Serum Calcium-decreasing Factor, Caldecrin, Inhibits Receptor Activator of NF-κB Ligand (RANKL)-mediated Ca^{2+} Signaling and Actin Ring Formation in Mature Osteoclasts via Suppression of Src Signaling Pathway*\(\textcircled{S}\)

Received for publication, March 3, 2012, and in revised form, March 28, 2012 Published, JBC Papers in Press, March 29, 2012, DOI 10.1074/jbc.M112.358796

Miné Tomomura, H., Hiroya Hasegawa, Naoto Suda, Hiroshi Sakagami, and Akito Tomomura

From the Division of Biochemistry, Department of Oral Biology and Tissue Engineering, the Division of Orthodontics, Department of Human Development and Fostering, and the Meikai Pharmaco-medical Laboratory, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado, Saitama 350-0283, Japan

Osteoclasts are essential for bone dynamics and calcium homeostasis. Recently, we reported that serum calcium-decreasing factor, caldecrin, which is a secretory-type serine protease isolated from the pancreas, inhibits osteoclast differentiation by suppression of NFATc1 activity regardless of its own protease activity (Hasegawa, H., Kido, S., Tomomura, M., Fujimoto, K., Ohi, M., Kiyomura, M., Kanegae, H., Inaba, A., Sakagami, H., and Tomomura, A. (2010) Serum calcium-decreasing factor, caldecrin, inhibits osteoclast differentiation by suppression of NFATc1 activity. J. Biol. Chem. 285, 25448–25457). Here, we investigated the effects of caldecrin on the function of mature osteoclasts by treatment with receptor activator of NF-κB ligand (RANKL). Caldecrin inhibited the RANKL-stimulated bone resorptive activity of mature osteoclasts. Furthermore, caldecrin inhibited RANKL-mediated sealing actin ring formation, which is associated with RANKL-evoked Ca^{2+} entry through transient receptor potential vanilloid channel 4. The inhibitors of phospholipase Cγ, Syk, and c-Src suppressed RANKL-evoked Ca^{2+} entry and actin ring formation of mature osteoclasts. Interestingly, caldecrin significantly inhibited RANKL-stimulated phosphorylation of c-Src, Syk, phospholipase Cγ1 and Cγ2, SLP-76, and Pyk2 but not that of ERK, JNK, or Akt. Caldecrin inhibited RANKL-stimulated c-Src kinase activity and c-Src/Syk association. These results suggest that caldecrin inhibits RANKL-stimulated calcium signaling activation and cytoskeletal organization by suppression of the c-Src/Syk pathway, which may in turn reduce the bone resorptive activity of mature osteoclasts. Thus, caldecrin is capable of acting as a negative regulator of osteoclastogenesis and osteoclast function of bone resorption.

Osteoclasts (OCs)\(^3\) are multinucleated giant cells with bone resorbing activity that contributes to calcium homeostasis, including the serum calcium concentration (reviewed in Refs. 1–3). OCs differentiate from hematopoietic monocyte/macrophage precursors, and this process is regulated by multiple factors, including the receptor activator of nuclear factor (NF)-κB ligand (RANKL). RANKL-RANK recruits cytosolic tumor necrosis factor receptor-associated factors (TRAFs), followed by activation of downstream signaling pathways, including extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase, transcription factor NF-κB, and activator protein-1 (AP-1), including c-Jun and c-Fos, and finally induces expression of the nuclear factor of activated T-cell cytoplasmic β (NFATc1), a key molecule for osteoclastogenesis (for a review, see Ref. 4). The RANKL-RANK signal also activates Ca^{2+} signaling through the activation of phospholipase Cγ (PLCy). RANKL evokes Ca^{2+} oscillation, leading to acceleration of NFATc1 nuclear translocation and gene expression (5). In cooperation with RANKL signaling pathways, immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptor molecules, DNA-activateing protein 12 (DAP12), and Fc receptor γ-chain (FcγR), enhance NFATc1 autoamplification and osteoclast-specific gene expression (6, 7).

The abbreviations used are: OC, osteoclast; ITAM, immunoreceptor tyrosine-based activation motif; NF-κB, nuclear factor-κB; PLC, phospholipase C; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; TRPV, transient receptor potential vanilloid channel; MEM, minimum essential medium; BMM, bone marrow macrophage; TCM, total cell lysate; RIPA, radioimmune precipitation assay; p-Src, phospho-Src; RAW-OC, RAW cell-derived mature OC; BBM-OC, BMM-derived mature OC; RR, ruthenium red; 4a-PDD, 4a-phorbol 12,13-didecanoate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid acetoxymethyl ester.

*This work was supported in part by Grants-in-Aid for Scientific Research C16591864 (to A. T.) and B21390546 (to N. S.) from the Japan Society for the Promotion of Science.
†This article contains supplemental Figs. S1 and S2.
‡To whom correspondence should be addressed. Tel.: 81-49-279-2766; Fax: 81-49-271-2503; E-mail: atomomu@dent.meikai.ac.jp.
Caldecrin Inhibits Osteoclast Function

The effects of RANKL and its signaling on osteoclastogenesis, including gene expression profiles, have been well documented. However, the role of RANKL in mature OCs is less well defined, although RANKL is required continuously for mature OC function (8). Mature OCs create an actin ring, a unique cytoskeletal structure at the sealing zone at sites of attachment to the bone surface, and secrete proteases and protons into the acidic microenvironment needed for bone matrix degradation (9, 10). Therefore, formation of the sealing actin ring is essential for the osteoclast function of bone resorption. Many molecules are involved in actin ring organization and maintenance during bone resorption. αβ3 integrin and associated proteins, such as talin, vinculin, and paxillin, form a complex and organize the plasma membrane and actin cytoskeleton. Indeed, β3 integrin-deficient mice show increased bone mass by OCs with reduced activity to resorb bone (11). In 1991, Soriano et al. (12) reported that c-Src-deficient mice develop severe osteopetrosis despite an increased number of OCs impairing actin ring formation and bone resorption. Proline-rich tyrosine kinase 2 (Pyk2)-deficient mice also show impairment of bone resorption (13). Pyk2 forms a complex with c-Src, linking integrin signaling to actin cytoskeletal organization in OCs (14). Another tyrosine kinase, spleen tyrosine kinase (Syk), which associates with phosphorylated ITAM by its Src homology 2 domain, forms a complex with c-Src and in turn activates downstream molecules and regulates actin cytoskeletal organization (15). It is uncertain how RANKL–RANK binding activates c-Src-Syk, and c-Src-Pyk2, both leading to cytoskeletal organization to perform its function in mature OCs.

