

Ca²⁺-independent F-Actin Assembly and Disassembly during Fc Receptor-mediated Phagocytosis in Mouse Macrophages

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Abstract. Phagocytosis of IgG-coated particles by macrophages is presumed to involve the actin-based cytoskeleton since F-actin accumulates beneath forming phagosomes, and particle engulfment is blocked by cytochalasins, drugs that inhibit actin filament assembly. However, it is unknown whether Fc receptor ligation affects the rate or extent of F-actin assembly during phagocytosis of IgG-coated particles. To examine this question we have used a quantitative spectrofluorometric method to examine F-actin dynamics during a synchronous wave of phagocytosis of IgG-coated red blood cells by inflammatory mouse macrophages. We observed a biphasic rise in macrophage F-actin content during particle engulfment, with maxima at 1 and 5 min after the initiation of phagocytosis. F-actin declined to resting levels by 30 min, by which time particle engulfment was completed. These quantitative increases in macrophage F-actin were reflected in localized changes in F-actin distribution. Previous work showed that the

number of IgG-coated particles engulfed by macrophages is unaffected by buffering extracellular calcium or by clamping cytosolic free calcium concentration ($[Ca^{2+}]_i$) to very low levels (Di Virgilio, F., B. C. Meyer, S. Greenberg, and S. C. Silverstein. 1988. *J. Cell Biol.* 106: 657-666). To determine whether clamping $[Ca^{2+}]_i$ in macrophages affects the rate of particle engulfment, or the assembly or disassembly of F-actin during phagocytosis, we examined these parameters in macrophages whose $[Ca^{2+}]_i$ had been clamped to $\sim <3$ nM with fura 2/AM and acetoxymethyl ester of EGTA. We found that the initial rate of phagocytosis, and the quantities of F-actin assembled and disassembled were similar in Ca²⁺-replete and Ca²⁺-depleted macrophages. We conclude that Fc receptor-mediated phagocytosis in mouse macrophages is accompanied by an ordered sequence of assembly and disassembly of F-actin that is insensitive to $[Ca^{2+}]_i$.

DURING Fc receptor-mediated phagocytosis macrophages undergo profound shape changes embodied in the formation of pseudopods that engulf IgG-coated particles. The identification of F-actin as a key cytoskeletal element of pseudopods (Boxer et al., 1974) was soon followed by the demonstration that cytochalasins inhibit particle engulfment (Zigmond and Hirsch, 1972; Axline and Reaven, 1974). Later work established that phagocytosis of unopsonized erythrocytes by *E. histolytica* (Bailey et al., 1985) and of *C. albicans* hyphae and opsonized zymosan by neutrophils (Kolotila and Diamond, 1988) is accompanied by a net polymerization of actin. Furthermore, ingestion of antigen-IgG complexes in neutrophils is accompanied by a net rise in F-actin (Sheterline et al., 1984; Brennan, P. J., S. H. Zigmond, A. D. Schreiber, and F. S. Southwick. 1989. *J. Cell Biol.* 107[No. 6, Pt. 3]:452a [Abstr.]). Together, this evidence suggests that actin polymerization is an important cytoskeletal alteration that accompanies phagocytosis. De-

spite this knowledge, we have scant information on the relationship between the kinetics of actin polymerization and pseudopod extension, and on the signals transmitted from ligated Fc receptors to the macrophage cytoplasm that control these events. For example, since Fc receptor ligation causes a rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$)¹ (Young et al., 1984; Di Virgilio et al., 1988b), and calcium ionophores cause a rise in F-actin content in neutrophils (Howard and Wang, 1987), it seemed reasonable to assume that a transient rise in $[Ca^{2+}]_i$ provided a stimulus for actin polymerization and particle engulfment. However, further studies showed that phagocytosis can proceed in the absence

1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; EB, ethidium bromide; EGTA/AM, acetoxymethyl ester of EGTA; HBS, HEPES-buffered saline; IgG-RBC, sheep erythrocytes coated with rabbit anti-sheep erythrocyte IgG; RP, rhodamine phalloidin; thio-macrophages, mouse peritoneal macrophages elicited after the intraperitoneal injection of thioglycollate broth.

of Ca^{2+} transients (McNeil et al., 1986; Di Virgilio et al., 1988b). The latter findings cast doubt on the hypothesis that $[\text{Ca}^{2+}]_i$ provides a link between receptor ligation and phagocytosis. While these experiments indicated that Ca^{2+} cannot be the sole intracellular messenger mediating communication between Fc receptors and the cells' phagocytosis-promoting machinery, they did not exclude the possibility that Ca^{2+} -dependent events play a regulatory role in F-actin assembly and phagocytosis, analogous to results in some (Meshulam et al., 1986; Simchowicz and Cragoe, 1990), but not all (Sha'afi et al., 1986; Zigmond et al., 1988; Perez et al., 1989) studies of chemotaxing neutrophils. For Fc receptor-mediated events, such information can only be obtained by examining the effects of altered $[\text{Ca}^{2+}]_i$ on the early rates of particle engulfment, and of assembly and disassembly of F-actin. The present experiments were designed to explore these possibilities. Here we describe dynamic changes in the assembly and disassembly of F-actin during phagocytosis that correspond to discrete changes in phagocytic cup formation; we have found that neither the rate of ingestion of IgG-coated particles, nor the rates and extent of assembly and disassembly of F-actin, are affected by alterations in the levels of intracellular and extracellular calcium.

Materials and Methods

Chemicals

Rhodamine-phalloidin, fura-2/AM, and acetoxymethyl ester of EGTA (EGTA/AM) were obtained from Molecular Probes Inc. (Eugene, OR). Texas red goat anti-rabbit IgG was from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). All other chemicals were reagent grade.

Cells

Macrophages were harvested from the peritoneal cavities of mice after the intraperitoneal injection of thioglycollate broth as previously described (Di Virgilio et al., 1988b). $1-2 \times 10^7$ cells were harvested from each mouse. J774 cells were maintained in spinner cultures as described (Di Virgilio et al., 1988b).

Binding and Phagocytosis of IgG-coated Red Blood Cells by Macrophages

100 μl aliquots of a suspension of peritoneal exudate cells (6×10^5 cells/ml in DM10F) were plated onto the distal third of rectangular coverslips (10×27 mm coverslips cut with a diamond knife from 48×65 mm No. 1 coverslips), and allowed to adhere at 37°C for 15 min. The coverslips were washed to remove nonadherent cells, fresh DM10F was added to cover the coverslips, and the macrophages were maintained overnight at 37°C in a CO_2 incubator. At the onset of the experiment, the medium was replaced with ice cold Hepes-buffered saline containing 125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 5 mM glucose, 10 mM NaHCO_3 , 1 mM MgCl_2 , and 20 mM Hepes, pH 7.4 (HBS), and the macrophages were incubated on ice for 10 min. The medium was replaced by either fresh ice cold HBS or HBS containing sheep erythrocytes opsonized with a subagglutinating titer of rabbit anti-sheep RBC (IgG-RBC). After 10 min to allow the IgG-RBC to adhere to the macrophages, the cultures were washed three times with ice-cold HBS. To initiate phagocytosis, the cold buffer was replaced with HBS prewarmed to 37°C . The cells were further incubated at 37°C for the times indicated, and then fixed with 3.7% formaldehyde at 37°C for 20 min.

