Oscillatory Pericellular Proteolysis and Oxidant Deposition During Neutrophil Locomotion

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ABSTRACT To better understand the mechanism of leukocyte migration in complex environments, model extracellular matrices were prepared using gelatin, Hanks' solution, Bodipy-BSA (fluorescent upon proteolysis), and dihydrotetramethylrosamine or hydroethidine (fluorescent upon oxidation). Using quantitative microfluorometry, neutrophil-mediated extracellular pulses of reactive oxygen metabolites (ROMs) and pericellular proteolysis were periodically observed showing that these functions occur as quantal bursts. However, chronic granulomatous disease neutrophils, which do not produce ROMs, did not display ROM deposition. Matrices show an alternating pattern of green (proteolytic) and red (oxidative) fluorescence, indicating these functions are out of phase. Electric fields phase-matched with metabolic oscillations, which increase the amplitude of intracellular NAD(P)H oscillations, increase ROM deposition and pericellular proteolysis; this further supports the link between intracellular chemical oscillators and extracellular functions. This phase relationship may allow ROMs to inactivate protease inhibitors, followed by protease activation.

INTRODUCTION

Neutrophil infiltration of tissues and their accompanying activation are hallmarks of host resistance to infectious agents and numerous noninfectious disease states including arthritis and ischemia-reperfusion-dependent tissue damage (e.g., myocardial infarction, stroke, and organ transplantation) (Malech and Gallin, 1987). Cellular events in inflammation begin with loose selectin-mediated neutrophil-endothelial cell binding followed by tight β_2 integrin-mediated adherence and diapedesis across the endothelium (Lawrence and Springer, 1991; Springer, 1990; Rosales and Juliano, 1995). In addition to penetrating tissue planes, neutrophils must also traverse extracellular matrices, i.e., basement membranes and interstitial tissues. Although the mechanisms of neutrophil attachment to endothelial cells are beginning to be appreciated in some detail, the mechanisms responsible for leukocyte transmigration of cell layers and locomotion through connective tissues are poorly understood.

Neutrophil locomotion involves a series of tightly choreographed events including microfilament assembly from actin pools, cell shape change/extension, integrin-mediated adherence events, and local proteolysis, among others. It is now well known that several neutrophil functions oscillate in time, particularly in response to chemotactic factors (Wymann et al., 1989a, b; Omann et al., 1989, 1995; Ehrengruber et al., 1995; Hartman et al., 1994). Previous workers have reported oscillations in actin assembly (Wymann et al., 1989a; Omann et al., 1989, 1995), respiratory burst (Wymann et al., 1989b), shape change (Wymann et al., 1989a, b; Ehrengruber et al., 1995), velocity change (Hartman et al., 1994), and cytosolic calcium levels (Marks and Maxfield, 1990; Kruskal and Maxfield, 1987). We have recently reported that interreceptor binding between CR4 and urokinase receptors oscillates and that these oscillations may be traced to an oscillatory signal transduction apparatus associated with metabolic oscillations (Kindzelskii et al., 1997). We have speculated that intracellular chemical oscillators (e.g., signal transduction and metabolic machineries) represent how a cell mechanistically drives its oscillatory locomotory/effector functions. Our oscillatory model of transmembrane signaling in cell migration is a dramatic departure from conventional signaling models (e.g., reaction-diffusion signaling) and requires confirmation of numerous derivative hypotheses. We have recently shown that application of an external electric field 180° out of phase with cytosolic NAD(P)H autofluorescence triggers metabolic resonance, as defined by heightened NAD(P)H oscillatory amplitudes, wherein neutrophil extension and actin assembly are greatly exaggerated (Kindzelskii and Petty, 1997 and unpublished). Using gelatin as a model of the collagen-rich extracellular matrix, we now show that ROM production and pericellular proteolysis temporally oscillate, as judged by spatial deposition of reaction products in the gel matrix. We further show that these oscillations are 180° out of phase, suggesting that they are associated with different metabolites.

Received for publication 31 December 1996 and in final form 15 September 1997.