Calcium signaling pathways have been shown to play a role not only in differentiation but also in resorption, exerting effects on actin metabolism, cytoskeletal organization, and cell-matrix interactions. Differentiation of OCs is triggered by Ca2+ oscillation, which requires Ca2+ release from intracellular stores and Ca2+ influx via calcium channels. Transient receptor potential vanilloid channel 2 (TRPV2), TRPV4, and TRPV5 are involved in Ca2+ influx of OCs (16–18). TRPV channel activities are regulated not only by various physical and chemical stimuli, including heat, mechanical stress, and synthetic and endogenous compounds, but also by phosphorylation by protein kinase C and Src family kinase (19, 20).

We have purified and cloned the serum calcium-decreasing factor, caldecrin, from the pancreas (21–24). Caldecrin is a secretory-type serine protease, which has chymotryptic activity and is also known as chymotrypsin C (EC 3.4.21.2) (22, 23). We originally reported that the administration of caldecrin decreases mouse serum calcium concentration in a dose-dependent manner, and serum calcium-decreasing activity is correlated with a decrease in serum hydroxyproline, which is included as a component of collagen and is a marker of bone resorption (21). Recently, we reported that caldecrin inhibits osteoclast differentiation by inhibition of RANKL-mediated Ca2+ oscillation, leading to suppression of NFATc1 activation (25). Interestingly, the inhibitory effect of caldecrin on OC differentiation does not depend on its protease activity. We have also demonstrated that caldecrin suppresses bone resorption by mature rabbit OCs (26). These results, including the previous in vivo finding of rapid serum calcium decrease after intravenous injection of caldecrin, suggest that caldecrin may be functional not only in the OC differentiation step but also in the bone resorption step of mature OCs.

In this study, we extended our investigation of the role of caldecrin in mature OC function. Caldecrin inhibited RANKL-induced bone resorptive activity by suppression of RANKL-induced c-Src-Syk activation, thereby affecting downstream signaling, including intracellular calcium signaling needed for actin ring formation following bone resorption.

EXPERIMENTAL PROCEDURES

Preparation of Caldecrin—Recombinant wild-type caldecrin was prepared as described previously (27). Briefly, cultured HEK293T cells were transfected with rat caldecrin-3×FLAG cDNA. On day 3 of culture, the secreted recombinant protein was purified from culture medium by anti-FLAG M2 affinity gel chromatography according to the manufacturer’s instructions (Sigma-Aldrich). The recombinant caldecrin was freshly activated by treatment with trypsin (50:1) for 30 min at room temperature followed by treatment with 0.1 mM α-aminophenyl-methanesulfonyl fluor hydrochloride (Sigma-Aldrich) to terminate activation.

Preparation of Mature Osteoclasts and Assessment of Bone Resorption—Mouse bone marrow cells (5–10 × 10⁶ cells/ml) were seeded with α-MEM (Sigma-Aldrich) containing 10% FBS (Sigma-Aldrich) and macrophage-colony stimulating factor (M-CSF 10 ng/ml; R&D Systems, Inc., Minneapolis, MN) for 3 days to produce bone marrow macrophages (BMMs). The medium of BMMs was replaced with differentiation medium containing M-CSF and RANKL (10 ng/ml; R&D Systems) and cultured for 3–4 days to generate mature OCs. All procedures were approved by the Animal Care and Use Committee of the Meikai University School of Dentistry (Saitama, Japan). RAW264.7 (mouse leukemic monocyte macrophage cell line) cells (5 × 10⁵ cells/ml) were differentiated by culturing with α-MEM containing 10% FBS and RANKL for 3 days. Both types of cells were incubated on 96-well plates (0.1 ml/well), 24-well plates (0.5 ml/well), or plastic 35-mm μ-dishes (Ibidi, Nippon Genetics Co., Ltd., Tokyo, Japan). Multinucleated cells (>3 nuclei) were used as mature OCs. In some experiments, cells were cultured on RepCell (CellSeed Co., Ltd., Tokyo, Japan) with differentiation medium for 4 days, and the mature OCs on RepCell were collected by incubation at room temperature and 4 °C for detachment from the temperature-responsive culture dishes as reported elsewhere (28). The same numbers of mature OCs (500 cells/0.1 ml/well) were seeded on discs coated with submicron calcium phosphate (Osteologic discs; BD Biosciences) or dentin slices (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After 2 h of attachment on the matrices, cells were treated with RANKL or RANKL + caldecrin and cultured for 2 days. The cells on the Osteologic discs were removed by washing with 5% sodium hypochlorite, followed by extensive washing with distilled water and air drying. The cells were scraped off the dentin slices, and the resorption pits on the slices were stained for 10 min with acid-hematoxylin. The absorbed areas on the discs or slices were observed by phase-contrast inverted light microscopy (ECLIPSE TS100, Nikon Instech, Co., Ltd., Tokyo, Japan) with a charge-coupled device.
Caldecrin Inhibits Osteoclast Function

Cell Viability Assay—The same number of OCs collected from RepCell were cultured with or without RANKL or RANKL + caldecrin (100 nM) for 1 day, as described above, fixed with 4% paraformaldehyde for 5 min, and stained for tar-taric acid phosphatase (TRAP) as described previ-
ously (25). To evaluate the effects of caldecrin on OC survival, terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining was performed with an In Situ Cell Death Detection kit (Roche Applied Science). After nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), TUNEL-positive apoptotic cells were counted using fluorescence microscopy.

