Membrane-Proximal Calcium Transients in Stimulated Neutrophils Detected by Total Internal Reflection Fluorescence

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ABSTRACT A novel fluorescence microscope/laser optical system was developed to measure fast transients of membrane-proximal versus bulk cytoplasmic intracellular calcium levels in cells labeled with a fluorescent calcium indicator. The method is based on the rapid chopping of illumination of the cells between optical configurations for epifluorescence, which excites predominantly the bulk intracellular region, and total internal reflection fluorescence, which excites only the region within ~100 nm of the cell-substrate contact. This method was applied to Fluo-3-loaded neutrophils that were activated by the chemoattractant *N*-formyl-met-leu-phe. Chemoattractant-activated cells showed 1) transient increases in both membrane-proximal and bulk cytosolic Ca^{2+} that peaked simultaneously; 2) a larger fractional change (20–60%) in membrane-proximal Ca^{2+} relative to bulk cytosolic Ca^{2+} that peaked at a time when the main Ca^{2+} transient was decreasing in both regions and that persisted well after the main transient was over. This method should be applicable to a wide variety of cell types and fluorescent ion indicators in which membrane-proximal ionic transients may be different from those deeper within the cytosol.

INTRODUCTION

Cellular Ca^{2+} is widely recognized as an important intermediate messenger in signal transduction systems (Ghosh and Greenberg, 1995; Felder et al., 1994; Clapham, 1995; Bootman and Berridge, 1995; Cheek and Barry, 1993). In some cases, such as G-protein-mediated hormonal responses, a cytosolic Ca^{2+} increase results from the release of Ca^{2+} from intracellular stores via phospholipase C activation. In other cases, such as receptor- or voltage-regulated Ca^{2+} channels, a cytosolic Ca^{2+} increase results from the uptake of Ca^{2+} from the extracellular medium. In either case, the resulting cytosolic Ca^{2+} increase is important for regulating events such as protein phosphorylation and fusion of secretory vesicles with membranes that result in diverse functions such as production of oxidants and release of neurotransmitters and other secretory granule contents. In addition, it has been hypothesized that there is a membrane-bound pool of Ca^{2+} that may be important for regulating events occurring at the plasma membrane, such as fusion of secretory vesicles with the plasma membrane (Smolen et al., 1982; Chandler and Williams, 1978; Taljedal, 1978) and polymerization of actin (Bengtsson, 1990).

Cytosolic Ca^{2+} levels are most commonly detected by fluorescent Ca^{2+} chelators that can be loaded into cells and whose spectral properties are sensitive to Ca^{2+} binding (Tsien, 1980, 1981). These probes label the cell cytoplasm and therefore detect a cytosolic pool of Ca^{2+}, the concentration of which is generally reported in the range of 10^{-7} M. Evidence exists that suggests that measurement of the bulk cytosolic Ca^{2+} does not reflect the concentration of Ca^{2+} proximal to the plasma membrane. In vitro studies of secretion from chromaffin granules show that 1–100 μM free Ca^{2+} is required to induce secretion; however, this concentration range is much greater than the levels measured in the bulk cytosol of stimulated cells (Augustine and Neher, 1992). Measurement of presynaptic Ca^{2+} currents in neurons predicts that the intracellular free Ca^{2+} concentration near the Ca^{2+} channels could be several hundred micromolar (Llinas et al., 1981). Using a fluorescent Ca^{2+} indicator whose minimum Ca^{2+} sensitivity was 100 μM, Llinas et al. (1992) have shown that electrical stimulation of giant squid axons results in the generation of small Ca^{2+} microdomains containing 100 μM Ca^{2+} or more.

Evidence for a membrane-bound pool of Ca^{2+} has been based on the ability of the compound chlortetracycline (CTC) to detect Ca^{2+} at the membrane. CTC partitions into membranes, and binding of Ca^{2+} increases its fluorescence (Caswell and Hutchison, 1971a,b). Stimulation by secretogogs of CTC-loaded neutrophils (Smolen et al., 1982; Naccache et al., 1979), pancreatic acinar cells (Chandler and Williams, 1978), and mast cells (White and Pearce, 1983) causes a decrease in fluorescence that has been interpreted as a release of Ca^{2+} from the membrane that would presumably increase the free Ca^{2+} proximal to the membrane. However, many uncertainties remain regarding the processes that regulate CTC fluorescence (White and Pearce, 1982, 1983). Conditions that obliterate the increase in fluorescence of bulk cytosolic Ca^{2+} indicators do not alter the CTC response (Bengtsson, 1990), and the dose-response characteristics of CTC fluorescence changes and cytosolic Ca^{2+} indicator changes are different (Korchak et al., 1983), suggesting that the Ca^{2+} released from the membrane-bound pool is an insignificant fraction of the bulk cytosolic Ca^{2+} increase.