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0006-3495/98/01/90/08 $2.00

MATERIALS AND METHODS

Neutrophil preparation

Peripheral blood was obtained from normal healthy adults by using heparinized tubes or from the American Red Cross (Detroit, MI). Neutrophils were isolated as described (Xue et al., 1994). Purified cells were >95% viable as judged by trypan blue exclusion.

Proteolytic action

Bodipy-BSA (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indacene-3-propionic acid-conjugated BSA; Molecular Probes, Eugene, OR) at 20 μg/ml was incubated for 60 min at 37°C with plasmin, proteinase K, thermolysin, and elastase (Sigma Chem., St. Louis, MO) at 1 U/ml. Fluorescence was measured using a CytoFluor 2350 (Perseptive BioSystems, Bedford, MA) with excitation at 485 nm and emission at 530 nm.

Spectrophotometry

Spectrophotometry was performed using a SLM Aminco SPF-550 TMC spectrofluorimeter (SLM Instruments, Urbana, IL). Solutions of BSA conjugates and reference dyes were prepared at the same optical densities at their absorption peaks. The emission spectra were recorded with the excitation monochromator adjusted to 493 nm.

Matrix preparation

Hanks' gel matrices were prepared in a fashion similar to that previously described (Zigmond, 1977). Matrices containing 2% gelatin and various compounds were prepared at 45°C then allowed to cool to 37°C, where they demonstrated properties of a semi-solid. Gel mixtures contained 100 ng/ml dihydrotetramethylrosamine (Molecular Probes, Eugene, OR), 3 μM hydroethidine (Sigma, St. Louis, MO), and/or 25 μg/ml Bodipy-BSA. Previous studies have illustrated the utility of these compounds (Royall and Ischiropoulos, 1993; Rothe and Valet, 1990; Carter et al., 1994; Cao et al., 1993; Kindzelskii et al., 1996b).

Electric field exposure

In some cases electric fields were applied during microscopic observations as previously described (Friend et al., 1975; Kindzelskii and Petty, 1997). Pulsed square wave electric fields (20 ms, 10 V/m) were applied using a power supply and platinum or Ag/AgCl electrodes (Bioanalytical Systems, Inc., West Lafayette, IN). The electric field was assessed by measuring the current using an electrometer (Keithley, Cleveland, OH; model 6517A). When these electric fields are applied at the troughs in NAD(P)H autofluorescence intensity, the NAD(P)H intensity grows in amplitude.

Microscopy

Cells were examined using an automated axiovert inverted fluorescence microscope (Carl Zeiss, New York, NY) with mercury illumination interfaced to a Perceptics Biovision system (Knoxville, TN). All experiments were performed using a Zeiss temperature stage set to 37°C. DIC and fluorescence images were collected as described (Xue et al., 1994). Briefly, Bodipy fluorescence was detected using a 485DF22 nm and 530DF30 nm filter combination and a 510 long-pass dichroic mirror (Omega Optical, Brattleboro, VT). TMRos fluorescence was detected using a 540DF20 nm and 590DF30 filter set with a 560 long-pass dichroic mirror. EB was detected using a 540DF20 nm and 590DF30 filter set with a 560 nm dichroic mirror; due to the large Stoke's shift, EB was not imaged with TMRos or Bodipy labels. NAD(P)H autofluorescence was detected using 365DF20 and 405DF35 filters and a 405 long-pass dichroic mirror. Fluorescence levels were quantitated using a photon counting apparatus (Photochemical Research Associates, Inc.; London, Ont.) coupled to the microscope (Maher et al., 1993). Cells were illuminated individually to ensure that each quantitative experiment corresponded to just one cell. In addition, background photon count rates were taken from an adjacent area on the slide that contained no cells. A digital oscilloscope was used to monitor kinetic changes in fluorescence levels.