Assessment of Actin Ring Formation—Mature OCs were prepared from RAW cells or BMMs by treatment with RANKL for 4 days on μ-dishes. After OCs were cultured for 17 h in RANKL-free α-MEM containing 10% FBS and M-CSF (as in BMM culture), the cells were either unstimulated or stimulated with RANKL alone or RANKL + caldecrin (100 nM) for 24 h and stained for actin rings. For the experiment without RANKL, mature OCs were also further cultured with or without caldecrin for 7 or 24 h in the presence or absence of RANKL and stained for actin rings. In some experiments with various inhibitors, the calcium chelator BAPTA-AM (20 μM; Merck), the PLCγ inhibitor U73122 and negative control U-73343 (5 μM; Sigma-Aldrich), Syk inhibitor ER-27319 (15 μM; Sigma-Aldrich), Src inhibitor PP2 (0.5 μM; Enzo Life Sciences International, Farmingdale, NY), and TRPV inhibitor ruthenium red (2 μM; Sigma-Aldrich) were used. After incubation, the cells were fixed for 3 min with Cytoskelfix (Cytoskeleton, Inc., Denver, CO) at −20 °C. F-actin in the OC was stained with MFR 488-
conjugated phalloidin (MoBiTec GmbH, Göttingen, Germany) for 30 min at room temperature. Actin rings were visualized using an LSM 510 confocal microscope (Carl Zeiss MicroImaging Co., Tokyo, Japan).

Intracellular Ca2+ Measurement—Intracellular Ca2+ was monitored using a Fluo-4NW calcium assay kit (Invitrogen) and Fura Red-AM according to the manufacturer’s instructions and as described previously (25). RAW264.7 cell-derived mature OCs were cultured in the presence of RANKL on μ-dishes. Cells were treated with RANKL starvation for a further 17 h and then loaded with 4 μM Fluo-4NW and 2.3 μM Fura Red-AM “cell permeant” (Invitrogen) in loading solution for a final 30 min. After washing, cells were incubated with α-MEM containing 10% FBS and stimulated with RANKL. Cells were excited at 488 nm, and fluorescence images with emission at 505–530 nm for Fluo-4 and 500–580 nm for Fura Red were monitored by laser-scanning confocal microscopy at 1-s intervals. For the inhibition assay, caldecrin or various inhibitors were subsequently added to the RANKL-containing medium after the first Ca2+ spike was observed, and additional RANKL treatments were performed. For ratiometric measurement of Ca2+ in a single cell, the fluorescence intensity of Fluo-4/Fura Red was calculated and expressed as the percentage maximum ratio increase, which was obtained by the addition of 10 μM ionomycin (Sigma-Aldrich) at 30 min to terminate the experiment. For Ca2+ imaging of BMM-derived mature OCs (BMM-
OCs), mature OCs were cultured with α-MEM, 10% FBS, and M-CSF for the RANKL-depleted condition (21 h), followed by replacement with medium containing 0.5 mM EGTA (3 h). M-CSF and EGTA were added to the manufacturer’s loading solution and the assay solution.

Immunoblotting and Immunoprecipitation—Mature OCs differentiated on culture dishes were further cultured with RANKL-free medium for 12 h. Thereafter, cells were incubated with or without RANKL or RANKL + caldecrin in the presence (BMMs) or absence (RAW) of M-CSF for 15 min. Total cell lysates (TCLs) were prepared with SDS sampling buffer or RIPA I buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerophosphate, 10 mM NaF, 5 mM Na3VO4, and protease inhibitor mixture; Roche Applied Science). Samples were separated by SDS-PAGE, transferred onto Immobilon-P PVDF membranes (Millipore, Billerica, MA), and then immunoblotted with antibody against Akt, phospho-Akt (Ser-473), phospho-Syk (Tyr-525/526), PLCγ1, phospho-
PLCγ1 (Tyr-783), PLCγ2, phospho-PLCγ2 (Tyr-1217), Src homology 2 domain-containing leukocyte (SLP)-76, ERK, phospho-ERK (Thr-202/Tyr-204), JNK, phospho-JNK (Thr-183/Tyr-185) (Cell Signaling Technology, Danvers, MA), c-Src (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Cell Signaling Technology, and R&D Systems), phospho-Src (p-Src) (human Tyr(P)-418 corresponding to mouse Tyr(P)-416; Invitro-
gen), Syk (N-19; Santa Cruz Biotechnology), phospho-
SLP-76 (Tyr-128; Assay Biotechnology Co., Sunnyvale, CA), Pyk2 (BD Transduction Laboratories Inc., Lexington, KY), phospho-Pyk2 (Tyr-402; Signalway Antibody, Pearlard, TX), or β-actin (Medical and Biological Laboratories, Nagoya, Japan). The blots were incubated with horseradish peroxidase-
conjugated secondary antibodies (Pierce) for 1 h, and chemiluminescence was detected with an Immobilon system (Milli-
pore). For immunoprecipitation, TCLs extracted with RIPA I buffer were incubated with control IgG or anti-Syk, anti-c-Src (R&D Systems), anti-TRAF6 (H-274, Santa Cruz Biotechnol-
dy), or anti-TRAF6 antibody. Immunoprecipitation was performed with anti-c-Src antibody. Cell lysates (TCLs) were prepared in RIPA buffer containing 1% sodium deoxycholate and 0.1% SDS) and immunoprecipitated with anti-c-Src antibody. The immunoprecipitate was washed three times with RIPA II buffer and twice with kinase assay buffer (50 mM HEPES, pH 7.5, 5 mM MnCl2, 0.1 mM Na3VO4) in the presence or absence of the Src-
specific inhibitor PP2 (10 μM). The kinase reaction was initiated by the addition of ATP (0.5 mM), incubated for 20 min at 30 °C, and terminated by the addition of 2× SDS sample buffer. The reactions were analyzed by Western blotting with anti-phos-
phototyrosine antibody PY99 (Santa Cruz Biotechnology). The same filter was stripped and reblotted with anti-p-Src or anti-
c-Src antibody.

