Calcium spikes in activated macrophages during Fcγ receptor-mediated phagocytosis

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Abstract: Rises in intracellular-free calcium ($[Ca^{2+}]_i$) have been variously associated with Fcγ receptor (FcR)-mediated phagocytosis in macrophages. We show here that activation of murine bone marrow-derived macrophages increases calcium spiking after FcR ligation. Ratiometric fluorescence microscopy was used to measure $[Ca^{2+}]_i$ during phagocytosis of immunoglobulin G (IgG)-opsonized erythrocytes. Whereas 13% of nonactivated macrophages increased $[Ca^{2+}]_i$ in the form of one or more spikes, 56% of those activated with lipopolysaccharides (LPS; 18 h at 100 ng/ml) and interferon-γ (IFN-γ; 100 U/ml) and 73% of macrophages activated with LPS, IFN-γ, interleukin (IL)-6 (5 ng/ml), and anti-IL-10 IgG (5 μg/ml) spiked calcium during phagocytosis. Calcium spikes were inhibited by thapsigargin (Tg), indicating that they originated from endoplasmic reticulum. The fact that activated macrophages showed a more dramatic response suggested that calcium spikes during phagocytosis mediate or regulate biochemical mechanisms for microbicidal activities. However, lowering $[Ca^{2+}]_i$ with ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid or inhibiting calcium spikes with Tg did not inhibit phagosome-lysosome fusion or the generation of reactive oxygen or nitrogen species. Thus, the increased calcium spiking in activated macrophages was not directly associated with the mechanism of phagocytosis or the increased antimicrobial activities of activated macrophages. *J. Leukoc. Biol.* 72: 677–684; 2002.

Key Words: *phagosome-lysosome fusion · ROS · RNS · activation*

INTRODUCTION

Calcium is a commonly used intracellular signaling molecule that participates in a wide range of cellular activities [1], including Fcγ receptor (FcR)-mediated phagocytosis in macrophages. Although intracellular-free calcium ($[Ca^{2+}]_i$) sometimes increases during FcR-mediated phagocytosis by macrophages [2–4] and is necessary for optimal particle internalization in neutrophils [5–8], macrophages can engulf immunoglobulin G (IgG)-opsonized particles after $[Ca^{2+}]_i$ has been chelated to very low levels [3]. Thus, the role that calcium plays during FcR-mediated phagocytosis in macrophages remains ambiguous.

One potential function for calcium during phagocytosis is the regulation of macrophage microbicidal activities. After a macrophage has ingested a microorganism such as a bacterium, the phagosome traffics to the lysosomes. The pH in the phagosome drops, and the macrophage produces reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can kill microbes or inhibit their activities. Generation of ROS and RNS is greatly enhanced by activation, an inducible differentiation of macrophages originally described by Mackaness [9]. Macrophage activation is primarily mediated by interferon-γ (IFN-γ) but can be modulated by other factors. Lipopolysaccharide (LPS) and cytokines such as interleukin (IL)-6 can enhance activation when combined with IFN-γ [10–12], whereas IL-10 down-modulates activation. If calcium has a role in microbicidal activity, one might expect to see differences in the calcium responsiveness of activated macrophages.

We hypothesized that rises in $[Ca^{2+}]_i$ during FcR-mediated phagocytosis aid in signaling of downstream microbicidal functions. Monitoring $[Ca^{2+}]_i$ during phagocytosis in activated macrophages, we found that activated macrophages increased $[Ca^{2+}]_i$ more readily during phagocytosis than did nonactivated macrophages and that the degree of calcium responsiveness correlated with the degree of activation. Calcium was not necessary for the oxidative burst, and although macrophages did require a basal amount of calcium to generate RNS, rises in calcium were not necessary for generation of nitric oxide (NO). Finally, phagosome-lysosome fusion occurred normally in low calcium. Thus, calcium spikes do not appear to aid in the immediate microbicidal response, although they may act over a longer period of time by controlling gene expression.

MATERIALS AND METHODS

Cells

Bone marrow-derived macrophages were obtained from femurs and tibias of C56BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and cultured for 5–8 days as previously described [13]. Cells were harvested and replated onto

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25-mm diameter circular coverslips or 12-mm coverslips and were cultured overnight in 6- or 24-well plates containing Dulbecco's modified Eagle's medium, 10% heat-inactivated goat serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (DME-10F; Gibco-BRL, Gaithersburg, MD). Macrophages were activated by overnight incubation with 100 U/ml recombinant IFN-γ (Genzyme, Cambridge, MA), 100 ng/ml LPS (List Biological, Campbell, CA), and 5 μg/ml neutralizing antibody against IL-10 (α-IL-10; R&D Systems, Minneapolis, MN). IL-6 (5 ng/ml; Calbiochem, San Diego, CA) and α-IL-10 were then included in all solutions during the experiment (I, L, α10, 6 activation). In some cases, macrophages were activated with only IFN-γ and LPS (I, L activation). Sheep erythrocytes (Reproductive Sciences Program, University of Michigan, Ann Arbor) were opsonized with anti-sheep erythrocyte IgG (ICN Biomedical, Aurora, OH) as previously described [14].

