Calcium Transients during Fc Receptor-mediated and Nonspecific Phagocytosis by Murine Peritoneal Macrophages

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Abstract. Studies with populations of macrophages have produced conflicting results concerning the possibility that the concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) may act as an important mediator for phagocytosis. Since asynchronous changes in $[Ca^{2+}]_i$ in individual cells undergoing phagocytosis may be averaged to undetectability in population studies, we studied single adhering murine macrophages using fura-2 and our previously described digital imaging system. The proportion of macrophages phagocytosing IgG-coated latex beads was greater than for uncoated beads (percent phagocytosing cells: 71 ± 7 vs. 27 ± 7, $P < 0.01$). Phagocytosis of IgG-coated and uncoated beads was always associated with a calcium transient that preceded the initiation of phagocytosis. No calcium transients were detected in cells that bound but did not phagocytose beads. Four major differences between Fc receptor-mediated and nonspecific phagocytosis were detected: (a) the duration of calcium transients was longer for nonspecific phagocytosis compared with Fc receptor-mediated phagocytosis (69.9 ± 10.2 vs. 48.7 ± 4.7 s, $P < 0.05$) and the magnitude of calcium transients was less for nonspecific phagocytosis (178 ± 43 vs. 349 ± 53 nM, $P < 0.05$); (b) removal of extracellular calcium abolished the calcium transients associated with nonspecific phagocytosis but had no effect on those associated with receptor-mediated phagocytosis; (c) in the absence of extracellular calcium, buffering intracellular calcium with a chelator reduced Fc receptor-mediated phagocytosis but had no additive inhibitory effect on nonspecific phagocytosis; and (d) inhibition of protein kinase C (PKC) with staurosporine inhibited nonspecific phagocytosis but had no effect on receptor-mediated phagocytosis. Our observations suggest that despite both types of phagocytosis being associated with intracellular calcium transients, the role played by intracellular calcium in the signaling pathways may differ for Fc receptor-mediated and nonspecific phagocytosis by elicited murine macrophages.

BINDING and endocytosis of immune complexes by macrophages are mainly mediated by membrane receptors for the Fc portion of IgG (Fc receptors) (20). Transmembrane signaling for the initiation of phagocytosis of bound immune complexes is not currently understood. Since calcium regulates the assembly and disassembly of cytoskeletal elements (7, 18, 22), it has long been proposed that a transient increase in the concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) acts as a second messenger for phagocytosis. However, available studies with populations of macrophages have shown conflicting results concerning this possibility (8, 14, 26). Studies of cell populations in which no calcium transients were observed may have been hampered by the fact that phagocytosis is an asynchronous event in different cells (14). Thus, calcium transients occurring in different cells at different times might have been averaged to undetectability as previously postulated by Kruskal and Maxfield (12). To overcome these difficulties, we used our previously described digital video imaging system (5, 15, 25) to simultaneously measure phagocytic events and $[Ca^{2+}]_i$ in

single elicited murine macrophages. In addition, we have devised experimental manipulations to further define the roles of extracellular calcium, intracellular calcium stores, and protein kinase C (PKC) activity in Fc receptor-mediated and nonspecific phagocytosis.

Materials and Methods

Coating Latex Beads with IgG and BSA

Human IgG was prepared from normal serum using 30% saturated ammonium sulfate salt precipitation followed by column chromatography with DEAE Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ). IgG was then dialyzed against 0.01 M PBS (pH 7.3). Latex beads (3.2 μm diameter, Sigma Chemical Co., St. Louis, MO) were coated by incubating beads (3.9 × 10⁹/ml) with IgG at a final concentration of 20 mg/ml in PBS for 90 min at 37°C. IgG-coated beads were then washed with HBSS (pH 7.3) containing 5% BSA (Sigma Chemical Co.) and then suspended and stored in this buffer at a final concentration of 5 × 10⁹/ml. Control (uncoated) latex beads were treated in a similar fashion except that they were initially incubated in PBS without IgG.

Isolation of Murine Peritoneal Macrophages

Female BALB/c mice were injected intraperitoneally with 0.3 ml of complete Freund's adjuvant (Sigma Chemical Co.). Mice were killed at 24 h by cervical dislocation and peritoneal cells were harvested by peritoneal lavage with HBSS. Cells were then washed three times with Eagle's minimum essential medium (MEM) buffered by bicarbonate containing gentamicin 50 μg/ml and 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY). Cells were resuspended at a concentration of 5×10^5/ml in MEM containing 10% FBS and 1.0-ml aliquots were plated on glass coverslips. Coverslips were incubated at 37°C for 2 h in humidified 5% CO_2, and nonadherent cells were removed by vigorous pipetting. Adherent cells were $\sim$95% macrophages morphologically and bound IgG-coated latex beads (85–92%). Polymorphonuclear granulocytes were not in evidence after the overnight culture period.

Measurement of Binding and Phagocytosis of Latex Beads by Populations of Adhering Macrophages

Medium was aspirated from the coverslips of cultured macrophages and replaced with 0.25 ml of HBSS (without BSA) containing 5×10^7 beads. Cells were then incubated for 15 min at 37°C and unbound beads were removed by gentle washing with HBSS. Binding and phagocytosis were then measured by counting the number of cells binding and phagocytosing beads for 100 consecutive cells per coverslip using an Olympus BHT microscope. Beads were scored as phagocytosed when the beads were at the same depth of field as the cell. Beads were scored as bound when they were seen to be attached to the cell membrane and either at different depth of field compared with the cell (for bound beads overlying the cell body) or were seen in profile to be outside the cell membrane (for beads bound to the edges of cells). The data were presented as percent binding cells, percent phagocytosing cells and/or phagocytic index which was calculated as follows; number of beads phagocytosed/number of cells scored multiplied by the percent phagocytosing cells.

