Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5430–5434, September 1984
Cell Biology

The increase in intracellular free calcium associated with IgGγ2b/γ1 Fc receptor–ligand interactions: Role in phagocytosis

(receptor aggregation/quin-2/monoclonal antibody/transmembrane ion flux/ion channel)

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Contributed by Zanvil A. Cohn, April 12, 1984

ABSTRACT **The concentration of cytosolic free calcium, $[Ca^{2+}]_i$, was measured in J774 mouse macrophages by use of the fluorescent indicator quin-2. Resting $[Ca^{2+}]_i$ was 87 nM. Addition of a number of specific ligands to the immunoglobulin γ2b/γ1 Fc receptor resulted in a transient increase in $[Ca^{2+}]_i$, the magnitude of which depended on the extent of receptor aggregation. Monovalent ligands gave only a small Ca^{2+} signal but blocked cell reponse to subsequent addition of multivalent ligands. Incubation with antibody-coated erythrocytes raised macrophage $[Ca^{2+}]_i$ to micromolar levels. $[Ca^{2+}]_i$ changes were only partially inhibited by the absence of external Ca^{2+}, suggesting the release of Ca^{2+} from internal stores in addition to an influx of external Ca^{2+}. These internal stores were not limited to mitochondria. An optimal range of $[Ca^{2+}]_i$ was required for phagocytosis. Buffering $[Ca^{2+}]_i$ with quin-2 and treating cells with quinine in the absence of external Ca^{2+} resulted in inhibition of phagocytosis. Increasing $[Ca^{2+}]_i$ to micromolar levels with the calcium ionophore A23187 also resulted in similar inhibitory effects. We suggest the involvement of localized cytosolic Ca^{2+} gradients in generating the signals necessary for phagocytosis.**

The binding of ligands to the Fc receptor (FcR) of mouse macrophages initiates the generation of a phagosome, the formation of toxic oxygen intermediates, and the secretion of arachidonic acid metabolites (1). We reported that the purified mouse FcR with IgGγ2b and IgGγ1 specificities (γ2b/γ1 FcR) functions as a ligand-dependent ion channel in model lipid membranes (2, 3). The receptor mediated ion flux is observed in intact cells as a transient plasma membrane depolarization (4). It seemed possible that these transmembrane ion fluxes might result in changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the subsequent internalization and movement of plasma membrane.

Changes in $[Ca^{2+}]_i$ are associated with a number of cell-surface activated responses (for review, see ref. 5). To measure $[Ca^{2+}]_i$, we used the fluorescent membrane-permeable acetoxymethyl ester quin-2/AM, which is trapped intracellularly as the impermeant anionic quin-2 (6). Quin-2 has a K_d value of $\approx 0.1 \times 10^{-6}$ M for Ca^{2+} and shows no binding to membranes or mitochondria, thereby providing a sensitive assay for $[Ca^{2+}]$. Here, we report on increases in $[Ca^{2+}]_i$ in macrophages associated with binding of FcR by ligands. In addition, we demonstrate the requirement of free ionized Ca^{2+} in the phagocytic process.

MATERIALS AND METHODS

Cells. The J774 macrophage cell line was maintained in spinner cultures in Dulbecco's modified Eagle's medium (DME medium)/fetal bovine serum as described (4). Resident mouse peritoneal macrophages were obtained by lavage of the peritoneal cavity with phosphate-buffered saline.

Reagents. Low- and high-K^+ buffers were as described elsewhere (4). Additions of Ca^{2+} and EGTA were made from concentrated stock solutions of $CaCl_2$ (0.25 M) and K_2H_2 EGTA. Ca^{2+} levels <50 μM were given by K_2H_2 EGTA and K_2Ca EGTA solutions, using dissociation constants published elsewhere (7). K_2Ca EGTA solutions were prepared by a pH-metric method (8). The intracellular pH and Mg^{2+} of J774 macrophages were taken as 6.8 and 1 mM, respectively, as measured in freeze–thawed cell suspensions. Calibration of quin-2 signal was done in high-K^+ buffer (different $[Ca^{2+}]$), pH 6.8, at 37°C.

The monoclonal antibodies 2.4G2 IgG and Fab fragments, 1.21J, 2D2C, and 2E2A and the immunocomplexes were prepared as described (2–4). Goat erythrocytes coated with rabbit anti-goat IgG (Cordis, Miami, FL) were prepared as described (9) and resuspended in Ca^{2+}-free low-K^+ buffer prior to use (9).

Quin-2 and its tetraacetoxymethyl ester derivative (quin-2/AM) were purchased from Lancaster Synthesis (Lancashire, England) and quantitated spectrophotometrically at 360 nM prior to use; A23187 came from Calbiochem; valinomycin, oligomycin, and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma.