Calcium imaging

Macrophages plated on 25-mm circular No. 1 coverslips (2×10^5/coverslip) were incubated for 30 min at 37°C in Ringer's buffer (RB; 155 mM NaCl, 5mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM Hepes, and 10 mM glucose, pH 7.2) with 1 μM fura-PE3/AM (TEF Labs, Austin, TX) or 1 μM FFP-18/AM (TEF Labs) and 0.1% Pluronic F-127. Cells were then washed with RB for 10 min, and the coverslips were mounted in a Leiden chamber (Harvard Apparatus, Cambridge, MA) with 1 ml RB. The chamber was then placed on the heated stage of an inverted microscope (TE-300; Nikon, Tokyo, Japan) equipped with a cooled CCD camera (Quantix; Photometrics, Tuscon, AZ) and a 40× phase-contrast, UV objective (N.A. 0.9). Metamorph software (Universal Imaging, West Chester, PA) was used to control a shutter (Uniblitz, Rochester, NY) and filter wheel (Lambda 10-2; Sutter Instruments, Novato, CA), allowing sequential imaging of phase-contrast and 510-nm fluorescence emission excited at 340 nm and 380 nm (called 340 and 380 images). Background subtraction was performed by acquiring an image with all shutters closed to compensate for dark-current artifacts. This image was subtracted from the 340 and 380 images and provided uniform, background intensity. In addition, the average pixel intensity of cell-free regions from the 340 and 380 images was measured, and this number was subtracted from the 340 and 380 images.

For time-lapse imaging of phagocytosis, IgG-opsonized erythrocytes (~5/macrophage) were added to the chamber; then, phase-contrast, 340-nm excitation and 380-nm excitation images were taken every 10 or 15 s for 10–20 min. For experiments in which $[Ca^{2+}]_i$ was reduced to low levels, macrophages were incubated in Ca^{2+}-free RB + 2 mM ethyleneglycol-bis(β-aminoethyl-ether)-*N,N′*-tetraacetic acid (EGTA-RB) for 60–75 min prior to the experiment. Images were calibrated according to Grynkiewicz et al. [15]. R_{min} was obtained after incubating macrophages for 5 min in EGTA-RB + 10 μM ionomycin (Calbiochem). R_{max} was then determined by replacing the R_{min} buffer with RB (2 mM Ca^{2+}) + 10 μM ionomycin and acquiring 340 and 380 images every minute for 10 min.

For experiments to determine the sources of calcium, an image intensifier (VSH-1845, Videoscope Intl., Dulles, VA) was used to limit light exposure of the cells. IgG-opsonized erythrocytes [IgG-red blood cells (RBC)] were added to macrophages; then, 340 and 380 images were acquired every 10 s for 10 min. After 4.5 min, RB was replaced with RB, RB + 1 μM thapsigargin (Tg; Molecular Probes, Eugene, OR), EGTA-RB, or RB + 10 mM NH_4Cl.

Phagocytosis assay

Nonactivated or I-, L-, α10-, 6-activated macrophages on 13-mm circular No.1 coverslips (0.75×10^5/coverslip) were incubated at 37°C for 60 min in EGTA-RB prior to IgG-RBC addition. IgG-RBC were then added (~3×10^7/well), and macrophages were allowed to phagocytose for 5 min at 37°C. Coverslips were then washed 3× with EGTA-RB, and half the coverslips were then dipped in water for 30 s to lyse all erythrocytes, except those internalized by macrophages. Coverslips were scored by counting the number of erythrocytes per 50 macrophages.

Measurement of ROS

Macrophages plated onto 25-mm coverslips (2×10^5/coverslip) were mounted in an inverted microscope as described above. Macrophages were I-, L-, α10-, 6-activated. 2′,7′-Dichlorofluorescein (H_2DCF)-bovine serum albumin (BSA)-IgG (10 μL of 250 μg/ml; FcOxyBurst, Molecular Probes) was added directly to the coverslip. Phase-contrast and 485-nm excitation images were then acquired every minute for 20–30 min. Fluorescence was measured by drawing a region around the H_2DCF-BSA-IgG particle in the 485 ex. image and subtracting the signal from a similar size region next to the H_2DCF-BSA-IgG particle.

Nitrite measurement

Nitrite levels were measured using the Griess reaction [16]. Macrophages were plated on a 96-well plate (1.5×10^5/well) and activated with I, L, α10, 6, as described above. Some wells were then washed and incubated for 70 min in EGTA-RB to reduce $[Ca^{2+}]_i$ or 45 min in RB + 1 μM Tg to deplete endoplasmic reticulum (ER) calcium stores. Solutions were then replaced with 100 μL fresh solution + stimulus and were incubated for 1 h at 37°C. Griess reagent (100 μL; 0.5% sulfanilamide, 0.05% napthylethylenediamine-HCl in 1.25% H_3PO_4) was added to each well, and Abs_{550} was measured on a microplate reader (Molecular Devices, Sunnyvale, CA) after 5 min. Nitrite concentration was determined using a standard curve prepared with $NaNO_2$. All tests were done in triplicate; RB was supplemented with 398 μM L-arginine.

Phagosome-lysosome fusion

To label lysosomes and late endosomes, macrophages on 13-mm coverslips were incubated for 30 min at 37°C with Texas Red-Dextran (TR-Dx) conjugate (molecular weight 10,000; 0.5 mg/ml in DME-10F; Molecular Probes) and then were washed 2× with RB and incubated for 60 min in DME-10F. Macrophages were then incubated with EGTA-RB for 70 min, 1 μM Tg for 45 min, or RB. IgG-RBC (~5 RBC/macrophage) were added and spun down onto the coverslips at 4°C (1 min, 60 G). Coverslips were washed 3× with cold RB and then placed at 37°C for 10 min before fixing in 40 mM HEPES, 400 mM sucrose, and 4% paraformaldehyde for 60 min. Lysosomal fusion was then scored on a Zeiss Axioplan II microscope (Carl Zeiss, Thornwood, NY) by determining the percent of erythrocytes completely surrounded by rings of TR.