A measurably long-lasting excess of membrane-proximal Ca^{2+} (due to release from the membrane or entering the cell

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through membrane channels) relative to cytosolic Ca^{2+} can develop only if the rate at which the Ca^{2+} diffuses into the bulk cytosol is somehow restricted. Allbritton et al. (1992) measured Ca^{2+} diffusion in cytosolic extracts from *Xenopus laevis* oocytes and determined that the movement of free Ca^{2+} in cells is highly restricted because of the Ca^{2+} buffering capacity of the cytosol. They estimate that the effective range of free Ca^{2+} in the cells is only ~100 nm. Thus detection of free Ca^{2+} released from the membrane or entering the cell through membrane channels requires high spatial resolution. Attempts to identify membrane-proximal Ca^{2+} by confocal microscopy are limited by the depth of resolution of ~0.6 μm and have low temporal resolution. Thus alternative methods are required. Membrane-tethered derivatives of Ca^{2+} indicators have been developed in an attempt to monitor membrane-proximal Ca^{2+} (Etter et al., 1994; Lloyd et al., 1995). This approach has some limitations in that the compound must be microinjected into cells, and not all cells are amenable to microinjection. In addition, the probe may enter the membranes of intracellular organelles, making it difficult to determine whether changes are occurring at the plasma membrane or at the membranes of organelles.

In an effort to identify membrane-proximal Ca^{2+} gradients, we have developed a real-time technique combining total internal reflection (TIR) fluorescence (TIRF) (Axelrod et al., 1992) and epi-illumination (EPI) fluorescence in a microscope to determine the relationship between the membrane-proximal and cytosolic pools of Ca^{2+} on adherent, single living cells loaded with the bulk cytosolic Ca^{2+} indicator Fluo-3. TIR illumination creates a thin (~100 nm) exponentially decaying field near the glass substrate called the "evanescent field." The evanescent field selectively illuminates just those regions of a cell at or near cell-substrate contacts, including a thin layer of the submembrane cytosol at the contact regions. Most of the Fluo-3 fluorescence excited by TIR comes from the membrane-proximal regions. Thus the depth resolution is similar to the effective range of free calcium predicted by Allbritton et al. (1992). EPI (also called "trans-") illumination, on the other hand, illuminates the entire depth of the cell. Most of the Fluo-3 fluorescence excited by EPI comes from regions of the cytosol much deeper in the cell.

The cell system examined in these experiments was the human neutrophil. Human neutrophils exhibit increased cytosolic Ca^{2+} when stimulated with numerous chemoattractants, including *N*-formyl peptides. Phospholipase C is activated via a G-protein, and the inositol triphosphate (IP_3) thereby produced releases Ca^{2+} from intracellular stores (Lad et al., 1992). This is followed by a Ca^{2+}-induced influx of Ca^{2+} from the extracellular medium (Lagast et al., 1984; von Tscharner et al., 1986; Chandler and Kazilek, 1987). The changes in cytosolic Ca^{2+} may be related in part to changes in a hypothesized membrane-bound pool of Ca^{2+} upon ligand-receptor binding. This hypothesis has been based on the use of CTC in human neutrophils (Smolen et al., 1982; Bengtsson, 1990; Naccache et al., 1979). In addition, membrane-proximal Ca^{2+} has been detected in human neutrophils as pyroantimonate-precipitable Ca^{2+} by using microscopy techniques (Cramer and Gallin, 1979). The relationship between this membrane-associated pool of Ca^{2+} and the general cytosolic pool of Ca^{2+} is not yet well characterized. It has also been hypothesized that the membrane-bound pool of Ca^{2+} is important for regulating *N*-formylpeptide-induced secretion (Smolen et al., 1982) and cytoskeletal changes, measured as changes in the F-actin levels and generally called the actin polymerization response (Bengtsson, 1990). Studies using cytosolic Ca^{2+} indicators and buffers have shown that sequestration of the chemoattractant-induced cytosolic Ca^{2+} increase does not inhibit the actin polymerization response (Sklar et al., 1985). However, a recent report (Bengtsson, 1990) has shown a strong correlation between the release of membrane-bound Ca^{2+} (measured with CTC) and the actin polymerization response (measured as right-angle light scatter, which is an indicator of actin polymerization in these cells). Thus in this work we sought to develop a method to define the relationship between the bulk cytosolic and membrane-proximal Ca^{2+} pools and their role in regulating neutrophil responses.

MATERIALS AND METHODS

Configuration of optics

The purpose of the optical configuration is to rapidly chop the illumination between the two alternatives of TIR and EPI so that fluorescence transients in both can be followed simultaneously for comparison on the same cell. We describe first the optical illumination paths and then the chopping system.

The EPI beam is incident normally upon the sample by propagating up through the inverted microscope (Leitz Diavert) objective via the conventional dichroic mirror system. The beam is defocused at the field diaphragm sufficiently to illuminate a circular region (~30 μm in diameter) several times wider than the diameter of a neutrophil. The TIR beam is deflected, as shown in Fig. 1, to travel vertically downward toward the sample plane but off-center through the base of a trapezoidal prism made by truncating and polishing a 60° equilateral triangle prism made of flint glass (n = 1.62; Rolyn Optics). The TIR beam thereby internally reflects at a side wall of the prism, exits the prism at its truncated face at an oblique angle, propagates through a layer of immersion oil placed there to establish optical contact, enters the glass wall of a microcapillary tube containing the cells, and totally reflects at the glass/water interface to which the cells are adhered upside-down. The TIR beam is focused at the cell adhering surface to produce an elongated region of illumination about 20 μm wide and 80 μm long. The trapezoidal prism is mounted on the microscope's own condenser holder/translator, so it can be withdrawn and put back in place easily and reproducibly.

