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22 November 1982; revised 18 August 1983

Is Cytosolic Ionized Calcium Regulating Neutrophil Activation?

Abstract. *The concentration of cytosolic ionized calcium, $[Ca^{2+}]_i$, was measured in intact neutrophils by use of a fluorescent indicator trapped in the cytoplasm. A given rise of $[Ca^{2+}]_i$ elicited by the chemotactic peptide formylmethionylleucylphenylalanine (FMLP) was associated with a much greater degree of superoxide generation and myeloperoxidase secretion than was the same or larger $[Ca^{2+}]_i$ produced by a specific calcium ionophore, ionomycin, which bypasses cell surface receptors. Thus, FMLP appears to generate some important excitatory signal in addition to a rise in $[Ca^{2+}]_i$, and exocytosis and superoxide generation in neutrophils may not be simply dependent on $[Ca^{2+}]_i$ as is widely supposed.*

The production of oxygen radicals and secretion of lysosomal enzymes by neutrophil leukocytes are vital links in animal defenses against infections. These neutrophil responses are conveniently elicited in vitro by various compounds that bind to specific cell surface receptors. It is generally thought that neutrophil activation occurs by raising the concentration of cytoplasmic free Ca^{2+}, $[Ca^{2+}]_i$, which then triggers superoxide generation (*1*), enzyme secretion (*2*), actin gel-sol transitions (*3*), and locomotion (*4*).

However, the difficulty of measuring $[Ca^{2+}]_i$ directly in small mammalian cells in suspension has been a major obstacle in studying the role of $[Ca^{2+}]_i$ in the mechanism of transmembrane signaling. New methods for loading either fluorescent Ca^{2+} indicators (*5*) or Ca^{2+}-sensitive photoproteins (*6, 7*) into the cytoplasm of small intact cells have been described. Using erythrocyte-leukocyte hybrids, loaded with the photoprotein obelin, Hallett and Campbell (*6*) detected a rise of $[Ca^{2+}]_i$ when the cells were exposed to a number of stimuli. However, the use of virally fused cell hybrids is a laborious technique that involves a major rearrangement of the plasma membrane, the main target in neutrophil activation. Moreover, the quantitative and causal relationships between $[Ca^{2+}]_i$ and neutrophil responses have not yet been shown.

In neutrophils $[Ca^{2+}]_i$ can now be easily measured with a fluorescent indicator trapped in the cytoplasm of intact cells according to the method described by Tsien *et al.* (*5*). Using this procedure, we investigated the effect on exocytosis and superoxide (O_2^-) generation of increasing $[Ca^{2+}]_i$ either with ionomycin, a specific Ca^{2+} ionophore, or with the chemo-