RESULTS

Measurement of $[Ca^{2+}]_i$

Ratiometric imaging of the fura-based probes, fura-PE3 and FFP-18, was used to measure $[Ca^{2+}]_i$ in individual macrophages during phagocytosis. These dyes have the same excitation and emission profile as fura-2 but have beneficial modifications. Unlike fura-2, fura-PE3 has been modified so that it is not as quickly pumped out of the cell or into intracellular compartments by organic anion transporters [17, 18]. FFP-18 has a hydrocarbon tail that lets it associate with membranes [17]. This gives better spatial resolution of calcium changes when looking at subcellular processes and also prevents leakage of the probe. Cells loaded with either dye could be observed for up to 1 h without any noticeable decrease in fluorescence intensity or probe redistribution. To verify that we could detect changes in $[Ca^{2+}]_i$, we measured the response to platelet activating factor (PAF) in fura-PE3-loaded macrophages. Addition of PAF stimulated an immediate rise in $[Ca^{2+}]_i$, followed by a sustained elevation of $[Ca^{2+}]_i$, which lasted for the duration of the experiment (2 min; data not shown).

Calcium transients accompany phagocytosis by activated macrophages

To monitor $[Ca^{2+}]_i$ changes during phagocytosis, IgG-RBC were added to variously activated probe-labeled macrophages, and $[Ca^{2+}]_i$ levels were recorded in individual macrophages for

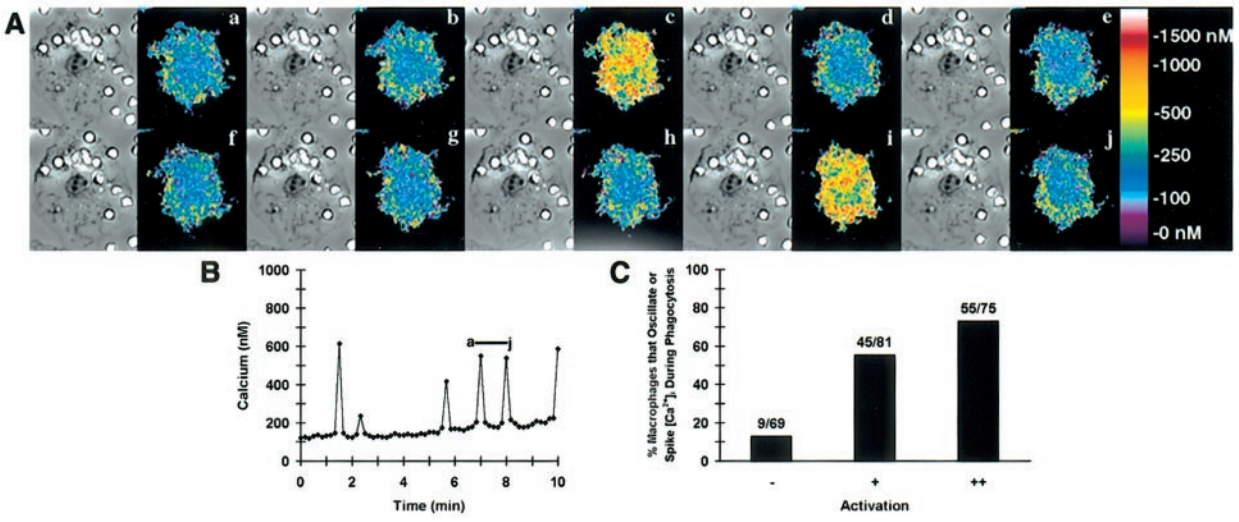

Fig. 1. Increased calcium responses during phagocytosis by activated macrophages. IgG-RBC were added to macrophages, and $[Ca^{2+}]_i$ was monitored for 10 min. (A) Phase-contrast and $[Ca^{2+}]_i$ images in a representative, activated macrophage during phagocytosis. (B) The corresponding $[Ca^{2+}]_i$ trace. [The region of the trace represented in panels a–j of (A) is indicated.] (C) Activated macrophages exhibit more $[Ca^{2+}]_i$ spikes. $[Ca^{2+}]_i$ was monitored during phagocytosis, and the percentage of macrophages phagocytosing IgG-RBC that oscillated or spiked $[Ca^{2+}]_i$ was recorded. Macrophages were not activated (−), activated with 100 ng/ml LPS and 100 U/ml IFN-γ (+), or activated with LPS, IFN-γ, 5 μg/ml anti-IL-10, and 5 ng/ml IL-6 (++).

10–20 min during the course of phagocytosis. Some macrophages showed no change of $[Ca^{2+}]_i$ during phagocytosis, and others increased $[Ca^{2+}]_i$ in the form of one or more transient rises or spikes (**Fig. 1, A** and **B**). Nonactivated macrophages generally did not increase $[Ca^{2+}]_i$ during FcR-mediated phagocytosis: Only 9 of 69 observed macrophages that bound IgG-RBC spiked $[Ca^{2+}]_i$ (Fig. 1C). When macrophages activated with IFN-γ and LPS were used, the $[Ca^{2+}]_i$ response was more pronounced. Over half of these macrophages increased $[Ca^{2+}]_i$ during phagocytosis of IgG-RBC (Fig. 1C). The experiment was then repeated using macrophages activated with a four-component cocktail consisting of IFN-γ, LPS, α-IL-10, and IL-6 (I, L, α10, 6 activation). Over 70% of these macrophages showed concomitant increases in $[Ca^{2+}]_i$ upon binding IgG-RBC (Fig. 1C).