Control experiments were performed to verify that our criteria with bright field microscopy were adequate for determining phagocytosis using an approach based on methods described by others to study the phagocytosis of bacteria (10) or yeast particles (11, 13). Briefly, latex beads that were coated with FITC-labeled IgG or with FITC only were used in phagocytosis experiments in the usual manner. After phagocytosis was determined by bright field microscopy, 0.5 ml of 0.2% trypan blue in PBS was added to each coverslip and the cells were reexamined using fluorescence microscopy. Since trypan blue quenches the fluorescence of extracellular beads, only intracellular (phagocytosed) beads would fluoresce normally (10, 11, 13). Our results for assessing phagocytosis were very similar for transmission light microscopy and fluorescence-quenching methods. For example, the average percent phagocytosing cells for IgG-coated beads in three experiments was 68.6% by the bright field method and 66.7% by the fluorescent method while the same parameter for FITC coated beads (without IgG coating) was 28.4 and 34.0%, respectively.

Digital Imaging System

The assembly and performance characteristics of our digital imaging system for the study of events occurring in a single cell have been extensively described previously (5, 15, 25). Briefly, the system consists of a SPEX dual wavelengths fluorimeter (SPEX Industries, Edison, NJ), a Zeiss IM 35 inverted microscope, and an intensified charge-coupled device (CCD) video camera (model 3000 F; Fairchild, Sunnyvale, CA). The microscope is equipped with a KT stage temperature controller, a glycerin immersion objective (Zeiss Neofluar 63X/1.25 NA) and a 60-W epi-illuminator with which we used to observe binding and phagocytosis (see below). The SPEX fluorimeter provides two excitation wavelengths (350 nm and 380 nm; 9-nm bandpass) which are alternated at video rate (33.3 ms per wavelength change) by means of a rotating mirror ("chopper"). Both white light and fluorescence light pass through a band pass filter (505 nm ± 20 nm; Ditric Optics, Hudson, MA) before arrival at the faceplate of the intensified CCD video camera. Fluorescence images (170 pixels × 240 lines) are digitized on-line at 8 bits per pixel by a video digitizing board (EPIX, Chicago, IL) in an IBM AT personal computer. Data are stored initially in system RAM

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1. *Abbreviations used in this paper*: CCD, charge-coupled device; fura-2/AM, fura-2 acetoxymethyl ester; MAPT/AM, 1,2-bis-5-methyl-amino-phenoxylethane-N,N,n^1-tetra-acetoxylmethylacetate.

memory and subsequently transferred to a Bernoulli box system for permanent storage and off-line analysis. The chopper, CCD video camera and video digitizing board are synchronized and there is no detectable spillover between frames of excitation wavelength 350 nm and those of 380 nm. The overall system has an intrascene dynamic range of 50:1 and a two-dimensional x-y resolution of 1,000 line pairs/mm or 0.5 μm. Over the range of intracellular fluorescence measured, the intensified CCD camera is always operating in the linear response region (5).

Loading Macrophages with fura-2

Medium was aspirated from the coverslips of cultured macrophages and replaced with 2.0 ml of HBSS containing 2 μM fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes Inc., Eugene, OR) for 15 min at 37°C. After incubation, cells were washed twice with HBSS to remove extracellular fura-2/AM and used immediately for $[Ca^{2+}]_i$ measurements and phagocytosis experiments.

To ensure that no significant amounts of fura-2 were leaked or pumped out of the macrophages, fluorescence intensity at 365 nm excitation (isobestic point of fura-2) was measured for 10 cells at 0 time and after 15 min; no detectable fall in fluorescence intensity occurred during the 15 min period. Similarly, to ensure that fura-2 was not sequestered in intracellular organelles, fluorescence intensity was measured at 365 nm for fura-2 loaded cells before and after permeabilization of the plasma membrane with digitonin 6 μM ($n = 7$ cells) or 4 μM ($n = 12$ cells). Digitonin at these concentrations selectively permeabilizes the plasma membrane but the permeabilities of mitochondrial and endoplasmic reticular membranes remain intact (4, 16). Fluorescence intensity was reduced by a mean of 97.1 and 95.3%, respectively. Thus, significant subcellular sequestration of fura-2 did not occur to affect our data appreciably.

Measurement of $[Ca^{2+}]_i$ during Binding and Phagocytosis of Latex Beads by Single Cells

Coverslips with fura-2 loaded macrophages were mounted in a Dvorak-Stotler chamber (Nicholson Precision Instruments, Gaithersburg, MD) and placed on the thermoregulated stage (37°C). A single cell with good morphological characteristics was chosen by bright field microscopy and adequate fura-2 loading was checked by fluorescence microscopy. Medium was then aspirated and replaced with 0.25 ml of HBSS containing 5×10^7 latex beads. The selected cell was observed throughout as beads settled onto the cell. Binding was defined as the initial time when a bead (which subsequently did not become disengaged) first touched the cell membrane. At this point, the bead was clearly out-of-focus when compared with the cell at optimal focus (Fig. 2 a). Data acquisition was then started and both bright field images and fluorescence images (350 and 380 nm excitation) were recorded. Each set of 3 images at each time-point required $\sim$12 s to acquire, and data for each selected cell were collected for 300 s. The initiation of phagocytosis was determined from the bright field images and was defined as the first detectable motion of the bead in the Z-axis (bead became less out-of-focus, Fig. 2 e) followed by complete ingestion of the particle (bead in same depth-of-field as the cell, Fig. 2 g). The initiation of phagocytosis usually occurred within 96 s after binding (range 37-174 sec). After its initiation, phagocytosis was usually complete within 120 sec (range 75-300 sec). Failure to phagocytose a bound latex bead was defined as no motion of the bead within 180 s after binding. After the 180-s recording, most of the cells were also observed under bright field microscopy after 5 and 10 min; in no case did a cell which had not initiated phagocytosis within 180 s subsequently successfully phagocytose a latex bead.