Loading Quin-2 into Cells and Fluorescence Measurements. J774 cells at 10^7 cells per ml in DME medium/fetal bovine serum were incubated with 20 μM quin-2/AM for 90 min at 37°C. Cells were then washed (800 × *g*, 5 min) and resuspended in DME medium/fetal bovine serum. A cell density of 2×10^6 cells per ml was used for fluorescence studies. Fluorescence measurements were done at 37°C at 339 nm (excitation, 4 nm slits) and 492 nm (emission, 10 nm slits) on a Perkin–Elmer MPF-44A. Calibrations were done by determining first the intracellular concentration of quin-2 loaded into the cells. With this value of [quin-2] (0.21 mM) fixed, the K_d for the dissociation of quin-2-Ca^{2+} complex was obtained (10). For calibration of the signal after each experiment, maximum fluorescence (F_{max}) was taken from cell samples loaded with quin-2 and lysed in high-K^+ buffer (which contained excess Ca^{2+}), while minimum fluorescence (F_{min}) was obtained from cells lysed in Ca^{2+}-free high-K^+ buffer containing 2 mM EGTA (pH 9.0). The cell autofluorescence, obtained in the absence of quin-2 loading, was subtracted from all fluorescence readings. Cell lysis was produced either by sonication (20 sec; cell disruptor W-375, Ultrasonics) or by 0.05% Triton. $[Ca^{2+}]_i$ was then calculated by $[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F)$ (10), where F is the measured fluorescence. K_d was found to be 120×10^{-9} M.

Phagocytic Index. Macrophages were plated on coverslips at 37°C for 1 hr (J774) and 24 hr (resident peritoneal), washed 2 times with fresh medium, and challenged with IgG-coated erythrocytes, at an erythrocyte/macrophage ratio of 100:1.

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Abbreviations: FcR, Fc Receptor; γ2b/γ1 FcR, IgGγ2b/γ1 FcR; DME medium, Dulbecco's modified Eagle's medium; DNP, dinitrophenyl; HRP, horseradish peroxidase; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

The phagocytic index after 1 hr of coincubation was expressed as described (9). Noningested erythrocytes were lysed with 0.8% NH_4Cl. For scoring binding of IgG-coated erythrocytes to macrophages, cells were prepared as before except that after incubation at different time intervals, macrophages were washed 4 times with low-K^+ buffer, and the total number of erythrocytes bound to macrophages was scored. Binding was expressed as the average number of erythrocytes bound per macrophage.

RESULTS

Quin-2 Loading Conditions. Loading was complete after 60 min of incubation and showed an efficiency of 15%–20% at 20 μM quin-2/AM. With a cell volume of 1.50 μl per 10^6 J774 cells (4), this represented cell trapping of 0.18–0.24 mM quin-2. The maximum signal-to-noise ratio, as determined from the ratios of F_{max}/F_{min} was 4. $[Ca^{2+}]_i$ (at 20μM quin-2/AM) of J774 macrophages was 87 nM (±15 SEM; $n = 9$). Similar results were obtained with cells loaded with 10–70 μM quin-2/AM. $[Ca^{2+}]_i$ could be lowered to 29 nM (±9 SEM; $n = 6$) by incubating cells in Ca^{2+}-free medium, and to <20 nM if 0.2 mM EGTA was also added. The effect of EGTA was reversible, because subsequent addition of excess Ca^{2+} restored $[Ca^{2+}]_i$ to resting levels (not shown).

$[Ca^{2+}]_i$ Changes Produced by Surface Stimuli. Addition of 10 nM A23187, a Ca^{2+} ionophore, increased $[Ca^{2+}]_i$ to >1 μM within seconds (Fig. 1). The fluorescence increase decayed with a relaxation time of several minutes, the extent of which was dependent on the dose of A23187 used. Depletion of extracellular Ca^{2+} did not abolish the response elicited by A23187. In the absence of Ca^{2+} and with 0.2 mM EGTA the magnitude of the response corresponded to 40% of that observed in normal medium.

Addition of the monoclonal antibody 2.4G2 IgG (5×10^{-7} M), which binds to a functional site of FcR (1), increased $[Ca^{2+}]_i$ to 400 nM (±40 SEM; $n = 5$) within seconds (Fig. 2*a*). This response was transient, lasting 5–10 min, and showed strong dose dependence (Fig. 2*b*). The response showed saturation at 10^{-6} M 2.4G2. The relaxation time for complete recovery of baseline $[Ca^{2+}]_i$ also showed dependence on the dose of the IgG applied (Fig. 2*c*).

