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Regional and Generalized Changes in Cytosolic Free Calcium in Monocytes during Phagocytosis

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We measured and visualized cytosolic free calcium ($[Ca^{2+}]_i$) in individual human peripheral blood monocytes during phagocytosis by using the fluorescent indicator fura-2. Monocytes exhibit a rapid rise in $[Ca^{2+}]_i$ from a basal level of 75 ± 11 nM to a peak level of 676 ± 78 nM (means ± standard errors of the means; $P < 0.001$) by 34 ± 5 s after contact with opsonized zymosan particles, and a thin rim of high $[Ca^{2+}]_i$ was observed surrounding the ingested particle.

In neutrophils, cytosolic free calcium ($[Ca^{2+}]_i$) has been proposed as an important regulator for the activation of phagocytosis (3, 20, 21), degranulation, and the oxidative burst (10, 17, 19). We (16, 18) and Marks and Maxfield (13) have observed generalized $[Ca^{2+}]_i$ increases during phagocytosis in neutrophils and transient localized increases in $[Ca^{2+}]_i$ in the paraphagosomal region of phagocytosing neutrophils.

Studies of transient $[Ca^{2+}]_i$ increases in large numbers of monocytes during phagocytosis report conflicting results; two indicate an increase (4, 23), and one shows no change (14). In a single cell study, transient oscillations in the $[Ca^{2+}]_i$ were observed in macrophages during frustrated phagocytosis, and possible differences between neutrophil and macrophage signaling mechanisms or effector mechanisms were suggested (11). A previous study with single cells from our laboratory (7) indicated that there are transient $[Ca^{2+}]_i$ increases in monocytes during phagocytosis. We now extend these earlier studies by examining the kinetics and regional patterns of $[Ca^{2+}]_i$ by using the intracellular fluorescent calcium probe fura-2 to visualize $[Ca^{2+}]_i$ fluxes during phagocytosis of opsonized zymosan by single human peripheral blood monocytes.

Peripheral blood mononuclear cells were obtained from whole blood of healthy human volunteers by Ficoll-Hypaque (Flow Laboratories, McLean, Va.) density gradient centrifugation by the method of Böyum (2) as modified by Ferrante and Thong (5). The cell concentration was adjusted to 1×10^6 to 2×10^6/ml in Hanks balanced salt solution (HBSS; M. A. Bioproducts, Walkersville, Md.) supplemented with 5% autologous human serum containing 1.3 mM Ca^{2+} (Ca^{2+}-containing incubation medium). A sample (100 μl) of cell suspension was placed onto a coverslip and incubated at 37°C for 30 min in a 5% CO_2 incubator. Nonadherent cells were removed by three washes with 37°C HBSS. Serum-treated zymosan (STZ; Sigma Chemical Co., St. Louis, Mo.) particles were prepared as previously described (16).

Monocytes that adhered to coverslips were incubated with 5 μM fura-2 acetoxy methyl ester {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester; Calbiochem-Behring, La Jolla, Calif.} in HBSS at 37°C for 30 min. After incubation, the cells were rinsed with HBSS (37°C) and held at room temperature for 30 min, allowing ester hydrolysis to occur. The coverslips with attached fura-2-loaded cells (1×10^5 to 2×10^5) were inverted onto glass slides over a suspension of STZ particles (1×10^6 to 2×10^6), and the edges of the coverslips were secured with a melted paraffin-petroleum jelly mixture for examination with a microscope. For experiments in the absence of extracellular Ca^{2+}, fura-2-loaded cells were incubated in Ca^{2+}-free HBSS containing 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Sigma) and 0.8 mM $MgSO_4$ at 37°C for 1 min just before microscopy. Particles were also washed and resuspended with Ca^{2+}-free HBSS containing 5 mM EGTA and 0.8 mM $MgSO_4$.