The $[Ca^{2+}]_i$ was calculated for each data aquisition point as follows. A ratiometric ($R_{350/380}$) image was generated by dividing the 350 nm fluorescence intensity by the 380-nm intensity on a pixel by pixel basis. The derived ratiometric images were displayed on a Sony RGB monitor. The $[Ca^{2+}]_i$ was then calculated as the mean of the individual pixel ratios for the entire cell as well as for a 20-μm^2 window of the cell immediately adjacent to the bound bead using the following expression originally described by Grynkiewicz et al. (9) and adapted by others (6, 19).

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where K_d is the apparent dissociation constant for Ca^{2+} (224 nM for fura-2) (9), R is the 350/380 ratio of fluorescence, R_{min} and R_{max} are the R values when intracellular fura-2 was clamped with ionomycin (2 μM) at zero Ca^{2+} (4 mM EGTA) and 4 mM Ca^{2+} (5, 15), respectively, and S_{f2}/S_{b2} is
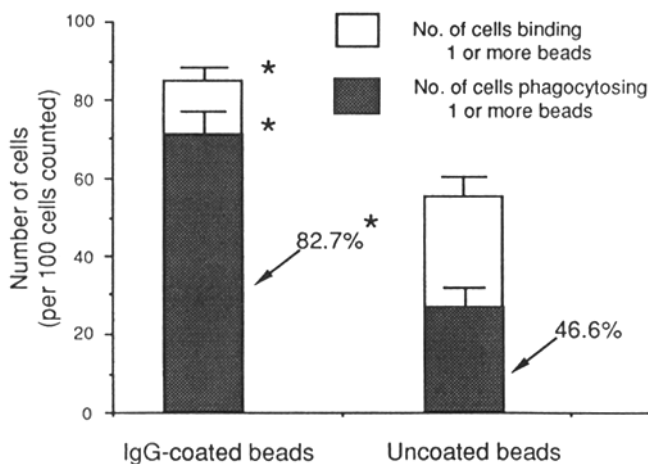
Figure 1. Binding and phagocytosis of IgG-coated beads by adherent macrophages. Elicited macrophages (5×10^5 cells per coverslip) were incubated with IgG-coated beads (four experiments) or uncoated beads (three experiments) as described in Materials and Methods. The number of cells binding and phagocytosing were counted for 100 cells per coverslip. Open bars represent the number of cells binding one or more beads; the shaded bars represent the number of cells phagocytosing one or more beads. Data are the mean ± SEM. The asterisk (*) indicates $P < 0.01$ comparing IgG-coated and uncoated beads by the unpaired t test.

the ratio of fura-2 fluorescence intensities at 380 nm excitation under R_{min} (S_{f2}) and R_{max} (S_{b2}) conditions (9). Before each experiment, background fluorescence was measured at each wavelength from an adjacent empty area of coverslip. These background values were subtracted from the cellular fluorescence values before the 350/380 ratios were calculated.

Depleting Macrophages of $[Ca^{2+}]_i$

Adherent macrophages were washed with calcium-free HBSS and then incubated for 15 min at 37°C in calcium–free HBSS containing 25 μM 1,2-bis-5-methyl-amino-phenoxylethane-N,N,n'-tetra-acetoxymethyl acetate (MAPT/AM; Calbiochem-Behring Corp., La Jolla, CA) and 1 mM EGTA, or 1 μM ionomycin and 4 mM EGTA. For some experiments, adherent macrophages were also incubated in calcium-free HBSS containing 5 mM ATP (Calbiochem-Behring Corp.) and 10 mM EGTA (pH 7.8) for 5 min at 37°C as described previously by Steinberg et al. (21). In this last case, cells were washed with calcium-free HBSS and incubated for an additional 10 min to allow desensitization of the cell membrane before the phagocytosis assay.

Treatment of Macrophages with Staurosporine

Staurosporine (Sigma Chemical Co.) was dissolved to a stock concentration of 50 μM in DMSO. For initial experiments with staurosporine, adhering macrophages were incubated for 15 min at 37°C with staurosporine at a final concentration of 50–500 nM (0.1% to 1.0% DMSO) in HBSS containing 1 mM calcium. Since staurosporine had similar effects over this range of concentrations tested, later experiments were done at a final concentration of 100 nM. In control experiments, DMSO (vehicle) alone had no effect on binding and phagocytosis of IgG-coated and uncoated latex beads.

Results

Binding and Phagocytosis of Latex Beads by Populations of Adherent Macrophages

Fig. 1 shows the binding and phagocytosis of IgG and BSA-coated latex beads and beads coated with only BSA by populations of adherent elicited macrophages. IgG-coated beads

were bound in greater numbers than uncoated beads. More importantly, phagocytosis of bound IgG-coated beads was greater than that of bound uncoated beads (82.7 ± 4.8 vs. 46.6 ± 6.9%, p < 0.01). These results are most consistent with Fc receptor-mediated mechanism for binding and phagocytosis of IgG-coated beads, since it is well recognized that F(ab')$_2$ fragments of IgG do not promote specific binding or phagocytosis (20).

