca^{2+}]_i$ was monitored by microspectrofluorimetry. *A*, RANKL (100 ng/ml) was applied to cells for 60 s, as indicated by the *bar* below the Ca^{2+} trace. *B*, illustrated is the response of one osteoclast to 2 successive stimulations with RANKL (10 ng/ml), where Ca^{2+} transients diminished in amplitude upon successive stimulation. *C*, the percentage of osteoclasts responding to the single application of RANKL increased with increasing RANKL concentration (10^{-3} to 10^2 ng/ml, applied locally, $n = 12$ osteoclasts for each concentration, except 10^{-3} ng/ml, where $n = 6$). Elevations in $[Ca^{2+}]_i$ >25 nM above basal were considered to be responses. *D*, amplitudes of Ca^{2+} transients were quantified as maximum elevations above basal levels. The *curve* illustrates dependence of amplitude on RANKL concentration. Data are mean ± S.E. of three to eight responsive osteoclasts for concentrations of RANKL $\geq 10^{-2}$ ng/ml.

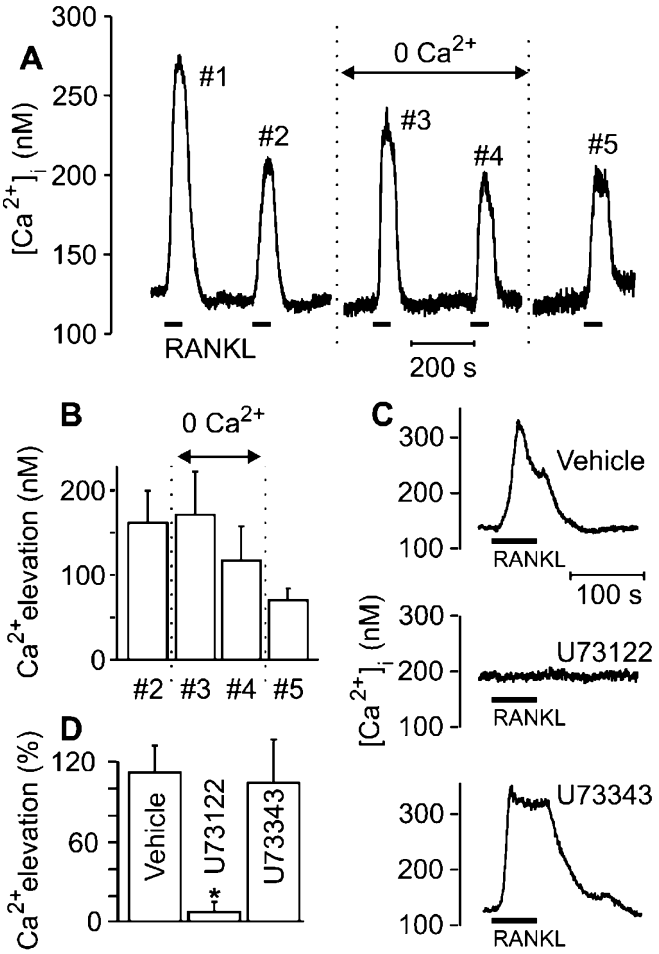

FIG. 2. **RANKL causes release of Ca^{2+} from intracellular stores.** *A*, to examine the source of Ca^{2+} contributing to the response, RANKL was applied locally to rat osteoclasts in the presence or absence of extracellular Ca^{2+}. Illustrated are responses of one osteoclast to RANKL (100 ng/ml, 60 s applications) superfused with physiological buffer containing 1 mM Ca^{2+}. Where indicated, cells were superfused with nominally Ca^{2+}-free buffer containing 0.5 mM EGTA (0 Ca^{2+}). *Dotted lines* indicate when the recording was interrupted for 60 s to allow buffer exchange. *B*, histogram illustrates the amplitude of Ca^{2+} transients during applications of RANKL (100 ng/ml) in the presence and absence of extracellular Ca^{2+} (means ± S.E., $n = 5$ cells). Withdrawal of Ca^{2+} did not significantly affect the amplitude of the response. *C*, osteoclasts were stimulated twice with RANKL (100 ng/ml) before application of test compounds to ensure responsiveness to repeated stimulation with RANKL. Cells were then treated for 10 min with vehicle (dimethyl sulfoxide, 0.1%), U73122, or U73343 (1 μM in the bath). *Traces* illustrate responses of separate osteoclasts challenged with RANKL (100 ng/ml) following treatment with the indicated test compound. *D*, histogram illustrates the amplitude of RANKL-induced Ca^{2+} transients following treatment with the indicated test compound. Data are expressed as a percentage of response to RANKL in the same cell prior to treatment. Data are means ± S.E. of 5 cells for each condition. U73122 significantly inhibited RANKL-induced elevations of $[Ca^{2+}]_i$ when compared with responses elicited by RANKL in osteoclasts treated with vehicle or U73343 ($p < 0.05$).