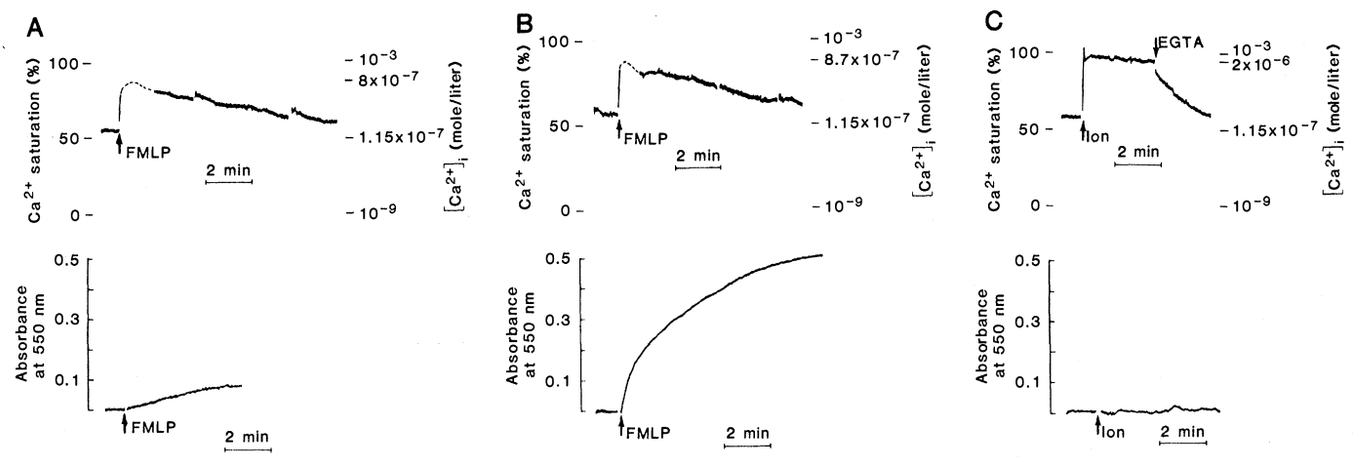

Fig. 1. Effect of FMLP and ionomycin on $[Ca^{2+}]_i$ and O_2^- generation in Ca^{2+} medium [138 m*M* NaCl, 6 m*M* KCl, 1.2 m*M* P_i, 1.2 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 5.6 m*M* glucose, 5 m*M* $NaHCO_3$, and 20 m*M* Hepes (*p*H 7.4 at 37°C)]. Extracellular calcium concentration, 10^{-3}*M*. (Top) Quin2 fluorescence. (Bottom) Cytochrome c reduction. (A) Quin2 content 0.6 nmole per 10^6 cells. FMLP when added was 0.34 μ*M*. (B) Cells from the same batch of quin2-loaded neutrophils were treated with cytochalasin B (5 μg/ml) for 5 minutes at 37°C. This treatment had no effect on $[Ca^{2+}]_i$ as such. FMLP concentration was the same as in (A). (C) Cells from the same batch of quin2-loaded neutrophils. Ionomycin (*Ion*) when added, was 1 μ*M*; EGTA when added was 4 m*M*. Neither $[Ca^{2+}]_i$ nor O_2^- production in response to ionomycin addition were affected by prior treatment with cytochalasin B. The cell numbers were 5×10^6 and 2.5×10^6 cells per milliliter for quin2 fluorescence and cytochrome c reduction, respectively. The dotted lines represent the graphically redrawn kinetics of $[Ca^{2+}]_i$ rise, since FMLP induces a decrease of cell autofluorescence that is larger and is irreversible in the presence of cytochalasin B. The autofluorescence change is complete in about 60 seconds. The correction was made as follows. At 10-second intervals, the actual fluorescence rise of quin2-loaded cells was corrected for the corresponding decrease of fluorescence in a sample of unloaded cells, at the same excitation and emission wavelengths. The autofluorescence represents 25 percent of the total signal. The autofluorescence decrease is 8 percent of the net fluorescence increase induced by FMLP in quin2-loaded cells in (A) and 35 percent of the net increase in (B). The higher the concentration of quin2 trapped in the cells, the smaller is the correction necessary. No effect on autofluorescence was observed after ionomycin. Interruptions occurred in the traces when the sample compartment was opened and the suspension stirred. The calibration of quin2 fluorescence as a function of $[Ca^{2+}]_i$ was similar to that used for lymphocytes (*5*); that is, quin2 was released from the cells with 0.1 percent Triton-X100 and minimum and maximum fluorescence were recorded at 10^{-9}*M* Ca^{2+} and 10^{-3}*M* Ca^{2+}, respectively. Intermediate values of $[Ca^{2+}]_i$ were calculated by assuming an effective dissociation constant of 115 n*M* for Ca^{2+} binding to quin2, the same as that used for lymphocytes (*5*), on the assumption that intracellular *p*H and $[Mg^{2+}]$ in neutrophils are not significantly different from those of lymphocytes. Excitation and emission wavelengths were 339 and 492 nm, respectively (*5*). The mean resting $[Ca^{2+}]_i$ was 126 ± 14 n*M* ($N = 20$). Some variability in the magnitude of $[Ca^{2+}]_i$ rise, O_2^- production, and MPO release stimulated by FMLP was observed between various batches of cells and from different donors. We do not know whether these differences are due to different proportions of cells responding to FMLP, or to intrinsic differences between batches of cells or donors, or both. The effects of ionomycin were quite constant.

tactic peptide formylmethionylleucylphenylalanine (FMLP) (*8*).