To examine the timing and duration of responses to a single phagocytic event, $[Ca^{2+}]_i$ was measured in activated macrophages during phagocytosis of individual erythrocytes (**Fig. 2**). Of 71 phagocytic events observed, 26 (37%) were accompanied by $[Ca^{2+}]_i$ spikes. The initial rise in $[Ca^{2+}]_i$ generally occurred within 1 min of the particle landing on the macrophage, prior to or during engulfment of the particle. Erythrocyte binding appeared to be sufficient for initiation of the calcium response, as $[Ca^{2+}]_i$ spikes were commonly observed immediately after opsonized erythrocytes landed on macrophages. The number of $[Ca^{2+}]_i$ spikes generated by a single IgG-RBC was variable. Most phagocytic events that generated a $[Ca^{2+}]_i$ rise produced one or two $[Ca^{2+}]_i$ spikes. Occasionally, phagocytosis of a single IgG-RBC led to repeated spikes or $[Ca^{2+}]_i$ oscillations that lasted for 6 min—the duration of the observation. These spikes were separated by intervals of 30–60 s.

Calcium is not required for particle internalization

To test the requirement of calcium for various processes, it was necessary to inhibit calcium rises during phagocytosis. This was accomplished by prolonged incubation of macrophages in EGTA-RB. After 70 min in EGTA-RB, average resting $[Ca^{2+}]_i$ was reduced from 209 nM to 14 nM (**Fig. 3A**). Depleting $[Ca^{2+}]_i$ in this manner prevented calcium signaling during phagocytosis; 0/38 Ca^{2+}-depleted macrophages increased $[Ca^{2+}]_i$ during phagocytosis of IgG-RBC. A time-lapse sequence of a representative Ca^{2+}-depleted macrophage is shown in Figure 3D with the corresponding trace in Figure 3C. Ca^{2+} depletion did not impair the ability of I-, L-, α10-, 6-activated or nonactivated macrophages to ingest IgG-RBC

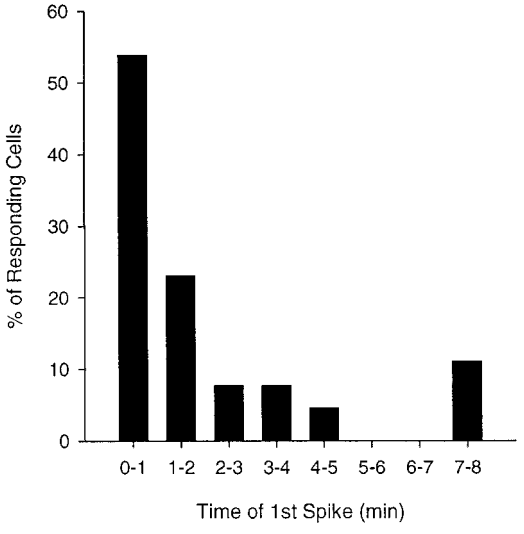

Fig. 2. Calcium increases followed shortly after the initiation of phagocytosis. $[Ca^{2+}]_i$ were measured in activated macrophages (I, L, α10, 6) during phagocytosis of individual IgG-RBC. Only macrophages that bound a single IgG-RBC were analyzed. The time of the first calcium spike, relative to the initial contact of the particle and the macrophage (time 0), was recorded for those macrophages that showed increased $[Ca^{2+}]_i$ (n=26).