responsive osteoclasts of 24 tested). We also assessed changes in Ca^{2+} upon application of RANKL to rat osteoclast precursors. Even at concentrations of 1 μg/ml, RANKL caused elevation of $[Ca^{2+}]_i$ in only 3 of 27 osteoclast precursors tested, whereas 11 of 17 multinucleated osteoclasts, tested in the same preparations, responded with elevation of Ca^{2+}. As a negative control, we tested the responses of spindle-shaped stromal cells and found that none of the 12 cells tested responded to RANKL. Thus, a proportion of osteoclast precursors responded to RANKL with elevation of $[Ca^{2+}]_i$, although the percentage of responsive precursors was significantly lower than that of mature osteoclasts. All subsequent studies were performed using multinucleated osteoclasts.

We next investigated the source of Ca^{2+} contributing to RANKL-induced elevation of $[Ca^{2+}]_i$ in osteoclasts. RANKL elicited Ca^{2+} elevations of comparable amplitude in Ca^{2+}-containing and Ca^{2+}-free extracellular solutions, consistent with release of Ca^{2+} from intracellular stores (Fig. 2, *A* and *B*, $n = 5$). Ca^{2+} release from stores often involves PLC-mediated production of inositol 1,4,5-trisphosphate. We have shown previously that the PLC inhibitor U73122 blocks P2Y nucleotide receptor-mediated elevation of $[Ca^{2+}]_i$ in osteoclasts (21). Treatment of osteoclasts with U73122 (1 μM for 10 min) abolished the RANKL-induced rise of $[Ca^{2+}]_i$, whereas RANKL still elicited $[Ca^{2+}]_i$ elevations in the presence of the control compound U73343 or vehicle (Fig. 2, *C* and *D*, $n = 5$). Taken together, these data indicate that RANKL signals through PLC leading to release of Ca^{2+} from intracellular stores and transient elevation of $[Ca^{2+}]_i$. Our findings are in contrast to previous observations that RANKL caused sustained elevation of $[Ca^{2+}]_i$ in osteoclasts (23).

RANKL Activates Ca^{2+}-dependent K^+ Current—An independent approach was used to verify the effect of RANKL on $[Ca^{2+}]_i$ in osteoclasts. Rat, rabbit, and human osteoclasts possess intermediate conductance Ca^{2+}-dependent K^+ channels (24). Because only a subpopulation of rat osteoclasts

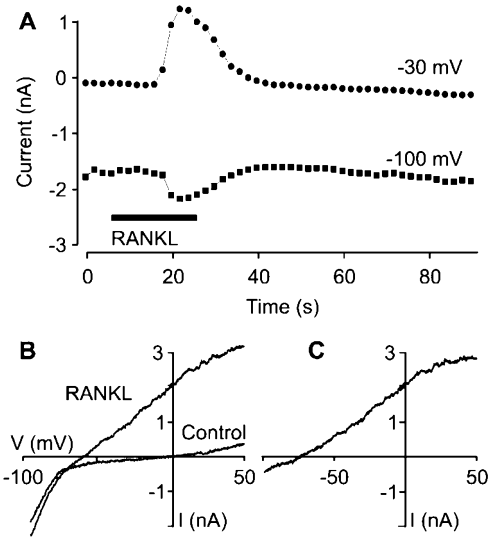

FIG. 3. **RANKL activates Ca^{2+}-dependent K^+ current in osteoclasts.** Rabbit osteoclast was held under voltage clamp at −30 mV, and voltage ramps from −100 to +100 mV in 340 ms were commanded every 2 s. *A*, RANKL (100 ng/ml), applied locally for 20 s where indicated by the *bar*, caused outward current at −30 mV, and inward current at −100 mV. *B*, current-voltage (I-V) relationship for the same osteoclast prior to stimulation (*Control*) and at the peak of the response to RANKL. Control I-V relationship displayed inwardly rectifying K^+ current at negative potentials. RANKL activated a large outward current. *C*, the RANKL-induced current was determined by subtraction of control current from the peak current during application of RANKL. The RANKL-induced current exhibited a linear I-V relationship and reversed direction close to −70 mV, consistent with activation of the Ca^{2+}-dependent K^+ current. Data are representative of 6 responsive osteoclasts of 14 tested.