RESULTS

In the present study we test the hypothesis that neutrophils elaborate oxidative molecules and display pericellular proteolytic activity during migration through complex environments. We employ gelatin including Hanks' balanced salt solution (HBSS) as a simple model of the collagen-rich connective tissue. While in a fluid state, we incorporated molecules into the matrix including dihydrotetramethylrosamine (H_2-TMRos), hydroethidine (HE), and Bodipy-BSA; these molecules become fluorescent upon exposure to hydrogen peroxide (Royall and Ischiropoulos, 1993; Rothe and Valet, 1990; Carter et al., 1994), superoxide anions (Rothe and Valet, 1990; Carter et al., 1994), and proteolytic activity (Kindzelskii et al., 1996b). In the case of Bodipy-BSA, its fluorescence is quenched by >95% within intact BSA, but is dequenched after proteolytic disruption, as described below. The nonfluorescent H_2TMRos becomes oxidized to the highly fluorescent TMRos, which has an emission spectrum similar to tetramethylrhodamine. HE is oxidized by superoxide to form ethidium bromide (EB). The gel matrix thus serves as a model for the extracellular matrix while immobilizing reporters for oxidant and proteolytic detection.

We have previously suggested that pericellular proteolysis may oscillate during neutrophil locomotion (Kindzelskii et al., 1997). To test this hypothesis, we have developed a methodology for studying extracellular proteolysis in three-dimensional matrices. Others have previously reported that the fluorescence of FITC-conjugated proteins, which is significantly quenched on certain proteins, can be used as a means of detecting proteolytic action (Twining, 1984; Hormer and Beighton, 1990; Farmer and Yuan, 1991). Fig. 1 *A* shows the fluorescence emission spectra of Bodipy-BSA and the unconjugated Bodipy molecule. In this case 16 dye molecules were incorporated into each protein. As this spectrum shows, the fluorescence of Bodipy-BSA is quenched by >95%. However, when Bodipy-BSA at 20 μg/ml in phosphate buffer was exposed to proteases including plasmin (1 U/ml for 60 min at 37°C), the fluorescence intensity increased dramatically (Fig. 1 *B*). Positive results were obtained using several proteases suggesting that Bodipy-BSA has a multiple substrate specificity. Since plasminogen activators and plasmin have been associated with breakdown of the extracellular matrix and cell migration (Saksela and Rifkin, 1988), we tested the concentration-dependence of fluorescence emission from Bodipy-BSA. Fig. 1 *C* shows the plasmin dose-dependent increase in fluorescence from 10 μg/ml Bodipy-BSA in phosphate buffer (37°C for 2 h); plasmin activities of <0.01 U/ml can be detected. Since uPAR focuses uPA into a small volume at the lamellipodium's surface during neutrophil migration (Estreicher et al., 1990; Kindzelskii et al., 1996a), the local proteolytic activity near the cell's leading edge is expected to be enhanced in comparison to that in solution phase.

When unstimulated neutrophils migrate within H_2TMRos, HE, or Bodipy-BSA-containing matrices, a se-