Fluorescence microscopy and video image analysis were performed as previously described (16). Images of a single cell were recorded at 5- to 15-s intervals for at least 5 min during phagocytosis. To minimize photobleaching and pho-

TABLE 1. $[Ca^{2+}]_i$ changes in monocytes during phagocytosis of opsonized zymosan[a]

Medium (*n*)	Fraction	$[Ca^{2+}]_i$ (nM)	Time (s) to:	
			Peak level	Baseline level[b]
With Ca^{2+} (17)	Whole cell	676 ± 78	34 ± 5	115 ± 15
	Paraphagosomal	720 ± 103	35 ± 5	
	Cell body	665 ± 83	35 ± 6	
Without Ca^{2+} (16)	Whole cell	162 ± 42	22 ± 3	83 ± 14
	Paraphagosomal	230 ± 63	22 ± 4	
	Cell body	146 ± 42	22 ± 3	

[a] Data are expressed as the means ± SEM of 17 and 16 cells, each from a different donor. $[Ca^{2+}]_i$ increased significantly from the basal level to peak level in both Ca^{2+}-containing and Ca^{2+}-free medium ($P \leq 0.001$). The peak $[Ca^{2+}]_i$s are slightly higher in the paraphagosomal regions in both Ca^{2+}-containing and Ca^{2+}-free medium, but the differences are not significant ($P > 0.5$ and $P > 0.1$, respectively). The basal $[Ca^{2+}]_i$ decreased and the peak $[Ca^{2+}]_i$ was blunted in Ca^{2+}-free medium ($P < 0.01$ and $P < 0.001$, respectively). The time until the peak and time until the level returned to near the baseline level were not significantly shorter in Ca^{2+}-free medium than in Ca^{2+}-containing medium ($P \geq 0.2$).

[b] Time to a stable level slightly above the basal levels, which were 75 ± 11 and 32 ± 9 nM in media with and without Ca^{2+}, respectively.

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totoxicity, the illumination time during the measurement of fluorescence at each wavelength was limited to 0.5 to 1.0 s. Paired images were separated in time by less than 0.5 s. To measure the $[Ca^{2+}]_i$, each raw fluorescence image was digitized to 640 by 480 by 8 bits with gray scale intensity values ranging from 0 to 255, and eight consecutive digitized frames were averaged with background subtraction. Regional intensity readings were taken to examine localized changes in $[Ca^{2+}]_i$ at the corresponding pixels at 340- and 380-nm fluorescence images. Three to five reading blocks (1 to 9 μm^2 per block) for localized density readings were appropriately chosen in the cell body and in the paraphagosomal region (which was defined as the cytoplasmic area within 3 μm of the edge of the attached or ingested particle [16]). The ratio of fluorescence intensity at 340 nm to that at 380 nm was calculated as previously described (6). Calibration of $[Ca^{2+}]_i$ was done by using Ca^{2+}-depleted and Ca^{2+}-saturated cells prepared as previously described (8, 16). This ratio measurement method largely cancels out the effects of cell thickness, variations in intracellular free fura-2 concentration, and instrumental efficiency; these uncertainties can jeopardize measurements at single wavelengths (22).

Transient and regional $[Ca^{2+}]_i$ changes were determined in single peripheral blood monocytes during phagocytosis of STZ. The data were obtained from cells that ingested only one opsonized particle. Within 5 s of contact with STZ, the generalized $[Ca^{2+}]_i$ began to increase from a basal level of 75 ± 11 nM and reached a peak of 676 ± 78 nM (mean ± standard error of the mean [SEM]; $P < 0.001$) within 2 min (34 ± 5 s; mean ± SEM). After the maximum value, the $[Ca^{2+}]_i$ decreased with minor fluctuations to a level slightly above the basal level within 3 min (115 ± 15 s; mean ± SEM) and then recovered to the basal level over a 5- to 15-min period (Table 1; Fig. 1A and 1C).