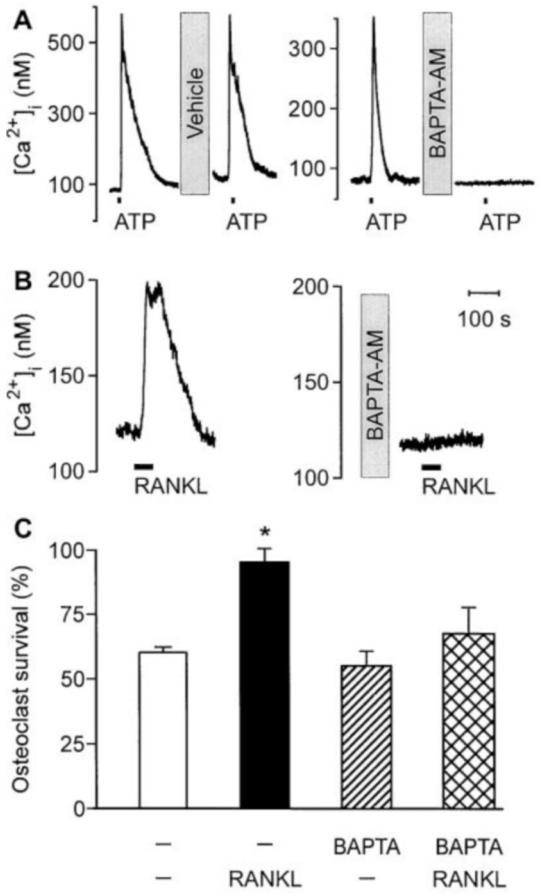

FIG. 4. **Effect of intracellular Ca^{2+} chelator, BAPTA, on osteoclast survival.** *A*, the ability of BAPTA to suppress P2Y nucleotide receptor-induced elevation of $[Ca^{2+}]_i$ was demonstrated in rat osteoclasts. Single osteoclasts were first stimulated with ATP (100 μM), then incubated with BAPTA-AM (50 μM) or vehicle (0.05% dimethyl sulfoxide) for 10 min before rechallenging with ATP. BAPTA abolished Ca^{2+} responses to ATP. Data are representative of 5 osteoclasts treated with vehicle and 8 osteoclasts loaded with BAPTA. *B*, parallel coverslips were incubated with BAPTA-AM (50 μM) or vehicle for 10 min, then the medium was changed and the osteoclasts were challenged with RANKL (100 ng/ml). RANKL elicited Ca^{2+} responses in 4 of 9 osteoclasts treated with vehicle, whereas 0 of 9 BAPTA-loaded osteoclasts responded to RANKL. *C*, to examine the role of $[Ca^{2+}]_i$ in osteoclast survival, rat osteoclasts were treated with BAPTA-AM or vehicle as described above. The medium was then changed and osteoclasts were incubated with RANKL (100 ng/ml) or vehicle at 37 °C for 24 h. The number of osteoclasts per dish at 24 h was expressed as a percentage of the initial number of osteoclasts in the same dish. Osteoclast survival was significantly greater in cultures treated with RANKL alone than under all other conditions ($p < 0.05$). Initial cell numbers (100%) were 182 ± 61, 169 ± 27, 125 ± 31, and 159 ± 39 osteoclasts/dish for samples treated with vehicle, RANKL alone, BAPTA alone, or BAPTA and RANKL, respectively. Data are means ± S.E. from four independent experiments.

exhibits this current (22), we used patch clamp techniques to monitor the effects of RANKL on membrane currents of rabbit osteoclasts, which all demonstrate the current (25). Cells were held at −30 mV and voltage ramp commands were applied every 2 s. RANKL (100 ng/ml) evoked outward current after a delay of ~10 s ($n = 6$ out of 14 osteoclasts tested) with inward current apparent at −100 mV (Fig. 3*A*). Current-voltage (I-V) relationships were determined from the voltage ramp commands. Basal current prior to application of RANKL was dominated by the inwardly rectifying K^+ current Kir2.1 that has been identified previously in osteoclasts (Fig. 3*B*, *Control*). Subtraction of the control current from that recorded at the peak of the response to RANKL showed that the RANKL-induced current was linear and reversed close to −70 mV, indicating K^+-selective current (Fig. 3*C*). A similar, linear K^+ current has been shown previously to closely follow elevations of $[Ca^{2+}]_i$ in osteoclasts (22). Thus, RANKL-induced current likely represents activation of Ca^{2+}-dependent K^+ channels because of rise of $[Ca^{2+}]_i$. Hence, the voltage-clamp data independently confirm that RANKL induces elevation of $[Ca^{2+}]_i$ in osteoclasts.

Role of Ca^{2+} in Osteoclast Survival—It was shown previously that RANKL prolongs osteoclast survival *in vitro* (26). We investigated the role of Ca^{2+} in this process using the intracellular Ca^{2+} chelator BAPTA. To establish conditions for effective buffering of Ca^{2+} by BAPTA, we used ATP, which activates P2Y nucleotide receptors on osteoclasts leading to release of Ca^{2+} from intracellular stores and reproducible elevation of $[Ca^{2+}]_i$ (21). Osteoclasts were stimulated with ATP (100 μM) to ensure their responsiveness, then treated with different concentrations of BAPTA-AM and rechallenged with ATP. We established that loading with 50 μM BAPTA-AM for 10 min at room temperature was optimal for suppressing elevation of $[Ca^{2+}]_i$ induced by ATP (Fig. 4*A*, $n = 8$). We then confirmed that, under these conditions, BAPTA was effective in preventing RANKL-induced elevation of $[Ca^{2+}]_i$ (Fig. 4*B*).

