Fc Receptor–mediated Phagocytosis Occurs in Macrophages at Exceedingly Low Cytosolic Ca^{2+} Levels

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Abstract. Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) homeostasis was investigated in mouse peritoneal macrophages and in the macrophage-like cell line J774. $[Ca^{2+}]_i$ measurements were performed in both cells in suspension and cells in monolayers loaded with either quin2 or fura-2. Resting $[Ca^{2+}]_i$ was 110–140 and 85–120 nM for cell suspensions and monolayers, respectively. There were no significant differences in $[Ca^{2+}]_i$ between the two macrophage populations whether quin2 or fura-2 were used as Ca^{2+} indicators. Addition of heat-aggregated IgG, IgG-coated erythrocyte ghosts, or a rat monoclonal antibody (2.4G2) directed against mouse Fc receptor II induced a rise in $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$ increase was consistently observed in J774 and peritoneal macrophage suspensions and in J774 macrophage monolayers; in contrast it was observed inconsistently in peritoneal macrophages in monolayer cultures. The increase in $[Ca^{2+}]_i$ induced by ligation of Fc receptors was inhibited totally in macrophages in suspension and by 80% in macrophages in monolayers by a short preincubation of macrophages with PMA; however, phagocytosis itself was unaffected. The effect of reducing cytosolic Ca^{2+} to very low concentrations on Fc receptor-mediated phagocytosis was also investigated. By incubating macrophages with high concentrations of quin2/AM in the absence of extracellular Ca^{2+}, or by loading EGTA into the cytoplasm, the $[Ca^{2+}]_i$ was buffered and clamped to 1–10 nM. Despite this, the phagocytosis of IgG-coated erythrocytes proceeded normally. These observations confirm the report of Young et al. (Young, J. D., S. S. Ko, and Z. A. Cohn. 1984. *Proc. Natl. Acad. Sci. USA.* 81:5430–5434) that ligation of Fc receptors causes Ca^{2+} mobilization in macrophages. However, these results confirm and extend the findings of McNeil et al. (McNeil, P. L., J. A. Swanson, S. D. Wright, S. C. Silverstein, and D. L. Taylor. 1986. *J. Cell Biol.* 102:1586–1592) that a rise in $[Ca^{2+}]_i$ is not required for Fc receptor–mediated phagocytosis; and they provide direct evidence that Fc receptor–mediated phagocytosis occurs normally even at exceedingly low $[Ca^{2+}]_i$.

PHAGOCYTOSIS by macrophages is initiated when particles coated with appropriate ligands (e.g., IgG) bind to the corresponding receptors (e.g., Fc receptors) on the cells' plasma membrane (25). The mechanism by which Fc receptor ligation signals pseudopod extension and particle engulfment is not known. The activities of cytoskeletal proteins presumably involved in pseudopod extension, such as actin and gelsolin, are regulated in vitro by physiological calcium concentrations (0.1–10 μM) (27, 29). Therefore several investigators (13, 34, 35) have suggested that changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] initiated by ligation of cell surface receptors might regulate the function of cytoskeletal proteins that effect pseudopod movement. The involvement of Ca^{2+} in phagocytosis, however, is still a matter of controversy. Lew and colleagues reported that Fc receptor–mediated phagocytosis in human neutrophils is accompanied by an elevation in $[Ca^{2+}]_i$ (13), but McNeil et al. were unable to confirm this observation in mouse macrophages (16). Lew et al. also showed that clamping of $[Ca^{2+}]_i$ at very low (1 nM) or high (>10 μM) levels greatly reduced Fc receptor–mediated phagocytosis while it did not affect phagocytosis mediated by the C3b/C3bi receptor (13). A significant difference between the studies of Lew et al. and those of McNeil et al., besides the use of two different cell types, was that in the former case the particles were presented to phagocytes in suspension while in the latter the phagocytes were plated on glass coverslips. Furthermore, Lew et al. measured $[Ca^{2+}]_i$ changes with the fluorescent indicator quin2, while McNeil et al. loaded the macrophages with aequorin by scrape loading.

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1. *Abbreviations used in this paper*: Agg. IgG or aggregated IgG, heat-aggregated immunoglobulins; $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; DMI0F, Dulbecco's modified Eagle medium containing 10% heat-inactivated FBS; DTPA, diethylenetriaminepentaacetic acid; IgG-ghosts, IgG-coated erythrocyte ghosts; IgG–RBC, IgG-coated erythrocytes; PAF, platelet-activating factor; thio-macrophages, thio-glycollate broth–elicited mouse peritoneal macrophages; TPEN, *N,N,N′,N′*,-tetrakis (2-pyridymethyl)ethylenediamine.

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In this paper we describe the results of a further examination of the role of $[Ca^{2+}]_i$ in phagocytosis in primary macrophages obtained from inflammatory exudates from the mouse peritoneum, and in the macrophage-like cell line J774. $[Ca^{2+}]_i$ changes were measured with the fluorescent indicators quin2 and fura-2. Experiments were performed with macrophages both in suspension and plated on glass coverslips. We used as agonists IgG-coated erythrocyte ghosts, heat-aggregated IgG (Agg. IgG), and the monoclonal antibody 2.4G2 which is directed against macrophage Fc receptors for aggregated IgG (21). In agreement with the report of Young et al. (35), these ligands caused an increase in $[Ca^{2+}]_i$ when added to peritoneal macrophages and J774 macrophages in suspension. A rise in $[Ca^{2+}]_i$ was also induced when aggregated IgG was added to J774 macrophages in monolayer culture. However, a rise in $[Ca^{2+}]_i$ played no apparent role in phagocytosis of IgG-coated erythrocytes since it was possible to abolish completely the $[Ca^{2+}]_i$ increase stimulated by Fc receptor ligation without blocking Fc receptor-mediated phagocytosis. Moreover, phagocytosis was unaffected by reduction of $[Ca^{2+}]_i$ to levels (10^{-9}–10^{-8} M) at which most Ca^{2+}-dependent reactions do not occur (6).

Materials and Methods

Cells

Thioglycollate-elicited mouse macrophages (thio-macrophages) were obtained as described previously (30). Briefly, female white mice (Institute for Cancer Research, Trudeau Institute, Saranac Lake, NY) were injected intraperitoneally with thioglycollate broth 4 d before harvest. Mice were sacrificed and their peritoneal cavities were rinsed with 5 ml cold divalent cation-free phosphate-buffered saline (PD). Cells suspended in PD were washed twice by centrifugation, and resuspended in Dulbecco's modified Eagle medium containing 10% heat-inactivated FBS (DM10F) at a concentration of 5–10 × 10^6 cells/ml. The cells were then either plated on glass coverslips or kept in suspension in polypropylene tubes on ice. Recovery of thio-macrophages kept in suspension was >90%. The macrophage suspensions were used within 4 h and the monolayers within 24 h from the time of harvest.

J774 macrophages were cultured in suspension at 37°C in DM10F. They were harvested, centrifuged, and suspended at a concentration of 10^7 cells/ml, and either plated on glass coverslips or kept in suspension until used. J774 monolayers were used 12–18 h after the plating.

All experiments with both J774 and thio-macrophages were carried out in a saline medium containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM $NaHCO_3$, 20 mM Hepes, pH 7.4. This medium is referred to as standard saline throughout this work. Ca^{2+}-free medium is standard saline from which $CaCl_2$ was omitted.