Monocytes are relatively slow-moving cells compared with neutrophils. There was less movement of monocytes in Ca^{2+}-free medium than in medium containing Ca^{2+}. There was less active ruffling and pseudopod extension in the absence of Ca^{2+}; however, once the cell contacted an opsonized zymosan particle, the rate of ingestion seemed to be little affected by the absence of extracellular calcium. Monocytes incubated in the absence of Ca^{2+} ($[Ca^{2+}]_o < 10$ nM) showed transient $[Ca^{2+}]_i$ increases during phagocytosis that were similar to those of cells in Ca^{2+}-containing medium, but the values were lower (Table 1).

Monocytes consistently showed a thin rim of increased $[Ca^{2+}]_i$ surrounding the ingested particle during the early phase of phagocytosis (within 1 min after contact) (Fig. 2B). However, there were no significant differences in mean $[Ca^{2+}]_i$ between the paraphagosomal region and the cell body in monocytes incubated with or without Ca^{2+} (Table 1; Fig. 1B).

Phagocytosis in monocytes is accompanied by a generalized rise in $[Ca^{2+}]_i$; this mobilization of cytosolic free calcium occurs in the absence of extracellular Ca^{2+}, albeit at lower levels. This indicates that the release of Ca^{2+} from intracellular pools is responsible for some of the rise in $[Ca^{2+}]_i$ during phagocytosis, even though most of the increase in $[Ca^{2+}]_i$ comes from extracellular sources. Regional transient increases in $[Ca^{2+}]_i$ in ingesting monocytes were characterized by a thin rim of paraphagosomal increases in $[Ca^{2+}]_i$ which were different from the broader paraphagosomal increases in $[Ca^{2+}]_i$ observed in ingesting neutrophils (16, 18). The data suggest that the fine zone of paraphagosomal increased $[Ca^{2+}]_i$ is not an artifact caused by changes in the thickness of the cytoplasm surrounding the phagosome. Our imaging system showed at least 15 arbitrary light units of fluorescence (at a 380-nm wavelength) over background fluorescence even in the thinnest parts of the monocytes. Thus, there should be enough cytoplasm for accurate ratio imaging with fura-2 in this region. Moore et al. (15)

