Ca$^{2+}$ influx through the osteoclastic plasma membrane ryanodine receptor

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IT HAS BECOME CLEAR OVER the last decade that Ca$^{2+}$ can function as an extracellular or “first” messenger (6). Ca$^{2+}$ receptors generally fall into a closely related family of G protein-coupled, seven-pass membrane receptors (7). Several genes for this receptor have been cloned and sequenced (7, 26, 27), and the list of cells sensitive to changes in ambient (extracellular) Ca$^{2+}$ concentration ([Ca$^{2+}$]) is expanding. This list includes renal tubular cells, neurons, gastric cells, enterocytes, osteoclasts, osteoblasts, and testicular cells. It is generally accepted that the bone-resorbing osteoclast monitors changes in its ambient [Ca$^{2+}$] through a Ca$^{2+}$ sensor (19, 40) that does not appear to be the traditional Ca$^{2+}$ receptor.

In the osteoclast, high extracellular Ca$^{2+}$ triggers a rise in cytosolic [Ca$^{2+}$] through both transmembrane Ca$^{2+}$ influx and intracellular Ca$^{2+}$ release (4, 31, 39). The elevated cytosolic [Ca$^{2+}$] results in cell-matrix detachment, margin retraction, reduced enzyme and acid secretion, and bone resorption inhibition (12, 19–21a, 40). These correlates are the basis of a negative-feedback mechanism through which an osteoclast monitors its own activity using resorbed Ca$^{2+}$ as an extracellular signal (39). Furthermore, the agonist effects of Ca$^{2+}$ are mimicked by several “surrogate” cations that follow a rank order of potency: Cd$^{2+}$ > Ni$^{2+}$ > Ca$^{2+}$ > Ba$^{2+}$ > Sr$^{2+}$ > Mg$^{2+}$ (29–31). Although we have assumed that these cations act at the osteoclastic surface, this has never been proven. It is critical to confirm this, as the cations may alternatively permeate the cell membrane and release stored Ca$^{2+}$. We hypothesize that these effects occur through interaction with intracellular ryanodine receptors (RyRs) and/or inositol 3,4,5-trisphosphate receptors (IP$_3$Rs).

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We have shown recently that a type 2 RyR isoform (RyR-2) resides in the osteoclastic plasma membrane and functions in extracellular Ca$^{2+}$ sensing (43). We previously observed intense, strictly peripheral immunostaining with a highly specific anti-RyR-2 antibody raised to a putatively extracellular epitope (43). Importantly, the antibody potentiated the Ca$^{2+}$ release triggered by Ni$^{2+}$, suggesting that Ni$^{2+}$ interacted with the surface RyR (43). Furthermore, several known RyR modulators, including ryanodine (41), ruthenium red (1), caffeine (33), and cADP-ribose (1), all attenuated cation-induced Ca$^{2+}$ release. Notably, the effect of ryanodine was modulated by membrane voltage, indicating that the triggering events for Ca$^{2+}$ release occurred at or near the plasma membrane (41). Furthermore, Ni$^{2+}$ displaced $[^{3}$H]ryanodine bound to osteoclasts, strengthening the link between cation sensing and RyR activation (43). These sets of biophysical evidence suggest that surface RyR-2 may be a functional component of the extracellular Ca$^{2+}$-sensing system.

Ordinarily located in endoplasmic reticular membranes, RyRs gate Ca$^{2+}$ release from intracellular stores into the cytosol (5). Our demonstration that RyR-2 is situated in the osteoclastic plasma membrane (43) represents the only known plasma membrane location for an RyR. In contrast, for IP$_3$Rs there is strong electrophysiological evidence for surface localization, whereas their function still remains unclear (22, 37). Recently, both RyRs and IP$_3$Rs have been assigned a role in extracellular Ca$^{2+}$ sensing (43). We have shown recently that a type 2 RyR isoform (RyR-2) resides in the osteoclastic plasma membrane (41). Furthermore, Ni$^{2+}$-sensitive, divalent cation-selective conductance in inside-out patches of the osteoclastic membrane is highly specific for the RyR-2 (3) should be expressed extracellularly in a plasma membrane configuration. Nevertheless, a role for the surface RyR-2 as a Ca$^{2+}$ sensor remains unclear.

Here, we have characterized the putative functional domains of the surface RyR-2 in osteoclasts. We first provide microfluorometric and electrophysiological evidence for Ca$^{2+}$-sensitive, divalent cation-selective conductance in inside-out osteoclastic membrane patches. Taken together, the results are compelling enough to assign a tentative role for the surface RyR-2 as a Ca$^{2+}$ influx channel. Second, we show that the divalent cations, such as Ni$^{2+}$ and Cd$^{2+}$, trigger cytosolic Ca$^{2+}$ release through interaction with a surface, rather than an intracellular, site. Finally, we find that intracellular application of the anti-RyR antibody highly specific for the cytosolic calmodulin (CaM)-binding RyR domain (38) attenuates Ni$^{2+}$-induced Ca$^{2+}$ release. This provides additional evidence that the surface RyR-2 is itself the cation sensor. Topologically, this makes sense because the known intraluminal, low-affinity Ca$^{2+}$-binding site of the surface RyR-2 (3) should be expressed extracellularly in a plasma membrane configuration.
Briefly, the cells were incubated in α-MEM and allowed to settle for 60 min on 0-grade, 22-mm glass coverslips (Fisher; see the next subsection). Contaminating cells were removed by gently rinsing the coverslips.