Loading Cells with quin2 and fura-2

As previously reported, fura-2 in macrophages is secreted into the extracellular medium and sequestered within cytoplasmic vacuoles (7). These processes are especially rapid in J774 macrophages, less so in thio-macrophages. We have shown that probenecid, an inhibitor of anion transport, inhibits both secretion and sequestration of fura-2 without any untoward effects on resting and stimulated $[Ca^{2+}]_i$ or on phagocytosis. Therefore we added 2.5 mM probenecid to the standard saline, and measurements of $[Ca^{2+}]_i$ and phagocytosis were performed in the presence of probenecid. Resting $[Ca^{2+}]_i$ was slightly higher (10–20%) in the probenecid-treated cells compared with controls. Secretion of quin2 into the extracellular medium did not seem to be a problem and we could not establish whether quin2 was sequestered intracellularly similarly to fura-2 because of the very dim fluorescence visible in quin2-loaded cells. Nonetheless we also used 2.5 mM probenecid when quin2 was used as a Ca^{2+} indicator.

Macrophage suspensions were loaded in DM10F containing 30 μM quin2 acetoxymethylester (quin2/AM) or 5 μM fura-2 acetoxymethylester (fura-2/AM) according to standard procedures (6, 7, 20, 32). The amount of quin2 taken up into the cells varied between 200 and 300 and between 300 and 400 pmoles/10^6 cells for thio and J774 macrophages, respectively. For fura-2, the amounts were 200 and 60 pmoles/10^6 cells for thio and J774 macrophages, respectively.

For measurements of $[Ca^{2+}]_i$ of macrophages in confluent monolayers, 4 × 10^5 cells were plated on rectangular (24 × 10 mm) No. 1 glass coverslips in 60-mm petri dishes. Cells were loaded in DM10F containing 30 μM quin2/AM or 5–10 μM fura-2/AM and 2.5 mM probenecid. After 30 min of incubation at 37°C in a 5% CO_2 atmosphere, coverslips were rinsed, transferred to fresh DM10F medium containing 2.5 mM probenecid and 20 mM Hepes and kept at room temperature until used (usually 1–2 h).

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

Fluorescence measurements were performed in a fluorescence spectrophotometer (model 650-40; Perkin-Elmer Corp., Norwalk, CT) equipped with a thermostatically controlled cuvette holder and magnetic stirrer. $[Ca^{2+}]_i$ with quin2 and fura-2 was measured in macrophage suspensions at 37°C as described (6, 32). The excitation/emission wavelength pair used to monitor quin2 fluorescence was 339:492 and that used for fura-2 was 340:500. $[Ca^{2+}]_i$ was calculated according to the general formula (32):

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F),$$

where the K_d is the dissociation constant for Ca^{2+} binding, 115 and 224 nM for quin2 and fura-2, respectively; F is the fluorescence of the intracellular indicator; F_{min} is the fluorescence after lysis of the cells with 0.05% Triton X-100 in the presence of 5 mM EGTA and 40 mM Tris (final pH 8.5); and F_{max} is the fluorescence of the lysed cells after addition of 5 mM $CaCl_2$. Changes in intrinsic fluorescence of unloaded cells (autofluorescence) caused by the various additions (especially Triton) were taken into account in the calculation of $[Ca^{2+}]_i$.

In fura-2-loaded macrophages, resting $[Ca^{2+}]_i$ was also calculated by measuring the fluorescence intensity (500 nm emission) with excitation at 340 and 385 nm as described by Grynkiewicz et al. (11) and according to the following equation:

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)\ (F_o/F_s),$$

where K_d is 224 nM, R is the 340:385 ratio of fluorescence of the intracellular indicator, R_{min} is the 340:385 ratio of fura-2 at 1 nM Ca^{2+}, R_{max} is the 340:385 ratio of fura-2 fluorescence intensity in the presence of saturating Ca^{2+} concentrations, and F_o/F_s is the ratio of fura-2 fluorescence emission at 1 nM Ca^{2+} and at saturating Ca^{2+} concentrations (excitation 385 nm). No correction was applied to allow for the effect of cytoplasmic microviscosity, therefore our values may slightly (10–15%) underestimate the true $[Ca^{2+}]_i$ (19). However there was good overall agreement between determinations of $[Ca^{2+}]_i$ performed with fura-2 and quin2 and by Triton lysis and 340:385 ratio.

To minimize light scattering coverslips were placed into the fluorimeter

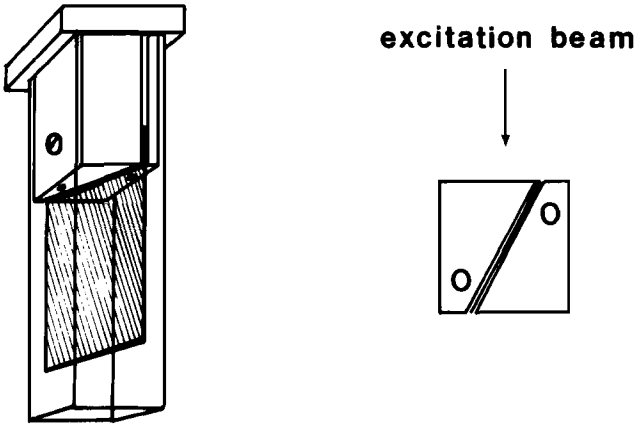

Figure 1. Schematic drawing of the device used to hold coverslips in the fluorimeter cuvette. Side view (*left*), bottom view (*right*). The coverslip (*cross-hatched*) was aligned at 30° to the excitation beam to minimize the effect of light scattering. Additions to the cuvette were performed with a microsyringe through the ports in the teflon plug.

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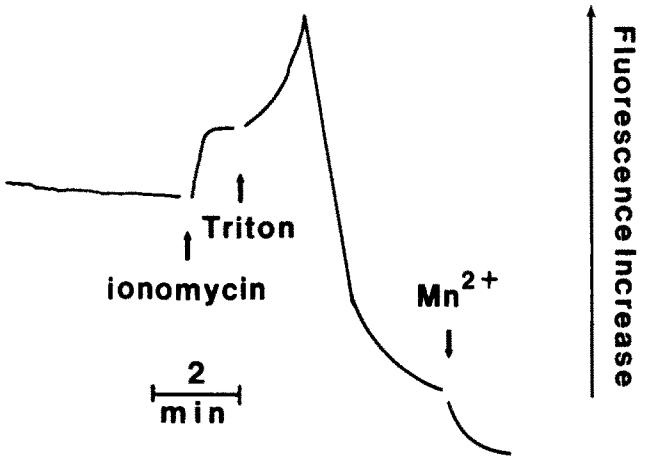

Figure 2. Calibration of $[Ca^{2+}]_i$ in adherent J774 macrophages by lysis of the cells with Triton X-100. J774 macrophages on coverslips were loaded with quin2 as described in Materials and Methods. A similar calibration procedure was followed with fura-2-loaded macrophages. Ionomycin was 2 μM; Triton X-100, 0.025%; Mn^{2+}, 200 μM.