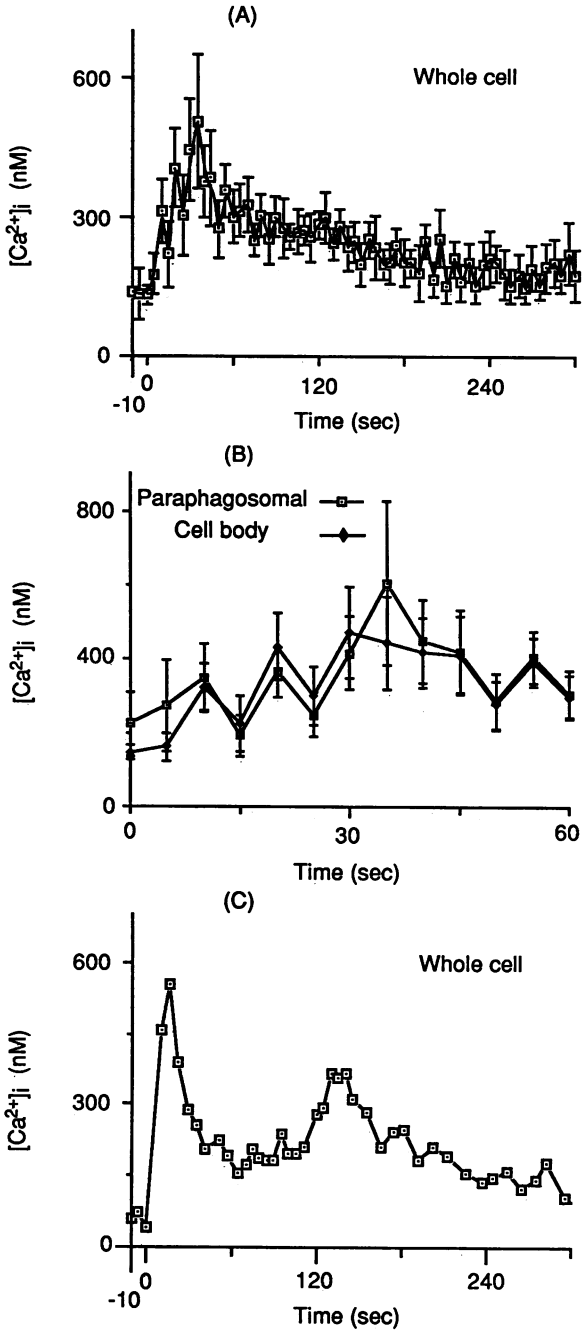

FIG. 1. Time course of $[Ca^{2+}]_i$ changes in monocytes during ingestion of opsonized zymosan in Ca^{2+}-containing medium. (A) Whole cell $[Ca^{2+}]_i$. (B) Paraphagosomal and cell body $[Ca^{2+}]_i$ during the first 60 s after phagocytosis. Contact with the particle is at 0 s. Each point up to 180 s indicates the mean ± SEM of at least seven cells each from a different donor. (C) An example of whole cell $[Ca^{2+}]_i$ changes in a single cell. This cell shows an initial major peak followed by a minor peak in $[Ca^{2+}]_i$.