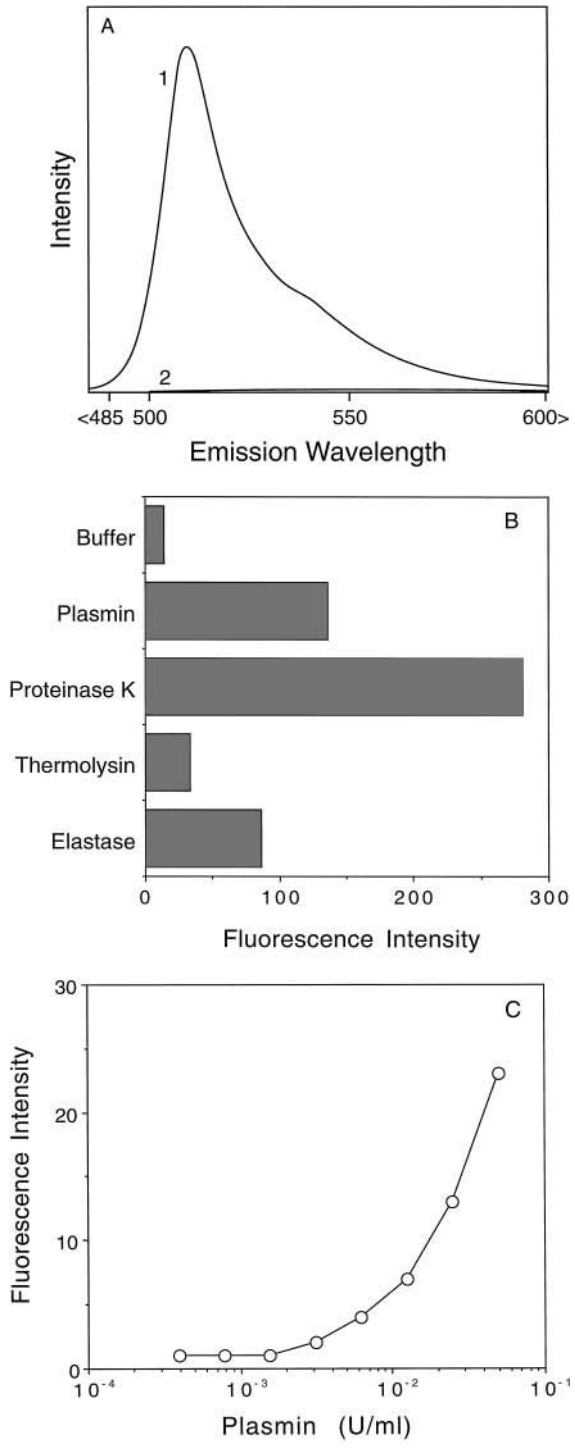

FIGURE 1 Quantitative analyses of the fluorescence emission of Bodipy-BSA as a protease substrate. (*A*) Fluorescence emission spectra of unconjugated Bodipy dye (*trace 1*) and Bodipy-BSA (*trace 2*). Emission intensity is shown at the ordinate, whereas emission wavelength is plotted at the abscissa. The fluorescence emission maximum is 517 nm. Bodipy is strongly quenched when attached to BSA. (*B*) Effect of incubation of a variety of proteases on the fluorescence emission of Bodipy-BSA samples. Proteolytic action on Bodipy-BSA yields fluorescent peptides, thus relieving fluorescence quenching in the intact molecule. One of three representative experiments is shown. (*C*) The plasmin dose-dependent acquisition of fluorescence emission from Bodipy-BSA samples. Sample fluorescence intensity is plotted at the ordinate, whereas plasmin concentration is given at the abscissa. (One of three similar experiments is shown in each panel.)

ries of fluorescent stripes is observed (Fig. 2, *A–C*). These stripes coincide with positions of the lamellopodia. The stripes are spatially separated by ~5 μm and temporally separated by a period of 20 s. These data show that hydrogen peroxide and related oxidant deposition and extracellular proteolysis oscillate during cell migration. Although extracellular proteolysis has been generally linked with leukocyte locomotion (Estreicher et al., 1990; Kirchheimer and Remold, 1989; Gyetko et al., 1994), the production of oxidants during cell locomotion has not been appreciated. The high sensitivity of modern intensified charge-coupled device cameras coupled with the high sensitivity of the fluorophores and their stabilization in the matrix allow these oxidant signals to be detected. Furthermore, these micrographs suggest that cell functions can be associated with the lamellipodia of migrating cells.

To obtain quantitative data, cells were examined using a photomultiplier tube and housing attached to the microscope. Fig. 3 shows quantitative data of fluorescence emission from matrices labeled with H_2TMRos, HE, or Bodipy-BSA during neutrophil infiltration. Fig. 3 *a* shows the rhythmic delivery of ROMs to the extracellular environment. The stepwise increase in TMRos formation takes place at 21.9 ± 1.8 s intervals, as does EB formation (22.1 ± 1.8) (Fig. 3 *b*). Similarly, pericellular proteolysis takes place in a similar quantitative fashion (22.1 ± 1.9 s) (Fig. 3 *c*). Neutrophils from chronic granulomatous disease (CGD) patients are genetically deficient in the NADPH oxidase and are therefore unable to generate ROMs (Orkin, 1989). CGD neutrophils were unable to cause the formation TMRos or EB in these matrices (Fig. 3, *d* and *e*), thus indicating that these probes required cellular production of ROMs to trigger the production of fluorescent derivatives. However, as anticipated, CGD neutrophils retained the ability to mediate pericellular proteolysis (Fig. 3 *f*). Thus, during migration through a model extracellular environment, ROMs and proteolytic activation are delivered in an oscillatory, not continuous, fashion. Functional cell oscillations take place at the same frequencies as metabolic oscillations (Kindzelskii et al., 1997; Kindzelskii and Petty, 1997).