In Vitro Kinase Assay—TCLs were prepared in RIPA II buffer (RIPA I buffer containing 1% sodium deoxycholate and 0.1% SDS) and immunoprecipitated with anti-c-Src antibody. The immunoprecipitate was washed three times with RIPA II buffer and twice with kinase assay buffer (50 mM HEPES, pH 7.5, 5 mM MnCl2, 0.1 mM Na3VO4) in the presence or absence of the Src-specific inhibitor PP2 (10 μM). The kinase reaction was initiated by the addition of ATP (0.5 mM), incubated for 20 min at 30 °C, and terminated by the addition of 2× SDS sample buffer. The reactions were analyzed by Western blotting with anti-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology). The same filter was stripped and reblotted with anti-p-Src or anti-c-Src antibody.
Caldecrin Inhibits Osteoclast Function

lized with 0.2% Triton X-100 for 15 min. After blocking of non-specific binding with 1% BSA for 30 min, cells were incubated with anti-c-Src or anti-Syk antibody or control IgG in PBS overnight. The cells were washed with PBS and then incubated for 1 h with Alexa Fluor 594-labeled secondary antibodies (Invitrogen). F-actin was identified using MFP 488-labeled phalloidin. The fluorescence images were then visualized and photographed by confocal microscopy.

Statistics—Data were analyzed by analysis of variance and are shown as means ± S.D. from at least three independent experiments. In all analyses, p < 0.05 was taken to indicate statistical significance.

RESULTS

Caldecrin Inhibits Bone Resorption by Mature OCs—Differentiated mature OCs can form a sealing zone/actin ring and resorb the mineral substratum on discs coated with hydroxyapatite (Osteologic discs) as well as dentin discs (29–31). To prepare multinucleated mature OCs for bone resorption assay, RAW264.7 cells or BMMs were cultured with RANKL or M-CSF + RANKL, respectively, for 4 days in temperature-responsive culture dishes, RepCell, as described under “Experimental Procedures.” Mature OCs recovered from RepCell dishes were further cultured on Osteologic discs or dentin slices for 48 h with or without RANKL (10 ng/ml) or caldecrin in the presence (BMMs) or absence (RAW cells) of M-CSF. As shown in Fig. 1A, the resorption area of RANKL-treated RAW cell-derived mature OCs (RAW-OCs) was larger than that of RANKL-starved control OCs, whereas the resorption area of caldecrin-treated cells was small, similar to that of RANKL-free control OCs. We quantified the resorption area per cell, indicating that caldecrin (100 nm) significantly inhibited the resorption area to 39% of the RANKL-induced resorption in RAW-OCs (Fig. 1B). BMM-OCs cultured on dentin slices showed increased absorption area by RANKL treatment and inhibited to the control level by treatment with caldecrin even in the presence of RANKL (Fig. 1, C and D). These results suggest that caldecrin inhibits RANKL-stimulated osteoclastic bone resorption.

We determined the viability of the cells to examine whether caldecrin specifically inhibits the bone resorptive activity of mature OCs. RepCell-purified mature OCs were cultured for 24 h with or without RANKL or RANKL + caldecrin in the presence (BMMs) or absence (RAW cells) of M-CSF, followed by TRAP staining. TRAP-positive mature OCs were analyzed for apoptosis by TUNEL staining (Fig. 2A). The numbers of TUNEL-positive apoptotic cells were increased after RANKL depletion. Caldecrin did not significantly affect the number of TRAP-positive apoptotic cells (Fig. 2B).

Caldecrin Inhibits RANKL-induced Actin Ring Formation in Mature OCs—The actin sealing ring in the OCs is essential for bone resorption. RANKL induces actin ring formation and maintains ring assembly. Therefore, we used two protocols to examine whether caldecrin inhibits RANKL-mediated actin ring assembly in mature OCs.

First, RAW264.7 cells were differentiated by RANKL on plastic μ-dishes for 4 days, and then mature OCs were subsequently depleted of RANKL for 17 h to abolish RANKL signaling. The cells were further cultured with or without RANKL or RANKL + caldecrin. After staining with fluorescence-labeled phalloidin, the actin ring was evaluated by converting fluorescence intensity to color images and confocal z axis reconstruction images. As shown in Fig. 3A (a), RAW-OCs produced a well defined actin sealing ring with a higher intensity ring height at the cell margin. After RANKL depletion, mature OCs showed loss of the peripheral actin ring with low intensity ring height (Fig. 3A (b)). RANKL restimulation caused rapid actin ring formation at 1 h poststimulation (Fig. 3A (c)) and almost complete recovery of actin ring construction at 24 h poststimulation (Fig. 3A (d)). However, caldecrin inhibited actin ring formation at 24 h poststimulation even in the presence of RANKL (Fig. 3A (e)). The majority of cells restimulated with RANKL for 24 h showed more than medium intensity actin rings (yellow column) consisting of well defined actin rings (red), whereas caldecrin significantly decreased the number of well defined actin rings and reduced the intensity (Fig. 3A (f)).

Another protocol was used, in which RAW267.4 cells were differentiated to mature OCs by RANKL as in the above protocol and then further cultured with or without caldecrin in the presence of RANKL without prior depletion. RAW-OCs treated with caldecrin for 7 h showed partial actin ring loss with a low intensity of ring height, similar to RANKL-depleted control OCs (supplemental Fig. S1). RAW-OCs depleted of
RANKL for 24 h showed complete loss of actin ring construction as compared with the 24-h RANKL stimulation control (Fig. 3B, b and a, respectively). Caldecrin inhibited actin ring assembly even in the presence of RANKL (Fig. 3B c)). To examine whether calcium mobilization is involved in RANKL-mediated actin ring assembly, the effects of the calcium chelator BAPTA-AM, TRPV inhibitor ruthenium red (RR), and PLCγ inhibitor U-73122 were investigated. BAPTA-AM and inhibitor U-73122 were investigated. BAPTA-AM and U-73122, but not the inactive analog U-73343, inhibited RANKL-mediated actin ring assembly in mature OCs (Fig. 3B, d–g). The Syk inhibitor ER-27319 and Src inhibitor PP2 also abolished RANKL-mediated actin ring assembly (Fig. 3B, h and i)). Quantitative analysis of actin rings is summarized in Fig. 3B (j). These inhibitors and caldecrin demonstrated similar results already at 7 h of culture in RAW-OCs (supplemental Fig. S1). Next, the actin ring of BMM-OCs was also investigated according to the above-described RANKL non-starvation protocol. As shown in Fig. 3C, RANKL withdrawal for 24 h caused actin ring loss as compared with RANKL exposure (Fig. 3C, b and a, respectively). RR or U-73122 in the presence of RANKL also inhibited actin ring assembly as well as that under conditions of RANKL starvation (Fig. 3C, c and d). Treatment with caldecrin or PP2 also inhibited actin ring assembly in BMM-OCs (Fig. 3C, e and f). These results suggest that the actin ring cytoskeletal organization of mature OCs is required for RANKL exposure, in which the mechanisms involve Ca2+ influx mediated by signaling molecules, such as Src, Syk, PLCγ, and TRPV. Caldecrin may inhibit the actin ring assembly of mature OCs by inhibition of the signaling pathways of these molecules.