The monovalent ligand 2.4G2 Fab at 10^{-6} M was only capable of increasing $[Ca^{2+}]_i$ to 120 nM (±20 SEM; $n = 4$), but it blocked cell response to subsequent addition of 2.4G2 IgG (Fig. 3). Addition of A23187 (10 nM) at this point could trigger a burst in $[Ca^{2+}]_i$ (Fig. 3), indicating that A23187 could bypass the receptor binding and, furthermore, that cells bound with Fab were not incapacitated in eliciting a normal Ca^{2+} response. This result resembles previous observations made on FcR-mediated arachidonate metabolite release (11)

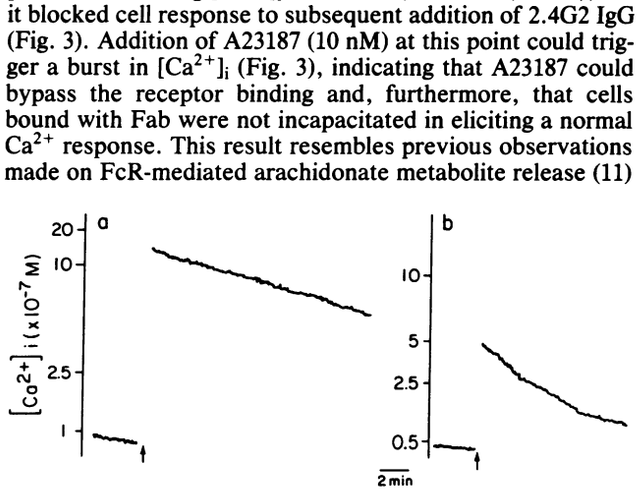

FIG. 1. Effect of A23187 on quin-2 signal. J774 cells (2×10^6 cells per ml), loaded with quin-2/AM, were treated with A23187 (10 nM) (arrow) in the presence of 1 mM Ca^{2+} (*a*) or no Ca^{2+} and 0.1 mM EGTA (*b*). In *b*, cells were washed in Ca^{2+}-free low-K^+ buffer and incubated for 10 min prior to addition of A23187. Resting $[Ca^{2+}]_i$ was 89 nM in *a* and 30 nM in *b*. The noise associated with addition of ligands (<10 sec) was deleted. The cell autofluorescence has been subtracted from the scales.

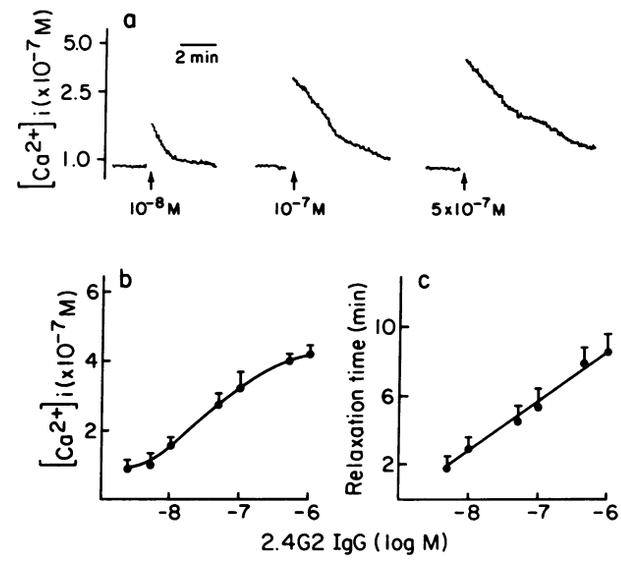

FIG. 2. Quin-2 signal in response to 2.4G2 IgG. (*a*) Cells (2×10^6 cells per ml) in low-K^+ buffer, loaded with quin-2, were treated with 2.4G2 IgG (arrows). (*b*) Peak $[Ca^{2+}]_i$ as a function of [2.4G2 IgG], determined as in *a*. (*c*) Time for recovery of $[Ca^{2+}]_i$ to baseline levels as a function of the concentration of 2.4G2 IgG added.

and transmembrane ion fluxes (4) in that binding to the receptor alone, without concomitant aggregation, is not sufficient to trigger a functional cellular response.