**Microspectrophotometric measurements of cytosolic Ca**$^{2+}$

For measuring the cytosolic [Ca$^{2+}$] in single rat osteoclasts, coverslips containing freshly isolated osteoclasts were incubated for 30 min at 37°C with 10 μM fura 2 acetoxymethyl ester (Molecular Probes, San Diego, CA) in serum-free medium. They were then washed in α-MEM and transferred to a Perspex bath positioned on the stage of a microspectrophotometer. The latter was previously constructed from an inverted microscope (Diaphot, Nikon, Telford, UK). The cells were exposed alternatively to excitation wavelengths of either 340 or 380 nm approximately every second. Instead of pipetting compounds into the experimental chamber, we used a constant-flow superfusion system that avoided experimental artifacts.

For recording of the emitted fluorescence, a single rat osteoclast was selected for each experiment by narrowing an optical lie to approximate the cell's boundary. The cell was then removed from the field, and a background count was obtained for 10 s from the same field. The average background fluorescence (counts/s) was then subtracted automatically from subsequent counts obtained from the selected cell. This emitted fluorescence was collected at 510 nm and directed to a photomultiplier tube (PM28B; Thorn EMI, London, UK) that converted the signal to 25-ns, 5-V transistor-transistor logic pulses. The pulses were counted by a dual photon counter (Newcastle Photometrics, Newcastle, UK). Photon counts were recorded every second on a computer to give the ratio of emitted intensities due to excitation at 340 and 380 nm ($F_{340}/F_{380}$).

Osteoclasts were bathed in one of the following solutions. The control solution was α-MEM ([Ca$^{2+}$] = 1.25 mM). A modified Ca$^{2+}$-free, EGTA-containing solution ([in mM] 130 NaCl, 5 KCl, 0.8 MgCl$_2$, 10 HEPES, 10 glucose, and 1.2 EGTA) was used in selected experiments. For experiments requiring cell membrane depolarization, we further modified the above solution to include 100 mM KCl (with or without CaCl$_2$ and EGTA).

The cytosolic Ca$^{2+}$-measuring system was calibrated using an established protocol for intracellular calibration (31). Briefly, fura 2-loaded osteoclasts were bathed in Ca$^{2+}$-free, EGTA-containing solution containing (in mM) 130 NaCl, 5 KCl, 5 glucose, 0.8 MgCl$_2$, 10 HEPES, and 0.1 EGTA. Ionomycin (5 μM) was first applied to obtain the minimum ratio due to the lowest cytosolic Ca$^{2+}$ ($R_{\text{min}}$) and the maximum fluorescence intensity at 380 nm ($F_{\text{max}}$). CaCl$_2$ (1 mM) was then applied with 5 μM ionomycin to obtain values of the maximum ratio due to an elevated cytosolic Ca$^{2+}$ ($R_{\text{max}}$) and the minimum fluorescence intensity at 380 nm ($F_{\text{min}}$). The dissociation constant ($K_d$) for Ca$^{2+}$ and fura 2 at 20°C, an ionic strength of 0.1 M, and a pH of 6.85 is 224 mM. The values were substituted into the equation $[\text{Ca}^{2+}] = K_d \times ([R - R_{\text{min}}]/(R_{\text{max}} - R)) \times (F_{\text{max}}/F_{\text{min}})$. For certain experiments, mean changes (Δ) in the peak cytosolic [Ca$^{2+}$] were calculated by subtracting the peak cytosolic Ca$^{2+}$ level from basal and are reported as mean changes ± SE. These values were compared by ANOVA and, in instances with Student's unpaired t-test, with Bonferroni's correction for inequality.

**Electrophysiological measurements.** Ionic currents were monitored in both rat and rabbit osteoclasts using the patch-clamp recording technique in cell-attached and inside-out excised configurations (16). Pipettes were filled with Baraspartate (Aasp; 110 mM Ba-Aasp and 10 mM HEPES), and the bath contained either Ba-Aasp or Na-Aasp (153 mM Na-Aasp, 0.01 mM Ca-Asp, and 10 mM HEPES/KOH). Cells were viewed on an inverted microscope (at ×600) equipped with Nomarski or contrast modulation optics (model IMT-2, Olympus, Tokyo, Japan). Patch pipettes were pulled from borosilicate capillary tubing (Drummond Scientific, Broomall, PA), coated with Sylgard (Dow-Corning, Midland, MI), and firepolished. To standardize patch geometry, only pipettes with resistances ranging from 4 to 12 MΩ were used. Current-voltage (I-V) relationships and voltage dependences were examined either during steady-state conditions or during the application of instantaneous voltage steps or voltage ramps (at 20°C).

