Simultaneous Measurement of Stimulus-induced Changes in Cytoplasmic Ca$^{2+}$ and in Membrane Potential of Human Neutrophils

(Received for publication, February 5, 1986)

Kristina G. Lazzari, Philip J. Proto, and Elizabeth R. Simons
From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

The activation of human neutrophils by chemotactic peptides evokes a rapid change in membrane potential and an increase in cytoplasmic Ca$^{2+}$ levels. These events are followed up to a minute later by detectable levels of microbicidal agents formed by the oxidative burst. Except for the latter, the sequence of events has remained unclear. We report here that a new fluorescent Ca$^{2+}$ indicator developed by R. Tsien, Indo-1, has allowed us to resolve the temporal relationship between the rapid and transient cytoplasmic Ca$^{2+}$ rise and the membrane potential change and to do so on very small samples by using a fluorescence-activated cell sorter. We have adapted a FACS 440 for simultaneous single cell membrane depolarization and cytoplasmic [Ca$^{2+}$]$_i$ detection in human neutrophils upon stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP). A membrane potential probe, dipentyloxacarbocyanine, allows us to determine that the membrane potential change is fMLP dose-dependent and apparently biphasic. The depolarization is maximal 40 s after stimulation. In contrast, cytosolic [Ca$^{2+}$], while fMLP-dose dependent, is maximal at 10 s and already decreasing rapidly when the cell has reached its lowest potential. It can be measured with Indo-1 which has a fluorescence emission ($\lambda_{em}$ = 537 nm) maximum at 485 nm when Ca$^{2+}$-free and 405 nm when Ca$^{2+}$-liganded. The ratio of these fluorescent intensities at two suitable wavelengths may be calibrated in terms of cytoplasmic Ca$^{2+}$ levels. Thus, Ca$^{2+}$ release into the cytoplasm becomes the earliest evidence of neutrophil stimulation by fMLP and occurs in close association with an apparent membrane hyperpolarization.

The stimulus response of human neutrophils has been studied extensively in vitro in cell suspensions and has been shown to involve changes in transmembrane cation gradients within seconds of exposure to soluble or particulate stimuli (1-5). Simultaneous measurement of these changes, however, has been difficult. The fluorescence-activated cell sorter (FACS$^3$) has recently been useful in investigating changes in membrane potential alone (6-8) or simultaneously with oxidative product formation (9). In these experiments, the data for individual cells were collected and analyzed after a minimum time interval of 1.5 min, significantly prolonged when compared to the speed of the neutrophil response. We report here that a FACS may be used to monitor rapidly (at 10-s intervals) and simultaneously the chemotactic peptide formyl-methionyl-leucyl-phenylalanine-induced changes in neutrophil cytoplasmic Ca$^{2+}$ concentrations and membrane potential as a function of stimulus concentration and elapsed time.

Membrane potential changes, oxidative burst product formation, and degranulation are among the earliest stimulus responses of the neutrophil and are parameters often used as measures of cell activation. Cytoplasmic Ca$^{2+}$ transients have recently been reported to occur within seconds of ligand binding and may be involved in the ensuing activation of the neutrophil (10-14). It is not yet known whether any of these changes are essential for cell activation, nor has their temporal interrelationship been elucidated. However, the importance of Ca$^{2+}$ in the process of signal transduction from membrane receptors to the demonstration of a cellular response remains undefined. Evidence obtained from studies of unopsonized Candida albicans hyphae stimulation of human neutrophils suggests that a Ca$^{2+}$ signal can occur when depolarization and degranulation remain absent. These results imply that the Ca$^{2+}$ signal cannot be sufficient to stimulate complete activation of the cellular functions.