The chopping is done by a system of three acousto-optic modulators (AOM 1–3) responding to commands from a 486/50 PC (Fig. 1). Fluorescence photons detected during each phase (HI and LO) of the square chopping signal are counted and stored by the PC as the EPI and TIR transient signals, respectively. The minimum counting (and chopping) time interval can be set as low as 15 ms in our system (limited mainly by the speed of reading the counter/timer interface board in the computer and the rise time of the AOM response), so that in principle very rapid changes can be followed. The counting/chopping interval used in these experiments was 30 ms or 100 ms. The size of the square image plane diaphragm was adjusted to gather light from about one-third of the lateral area projection of a single cell, around its center.

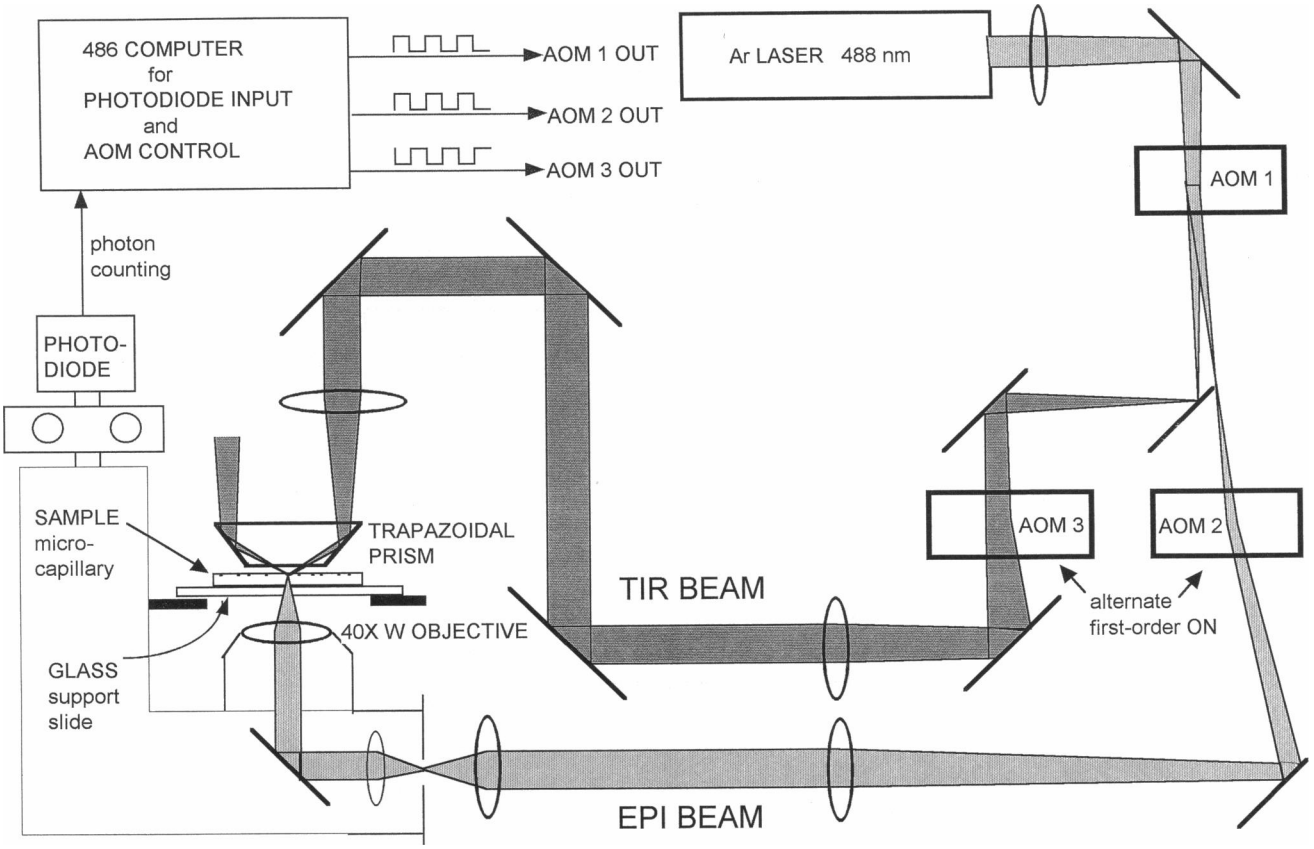

FIGURE 1 Optical and electronic system for fast TIR/EPI chopping.

A 50% duty cycle chopping signal of programmable period is generated from the clock of a custom-programmed counter/timer board (Keithley/Metrabyte CTM-05) in the I/O bus of a 486/33-based computer. The controller units for AOM 1 and 2 receive this signal; the controller for AOM 3 receives an inverted version. AOM 1 thereby chops between a deflected first-order diffraction beam (which becomes the EPI path) and an undeflected zero-order beam (which becomes the TIR path). AOM 2 and AOM 3 each are in line with these two paths, respectively; they chop their output first-order diffraction beams in synchrony with the intensity of their respective input beams to increase the on/off contrast of each to at least 10^6:1.

The fluorescence is gathered by a water-immersion objective (40×, NA 0.75; Zeiss). This microscope objective has a free working distance of 1.4 mm, sufficient to allow viewing through the thickness of the microcapillary wall and its supporting glass slide. Fluorescence photons are detected by a commercial avalanche photodiode (SPCM-100; EG&G Ortec). The sequence of TTL pulses from that device are directly counted by the same counter/timer board used to generate the AOM control signals. A custom Fortran/Assembly program separated the numbers of photons counted during the alternating TIR and EPI intervals into two variable arrays for display and data file storage.