To examine the role of $[Ca^{2+}]_i$ in osteoclast survival, cells were treated with BAPTA-AM or vehicle. The medium was changed and osteoclasts were incubated with RANKL (100 ng/ml) or vehicle at 37 °C for 24 h. The number of osteoclasts per dish at 24 h was expressed as a percentage of the initial number of osteoclasts in the same dish. As expected, RANKL significantly increased the number of osteoclasts that survived 24 h (Fig. 4*C*, $n = 4$ independent experiments). BAPTA suppressed this effect of RANKL, but did not affect osteoclast survival in the absence of RANKL (Fig. 4*C*). Thus, elevation of

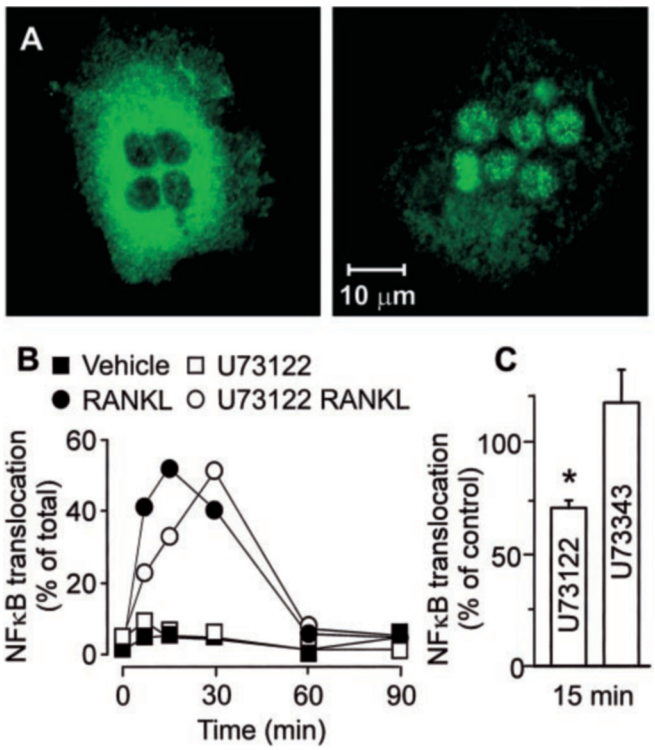

FIG. 5. **Effect of PLC inhibitor on RANKL-induced nuclear translocation of NFκB.** Rat osteoclasts were pretreated with test compounds prior to addition of RANKL (100 ng/ml) or its vehicle at time 0. Samples were fixed at the indicated times and localization of the p65 subunit of NFκB was determined by immunofluorescence. *A*, confocal image on *left* illustrates cytoplasmic localization of NFκB in vehicle-treated osteoclast. Image on *right* illustrates nuclear localization of NFκB in a different osteoclast treated with RANKL (100 ng/ml) for 30 min. *Calibration bar* of 10 μm applies to both panels. Micrographs are representative of results from 12 independent experiments. *B*, kinetics of RANKL-induced nuclear translocation of NFκB. Cells were pretreated with the PLC inhibitor U73122 (1 μM) or dimethyl sulfoxide (vehicle for inhibitors, 0.1% final) for 1 h at 37 °C prior to addition of RANKL (100 ng/ml) or its vehicle. The number of osteoclasts exhibiting nuclear localization of NFκB was expressed as a percentage of the total number of osteoclasts on the coverslip. RANKL caused rapid translocation of NFκB to the nuclei. Treatment with U73122 delayed nuclear translocation of NFκB. Data are representative of six independent experiments. *C*, average nuclear translocation of NFκB 15 min following addition of RANKL to cells pretreated with U73122 or inactive control U73343 (1 μM). Data are expressed as percentage of translocation observed 15 min following addition of RANKL to parallel samples of control cells pretreated with vehicle. Treatment with U73122 ($n = 6$), but not with U73343 ($n = 3$), significantly reduced RANKL-induced nuclear translocation of NFκB ($p < 0.05$). 100% (*control*) value was 44 ± 6% of total number of osteoclasts.

$[Ca^{2+}]_i$ appears to be necessary for RANKL to promote osteoclast survival.