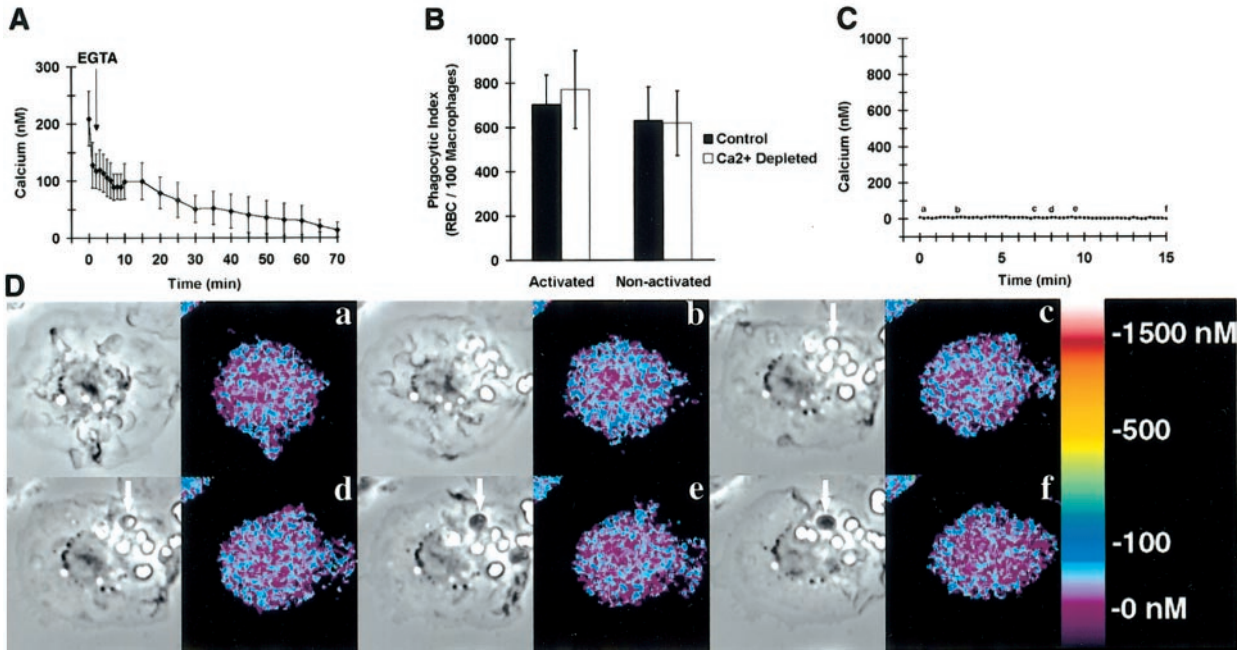

Fig. 3. Calcium depletion did not affect internalization of IgG-RBC. (A) $[Ca^{2+}]_i$ was measured in macrophages, and the buffer was replaced with EGTA-RB after the first time point. (A) $[Ca^{2+}]_i$ was depleted by incubating macrophages in EGTA-RB, which was added after the first time point, and $[Ca^{2+}]_i$ was monitored periodically thereafter. Results are expressed as mean ± SD (n=29). (B) Nonactivated macrophages and activated macrophages (I, L, α10, 6) were pretreated by incubation in EGTA-RB for 60 min. A phagocytosis assay was then performed as described in Materials and Methods. Results are expressed as mean ± SEM (n=3). (C) $[Ca^{2+}]_i$ trace of a representative $[Ca^{2+}]_i$-depleted macrophage during phagocytosis of IgG-RBC and (D) the corresponding phase contrast and $[Ca^{2+}]_i$ images. Arrows show a particle ingested (changing from phase-bright to phase-dark) during the recording. Panels a–f are indicated on the trace in (C).

(Fig. 3B), consistent with earlier observations [3]. There were no morphological differences between normal and Ca^{2+}-depleted macrophages using this method of Ca^{2+} depletion.

$[Ca^{2+}]_i$ rises are inhibited by Thapsigargin g

To determine the source of the calcium spikes seen during phagocytosis, the effects of various inhibitors on $[Ca^{2+}]_i$ spiking were measured in activated macrophages. The two most common sources of $[Ca^{2+}]_i$ are ER and extracellular medium ($[Ca^{2+}]_e$). $[Ca^{2+}]_e$ can be tested as a source of calcium by short-term replacement of RB with buffer containing EGTA. Likewise, ER as a source of $[Ca^{2+}]_i$ spikes could be tested by treatment with Tg, which depletes ER of calcium by inhibiting its reuptake via the SERCA pump. Recently, this laboratory identified lysosomes as another potential source for intracellular calcium [19]. When lysosomal pH is raised by addition of NH_4Cl, lysosomal calcium ($[Ca^{2+}]_{lys}$) drops from 600 μM to 100 nM, and this is accompanied by a slight increase in $[Ca^{2+}]_i$. Therefore, $[Ca^{2+}]_{lys}$ could be examined as a source of the $[Ca^{2+}]_i$ spikes by testing the ability of IgG-RBC to increase $[Ca^{2+}]_i$ in macrophages treated with NH_4Cl (Ca^{2+}-depleted lysosomes).

$[Ca^{2+}]_i$ was measured during addition of IgG-RBC to macrophages in RB. Macrophages were allowed to phagocytose particles for 4.5 min to ensure they were spiking $[Ca^{2+}]_i$ normally. RB was then replaced with RB, RB + 10 mM EGTA, RB + 10 mM NH_4Cl, or RB + 1 μM Tg (**Fig. 4**). Replacement of RB with RB led to an increased number of calcium spikes (Fig. 4A), probably because replacing the buffer stirred up RBC that had settled on noncellular regions and increased the number of cell-bound IgG-RBC. Macrophages still spiked $[Ca^{2+}]_i$ after RB was replaced with calcium-free buffer containing 10 mM EGTA (Fig. 4B). Although the total number of $[Ca^{2+}]_i$ spikes was reduced after EGTA addition, the number of phagocytosing macrophages that increased $[Ca^{2+}]_i$ was unchanged (data not shown). This indicated that $[Ca^{2+}]_e$ was not necessary for generation of $[Ca^{2+}]_i$ spikes during phagocytosis of IgG-RBC but may be important for refilling ER stores to enable repeated $[Ca^{2+}]_i$ rises. Replacement with RB containing 10 mM NH_4Cl resulted in a response similar to that seen when RB was replaced with just RB, which indicates that $[Ca^{2+}]_{lys}$ did not contribute to the $[Ca^{2+}]_i$ spikes (Fig. 4C). Treatment of macrophages with Tg completely abrogated all $[Ca^{2+}]_i$ (Fig. 4D). Calcium spikes might have been hidden by the $[Ca^{2+}]_i$ rise induced by Tg. For that reason, we pretreated macrophages for 45 min with 1 μM Tg. This allowed $[Ca^{2+}]_i$ to return close to basal levels (Fig. 4E). After Tg pretreatment, macrophages did not increase $[Ca^{2+}]_i$ during phagocytosis (0/19 cells observed; data not shown). Thus, the $[Ca^{2+}]_i$ spikes generated during phagocytosis of IgG-RBC were most likely a result of the release of calcium from ER stores. Ca^{2+}-spike inhibition by treatment with Tg could now be used to study the contribution of $[Ca^{2+}]_i$ spikes to microbicidal chemistries that accompany FcR-mediated phagocytosis.