This system measures relative time-dependent changes in Ca^{2+}, not absolute concentrations. The ratio of the two signals after subtraction of background (TIR/EPI) reports whether transients in the membrane-proximal Ca^{2+} are fractionally greater or less than transients in cytosolic Ca^{2+}. A flat ratio of TIR/EPI indicates that changes in TIR are fractionally the same as changes in EPI, with the same time course.

Preparation of cells

Human peripheral blood was obtained from volunteers using procedures approved by the Internal Review Board of the University of Michigan Medical School. Neutrophils were purified by counterflow centrifugation (Tolley et al., 1987) and loaded with the Ca^{2+} indicator Fluo-3 (Molecular Probes, Eugene, OR) as previously described (Omann and Harter, 1991). Cells were suspended at 1×10^7/ml in modified Gey's buffer containing 1.5 mM Ca^{2+} as described (Omann and Harter, 1991).

Cells were loaded with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF) (Molecular Probes) by incubation with 5 μM BCECF-acetoxymethyl ester for 45 min at room temperature and then washed three times with buffer to remove the external indicator. The cells were resuspended at a concentration of 1×10^7/ml.

Neutrophils loaded with fluorescent indicator were drawn up into a 0.3-mm internal thickness rectangular microcapillary tube (Vitro Dynamics, Rockaway, NJ) and allowed to settle by gravity and adhere to one surface for at least 3 min (Fig. 2). The capillary was then inverted (so the cells were on the inside of the top surface) and glued to a microscope slide on which two rings of silicon grease had been applied. These rings created input and output reservoirs for solutions that could replace the existing fluid in the microcapillary while a cell was under observation. For this purpose, a droplet of replacement fluid was deposited at one end of the capillary while a rolled-up paper tissue soaked up solution at the other end of the capillary. After a delay time, the solution around any cell changed over with a characteristic time of 0.2 s. This time was determined in separate experiments with the same optical and sample holder system in which fluorescein-containing buffer was introduced into a microcapillary tube containing pure buffer. The characteristic changeover time is defined as the duration between the initial onset of detected fluorescein and the time at which the fluorescence reaches its half-maximum value. The cells were placed on the microscope and activated within 10–30 min of adherence to the slide. It has been shown that spreading of neutrophils on glass is preceded by a large increase in cytosolic free calcium that returns to baseline within ~2 min (Kruskal et al., 1986). It has also been shown that adherence of neutrophils to glass causes an increase in F-actin that recovers