In Fig. 1A, the effects of FMLP on $[Ca^{2+}]_i$ and O_2^- production are compared. The increase in $[Ca^{2+}]_i$ began in less time than was required to stir the solution manually and close the fluorimeter sample compartment and the increase was mainly complete in less than 10 seconds. Superoxide production had a noticeable, but short, lag phase—less than 5 seconds—and was completed in about 5 minutes. Cytochalasin B is well established to increase most neutrophil responses (*9*). Figure 1B shows $[Ca^{2+}]_i$ and O_2^- generation in neutrophils that had been treated with cytochalasin B for 5 minutes. Comparison with Fig. 1A shows that the drug has a negligible effect on the FMLP-stimulated $[Ca^{2+}]_i$ transient, but increases by a factor of 5 the rate and extent of O_2^- production. It is evident that the effects of cytochalasin B are primarily at a later stage than, or independent of, the rise of $[Ca^{2+}]_i$ (*9*).

Figure 1C shows the effects of ionomycin on $[Ca^{2+}]_i$ and O_2^- production. An ionomycin dose of 1 μM increases $[Ca^{2+}]_i$ even more than FMLP does, yet it causes negligible superoxide generation. Neither $[Ca^{2+}]_i$ nor O_2^- production in response to ionomycin is affected by prior treatment with cytochalasin B. Only at very high concentrations (5 to 10 μM) is ionomycin able to increase O_2^- production significantly, although that response (not shown) remains much smaller than the response inducible by FMLP. This experiment shows that a $[Ca^{2+}]_i$ transient like that generated by ionomycin is not a sufficient stimulus for O_2^- generation in neutrophils.

Cockroft *et al.* (*10*) showed that ionomycin is a potent stimulator of lysosomal enzyme secretion. Ionomycin (1 μM) under the same conditions as were used in Fig. 1, in the presence of cytochalasin B, releases 60 to 70 percent of total myeloperoxidase (MPO) content, an effect similar to that obtained with 0.34 μM FMLP under these conditions.

The above experiments were done with cells in a medium containing a normal level of calcium, 1 mM. We then tested the effect of zero extracellular calcium, both to see whether internal calcium stores alone could account for rises in $[Ca^{2+}]_i$ and to determine the effects on cell function of a reduced $[Ca^{2+}]_i$ transient. In the experiment shown in Fig. 2, FMLP and ionomycin were added to neutrophils previously treated with cytochalasin B in Ca^{2+}-free medium plus EGTA. Comparison of Fig. 2A with Fig. 1A shows that removal of external Ca^{2+} reduces, but does not eliminate, the FMLP-induced rise in $[Ca^{2+}]_i$: FMLP increased $[Ca^{2+}]_i$ from 120 to 800 nM in Ca^{2+} medium and from 110 to 270 nM in Ca^{2+}-free medium. Thus FMLP causes both a release of Ca^{2+} from internal stores and an increased influx from the extracellular medium, in agreement with earlier deductions from tetracycline experiments and ^{45}Ca flux studies (*11*). Figure 2A shows that FMLP efficiently stimulated exocytosis and O_2^- generation in Ca^{2+}-free medium despite the diminished amplitude of the $[Ca^{2+}]_i$ rise (*12*). After 5 minutes, ionomycin caused another small rise in $[Ca^{2+}]_i$ but had no stimulatory effect on MPO and O_2^- release. An even more dramatic dissociation between $[Ca^{2+}]_i$ and these cell responses is shown in Fig. 2B. Since ionomycin in Ca^{2+}-free medium can still increase $[Ca^{2+}]_i$, the Ca^{2+} must be coming from intracellular stores accumulated within membrane-enclosed organelles. No MPO or O_2^- release was observed. However, addition of FMLP 5 minutes after ionomycin caused no further rise in $[Ca^{2+}]_i$, yet induced O_2^- generation and enzyme secretion. This ability of FMLP argues that the difference in efficacy between ionomycin and FMLP lies not in some inhibitory side effect of ionomycin but rather in some additional excitatory signal triggered by FMLP. The inability of FMLP to increase $[Ca^{2+}]_i$ after ionomycin treatment suggests that the FMLP-releasable pool lies within compartments discharged by ionomycin and is not simply bound to membrane surfaces in contact with cytoplasm (*11*). Alternatively, the inability of FMLP to increase $[Ca^{2+}]_i$ after ionomycin may reflect attenuation of cellular Ca^{2+} gradients promoted by ionomycin. Against this last possibility, however, is the observation that FMLP does not increase $[Ca^{2+}]_i$ even if enough time is allowed for $[Ca^{2+}]_i$ to return to the basal level (results not shown).