Single-channel currents were monitored with an Axopatch 1-C (Axon Instruments, Foster City, CA) and stored on tape using a VHS video recorder and a modified digital audio processor (model PCM-701 ES, Sony, Tokyo, Japan). For kinetic analyses, analog signals were filtered with a six-pole, low-pass Bessel filter (Frequency Devices, Haverhill, MA) at a cutoff frequency of 0.1–1 Hz and digitized at a sampling rate of 5–2 kHz using PClamp (Axon Instruments). Amplitudes of single-channel currents were detected by automated analysis software (IPROC, Axon Instruments) or by visual inspection. $E_{\text{rev}}$ values were obtained by either interpolation or extrapolation of the I-V relationship using standard linear regression analysis (Plot-IT, Haslet, MI). Recorded lengths ranged typically from 0.5 to 3 min, depending on the number of events and our ability to maintain the patch. The membrane potential was defined as the negative of the pipette potential, with respect to the bath as the ground. Thus, in both cell-attached and inside-out configurations, a positive value for the membrane potential represented depolarization of the patch, whereas a negative value indicated hyperpolarization of the patch. In all results, inward current is plotted as a downward deflection.

**Antibody experiments.** We used the antibody Ab34 to examine the role of the CaM-binding site of the RyR in Ca$^{2+}$ sening in the osteoclast. The antibody was raised previously in rabbit against the consensus CaM-binding region of the three RyR isoforms. Thus the antibody does not discriminate between the receptor isoforms (43).

Osteoclasts from both rabbit and rat were gently made permeable with Triton X-100 (0.1% vol/vol, Sigma) for 30 s in PBS (10 mM). Triton X-100 was then removed, and the cells were immediately exposed to antibody Ab34 (1:100) in PBS. Sixty minutes after the application of PBS, cell impermeability was restored by replacing the PBS solution with medium 199 supplemented with 10% FCS. The cells were incubated at 37°C for ~5 h. To confirm the integrity and impermeability of the cells, they were loaded with fura 2 and monitored for cytosolic [Ca$^{2+}$] changes (due to influx across a [Ca$^{2+}$] gradient) and fura 2 efflux. For up to 2 h after the loading with fura 2, there was no significant change in cytosolic [Ca$^{2+}$], no dye quenching (monitored for excitation of 380 nm), and no apparent cell leakage uptake, confirming the integrity of the cells. Under these aforementioned permeability conditions, we have previously shown that Ab34 permeates the cell (as evident by immunocytochemical staining) (43). It is apparent that incubating the cells in physiological media with FCS restored cellular impermeance.

**RESULTS**

Ni$^{2+}$ is required for cytosolic Ca$^{2+}$ influx in response to depolarization. Figure 2 illustrates typical results from test maneuvers that investigated the effects of valinomycin given with Ni$^{2+}$ preceding elevations of extracellular [K$^+$] (Fig. 2, A–C), as well as control
procedures that investigated the effects of the component agents given by themselves (Fig. 2, D—F) (extracellular \([\text{Ca}^{2+}] = 1.25 \text{ mM}\)). Hyperpolarization of the osteoclastic membrane by valinomycin (5 \(\mu\text{M}\) in 5 mM K\(^+\)), accompanying or followed by the application of 50 \(\mu\text{M} \text{Ni}^{2+}\), resulted in a gradually increasing cytosolic \([\text{Ca}^{2+}]\) (\(\Delta = 436 \pm 125 \text{ nM}, n = 4\)) (Fig. 2A). This was qualitatively similar to and not significantly different (\(P = 0.288\)) from the response to valinomycin itself (\(\Delta = 418.5 \pm 89.8 \text{ nM}, n = 11\)) (Fig. 2E). Subsequent
elevation of extracellular [K+] to 100 mM in the presence of both valinomycin, which would be expected to result in cellular depolarization, and Ni2+ produced a sharp, sustainable rise in cytosolic [Ca2+] to ~1,500 nM (Fig. 2B). This sustained rise was reversible when extracellular [K+] was switched back to 5 mM [Fig. 2C, arrow (iv)]. However, both Ni2+ and valinomycin, as opposed to their separate application, were required if a subsequent addition of [K+] was to modify measured levels of cytosolic [Ca2+] (Fig. 2, D–G). In the absence of valinomycin, 100 mM [K+] produced a much smaller cytosolic [Ca2+] change (mean Δ = 76.2 nM), likely indicating that the cells were not depolarized (Fig. 2D; note the difference of scale between Fig. 2D and Fig. 2, B and C). Addition of 50 μM [Ni2+] alone in the absence of valinomycin provided only a small change in cytosolic Ca2+ (32) (Δ = 92 ± 17 nM) (Fig. 2F). In the absence of 50 μM [Ni2+], although the gradual valinomycin-induced cytosolic Ca2+ rise persisted, the K+-induced sharp rise was not sustained (Fig. 2G), in contrast to Fig. 2, B and C, where the K+-induced increase in [Ca2+] was sustained. This latter finding suggested that Ni2+, a known Ca2+-receptor agonist, is required for the sharp and sustained rise of cytosolic [Ca2+] in depolarized osteoclasts.