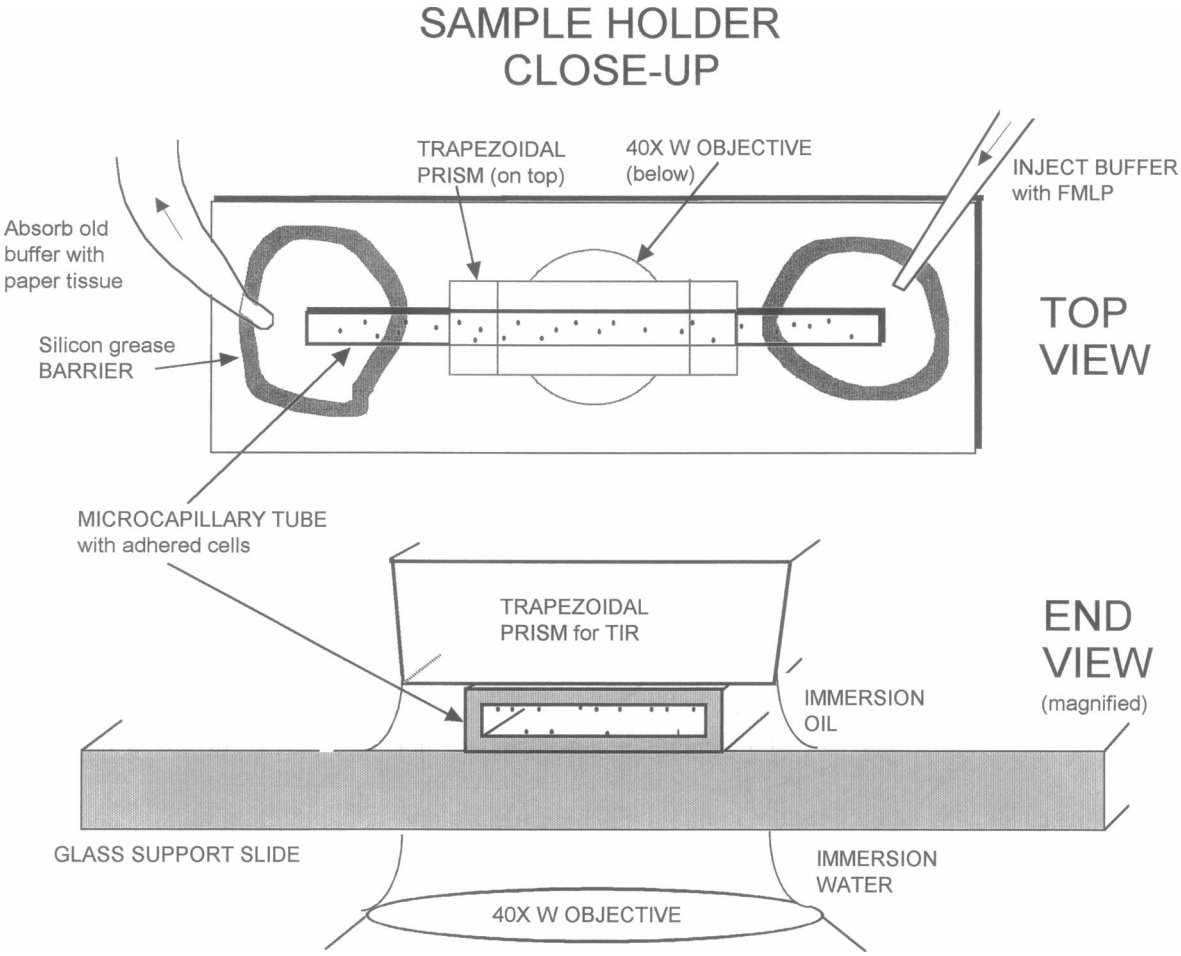

FIGURE 2 Preparation of cells for microscopy

to baseline within 10 min (Bengtsson et al., 1993). Thus we chose a time after adherence where the neutrophils were expected to have returned to a resting state. In addition, it has previously been shown with epi-illumination techniques that adherent neutrophils can subsequently be activated by *N*-formyl-met-leu-phe (FMLP) to give a Ca^{2+} rise (Jaconi et al., 1988).

RESULTS AND DISCUSSION

Under EPI illumination, each cell appeared as a sphere with the brightest fluorescence in the middle; under TIRF illumination, each cell appeared somewhat more uniform in brightness but spread over a larger and slightly irregular circular area, indicating that the cell had spread at the glass surface. The brightness was somewhat variable cell to cell, perhaps as a result of partial triggering in response to making contact with the glass. We chose cells whose prestimulation fluorescence was at the dimmer end of the distribution.

The kinetics of activation were measured using the chemoattractant FMLP (Fig. 3). The addition of FMLP caused a transient increase in Ca^{2+} detected by both EPI and TIR illumination. The response was heterogeneous in magnitude, with some cells showing a fourfold increase in fluorescence and others showing only a twofold increase or less. Heterogeneity in kinetics also existed, with the time for going from resting level to half-maximum response ranging from 2.5 s to 11 s. In all cases this was significantly longer than the typical characteristic time of 0.2 s required to replace the media around a given cell. Thus the heterogeneity in kinetics appears to represent cell-to-cell variations in responses and not solution changeover times. The maximum Ca^{2+} elevation was reached at the same time for EPI and TIR traces. In both regions probed, addition of the calcium ionophore, ionomycin, caused a substantial increase in fluorescence. Subsequent combined addition of ionomycin and cobalt ion (Co^{2+}) quenched the fluorescence, consistent with the interpretation that Fluo-3 was the source of the fluorescence signal being measured.

The ratio of TIR to EPI fluorescence showed that FMLP caused a significant increase in membrane-proximal Ca^{2+} relative to the bulk cytosolic Ca^{2+} increase (Fig. 4, *a* and *b*). However, this ratio reached a peak well after the initial Ca^{2+} transient, when each region's Ca^{2+} level had begun its return to the prestimulus baseline level. This indicated that a relatively higher concentration of membrane-proximal Ca^{2+} developed at the tail end of the response. In most cases the TIR/EPI ratio remained elevated at times when the

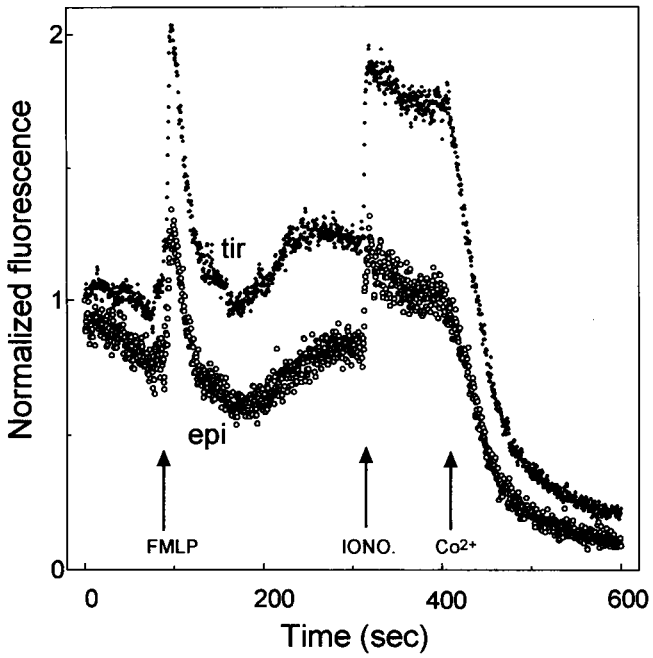

FIGURE 3 FMLP-induced Ca^{2+} transients in bulk cytosol and membrane-proximal regions. Fluo-3-loaded cells were viewed by TIR (●) and EPI (○) during the course of buffer-specific replacements. The first arrow indicates the time of introduction of buffer containing 100 nM of the *N*-formyl peptide chemoattractant FMLP. At the second arrow the buffer was replaced with buffer containing 26 μM ionomycin, the channel-forming drug, which nonspecifically allows extracellular Ca^{2+} to rush into the cell. At the third arrow the buffer was again replaced with 26 μM ionomycin and 0.12 mM cobalt ion, which enters the cell through the ionomycin channels and binds and quenches Fluo-3 fluorescence. The photon counting interval here was 100 ms.