Caldecrin Inhibits RANKL-evoked [Ca2+]i Increase in Mature OCs—Because caldecrin inhibits RANKL-mediated Ca2+ oscillation during osteoclastogenesis (25) and Ca2+ mobilization-associated actin ring organization (Fig. 3), we next examined whether caldecrin also affects [Ca2+]i of mature OCs. RAW264.7 cells were cultured with RANKL to generate mature OCs and then further cultured without RANKL for 17 h to remove its signal. Mature OCs stimulated with RANKL showed transient elevation of [Ca2+]i (i.e. Ca2+ spikeline mobilization)
Caldecin Inhibits Osteoclast Function
(Fig. 4A (a)). Multiple [Ca^{2+}]_{i} transients were elicited by successive applications of RANKL. This RANKL-evoked Ca^{2+} spike was inhibited in a time-dependent manner by 10 \text{nm} caldecrin and was completely inhibited by a higher concentration of 100 \text{nm}, indicating that caldecrin inhibits the Ca^{2+} spike in a dose-dependent manner (Fig. 4A, b and c). To investigate the mechanism underlying the inhibitory action of caldecrin on the RANKL-evoked Ca^{2+} transient, we examined the RANKL-evoked Ca^{2+} spike using specific inhibitors. We first investigated whether the Ca^{2+} spike is due to intracellular calcium mobilization using BAPTA-AM and U-73122. The RANKL-induced Ca^{2+} influx was blocked by BAPTA-AM and U-73122, whereas U-73343 showed no such effect (Fig. 4A, d–f). RR abolished the RANKL-evoked Ca^{2+} spike, and the TRPV4-selective agonist 4α-phorbol 12,13-didecanoate (4α-PDD) caused a transient Ca^{2+} influx in mature OCs (Fig. 4A, i and j). We next examined whether caldecrin inhibits the 4α-PDD-evoked Ca^{2+} spike of mature OCs. A lower concentration (10 \text{nm}) of caldecrin did not inhibit the 4α-PDD-evoked Ca^{2+} spike (data not shown). A higher concentration of caldecrin (100 \text{nm}) slightly suppressed the 4α-PDD-evoked Ca^{2+} spike, although the RANKL-mediated Ca^{2+} spike was completely abolished by caldecrin at the same concentration (Fig. 4A, k and c). Treatment with ER-27319 and PP2 also inhibited the RANKL-evoked Ca^{2+} spike in mature OCs (Fig. 4A, g and h).

RANKL-evoked Ca^{2+} mobilization was also investigated in BMM-OCs. The RANKL-mediated Ca^{2+} spike was insufficient and failed to respond to a second RANKL stimulation (supplemental Fig. S2A). Treatment with 4α-PDD caused a Ca^{2+} spike, but the response to the successive stimulation was also diminished (supplemental Fig. S2A). The addition of ionomycin, a Ca^{2+} ionophore, still caused a significant increase in intracellular Ca^{2+}, indicating that Fluo-4 signaling is still active. Most TRPV channels are down-regulated by an increase in the intracellular Ca^{2+} concentration (32). We thus investigated the RANKL-evoked Ca^{2+} mobilization in BMM-derived mature OCs in the extracellular lower Ca^{2+} condition. When EGTA (0.5 \text{mm}) was added during the final 3 h of culture under conditions of RANKL starvation followed by Ca^{2+} indicator loading and assay, a Ca^{2+} spike was evoked by a second stimulation with RANKL or 4α-PDD (Fig. 4B, a and e). Under these assay conditions, RR abolished not only 4α-PDD-evoked but also RANKL-evoked Ca^{2+} influx (Fig. 4B, f and b). Caldecrin inhibited RANKL-evoked Ca^{2+} influx but not that evoked by 4α-PDD, suggesting that caldecrin inhibits TRPV4 indirectly (Fig. 4B, c and g). U-73122 and PP2, but not U-73343, inhibited RANKL-evoked stimulation (Fig. 4B (d) and supplemental Fig. S2B). Taken together, these findings indicated that TRPV4 mediates RANKL-evoked Ca^{2+} influx, which is involved in the c-Src-Syk-PLCγ signaling pathway. Caldecrin inhibits RANKL-evoked Ca^{2+} influx by inhibition of this pathway in mature OCs.

**Caldecrin Inhibits RANKL-stimulated Activation of c-Src and Syk in Mature OCs**—To further investigate the target signaling molecules of caldecrin, which are involved in calcium mobilization and actin ring organization, we next examined the levels of protein phosphorylation in RAW264.7 cell (RAW) and BMM-derived mature OCs activated for 15 min by RANKL (Fig. 5). As shown in the left panels of Fig. 5, RANKL stimulation increased the phosphorylation of c-Src and Syk kinases in both OCs as compared with RANKL-free control (N). Phosphorylation of PLCγ1 and PLCγ2, which are downstream of Syk, were increased in both OCs by RANKL stimulation. RANKL also increased the phosphorylation of SLP-76 and Pyk2, which are phosphorylated by Syk and c-Src, respectively. Caldecrin inhibited the RANKL-stimulated phosphorylation of these signaling molecules (Fig. 5, left, R+C).