We next sought to determine the phase relationship between oxidant deposition and extracellular proteolysis. H_2TMRos and Bodipy-BSA were simultaneously incorporated into 2% gelatin matrices. Neutrophils were observed during migration within these matrices. Fig. 4 shows a gallery of color photomicrographs showing the spatial locations of TMRos and the fluorescent peptides that result from proteolysis of Bodipy-BSA. Notably, an alternating pattern of red and green fluorescence is observed. Fig. 4, *A–M* show the variety of trails neutrophils leave during migration through these matrices. Fig. 4, *N* and *O* show two higher magnification views of these trails, which suggest that substructures may be present within the bands. To provide compelling kinetic evidence for the formation of these alternating patterns, Fig. 4, *P–W* show the time-dependent formation of successive oxidative/proteolytic bands as neutrophils proceed through the matrix. Thus, deposition

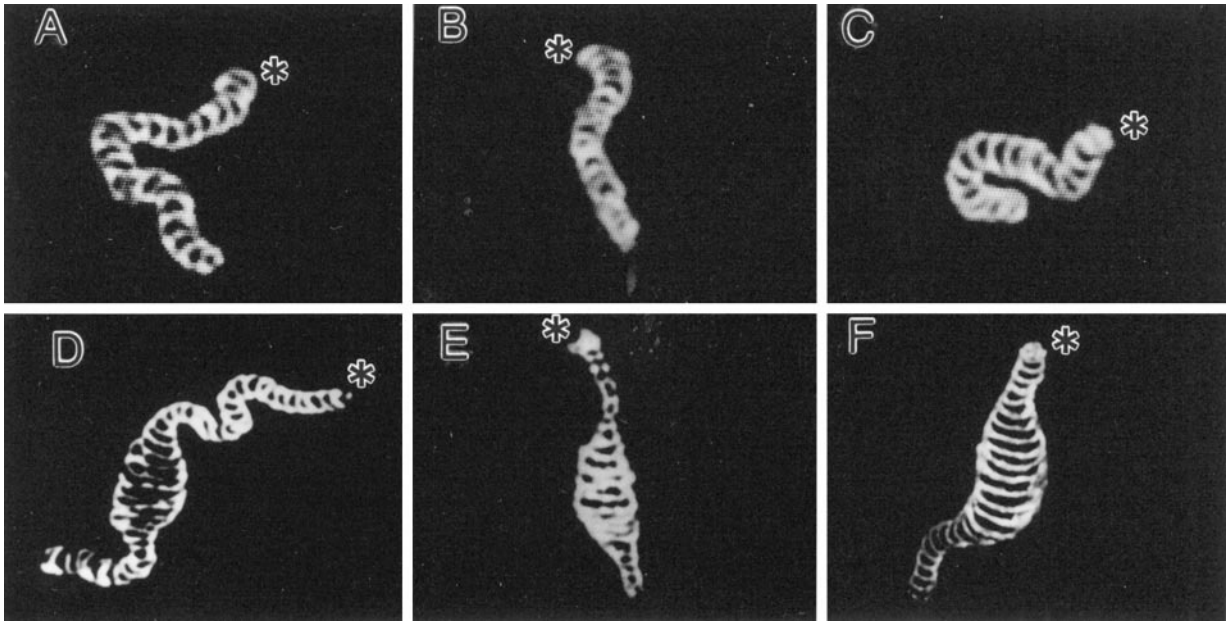

FIGURE 2 Oscillatory properties of neutrophils during spontaneous migration through gelatin matrices. Unlabeled neutrophils were placed in matrices containing 100 ng/ml H_2TMRos (*A, D*), 3 μM HE (*B, E*), or 25 μg/ml Bodipy-BSA (*C, F*). Extracellular release of reactive oxygen metabolites including hydrogen peroxide and hydroxyl radical oxidizes H_2TMRos to TMRos yielding fluorescence emission. Superoxide anions oxidize HE to EB. When Bodipy-BSA (nonfluorescent) is cleaved by proteases, its fluorescence intensity is dramatically increased, thus providing an indication of extracellular proteolytic action. (*A–C*) Representative examples of oscillatory oxidant deposition (*A, B*) and pericellular proteolysis (*C*) within matrices during spontaneous neutrophil migration. (*D–F*) When a phase-matched electric field is applied during cell migration, the extent of oxidant release (*D, E*) and proteolytic action (*F*) are increased in physical amplitude (note increases in the width of trails, which correspond to the durations of electric field exposures). (The position of the cell when the photograph was recorded is indicated by an *asterisk*.) (*A–C, F*: ×600; *D*: ×480; *E*: ×450).