Ligands of higher valence (soluble and precipitable immune complexes) were more effective in increasing $[Ca^{2+}]_i$ at much lower concentrations. Binding of dinitrophenyl $(DNP)_{11}$-albumin complexed with rabbit anti-DNP IgG (1:6 molar ratio) with IgG at 1 μM (Fig. 4*a*), and of horseradish peroxidase (HRP)-rabbit anti-HRP complexes (40 μg of HRP) (Fig. 4*b*) increased $[Ca^{2+}]_i$ to 550 nM and 700 nM, respectively. The exposure of cell suspensions to IgG-coated erythrocytes also increased $[Ca^{2+}]_i$ to micromolar levels within seconds (the erythrocyte autofluorescence was subtracted from total cell fluorescence) (Fig. 4*c*). These results suggest that $[Ca^{2+}]_i$ may increase with functional binding of the FcR by different ligands and may further indicate that the magnitude of the response is dependent on the extent of multivalent aggregation of the FcR.

To verify whether the effects observed here were specific to FcR ligands, three other monoclonal antibodies [1.21J, 2D2C, and 2E2A; all directed toward major membrane proteins of mouse macrophages (12)] were tested. At 2 μM

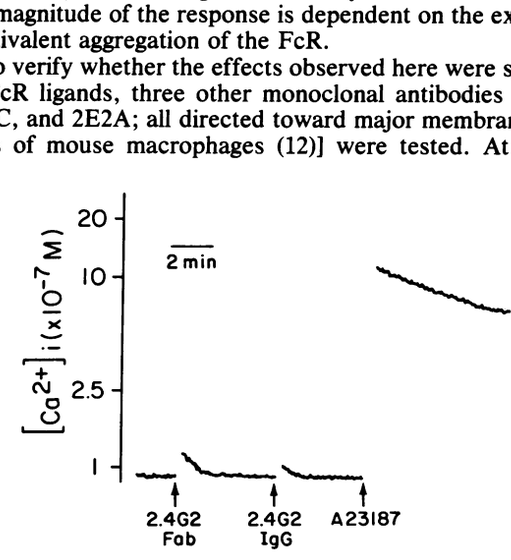

FIG. 3. Effect of 2.4G2 Fab on quin-2 signal. Cells (2×10^6 cells per ml), loaded with quin-2 in low-K^+ buffer, were treated with 1 μM 2.4G2 Fab (arrow). $[Ca^{2+}]_i$ increased to 120 nM. 2.4G2 IgG (500 nM) was added 5 min later, which increased $[Ca^{2+}]_i$ to 100 nM. A23187 (10 nM) was added 4 min later, increasing $[Ca^{2+}]_i$ to >1 μM.

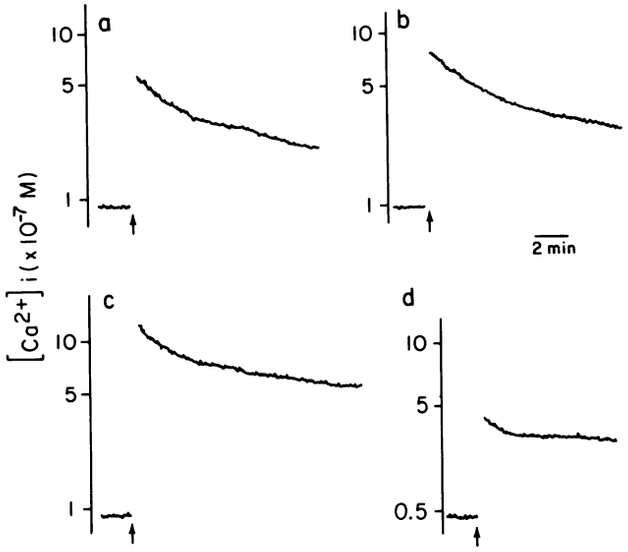

FIG. 4. Response of $[Ca^{2+}]_i$ to different ligands of the FcR. Cells (2×10^6 cells per ml) loaded with quin-2 in low-K^+ buffer were exposed to the following: (*a*) DNP_{11}-albumin complexed with rabbit anti-DNP IgG (1:6 molar ratio) with IgG at 1.5 μM; (*b*) HRP complexed with rabbit anti-HRP (50 μg of HRP per ml); and (*c*) sheep erythrocytes coated with rabbit anti-sheep erythrocytes IgG at 5×10 erythrocytes per ml. (*d*) Cells were washed 2 times and incubated (10 min) in Ca^{2+}-free buffer with 0.1 mM EGTA prior to addition of IgG-coated erythrocytes (5×10^7 erythrocytes per ml). Scales in *c* and *d* were corrected for autofluorescence of erythrocytes in addition to that of J774 cells.

each, $[Ca^{2+}]_i$ increased to 210, 180, and 150 nM for 1.21J, 2D2C, and 2E2A, respectively (not shown).