The present study shows that the specific fluorescent Ca^{2+} indicator quin2 can be trapped by intact human neutrophils without impairment of their functional activities. Evidence has been provided that the indicator is trapped in the cytoplasm (*8*) and this allows the use of quin2 as a quantitative probe of cytosolic free calcium.

These direct measurements of $[Ca^{2+}]_i$

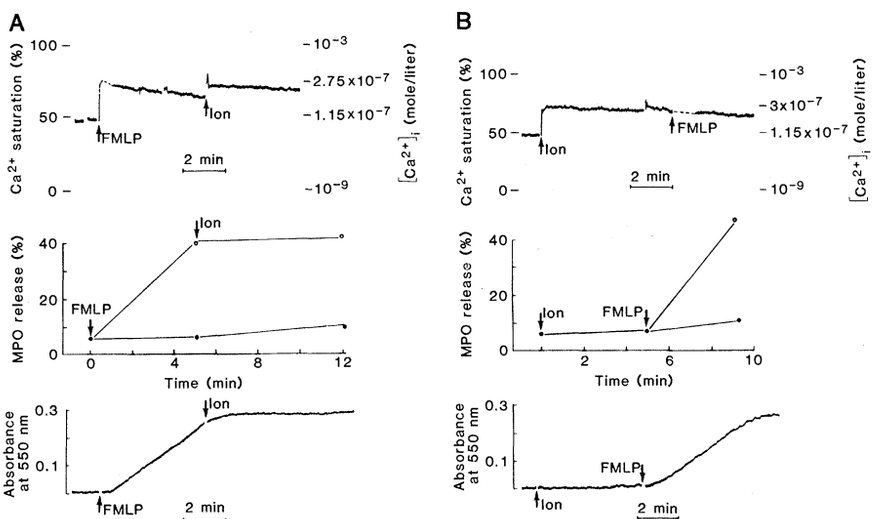

Fig. 2. Effect of FMLP and ionomycin (*Ion*) on $[Ca^{2+}]_i$, O_2^- production, and MPO release in Ca^{2+}-free medium. Extracellular calcium concentration, $10^{-9}M$. (A) FMLP preceded ionomycin. (B) Ionomycin preceded FMLP. Experiments were performed on portions of the same neutrophils as were used in Fig. 1. The medium was the same as that used in the experiment of Fig. 1, except that $CaCl_2$ was omitted (contaminating calcium, $10^{-5}M$). Quin2-loaded cells were incubated in this medium for 5 minutes with cytochalasin B (5 μg/ml); then 1 mM EGTA was added (free calcium, $10^{-9}M$), and 2 minutes later, the first stimulus, 0.34 μM FMLP in (A) or 1 μM ionomycin in (B), was added. (Top) Quin2 fluorescence. (Middle) MPO release. (Bottom) Cytochrome c reduction. Quin2 cell content was 0.4 nmole per 10^6 cells. The cell numbers were 5×10^6 per milliliter in quin2 fluorescence studies and 2.5×10^6 per milliliter in MPO release and cytochrome c reduction studies. MPO release is expressed as percent of the MPO activity in the supernatant after sonication of the cells at 0°C, as described by Boxer *et al.* (*18*). We noticed, however, that this procedure gives an underestimation of total MPO activity compared to the method described by Fehr and Jacob (*19*), in which MPO is extracted with 0.1 percent Triton-X100 in 1.5M NaCl. The lines joining the points do not represent the kinetics of MPO release, which is completed in 30 to 60 seconds. (●) Either unstimulated controls or ionomycin. (○) After FMLP treatment. The dashed lines represent quin2 fluorescence corrected for the autofluorescence decrease.

and comparisons with cell functions lead to the following conclusions.

1) FMLP, which acts through cell surface receptors, causes $[Ca^{2+}]_i$ to rise, both by influx through the plasma membrane and by release from intracellular membrane-enclosed compartments. It remains unclear how the message to release $[Ca^{2+}]_i$ is so quickly transmitted from the exterior receptors to the sites of sequestration.