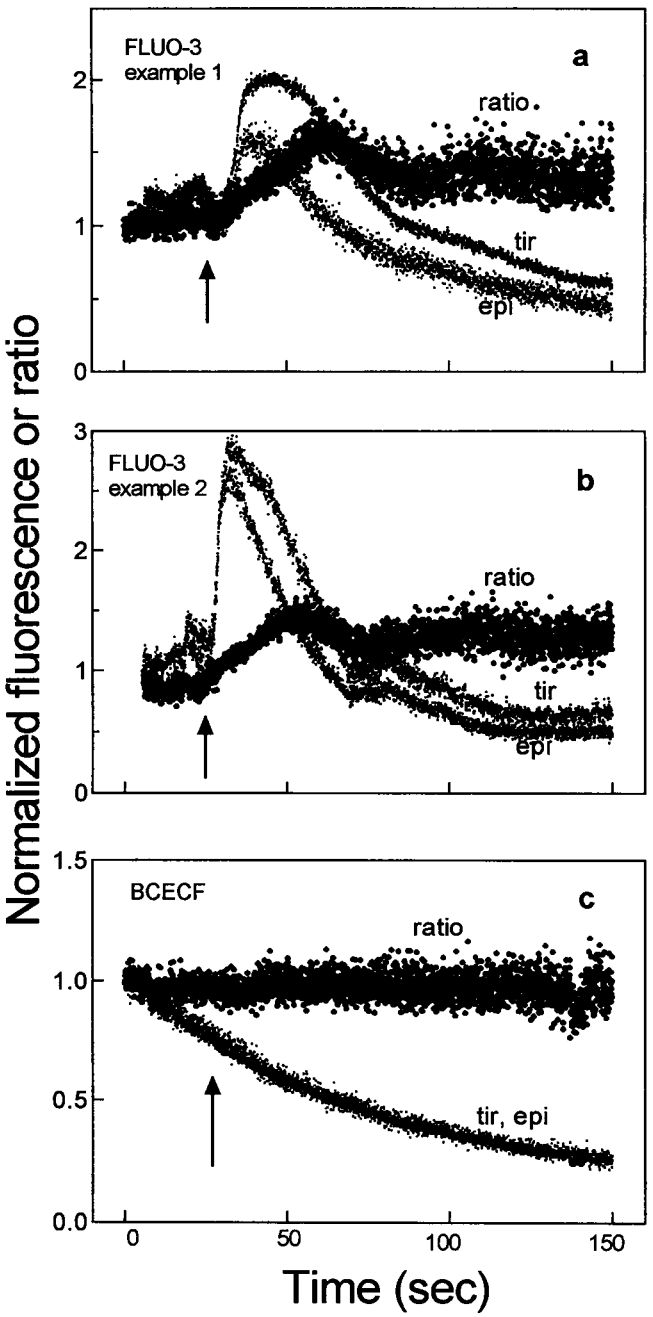

FIGURE 4 (*a* and *b*) FMLP-induced transient changes in the membrane-proximal/cytosolic Ca^{2+} ratio. Two examples are shown of Fluo-3-loaded cells activated with 100 nM FMLP added at the arrow. After the addition, a slight delay ensues before the FMLP reaches the cell under view. The individual traces for TIR and EPI are indicated by thin dots, and the ratio of TIR/EPI by thicker dots. (*c*) Lack of change in the TIR/EPI ratio for the fluorophore BCECF. Cells were loaded with BCECF as decribed in the text; 100 nm FMLP was added at the arrow. The photon counting interval for these three graphs was 30 ms.

individual transients approached baseline. There was some hetergeneity in this aspect of the response, with the TIR/EPI ratio of some cells returning to baseline within a minute, but in most cells the ratio remained elevated as long as the response was observed (~5 min). The average maximum increase in the TIR/EPI ratio for Fluo-3 (based on 45 different cells and data collected on 13 different days) was 36 ± 24 (SD)%.

Experiments designed to ensure that changes in fluorescence were a result of Ca^{2+} fluxes and not instrumental artifacts or morphological changes utilized BCECF, a fluorescent pH indicator that exhibits little intensity change (relative to that for Fluo-3) upon triggering in these cells. In cells loaded with BCECF (Fig. 4 *c*), the individual EPI and TIR signals decreased steadily because of photobleaching, and little additional change occurred with the addition of FMLP. In addition, FMLP induced no significant change in the TIR/EPI ratio. The average maximum increase in the TIR/EPI ratio for BCECF (based on four different cells and data collected on two different days) was 2.5 ± 2.8 (SD)%. These results indicate that morphological changes or instrumental artifacts were not responsible for the changes seen in Fluo-3 labeled cells.

There was often significant photobleaching during the time course of the experiments, and unstimulated cells sometimes exhibited spontaneous fluctuations in fluorescence, presumably because of spontaneous calcium fluctuations. However, these effects, which made it difficult to establish a precise baseline, did not by themselves lead to increases in the TIR/EPI ratio. The ratio was generally

unaffected by spontaneous fluctuations, and it tended to decrease slightly with photobleaching (perhaps because membrane-proximal regions were exposed to light during both the TIR and EPI phases of illumination).