Effect of PLC Inhibitor on RANKL-induced Nuclear Translocation of NFκB—NFκB is one of the major downstream effectors of RANK signaling and activation of NFκB enhances cell survival in many cell types (13, 26). Therefore, we investigated the possible involvement of the PLC/Ca^{2+} pathway in the nuclear translocation of NFκB in osteoclasts. Activation of NFκB was assessed at the single-cell level using immunofluorescence to monitor the spatial distribution of the p65 subunit of NFκB. In the majority of untreated rat or rabbit osteoclasts, NFκB was located in the cytoplasm for the duration of the experiment (Fig. 5*A*, *left*). In a proportion of osteoclasts, RANKL induced redistribution of NFκB to the nuclei, most often to all the nuclei within a single osteoclast (Fig. 5*A*, *right*). Nuclear translocation of NFκB was rapid (within 7 min after addition of RANKL) and transient, reversing within 60 min (Fig. 5*B*). Maximal translocation was evident by 15 min, with 52 ± 4% of osteoclasts exhibiting nuclear localization of NFκB ($n = 12$ independent experiments), compared with 4 ± 1% in untreated osteoclasts ($n = 7$ independent experiments).

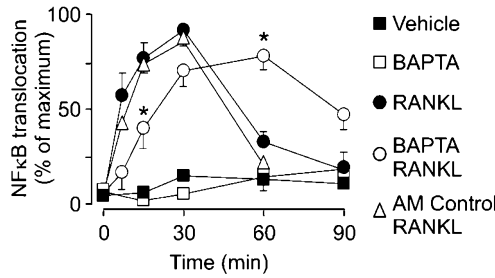

FIG. 6. **Effect of BAPTA on the kinetics of RANKL-induced nuclear translocation of NFκB.** Rat osteoclasts were treated with BAPTA-AM (50 μM), calcein blue-AM (50 μM, *AM Control*) or dimethyl sulfoxide (0.05%, *vehicle*) for 10 min. The medium was then changed and cells were incubated with or without RANKL (100 ng/ml) at 37 °C for the indicated times. The number of osteoclasts exhibiting nuclear localization of NFκB was expressed as a percentage of maximum translocation observed in each experiment. Loading with BAPTA, but not calcein blue, significantly delayed RANKL-induced nuclear translocation of NFκB. Data are means ± S.E. of seven independent experiments, except for AM control where $n = 3$. 100% value was 59 ± 4% (of total number of osteoclasts). *, indicates significant difference of BAPTA/RANKL compared with RANKL alone, $p < 0.05$.

To examine a role of the PLC signaling pathway, we determined the kinetics of RANKL-induced NFκB translocation in osteoclasts treated with the PLC inhibitor U73122, which prevents $[Ca^{2+}]_i$ elevations induced by RANKL. U73122 markedly delayed translocation of NFκB from the cytoplasm to the nuclei in response to RANKL. In U73122-treated cells, maximum translocation was delayed until 30 min following addition of RANKL (*versus* 15 min in parallel samples treated with RANKL alone) (Fig. 5*B*). U73122 did not significantly affect the maximum proportion of osteoclasts exhibiting nuclear translocation of NFκB in response to RANKL (100 ng/ml) (47 ± 6% in control *versus* 44 ± 5% in U73122-treated, based on parallel samples from six independent experiments). U73343, a closely related analog of U73122, which does not inhibit PLC or block RANKL-induced elevation of $[Ca^{2+}]$i (Fig. 2, *C* and *D*), had no significant effect on RANKL-induced translocation of NFκB (Fig. 5*C*). Thus, we provide evidence that RANKL signaling through PLC affects the kinetics of NFκB translocation.

Effect of BAPTA on RANKL-induced Nuclear Translocation of NFκB—We next examined whether chelation of intracellular Ca^{2+} using BAPTA affected the kinetics of RANKL-induced translocation of NFκB. Using loading conditions established above, we found that BAPTA delayed nuclear translocation of NFκB induced by RANKL (Fig. 6, data based on parallel samples from seven independent experiments). In BAPTA-loaded osteoclasts, maximum translocation was observed 30–60 min following treatment with RANKL (100 ng/ml) *versus* 15–30 min in cells treated with RANKL alone. Loading of cells with BAPTA significantly reduced the proportion of osteoclasts exhibiting nuclear localization of NFκB at 15 min, whereas the proportion of cells exhibiting nuclear localization at 60 min was significantly increased (as indicated by *asterisks* in Fig. 6). Furthermore, BAPTA reduced the maximum proportion of osteoclasts exhibiting nuclear translocation of NFκB in response to treatment with RANKL (41 ± 6% for BAPTA-loaded osteoclasts *versus* 57 ± 5% for control osteoclasts). In the absence of RANKL, BAPTA did not significantly affect NFκB distribution (Fig. 6).