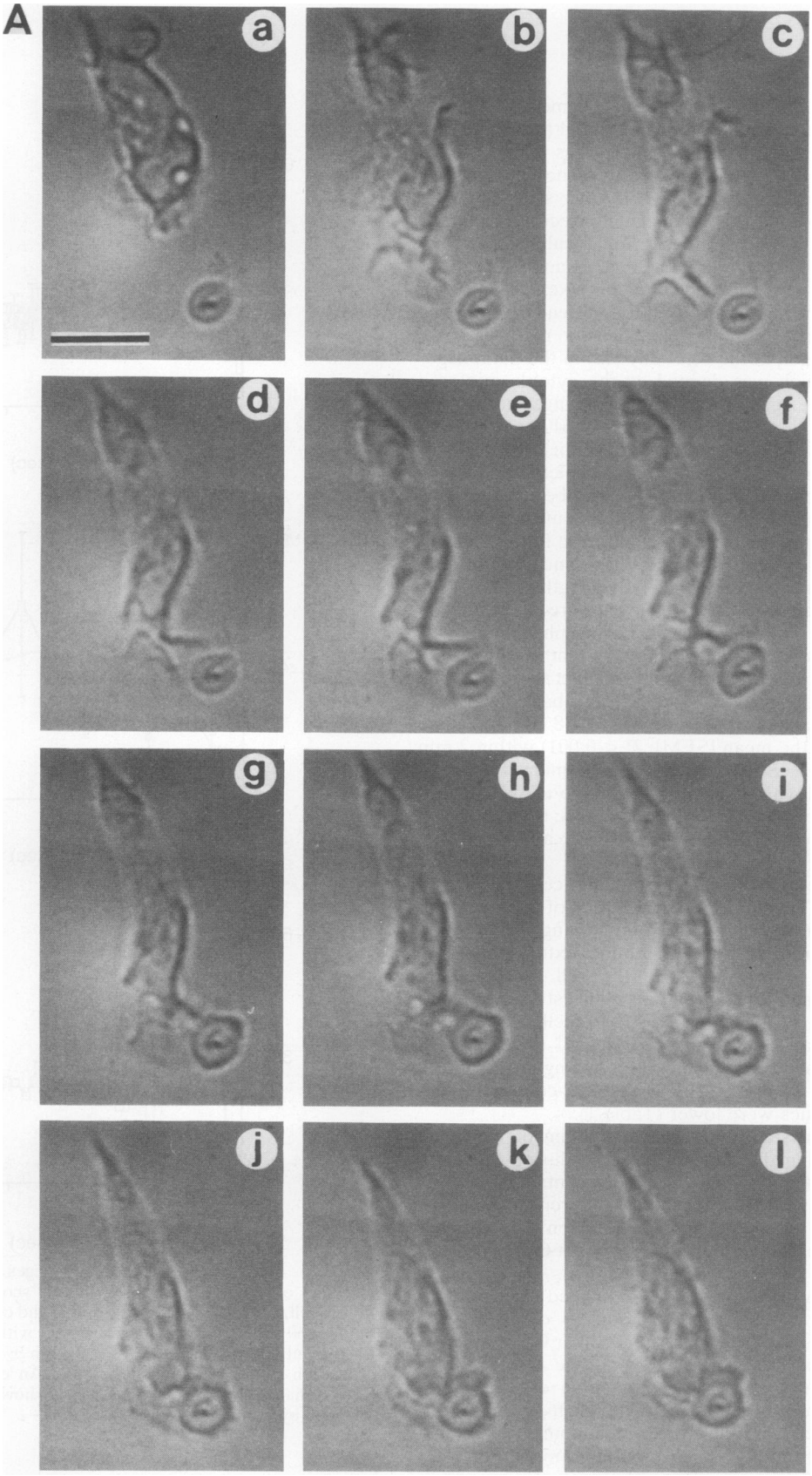

FIG. 2. $[Ca^{2+}]_i$ changes and regional distribution in a typical monocyte undergoing phagocytosis of opsonized zymosan: bright-field images (A) and corresponding monochromic ratio images (B) in the same cell at 212 (a) and 52 (b) s before contact with STZ and 0 (c), 12 (d), 20 (e), 32 (f), 42 (g), 55 (h), 65 (i), 75 (j), 87 (k), and 95 (l) s after attachment to an STZ particle. The oblique arrows in panel B, image f, indicate the approximate $[Ca^{2+}]_i$ in the paraphagosomal region (lower arrow, 900 nM), the intervening zone between the paraphagosomal area and the cell body (middle arrow, 800 nM), and the cell body (upper arrow, 1100 nM). The scale indicates the approximate nanomolar $[Ca^{2+}]_i$. Horizontal wedges indicate the location of the ingested particle. Note the thin rim of increased $[Ca^{2+}]_i$ around the ingested particle. Bars, 10 μm.