Sources of $[Ca^{2+}]_i$ Mobilized by FcR Binding. Extracellular Ca^{2+} could represent a major source of mobilized $[Ca^{2+}]_i$ as a result of binding to the FcR. To investigate the importance of this pool, cell suspensions loaded with quin-2 were incubated in the absence of extracellular Ca^{2+} and with 0.2 mM EGTA prior to the addition of FcR ligands. Fig. 4*d* shows such an experiment. Depletion of external Ca^{2+} inhibited the IgG-coated erythrocyte-elicited $[Ca^{2+}]_i$ increase by $\approx$70% (Fig. 4*d*) and similarly inhibited the response elicited by immunocomplexes (not shown). These results suggest that Ca^{2+} changes observed in the cells must depend on both the Ca^{2+} found in the extracellular medium and in intracellular stores.

Likely sources of internal Ca^{2+} include mitochondria. To assess the contribution of mitochondria to $[Ca^{2+}]_i$ changes, cells were treated with valinomycin or FCCP (5 μM; 30 min) in the absence of external Ca^{2+} and prior to addition of 2.4G2 IgG (Fig. 5). Treatment with valinomycin or FCCP increased $[Ca^{2+}]_i$ to 140–160 nM, but they were incapable of blocking Ca^{2+} signals after addition of antibody-coated erythrocytes (Fig. 5) or the monoclonal antibody 2.4G2 IgG (not shown). Similar results were obtained with cells treated with oligomycin (2 μM; 30 min) (not shown). These data imply the existence of other intracellular stores of releasable Ca^{2+}.

Binding of the FcR results in a transient membrane depolarization of the intact macrophage (4). This depolarization effect could trigger opening of voltage-dependent Ca^{2+} channels on the plasma membrane of macrophages, increasing cytosolic Ca^{2+} levels. To test this hypothesis, cells bathed in normal medium were depolarized with 50 mM KCl, resulting in a drop of 70% of the resting membrane potential, as measured by [^{3}H]tetraphenylphosphonium uptake (as in ref. 4; data not shown). Under such conditions, $[Ca^{2+}]_i$ increased only to 180 nm.

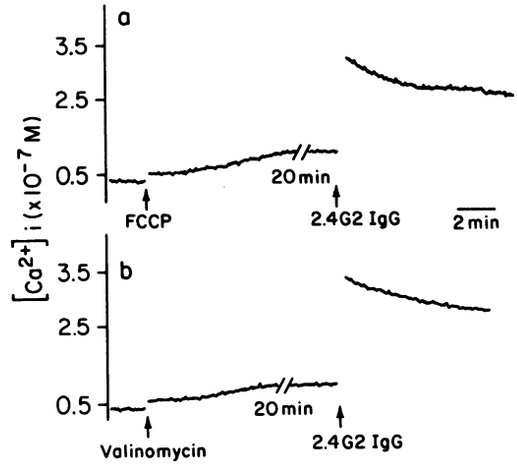

FIG. 5. Effect of mitochondrial inhibitors on quin-2 signal. Cells (2×10^6 cells per ml) in Ca^{2+}-free low-K^+ buffer were incubated with 5 μM FCCP (*a*) or 5 μM valinomycin (*b*) (parallel bars indicate 20 min). At the times indicated (arrows), 2.4G2 IgG (1 μM) was added, increasing $[Ca^{2+}]_i$ to 320 nM, comparable to that obtained in Ca^{2+}-free buffer and in the absence of FCCP or valinomycin.

Effect of $[Ca^{2+}]_i$ on Phagocytosis. To investigate the role of cytoplasmic $[Ca^{2+}]$ on phagocytosis, $[Ca^{2+}]_i$ was varied and the ingestion of opsonized erythrocytes was assessed. In the absence of external Ca^{2+} and with 0.2 mM EGTA, cells were still capable of ingesting, although at 40% of control levels (Table 1), consistent with published data (13). This effect was not simply due to inhibition of particle attachment, because in the absence of extracellular Ca^{2+} and with 0.2 mM EGTA, opsonized erythrocytes bound equally to macro-

Table 1. Effect of decreasing $[Ca^{2+}]$ on phagocytosis of opsonized erythrocytes by macrophages

Macrophage	Treatment*†	Phagocytic index	% control	Viability‡
J774	1 mM Ca^{2+}	1350	100	99
	No Ca^{2+}, 0.2 mM EGTA	810	40	95
	No Ca^{2+}, 0.2 mM EGTA, 100 μM quin-2/AM	55	4	93
	No Ca^{2+}, 0.2 mM EGTA, 100 μM quinine	41	3	92
Peritoneal	1 mM Ca^{2+}	980	100	97
	No Ca^{2+}, 0.2 mM EGTA, 100 μM quin-2/AM	50	5	94
	No Ca^{2+}, 0.2 mM EGTA, 100 μM quinine	41	4	91

Macrophages (10^5 per coverslip) were exposed to erthyrocytes in low-K^+ buffer with given amounts of Ca^{2+} and drugs. Data represent mean of triplicate determinations.