2) The kinetics of $[Ca^{2+}]_i$ rise are compatible with its role as a second messenger (both for O_2^- generation and exocytosis). Paradoxically, a $[Ca^{2+}]_i$ rise like that generated by ionomycin, which bypasses cell surface receptors, is itself insufficient to stimulate O_2^- generation.

3) A dissociation can be demonstrated between FMLP-elicited exocytosis and the reduced, submicromolar $[Ca^{2+}]_i$ transients generated in Ca^{2+}-free media. However, since ionomycin in normal Ca^{2+}-containing media is adequate to trigger exocytosis [(*10*), as well as experiments not shown here], $[Ca^{2+}]_i$ at micromolar levels is apparently a sufficient trigger. Alternatively, ionomycin might be generating some additional stimulatory messenger in normal but not in Ca^{2+}-free medium.

4) There is a clear dissociation between lysosomal enzyme secretion and O_2^- production. While enzyme secretion can be triggered by increasing cytosolic Ca^{2+} to micromolar levels, superoxide generation is not elicited even at high cytosolic Ca^{2+}.

5) The efficiency of FMLP at stimulating superoxide generation and exocytotic secretion requires a signaling mechanism in addition to $[Ca^{2+}]_i$. These results do not show what this mechanism might be or whether it is also responsible for release of intracellularly sequestered Ca^{2+}. Cyclic nucleotides might have been obvious candidates, but the increase in adenosine 3′,5′-monophosphate that follows FMLP stimulation seems to inhibit rather than promote cell activation (*13, 14*). Changes in guanosine 3′,5′-monophosphate could not be detected in response to FMLP (*14*). There are a few indications that changes in sodium or H^+ concentrations, arachidonic acid metabolites, or membrane methylation may be involved (*15*). An analogous partial dissociation between increases in $[Ca^{2+}]_i$ and cell activation was demonstrated in platelets (*16*). It remains to be established what role elevations in $[Ca^{2+}]_i$ play in neutrophil function and whether an increase in $[Ca^{2+}]_i$ is always an obligatory event in exocytosis and O_2^- generation.

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21. We thank G. Ronconi for technical assistance, and P. Arslan and G. F. Azzone for discussions. This work was supported by a Consiglio Nazionale delle Richerche grant to the Unit for the Study of the Physiology of Mitochondria, by grants 3.986-082, 869-3, 869-081, 3.869-1.81, 3.487-0.79 SR, and 3.246-0.82 SR from the Swiss National Science Foundation, and by USPHS grants GM31004 and EY04372. D.P.L. is recipient of a Max Cloetta career development award.

7 April 1983; revised 15 June 1983