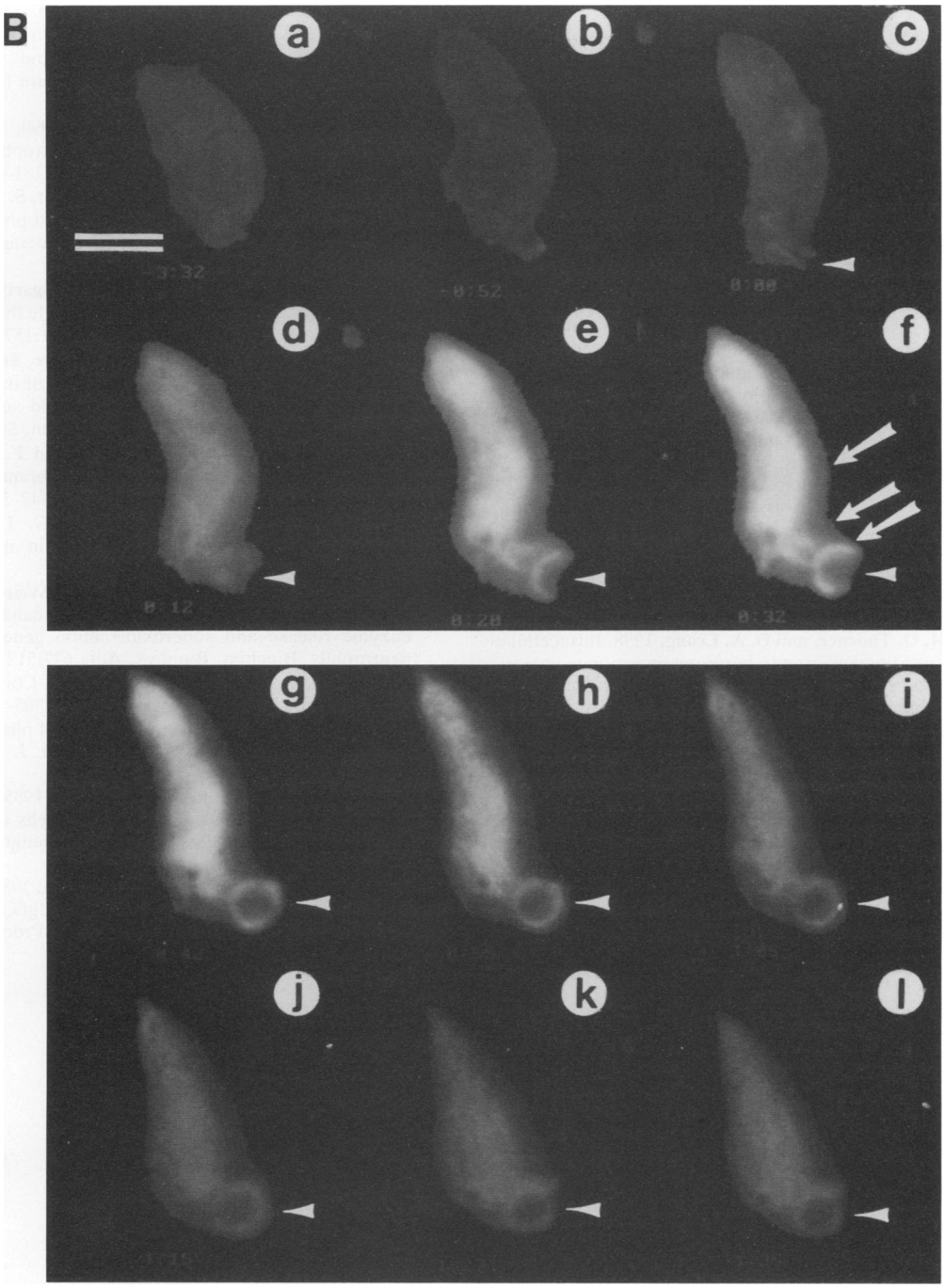

FIG. 2—*Continued.*

concluded that the measurement of $[Ca^{2+}]_i$ in a small compartment within the cytoplasm works best with very thin cells.

Previous data from our laboratory demonstrated that localized increases in $[Ca^{2+}]_i$ in the paraphagosomal area of neutrophils during phagocytosis are closely associated with degranulation and localized activation of the respiratory burst (16). The thin rim of increased $[Ca^{2+}]_i$ in the monocyte paraphagosomal area may reflect localized activities related to phagosomal events. The respiratory burst is less dramatic in monocytes (1), in which there are fewer granules than in neutrophils. In neutrophils, Fc receptor-mediated phagocytosis is a Ca^{2+}-dependent process (12) and $[Ca^{2+}]_i$ elevation is necessary to trigger subsequent phagosome-lysosome fusion during phagocytosis (9).

Regional $[Ca^{2+}]_i$ responses during phagocytosis differ, depending on the type of phagocyte (monocyte, macrophage, or neutrophil) and the particle. The observed dissimilarities may suggest fundamental differences in the sources and functions of cytosolic free calcium in the phagocytes.