It has only recently become possible to study the nanomolar levels of Ca$^{2+}$ within a viable cell without significant perturbation of the system through the development of cell-permeant, fluorescent Ca$^{2+}$ indicators such as Quin 2, Fura-2, and Indo-1 (16-18). Whereas useful information has been obtained from Quin 2, the probe suffers from two severe disadvantages: a low extinction coefficient and quantum yield and a significant Ca$^{2+}$ buffering capability which obscures small intracellular Ca$^{2+}$ transients. Other interferences such as instrument variation, intracellular dye concentration, and cell thickness also influence measurements of absolute Ca$^{2+}$ concentration with Quin 2 (17-20). Indo-1 and Fura-2 exhibit a spectral shift upon binding to Ca$^{2+}$ and therefore do not have many of the problems associated with Quin 2. The ratio of fluorescent intensities at two suitable wavelengths may then be used to calculate [Ca$^{2+}$], independently of variations in dye concentration or path length (16, 20).

To date, the analyses of intracellular Ca$^{2+}$ concentration changes have involved spectrofluorometric measurements in cell suspensions which require averaging over 10$^6$ cells/ml. Since separate experiments must be performed for each parameter being measured, it has been difficult to resolve the sequence of events occurring within the first 30 s after exposure of neutrophils to a specific stimulus. We report here success in resolving this issue, utilizing a flow cytometer...
equipped with dual lasers and three detection photomultipliers to determine membrane potential and intracellular Ca²⁺ concentration changes simultaneously within 10 s of stimulation.

We have analysed the kinetics of Ca²⁺ fluxes and membrane potential changes upon stimulation of neutrophils using a FACS and have resolved the temporal sequence of events, beginning within 10 s of stimulation. The cytoplasmic Ca²⁺ concentration peaks within 10 s, whereas the membrane potential change reaches a maximum after 60 s of exposure to formyl-methionyl-leucyl-phenylalanine. Both effects are dose-dependent, with [Ca²⁺]i, increasing with FMLP concentration to saturation at 10⁻¹⁰ M, whereas the membrane potential change reaches a maximum after 60 s from more negative (hyperpolarized) to less negative (depolarized) at low doses of FMLP (<10⁻⁸ M) to less negative (depolarized) at higher doses (>10⁻⁶ M).

### MATERIALS AND METHODS

**Leukocyte Isolation**—Peripheral blood polymorphonuclear leukocytes from citrated venous blood of normal human subjects were prepared by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation and hypotonic lysis to remove contaminating erythrocytes (27). The resulting cells (95% polymorphonuclear leukocytes) were suspended in phosphate-buffered saline (125 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 25 mM KCl, 5 mM glucose, pH 7.4, and stored on ice in a Ca²⁺- and Mg²⁺-free medium to minimize aggregation. Ca²⁺ influx was determined by an adaptation of the technique used for Indo-2 (17). The relationship [Ca²⁺] and membrane potential of stimulation. The cytoplasmic Ca²⁺, the ratio of mean channel fluorescence at 405 and 485 nm in the resting cell was calculated and subtracted from the ratio after stimulation. The percent change in ratio was calculated and described as above in order to calculate the percent change of [Ca²⁺].

**RESULTS**

Alterations in the membrane potential can be measured by changes in the intracellular concentration of fluorescent, lipophilic cations such as the cyanines (21). For observations of cell suspensions, it is preferable to use probes such as di-S-C₃(5) which self-associate and hence are quenched inside the cell so that only the residual, extracellular probe fluorescence is observed as an indicator (by difference) of the probe's distribution between the cells and the suspension medium (22, 23). Correlation with membrane potential has been well established with other types of membrane potential measurements (24, 25). In contrast, for observations of single cells in a flow cytometer, a non-self-associating probe such as di-O-C₃(3) or di-I-C₃(3) must be used such that the observed fluorescence is directly proportional to the internal probe concentration (7, 9, 26).

As Fig. 1 (right) indicates, the cytoplasmic Ca²⁺ increase reaches a maximum by 10 s. In contrast, whereas the membrane potential changes as rapidly (Fig. 1, left), the change appears to be biphasic. At very low fMLP concentrations, one observes a rapid increase in fluorescence which appears to represent a hyperpolarization of the cell. At higher fMLP concentrations, this hyperpolarization is followed by a slower, but larger loss in fluorescence corresponding to a membrane depolarization which, at high fMLP concentrations, tends to obscure the hyperpolarization and which is maximal at approximately 60 s.