Loading of Fura-2/AM and EGTA/AM

Adherent macrophages were incubated in HBS containing 3 μM fura-2/AM in the presence of 0.3% pluronic detergent and 1.5% rabbit serum at 8°C for 15 min. It was necessary to perform dye loading at this temperature to minimize endocytosis that might lead to dye sequestration within cytoplasmic vacuoles (Malgaroli et al., 1987). The fura-2-loaded macrophages

were incubated in HBS for a further 30 min at room temperature to allow complete hydrolysis of fura-2/AM to fura-2 free acid. All HBS solutions contained 1 mM sulfapyrazone, an inhibitor of organic anion secretion, to prevent dye secretion and sequestration (Di Virgilio et al., 1988a, 1990). We verified that this loading protocol afforded complete dye hydrolysis by confirming spectrofluorometrically that the spectrum of fura-2 released by lysing the macrophages was nearly identical to that of purified fura-2 free acid at saturating Ca^{2+} conditions. We also verified that the fura-2 loaded at reduced temperature and in the presence of sulfapyrazone was restricted to the cytosol, since no organellar dye sequestration was observed by fluorescence microscopy. The presence of fura-2 and sulfapyrazone did not affect the ability of the macrophages to phagocytose IgG-RBC, and did not influence the F-actin kinetics during phagocytosis (not shown). In experiments in which the $[\text{Ca}^{2+}]_i$ was clamped, the fura-2-loaded cells were incubated with 20 μM EGTA/AM in Ca^{2+} -free HBS containing 1 mM EGTA for 30 min at room temperature, and washed, and further incubated in Ca^{2+} -free HBS + 1 mM EGTA for 30 min to allow complete hydrolysis of the EGTA/AM to EGTA.

Determination of $[\text{Ca}^{2+}]_i$

Rectangular coverslips bearing fura-2-loaded macrophages (described above) were inserted into a specially designed cuvette holder (Di Virgilio et al., 1988b), and the fura-2 fluorescence was monitored in a fluorescence spectrophotometer (model 650-40; Perkin-Elmer Corp., Pomona, CA). Calibration was performed as described previously (Di Virgilio et al., 1988b), except ionomycin (2 μM) and ATP (1 mM) were added instead of digitonin to obtain F_{max} . ATP was used because it always caused an additional increase in the fluorescence over that produced by ionomycin alone, which reflects its ability to induce a large influx of extracellular Ca^{2+} in mouse macrophages and thus act as a potent Ca^{2+} ionophore (Greenberg et al., 1988). To monitor $[\text{Ca}^{2+}]_i$ changes in single cells undergoing phagocytosis, macrophages were plated on 22-mm² No. 1 glass coverslips (Fisher Scientific Co., Pittsburgh, PA) and loaded with fura-2/AM as described above; the fluorescence at 350 and 380 nm was monitored using a Zeiss Axioplan fluorescence microscope equipped with a photomultiplier tube interfaced with a microscope systems processor (Zeiss), and the 350:380 ratio was monitored before and after the addition of IgG-RBC. IgG-RBC were added at a ratio of 10 particles per macrophage. The $[\text{Ca}^{2+}]_i$ was usually monitored for 2 min after the addition of IgG-RBC, and the presence or absence of a " Ca^{2+} transient" (described in text) was noted in macrophages that were in the process of engulfing at least one IgG-RBC. Controls in which unopsonized RBC were added showed no significant change in $[\text{Ca}^{2+}]_i$ of the macrophages. $[\text{Ca}^{2+}]_i$ values were calculated according to Grynkiewicz et al. (1985).

Phagocytosis Assays

We were unable to use hypotonic lysis to distinguish internalized from bound RBC in macrophages whose cytosol was loaded with EGTA/AM in the presence of 1 mM extracellular EGTA because many of these macrophages were lysed by this procedure, leading to gross underestimations of the phagocytosis index. The susceptibility to hypotonic lysis of macrophages whose $[\text{Ca}^{2+}]_i$ is clamped to low levels is most likely the result of a non-selective increase in plasma membrane permeability (Picello et al., 1990). To circumvent this problem, we devised an alternative assay utilizing acridine orange to stain the cytoplasm, and Texas red-anti-rabbit IgG to stain the RBC. This method, described in detail elsewhere (Greenberg et al., 1991), provides an accurate assessment of particle uptake in macrophages whose $[\text{Ca}^{2+}]_i$ has been clamped at low (<10 nM) levels. Briefly, the cells were plated on round 12 mm No. 1 glass coverslips (Fisher Scientific Co.) and processed as described above. The formaldehyde-fixed cells were stained with 1 $\mu\text{g}/\text{ml}$ acridine orange for 20 min and, after permeabilization with 0.2% Triton X-100, counterstained with Texas Red anti-rabbit IgG diluted 1:50 in HBS. Ingested red cells were counted by visualizing dark phagocytic vacuoles delimited by a bright rim of acridine orange-stained cytoplasm when viewed under fluorescein optics. To distinguish phagocytic vacuoles from other types of vacuoles, only those vacuoles that contained Texas red-anti-rabbit IgG were scored as phagocytic. In addition, under Texas Red optics, the round shape of the ingested red cells distinguished them from the crenated erythrocytes that were bound but not ingested. Phagocytosis assays were carried out in triplicate.

Fluorescence Microscopy

Adherent macrophages were allowed to phagocytose IgG-coated red cells

as described above, fixed in formalin, and stained with 0.33 μM rhodamine-phalloidin (RP) in HBS containing 100 $\mu\text{g}/\text{ml}$ lysophosphatidylcholine for 30 min at 23°C. The stained cells were examined under phase contrast and fluorescence microscopy using a Zeiss Photomicroscope III, and photographed with Tri-X film processed at 800 ASA.

F-Actin Determination

Formalin-fixed cells were stained with 0.33 μM RP in HBS containing 100 $\mu\text{g}/\text{ml}$ lysophosphatidylcholine for 30 min at 23°C, and washed twice with HBS. The rectangular coverslips were placed in a cuvette holder (Di Virgilio et al., 1988b), and the fluorescence of RP-stained cells was measured in a fluorescence spectrophotometer (excitation, 540 ± 10 ; emission, 580 ± 15) (model 650-40; Perkin-Elmer Corp.). The RP fluorescence measurement was specific for F-actin since cells preincubated with unlabeled phalloidin could not be detectably stained with RP. To compensate for small differences in the number of cells traversed by the excitation beam, 10 mM ethidium bromide (EB) was added to stain the nucleus of each cell, and the fluorescence (excitation, 360 ± 10 ; emission, 580 ± 15) was recorded. All RP values were corrected for autofluorescence of cells and IgG-RBC, and all EB values were corrected for fluorescence of RP at the EB wavelengths and autofluorescence of EB itself. The assays were performed in quadruplicate. The data are expressed either as the ratio of RP fluorescence to EB fluorescence (RP/EB), which reflects the F-actin content per macrophage in arbitrary units, or as the difference between RP/EB of cells undergoing phagocytosis, and that of cells maintained in saline alone: $\Delta \text{RP/EB} = (\text{RP/EB})_{\text{experimental}} - (\text{RP/EB})_{\text{control}}$.

Calibration of the F-Actin Assay

To determine the optimal number of macrophages to use to quantitate F-actin, we plated macrophages at various concentrations on rectangular coverslips and determined RP and EB alone, as well as RP/EB. Control experiments demonstrated that the density at which the cells were plated and the density of cells that remained adherent were linearly related (not shown). RP and EB were linearly correlated when cells were plated between 0.3 and 1.5×10^6 cells/ml (Fig. 1; $r^2 = 0.95$). This indicated that the amount of RP present was sufficient to saturate the F-actin present in the macrophages in the monolayer. Since we were interested in detecting rises in F-actin content over baseline, we chose a final plating concentration of $6\text{--}7 \times 10^5$ cells/ml, which allowed detection of at least a twofold net increase in the F-actin content in macrophages undergoing phagocytosis.