REFERENCES

1. **Beaman, L., and B. L. Beaman.** 1984. The role of oxygen and its derivatives in microbial pathogenesis and host defense. Annu. Rev. Microbiol. **38:**27–48.
2. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77–89.
3. **Campbell, A. K.** 1983. Intracellular calcium: its universal role as regulator. John Wiley and Sons, Chichester, United Kingdom.
4. **Di Virgillio, F., B. C. Meyer, S. Greenberg, and S. C. Silverstein.** 1988. Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. J. Cell Biol. **106:**657–666.
5. **Ferrante, A., and Y. H. Thong.** 1980. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. J. Immunol. Methods **36:**109–117.
6. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
7. **Hoffman, T., E. F. Lizzio, J. Suissa, D. Rotrosen, J. A. Sullivan, G. L. Mandell, and E. Bonvini.** 1988. Dual stimulation of phospholipase activity in human monocytes: role of calcium-dependent and calcium-independent pathways in arachidonic acid release and eicosanoid formation. J. Immunol. **140:**3912–3918.
8. **Holl, R. W., M. O. Thorner, and D. A. Leong.** 1988. Intracellular calcium concentration and growth hormone secretion in individual somatotropes: effect of growth hormone-releasing factor and somatostatin. Endocrinology **122:**2927–2932.
9. **Jaconi, M. E. E., D. P. Lew, J. L. Carpentier, K. E. Magnusson, M. Sjögren, and O. Stendahl.** 1990. Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. J. Cell Biol. **110:**1555–1564.
10. **Korchak, H. M., K. Vienne, L. E. Rutherford, C. Wilkenfeld, M. C. Finkelstein, and G. Weissmann.** 1984. Stimulus response coupling in the human neutrophil. II. Temporal analysis of changes in cytosolic calcium and calcium efflux. J. Biol. Chem. **259:**4076–4082.
11. **Kruskal, B. A., and F. R. Maxfield.** 1987. Cytosolic free calcium increases before and oscillates during frustrated phagocytosis in macrophages. J. Cell Biol. **105:**2685–2693.
12. **Lew, D. P., T. Andersson, J. Hed, F. D. Virgillio, T. Pozzan, and O. Stendahl.** 1985. Ca^{2+}-dependent and Ca^{2+}-independent phagocytosis in human neutrophils. Nature (London) **315:**509–511.
13. **Marks, P. W., and F. R. Maxfield.** 1990. Local and global changes in cytosolic free calcium in neutrophils during chemotaxis and phagocytosis. Cell Calcium **11:**181–190.
14. **McNeil, P. L., J. A. Swanson, S. D. Wright, S. C. Silverstein, and D. L. Taylor.** 1986. Fc receptor-mediated phagocytosis occurs in macrophages without an increase in average $[Ca^{++}]_i$. J. Cell Biol. **102:**1586–1592.
15. **Moore, E. D. W., P. L. Becker, K. E. Fogarty, D. A. Williams, and F. S. Fay.** 1990. Ca^{2+} imaging in single living cells: theoretical and practical issues. Cell Calcium **11:**157–179.
16. **Murata, T., J. A. Sullivan, D. W. Sawyer, and G. L. Mandell.** 1987. Influence of type and opsonization of ingested particle on intracellular free calcium distribution and superoxide production by human neutrophils. Infect. Immun. **55:**1784–1791.
17. **Romeo, D., G. Zabucchi, N. Miani, and F. Rossi.** 1975. Ion movement across leukocyte plasma membrane and excitation of their metabolism. Nature (London) **253:**542–544.
18. **Sawyer, D. W., J. A. Sullivan, and G. L. Mandell.** 1985. Intracellular free calcium localization in neutrophils during phagocytosis. Science **230:**663–666.
19. **Smolen, J. E., H. M. Korchak, and G. Weissmann.** 1981. The roles of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils. Biochim. Biophys. Acta **677:**512–520.
20. **Southwick, F. S., and T. P. Stossel.** 1983. Contractile protein in leukocyte function. Semin. Hematol. **20:**305–321.
21. **Stossel, T. P.** 1973. Quantitative studies of phagocytosis: kinetic effects of cations and heat-labile opsonin. J. Cell Biol. **58:**346–356.
22. **Tsien, R. Y., T. J. Rink, and M. Poenie.** 1985. Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. Cell Calcium **6:**145–157.
23. **Young, J. D.-E., S. S. Ko, and Z. A. Cohn.** 1984. The increase in intracellular free calcium associated with $IgG_{\gamma}2b/_{\gamma}1$ Fc receptor-ligand interactions: role in phagocytosis. Proc. Natl. Acad. Sci. USA **81:**5430–5434.