cuvette at an angle of 30° to the exciting light beam; this was preferable to an angle of 45°. Coverslips were kept in position by a teflon coverslip holder designed for this purpose (Fig. 1). Coverslips were inserted in the slot and secured by tightening the screw on the side of the plug. The coverslip was suspended ~1 cm above the bottom of the cuvette to allow efficient stirring of the medium with a 2 × 7 mm magnetic bar. Two ports in the teflon holder permitted additions to the solution in the cuvette without repeatedly opening the cuvette. This minimized equilibration of the solution in the cuvette with the external atmosphere, thus preventing pH changes due to loss of CO_2 from the $NaHCO_3$-containing solution routinely used for the experiments. Calibration of $[Ca^{2+}]_i$ was performed as described by Moolenar et al. and by Conrad and Rink (3, 18). We also tried to calibrate $[Ca^{2+}]_i$ with ionomycin and $MnCl_2$ but this method proved to be very unreliable in our hands since, as shown in Fig. 2, ionomycin did not give a maximal increase in fluorescence. Therefore $[Ca^{2+}]_i$ was calibrated by adding 0.025% Triton X-100 to the cuvette, followed by 200 μM $MnCl_2$ (Fig. 2). The addition of Triton caused a fast increase in fluorescence due to the initial permeabilization of the plasma membrane to external Ca^{2+}. This increase in fluorescence was followed by a decrease due to diffusion of the dye out of the cells. Finally the addition of 100 μM $MnCl_2$ quenched the fluorescence of the indicator. $MnCl_2$ had no effect on autofluorescence. The residual fluorescence signal was a measure of autofluorescence. F_{min} was calculated according to the following equation:

$$F_{min} = F_{Mn^{2+}} + \alpha(F_{max} - F_{Mn^{2+}}),$$

where $F_{Mn^{2+}}$ is fluorescence after the addition of Mn^{2+} and α is 0.2 and 0.416 for quin2 and fura-2, respectively, under our experimental conditions. We also tested digitonin as a permeabilizing agent, but eventually chose Triton X-100 because the initial permeabilization (and therefore the rise in fluorescence) was faster with Triton X-100. We acknowledge that this calibration procedure may lead to a variable underestimation of F_{max}. However $[Ca^{2+}]_i$ determined by Triton X-100 lysis did not differ significantly from that determined with the 340:385 ratio in the very same fura-2-loaded macrophages. Correction for external dye was not needed in our experiments since probenecid prevented the release of the indicator from the cells (7).

Quenching of quin2 and fura-2 by Heavy Metals

The extent of quenching of quin2 and of fura-2 by intracellular heavy metals depends on the intracellular dye content; therefore it had to be determined for each batch of cells. At the beginning of each experiment the potential quenching of the intracellular dye was checked by adding 20 μM *N,N,N',N'*-tetrakis (2-pyridymethyl) ethylenediamine (TPEN), a membrane-permeant chelator of heavy metals (1) in the presence of 100 μM diethylenetriaminepenta acetic acid (DTPA). If the quenching was found to be significant (>5–10% of the intracellular dye), TPEN and DTPA were added to the medium. The presence of DTPA + TPEN is indicated in the figure and table legends.

Stimulants

Sheep erythrocytes were coated with rabbit anti-sheep red blood cell IgG (Cordis Laboratories, Inc., Miami, FL) as previously described (30). Erythrocyte ghosts were prepared from IgG-coated or uncoated erythrocytes by osmotic lysis followed by resealing in standard saline. Heat-aggregated IgG was prepared according to standard procedures (5). Monoclonal antibody 2.4G2 was a kind gift of Dr. J. Unkeless (Mount Sinai School of Medicine, New York).

Phagocytosis

IgG-coated erythrocytes were resuspended in standard saline containing 2.5 mM probenecid at an hematocrit of 0.5% and kept on ice. Fura-2 or quin2-loaded macrophage monolayers were placed on ice for 10 min, at which time an ice-cold suspension of IgG-coated erythrocytes was added for an additional 10 min. The coverslip cultures were then incubated in saline at 37°C for 20 min and washed with ice-cold saline. Uningested erythrocytes were lysed with ice-cold distilled water and ingested erythrocytes counted using a Zeiss phase-contrast microscope and a 40× objective. At least 100 macrophages were counted in three different microscopic fields for each coverslip.

Plasma Membrane Permeabilization

The plasma membranes of J774 and thio-macrophages were made permeable to aqueous solutes of molecular weight up to 830 by a brief treatment with ATP, as previously described (28). Cell monolayers were incubated for 5 min at 37°C in Ca^{2+}-free standard saline (1 mM Mg^{2+}), pH 7.8, with or without EGTA, after which they were washed and resuspended in DM10F or EGTA-containing DM10F. After a recovery time of 10 min, macrophages were washed again and presented with IgG-coated erythrocytes.

Chemicals

Fura-2/AM, fura-2 pentapotassium salt (fura-2), and TPEN were obtained from Molecular Probes, Inc., Eugene, OR; DTPA, platelet activating factor (PAF), and probenecid were from Sigma Chemical Co., St. Louis, MO; quin2/AM and quin2 tetrapotassium salt were from Calbiochem-Behring Corp., San Diego, CA. All other chemicals were of analytical grade.

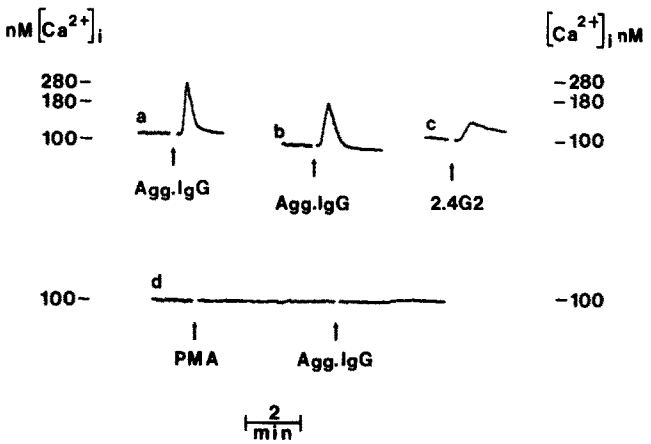

Figure 3. Ligation of Fc receptors causes an increase in $[Ca^{2+}]_i$ of J774 macrophages in suspension. Fura-2-loaded J774 macrophages were suspended in standard saline at a concentration of 2.5 × 10^6 cells/ml. *Trace a*, 500 μg/ml aggregated IgG; *trace b*, 500 μg/ml aggregated IgG in saline containing 1 mM EGTA; *trace c*, 20 μg/ml mAb 2.4G2; *trace d*, 500 μg/ml aggregated IgG in saline containing 60 nM PMA.

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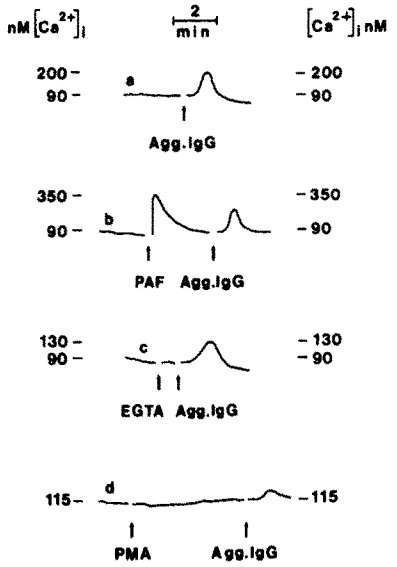

Figure 4. Ligation of Fc receptors causes an increase in $[Ca^{2+}]_i$ in substrate adherent J774 macrophages. 4×10^5 J774 macrophages were plated on coverslips and kept in culture for 12 h. They were then loaded with fura-2 as described in Materials and Methods, rinsed, and placed in standard saline. Aggregated IgG was 500 μg/ml; PAF, 40 ng/ml; EGTA, 1 mM; PMA, 60 nM.

Results

Ligation of Fc Receptors Is Linked to Ca^{2+} Mobilization in J774 Macrophages

J774 macrophages in suspension and monolayer culture responded to ligation of their Fc receptors with an increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ was minimally affected by chelation of external Ca^{2+} by EGTA (compare Fig 3, *a* and *b*) and therefore was due mainly to release of Ca^{2+} from intracellular stores. Monoclonal anti-Fc receptor IgG 2.4G2 also caused a clear albeit small rise in $[Ca^{2+}]_i$ (Fig. 3 *c*). The transient rise in $[Ca^{2+}]_i$ caused by ligation of Fc receptors was totally abolished by a 5-min preincubation of the J774 cells in suspensions with PMA (Fig. 3 *d*). Addition of aggregated IgG to J774 macrophages in monolayer cultures on glass coverslips induced a transient rise in $[Ca^{2+}]_i$ (Fig. 4, *trace a*). The rate of increase in $[Ca^{2+}]_i$ induced by aggregated IgG was slow when compared with that induced by PAF (Fig. 4, *trace b*), and was preceded by a lag phase of 20–30 s. Previous stimulation with PAF had no detectable effect on the rise in $[Ca^{2+}]_i$ caused by aggregated IgG. Chelation of external Ca^{2+} had no effect on the size or speed of the Ca^{2+} response (Fig. 4, *trace c*).