RANKL-RANK stimulates other signaling pathways in OCs, including MAPKs, during osteoclastogenesis. We next examined whether caldecrin inhibits RANKL-activated MAPKs, including ERK1/2 and JNK, of RAW-OCs and BMM-OCs. As

**FIGURE 4.** RANKL-mediated Ca^{2+} spike in mature OCs was inhibited by caldecrin or various inhibitors. A, RAW264.7 cells were differentiated to mature osteoclasts in the presence of RANKL for 4 days. The cells were incubated without RANKL for 17 h, and the [Ca^{2+}]_{i} spike induced by RANKL was monitored every 10 min (a). After the first [Ca^{2+}]_{i} spike by RANKL stimulation was detected, RANKL stimulations were conducted in the presence of caldecrin (10 \text{nm} (b) or 100 \text{nm} (c)), BAPTA-AM (20 \text{μm} (d)), U-73122 (10 \text{μm} (e)), U-73343 (10 \text{μm} (f)), ER-27319 (15 \text{μm} (g)), PP2 (15 \text{μm} (h)), or RR (2 \text{μm} (i)). The cells were incubated as in A, and the [Ca^{2+}]_{i} spike induced by 4α-PDD stimulation was monitored every 10 min (j and k). 4α-PDD stimulation was conducted in the presence of caldecrin (100 \text{nm} (k)). Five colors show distinct cells in the same field. B, BMMs were differentiated with M-CSF and RANKL for 4 days. After RANKL starvation for 21 h, ETGA (0.5 \text{mm}) was added to the medium for 3 h, followed by BAPTA-AM indicator loading and assay. RANKL-stimulated [Ca^{2+}]_{i} spikes were monitored with control vehicle (a), RR (b), caldecrin (c), or PP2 (d) as in A. 4α-PDD-stimulated Ca^{2+} spikes were monitored with control vehicle (e), RR (f), or caldecrin (g) as in A. Each color indicates a different cell in the same field.

**FIGURE 5.** Caldecrin inhibits RANKL-stimulated phosphorylation (p-) of c-Src, Syk, and Pyk2 in mature OCs. RAW264.7 cells (RAW) or BMMs (BMM) were differentiated into mature OCs in the presence of RANKL or M-CSF and RANKL for 3–4 days and then in medium depleted of RANKL for 12 h. The cells were incubated without (N) or with RANKL alone (R) or RANKL + caldecrin (R + C) for 15 min. Cell lysates (20 \text{μg}) were subjected to Western blotting as described under "Experimental Procedures."
shown in the right panels of Fig. 5, RANKL activated the phosphorylation of ERK1/2 and JNK at 15 min post-RANKL stimulation in both OCs as compared with RANKL-free control (N). Caldecrin did not alter the RANKL-stimulated ERK and JNK activation (Fig. 5, right, R+C). RANKL also stimulated the phosphorylation of Akt, which was not altered by caldecrin treatment (Fig. 5, right). These results suggest that caldecrin may inhibit the RANKL-stimulated c-Src kinase activity and subsequent activation of downstream signaling molecules. Activation of c-Src kinase involves autophosphorylation at tyrosine 416 within the catalytic domain (33). Next, we measured c-Src activity by an in vitro kinase assay. RANKL-stimulated TCLs from BMM-derived mature OCs were immunoprecipitated with control IgG or anti-c-Src antibody, followed by an in vitro kinase assay performed in the presence or absence of PP2 for 20 min. c-Src kinase was specifically precipitated with anti-c-Src antibody but not with control IgG by immunoblotting with anti-pan-Src antibody (Fig. 6A, bottom, lane 1 versus lane 2). In vitro tyrosine phosphorylation of c-Src kinase was inhibited by the Src kinase inhibitor PP2 (Fig. 6A, top, lane 2 versus lane 3). To examine whether caldecrin inhibits RANKL-stimulated Src kinase activity, BMM-derived mature OCs were cultured either unstimulated or stimulated with RANKL alone or with RANKL + caldecrin after RANKL depletion, and Src kinase activity was measured by an in vitro kinase assay. Tyrosine phosphorylation of the c-Src was very low in all treated samples when an in vitro kinase assay was performed in the absence of ATP (Fig. 6B, top, lanes 1–3). On the other hand, the phosphorylation activity of c-Src was high in RANKL-treated cell extract as compared with that of untreated cells in the presence of ATP (Fig. 6B, top, lane 4 versus lane 5). RANKL-stimulated phosphorylation activity of c-Src was reduced by caldecrin treatment (Fig. 6B, top, lane 5 versus lane 6). Consistent with these results, autophosphorylation of c-Src (Tyr-416) was increased in the RANKL-treated cell extracts but not in caldecrin-treated cells (Fig. 6B, middle). These results suggest that caldecrin inhibits RANKL-stimulated c-Src kinase activity.

Next, we investigated the association of c-Src with Syk by immunoprecipitation experiments using anti-Syk or anti-c-Src antibodies. In RAW264.7 cell OCs, c-Src was coimmunoprecipitated with anti-Syk antibody by RANKL stimulation but not by caldecrin treatment (Fig. 7A (a)). Reciprocal immunoprecipitation and immunoblotting showed that RANKL stimulated c-Src-Syk complex formation, which was inhibited by caldecrin (Fig. 7A (b)). c-Src and Syk are associated with β3 integrin in BMM-OCs (15). To examine whether these complexes are affected by RANKL stimulation, BMM-OCs were unstimulated or stimulated with RANKL alone or RANKL + caldecrin for 15 min. TCLs were then immunoprecipitated with anti-Syk or irrelevant IgG. c-Src was associated with Syk by RANKL stimulation, which was inhibited by caldecrin (Fig. 7B (a), top). Syk-integrin association was induced by RANKL treatment, which was inhibited by caldecrin treatment (Fig. 7B (a), middle). In contrast, c-Src was apparently associated with β3 integrin in a RANKL-independent manner, which was unaffected by caldecrin (Fig. 7B (b), top). These results suggest that RANKL stimulates molecular complex formation between c-Src-integrin and Syk, and this complex formation is blocked by caldecrin.