Sequential Changes in $[Ca^{2+}]_i$ during Phagocytosis of an IgG-coated Latex Bead

Fig. 2 shows the sequence of bright field with corresponding $[Ca^{2+}]_i$ images of a single macrophage undergoing phagocytosis of a bound IgG-coated latex bead. $[Ca^{2+}]_i$ was low at initial attachment of the bead (Fig. 2 *a*) as shown by the ratiometric image (Fig. 2 *b*). Approximately 1 min after binding, $[Ca^{2+}]_i$ abruptly increased from 60 to 436 nM (Fig. 2 *d*) but no phagocytosis was evident (Fig. 2 *c*). Only after $[Ca^{2+}]_i$ had returned to or toward baseline (Fig. 2 *f*), was the latex bead phagocytosed by the cell (Figs. 2, *e* and *g*).

The time course of $[Ca^{2+}]_i$ changes during phagocytosis by the macrophage illustrated in Fig. 2 is shown in Fig. 3. Mean $[Ca^{2+}]_i$ is plotted both for the entire cell and for a 20-μm^2 area of the cell body immediately adjacent to the bound bead. $[Ca^{2+}]_i$ increased to a peak value at 72.6 s after binding of the bead and then returned to baseline. The initiation of phagocytosis clearly occurred after the calcium transient (*solid arrow*) and was complete after 3.1 min (*open arrow*). We could not detect differences in peak $[Ca^{2+}]_i$ attained between the area immediately adjacent to the phagocytosed bead and the rest of the cell (Figs. 2, *d* and 3). In addition, no "oscillations" in $[Ca^{2+}]_i$ were observed within the time frame of our experiments.

Peak $[Ca^{2+}]_i$ during Binding and Phagocytosis of IgG-coated Beads

Data from our experiments comparing $[Ca^{2+}]_i$ changes for IgG-coated beads and uncoated beads in the presence of extracellular calcium are summarized in Table 1 *A*. Peak $[Ca^{2+}]_i$ was significantly higher than baseline $[Ca^{2+}]_i$ for cells phagocytosing either IgG-coated or uncoated beads. The duration, the peak $[Ca^{2+}]_i$ and the $\Delta[Ca^{2+}]_i$ (peak-baseline) were different for IgG-coated beads compared with uncoated beads (P < 0.05). The time interval between attachment and the subsequent calcium peak and the time from the calcium peak to the initiation of phagocytosis were analyzed and were not different for IgG-coated and uncoated latex beads (binding to transient 83.4 ± 7.1 vs. 89.9 ± 9.4 s, P > 0.1; transient to phagocytosis 11.3 ± 2.9 vs. 8.9 ± 4.8 s, P > 0.1). In eight instances, IgG-coated beads ($n = 3$) and uncoated beads ($n = 5$) were observed to attach to cells, but no phagocytosis ensued. No calcium peak was detected for any cell that bound either type of beads but failed to phagocytose (data not shown). Conversely, no phagocytosis occurred that was not associated with a calcium transient.

The number of beads bound by single cells selected for study in the above experiments were purposely similar for IgG-coated and uncoated beads (Table I). Thus, observed differences in the calcium transients cannot be due to any possible effect of binding different numbers of beads. As expected, the percent of attached beads that were subsequently
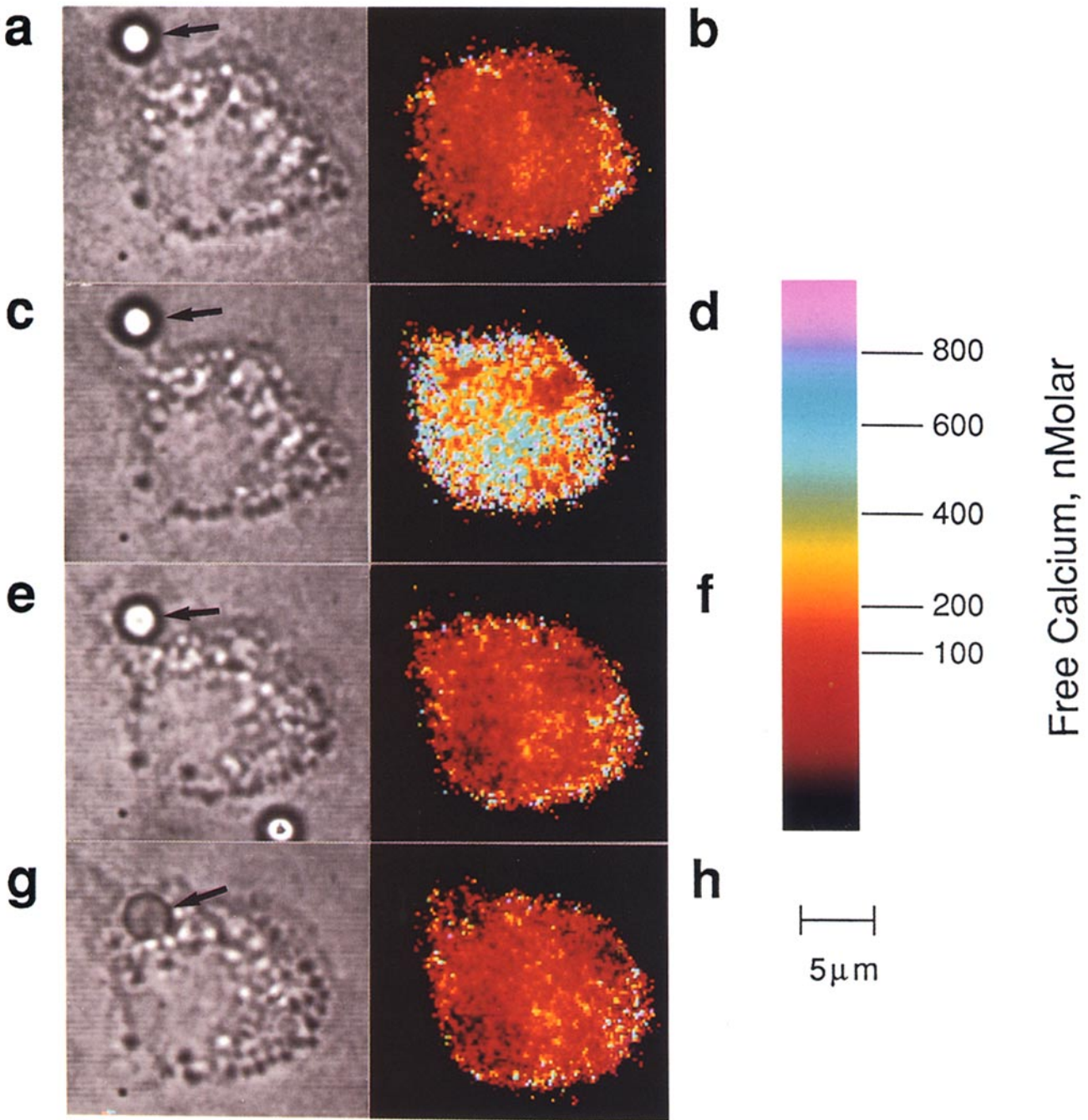
Figure 2. Bright field fluorescence ratiometric images of a macrophage during phagocytosis of a single IgG-coated bead. The macrophage is shown in a bright field image after binding a single IgG-coated bead (*a*) and the corresponding fluorescence ratiometric image at baseline $[Ca^{2+}]_i$ (*b*). After 66.6 s, the bound IgG-coated bead remains at the same location and same focus (*c*) but, the $[Ca^{2+}]_i$ has risen to a peak value (*d*), and subsequently returns to a near baseline level (*f*). After $[Ca^{2+}]_i$ returns to baseline, the bead moves to the interior of the cell as evidenced by both lateral movement relative to the cell and its becoming more sharply focussed (*e*). The bound bead has moved from margin of the cell to inside the cell (bead now at same depth of field as the cell) (*g*) but $[Ca^{2+}]_i$ remained at baseline (*h*). Arrow indicates the location of bead. The color scale indicates $[Ca^{2+}]_i$ in nanomolar.