Table I reports average resting $[Ca^{2+}]_i$ in J774 cells in monolayers and suspensions. Resting $[Ca^{2+}]_i$ was similar whether measured with quin2 or fura-2. $[Ca^{2+}]_i$ calculated from the ratio of the fluorescence intensity at 340:385 (emission 500 nm) in fura-2-loaded macrophages was not significantly different from the values determined by Triton lysis as described in Materials and Methods. Resting $[Ca^{2+}]_i$ was 25% lower in J774 macrophage monolayers compared with the same cells in suspension. This difference, though small, was observed consistently. Our $[Ca^{2+}]_i$ values for J774 macrophage suspensions are ~40% higher than those reported by Young et al. in these same cells (35). These authors, however, did not estimate quin2 quenching by heavy metals, which was likely to have had a significant effect under their experimental conditions (low intracellular quin2 concentration). As indicated in Table I, ligation of Fc receptors caused an increase in $[Ca^{2+}]_i$ to ~200–250 nM in J774 macrophages maintained in suspension, whether measured with quin2 or with fura-2. The increase in $[Ca^{2+}]_i$ in J774 macrophage monolayers was 30–40% less than that measured in the same cells in suspension.

Table I. Resting $[Ca^{2+}]_i$ and Peak Increases in $[Ca^{2+}]_i$ Induced by Ligation of Fc Receptors in J774 Macrophages

		Stimulated	
	Resting	IgG-ghosts	Agg. IgG
	nM	*nM*	*nM*
Suspension			
quin2	110 ± 20 (8)	170 ± 5 (3)	180 ± 10 (4)
fura-2	140 ± 20 (18)		290 ± 30 (7)
fura-2*	145 ± 30 (10)		
Monolayer			
quin2	90 ± 15 (3)		
fura-2	100 ± 30 (7)		200 ± 60 (8)
fura-2*	90 ± 20 (13)		

Cells were loaded with quin2/AM or fura-2/AM and $[Ca^{2+}]_i$ was measured in the presence of 100 μM DTPA and 20 μM TPEN as described in Materials and Methods. Numbers in parenthesis indicate the number of experiments performed. Cell concentration for macrophage suspensions was 2.5×10^6/ml. For $[Ca^{2+}]_i$ measurements of cells in monolayers, macrophages were plated at a density of 4×10^5 per coverslip.
* indicates values obtained from the 340:385 ratio.

Phagocytosis in J774 Macrophages Occurs in the Absence of a Detectable Change in $[Ca^{2+}]_i$

To determine whether the transient elevations in $[Ca^{2+}]_i$ induced by activation of Fc receptors played a role in Fc receptor-mediated phagocytosis, we examined phagocytosis in J774 macrophages in the absence of external Ca^{2+} and after depletion of the intracellular Ca^{2+} stores. Intracellular Ca^{2+} stores were depleted as described previously (8) by pretreating the cells with ionomycin in the absence of external Ca^{2+} and in the presence of EGTA. The addition of aggregated IgG

Table II. Fc Receptor-mediated Phagocytosis Occurs in J774 Macrophages in the Absence of Mobilization of Ca^{2+} from Intracellular Stores

	A	B	C	D
% macrophages ingesting IgG-RBC	92%	86%	86%	90%
IgG-RBC ingested/macrophage	8.7	6.7	7.7	7.6
Phagocytic index	793	580	667	680

3×10^5 J774 cells were plated on round glass coverslips. Macrophages were washed and kept throughout the phagocytic assay in standard saline containing the following: A, 10 mM EGTA and 1 μM A23187; B, standard saline containing 10 mM EGTA; C, standard saline containing 1 mM Ca^{2+}. In D macrophages were incubated for 5 min in standard saline containing 10 mM EGTA and 1 μM A23187, washed in DM10F plus 10 mM EGTA to remove the Ca^{2+} ionophore and then assayed for phagocytosis in standard saline plus 10 mM EGTA. Data are the average of triplicate determinations from a single experiment. Similar results were obtained on three separate occasions with three different cell preparations.

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to macrophages pretreated with ionomycin in the presence of EGTA failed to induce a rise in $[Ca^{2+}]_i$, confirming that intracellular Ca^{2+} stores had been depleted (not shown). Since extracellular Ca^{2+} was also lacking, we were able to investigate cellular responses at constant resting $[Ca^{2+}]_i$ levels. Table II shows that depletion of intracellular Ca^{2+} by this method had no inhibitory effect on phagocytosis.

Moreover, while pretreatment of J774 macrophages with PMA blunted (Fig. 4), or abolished (Fig. 3) changes in $[Ca^{2+}]_i$ stimulated by ligation of the Fc receptors, it did not inhibit Fc receptor-mediated phagocytosis (Table III).

To test the effect of depletion of $[Ca^{2+}]_i$ on phagocytosis we loaded J774 macrophages with high concentrations of quin2 in the presence of external EGTA or permeabilized their plasma membranes with ATP^{4-} in the presence of EGTA. The first approach, initially proposed by Tsien and colleagues (32) and later extended by other investigators (6, 13), exploits the Ca^{2+}-buffering power of quin2 and allows one to lower $[Ca^{2+}]_i$ 10–20-fold below resting levels. The second approach takes advantage of our observation that J774 macrophages can be made permeable to low molecular weight water soluble substances of <830 mol wt when these cells are exposed to micromolar concentrations of ATP^{4-} (28). As shown by Gomperts for mast cells (10) and by Steinberg et al. for J774 macrophages (28), the lesions generated by ATP^{4-} can be resealed simply by removing ATP^{4-} from the medium, thus trapping within the cytoplasm any molecule that had gained access to the intracellular space during treatment with ATP^{4-}. In order for EGTA or quin2 to be effective buffers of $[Ca^{2+}]_i$ two conditions must be fulfilled: (*a*) the chelators must be in excess of the intrinsic cytoplasmic molar buffers for Ca^{2+}; (*b*) the chelators must be uniformly distributed throughout the cytoplasm. Both requirements were met under our experimental conditions by loading the macrophages with high EGTA or quin2/AM concentrations and performing the phagocytosis assay in the presence of probenecid, as detailed in the Materials and Methods section (see also Figs. 7 and 9).

J774 macrophages in monolayer culture were loaded with quin2 in the presence of EGTA. This treatment lowered $[Ca^{2+}]_i$ to almost undetectable levels. Addition of 2 μM ionomycin to quin2-loaded macrophages did not cause any change in $[Ca^{2+}]_i$, thus demonstrating that the internal Ca^{2+} stores were depleted fully (Fig. 5). In cells that had been loaded with EGTA, confirmation that intracellular Ca^{2+} had been chelated was obtained by measuring $[Ca^{2+}]_i$ with fura-2 after the ATP^{4-} permeabilization. $[Ca^{2+}]_i$ in cells permeabilized in the presence of EGTA was 10–15 nM while $[Ca^{2+}]_i$ in cells permeabilized in Ca^{2+}-free medium in the absence of EGTA was 70–80 nM (Table V). Addition of aggregated IgG (Fig. 6) or of ionomycin (not shown) to the EGTA and fura-2-loaded J774 cells failed to cause any increase in fura-2 fluorescence, thus showing that the cells had been effectively depleted of mobilizable Ca^{2+}.