Since the phagocytic particles (sheep erythrocytes) themselves contain a small amount of F-actin, and a confluent lawn of erythrocytes plated on poly-L-lysine showed faint RP staining (not shown), we determined whether F-actin in the red cells interfered with the assay. Quantitative studies showed that F-actin in the erythrocytes made only a minor contribution to the total amount of RP bound. At time 0, the F-actin content of macrophages + IgG-RBC was $105 \pm 16\%$ (mean \pm SD, $n = 20$) of macrophages alone. In addition, Fig. 2 A and B show that at the 0 time point, staining of the erythrocytes with RP could not be discerned by fluorescence microscopy. Thus, at most 5% of the total signal could be attributed to the erythrocytes.

Results

Dynamic Changes in F-Actin Distribution and Content Occur during Fc Receptor-mediated Phagocytosis

Fig. 2 shows phase-contrast and fluorescence micrographs of macrophages with IgG-RBC bound at 4°C, and at various times after warming to 37°C to initiate phagocytosis. Macrophages with IgG-RBC attached at 0°C showed no discernable change in rhodamine-phalloidin staining (arrowheads in Fig. 2, A and B). By 1 min after the addition of 37°C buffer, early phagocytic cup formation was evident (Fig. 2, C and D). The phagocytic cups were heavily stained with RP at their bases (arrowheads in Fig. 2, C and D). At 2 min, extension of pseudopods and encirclement of the particles were evident, and the thick actin webs at the bases of the phagocytic cups had thinned somewhat, compared with those at 1 min (arrowheads in Fig. 2, E and F). Indeed, beneath some of the particles, the RP staining was not apparent at the base

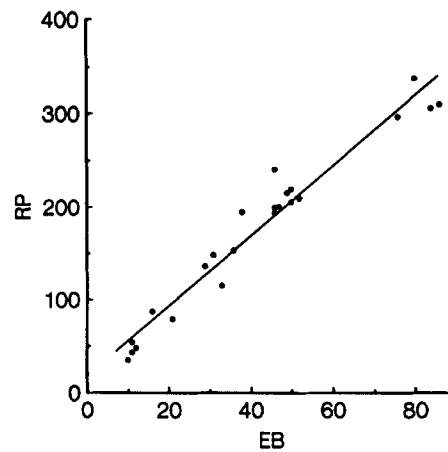
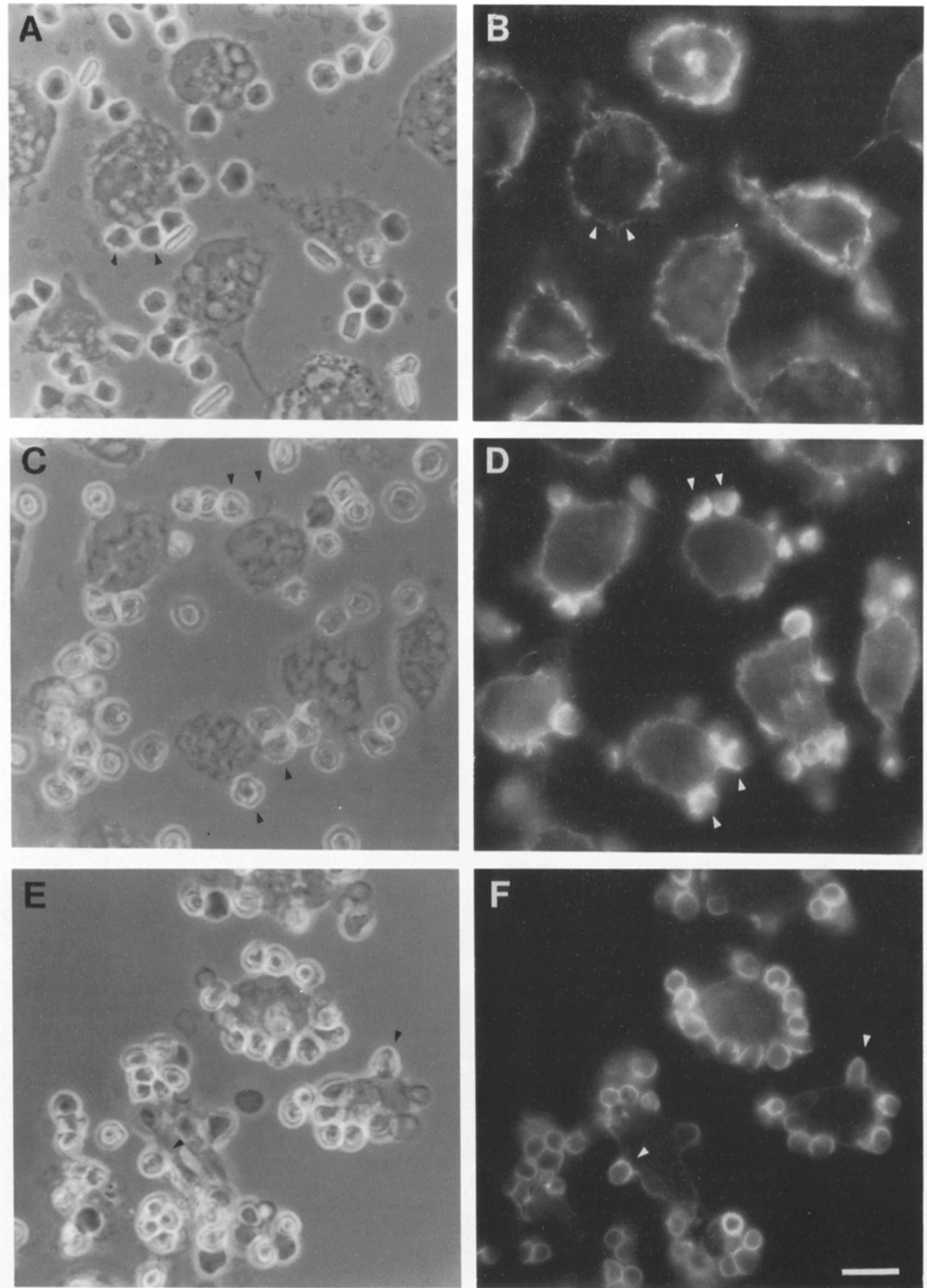


Figure 1. Calibration of the assay for F-actin. Thio-macrophages were plated at various concentrations between 0.3 and 1.5×10^6 cells/ml on glass coverslips. The cells were fixed and stained for F-actin content with rhodamine-phalloidin (RP) and for cell number with ethidium bromide (EB) as described in Materials and Methods. Each point represents a single coverslip. $r^2 = 0.95$.

of the cups, and was restricted to the distal ends of the pseudopods, giving rise to an "inverted cup" configuration; this configuration was commonly seen at the 5-min time point and, to a lesser extent, at the 10-min time point (data not shown). RP staining was most prominent at the 5-min time point (Fig. 3, A and B); however, by 10 min, most of the IgG-RBC had been internalized and were no longer surrounded by F-actin-rich cytoplasm (arrows in Fig. 3, C and D). A few peripherally located particles that had not been completely engulfed (Fig. 3, arrowheads) were still surrounded by F-actin. By 30 min, particle engulfment was complete (Fig. 4 B). F-actin, associated with the forming phagosomes, so prominent at the earlier time points, was barely visible (Fig. 3, E and F).