phagocytosed appeared to be greater for IgG-coated beads (83.3%) than for uncoated beads (59.1%). Further, phagocytosis was not an all or nothing event in that cells binding more than one bead might phagocytose one, some, or all of the bound beads. Moreover, phagocytosis of two beads occurred sequentially in some cases, and in these cases, each sequential phagocytic event was preceded by a separate calcium transient.

Effect of Extracellular Calcium Removal on Phagocytosis of IgG-coated and Uncoated Beads by Single Macrophages

Identical experiments as described above were also performed in the absence of extracellular calcium (Table I *B*). Cells in calcium-free buffer were noted to "round up" during the 2–3 min required for the latex beads to attach to cells.
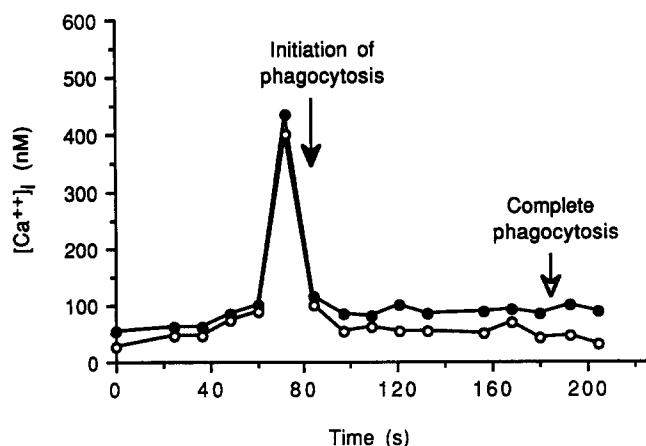
Figure 3. Time course of the change in $[Ca^{2+}]_i$ in a single macrophage during phagocytosis of an IgG-coated bead. The time course of $[Ca^{2+}]_i$ for the same experiment illustrated in Fig. 2 is shown. $[Ca^{2+}]_i$ is plotted both for the entire cell (●) and for the area immediately adjacent to phagocytosing bead (20 μm^2; o). Phagocytosis of the bead is first detected at the time indicated by solid arrow, and is completed at the time indicated by open arrow.

No differences were observed for IgG-coated beads with and without extracellular calcium for number of beads bound per cell, percent phagocytosed beads, baseline, peak and $\Delta[Ca^{2+}]_i$ and duration of calcium transient. In contrast to IgG-coated beads, macrophages binding uncoated beads failed to manifest a calcium transient and failed to phagocytose beads in the absence of extracellular calcium in 13 observations. Thus, the "peak" $[Ca^{2+}]_i$ was not different from baseline and the "peak" and $\Delta[Ca^{2+}]_i$ were significantly less than for uncoated beads in the presence of extracellular calcium (P < 0.05). This difference occurred despite similar baseline $[Ca^{2+}]_i$ and our choosing cells with similar numbers of bound beads (P > 0.1).

Calcium Sources and the Effect of Staurosporine on Phagocytosis of IgG-coated and Uncoated Beads by Populations of Macrophages

The effect on phagocytosis of changes in the availability of intra- and extracellular $[Ca^{2+}]$ was further elucidated by additional experiments (Fig. 4). Phagocytic events were scored for 100 adhering cells for untreated cells in HBSS (control), cells in calcium free HBSS with EGTA (low extracellular $[Ca^{2+}]$), and MAPT/AM-loaded cells in a calcium-free HBSS with EGTA (low intra- and extracellular $[Ca^{2+}]$). Adherent macrophages were also treated with staurosporine or staurosporine and MAPT/AM in EGTA buffer. Staurosporine is thought to be a potent inhibitor of PKC (17, 23). Staurosporine also causes cells to spread on the coverslips which makes assessment of phagocytosis more difficult when beads are observed under bright field illumination. Therefore, experiments with staurosporine were done using trypan blue quenching of fluoresceinated beads to assess phagocytosis, as described in Materials and Methods.