Role of calcium in ROS and RNS production

One possible explanation for increases in $[Ca^{2+}]_i$ during FcR-mediated phagocytosis is that they are required for enhanced production of ROS or RNS. ROS production by activated macrophages was measured with H_2DCF-BSA-IgG, which con-

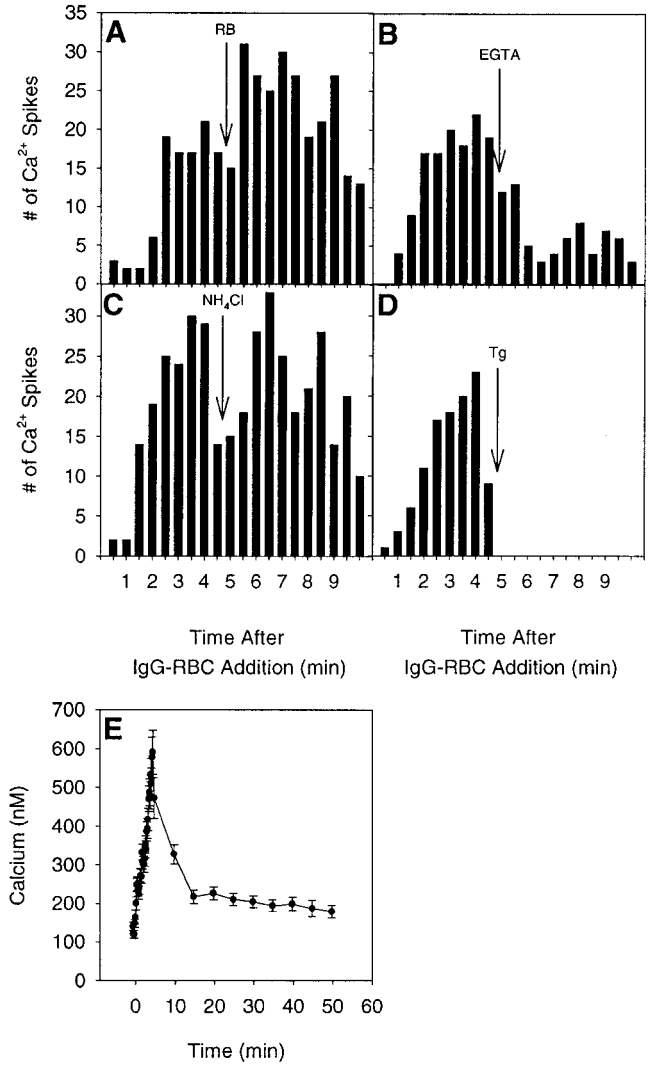

Fig. 4. Calcium spikes were inhibited by Tg. $[Ca^{2+}]_i$ was recorded following the addition of IgG-RBC (time 0), and the time of any $[Ca^{2+}]_i$ spikes relative to IgG-RBC addition was recorded. RB in the chamber was replaced 4.5 min after IgG-RBC addition, with RB (A), RB + 10 mM EGTA (B), RB + 10 mM NH_4Cl (C), or RB + 1 μM Tg (D). Each graph represents the cumulative $[Ca^{2+}]_i$ spikes generated by 45 macrophages, and each bar represents the total number of spikes recorded in that time interval. (E) Depletion of ER calcium stores with Tg. Tg (1 μM) was added to macrophages at the beginning of the time-lapse, and $[Ca^{2+}]_i$ was recorded for 50 min.

sists of the nonfluorescent molecule H_2DCF covalently linked to an insoluble, immune complex of IgG-BSA. H_2O_2 oxidizes H_2DCF to DCF [20], a fluorescent analog with excitation and emission spectra similar to fluorescein. Time-lapse sequences of phase-contrast and fluorescence (ex. 485) images were collected during phagocytosis of H_2DCF-BSA-IgG particles by activated macrophages. In control macrophages, phagocytosis was accompanied by large increases in fluorescence of the ingested H_2DCF-BSA-IgG particles. There was no difference in the rate or extent of DCF generation in phagosomes of Ca^{2+}-depleted macrophages (EGTA 70 min; **Fig. 5A**). Addition of the flavoprotein inhibitor diphenyleneiodonium (DPI), which prevents reduced nicotinamide adenine dinucleotide phosphate oxidase from generating superoxide [21], prevented an increase in fluorescence in phagosomes containing H_2DCF-BSA-IgG (Fig. 5A). Moreover, H_2DCF-BSA-IgG particles that did not land on cells remained relatively nonfluorescent during the course of the experiment (data not shown), indicating that the probe was not significantly oxidized by the excitation light source.

RNS are also important mediators of microbicidal activity in macrophages. Macrophages generate NO by the conversion of L-arginine to L-citruline, which is mediated by inducible NO synthase (iNOS). NO-derived nitrite was measured in culture media after allowing macrophages to phagocytose IgG-RBC for 1 h. In activated macrophages, nitrite generation was increased during phagocytosis of IgG-RBC, relative to cells receiving no stimulus (activated) or the addition of unopsonized erythrocytes (Act + RBC; Fig. 5B). Ca^{2+} depletion with EGTA inhibited RNS generation. Ca^{2+}-spike inhibition by pretreatment with Tg did not inhibit IgG-RBC-induced nitrite formation (Fig. 5B). This indicates that a basal level of $[Ca^{2+}]_i$ was required for generation of RNS but that increases in $[Ca^{2+}]_i$ above resting levels (e.g., in the form of $[Ca^{2+}]_i$ spikes) were not necessary for the RNS response.