The concentration dependence of the membrane potential and intracellular Ca²⁺ change is very similar, reaching half maximal response at 10⁻⁵ M fMLP and saturation at 10⁻⁴ M fMLP. It should be noted that at very low fMLP concentrations, only the apparent hyperpolarization occurs. The entire population of cells responds uniformly to stimulation, demonstrated on the FACS by the uniform shift seen in the 405/485 nm diagonal fluorescence plot of the cell population before and after stimulation.

Calculations of the concentration of intracellular, cytoplasmic Ca²⁺ indicate a resting level of approximately 100 nM Ca²⁺ in cells suspended in 1.0 mM external CaCl₂ and a maximal increase to approximately 3 μM Ca²⁺ 10 s after stimulation with a saturating dose of fMLP (Fig. 4).
FIG. 1. Left, changes in membrane potential in single cells stimulated with increasing doses of fMLP: 1 × 10^{-11} M (Φ), 1 × 10^{-10} M (O), 1 × 10^{-9} M (□), 1 × 10^{-8} M (A), and 1 × 10^{-7} M (Δ). These measurements were done simultaneously with Indo-1 fluorescence measurements of changes in cytoplasmic Ca^{2+} concentration (see right) on the FACS. Right, changes in cytoplasmic Ca^{2+} concentration measured with Indo-1 in single cells stimulated with increasing doses of fMLP: 1 × 10^{-11} M (Φ), 1 × 10^{-10} M (O), 1 × 10^{-9} M (□), 1 × 10^{-8} M (A), and 1 × 10^{-7} M (Δ). These measurements were done simultaneously with di-O-C6(3) fluorescence measurements of changes in membrane potential (see left) on the FACS.

DISCUSSION

Changes in transmembrane potential are among the earliest detectable events following ligand-receptor binding and have been interpreted by some as being necessary but not sufficient for the formation of the oxidative metabolic response (2–4, 22, 28). Calcium has been strongly implicated as a second messenger in this process of cell activation (10, 35) and is also thought to play a role in the regulation of cytoskeletal protein polymerization (6, 30). Increases in cytosolic Ca^{2+} have been demonstrated to be involved in, but insufficient for, the triggering of depolarization, degranulation, or superoxide production (12). Evidence that cell stimulation occurs in the presence of a chelator of extracellular Ca^{2+} has indicated that an influx of divalent cations is not essential for activation, and an intracellular calcium reservoir appears to be responsible for the cytosolic Ca^{2+} increase observed after stimulation by fMLP (12, 14, 31, 35).

Studies of chronic granulomatous disease patients whose neutrophils, in most cases, are unable to produce a normal oxidative response or a normal depolarization have indicated that these two responses are interrelated (2, 22, 32), whereas in all cases, the influx in intracellular Ca^{2+} upon stimulation appeared to be normal (32). From other studies involving the artificial reduction of transmembrane potential using high K^+/low Na^+ buffers (3, 33) and from the measurement of simultaneous depolarizations and oxidative product formation...
cells exhibit a similar increase in di-O-C₆(3) fluorescence (measured on a FACS) in response to saturating doses (10⁻⁶ M) of fMLP. As these cells differentiate in culture over a period of 8 days, their response to fMLP stimulation gradually becomes more characteristic of a mature neutrophil; the detectable initial hyperpolarization decreases as the later, larger depolarization increases. In view of these findings, we believe that there may indeed be a hyperpolarization occurring within the first 10 s of stimulation which is observable only at very low doses of fMLP and is indicative of a biphasic response.

The studies reported here demonstrate that, by using a FACS and simultaneous detection of Ca²⁺ concentration and membrane depolarization, we can document a fMLP dose-dependent rise in cytoplasmic Ca²⁺ and a change in membrane potential which are uniform in the entire population of human neutrophils. At low doses, the cells appear to hyperpolarize as the cytoplasmic Ca²⁺ increases until both responses reach a maximum at 10 s. With increasing fMLP, the maximum [Ca²⁺]i change increases, but the apparent hyperpolarization decreases as the slower depolarization becomes larger. These events taken together indicate that the [Ca²⁺]i increase is independent of and precedes the fMLP-induced depolarization of human neutrophils, but may be concomitant with a faster hyperpolarization.

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