To determine whether the morphological changes described above reflected newly polymerized actin, as opposed to F-actin redistribution within the macrophages, we monitored F-actin content during a synchronous "wave" of phagocytosis. This enabled us to discern the kinetics of actin polymerization at early phases of phagocytosis. Fig. 4 shows the results of a typical experiment. Note that there was little or no change in F-actin content of control macrophages after warming to 37°C. In contrast, there was a rapid rise in the F-actin content of phagocytosing macrophages during the first minute of particle engulfment. This was followed by a relative decrease in F-actin during the second minute. This relative decrease in F-actin was followed by a second burst of actin assembly which reached its maximum at 5 min after the initiation of phagocytosis. In all experiments the F-actin content of phagocytosing macrophages was maximal at 5 min. By 30 min, a time point at which phagocytosis was complete, the F-actin content of macrophages that underwent phagocytosis had declined to control values. The increase in F-actin due to phagocytosis per se can be expressed as the difference between RP/EB in the control and experimental groups ($\Delta \text{RP/EB}$; Fig. 4 B). To correlate the F-actin dynamics with the extent of phagocytosis, phagocytosis assays were carried out in parallel (Fig. 4 B). Note that at 5 min, 66% of all the particles that were engulfed by 30 min had



been ingested. Thus, the increase in macrophage F-actin content between 0 and 5 min occurred during the same period as the major portion of phagocytosis. Macrophage F-actin content declined between 5 and 30 min despite continued particle engulfment during this period (Fig. 4 B).

When the F-actin content of phagocytosing macrophages was expressed as a percentage of the F-actin content of control macrophages, the biphasic rise in F-actin was still evident. The peak increases in F-actin during phagocytosis represented 135 ± 5 and $158 \pm 12\%$ of control values at 1 and 5 min, respectively ($n = 6$). Thus, there was a net increase in total macrophage F-actin content during Fc receptor-mediated phagocytosis.

The Increase in F-Actin Content Is Dependent upon the Number of Particles Engulfed

The relative increase in F-actin was proportional to the number of IgG-coated particles ingested by the macrophages (Fig. 5). Addition of twice the usual number of IgG-RBC yielded a phagocytosis index at 30 min of 2,370, and a larger than usual increase in F-actin. Addition of half the usual number of IgG-RBC yielded a phagocytosis index at 30 min of 530 (or approximately five erythrocytes per macrophage). Under the last condition it was not always possible to detect an increase in F-actin. We consider this amount of particle engulfment to cause a rise in F-actin which is barely detectable above the F-actin content of unstimulated cells, and therefore to define the lower limit of sensitivity of the assay.

The Increase in F-Actin Content after Fc Receptor Ligation Is Inhibitable by Cytochalasin D

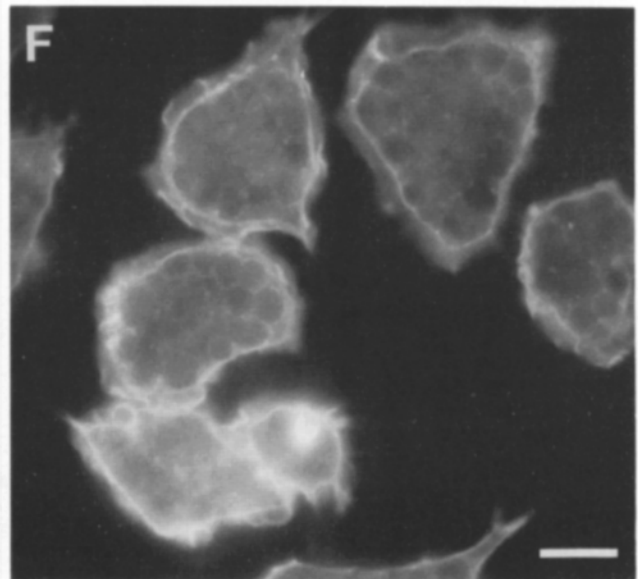
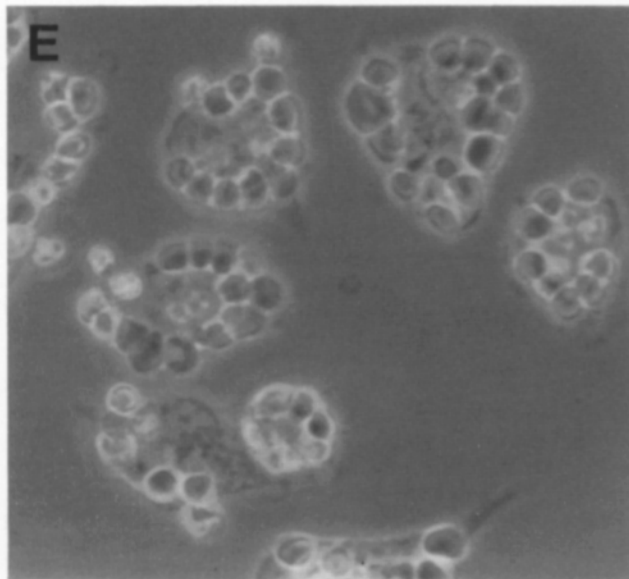
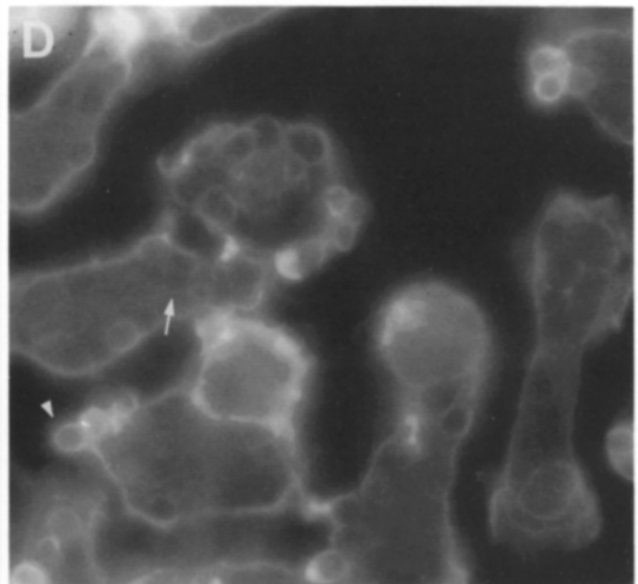
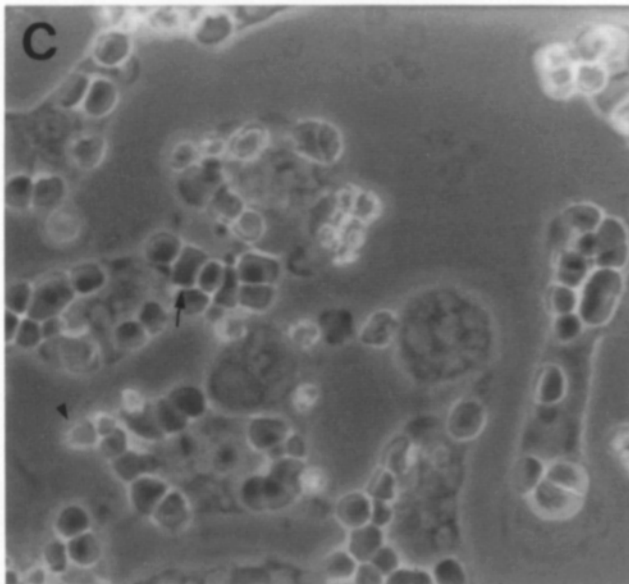
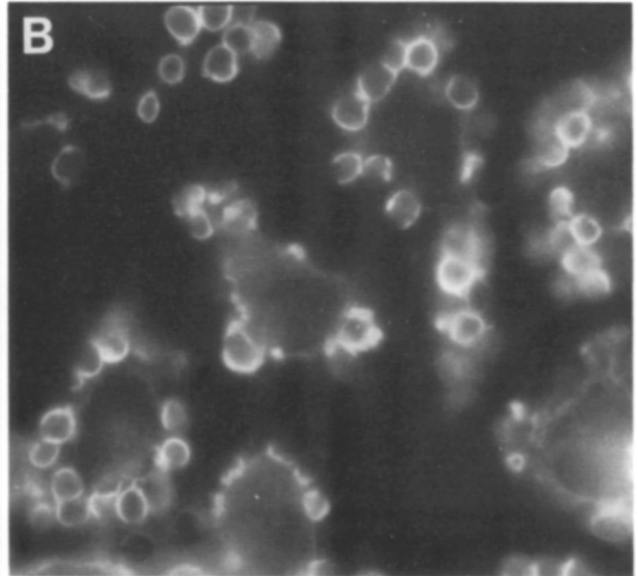
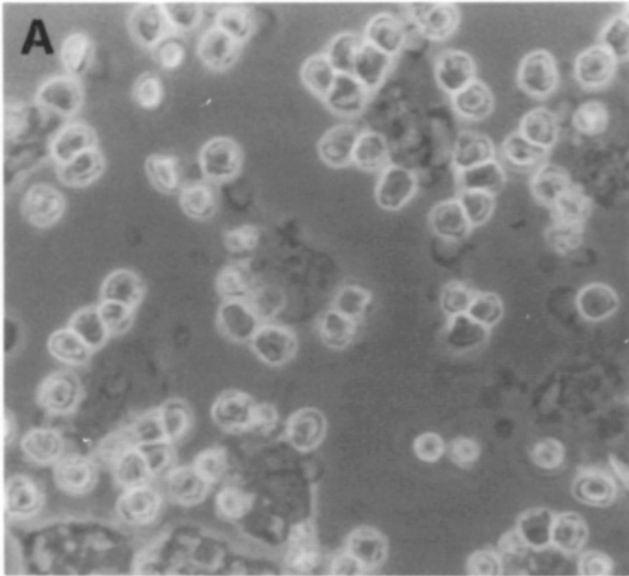
Addition of cytochalasin D did not affect binding of IgG-RBC to the macrophages. However, the presence of $1 \mu\text{M}$ cytochalasin D during 37°C incubation of macrophage-IgG-RBC complexes inhibited particle engulfment by $92 \pm 10\%$ (% inhibition of phagocytosis in the presence of $1 \mu\text{M}$ cytochalasin D as compared to DMSO-treated controls [mean \pm SD, $n = 3$]). Fluorescence microscopy of RP-stained, cytochalasin-treated macrophages showed that there were detectable focal areas of enhanced staining for F-actin beneath the IgG-RBC but they appeared irregularly shaped as compared to controls. When the concentration of cytochalasin D was increased to $2 \mu\text{M}$, the appearance of focal accumulations of F-actin beneath IgG-RBC was delayed. This effect was more pronounced as the concentration of cytochalasin D was increased to $10 \mu\text{M}$. At this concentration, there were no detectable focal accumulations of F-actin at the early (2.5 min or less) time points after incubation at 37°C ; at 5 min there were barely detectable accumulations of F-actin beneath the IgG-RBC in $\sim 20\%$ of the cells, and at 10 min, the staining for F-actin beneath IgG-RBC was more evident, although it appeared irregularly shaped (not shown). By