RANKL stimulation initiates association of c-Src with TRAF6 (34). To investigate whether caldecrin affects TRAF6-c-Src association, BMM-OCs were treated with or without RANKL or RANKL + caldecrin and then immunoprecipitated...
with anti-c-Src antibody and subjected to immunoblotting for TRAF6. RANKL stimulated the formation of TRAF6/c-Src association as compared with M-CSF alone, which was inhibited by caldecrin (Fig. 7B (c), top). These results suggest that TRAF6/c-Src interaction is stimulated by RANKL, which is inhibited by caldecrin in mature OCs.

Caldecrin Inhibits Colocalization of Syk, Src, and Actin Ring in Mature OCs—Caldecrin inhibits RANKL-induced c-Src and Syk interaction in mature OCs. Thus, we demonstrated the subcellular localization of Syk and Src in mature OCs by treatment with RANKL alone or RANKL + caldecrin by confocal microscopy. Actin rings were apparent with thick and uniformly stained bands in RANKL-stimulated OCs (Fig. 8, A and B, F-actin). This structure was disrupted by caldecrin. Syk was predominantly localized to the margins of OC adjacent to the actin ring in RANKL-stimulated OCs (Fig. 8A, Syk). However, Syk of caldecrin-treated and untreated control OCs showed a diffuse distribution, including partial overlap with actin (Fig. 8A, merged). c-Src was also distributed in the periphery, where actin rings were present in RANKL-stimulated OCs. On the other hand, c-Src was distributed diffusely over the cell rather than at the periphery in caldecrin-treated cells (Fig. 8B, Src). Although actin rings by staining with fluorescent phalloidin were clearly present in RANKL-treated OCs, immunofluorescence signals were very low when control IgG was used instead of primary antibody (Fig. 8D), suggesting the specificity of the primary antibody. p-Src was localized exclusively on the outer edge of the RANKL-treated OCs, where c-Src was localized. In OCs treated with RANKL + caldecrin, however, p-Src was barely detectable, although c-Src showed a diffuse distribution throughout the cell. These results suggested that c-Src is localized with the actin ring in phosphorylated form (active Src) under conditions of RANKL stimulation but not caldecrin treatment.

**DISCUSSION**

We recently reported that caldecrin inhibits RANKL-mediated OC differentiation of bone marrow cells and RAW264.7 cells by inhibition of Syk-PLCγ-mediated Ca²⁺ oscillation, followed by inhibition of calcineurin activation and NFATc1 activity and osteoclastogenesis (25). We also found that caldecrin suppresses bone resorption of freshly isolated rabbit mature OCs (26). In the present study, we used mature OCs prepared from RANKL plus M-CSF-treated BMMs and RANKL-treated RAW264.7 cells in the absence of M-CSF to elucidate the suppression mechanism of caldecrin on the OC function. Here, we showed that caldecrin suppresses RANKL-stimulated bone resorptive activity of mature OCs derived from RAW264.7 cells and BMMs (Fig. 1). This effect seems not to be due to the toxicity of caldecrin, because caldecrin treatment did not alter the cell apoptosis significantly compared with RANKL alone (Fig. 2). In addition, caldecrin suppressed the RANKL-induced phosphorylation of a subgroup of signaling proteins, but not of all proteins (Fig. 5), supporting the suggestion that caldecrin may impact a certain signaling pathway(s) of RANKL-mediated OC function.

Actin cytoskeletal organization includes the ruffled membrane, and the actin ring or sealing zone is essential for mature OCs. This cytoskeleton plays a key role in OC function, including bone resorption, adhesion, and migration. The actin ring is formed by the interaction of myosin and actin filaments, and it facilitates the formation of the ruffled membrane. The actin ring is essential for the formation of the sealing zone, which is a critical component of OC function. The sealing zone is composed of several proteins, including calponin, vinculin, and talin, which are involved in the regulation of actin filaments. The formation of the sealing zone is essential for the efficient resorption of bone, as it allows OCs to create a high negative pressure in the bone resorption cavity, which facilitates the removal of bone matrix.

**FIGURE 8. Effects of caldecrin on intracellular localization of Src and Syk in mature OCs.** RAW264.7 cell-derived mature OCs were cultured without RANKL for 8 h and further cultured with or without RANKL or RANKL + caldecrin for 16 h. Cells were fixed and stained with anti-Syk (A), anti-c-Src (B) or control IgG (D) antibody or Alexa Fluor 594-labeled secondary antibody. Staining for F-actin was performed using MFP 488-labeled phalloidin (A, B, and D; C, cells were stained with anti-p-Src and anti-c-Src antibodies followed by staining with Alexa Fluor 594-labeled anti-mouse and 488-labeled anti-rabbit second antibody, respectively. Bars, 50 μm.
Caldecrin Inhibits Osteoclast Function

Ocets to perform bone resorption (9, 10, 30, 31). Mice deficient in c-Src, Syk, and PLCγ2 genes show impaired actin cytoskeletal organization in Ocets (12, 15, 34). In this study, RANKL stimulated the phosphorylation of c-Src, Syk, PLCγ1, and PLCγ2 (Fig. 5), and its inhibitors PP2, ER-27319, and U-73122 prevented RANKL-mediated actin ring formation of mature Ocets (Fig. 3), suggesting that tyrosine kinase activities of c-Src and Syk, and PLCγ activity are involved in the regulation of RANKL-stimulated actin ring formation. Zou et al. (15) reported that integrin occupancy activates c-Src, which in turn phosphorylates ITAM. Docking of the Src homology 2 domain of Syk to phosphorylated ITAM causes phosphorylation and activation of Syk by c-Src, leading to Vav activation, and promotes actin cytoskeletal organization in Ocets. The Syk substrate SLP-76 is also phosphorylated and recruits downstream molecules, including Vav, and PLCγ, and regulates the actin cytoskeleton (35, 36). We also demonstrated that SLP-76 in addition to c-Src and Syk was phosphorylated in response to RANKL (Fig. 5). Thus, the signaling pathway, in which integrin occupancy-mediated c-Src-Syk/ITAM complex formation activates the SLP-76-PLCγ or SLP-76-Vav axis, may be involved in RANKL-mediated actin ring organization of mature Ocets. Caldecrin inhibited RANKL-stimulated phosphorylation of these proteins (Fig. 5), suggesting that the signaling pathway leading to cytoskeletal organization was impaired by caldecrin.