To differentiate whether the cytosolic [Ca2+] change arose from transmembrane Ca2+ influx or intracellular Ca2+ release, we performed the above experiments in a Ca2+-free, EGTA-containing solution. In this case, any inwardly directed Ca2+ gradient across a putative channel should be reduced so as not to allow Ca2+ entry. Figure 3B shows that the gradual change in cytosolic [Ca2+] induced by valinomycin ([K+] = 5 mM) alone, as well as the sharp rise induced by high extracellular [K+] (100 mM) shown in Fig. 3A, were both abolished. This suggested that the [Ca2+] change was dependent on extracellular Ca2+ influx. In a separate experiment, the sharp rise in cytosolic [Ca2+] induced by high [K+] in the presence of extracellular Ca2+ (Fig. 3C, iii) was intercepted by EGTA (Fig. 3C, iv); this triggered a slowly declining cytosolic [Ca2+], again confirming that extracellular Ca2+ influx is vital for the rise in cytosolic [Ca2+].

Depolarization-induced Ca2+ influx is ryanodine sensitive. The effect of ryanodine on the cytosolic [Ca2+] elevation in depolarized cells was evaluated microspectrophotometrically in Figs. 4 and 5. Such results were observed when osteoclasts, exposed to 5 μM valinomycin (i) followed by 50 μM [Ni2+] (ii), which led to prolonged elevation of cytosolic [Ca2+], were subjected to an addition of 100 mM [K+] together with 4 μM ryanodine (iii); this led to an abolition of the expected rise in cytosolic Ca2+ triggered by 100 mM [K+] (Fig. 4A). The latter was particularly clear in comparisons with Fig. 4B, which illustrates a similar experiment in which 50 μM [Ni2+] was added to valinomycin-pre-treated osteoclasts (i) to elicit the initial gradual elevation of cytosolic [Ca2+], and this was followed by addition of 100 mM-[K+] (ii) to produce a further sharp increase in cytosolic [Ca2+]. Thus the latter increase was also abated by addition of 4 μM [ryanodine] (iii).

The smaller changes in cytosolic [Ca2+] triggered by 100 mM [K+] without valinomycin were also blocked by ryanodine (4 μM) (Fig. 4C). These results suggest that the changes in cytosolic [Ca2+] were sensitive to ryanodine application.

We next examined the effect of ryanodine (4 μM) on cytosolic [Ca2+] in osteoclasts at resting membrane potential. Ryanodine itself caused both transmembrane Ca2+ influx and intracellular Ca2+ release in ~75% (11 of 15) of the cells studied. Each cytosolic Ca2+ transient was typically biphasic, i.e., a rapid increase to a peak, followed by a slow decline to a plateau (Fig. 5A). Only the transient rise in cytosolic Ca2+ was present in the Ca2+-free, EGTA-containing solution (Fig. 5B). Oscillatory responses occurred in ~40% of the cells. Taken together, the results show that, in addition to inhibiting Ca2+ influx, ryanodine itself can cause both transmembrane Ca2+ influx and
intracellular Ca$^{2+}$ release. Note that we have previously documented the inhibition of Ca$^{2+}$ release by a higher concentration of ryanodine (50 µM) in hyperpolarized osteoclasts (41).

**Electrophysiological evidence for an RyR-like cation conductance.** To confirm and further explore the characteristics of the detected Ca$^{2+}$ influx pathway, we performed electrophysiological studies on cell-attached and inside-out excised patches. Ba$^{2+}$ was used as a divalent carrier ion in the pipette, and the bath contained either Ba-Asp (110 mM) or Na-Asp (153 mM; see MATERIALS AND METHODS). The cells were held at voltages between 0 and −60 mV. A divalent cation-permeable channel was identified in 9 of 52 (18%) osteoclasts (Fig. 6). This suggests that either channel density was relatively low or that the channels were mostly inactive. The kinetic behavior of this channel was variable; it frequently displayed one or more substates (partially open states) during the course of recording. Note that at a membrane potential of −60 mV, a substate was observed. The latter feature is reminiscent of record-
the open probability \((P_o)\) values increased from 0.5 at −60 mV to −0.95 at −20 mV.

The \(I-V\) relationship of the channel is illustrated in Fig. 7. In symmetric solutions of Ba-Asp, slope conductance of the channel averaged \(63 \pm 15 \text{ pS} (\text{mean} \pm \text{SD}, n = 4)\), and the \(E_{\text{rev}}\) was close to 0 (2.3 \(\pm\) 1.7). That the channel was a divalent cation-selective, rather than a nonselective cation, channel was established by comparing recordings obtained in symmetrical solutions of Ba-Asp with recordings obtained in the presence of a Ba\(^{2+}\) gradient. Thus in separate recordings made with Ba-Asp in the pipette and Na-Asp in the bath, channel conductance increased to \(\sim 120 \text{ pS}\) and \(E_{\text{rev}}\) shifted to more positive values (21.6 mV).

Finally, we examined the effect of the cell-impermeant RyR modulator ruthenium red (250 \(\mu\)M) and a polyclonal antibody, Ab\(^{34}\) (1:200), raised to the cytosolic CaM-binding region of the RyR, on channel activity in inside-out patches in symmetric solutions of Ba-Asp, at 0 mV. Nonimmune rabbit serum (NIRS) was used as control for the latter experiments. In the absence of ruthenium red, the \(P_o\) of the channel was −0.95 (Fig. 8, control trace). After the addition of ruthenium red, channel activity diminished rapidly \((P_o < 0.01, n = 4)\) (Fig. 8). Ruthenium red has previously been shown to modulate osteoclastic RyRs (1) and skeletal muscle RyRs (13). Figure 8 shows that the \(P_o\) of the channel decreased from 0.86 to 0.07 after the application of Ab\(^{34}\). This, together with the observed ruthenium red sensitivity, clearly pointed to the existence of an intracellular regulatory site for the plasma membrane RyR (see inset for membrane topology).