30 min, most of the F-actin present in these cytochalasin D-treated macrophages was condensed at the periphery of the cells, and there were no detectable focal areas of staining for F-actin beneath the IgG-RBC attached to the cells' surface (not shown). When the concentration of cytochalasin was increased to $50 \mu\text{M}$, there were no detectable focal areas of enhanced staining for F-actin at any time point. Thus, depending upon the concentration of cytochalasin D used, the appearance of focal areas of enhanced F-actin staining beneath the test particles was delayed ($2\text{--}10 \mu\text{M}$ cytochalasin D) or abolished ($50 \mu\text{M}$ cytochalasin D). The presence of $1 \mu\text{M}$ cytochalasin D inhibited most of the quantitative increase in F-actin that otherwise occurred during phagocytosis (Fig. 6). At the 5-min time point, the presence of cytochalasin D inhibited the IgG-RBC-stimulated rise in F-actin content by $75 \pm 22\%$ (mean \pm SD, $n = 3$). These observations confirm that the increase in RP staining measured during Fc receptor-mediated phagocytosis (Fig. 4) reflects net polymerization of actin; they suggest that this polymerization is occurring primarily at the barbed (rapidly growing) end of the actin molecule (Cooper, 1987), and not by recruitment of preexisting filaments.

Fc Receptor-mediated Phagocytosis in Thio-macrophages Is Seldom Associated with an Increase in $[\text{Ca}^{2+}]_i$

McNeil et al. (1986) reported that aequorin-loaded adherent thio-macrophages (i.e., mouse peritoneal macrophages elicited after the intraperitoneal injection of thioglycollate broth) do not manifest a rise in $[\text{Ca}^{2+}]_i$ during phagocytosis of IgG-RBC. In contrast, Di Virgilio et al. (1988b) found a variable rise in $[\text{Ca}^{2+}]_i$ in response to Fc receptor ligation in an adherent population of thio-macrophages as assessed by fura-2 fluorescence. We measured $[\text{Ca}^{2+}]_i$ in single thio-macrophages undergoing phagocytosis of IgG-RBC. We found that these cells often ingested IgG-RBC without a detectable rise in $[\text{Ca}^{2+}]_i$; only 4/21 cells ingesting one to six IgG-RBCs exhibited a transient increase in $[\text{Ca}^{2+}]_i$. There was no relationship between the presence of a $[\text{Ca}^{2+}]_i$ transient and the number of IgG-RBC ingested. All of these macrophages were capable of manifesting a rise in $[\text{Ca}^{2+}]_i$, since they responded to $100 \mu\text{M}$ ATP, a concentration that causes an increase in $[\text{Ca}^{2+}]_i$ without "permeabilizing" the cell membrane (Greenberg et al., 1988), with a marked increase in $[\text{Ca}^{2+}]_i$ (Fig. 7 A). To confirm that we could detect changes in $[\text{Ca}^{2+}]_i$ during phagocytosis, we monitored $[\text{Ca}^{2+}]_i$ during phagocytosis in J774 cells. In contrast to thio-macrophages, the vast majority of J774 cells (21/25 cells) manifested a rise in $[\text{Ca}^{2+}]_i$ during ingestion of IgG-RBC, confirming our previous results obtained by monitoring $[\text{Ca}^{2+}]_i$ in a population of J774 cells stimulated with heat-aggregated IgG (Di Virgilio et al., 1988b). A typical recording of $[\text{Ca}^{2+}]_i$ during phagocytosis of four

Figure 2. Localization of F-actin during phagocytosis of IgG-coated erythrocytes after 0, 1, and 2 min of phagocytosis. A, C, and E are phase-contrast micrographs and B, D, and F are corresponding fluorescence micrographs of the same cells. (A and B) IgG-RBC were attached to thio-macrophages at 4°C . Note the paucity of RP staining in the erythrocytes themselves, and the uniform RP staining in the cortical cytoplasm beneath the opsonized particles (arrowheads). (C and D) After 1 min at 37°C , phagocytic cup formation is evident. Note the intense staining of F-actin by RP at the base of the cups (arrowheads). (E and F) After 2 min at 37°C , extension of the RP-stained pseudopods is evident. Note the thin rim of F-actin containing cytoplasm surrounding many IgG-RBCs (arrowheads; cf. C and D). Staining with RP as described in Materials and Methods. Bar, $10 \mu\text{m}$.