On the other hand, Pyk2 is the main adhesion-induced kinase in Ocets. Mutation at the autophosphorylation site of Pyk2 (Y402F) or BAPTA-AM treatment abolishes Pyk2-c-Src association and subsequent phosphorylation and activation of Pyk2 (14, 37), suggesting that tyrosine 402 phosphorylation in the presence of intracellular Ca2+ is a prerequisite for the association with c-Src and OC spreading. The autophosphorylation of Pyk2 is stimulated by increases in intracellular Ca2+ concentration (37). We found that Pyk2 (Tyr-402) phosphorylation was increased by RANKL treatment, which was inhibited by caldecrin (Fig. 5). Furthermore, compounds inhibiting intracellular Ca2+ increase, such as BAPTA-AM and U-73122, caused actin ring disruption (Fig. 3), suggesting that Ca2+ also plays a crucial role in actin organization and that caldecrin may suppress Ca2+ signals.

The calcium dynamics changed during OC differentiation. TRPV4 and TRPV5 are involved in Ca2+ influx in mature Ocets, in the late stage of differentiation when Ca2+ oscillation disappears (17, 18). In this study, we observed that the RANKL-mediated Ca2+ spike was inhibited by the TRPV inhibitor RR, and the TRPV4-selective agonist 4α-PDD caused a Ca2+ spike as well as RANKL stimulation (Fig. 4). It has been reported that TRPV5 also mediates RANKL-induced Ca2+ influx. However, it down-regulates bone resorption and is active even in the presence of the PLCγ inhibitor U-73122 (18). Taken together, these findings suggested that RANKL-mediated Ca2+ influx probably occurs through TRPV4 in our mature Ocets. Our results, in which the RANKL-mediated Ca2+ spike was blocked by U-73122, ER-27319, and PP2, support the involvement of the c-Src-Syk-PLCγ pathway in the TRPV4-mediated Ca2+ spike in mature Ocets. We also demonstrated that caldecrin had no effect on 4α-PDD-induced TRPV4 activation even when caldecrin completely abolished RANKL-induced Ca2+ influx, suggesting that caldecrin may not inhibit TRPV4 channels directly but rather inhibits RANKL-mediated indirect TRPV4 activation through the RANKL-mediated Src pathway.

The signaling events downstream of integrin and Src in the Ocets have been investigated extensively. The c-Src-Pyk2-Cbl signaling complex links αvβ3 integrin signaling and promotes actin ring formation (37). PLCγ2 promotes β3 integrin-Src-Pyk2 association (38). c-Src and Syk bind directly to β3 integrin and form a complex with ITAM (15, 39). In both studies, c-Src kinase was shown to play a pivotal role in actin cytoskeletal organization (40). In this study, we demonstrated that c-Src constitutively associates with β3 integrin regardless of RANKL stimulation. RANKL treatment stimulates c-Src phosphorylation and kinase activation concomitantly and forms a ternary complex of integrin-Src-Syk (Fig. 7), which may facilitate cytoskeletal organization. Importantly, caldecrin inhibited c-Src kinase activity, c-Src-Syk association, and phosphorylation of Syk but not β3 integrin-c-Src association (Fig. 7). Integrin receives an inside-out signal in collaboration with M-CSF or outside-in signal by integrin ligands (41). In our attached culture conditions where the integrin ligand may be continually engaged with αvβ3 integrin, c-Src was activated by RANKL through interaction with TRAF6 under integrin-c-Src association.

TRAF, especially TRAF6, has been implicated in RANKL-mediated signaling in mature Ocets. TRAF6-deficient mice show severe osteopetrosis, indicating that TRAF6 is the main adaptor molecule involved in osteoclastogenesis (42). TRAF6 initiates recruitment to RANK on RANKL stimulation and increases the activation of downstream signaling pathways, such as NF-κB, MAPK (p38, ERK, and JNK), and Akt (43). In this study, RANKL stimulated the phosphorylation of ERK1/2, JNK, and Akt in mature Ocets, which was unaffected by caldecrin (Fig. 5). These results suggested that RANKL-RANK binding ability may not be affected by caldecrin. In addition, TRAF6 also associates with c-Src, which is essential for OC function (44). We demonstrated that TRAF6 was associated with c-Src upon RANKL exposure, and this association was blocked by caldecrin in mature Ocets (Fig. 7).

We propose a model (Fig. 9) in which RANKL activates c-Src upon integrin association through TRAF6, resulting in the phosphorylation of ITAM for recruitment of Syk and forming an integrin-c-Src-Syk complex. In turn, c-Src phosphorylates Syk. Activated Syk phosphorylates PLCγ via SLP-76, which leads to activation of TRPV4 channels and evokes Ca2+ influx and promotes actin cytoskeletal organization. Increased Ca2+ activates Pyk2 and associates with c-Src, leading to cytoskeletal organization. In this scenario, caldecrin may inhibit RANKL-mediated c-Src activation through TRAF6, which results in suppression of actin ring formation and bone resorption in mature Ocets. If caldecrin has an inhibitory effect on the same paradigm in both osteoclastogenesis and osteoclast function, signaling molecules downstream of RANK, including TRAF6 and Src family kinase, or molecules that affect TRAF6-c-Src complex formation may be targets of caldecrin. c-Src expressed in the late stage of differentiation is the only Src family kinase known to be functionally important for osteoclast function. Other Src family kinases may link TRAF6 with ITAM in oste-
Caldecrin Inhibits Osteoclast Function

function. Role of podosomes in regulation of bone-resorbing activity. Am. J. Physiol. 261, C1–C7
Caldecrin Inhibits Osteoclast Function

calcium-decreasing factor, caldecrin, ameliorates muscular dystrophy in dy/dy mice. In vivo 25, 157–163