The Ca\(^{2+}\) influx and single channel studies provide complementary evidence for a plasma membrane Ca\(^{2+}\) channel that displays features of the RyR in terms of its conductivity, ion selectivity, existence of substates, modulation by the classic modulators ryanodine and ruthenium red, and more specifically, modulation by the anti-RyR antibody Ab\(^{34}\). It is thus possible that the surface RyR-2 (43) represents a major pathway for Ca\(^{2+}\) influx in the osteoclast.

Evidence for a divalent cation-sensitive site on the osteoclastic surface. We have reported substantial biophysical data suggesting that the surface RyR-2 may function as the Ca\(^{2+}\) and possibly Ni\(^{2+}\) sensor (1, 31, 33, 41). To examine whether such divalent cation-sensing occurred on the extracellular osteoclastic surface rather than intracellularly, we performed protease protection assays using the proteolytic enzyme pronase. Osteoclasts were exposed to 5 mM [Ni\(^{2+}\)] or 50
μM [Cd^{2+}] after preincubation with either 10 or 25 mg/l pronase for intervals between 0 and 40 min. We used 5 mM [Ni^{2+}], rather than 50 μM [Ni^{2+}], because this was the maximally effective concentration and we expected gradual reduction in the cytosolic Ca^{2+} response to zero from a maximal response magnitude (31). Figures 9 and 10 show a time-dependent attenuation of the peak cytosolic response to the divalent cations applied, Ni^{2+} or Cd^{2+}, respectively, in pronase-pretreated cells. With 10 mg/l pronase (Fig. 9, A and C), maximal attenuation occurred after a 20-min preincubation, which was well before membrane damage set in, as evidenced by trypan blue uptake at ~28 min. With 25 mg/l pronase (Fig. 9, B and D), maximal attenuation occurred within 5 min, whereas trypan blue uptake was positive at 15 min. The results with Cd^{2+} were identical to those with Ni^{2+} with an ~80% attenuation occurring within 10 min of pronase application (Fig. 10). Table 1 gives the mean cytosolic [Ca^{2+}] changes for Fig. 10C, thus showing that the application of one divalent cation significantly attenuates subsequent responses to the other.

For each experiment, we scrupulously monitored for any pronase-induced membrane damage that would allow cation permeation into the cell. First, we monitored for any time-related drifts in cytosolic [Ca^{2+}]. In some instances, cytosolic Ca^{2+} became elevated, but only at advanced time periods (>20 min after pronase treatment). Second, at the conclusion of each experiment, we assessed the uptake of trypan blue (1 mM; molecular mass ~961 Da). Dye uptake became positive considerably after maximal attenuation of the Ca^{2+} signal. At the times of maximal attenuation of the cytosolic Ca^{2+} signal (i.e., 5 min with 25 mg/l pronase and at 20 min with 10 mg/l pronase), there was no change in basal cytosolic [Ca^{2+}]. The plasma membrane remained relatively impermeable to Ca^{2+} at the time when maximal attenuation of cytosolic [Ca^{2+}] in the presence of pronase was seen, strongly suggesting that the cell was also impermeable to the applied Ni^{2+}. Thus the effects of Ni^{2+} on other divalent cations such as Cd^{2+} are likely exerted extracellularly.

Next, we monitored for any leakage of fura 2 (molecular mass ~1 kDa) at excitation of 380 nm. No significant drift in count rate was seen over the experimental periods. Most importantly, fura 2 did not quench; quenching would be expected if Ni^{2+} or Cd^{2+} permeated the cell. Together, the results suggest that during the observed attenuation in cytosolic [Ca^{2+}], pronase did not cause significant membrane damage that would allow intracellular cation access. They also suggest that the events that trigger the release of intracellularly stored Ca^{2+} in response to divalent cation application occur at the osteoclastic surface, rather than intracellularly.