Phagosome-lysosome fusion does not require calcium

The effects of Ca^{2+} depletion and inhibitions of $[Ca^{2+}]_i$ spikes on phagosome-lysosome fusion were measured (**Fig. 6**). Lysosomes were labeled by incubating macrophages for 30 min in TR-Dx and then chasing for 1 h in RB. Macrophages were then treated with EGTA (Ca^{2+}-depleted), Tg (Ca^{2+}-spike inhibition), or NH_4Cl ($[Ca^{2+}]_{lys}$ depletion). Neither Tg treatment nor EGTA decreased fusion with lysosomes. NH_4Cl, which increases the pH of the phagosome, was used as a positive control for inhibition of phagosome-lysosome fusion [22]. Pretreatment of macrophages with 10 mM NH_4Cl significantly reduced fusion. Thus, phagosome-lysosome fusion following FcR-mediated phagocytosis was not dependent on calcium.

DISCUSSION

There is still much that is unknown about the signaling mechanisms that accompany phagocytosis. In particular, rises in calcium that accompany phagocytosis suggest that it is an intracellular messenger in the phagocytic process. To date, clear demonstrations of a requirement for calcium rises in FcR-mediated phagocytosis or related processes in macrophages are lacking. Here, using fluorescence microscopy to analyze the calcium response of individual macrophages during FcR-mediated phagocytosis, we report a novel increase in the prevalence of calcium spikes in activated macrophages.

The calcium responses were similar to those seen previously [3, 4, 23]. Intracellular calcium rises were a result of calcium release from ER stores, and calcium was not required for particle internalization. Generally, the phagocytosis-induced calcium response consisted of a single spike. Some macrophages, however, continued spiking calcium at regular intervals for many minutes during phagocytosis, similar to the response seen by Kruskal and Maxfield [23] in macrophages undergoing frustrated phagocytosis.

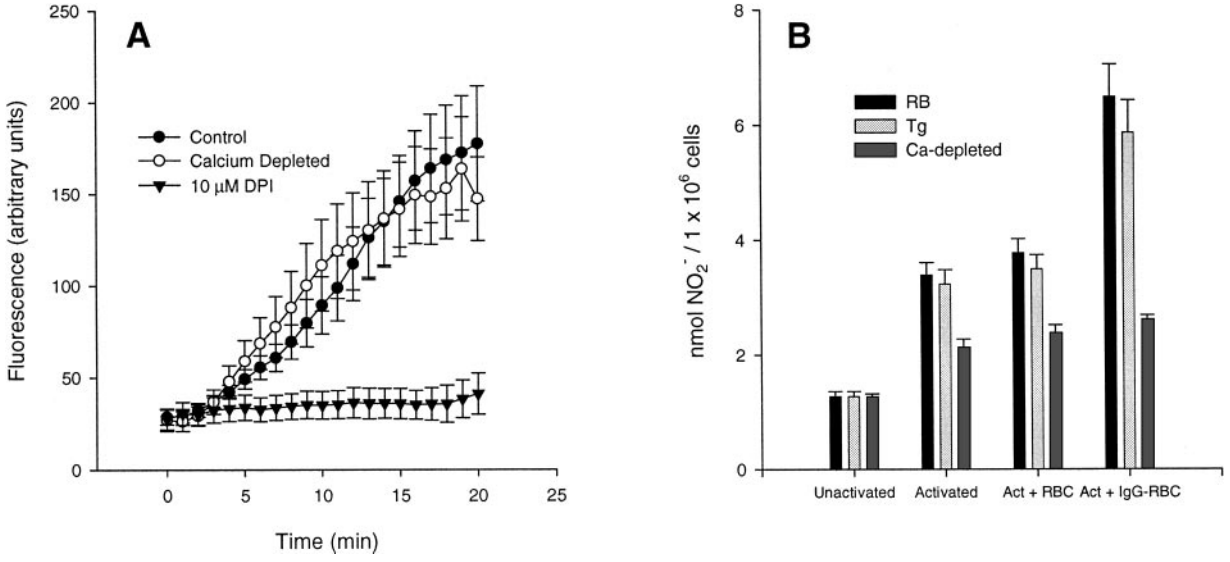

Fig. 5. Contributions of calcium to the generation of ROS and RNS during phagocytosis. (A) ROS generation was measured in macrophages using the H_2O_2-sensitive probe H_2DCF-BSA-IgG. H_2DCF-BSA-IgG particles were added to control macrophages (●), Ca^{2+}-depleted macrophages (○), or macrophages + 10 μM DPI (▼), and the fluorescence intensity of individual particles was measured for 30 min. (B) Nitrite generation was measured in macrophages after 1 h. Results are expressed as mean ± SEM (n=3). Macrophages were Ca^{2+}-depleted or pretreated with Tg (1 μM) as indicated and were then stimulated with IgG-RBC. Results are expressed as mean ± SEM (n≥7).