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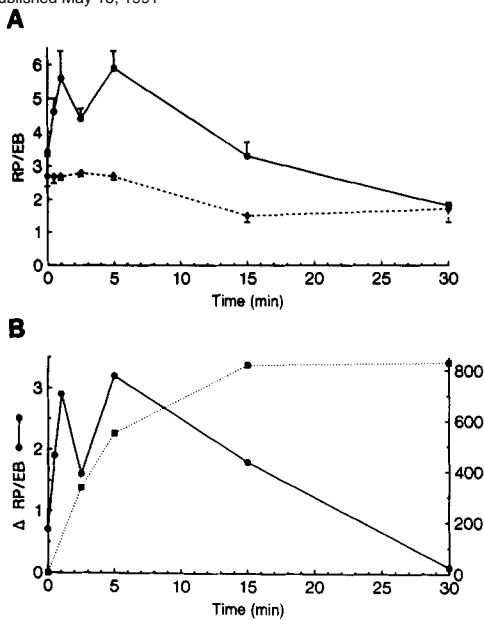


Figure 4. Fc receptor-mediated phagocytosis is accompanied by a rise in F-actin. (A) F-actin content of control macrophages (◆) and macrophages undergoing phagocytosis of IgG-RBC (●). Results are the mean \pm SD of a typical experiment performed in quadruplicate, and is representative of 15 such experiments. (B) Net increase in F-actin during phagocytosis (the data are derived from A) and are expressed as the difference between RP/EB of cells undergoing phagocytosis, and that of cells maintained in saline alone: Δ RP/EB = (RP/EB)_{experimental} - (RP/EB)_{control}. Phagocytosis index of macrophages performed in parallel (■). F-actin content and phagocytosis index were determined as described in Materials and Methods.

IgG-RBC in a single J774 cell is depicted in Fig. 7 B. Thus, Fc receptor-mediated phagocytosis in J774 cells is often accompanied by a transient rise in $[Ca^{2+}]_i$, whereas it is usually absent and therefore not necessary for particle engulfment in thio-macrophages.

Fc Receptor-mediated Phagocytosis Is Unimpaired in Thio-macrophages Whose $[Ca^{2+}]_i$ is Clamped to <3 nM

We used fura-2/AM and EGTA/AM to clamp the $[Ca^{2+}]_i$ in adherent thio-macrophages. The resting $[Ca^{2+}]_i$ under these conditions was often indistinguishable from "F_{min}" (Fig. 8 B and Table I), and these cells did not manifest a rise in $[Ca^{2+}]_i$ when stimulated with 2 μ M ionomycin (compare Fig. 8, B with A). We compared the ability of these $[Ca^{2+}]_i$ -clamped thio-macrophages to phagocytose IgG-RBC with that of macrophages phagocytosing in the presence or absence of extracellular Ca^{2+} (Fig. 9). Both the rate and extent of phagocytosis were unaffected in macrophages in which extracellular

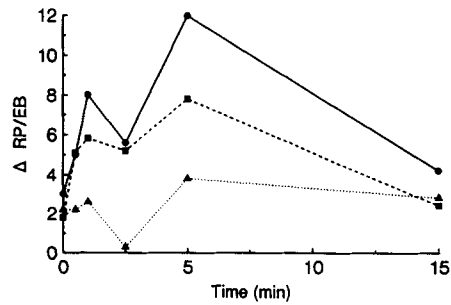


Figure 5. Maximal rise in F-actin is dependent on the number of IgG-RBC added at time 0. 1.25×10^8 IgG-RBC/ml added at time 0, phagocytosis index at 30 min = 530 (▲). 2.5×10^8 IgG-RBC/ml added at time 0, phagocytosis index at 30 min = 1280 (■). 5×10^8 IgG-RBC added at time 0, phagocytosis index at 30 min = 2370 (●).

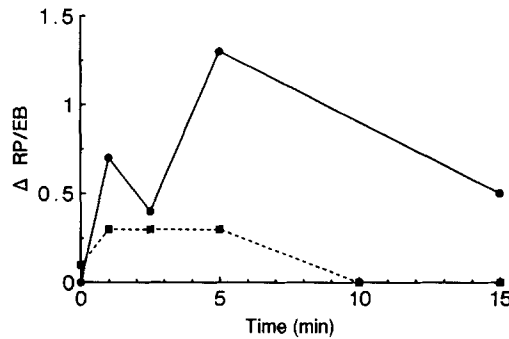


Figure 6. Cytochalasin D inhibits the increase in F-actin induced by Fc receptor ligation. Control macrophages phagocytosing in the presence of vehicle (DMSO) (●); macrophages treated with 1 μ M cytochalasin D from the time of addition of IgG-RBC onwards (■). Results are the mean of three experiments.

Ca^{2+} or extracellular and intracellular Ca^{2+} had been chelated. Thus, neither $[Ca^{2+}]_i$ transients nor a normal resting $[Ca^{2+}]_i$ were needed for efficient Fc receptor-mediated phagocytosis in thio-macrophages.

The Effect of Chelating the Extra- and Intracellular Ca^{2+} on F-Actin Kinetics during Fc Receptor-mediated Phagocytosis in Thio-macrophages

The mean F-actin content in control cells maintained in EGTA-containing buffer, or in cells whose $[Ca^{2+}]_i$ was chelated to <3 nM was not significantly different than that of cells maintained in Ca^{2+} -containing buffer; for example, the F-actin content (expressed in RP/EB units, mean \pm SD, $n = 23$) during the first 10 min at 37°C incubation was $2.7 \pm$

Figure 3. Localization of F-actin during phagocytosis of IgG-coated erythrocytes after 5, 10, and 30 min of phagocytosis. A, C, and E are phase-contrast micrographs and B, D, and F are corresponding fluorescence micrographs of the same cells. (A and B) After 5 min at 37°C phagocytic cup formation is maximal and RP is evident in these zones. (C and D) After 10 min at 37°C, most of the particles are completely ingested and are surrounded by a very faint rim of F-actin (arrows). A few peripherally located particles had not yet been ingested and are surrounded by a more prominent ring of F-actin-containing cytoplasm (arrowheads). (E and F) By 30 min, particle engulfment is complete and the F-actin is barely detectable around the phagosomes. Staining with RP as described in Materials and Methods. Bar, 10 μ m.