The percent of cells binding latex beads was little affected by the various treatments although the EGTA treated cells had a slightly increased percent of cells binding IgG-coated beads while staurosporine-treated cells had a greater percent of cells binding uncoated beads. This latter observation may be due to staurosporine increasing the tendency of adhering macrophages to spread, thus increasing the surface area available for nonspecific binding of uncoated latex beads. The total number of beads bound per 100 cells was also little affected by the various treatments, but the same trends were observed as for the percent of cells binding beads. (See figure legend for mean values.)

Removing extracellular calcium did not affect the percent of cells phagocytosing at least one IgG-coated (Fig. 4 *a*, group *I*) as seen in the single cell studies described earlier. The phagocytic index, however, was decreased for IgG-coated beads. It is important to recognize that the single cell studies were performed by observing selected cells that had bound one to three beads. In contrast, the population studies were done by scoring 100 consecutive cells for phagocytosis

Table I. Peak $[Ca^{2+}]_i$ in Individual Adherent Macrophages during Binding and Phagocytosis of IgG-coated Latex Beads

Latex beads	Cells studied	No. of beads bound/cell	Bound beads phagocytosed	[Ca²⁺]ᵢ Baseline	[Ca²⁺]ᵢ Peak	[Ca²⁺]ᵢ Δ	Duration of calcium transient
	n	*n*	%	*nM*			*s*
A. 1 mM Ca²⁺							
IgG-coated	30	2.3 ± 0.4	83.3	166.0 ± 18.7	514.7 ± 63.2*‡	348.7 ± 52.8‡	48.7 ± 4.7‡
Uncoated	17	2.6 ± 0.5	59.1	144.0 ± 22.4	321.7 ± 50.0*	177.7 ± 43.1	69.9 ± 10.2
B. 0.0 mM Ca²⁺, 1 mM EGTA							
IgG-coated	14	1.6 ± 0.2	77.3	171.5 ± 18.2	488.1 ± 113.2*	316.7 ± 107.9	48.0 ± 5.2
Uncoated (no phagocytosis)	13	2.7 ± 0.7	—	120.0 ± 12.2	144.1 ± 19.7§	24.2 ± 11.2‡	—

Elicited macrophages (5 × 10⁵ cells per coverslip) were loaded with fura-2 in HBSS and incubated with IgG-coated or uncoated latex beads (5 × 10⁷ particles/coverslip). Phagocytosis and $[Ca^{2+}]_i$ were measured as described in Materials and Methods. The % of bound beads phagocytosed was calculated as the number of beads phagocytosed/number of beads bound ×100 for the total number of cells studied. The duration of calcium transients was measured as the time period between the onset of the $[Ca^{2+}]_i$ increase and the end of its decay. Values given represent the mean ± SEM. In all cases, the single asterisk (*) indicates P < 0.001 comparing peak $[Ca^{2+}]_i$ with baseline using *t* test for paired data. (A) The double dagger (†) indicates P < 0.05 comparing IgG-coated and uncoated beads in the presence of 1 mM Ca²⁺ using *t* test for unpaired data. (B) Data obtained in the absence of calcium were compared with data for the same type of beads obtained in the presence of calcium using *t* test for unpaired data. For IgG-coated beads, there were no statistical differences (P > 0.1) comparing number of beads bound/cell, baseline, peaks, and $\Delta[Ca^{2+}]_i$ and duration of calcium transient in the presence and absence of calcium. For uncoated beads, the number of beads bound/cell and the baseline $[Ca^{2+}]_i$ were similar but peak and $\Delta[Ca^{2+}]_i$ were significantly less (§) for uncoated beads in the absence of calcium (where no phagocytosis occurred).
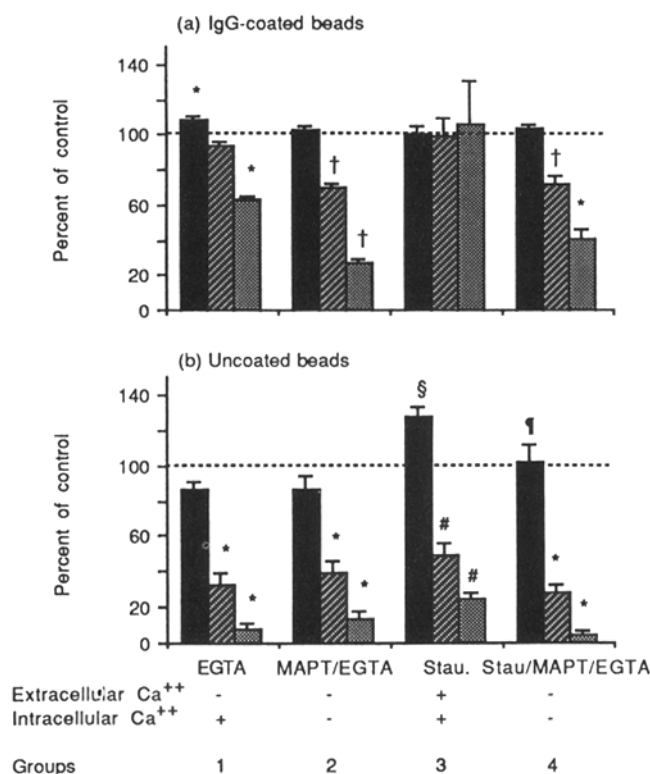
(a) IgG-coated beads

(b) Uncoated beads

	EGTA	MAPT/EGTA	Stau.	Stau/MAPT/EGTA
Extracellular Ca++	-	-	+	-
Intracellular Ca++	+	-	+	-
Groups	1	2	3	4