*Cells were washed (3 times) with cold Ca^{2+}-free low-K^+ buffer, incubated in the given buffer for 1 hr at 37°C, and exposed to opsonized erythrocytes for phagocytic index studies.

†$[Ca^{2+}]_i$ was determined for cells in suspension (2×10^7 cells per ml) receiving similar treatments as described here. $[Ca^{2+}]_i$ for J774 cells in low-K^+ buffer was 87 ± 15 nM (SEM; $n = 4$), 10 ± 8 nM in 0.2 mM EGTA (no Ca^{2+}), and <5 nM for cells in 0.2 mM EGTA/100 μM quinine (no Ca^{2+}). $[Ca^{2+}]_i$ for other data-points were not determined.

‡% total cells that excluded Trypan blue, determined in at least 100 cells.

Table 2. Effect of extracellular Ca^{2+} on binding of opsonized erythrocytes to macrophages

Treatment	Incubation time with erythrocytes, min	Erythrocytes bound/ J774 cell, no.	Cells counted, no.*
Low-K^+ buffer	1	0.75	310
	5	1.71	319
	10	12.44	303
	15	20.20	320
Ca^{2+}-free low-K^+ buffer	1	2.09	321
	5	2.75	316
	10	13.28	309
	15	18.80	318
Ca^{2+}-free low-K^+ buffer, 0.2 mM EGTA	1	0.98	229
	5	1.79	317
	10	16.36	200
	15	19.87	304
Ca^{2+}-free low-K^+ buffer, 100 μM quin-2/AM†	5	1.88	339
	10	14.32	308
Ca^{2+}-free low-K^+ buffer, 500 nM A23187†	5	2.21	321
	10	13.72	298

J774 cells (10^5 per coverslip) were incubated in buffer (30 min; 37°C) and exposed to IgG erythrocytes (washed 2 times in Ca^{2+}-free low-K^+ buffer). After incubation (time indicated), cells were washed 4 times with corresponding buffer and fixed.

*Total number of cells were pooled from three coverslips for each data point.

†Cells were washed in Ca^{2+}-free low-K^+ buffer and incubated with quin-2/AM (100 μM) or A23187 (500 nM) for 30 min before addition of IgG-coated erythrocytes.

phages at 37°C (Table 2) and at 4°C (not shown). $[Ca^{2+}]_i$ could also be buffered with quin-2/AM (100 μM; 60 min; no Ca^{2+}), under which conditions the ingestion of opsonized erythrocytes was decreased to <5% (Table 1), while binding remained unaltered (Table 2). Similar results were obtained with quinine (100 μm; 30 min) in Ca^{2+}-free medium (Table 1). Quinine is a weak base known to block Ca^{2+}-activated responses (4). Recovery of the phagocytic activity in treated cells occurred with the addition of 1 mM Ca^{2+} (Table 1). Similar results were obtained with resident mouse peritoneal macrophages (Table 1).

We also investigated the effect of increasing $[Ca^{2+}]_i$ on phagocytosis of opsonized erythrocytes. Treatment of J774 and peritoneal macrophages with A23187 (500 nM; 10 min) prior to ingestion of opsonized erythrocytes, which did not affect binding of erythrocytes to cells (Table 2), inhibited phagocytosis (Table 3). This inhibitory effect was reversible. Cells pretreated with A23187 for 10 min, washed, and incubated for 1 hr showed almost complete recovery of phagocytic activity (Table 3). Concentrations of A23187 >2 μM, however, were increasingly toxic to macrophages, as assessed by lack of recovery of phagocytosis and of Trypan blue exclusion. $[Ca^{2+}]_i$ could also be increased by increasing Ca^{2+} concentrations in the extracellular medium, but this was much less effective than treatment with A23187. The effects of such an increase in external $[Ca^{2+}]$ on phagocytosis and resting $[Ca^{2+}]_i$ are shown in Table 3. At external concentrations >10 mM, the ingestion of opsonized erythrocytes was significantly inhibited. The changes in $[Ca^{2+}]_i$, however, were small: at 15 mM external Ca^{2+}, the resting $[Ca^{2+}]_i$ was only 25% higher than that observed at 1 mM Ca^{2+}. Increasing Ca^{2+} to 15 mM did not exert any toxic effect on J774 cells, as assessed by unaltered ATP and protein synthesis (not shown). Although $[Ca^{2+}]_i$ changed little with increases in external $[Ca^{2+}]$, $[Ca^{2+}]_i$ increased to much higher levels with a phagocytic stimulus (4–5 times higher with cells bathed in 10 mM vs. 1 mM), but such Ca^{2+} signals could not be quantitated with precision as nonlinearity occurred at levels >1 μM. Higher $[Ca^{2+}]_i$ resulting in inhibition of phagocytosis reflected the effect of A23187, which produced higher $[Ca^{2+}]_i$ and stronger inhibition of ingestion of opsonized erythrocytes (Table 3).