The addition of ionomycin, which makes the membrane permeable to calcium, led to large increases in both TIR and EPI signals, but unlike FMLP, caused the TIR/EPI ratio to decrease by approximately 25% (in 14 of 18 cells on six different days). Based on the amplitude of the fluorescence response to ionomycin, relative to FMLP, we could roughly estimate the amplitude of the calcium concentration increase due to FMLP (Omann and Harter, 1991). These calculations yielded estimates of the intracellular calcium concentrations of ~280 nM in resting cells and 1–5 μM in maximally stimulated cells (both EPI and TIR, $n = 3$). This is in reasonable agreement with estimates of intracellular calcium in cells in suspension (the "EPI" equivalent) of 195 nM and 1.2 μM for resting and maximally stimulated cells, respectively (Omann and Harter, 1991).

The kinetic pattern of the increased TIR/EPI signal in Fluo-3-labeled neutrophils is more typical of secretory responses (slower onset and longer duration) (Smolen et al., 1982) than cytoskeletal changes (more rapid onset and recovery) (Bengtsson, 1990; Sklar et al., 1985), suggesting that membrane-proximal Ca^{2+} may be important for regulating vesicle fusion, but not actin polymerization in neutrophils. It is also possible that the elevated membrane-proximal calcium reflects the influx of extracellular calcium as the cell rebuilds its calcium stores. The greater increase in $[Ca^{2+}]$ at the membrane relative to the cytoplasm may appear slight, given the predictions that the concentration of calcium needed to support vesicle fusion would need to be ~10-fold greater than the cytoplasmic increases (Smolen et al., 1986; Smolen and Stoehr, 1985, 1986). However, if the observed relative increase in membrane-proximal calcium is localized into submicroscopic "hot spots," either laterally or in a depth significantly less than the 100-nm illumination depth of the TIR measurements, then the calcium increase in these hot spots could be sufficient to support vesicle fusion.

The prolonged increase in TIR/EPI ratio that persists well after the initial transient has begun to recover is consistent with the hypothesis that the increase in membrane-proximal Ca^{2+} results from a late-occurring Ca^{2+}-stimulated Ca^{2+} influx, as observed by others (Lagast et al., 1984; von Tscharner et al., 1986; Chandler and Kazilek, 1987). Preliminary attempts to test this hypothesis were performed by measuring the Ca^{2+} fluxes in FMLP-stimulated cells in the presence of the calcium channel blocker La^{3+} (Demaurex et al., 1992). La^{3+} (100 μM) did not inhibit the rapid transient increases in Ca^{2+} seen by both TIR and EPI illumination. However, it reduced the increase in the TIR/EPI ratio induced by FMLP down to 3 ± 7% (SD, $n = 5$), supporting the hypothesis that the FMLP-induced increase in the TIR/EPI ratio is due to the influx of calcium from the extracellular medium.

In conclusion, the optical chopping scheme used here for comparing TIR versus EPI fluorescence signals appears to work well for comparing the time course of membrane-proximal versus cytosolic Ca^{2+} concentrations in stimulated neutrophils. The TIR/EPI chopping system should be applicable to a wide variety of cell types exhibiting responses that involve changes in intracellular Ca^{2+} (and other ions for which there are fluorescent indicators) that may differ between regions that are proximal to the plasma membrane versus regions that are deeper in the cell cytosol.

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REFERENCES

Allbritton, N. L., T. Meyer, and L. Stryer. 1992. Range of action of calcium ion and inositol 1,4,5-trisphosphate. *Science.* 258:1812–1815.

Augustine, G. J., and E. Neher. 1992. Calcium requirement for secretion in bovine chromaffin cells. *J. Physiol. (Lond.).* 450:247–271.

Axelrod, D., E. H. Hellen, and R. M. Fulbright. 1992. Total internal reflection fluorescence. *In* Fluorescence Spectroscopy: Principles and Applications, Vol. 3: Biochemical Applications. J. Lakowicz, editor. Plenum Press, New York. 289.

Bengtsson, T. 1990. Correlation between chemotactic peptide-induced changes in chlorotetracycline fluorescence and F-actin content in human neutrophils: a role for membrane-associated calcium in the regulation of actin polymerization? *Exp. Cell Res.* 191:57–63.

Bengtsson, T., M. E. E. Jaconi, M. Gustafson, K.-E. Magnusson, J.-M. Theler, D. P. Lew, and O. Stendahl. 1993. Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. *Eur. J. Cell Biol.* 62:49–58.

Bootman, M. D., and M. J. Berridge. 1995. The elemental principles of calcium signaling. *Cell.* 83:675–678.

Caswell, A. H, and J. D. Hutchison. 1971a. Selectivity of cation chelation to tetracyclines: evidence for special conformational of calcium chelate. *Biochem. Biophys. Res. Commun.* 43:625–630.

Caswell, A. H., and J. D. Hutchison. 1971b. Visualization of membrane bound cations by a fluoescent technique. *Biochem. Biophys. Res. Commun.* 42:43–49.

Chandler, D. E., and C. J. Kazilek. 1987. Calcium signals in neutrophils can be divided into three distinct phases. *Biochim. Biophys. Acta.* 931: 175–179.

Chandler, D. E., and J. A. Williams. 1978. Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling. *J. Cell Biol.* 76:371–385.

Cheek, T. R., and V. A. Barry. 1993. Stimulus-secretion coupling in excitable cells: central role for calcium. *J. Exp. Biol.* 184:183–196.