Fig. 7 shows phase-contrast and fluorescence photomicrographs of a monolayer of J774 cells permeabilized with ATP^{4-} in medium containing 10 mM EGTA and Lucifer Yellow (a fluorescent dye that is normally membrane imper-

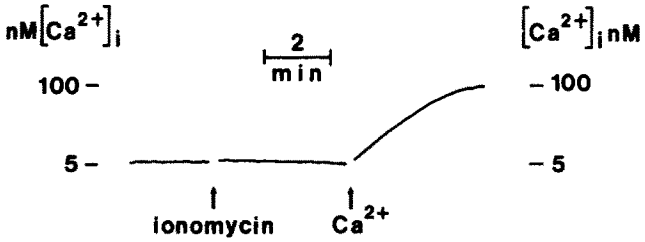

Figure 5. Loading of adherent J774 macrophages with quin2 in Ca^{2+}-free, EGTA-containing medium decreases $[Ca^{2+}]_i$ to 5 nM. 4 × 10^5 J774 cells were plated on coverslips and kept in culture for 12 h. They were then loaded with 50 μM quin2/AM in DM10F plus 10 mM EGTA, rinsed, and incubated in Ca^{2+}-free, EGTA-containing (1 mM) standard saline. Ionomycin was 2 μM; added Ca^{2+} was 2 mM. Intracellular quin2 concentration was ~1.5 mM.

Table III. Preincubation with PMA Does Not Inhibit Fc Receptor-mediated Phagocytosis in J774 Macrophages

	A	B
% macrophages ingesting IgG-RBC	98%	99%
IgG-RBC ingested/macrophage	14	13
Phagocytic index	1,350	1,300

A, control; B, macrophages preincubated with 60 nM PMA for 5 min and kept in the presence of PMA throughout the phagocytic assay.

Table IV. Chelation of $[Ca^{2+}]_i$ with quin2 Does Not Inhibit Fc Receptor Phagocytosis in J774 Macrophages

	A ($[Ca^{2+}]_i$ = 1 nM)	B ($[Ca^{2+}]_i$ = 70 nM)	C (control)
% macrophages ingesting IgG-RBC	85%	85%	90%
IgG-RBC ingested/macrophage	7.5	6	8
Phagocytic index	662	536	727

J774 macrophages were plated on round glass coverslips (3 × 10^4 per coverslip) for phagocytosis assays and on rectangular glass coverslips (4 × 10^5 per coverslip) for $[Ca^{2+}]_i$ measurements. For A and B, phagocytosis assays and $[Ca^{2+}]_i$ measurements were performed in parallel. A, macrophages were loaded with quin2/AM in Ca^{2+}-free, EGTA-containing (1 mM) medium and kept in the same medium throughout the experiment. Phagocytosis was assayed in standard saline supplemented with 1 mM EGTA. B, macrophages were loaded with quin2/AM in standard saline containing 1 mM Ca^{2+}. Phagocytosis was assayed in standard saline. C, phagocytosis was assayed in unloaded cells. Data are the average of triplicate determinations from a single experiment repeated with three different cell preparations.

Table V. Chelation of $[Ca^{2+}]_i$ with EGTA Does Not Inhibit Fc Receptor Phagocytosis in J774 Macrophages

	A ($[Ca^{2+}]_i$ = 10 nM)	B ($[Ca^{2+}]_i$ = 85 nM)
% macrophages ingesting IgG-RBC	90%	85%
IgG-RBC ingested/macrophage	4	4
Phagocytic index	351	363

J774 macrophages were plated on rectangular coverslips at a concentration of 2 × 10^6/ml. $[Ca^{2+}]_i$ (determined with 340:385 ratio) was measured in the same macrophage monolayers that were used for the phagocytic assays. Macrophages were permeabilized at 37°C with 5 mM ATP in Ca^{2+}-free medium plus 10 mM EGTA (A), or Ca^{2+}-free medium alone (B). After 5 min the coverslips were rinsed with DM10F supplemented with 10 mM EGTA (A) or DM10F alone (B), and loaded with fura-2/AM as described in Materials and Methods. Data are the average of triplicate determinations from a single experiment repeated with three different cell preparations.

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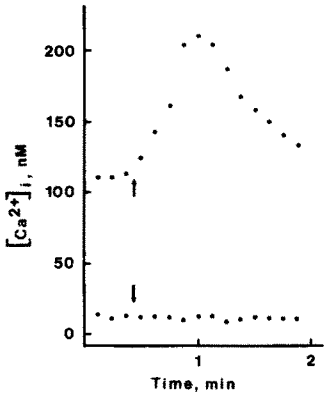

Figure 6. Loading of adherent J774 macrophages with EGTA abolishes the rise in $[Ca^{2+}]_i$ induced by aggregated IgG. J774 macrophages plated on rectangular coverslips at a concentration of 2×10^6/ml, were permeabilized at 37°C with 5 mM ATP in Ca^{2+}-free medium or Ca^{2+}-free medium plus 10 mM EGTA. After 5 min, coverslips were rinsed with DM10F or DM10F supplemented with 10 mM EGTA and loaded with fura-2/AM as described in Materials and Methods. $[Ca^{2+}]_i$ was calibrated according to the 340:385 ratio which was accomplished by repeated switching of the monochromator wavelengths. (*Upper tracing*) Control cells incubated in permeabilization buffer without EGTA. $[Ca^{2+}]_i$ measurements performed in standard saline with 1 mM Ca^{2+}. (*Lower tracing*) Cells incubated in permeabilization buffer supplemented with 10 mM EGTA. $[Ca^{2+}]_i$ measurements performed in Ca^{2+}-free standard saline in the presence of 1 mM EGTA. Arrows denote the addition of 500 μg/ml Agg. IgG.

Table VI. Resting $[Ca^{2+}]_i$ and Peak Increases in $[Ca^{2+}]_i$ Induced by Ligation of Fc Receptors in Thio-Macrophages

	Resting	Stimulated	
		IgG-ghosts	Agg. IgG
	nM	*nM*	*nM*
Suspension			
quin2	120 ± 20 (8)	200 ± 30 (4)	250 ± 70 (5)
fura-2	125 ± 30 (18)		320 ± 30 (5)
fura-2*	110 ± 30 (10)		
Monolayer			
quin2	85 ± 20 (10)		
fura-2	95 ± 30 (6)		Variable
fura-2*	120 ± 20 (9)		

$[Ca^{2+}]_i$ was measured as described in Materials and Methods in the presence of 100 μM DTPA and 20 μM TPEN. Numbers in parenthesis indicate the number of experiments performed. Cell concentration for macrophage suspensions was 10^6/ml. For $[Ca^{2+}]_i$ measurements in monolayers, macrophages were plated at a density of 4×10^5 per coverslip.
* indicates values obtained from the 340:385 ratio.

meant) and then resealed and presented with IgG-coated erythrocytes. Almost all the macrophages were brightly and uniformly fluorescent, thus confirming that the plasma membrane had been rendered permeabile to water soluble compounds in the medium. Lucifer Yellow and EGTA have similar molecular weights, 463 and 380, respectively, and are both polyanions. Therefore it is reasonable to assume that equivalent amounts of EGTA and Lucifer Yellow entered the cells' cytoplasm. All macrophages loaded with EGTA and Lucifer Yellow had also ingested erythrocytes, which were clearly visible as black spots against the bright fluorescent background. Tables IV and V show that chelation of $[Ca^{2+}]_i$ by either quin2 or EGTA had no effect on Fc receptor–mediated phagocytosis by J774 macrophages. These experiments provide strong support for the hypothesis that phagocytosis mediated by Fc receptors in J774 cells does not involve Ca^{2+}, and show by direct measurements that any cytoskeletal rearrangements that occur during phagocytosis must do so at very low (≤10 nM) $[Ca^{2+}]_i$.