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Fig. 9. Effect of various concentrations of pronase at different incubation times (0–40 min) on cytosolic Ca^{2+} responses for either 10 (A) or 25 mg/l pronase (B). Solid horizontal bars, duration of 5 mM [Ni^{2+}] application. C and D: effect of 10 and 25 mg/l pronase, respectively, on the change (Δ) in cytosolic [Ca^{2+}] (peak – basal) elicited in response to 5 mM [Ni^{2+}]. Trypan blue uptake was assessed for each experiment and was evident at 28 min for 10 mg/l and at 15 min for 25 mg/l pronase (open horizontal bars). *P < 0.05 and **P < 0.01 compared with time 0 (ANOVA).
Regulation of Ca\(^{2+}\) sensing through the cytosolic CaM-binding domain of the RyR. To test the hypothesis that a single surface-resident RyR-2 subserves the dual roles of Ca\(^{2+}\) sensor and Ca\(^{2+}\) influx channel, experiments were performed with Ab\(^{34}\), an inhibitory antibody to RyR’s cytosolically located CaM-binding sequence (43). The cytosolic introduction of Ab\(^{34}\) by gentle permeabilization (42) markedly attenuated the Ca\(^{2+}\) signals elicited by 5 mM [Ni\(^{2+}\)] (Fig. 11) (see MATERIALS AND METHODS). That this action was intracellular was examined by comparing this response to that triggered by extracellular Ab\(^{34}\) application. No attenuation of the Ca\(^{2+}\) signal was observed with extracellular Ab\(^{34}\) application, consistent with the cytosolic location of the CaM-binding site. It is also noteworthy that the antibody has been shown previously to stain permeabilized osteoclasts but not cells whose plasma membranes are intact (43). These results provide evidence for an intracellular CaM regulatory site on the RyR that modulates divalent cation sensing.

**DISCUSSION**

We have demonstrated previously that a type 2 RyR isoform positioned uniquely at the osteoclastic surface is involved in extracellular Ca\(^{2+}\) sensing (43). We now extend these studies to probe into its topology and functionality. We believe that the surface RyR-2 likely serves as a channel for the transcellular flux of Ca\(^{2+}\) during bone resorption. It is well known that Ca\(^{2+}\) concentrations in the resorptive hemivacuole rise to

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Conditioning [Ca(^{2+}), nM]</th>
<th>Test [Ca(^{2+}), nM]</th>
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</thead>
<tbody>
<tr>
<td>Cd(^{2+}) then Ni(^{2+})</td>
<td>252 ± 33.0</td>
<td>6.0 ± 2.5</td>
</tr>
<tr>
<td>Ni(^{2+}) then Cd(^{2+})</td>
<td>238 ± 15.8</td>
<td>28.6 ± 10.7</td>
</tr>
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Values are means ± SE. The mean change in (Δ) cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) is the difference between peak and pretreatment cytosolic [Ca\(^{2+}\)] (±SE). Comparisons were made between mean Δcytosolic [Ca\(^{2+}\)] levels in response to Cd\(^{2+}\) or Ni\(^{2+}\), when these were conditioning and test applications, respectively. *P < 0.05 in both cases (Student’s paired t-test).

![Fig. 10](image-url) Fig. 10. Effect of 25 mg/l pronase at different incubation times (0–20 min) on cytosolic [Ca\(^{2+}\)] responses (A) and on Δcytosolic [Ca\(^{2+}\)] (peak − basal; B) elicited in response to the extracellular application of 50 μM [Cd\(^{2+}\)]. *P < 0.01 compared with time 0 (ANOVA). C: effect of preconditioning osteoclasts with either 50 μM [Cd\(^{2+}\)] (open horizontal bars) or 5 mM [Ni\(^{2+}\)] (solid horizontal bars) on the cytosolic [Ca\(^{2+}\)] response to a subsequent application of the other cation (see Table 1).

![Fig. 11](image-url) Fig. 11. Effect of intracellular introduction, by gentle Triton X-100 permeabilization, of the anti-RyR antibody Ab\(^{34}\) on cytosolic Ca\(^{2+}\) levels in response to extracellular [Ni\(^{2+}\)] (5 mM). Control experiments involved incubating nonpermeabilized osteoclasts with Ab\(^{34}\) (extracellular) or permeabilized cells with NIRS (intracellular).
between 8 and 40 mM (34). This triggers an increase in cytosolic [Ca\(^{2+}\)] followed by margin and podosomal retraction (12, 19). The latter should itself serve as a critical mechanism for the outward diffusion of the increasing ambient [Ca\(^{2+}\)]. Nevertheless, it is conceivable that the surface RyR-2 also allows considerable Ca\(^{2+}\) influx. The rising cytosolic Ca\(^{2+}\) is then likely pumped out of the cell through the action of a Ca\(^{2+}\)-ATPase and a Na\(^+\)/Ca\(^{2+}\) exchanger that we have recently described (21).

This study provides the first evidence that the surface RyR acts as a divalent cation sensor and permits extracellular Ca\(^{2+}\) influx. Notably, an ~60-pS, divalent cation-selective, channel was observed that we tentatively identified as the RyR on the basis of two key considerations: the existence of metastable states (13) and its complete blockade by the RyR modulator ruthenium red and by Ab\(^{34}\), an antibody raised to the CaM-binding region of the RyR family of receptors (34). The use of ruthenium red and Ab\(^{34}\) for these experiments using inside-out patches has also allowed us to confirm the hypothesized topology of the surface RyR-2 (43). Because of its highly charged nature, ruthenium red cannot permeate cell membranes. Therefore, it should not activate RyR domains that face the interior of the patch pipette (Fig. 8, inset). The known location of the ruthenium red-binding site is on the cytosolic portion of the RyR. As noted above, Ab\(^{34}\) binds the CaM-binding region located cytosically in intact cells. Thus the blockade of Ba\(^{2+}\) conductance in inside-out patches, i.e., those with their cytosolic surface facing away from the pipette interior, would imply that the respective sites remain cytosolic in the surface membrane RyR-2. Information on topology in electrophysiological studies could not have been obtained using ryanodine as it permeates the plasma membrane freely and can cause contradictory effects on the channel \(P_0\) depending on its concentration.