of oxidants and extracellular proteolysis alternate in space and time as neutrophils migrate within complex environments. To provide further evidence regarding this phase relationship, quantitative fluorometry of samples was performed while switching between the optical set-ups for TMRos and Bodipy. Fig. 5, *a* and *b* show time-correlated kinetic traces of TMRos and Bodipy fluorescence during neutrophil migration through the gel. Note that upward steps in fluorescence intensity occur at 20 s intervals in both panels, but that the increases are 10 s out of phase in panel *a* versus *b*. Thus, these data indicate that oxidant release and pericellular proteolysis oscillate 180° out of phase.

Since the neutrophil's NADPH oxidase generates superoxide anions and downstream ROMs via electron donation from its substrate NADPH (Orkin, 1989) and the respiratory burst has been shown to oscillate at a similar frequency (Wymann et al., 1989), we examined the phase relationship between cytoplasmic NAD(P)H oscillations (Kindzelskii and Petty, 1997; Kindzelskii et al., 1997) and cellular oxidant release. Neutrophil migration through a 2% matrix containing H_2TMRos was monitored. Importantly, intervals of maximal oxidant delivery correspond to peaks in NAD(P)H oscillations (Fig. 5, *c* and *d*). Thus, oxidant production is in phase with NAD(P)H autofluorescence oscillations.

We have previously shown that application of electric fields that are phase-matched with metabolic oscillations leads to metabolic resonance, wherein maximal cytoplasmic levels of NAD(P)H (and perhaps other metabolites) are substantially increased (Kindzelskii and Petty, 1997). Thus, electric fields can be used to manipulate the amplitude of metabolic oscillations. To provide another means of linking metabolic oscillations with extracellular oxidant deposition and proteolysis, we exposed neutrophils migrating in matrices to electric fields. Thus, in this study we employ electric fields simply as a tool to manipulate metabolite levels without regard to its mechanism, which has been addressed elsewhere (Kindzelskii and Petty, 1997 and unpublished). Fig. 2, *c* and *d* show fluorescence micrographs of TMRos and the fluorescent peptides from Bodipy-BSA in the presence of an electric field (20 ms, 10 V/m) applied at NAD(P)H autofluorescence troughs. As described above in the absence of an electric field, a pattern of stripes separated by ~5 μm is observed for both oxidant deposition and local proteolysis. In comparing Fig. 2 *a* to *c* and *b* to *d*, one can see that the regions of the matrix undergoing oxidant deposition and proteolytic action are substantially increased. The effect is specific for the presence of an electric field since it appears and disappears with field application. The increased fluorescence intensities associated with ROM deposition and protease action cannot be accounted for by simple electrophoresis of reaction products, since the patterns are symmetrical about the direction of cell migration and independent of the direction of cell migration. At least two factors could contribute to the appearance of these micrographs. The total intensity of ROM