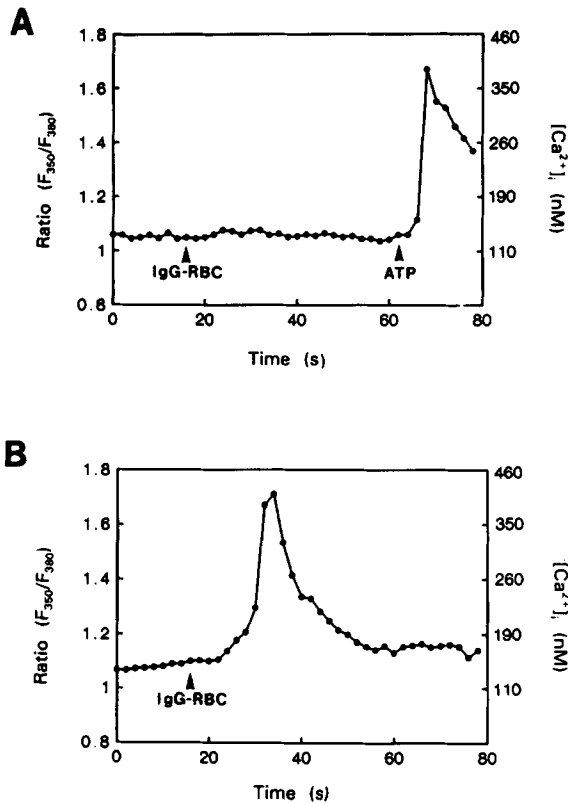


Figure 7. Single cell measurement of $[Ca^{2+}]_i$ in thio-macrophages and J774 macrophages during phagocytosis of IgG-coated erythrocytes. (A) A single thio-macrophage shows no $[Ca^{2+}]_i$ transient during phagocytosis of IgG-RBC, but does display an increase in $[Ca^{2+}]_i$ in response to 100 μ M ATP. A similar lack of rise in $[Ca^{2+}]_i$ was seen in 17/21 cells engulfing one to six particles during a time period of 80–160 s. (B) A single J774 cell shows a rise in $[Ca^{2+}]_i$ during the ingestion of four IgG-RBC. Both uniphasic and repetitive rises in $[Ca^{2+}]_i$ were seen in 21/25 cells ingesting up to six IgG-RBC.

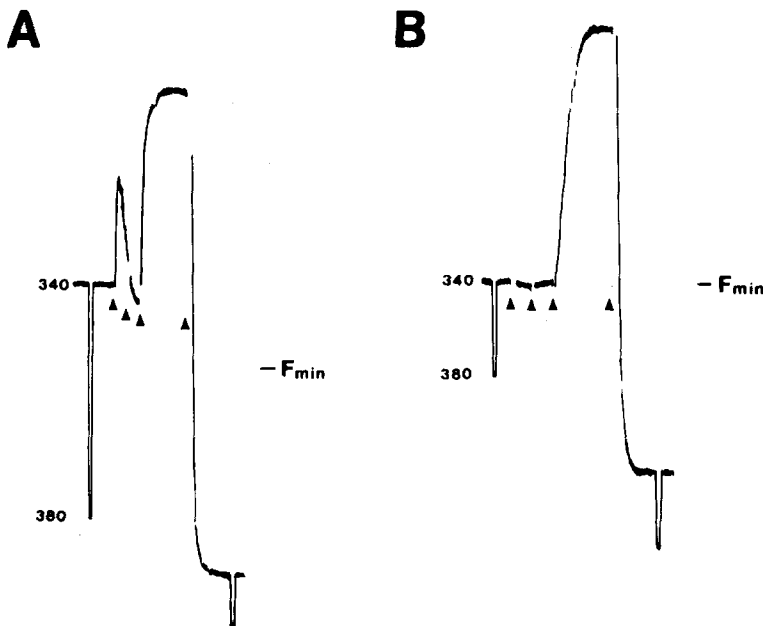


Figure 8. Chelation of $[Ca^{2+}]_i$ using fura-2/AM and EGTA/AM in the presence of extracellular EGTA. (A) $[Ca^{2+}]_i$ monitored in an adherent population of thio-macrophages incubated with fura-2/AM in the presence of extracellular Ca^{2+} and measured in the presence of 1 mM EGTA. Note the response to 2 μ M ionomycin (first arrowhead). Subsequent additions (arrowheads) were 1 mM ATP, 2 mM $CaCl_2$, and 1.5 mM $MnCl_2$. (B) $[Ca^{2+}]_i$ monitored in an adherent population of thio-macrophages incubated with fura-2/AM and EGTA/AM in the presence of 1 mM EGTA as described in Materials and Methods. Note the relatively low 340:380 ratio (cf. A) and the lack of response to 2 μ M ionomycin (first arrowhead). Other additions in the order and amounts indicated in A (arrowheads).

Table I. Average $[Ca^{2+}]_i$ of Adherent Populations of Thio-macrophages

Condition	$[Ca^{2+}]_i$, nM*	n
1 mM Ca^{2+}	131 \pm 41	8
1 mM EGTA	115 \pm 29	7
EGTA/AM;EGTA	3.0 \pm 3.6 [†]	7

$[Ca^{2+}]_i$ calculated according to Materials and Methods. First two conditions refer to extracellular concentrations of either Ca^{2+} or EGTA; final condition represents $[Ca^{2+}]_i$ -clamped cells as described in Materials and Methods.

* Mean \pm SD.

[†] This represents an upper limit estimate since five out of seven individual values for $[Ca^{2+}]_i$ were indistinguishable from F_{min} and were arbitrarily set to 1 nM (see text).

0.80 in the macrophages maintained in Ca^{2+} -containing medium, 2.5 ± 0.71 in macrophages maintained in EGTA-containing medium, and 2.6 ± 0.44 in the macrophages maintained in medium containing 1 mM EGTA in which the $[Ca^{2+}]_i$ was buffered with fura-2/AM and EGTA/AM (" $[Ca^{2+}]_i$ -clamped cells"). When phagocytosis was allowed to proceed in the presence of 1 mM extracellular EGTA, the kinetics of F-actin assembly during phagocytosis were similar to those observed in macrophages allowed to phagocytose in the presence of 1 mM extracellular Ca^{2+} (Fig. 10). We obtained similar results in $[Ca^{2+}]_i$ -clamped cells. As is evident from the data, we did not observe biphasic actin kinetics in macrophages phagocytosing IgG-RBC in medium containing EGTA, or in macrophages whose $[Ca^{2+}]_i$ had been clamped with fura-2/AM and EGTA/AM. However, the magnitude and extent of actin polymerization and depolymerization were similar in all cases, and there were no statistically significant differences between the $\Delta RP/EB$ values for all three groups at any given time point.

Discussion

The present study demonstrates that Fc receptor-mediated

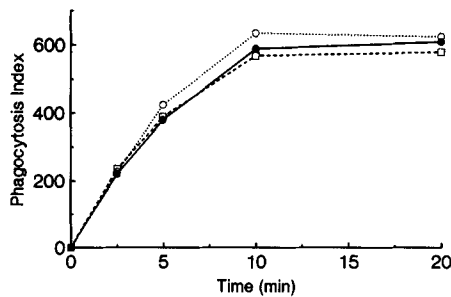


Figure 9. Phagocytosis proceeds unimpaired in macrophages whose extra- or intracellular $[Ca^{2+}]_i$ was chelated. Phagocytosis performed in the presence of 1 mM extracellular Ca^{2+} (●), 1 mM extracellular EGTA (○), and in $[Ca^{2+}]_i$ -clamped cells in EGTA-containing buffer (□) as described in Materials and Methods. Results are expressed as the mean of triplicate determinations, and are typical of three experiments.