Hydrolysis of BAPTA-AM results in release of small molecular weight products because of the degradation of the acetoxymethyl ester (AM) moieties. To determine whether these degradation products might affect NFκB translocation, we ex-

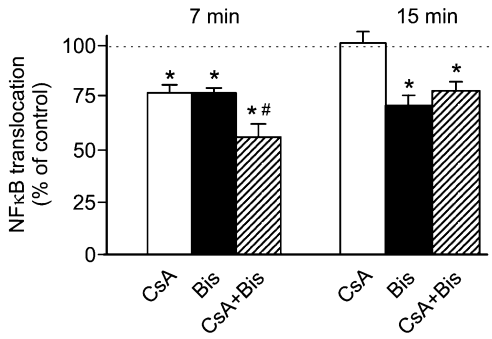

FIG. 7. **Effect of inhibitors of calcineurin and protein kinase C on RANKL-induced nuclear translocation of NFκB.** Parallel samples of rat osteoclasts were incubated with the indicated inhibitors or vehicle (0.05% dimethyl sulfoxide) for 30 min at 37 °C before addition of RANKL (100 ng/ml). Nuclear translocation of NFκB was assessed at 7 and 15 min following addition of RANKL. Data are expressed as percentages of translocation observed in parallel samples of vehicle-treated control cells at 7 and 15 min after addition of RANKL. *Dashed line* indicates 100% level. Calcineurin inhibitor, cyclosporin A (*CsA*, 1 μM, $n = 8$) significantly reduced RANKL-induced nuclear translocation of NFκB at 7 min ($p < 0.05$), but had no effect at 15 min. The PKC inhibitor bisindolylmaleimide I (*Bis*, 100 nM, $n = 7$), significantly reduced RANKL-induced nuclear translocation of NFκB at both 7 and 15 min ($p < 0.05$). The effects of cyclosporin A and bisindolylmaleimide I were additive at 7 min, however, cyclosporin A had no additional effect at 15 min. *, indicates significant difference compared with control, $p < 0.05$. #, indicates significant difference compared with samples treated with only one inhibitor, $p < 0.05$. Data are means ± S.E., 100% (control) values were 32 ± 3 and 39 ± 3% of total number of osteoclasts for 7 and 15 min, respectively.

amined cells treated with calcein blue-AM (a compound that bears the same AM modification as BAPTA-AM, but is ineffective as a Ca^{2+} chelator at physiological $[Ca^{2+}]_i$). Loading cells with calcein blue did not affect the kinetics or degree of RANKL-induced NFκB translocation (Fig. 6, $n = 3$), arguing against possible nonspecific effects of the AM degradation products. Taken together, these data indicate that RANKL-induced elevation of $[Ca^{2+}]_i$ accelerates nuclear translocation of NFκB.

Role of Calcineurin and Protein Kinase C in RANKL-induced Nuclear Translocation of NFκB—We examined the role of a Ca^{2+}-regulated effector known to contribute to activation of NFκB in other systems, the Ca^{2+}-calmodulin-dependent phosphatase, calcineurin. The calcineurin inhibitor, cyclosporin A (1 μM), applied 30 min before addition of RANKL, suppressed the initial NFκB translocation (7 min), with no significant effect at later times (15–30 min) (Fig. 7, data based on parallel samples from eight independent experiments). The structurally distinct calcineurin inhibitor, FK506, had similar effects. FK506 (10 nM) significantly suppressed RANKL-induced NFκB translocation at 7 min to 67 ± 7% of control, but had no significant effect at 15 min (94 ± 7% of control) ($n = 8$). These findings are consistent with RANKL-induced elevation of $[Ca^{2+}]_i$ causing transient activation of calcineurin, which in turn accelerates activation of NFκB.

PLC activation leads to the generation of 2 second messengers, Ca^{2+} and diacylglycerol, both of which contribute to activation of PKC. In other systems, PKC can activate IκB kinases, inducing translocation of NFκB (12). In osteoclasts, the PKC inhibitor bisindolylmaleimide I (100 nM) applied 30 min before addition of RANKL suppressed NFκB translocation at 7 and 15 min (Fig. 7, data based on parallel samples from seven independent experiments), with no significant effect at 30 min. In contrast, the control compound, bisindolylmaleimide V (100 nM) that does not inhibit PKC, had no significant effect on RANKL-induced NFκB translocation (107 ± 3 and 107 ± 4% of control at 7 and 15 min, respectively, $n = 6$). These findings are consistent with a role for PKC, activated following stimulation of phospholipase C, in mediating the effects of RANKL on NFκB.