Figure 4. The effect of intra- and extracellular calcium and or staurosporine on phagocytosis of IgG-coated beads (*a*) and uncoated beads (*b*). Adherent macrophages were incubated in calcium-free HBSS containing 1 mM EGTA alone, or EGTA and MAPT/AM and compared with cells in HBSS containing 1 mM calcium. Additional groups of cells were treated with staurosporine in HBSS containing 1 mM calcium, or both staurosporine and MAPT/AM in calcium free HBSS containing 1 mM EGTA. Values are expressed as the mean ± SEM of separated three to six experiments. Closed bars represent binding, striped bars and shaded bars represent phagocytosing cells and phagocytic index, respectively. The asterisk (*) indicates $P < 0.05$ compared with control, and staurosporine, and (‡) indicates $P < 0.05$ compared with control, EGTA, and staurosporine. (#) indicates $P < 0.05$ compared with control, and (§) and (¶) indicate $P < 0.05$ compared with any other groups and MAPT/EGTA, respectively. Statistical analysis is done by analysis of variance. Phagocytic index was calculated as follows: No. of beads phagocytosed/No. of cells scored multiplied by percent phagocytosing cells. Control values for IgG-coated beads are 89.1, 68.6, and 129.1%, and control values for uncoated beads are 72.8, 28.4, and 12.7% for percent binding, percent phagocytosing cells and phagocytic index, respectively. The mean total number of beads bound/100 cells was as follows: IgG-coated beads, control = 238, group *1* = 272, group *2* = 251, group *3* = 237, group *4* = 253; uncoated beads, control = 153, group *1* = 113, group *2* = 110, group *3* = 231, group *4* = 137.

after phagocytosis was complete. In the latter case, many cells were counted that had bound and ingested multiple beads. Thus, intracellular calcium stores are clearly sufficient to support one or two phagocytic events for IgG-coated beads. However, extracellular calcium appears necessary for multiple phagocytic events. One possible explanation is that intracellular stores may become depleted after phagocytosis of the first one or two beads. In contrast to IgG-coated beads, reducing extracellular [Ca²⁺] caused a reduction in the per-

cent phagocytosing cells of uncoated beads and an even greater reduction in the phagocytic index (Fig. 4 *b*, group 1, $P < 0.05$). Reducing both intra- and extracellular [Ca²⁺] with MAPT/AM and EGTA caused an additional reduction in phagocytic index for IgG-coated beads that appeared to be additive (Fig. 4 *a*, compare groups *1* and *2*, $P < 0.05$), whereas there was no additional reduction for uncoated beads (Fig. 4 *b*). Phagocytosis was also assessed in two otherwise identical experiments using IgG-coated and uncoated fluoresceinated latex beads and trypan blue to quench the fluorescence of extracellular (nonphagocytosed) beads. Virtually identical results were obtained as those shown in Fig. 4, *a* and *b*). Data similar to those in Fig. 4 were obtained in two experiments with IgG-coated beads that used two other methods to reduce [Ca²⁺]ᵢ. Thus, ionomycin and EGTA caused the phagocytic index to fall to a mean value of 35% of control while ATP and EGTA caused the phagocytic index to fall to a mean value of 38% of control. In one of these experiments, phagocytosis was restored to 95% of control for ionomycin/EGTA and 73% of control for ATP/EGTA by replenishing extracellular calcium with HBSS containing 1 mM calcium.

Staurosporine had no discernible effect on the phagocytosis of IgG-coated beads in the presence of calcium (Fig. 4 *a*, group *3*) and had no statistically significant additive effect to low intra- and extracellular [Ca²⁺] (Fig. 4 *a*, compare groups 2 and *4*). In contrast, with uncoated beads, the percent phagocytosing cells and the phagocytic index were markedly reduced by staurosporine in the presence of calcium (Fig. 4 *b*, group *3*). Moreover, staurosporine appeared to have an additive effect on the inhibition of phagocytosis of uncoated beads by reduced intra- and extracellular [Ca²⁺] (Fig. 4 *b* compare groups 2 and *4*).

Discussion

The principal finding of our studies is that phagocytosis of both IgG-coated latex beads and uncoated beads is associated with a transient increase in the concentration of intracellular ionized calcium ([Ca²⁺]ᵢ). The rise in [Ca²⁺]ᵢ appears to act as a triggering mechanism for phagocytosis, based on the following observations. First, each calcium transient was invariably followed by a phagocytic event. Conversely, no phagocytic events were observed that were not preceded by a calcium transient. Depletion of [Ca²⁺]ᵢ by MAPT/AM, ionomycin, or ATP treatment with extracellular calcium removal, led to a significant reduction of phagocytosis for both IgG-coated and uncoated beads. Moreover, replenishing calcium in calcium-depleted cells restored phagocytosis in two separate observations.

Published studies concerning changes in [Ca²⁺]ᵢ during phagocytosis by macrophages have focused on Fc receptor-mediated phagocytosis and have produced conflicting results. McNeil et al. (14) did not detect an increase in [Ca²⁺]ᵢ during challenge with various kinds of ligands using aequorin, which is an even more sensitive indicator of localized [Ca²⁺] than fura-2. Their study involved populations of adherent macrophages and it is possible that temporally asynchronous calcium transients in different cells might go undetected as initially postulated by Kruskal and Maxfield (12). Young et al. reported that binding of monomeric IgG2b/1 and IgG-coated erythrocytes caused a nearly instantaneous cal-

cium transient in suspensions of J774 cells, a macrophage cell line (26). Di Virgilio et al. (8) found that binding of aggregated IgG by J774 macrophages was consistently followed by a calcium transient that occurred ~1 min after challenge whether the cells were in suspension or monolayer. Calcium transients were also detected in suspensions of thioglycollate elicited macrophages but were only variably encountered with plated (cultured) macrophages. Binding of high concentrations of monomeric IgG and aggregated IgG to Fc receptors is probably relatively rapid particularly in cell suspensions, so that calcium transients in those circumstances might occur sooner and be more synchronous than for cell populations binding particulate ligands. Lew et al. (13) observed calcium transients associated with phagocytosis of IgG- and C3b/bi-coated yeast particles by neutrophils using quin2 to measure $[Ca^{2+}]_i$. The time course of the calcium transient was slower and the magnitude smaller when compared to our results. This may have been due to the recognized calcium-buffering effect of quin2.