Clapham, D. E. 1995. Calcium signaling. *Cell.* 80:259–268.

Cramer, E. B., and J. I. Gallin. 1979. Localization of submembranous cations to the leading end of human neutrophils during chemotaxis. *J. Cell Biol.* 82:369–379.

Demaurex, N., D. P. Lew, and K-H. Krause. 1992. Cyclopiazonic acid depletess intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.* 267:2318–2324.

Etter, E. F., M. A. Kuhn, and F. S. Fay. 1994. Detection of changes in near-membrane Ca^{2+} concentration using a novel membrane-associated Ca^{2+} indicator. *J. Biol. Chem.* 269:10141–10149.

Felder, C. C., D. Singer-Lahat, and C. Mathes. 1994. Voltage-independent calcium channels. Regulation by receptors and intracellular calcium stores. *Biochem. Pharmacol.* 48:1997–2004.

Ghosh, A., and M. E. Greenburg. 1995. Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science.* 268: 239–247.

Jaconi, M. E. E., R. W. Rivest, W. Schlegel, C. B. Wollheim, D. Pittet, and P. D. Lew. 1988. Spontaneous chemoattractant-induced oscillations of cytosolic free calcium in single adherent human neutrophils. *J. Biol. Chem.* 263:10557–10560.

Korchak, H. M., K. Vienne, C. Wilkenfeld, C. S. Roberts, L. E. Rutherford, K. A. Haines, and G. Weissmann. 1983. The role of calcium in neutrophil activation-mobilization of multiple calcium pools. *J. Cell Biol.* 97:160a.

Kruskal, B. A., S. Shak, and F. R. Maxfield. 1986. Spreading of human neutrophils is immediately preceded by a large increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 83:2919–2923.

Lad, P. M., J. S. Kaptein, C. K. Lin, C. I. Kalunta, S. J. Scott, and D. G. Gu. 1992. G-proteins and the role of second messengers in the regulation of the human neutrophil. *Immunol. Ser.* 57:107–136.

Lagast, H., P. D. Lew, and F. A. Waldvogel. 1984. Adenosine triphosphate-dependent calcium pump in the plasma membrane of guinea pig and human neutrophils. *J. Clin. Invest.* 73:107–115.

Llinas, R., I. Z. Steinberg, and K. Walton. 1981. Presynaptic calcium currents in squid giant synapse. *Biophys. J.* 33:289–322.

Llinas, R., M. Sugimori, and R. B. Silver. 1992. Microdomains of high calcium concentration in a presynaptic terminal. *Science.* 256:677–679.

Lloyd, Q. P., M. A. Kuhns, and C. V. Gay. 1995. Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium-sensitive fluorescent dye, calcium green C_{18}. *J. Biol. Chem.* 270:22445–22451.

Naccache, P. H., M. Volpi, H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1979. Chemtactic factor-induced release of membrane calcium in rabbit neutrophils. *Science.* 203:461–463.

Omann, G. M., and J. M. Harter. 1991. Pertussis toxin effects of chemoattractant-induced response heterogeneity in human PMNs utilizing Fluo-3 and flow cytometry. *Cytometry.* 12:252–259.

Sklar, L. A., G. M. Omann, and R. G. Painter. 1985. Relationship of actin polymerization and depolymerization to light scattering in human neutrophils: dependence on receptor occupancy and intracellular calcium. *J. Cell Biol.* 101:1161–1166.

Smolen, J. E., B. A. Eisenstat, and G. Weissmann. 1982. The fluorescence response of chlorotetracycline-loaded human neurophils. Correlation with lysosomal enzyme release and evidence for a "trigger pool" of calcium. *Biochim. Biophys. Acta.* 717:422–431.

Smolen, J. E., and S. J. Stoehr. 1985. Micromolar concentrations of free calcium provoke secretion of lysozyme from human neutrophils permeabilized with saponin. *J. Immumol.* 134:1859–1865.

Smolen, J. E., and S. J. Stoehr. 1986. Guanine nucleotides reduce the free calcium requirement for secretion of granule constituents from permeabilized human neutrophils. *Biochim. Biophys. Acta.* 889:171–178.

Smolen, J. E., S. J. Stoehr, and L. A. Boxer. 1986. Human neutrophils permeabilized with digitonin respond with lysosomal enzyme release when exposed to micromolar levels of free calcium. *Biochim. Biophys. Acta.* 886:1–17.

Taljedal, I-B. 1978. Chlorotetracyline as a fluorescent Ca^{2+} probe in pancreatic islet cells. *J. Cell Biol.* 76:652–674.

Tolley, J. O., G. M. Omann, and A. J. Jesaitis. 1987. A high yield, high purity elutriation method for preparing human granulocytes demonstrating enhanced experimental lifetimes. *J. Leukoc. Biol.* 42:43–50.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry.* 19:2396–2404.

Tsien, R. Y. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature.* 290:527–528.

von Tscharner, V., B. Prod'hom, M. Baggiolini, and H. Reuter. 1986. Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature.* 324:369–371.

White, J. R., and F. L. Pearce. 1982. Characterization of chlortetracycline (aureomycin) as a calcium ionophore. *Biochemistry.* 21:6309–6312.

White, J. R., and F. L. Pearce. 1983. Use of chlortetracycline to monitor calcium mobilization during histamine secretion from the mast cell: a cautionary note. *Anal. Biochem.* 132:1–5.