DISCUSSION

Loading of quin-2 provides a rapid and sensitive assay for cytosolic ionized calcium (8, 10). Advantages of this technique include good approximations to resting $[Ca^{2+}]_i$ by quin-2. However, for surface-activated responses that result in fast Ca^{2+} transients, quantitation of Ca^{2+} levels by quin-2 fluorescence becomes much less precise. Quin-2 chelates Ca^{2+} and, as such, should buffer considerably any Ca^{2+} transient. Although we have chosen to work with low levels of quin-2 (20 μM quin-2/AM), the buffering effect could still

Table 3. Effect of increasing $[Ca^{2+}]_i$ on phagocytosis of opsonized erythrocytes by macrophages

Macrophage	Treatment	PI	% control	Viability	$[Ca^{2+}]_i$, nM ± SEM
J774	1 mM Ca^{2+}	1420	100	98	91 ± 9*
	2 mM Ca^{2+}	1480	104	97	89 ± 16
	3 mM Ca^{2+}	1510	106	94	97 ± 18
	5 mM Ca^{2+}	1350	95	93	99 ± 7
	7 mM Ca^{2+}	1235	87	93	100 ± 9
	10 mM Ca^{2+}	1008	71	92	107 ± 12
	15 mM Ca^{2+}	724	51	89	116 ± 17
	1 mM Ca^{2+}, 500 nM A23187†	298	21	91	>1000
	1 mM Ca^{2+}, 500 nM A23187, washed, incubated 1 hr‡	1235	87	92	120 ± 21
Peritoneal	1 mM Ca^{2+}	910	100	95	ND
	1 mM Ca^{2+}, 500 nM A23187	291	32	92	ND
	1 mM Ca^{2+}, 500 nM A23187, washed, incubated 1 hr	828	91	93	ND

Cells (10^5 per coverslip) were incubated in low-K^+ buffer with $[Ca^{2+}]$ for 10 min at 37°C prior to phagocytic index (PI) studies. ND, not determined.

*$[Ca^{2+}]_i$ was determined in cell suspensions as in Table 1.

†Cells were treated with 500 nM A23187 for 10 min at 37°C before exposure to IgG-coated erythrocytes.

‡Cells treated with A23187 for 10 min were washed 3 times with low-K^+ buffer (1 mM Ca^{2+}) and incubated for 1 hr at 37°C before challenge with opsonized erythrocytes.

mask fast changes of cytosolic $[Ca^{2+}]$. We took advantage of this observation by using quin-2 to buffer $[Ca^{2+}]_i$ in the absence of external Ca^{2+} to study the functional role of intracellular Ca^{2+} in phagocytosis. Quin-2 loading results in at least a 10-fold concentration of quin-2 in the cells. The loading efficiency varies with each cell type, which is probably dependent on the levels of esterases found in the cell. For macrophages (J774), we obtained a loading efficiency of 15%–20% and a maximum signal-to-noise ratio of only 4. At the moment, these observations preclude us from drawing a more accurate quantitative picture of the Ca^{2+} signal associated with the receptor-ligand interaction studied here.

$\gamma 2b/\gamma 1$ FcR-mediated responses in mouse macrophage, such as phagocytosis and secretion of inflammatory metabolites, involve extensive membrane movement. It is conceivable that free intracellular Ca^{2+} may play a direct role in all these processes (5). We have previously shown that FcR functions as a ligand-dependent cation channel in both native and reconstituted states (2–4). An increase in $[Ca^{2+}]_i$ could result from one or more of the following: (*i*) Ca^{2+} influx could occur directly through the occupied Fc receptor-channel due to the large transmembrane Ca^{2+} gradient, despite its low permeability to Ca^{2+} as measured in lipid vesicles and planar bilayers (2, 3); (*ii*) release of Ca^{2+} from internal stores and displacement of membrane-bound Ca^{2+}; (*iii*) depolarization-activated inward Ca^{2+} conductances.