In the only published study of $[Ca^{2+}]_i$ in single macrophages, Kruskal and Maxfield (12) found that cells manifested oscillations in $[Ca^{2+}]_i$ of 29 s mean duration (range 19–69 s) during spreading of macrophages on glass coverslips. Spreading was used as a model of frustrated phagocytosis. The onset of oscillations in $[Ca^{2+}]_i$ appeared to precede spreading and oscillation could persist for up to 10–15 min (12). We did not observe oscillations in $[Ca^{2+}]_i$ for our cells studied after 24 h of culture. The design of our experiments to capture both bright field and fluorescence images of the same cell undergoing phagocytosis does not allow as rapid tracking of $[Ca^{2+}]_i$ changes as in our studies designed for much different purposes (5). Thus, rapid oscillations of <24 s would have likely been missed. Similarly, the time frame of our data acquisition might have missed oscillations in $[Ca^{2+}]_i$ of very low frequency. However, our system would not have missed oscillations of the duration observed by Kruskal and Maxfield.

While both Fc receptor-mediated and nonspecific phagocytosis required calcium, our data suggest that the signaling mechanism may differ. First, the presence of extracellular calcium is an absolute requirement for nonspecific phagocytosis. The longer duration and lower peak magnitude of calcium transients for uncoated beads are also most consistent with an extracellular source of calcium for nonspecific phagocytosis. In contrast, Fc receptor-mediated phagocytosis proceeds via mobilization of intracellular calcium stores, although repeated phagocytic events cannot be sustained as evidenced by a reduction in the phagocytic index. This latter observation may be due to depletion of intracellular stores in the prolonged absence of extracellular calcium. Finally, we observed differential sensitivity of phagocytosis (Fig. 4) to protein kinase C (PKC) inhibition by staurosporine (17, 23). Our observations suggest that there may be two mechanisms of phagocytosis in elicited murine macrophages: a $[Ca^{2+}]_i$-dependent but PKC-independent mechanism triggered by Fc receptor occupancy, and an extracellular calcium and PKC-dependent mechanism for nonspecific phagocytosis.

Our finding that Fc receptor-mediated phagocytosis in macrophages is dependent on $[Ca^{2+}]_i$ is in agreement with the findings of Lew et al. who used IgG-coated yeast particles to study calcium requirements in neutrophils (13). In

their study, they also found that phagocytosis of C3b/bi-coated yeast particles was not inhibited by buffering $[Ca^{2+}]_i$ with quin2. Lowering extracellular $[Ca^{2+}]$ tended to reduce phagocytosis of C3b/bi opsonized, endotoxin-coated oil droplets and buffering $[Ca^{2+}]_i$ with quin2 had no additive inhibitory effect. These findings for C3b/bi-mediated phagocytosis are analogous with our findings with uncoated latex beads, although inhibition of nonspecific phagocytosis by reducing extracellular $[Ca^{2+}]$ was more complete in our study.

Di Virgilio et al. (8) observed a 45% reduction in the phagocytic index and a smaller fall in percent phagocytosing cells when $[Ca^{2+}]_i$ was reduced by quin2; they attributed these changes to the "toxic" effects of quin2, but the buffering effect on $[Ca^{2+}]_i$ may have accounted for the reduced phagocytosis. Lowering extracellular $[Ca^{2+}]$ had no additive effect under these conditions. These authors also compared a slight lowering of $[Ca^{2+}]_i$ to 85 nM from approximately 120 nM (measured with fura-2) with a more pronounced reduction to 10 nM using ATP treatment of cells and EGTA or calcium-free buffers. They found no difference in the percent phagocytosing cells or the phagocytic index; however, an ATP-untreated control was not included for that experiment. The quantitative differences between their findings and ours may be explained in part by other methodological differences. In contrast to our elicited murine macrophages which did not "secrete" intracellularly trapped fura-2 (Materials and Methods), J774 macrophages and thioglycollate-elicited peritoneal macrophages rapidly leaked fura-2 into the medium such that the use of probenecid was necessary in their experiments. Second, they calibrated fura-2 after it was released into the medium by cell lysis because of acknowledged technical difficulties they encountered with ionomycin when attempting to calibrate the dye while intracellular. Such in vitro calibration may lead to an underestimation of $[Ca^{2+}]_i$ and to masking of calcium transients (19).

In summary, using single cell as well as population studies, we have identified two independent signaling mechanisms for phagocytosis in elicited murine macrophages: a $[Ca^{2+}]_i$-dependent and PKC-independent mechanism for Fc receptor-mediated phagocytosis, and an extracellular Ca^{2+}- and PKC-dependent mechanism for nonspecific phagocytosis. An attractive hypothesis is that Fc receptor-mediated phagocytosis uses the well-known inositol 1,4,5-trisphosphate signaling pathway (2, 3) while nonspecific phagocytosis depends on activation of plasma membrane Ca^{2+} channels, presumably via PKC, which is known to modulate Ca^{2+} channel activity (1, 24).

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