Effects of Fc Receptor Ligation on $[Ca^{2+}]_i$ in Mouse Peritoneal Macrophages

In a previous study, McNeil et al. (16) reported that Fc receptor ligation did not cause an increase in $[Ca^{2+}]_i$ in thyoglycollate broth–elicited mouse peritoneal macrophages (thio-macrophages). To further examine the differences in the effects of Fc receptor ligation on $[Ca^{2+}]_i$ in thio-macrophages and J774 macrophages, and to determine whether thio-macrophages have different calcium requirements for phagocytosis than J774 macrophages, we repeated many of the experiments described above with thio-macrophages. Resting $[Ca^{2+}]_i$ in thio-macrophages was similar whether

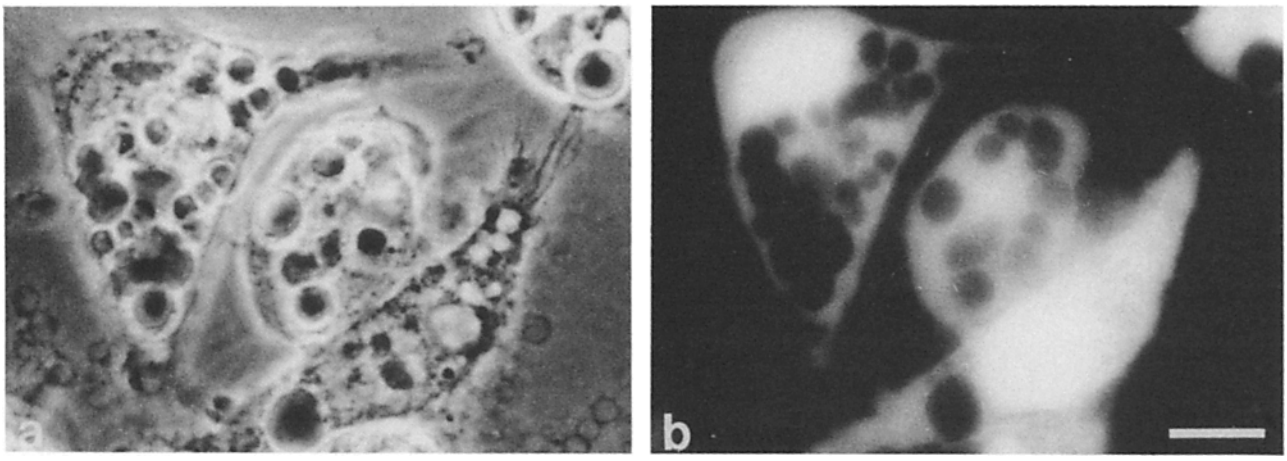

Figure 7. J774 macrophages permeabilized with ATP^{4-} in EGTA and Lucifer Yellow–containing medium ingest IgG-coated erythrocytes. 10^4 J774 macrophages, plated on round glass coverslips, were incubated for 5 min in Ca^{2+}-free standard saline containing 5 mM ATP, 10 mM EGTA, 1 mg/ml Lucifer Yellow, and 2.5 mM probenecid. They were then rinsed in DM10F, incubated in DM10F for 10 min, rinsed in standard saline, and incubated at 37°C with IgG-coated erythrocytes. (*a*) Phase; (*b*) fluorescence. Bar, 10 μm.

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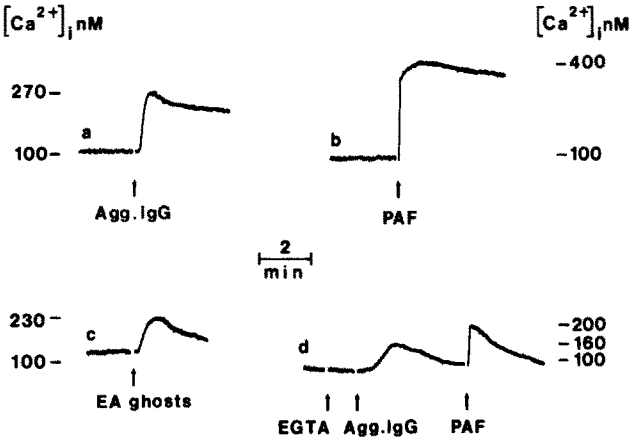

Figure 8. Ligation of Fc receptors causes an increase in $[Ca^{2+}]_i$ of thio-macrophages in suspension. Fura-2-loaded thio-macrophages were suspended in standard saline at a concentration of 10^6 cells/ml and assayed at 37°C. Aggregated IgG, 500 μg/ml; PAF, 40 ng/ml; EGTA, 1 mM; IgG-coated erythrocytes ghosts (*EA ghosts*) were added at a cytocrit of 0.2%.

measured with quin2 or fura-2, and whether measured by the ratio method or the Triton lysis method (Table VI). As noted for J774 macrophages, resting $[Ca^{2+}]_i$ was consistently 25% lower in monolayer cultures of thio-macrophages than with suspension cultures of these cells. Our values for $[Ca^{2+}]_i$ determined in thio-macrophage monolayers are in good agreement with those reported by McNeil et al. (16) for these same cells loaded with aequorin, but are significantly lower than those reported by Conrad and Rink (3) for quin2-loaded thio-macrophages. In agreement with our findings in J774 cells, addition of heat-aggregated IgG (Fig. 8, *trace a*) or antibody-coated erythrocyte ghosts (Fig. 8, *trace c*) to thio-macrophages in suspension induced an increase in $[Ca^{2+}]_i$ from a resting $[Ca^{2+}]_i$ of 100 nM to ~270 and 230 nM, respectively. Similar results were obtained when quin2 was used to measure the $[Ca^{2+}]_i$ response to Fc receptor ligation (Table VI).

The increase in $[Ca^{2+}]_i$ induced by these ligands was prolonged and preceded by a lag of 5–10 s, presumably reflecting the time required for the aggregated IgG and for the IgG-coated ghosts to bind to the macrophage Fc receptors. In contrast, the $[Ca^{2+}]_i$ rise elicited by PAF occurred without any appreciable lag (Fig. 8, *trace b*). An increase in $[Ca^{2+}]_i$, though much smaller, was also observed in Ca^{2+}-free, EGTA-supplemented medium (Fig. 8, *trace d*), indicating that ligation of Fc receptors induced release of Ca^{2+} from intracellular stores, as was the case in J774 cells. Addition of uncoated erythrocyte ghosts had no effect on $[Ca^{2+}]_i$ but a subsequent addition of PAF induced a normal $[Ca^{2+}]_i$ transient (not shown). Thio-macrophages responded to ligation of their Fc receptors with an increase in $[Ca^{2+}]_i$, which was prolonged compared with that observed in J774 cells (compare Fig. 8, *trace a* with Fig. 3, *trace a*).

In contrast to the consistent increase in $[Ca^{2+}]_i$ in thio-macrophages in suspension, Fc receptor ligation elicited highly variable $[Ca^{2+}]_i$ responses in thio-macrophages in monolayers. We tested 10 preparations of thio-macrophages in monolayers from 10 separate groups of mice: 3 preparations showed a rise in $[Ca^{2+}]_i$ to ~200 nM upon the addition of aggregated IgG, 4 showed no response, and 3 gave intermediate responses. Although we are unable to explain satisfactorily this variability, it probably accounts for the lack of increase in $[Ca^{2+}]_i$ in thio-macrophages in monolayers in response to Fc receptor ligation reported by McNeil et al. (16).