Our results showed that aggregation of the FcR resulted in a dramatic increase in $[Ca^{2+}]_i$ that appeared to correlate with the extent of receptor aggregation. This result was not simply due to aggregation of surface antigens, because binding to three other major surface proteins by monoclonal antibodies resulted in considerably lower Ca^{2+} signals. Binding of the FcR by a monovalent ligand (the monoclonal 2.4G2 Fab) also resulted in little increase in Ca^{2+} signal, a result consistent with previous observations on the relative inefficacy of 2.4G2 Fab in triggering arachidonate metabolite release (11) and transmembrane ion fluxes (2–4). In the absence of external calcium, the FcR-mediated Ca^{2+} response was only partially (70%) blocked, suggesting that release of Ca^{2+} from internal stores represented a substantial portion of the observed signal. Mitochondrial blockers did not abolish calcium transients observed in the absence of external Ca^{2+}, implying the existence of other releasable stores of Ca^{2+}. One possible site of Ca^{2+} release could involve the segment of membrane directly apposed to the bound particle, as had been shown for neutrophils (14). An influx of Na^+ into the cell, triggered by the aggregated FcR, might result in such local displacement of bound Ca^{2+} from membranes (4). Finally, regarding voltage-gated inward Ca^{2+} conductances on macrophage membranes, our results showed that depolarizing cells with high external K^+ only increased $[Ca^{2+}]_i$ 2-fold. Assessment of the role of such conductances at this point should be viewed as inconclusive, because it is possible that such conductances may only be activated by fast and transient membrane depolarizations.

Decreasing internal $[Ca^{2+}]$ inhibited phagocytosis. This inference could only be convincingly demonstrated by buffering internal Ca^{2+} with quin-2 and treating cells with quinine in the absence of extracellular Ca^{2+}, because macrophages were still capable of phagocytosis in Ca^{2+}-free medium (Table 1). Inhibition of phagocytosis by quin-2 and quinine could be reversed by subsequent addition of 1 mM external Ca^{2+} (Table 1), so that the result could not be explained by a cytotoxic effect.

Increasing $[Ca^{2+}]_i$ by means of the calcium ionophore A23187 also inhibited phagocytosis of opsonized erythrocytes (Table 2). This effect was reversible, indicating that A23187 was not toxic to cells at the levels used. Increasing external $[Ca^{2+}]$ to raise $[Ca^{2+}]_i$ was only effective at concentrations >10 mM, at which levels the resting $[Ca^{2+}]_i$ changed little and phagocytosis was partially inhibited. At high external Ca^{2+}, however, the $[Ca^{2+}]_i$ associated with a phagocytic stimulus was severalfold higher than that found for cells incubated in low external $[Ca^{2+}]$.

Other studies that suggest a role for Ca^{2+} in phagocytosis by macrophages include observations that gelsolin (M_r, ≈93,000) confers Ca^{2+} dependence on the contractile proteins of macrophages (15); description of a Ca^{2+}-binding protein that becomes phosphorylated during phagocytosis (16); and the observation that extracellular ATP, which inhibits phagocytosis, also increases $[Ca^{2+}]_i$ to micromolar levels (unpublished observations).

A local increase in $[Ca^{2+}]_i$ may be involved in initiating phagocytosis. Such localized gradients could occur along and directly under the plane of the membrane in contact with the particle, with higher concentrations occurring in and tapering from the regions apposed to the bound particle. Local gradients could give rise to a vectorial flow of Ca^{2+} along the cytosol, which would orient movement of membranes during phagocytosis. The need for continued interaction between macrophage membrane receptors and particle-bound ligands to ensure the repeated generation of intracellular phagocytic signals has been proposed (17). We suggest that these phagocytic signals could occur in the form of segmental transmembrane ion fluxes, gated by the occupied and aggregated receptor as well as by the local displacement of membrane-bound Ca^{2+}. Both lowering or increasing $[Ca^{2+}]_i$ could mask this local gradient and inhibit phagocytosis.

Receptor aggregation resulting in enhanced endocytosis and/or exocytosis is a property of a number of different receptors. It would be interesting to verify whether such transient changes in free intracellular Ca^{2+} changes as observed here may be a general response to aggregation of surface receptors.

We thank Drs. J. Brown, J. Unkeless, and S. Silverstein for helpful discussions and Dr. J. Unkeless for the monoclonal antibodies. Dr. C. Nathan (Rockefeller University) and members of the Laboratory of Physiology at Marine Biological Laboratory (Woods Hole, MA) have kindly allowed us to use their spectrophotometers. This work was supported by fellowships from the Grass Foundation and the Jane Coffin Childs Memorial Research Fund for Medical Research to J.D.-E.Y. during different parts of this research and by Grants CA 30198 and AI 070127 from the National Institutes of Health to Z.A.C.

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