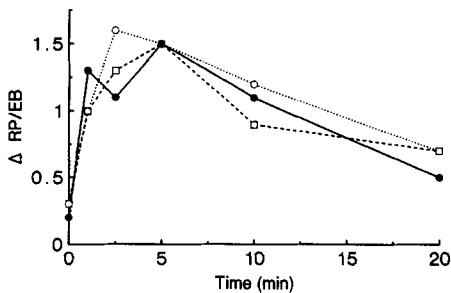


Figure 10. F-actin kinetics during phagocytosis are largely insensitive to extra- or intracellular $[Ca^{2+}]_i$. Phagocytosis and F-actin determinations performed in the presence of 1 mM extracellular Ca^{2+} (●), 1 mM extracellular EGTA (○), and in $[Ca^{2+}]_i$ -clamped macrophages maintained in EGTA-containing buffer (□) as described in Materials and Methods. Results are the mean of seven experiments.

phagocytosis in thio-macrophages is accompanied by a net increase in F-actin. This is consistent with the hypothesis that the localized accumulation of F-actin in the phagocytic cup region (as seen in Figs. 2 and 3, and Wang et al., 1984) is the result of newly polymerized actin and not just the redistribution of existing actin filaments. The fact that most of the increase in F-actin is sensitive to 1 μ M cytochalasin D implies that F-actin assembly is largely the result of growth of actin filaments from the barbed end of the molecule (Cooper, 1987). In these respects, F-actin assembly during Fc receptor-mediated phagocytosis in thio-macrophages resembles F-actin assembly after chemotactic peptide stimulation of human neutrophils (Wallace et al., 1984; Howard and Oresajo, 1985; Carson et al., 1986). The incomplete inhibition of F-actin assembly using 1 μ M cytochalasin D could reflect the inability of cytochalasins to completely block barbed-end assembly of F-actin (Bonder and Mooseker, 1986), especially at physiological salt concentrations (MacLean-Fletcher and Pollard, 1980). Alternatively, there may be some pointed-end assembly of F-actin that occurs during Fc receptor-mediated phagocytosis as well. The fact that both the quantitative increase in F-actin assembly (not shown), and the appearance of focal accumulations of F-actin beneath phagocytic cups were abolished by higher concentrations of cytochalasin may

reflect the ability of higher concentrations of cytochalasins to adversely affect actin network formation rather than to inhibit barbed-end assembly more completely (MacLean-Fletcher and Pollard, 1980).

The finding that the rate of phagocytosis was unaffected by chelating both extra- and intracellular Ca^{2+} , and was not dependent on $[Ca^{2+}]_i$ transients is consistent with the hypothesis that Fc receptor-mediated phagocytosis proceeds in a $[Ca^{2+}]_i$ -independent manner, confirming our previous results (Di Virgilio et al., 1988b), and in agreement with studies of phagocytosis of opsonized yeast in neutrophils (Rossi et al., 1989; Della Bianca et al., 1990). The lack of sensitivity of either actin polymerization or depolymerization to alterations in extra- or intracellular Ca^{2+} suggests that the transient rises in $[Ca^{2+}]_i$ seen during Fc receptor ligation in some, but not all macrophages, may reflect a signal transduction pathway that is "parallel" to, but independent of, the signal transduction pathway responsible for actin polymerization. The importance of $[Ca^{2+}]_i$ transients that occur during phagocytosis in some leukocytes is unclear, although they may be necessary for efficient phagosome-lysosome fusion (Jaconi et al., 1990).

The current study eliminates a normal $[Ca^{2+}]_i$ as a requirement for actin assembly during Fc receptor-mediated phagocytosis and is consistent with a model proposed by Stossel and co-workers that implicates a Ca^{2+} -independent dissociation of F-actin-gelsolin complexes as the initiating event in actin nucleation and increases in F-actin content (Stossel, 1989; Howard et al., 1990). Our results are also consistent with the recent demonstration that the increase in F-actin content in neutrophils after ionomycin stimulation is an indirect effect of LTB₄ receptor activation, and is not a direct effect of Ca^{2+} on actin or actin-binding proteins (Downey et al., 1990).

The biphasic rise in F-actin that accompanies phagocytosis, while interesting, does not appear necessary for particle engulfment since ingestion proceeded normally in cells that lacked the early (2 min) decline in F-actin (i.e., in cells whose extra- or intracellular Ca^{2+} was chelated; Figs. 9 and 10). It is unclear why this slight early decline in F-actin content was not seen in cells phagocytosing in the absence of either extra- or intracellular Ca^{2+} . In this one respect the actin dynamics were Ca^{2+} dependent. The early decline in F-actin may reflect remodeling of the newly assembled F-actin network that accumulates at the bases of phagocytic cups, as suggested by Fig. 2. Although its significance is unexplained, similar biphasic kinetics in F-actin assembly have been seen in PAF-stimulated (Shalit et al., 1987) and fMLP-stimulated (Sklar et al., 1985; Harvath, L., K. N. Prodou, and R. R. Aksamit. 1990. *J. Cell Biol.* 111[No. 5, Pt. 2]: 304a [Abstr.] neutrophils, and cAMP-stimulated *Dictyostelium* (Hall et al., 1988).

While the erythrocytes used in this study were opsonized with polyclonal IgG, it is likely that most of the "phagocytic signal" was generated through ligation of FcR II, since binding and phagocytosis was completely inhibitable by addition of mAb 2.4G2 (not shown). Since there are two subtypes of FcR II present on thio-macrophages (i.e., FcR IIB2 and IIA [Hunziker et al., 1990]), and both are recognized by mAb 2.4 G2, it is unknown which, if not both, is responsible for generating the "phagocytic signal," although a recent study

documents the independent phagocytic capacity of FcR IIA in mouse macrophages (Weinshank et al., 1988).

We have described dynamic $[Ca^{2+}]_i$ -independent changes in actin polymerization during phagocytosis; however, the question of whether and how actin polymerization translates into cellular movement is unexplained. The only clear-cut example of actin polymerization serving as a driving force for cell motility is in *Thyone sperm* (Tilney and Inoue, 1982). We suggest that during Fc receptor-mediated phagocytosis, actin polymerization itself is responsible for pseudopod extension. Newly formed F-actin filaments quickly become cross-linked by actin-binding protein into an isotropic gel. This process, if localized to the submembranous region as suggested by Figs. 2 and 3, might result in a distending force that acts to deform a relatively compliant plasma membrane. According to this view, movement is created by centripetal force applied to the cytosolic aspect of the plasma membrane. The centripetal force is generated by the growing isotropic network. This explanation could also account for the observation of *Listeria* movement in the cytosol of infected cells (Tilney and Portnoy, 1989; Dabiri et al., 1990), except that the distending force provided by the growing isotropic network is applied to the motile bacterium, and not the relatively compliant plasma membrane of the leukocyte. The posterior ends of the motile bacteria are followed by "tails" of F-actin arranged in an isotropic network; these tails elongate in the direction of bacterial movement. These findings led Tilney and Portnoy to suggest that the bacteria are propelled through the host cell's cytoplasm by the assembling actin filaments. Similarly, we predict that the force causing pseudopod extension during phagocytosis is the result of elongation and "pushing" by the actin network. This is conceptually opposite to myosin-based movements that depend on the "pulling" of actin cables by the myosin heads (Warrick and Spudich, 1987). One mechanical requirement for such a mechanism is that the network created by the newly polymerized actin is relatively stiff, and hence able to transmit force efficiently. This is consistent with both the physicochemical measurements of the viscoelasticity of gels containing F-actin and actin-binding protein (Janmey et al., 1990), and the decreased deformability measured in neutrophils stimulated with fMLP (Worthen et al., 1989), and is reminiscent of recent studies on the physical nature of membrane ruffles in fibroblasts (Felder and Elson, 1990). This mechanism also is consistent with the lack of requirement for myosin II in pseudopod extension in *Dictyostelium* (Warrick and Spudich, 1987). Although the molecular components of the pseudopod cytoskeleton is likely to be complex, involving multiple proteins (including F-actin and talin; Greenberg et al., 1990), the findings of this study support a central role for actin polymerization, and a lack of importance of $[Ca^{2+}]_i$ in the formation of pseudopods.²

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2. After acceptance of this manuscript increases in F-actin have been reported in human neutrophils in response to Fc receptor ligation (Salmon, J. E., N. L. Brogle, J. C. Edberg, and R. P. Kimberly. 1991. *J. Immunol.* 146:997-1004).

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