Phagocytosis was not always accompanied by a change in calcium. Heterogeneity of the calcium response was also observed by Greenburg et al. [4] in measurements of thioglycollate-elicited macrophages and the macrophage-like cell line J774. The present study extends those observations by correlating calcium spiking with the activation state of the macrophage. Murine macrophages express different types of FcRs. FcγRI and FcγRIII signal particle internalization and calcium rises, and FcγRII is inhibitory and down-regulates the immune response [24]. Macrophages from FcγRII knockout mice give a more robust calcium response upon ligation of FcγRIII [25]. It is possible that phagocytic calcium responses are determined by the relative ratios of the different receptor types on the cell surface. An interesting possibility that remains to be investigated is whether the increased calcium responsiveness of activated macrophages is a result of reduced expression of FcγRII.

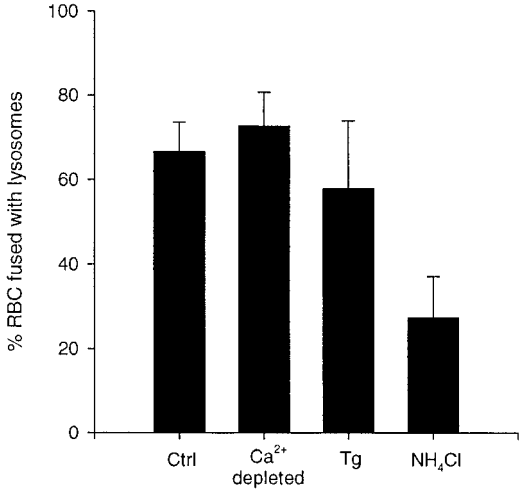

Fig. 6. Effects of Ca^{2+} depletion on phagosome-lysosome fusion. Lysosomes of activated macrophages were labeled with TR-Dx by pulsing for 30 min and chasing for 1 h in RB. Control macrophages, Ca^{2+}-depleted macrophages, or macrophages treated with Tg or NH_4Cl were allowed to phagocytose IgG-RBC for 10 min before fixing. RBCs that were completely surrounded by a ring of TR-Dx were judged as having fused with lysosomes. For each coverslip, 50 RBC from 50 different macrophages were scored for fusion. Results are expressed as mean ± SEM (n=3).

An advantage of using single-cell fluorescence microscopy to study calcium responses is that it provides subcellular resolution of spatial dynamics. The calcium response that we observed, however, was relatively delocalized. In most instances, $[Ca^{2+}]_i$ rose uniformly throughout the cell, although in larger macrophages, the calcium responses were sometimes restricted to the half of the cell on which the erythrocyte landed. Such delocalized responses indicate that the calcium spikes signal a more global response than restricted to the individual phagosome. However, it remains possible that a localized change in $[Ca^{2+}]_i$ was delocalized by the probe; that is, calcium could be binding to fura and then rapidly diffusing throughout the cell. We consider this is unlikely, however, as there was no observable difference in the spatial organization of the response when using FFP-18, a fura-based probe with a hydrophobic tail that inserts in membranes. If significant probe-facilitated diffusion was occurring, it would have been reduced with the membrane-bound probe.

The function of the calcium spikes remains unknown. Because of their increased presence in activated macrophages, we hypothesized that they played a role in microbicidal function. Therefore, we examined the requirement for calcium in three activities related to the killing of ingested microbes: generation of ROS and RNS and phagosome-lysosome fusion. Although previous studies have indicated a requirement for calcium for the respiratory burst in macrophages [26, 27], we observed no requirement for calcium in the ROS-dependent conversion of H_2DCF to DCF. ROS generation during phagocytosis of IgG-

opsonsized BSA occurred normally in Ca^{2+}-depleted macrophages. The differences in our findings can possibly be explained by differences in the stimulus (IgG-BSA vs. phorbol 12-myristate 13-acetate or opsonized glass beads) and type of macrophage (activated bone marrow-derived vs. RAW 264.7 or peritoneal-elicited).

FcR-mediated phagocytosis increased RNS generation, and this increase was inhibited in Ca^{2+}-depleted macrophages but not in macrophages that were pretreated with Tg to prevent calcium spikes. This is consistent with the fact that iNOS, the protein that generates NO, requires nanomolar $[Ca^{2+}]_i$ to become fully active. Unlike its homologs eNOS and nNOS, iNOS does not require calcium rises [28]. Therefore, the calcium spikes observed during FcR-mediated phagocytosis do not appear to be necessary for production of NO.

Phagosome-lysosome fusion was unimpaired in Ca^{2+}-depleted macrophages as well as Ca^{2+} spike-inhibited macrophages, reflecting a different signal transduction pathway than complement receptor-mediated phagocytosis, where calcium has been shown to play a role in phagosome-lysosome fusion [29, 30].

Our results demonstrate a correlation between the calcium response and macrophage activation. As activated macrophages are defined by their increased microbicidal activity, it is likely that the increased calcium signaling plays some role in the microbicidal response. Although we did not observe any effects from the inhibition of calcium signaling, our studies concerned only those events occurring within 15 min of particle uptake. The calcium spikes could instead be responsible for events that occur long after phagocytosis. The lack of tight spatial regulation of the calcium signal suggests a role in a broad cellular response such as altered gene expression, a change that would occur over a longer period. Calcium is known to play a regulatory role in the expression of many different genes, including iNOS, where it can increase or decrease gene expression depending on the degree of macrophage activation [31]. Secretion of tumor necrosis factor has also been shown to be linked to calcium in LPS-activated macrophages [32]. Further work remains to be done to determine if phagocytosis-associated calcium rises affect the expression of iNOS or other genes involved in the immune response.

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