The effects of cyclosporin A and bisindolylmaleimide I were additive at 7 min, however, the calcineurin inhibitor had no additional effect at 15 min (Fig. 7, data based on parallel samples from seven independent experiments). Similarly, NFκB translocation at 7 min in samples treated with FK506 together with bisindolylmaleimide I was 54 ± 6% of control, significantly less than in samples treated with bisindolylmaleimide I or FK506 alone (77 ± 3 and 67 ± 7%, respectively, $n = 7$). Like cyclosporin A, FK506 had no additional effect at 15 min (translocation in the presence of FK506 and bisindolylmaleimide I was 78 ± 4% of control *versus* 71 ± 5% in osteoclasts treated with bisindolylmaleimide I alone, $n = 7$). Taken together, these data indicate that calcineurin and PKC are downstream effectors of RANK that accelerate activation of NFκB.

DISCUSSION

We demonstrate that RANKL induces transient elevation of $[Ca^{2+}]_i$ in osteoclasts because of activation of PLC and release of Ca^{2+} from intracellular stores. The rise of $[Ca^{2+}]_i$ stimulates Ca^{2+}-dependent K^+ current, accelerates nuclear translocation of NFκB, and promotes osteoclast survival, indicating key functional roles for PLC and Ca^{2+} in RANK signaling in osteoclasts.

Half-maximal effects of RANKL on $[Ca^{2+}]_i$ were observed at ~0.1 ng/ml, with maximal actions at 10–100 ng/ml. These findings are in keeping with the concentration dependence reported for the induction of osteoclastogenesis by RANKL (27, 28), suggesting that elevation of $[Ca^{2+}]_i$ is mediated by RANK. Moreover, OPG prevented RANKL-induced elevation of $[Ca^{2+}]_i$, ruling out possible nonspecific effects. Repeated application of RANKL elicited multiple Ca^{2+} transients indicating lack of short term desensitization. *In vivo*, RANK signaling is thought to be mediated primarily by interaction of osteoclasts and their precursors with cells expressing RANKL (stromal cells, osteoblasts, and lymphocytes). Lack of desensitization would allow osteoclasts to receive multiple signals from neighboring cells, giving rise to temporal and spatial summation of RANKL signals.

Our data demonstrate that RANK signaling in osteoclasts involves PLC. PLC-γ is activated by growth factor receptor tyrosine kinases or nonreceptor tyrosine kinases linked to cytokine receptors (29). In this regard, RANK and TRAF6 interact with, and activate the nonreceptor tyrosine kinase c-Src (10), which may serve to recruit PLC-γ to the RANK-signaling complex in osteoclasts, as was shown for endothelial cells (30). Targeted disruption of c-*src* results in an osteopetrotic phenotype because of compromised resorptive function of osteoclasts (31). Interestingly, osteoclastogenesis is not impaired in c-*src* knockout mice, indicating a different requirement for c-Src signaling in osteoclasts and their precursors. In keeping with these differences, we observed that RANKL-induced Ca^{2+} signaling is more prominent in mature osteoclasts than in precursors. Furthermore, TRAF6-deficient mice have been reported to display osteopetrosis because of nonfunctional osteoclasts (32), also suggesting critical differences between RANKL signaling in precursors and mature osteoclasts. However, osteoclastogenesis was found to be impaired in another TRAF6 knockout model (33), leaving open the question of the precise role for TRAF6-dependent pathways in RANKL-induced osteoclastogenesis and activation of resorption.

As in other cell types, cytosolic Ca^{2+} likely plays important roles in regulating a number of osteoclast functions. We have shown that RANKL activates the intermediate conductance Ca^{2+}-dependent K^+ current (IK_{Ca}) in osteoclasts. Our previous studies have shown that elevation of $[Ca^{2+}]_i$ results in activation of this current (22), so our electrophysiological results

provide independent evidence to support RANKL-induced elevation of $[Ca^{2+}]_i$. *In vivo*, the membrane hyperpolarization resulting from activation of IK_{Ca} would modulate the activity of electrogenic ion transport systems and increase the driving force for influx of Ca^{2+}, a vital process in T cell signaling (34). Similarly, IK_{Ca} may play an important role in osteoclast regulation, especially considering that IK_{Ca} channels are selectively expressed in rat osteoclasts having the morphological characteristics of actively resorbing cells (22). Ca^{2+} also regulates cytoskeletal organization through Ca^{2+}-dependent actin-binding proteins, such as gelsolin, that play critical roles in osteoclast motility (35). Therefore, RANKL-induced alterations in $[Ca^{2+}]_i$ could have multiple downstream effects in osteoclasts. In this regard, we found that elevation of $[Ca^{2+}]_i$ is necessary for RANKL to prolong osteoclast survival. Although previous reports have suggested that elevation of $[Ca^{2+}]_i$ in osteoclasts leads to inhibition of resorption (36), more recent studies have implicated Ca^{2+}-dependent pathways in promoting osteoclast formation (37). In osteoclasts, as in other systems, the effects of $[Ca^{2+}]_i$ elevation likely depend upon the pattern, amplitude, and duration of the Ca^{2+} signal as well as its interactions with other signaling pathways.