In microspectrofluorometric experiments, application of ryanodine blocked Ca\(^{2+}\) influx in osteoclasts at concentrations similar to those that block cation permeation through skeletal muscle RyRs reconstituted in lipid bilayers (36). Alternatively, under certain conditions, such as a low concentration, ryanodine can activate skeletal muscle RyRs to cause Ca\(^{2+}\) release (36). In the osteoclast, it activates the RyR-2 to cause both Ca\(^{2+}\) release and Ca\(^{2+}\) influx. The induction of Ca\(^{2+}\) release by ryanodine was noted at resting membrane potentials of around −25 mV (Fig. 5), whereas the blockage of Ca\(^{2+}\) influx was noted in depolarized cells (Fig. 4). Indeed, voltage sensitivity of RyRs has also been documented in lipid bilayer studies (23), as well as by us in the osteoclast (40).

The present demonstration of both excitatory and inhibitory effects of ryanodine on cytosolic [Ca\(^{2+}\)] may be explained in terms of recent reports on the modulation of RyR conformation by changes in membrane potential (36a). Membrane potential, by altering the affinity of RyR for ryanodine, permits two different binding states that have been associated with the inhibitory and stimulatory effects of ryanodine (36a). New reports (19a, 36a) indicate that ryanodine does not lock the RyR into a ligand-insensitive open state. In agreement with the above findings, we show that ryanodine has dual actions depending on membrane potential and that the ryanodine-exposed RyR is modifiable by ligands such as Ni\(^{2+}\).

What might cause the surface RyR to become activated physiologically is not clear. Several possibilities exist (Fig. 1). Resorbed Ca\(^{2+}\) may itself activate RyR through its low-affinity Ca\(^{2+}\)-binding site (Ref. 3; see immediately below and Fig. 1, scheme 1). Alternatively, Ca\(^{2+}\) may first activate a hypothetical, possibly G protein-coupled, Ca\(^{2+}\) sensing receptor (18), resulting in IP\(_3\) formation and Ca\(^{2+}\) release (Fig. 1, scheme 2). The elevated cytosolic [Ca\(^{2+}\)] may then activate the surface RyR-2 via an action on its high-affinity cytosolic Ca\(^{2+}\)-binding site (3). Additionally, capacitative Ca\(^{2+}\) influx may ensue either because of intracellular Ca\(^{2+}\) store depletion (42) or due to conformational coupling between the surface and intracellular RyRs (17, 22).

We have previously shown that, in addition to Ca\(^{2+}\) influx, cation-induced Ca\(^{2+}\) release is inhibited by several RyR modulators, including ryanodine (41), caffeine (33), and ruthenium red (1). It has remained unclear whether these modulators interfere with the surface or intracellular actions of the applied cations. To rule out the possibility that divalent cations, such as Ni\(^{2+}\) and Cd\(^{2+}\), to which the plasma membrane is generally considered impermeant (8), could permeate and trigger Ca\(^{2+}\) release by interacting with intracellular RyRs and IP\(_3\)Rs, we conducted a protease protection assay. The proteolytic enzyme prorase, when applied at low concentrations, is known to cleave most cell surface proteins. Short of permeabilizing the cell membrane, prorase would be expected to abolish divalent cation sensing through any surface protein, including the surface RyR-2. We found that prorase abolished Ni\(^{2+}\)- and Cd\(^{2+}\)-induced Ca\(^{2+}\) release in a concentration- and time-dependent manner with membrane impermeability intact. This indicates that Ni\(^{2+}\) and Cd\(^{2+}\) act at the cell’s surface, rather than intracellularly.

That the plasma membrane remained impermeant after prorase treatment even to small cations and molecules was confirmed in four ways. Most importantly, there were no significant shifts in basal cytosolic [Ca\(^{2+}\)], confirming that the cell membrane remained impermeant even to a small divalent cation, Ca\(^{2+}\). Second, any leakage of the Ca\(^{2+}\)-sensitive fluorochrome fura 2 (molecular mass ~1 kDa) from the cell was assessed by continuous monitoring of background fluorescence at \(\lambda_{ex}\) of 380 nm. Fluorescence remained constant over the course of the experiment, virtually excluding dye extrusion and again confirming an intact plasma membrane. Third, addition of Ni\(^{2+}\) or Cd\(^{2+}\) did not result in a precipitous reduction in background fluorescence at \(\lambda_{ex}\) of 380 nm. Such a reduction would be expected if either cation permeated the cell membrane and quenched fura 2. Fourth, trypan blue (molecular mass ~961 Da) uptake remained negative up to 28 min after incubation with 10 mg/l prorase and up to
15 min after incubation with 25 mg/l pronase. Together, these results strongly suggest that the cations Ni$^{2+}$, Cd$^{2+}$, and possibly Ca$^{2+}$ interact with a cell-surface moiety, likely RyR-2, to release intracellularly stored Ca$^{2+}$.