Reduction of $[Ca^{2+}]_i$ to Very Low Levels (1–15 nM) in Thio-Macrophages in Monolayer Cultures Does Not Impair Their Capacity to Phagocytose IgG-coated Erythrocytes

To confirm that Fc receptor–mediated phagocytosis occurred in mouse peritoneal macrophages whose $[Ca^{2+}]_i$ had been buffered to very low levels (1–15 nM), we repeated the experiments described above for J774 macrophages with thio-macrophages. Buffering $[Ca^{2+}]_i$ with quin2 did reduce the efficiency of phagocytosis of IgG-coated erythrocytes by

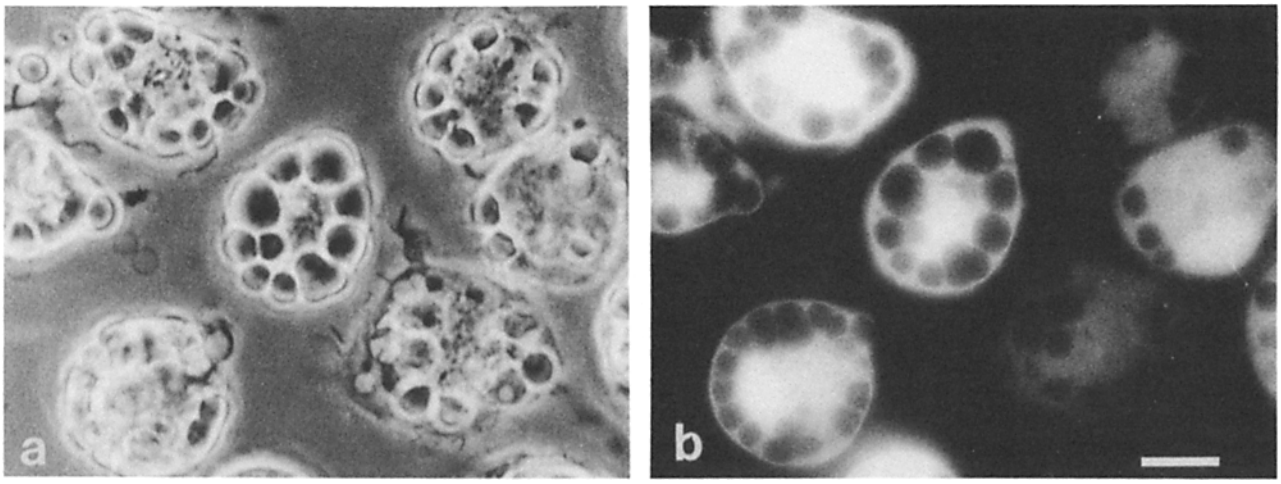

Figure 9. Thio-macrophages permeabilized with ATP^{4-} in EGTA and Lucifer Yellow–containing medium ingest IgG-coated erythrocytes. 10^4 thio-macrophages, plated on round glass coverslips, were incubated for 5 min in Ca^{2+}-free standard saline containing 5 mM ATP, 10 mM EGTA, 1 mg/ml Lucifer Yellow, and 2.5 mM probenecid. They were then rinsed in DM10F, incubated in DM10F for 10 min, rinsed in standard saline, and incubated at 37°C with IgG-coated erythrocytes. (*a*) Phase; (*b*) fluorescence. Bar, 10 μm.

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Table VII. Chelation of $[Ca^{2+}]_i$ with quin2 Does Not Inhibit Fc Receptor Phagocytosis in Thio-Macrophages

	A ($[Ca^{2+}]_i$ = 1)	B ($[Ca^{2+}]_i$ = 80 nM)	C (control)
Percent macrophages ingesting IgG–RBC	80%	80%	95%
IgG–RBC ingested/macrophage	7	8	12
Phagocytic index	580	640	1,150

Thio-macrophages were plated on round glass coverslips (3×10^4 per coverslip) for phagocytosis assays and on rectangular glass coverslips (4×10^5 per coverslip) for $[Ca^{2+}]_i$ measurements. For A and B, phagocytosis assays and $[Ca^{2+}]_i$ measurements were performed in parallel. A, macrophages were loaded with quin2/AM in Ca^{2+}-free, EGTA-containing (1 mM) medium and kept in the same medium throughout the experiment. Phagocytosis was assayed in standard saline supplemented with 1 mM EGTA. B, macrophages were loaded with quin2/AM in standard saline containing 1 mM Ca^{2+}. Phagocytosis was assayed in standard saline. C, phagocytosis was assayed in unloaded cells. Data are the average of triplicate determinations from a single experiment repeated with three different cell preparations.

these cells (Table VII). We believe that the reduction of phagocytosis observed in quin2-loaded, Ca^{2+}-depleted thio-macrophages is not related to Ca^{2+} chelation but rather to some other effect of quin2, since cells that were loaded with the same amount of quin2 in the presence of 1 mM free Ca^{2+} (and were therefore not Ca^{2+} depleted), exhibited a similar reduction of phagocytosis seen in quin2-loaded, Ca^{2+}-depleted cells. "Toxic" effects of quin2 on plated thio-macrophages were also described by McNeil et al. (16). Table VIII shows similar experiments in EGTA-loaded cells. Note that there is no decrease in the phagocytic index of cells that had been permeabilized in the presence of EGTA, and then resealed, thus buffering the $[Ca^{2+}]_i$ to 10 nM. To confirm that such treatment of thio-macrophages affords access to the cytosol of water-soluble compounds of ≤463 mol wt, including EGTA, we permeabilized these cells with ATP in medium containing 10 mM EGTA and Lucifer Yellow, and then allowed them to ingest IgG-coated erythrocytes. As expected, the Lucifer Yellow was diffusely distributed throughout the cytoplasm of these cells (Fig. 9).

These experiments confirm that there are no significant differences in the effects of buffering of intracellular calcium on Fc receptor–mediated phagocytosis in both primary mouse peritoneal macrophages and cells of the J774 macrophage-like cell line. For both cell types, phagocytosis proceeds unimpaired at very low $[Ca^{2+}]_i$ and in the presence of potent cytoplasmic buffers of calcium transients.

Discussion

Knowledge of the molecular mechanism of phagocytosis has increased enormously since the first description of this process by Metchnikoff a century ago (17). Several receptors that mediate phagocytosis have been identified, and recently two subtypes of Fc receptors have been cloned (15, 21). A number of cytoplasmic proteins responsible for pseudopod movement have been identified and their modes of interaction are being characterized (27, 29, 34). However, the mechanisms governing transfer of information from ligated surface receptors to the cytoplasmic contractile apparatus is still an unsolved problem. A widely accepted hypothesis suggests that spreading of the pseudopods around the particle is powered by the interaction of actin with other cytoskeletal and membrane-associated proteins and that the system is controlled by Ca^{2+} (29). According to this view, and in analogy with the general hypothesis for stimulus–response coupling, Ca^{2+} is thought to be the second messenger that is generated upon the ligation of plasma membrane receptors by particle-bound ligands, and that governs the assembly and disassembly of the cytoskeleton. However, recent studies of fMet-Leu-Phe–stimulated neutrophils (2, 24) suggest that a rise in $[Ca^{2+}]_i$ is not necessary for either nucleation of actin filament formation or association of actin with the cytoskeleton, two reactions believed crucial in pseudopod formation. In addition, Sklar et al. have shown that buffering $[Ca^{2+}]_i$ to vanishingly low levels with quin2 does not inhibit chemotactic peptide–stimulated actin polymerization in neutrophils (26). Taken together these reports call into question a direct role for Ca^{2+} in initiating or regulating early cytoskeletal changes induced by receptor activation.