NFκB is a key transcription factor that promotes cell survival in many systems (13). In the present study, we have shown that RANKL-induced Ca^{2+} signaling accelerates nuclear translocation of NFκB. Several mechanisms may be considered. Elevation of $[Ca^{2+}]_i$ alone appears to be insufficient to activate NFκB in osteoclasts. Nucleotides, such as ATP (10–100 μM), which bind to the P2Y class of G protein-coupled receptors causing even greater release Ca^{2+} from stores (21), do not activate NFκB in osteoclasts.[2] Similarly, elevation of $[Ca^{2+}]_i$ alone is insufficient to activate NFκB in immune cells (18, 38). Therefore, Ca^{2+} appears to act in concert with other canonical signaling pathways to accelerate activation of NFκB in osteoclasts.

RANK signaling involves a cascade of events, beginning with the recruitment of TRAFs, leading to activation of NFκB-inducing kinase and the IKK complex (5, 11, 14). Enhanced activity of the enzyme complex would increase phosphorylation of IκB, accelerating translocation of NFκB to the nucleus. Ca^{2+} may act to increase activity of the IKK complex, and indeed, IKKβ is reported to be activated synergistically by two Ca^{2+}-dependent mediators of T cell receptor signaling, calcineurin and PKC (18). We show in osteoclasts that RANK signaling involves these same mediators.

Calcineurin is a serine/threonine phosphatase that is stimulated by Ca^{2+}. The immunosuppressant drugs cyclosporin A and FK506 bind distinct immunophilins and inhibit the activity of calcineurin (38). We found that both of these inhibitors delayed the initial phase of NFκB activation in osteoclasts, without affecting maximal activation. Similar actions of distinct inhibitors support the interpretation that their effects are mediated by calcineurin. The actions of calcineurin inhibitors were restricted to early time points, which might be explained by the fact that elevation of $[Ca^{2+}]_i$ in response to RANKL is transient, giving rise to only brief activation of calcineurin. Our findings may have relevance to previously observed inhibitory effects of cyclosporin A on osteoclastic resorption *in vitro* (39).

Stimulation of PLC also leads to increased activation of PKC. To investigate the involvement of PKC, we used bisindolylmaleimide I, a selective inhibitor of the conventional and novel PKC isoforms, and carried out control studies using the inactive analog, bisindolylmaleimide V. Inhibition of PKC also caused delay in NFκB activation, again with no marked change in maximal activation. In contrast to the calcineurin blockers, PKC inhibition resulted in a greater delay in NFκB translocation, which could reflect the time course for production of diacylglycerol. Therefore, it would appear that early stages of NFκB activation involve the convergence of several signaling pathways. Moreover, the effects of calcineurin and PKC blockers were additive, and similar in amplitude to the effects of BAPTA, all supporting a critical role for Ca^{2+} in controlling the latency of NFκB activation.

Because the concurrence of multiple transcription factors regulates expression of genes, the kinetics of their activation is of critical importance. In this regard, the temporal pattern of NFκB activation has recently been shown to generate specificity in gene expression (40). In concert with other transcription factors, NFκB controls cell survival and the expression of genes encoding cytokines and adhesion molecules (12, 13, 41). RANK also activates AP-1, which requires cooperative interactions with other transcription factors and coactivators, including NFκB, to achieve stimulus-specific regulation of transcription (42, 43). In this regard, c-Jun and c-Fos have been shown to interact directly with the p65 subunit of NFκB, enhancing transactivation via both the κB and AP-1 response elements (44). In addition, NFκB interacts with the cancer-amplified transcription coactivator ASC-2 and other transcriptional regulators to control gene expression (45). Therefore, the relative activities of NFκB and other transcription factors at any point of time will determine the composition of multipartner transcription complexes and consequently gene expression. Thus, distinct responses will depend upon the kinetics of activation of NFκB and other transcription factors stimulated by RANKL or by other signaling molecules acting on the osteoclast. Whether the dependence of osteoclast survival on $[Ca^{2+}]_i$ elevation is because of changes in the kinetics of NFκB translocation or because of the activation of other Ca^{2+}-dependent pathways is yet to be determined. Nevertheless, the cross-talk between NFκB and Ca^{2+} signaling demonstrated here provides a novel mechanism for the temporal regulation of NFκB activity and gene expression in osteoclasts and other cell types.

Acknowledgments—We thank Dr. Lin Naemsch for performing preliminary electrophysiological and immunofluorescence studies on rabbit osteoclasts, and Caiqiong Liu for assistance in performing immunofluorescence labeling. We acknowledge Drs. John Hiscott (McGill University), David Litchfield (University of Western Ontario), and Michael Underhill (University of Western Ontario) for helpful comments on the manuscript. We thank Drs. William Boyle and Colin Dunstan (Amgen Inc., Thousand Oaks, CA) for providing soluble RANKL and OPG.

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