We could not use Ca$^{2+}$ in these studies to activate the Ca$^{2+}$ sensor because we would then be measuring a combination of events, namely, receptor activation, Ca$^{2+}$ influx, and Ca$^{2+}$ release. With Ni$^{2+}$ and Cd$^{2+}$ as surrogate cations, it becomes possible to separate these components. Note, however, that the Ni$^{2+}$ concentrations between the two sets of experiments to demonstrate Ca$^{2+}$ release and Ca$^{2+}$ influx are different. We have shown extensively in previous studies (31, 39–43) that 50 μM Ni$^{2+}$ triggers the Ca$^{2+}$ sensor to cause Ca$^{2+}$ release without blocking the channel. Therefore, in Figs. 2–4, we simply aim at triggering the RyR/Ca$^{2+}$ sensor while allowing the Ca$^{2+}$ influx under study to continue. In Figs. 9 and 10, however, we are using Ni$^{2+}$ at 5 mM as this is the concentration that elicits Ca$^{2+}$ release from intracellular stores and blocks Ca$^{2+}$ influx. It is the ideal concentration for selectively examining Ca$^{2+}$ release, as opposed to a combination of Ca$^{2+}$ release and Ca$^{2+}$ influx that would otherwise be difficult to separate. The concept of using surrogate divalent cations is not new. They have been elegantly used in early studies with parathyroid cells (see Ref. 39 for a review).

We used Cd$^{2+}$ to replicate and substantiate the observed effects of Ni$^{2+}$ in the pronase protection assay. In this assay, we examined Ca$^{2+}$ release alone rather than the combination of Ca$^{2+}$ release and Ca$^{2+}$ influx because Ni$^{2+}$ and Cd$^{2+}$ both block RyRs (36). Both Cd$^{2+}$ and Ni$^{2+}$ are also surrogate agonists of the osteoclastic Ca$^{2+}$ sensor/RyR (30, 39). Thus data presented in Table 1 show that Cd$^{2+}$ and Ni$^{2+}$ cross-react to desensitize each other’s effects. Cd$^{2+}$ also replicates the effect of Ni$^{2+}$ in the pronase protection assay (Fig. 10). Furthermore, Cd$^{2+}$ has a greater ionic radius and is used at a 100-fold lower concentration than Ni$^{2+}$. Hence, Cd$^{2+}$ is less likely than Ni$^{2+}$ to permeate the cell membrane. Together, the results strengthen our hypothesis for a surface action of both cations.

Finally, intracellular introduction of Ab$^{34}$ by gentle cell permeabilization (38) potently inhibited Ni$^{2+}$-induced Ca$^{2+}$ release. Expectedly, extracellular application of the antibody failed to attenuate Ca$^{2+}$ release. This was consistent with the blockade of Ba$^{2+}$ currents by the antibody in inside-out patches of osteoclastic plasma membranes, particularly as the antibody-binding site is expected to be cytosolic rather than extracellular. Taken together, these observations also suggest that the same epitope of the plasma membrane receptor was involved in modulating cytosolic Ca$^{2+}$ release and Ca$^{2+}$ influx. This antibody has previously been shown to stain permeabilized but not intact osteoclasts, suggesting a cytosolic action site (43). Whether the antibody also blocks the cytosolic CaM-binding site of organelle RyRs, however, remains to be determined. Nevertheless, these studies provide further and more compelling evidence that RyRs, in particular the surface RyR-2, play a role in Ca$^{2+}$ sensing and Ca$^{2+}$ influx in the osteoclast.

We thus demonstrate that the surface RyR-2 serves as both a Ca$^{2+}$ influx channel and a divalent cation sensor. It would seem that the topology of the surface RyR-2, predicted from our previous studies (43) and ruthenium red data, would suit its proposed dual role. Notably, portions of a molecule that face the cytosol in its endoplasmic reticular configuration should remain cytosolic in its plasma membrane configuration. In the case of RyR, these cytosolic domains would include its high-affinity Ca$^{2+}$-activation and CaM-responsive sites (38). Parts of the RyR that are normally intraluminal (3) should become extracellular. It is noteworthy that the cardiac muscle RyR-2 isoform possesses an intraluminal site that has a low millimolar affinity for Ca$^{2+}$ (3). At least conceptually, this low-affinity divalent cation-binding site could represent the sensor for Ca$^{2+}$ (Fig. 1, scheme 1). However, we cannot rule out the possibility of a separate Ca$^{2+}$ sensor, one linked, for example, to the G protein-phospholipase C-IP$_3$ pathway (Ref. 6; Fig. 1, scheme 2). Nevertheless, neither this sensor nor a similar molecule has been isolated from or identified in the osteoclast. Furthermore, the micromolar affinity of such a Ca$^{2+}$ receptor is not consistent with the sensing of the millimolar ambient Ca$^{2+}$ levels generated from hydroxyapatite dissolution. There are other possibilities. For example, a variant of the low-density lipoprotein receptor/Ca$^{2+}$ sensor previously isolated from the cytotoxoblast (39) may play a role, although we find no evidence of this in preliminary studies. Alternatively, a new Ca$^{2+}$-gated Ca$^{2+}$ channel, polycystin-L, has been identified in the intestine (10), and a similar molecule may exist in the osteoclast. These interesting possibilities require further investigation.

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REFERENCES

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