There have been conflicting reports as to the role of Ca^{2+} in phagocytosis. Even the basic question of whether the activation of Fc receptors is linked to Ca^{2+} mobilization is a matter of controversy. Young et al. reported that challenging quin2-loaded macrophages in suspension with a number of Fc receptor–directed ligands induced a sustained rise in $[Ca^{2+}]_i$ (35). Lew et al. confirmed these results in quin2-loaded human neutrophils in suspension and also provided the first evidence that phagocytosis mediated by complement receptor can occur in a completely Ca^{2+}-independent fashion (13). However, McNeil et al. were unable to detect any changes in $[Ca^{2+}]_i$ after activation of Fc receptors in adherent thio-macrophages loaded with aequorin by scrape loading (16).

Our results agree with the reports by Young et al. (35) and

Table VIII. Chelation of $[Ca^{2+}]_i$ with EGTA Does Not Inhibit Fc Receptor Phagocytosis in Thio-Macrophages

	A ($[Ca^{2+}]_i$ = 10 nM)	B ($[Ca^{2+}]_i$ = 85 nM)
% macrophages ingesting IgG–RBC	98%	85%
IgG–RBC ingested/macrophage	13	14
Phagocytic index	1,300	1,350

Thio-macrophages were plated on rectangular coverslips at a concentration of 2×10^6/ml. $[Ca^{2+}]_i$ (determined with 340:385 ratio) was measured in the same macrophage monolayers that were used for the phagocytic assay. Macrophages were permeabilized at 37°C with 5 mM ATP in Ca^{2+}-free medium plus 10 mM EGTA (A), or Ca^{2+}-free medium alone (B). After 5 min the coverslips were rinsed with DM10F supplemented with 10 mM EGTA (A) or DM10F alone (B), and loaded with fura-2/AM as described in Materials and Methods. Data are the average of triplicate determinations from a single experiment repeated with three different cell preparations.

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Lew et al. (13) that Fc receptor ligation usually is linked to Ca^{2+} mobilization, and are consistent with the findings of McNeil et al. (16) that Fc receptor-mediated phagocytosis can occur in the absence of a rise in $[Ca^{2+}]_i$. However, while the kinetics of the $[Ca^{2+}]_i$ rise observed by us are very similar to those reported by Lew et al., they are strikingly different from those reported by Young et al. In the experiments described by Young et al. (35) the addition of Fc receptor-directed ligands caused an instantaneous increase in $[Ca^{2+}]_i$, while in the experiments reported by Lew et al. (13) and in the present report the $[Ca^{2+}]_i$ rise was always slow and preceded by a lag phase. J774 macrophages consistently showed an increase in $[Ca^{2+}]_i$ in response to aggregated IgG, whether the cells were in suspension or plated on glass coverslips. Thio-macrophages, on the contrary, exhibited a reproducible increase in $[Ca^{2+}]_i$ only when they were in suspension. Even the same preparation of thiomacrophages that responded to aggregated IgG with a rise in $[Ca^{2+}]_i$ when in suspension often lost this response 10–12 h after plating. The variability of the response of thio-macrophages to aggregated IgG stimulation may explain why McNeil and colleagues did not detect any change in $[Ca^{2+}]_i$ upon ligation of Fc receptors in these cells though they used aequorin, a Ca^{2+} indicator theoretically more sensitive than fura-2. Another relevant difference between J774 and thio-macrophages was that the $[Ca^{2+}]_i$ rise caused by ligation of Fc receptors was due mainly to release of Ca^{2+} from intracellular stores in J774 cells and to influx of extracellular Ca^{2+} in thio-macrophages.

PMA abolished the $[Ca^{2+}]_i$ rise induced by Fc receptor ligation in cells in suspension and blunted it in cells in monolayers. Pretreatment with PMA has been shown to decrease resting and stimulated $[Ca^{2+}]_i$ in many cell types (4, 12, 22, 33). In those instances it was shown that PMA either activated the Ca^{2+}-ATPase of the plasma membrane (12) or inhibited receptor-activated hydrolysis of phosphatidylinositol(4,5)bisphosphate and therefore the formation of inositol trisphosphate (22, 33). At present, it is impossible to say whether PMA is inhibiting Fc receptor-induced $[Ca^{2+}]_i$ rise through the same mechanisms, in particular because a coupling of the Fc receptor to inositol trisphosphate formation has never been demonstrated.

The coupling of Fc receptors to Ca^{2+} mobilization, however, becomes an academic question with respect to phagocytosis since our experiments show that a detectable increase in $[Ca^{2+}]_i$ is not required during phagocytosis. We have used different techniques to lower $[Ca^{2+}]_i$ to levels that are well below the Km for Ca^{2+} of most Ca^{2+}-dependent enzymes, yet phagocytosis was negligibly affected. The compounds we have used (i.e., quin2 and EGTA) to buffer $[Ca^{2+}]_i$ at the 1–15-nM level also are used to buffer calcium in biochemical experiments. There is every reason to expect that these compounds have access to the entire intracellular soluble cytoplasmic compartment since compounds of similar molecular weight and charge (i.e., Lucifer Yellow and fura-2) have been observed to fill this compartment (Figs. 7 and 9). For this reason they should be available to chelate any calcium that might be released into the cytosol in the region of the forming phagocytic vacuole in response to Fc receptor ligation. Moreover, the experiment described in Fig. 5 confirms that the concentration of quin2 used to buffer $[Ca^{2+}]_i$ (50 μM external quin2/AM, ~1.5 mM intracellular quin2) was sufficient to buffer all calcium released from intracellular stores by ionomycin. Under these same conditions Fc receptor-mediated phagocytosis in J774 cells was unaffected. Although we could not easily measure the concentration of EGTA that was loaded into the cytoplasm of J774 cells, Steinberg et al. (28) have measured the accumulation of Lucifer Yellow in J774 cells during ATP permeabilization under nearly identical conditions as ours. After a 5-min incubation, the intracellular concentration of Lucifer Yellow was 40% that of the extracellular concentration. For 10 mM dye, this would provide an intracellular concentration of 4 mM. Since the molecular weight of EGTA (380 mol wt) is less than that of Lucifer Yellow (463 mol wt), 4 mM is almost certainly a lower estimate of the concentration of EGTA that accumulates in these cells under our loading conditions. At this concentration EGTA should buffer even transient local increases in $[Ca^{2+}]_i$ that may be induced during phagocytosis.

Secretory exocytosis in mast cells and neutrophils, a response long thought to be Ca^{2+}-dependent and/or activated, has been shown to occur in a completely Ca^{2+}-independent fashion when triggered by effectors that bypass plasma membrane receptors, such as phorbol myristate acetate and GTPγS (6, 9). However for exocytosis initiated via ligand stimulation of surface receptors, a normal resting $[Ca^{2+}]_i$ appears necessary (6, 14, 20). This is in sharp contrast to Fc receptor-mediated phagocytosis, a process which is regulated by membrane receptors, involves extensive cytoskeletal rearrangements (23, 36) and appears to occur in a Ca^{2+} independent fashion.

If a rise in $[Ca^{2+}]_i$ is not required for phagocytosis, why does it occur upon Fc receptor ligation? Although definitive evidence is lacking, it is likely that other processes signalled by Fc receptor ligation, such as superoxide anion formation, release of arachidonic acid, and lysosomal enzyme secretion, require Ca^{2+} transients. Whether or not this hypothesis proves correct, it is evident that ligated Fc receptors, like fMet-Leu-Phe receptors, generate at least two cytoplasmic signals/second messengers: One is the influx of extracellular Ca^{2+} (cf. Fig. 8, *a* and *d*) and the release of Ca^{2+} from intracellular stores (Figs. 3 *c* and 8 *d*). The second is the assembly of filamentous actin (23, 36) and the engulfment of ligand-coated particles. The capacity of a single membrane receptor to transmit two independent signals to the cytoplasm suggests added levels of complexity in the regulation of cellular functions by individual plasma membrane receptors.

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