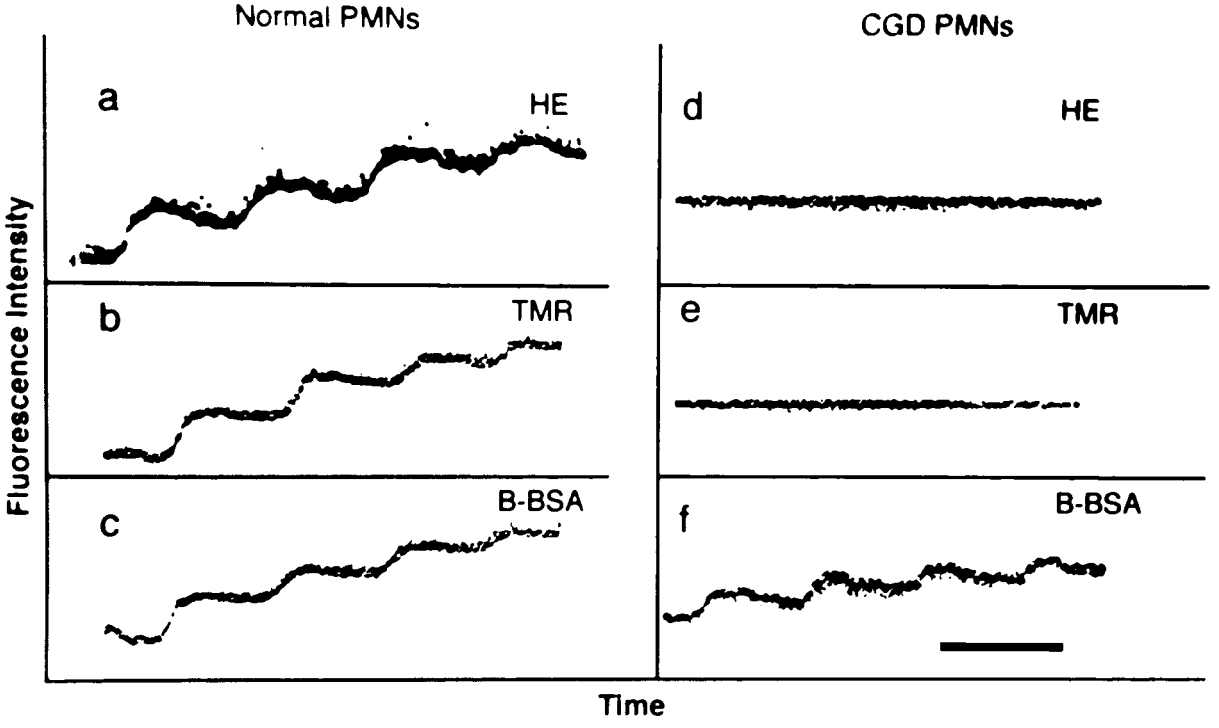

FIGURE 3 Quantitative analysis of oscillatory neutrophil functions during migration within gelatin matrices. Quantitative microfluorometry was performed on samples. The fluorescence intensity is plotted versus time. Normal neutrophils (*left*) and CGD neutrophils (*right*) were analyzed. Although normal neutrophils supported the oxidation of HE and H_2TMRos (*traces a* and *b*), CGD neutrophils were unable to mediate the oxidation of these probes (*traces d* and *e*). In contrast, both normal and CGD neutrophils were able to mediate proteolytic destruction of the extracellular matrix as indicated by the unquenched forms of Bodipy-BSA (*traces c* and *f*). Thus, quantitative fluorescence analysis supports the qualitative micrographs shown above. (Bar = 20 s).

product formation is greater because of the greater intensity of metabolic oscillations. In addition to increased product formation, the increased diameter could also be related to exaggerated cell shape changes accompanying metabolic resonance in an electric field (Kindzelskii and Petty, 1997). In either case, the experimental manipulation of intracellular oscillatory metabolite levels using electric fields affects extracellular oscillatory neutrophil functions during locomotion.

DISCUSSION

In the present study we have shown that migrating human neutrophils deposit ROMs and cleave extracellular protein substrates in a regular oscillatory fashion, as anticipated by our previous studies of metabolic oscillations (Kindzelskii et al., 1997). This is evident in the alternating red-green pattern laid down by cells as they migrate through matrices. These oscillatory cell functions are consistent with previous studies showing oscillations in neutrophil actin assembly, shape change, velocity change, and respiratory burst (Wymann et al., 1989a, b; Omann et al., 1989, 1995; Ehrengruber et al., 1995; Hartman et al., 1994). Our previous studies have suggested that functional oscillations are driven by the cadence of an oscillating signal transduction/metabolic apparatus during cell migration (Kindzelskii et al., 1997; Kindzelskii and Petty, 1997). Such oscillations may have numerous advantages in signaling, efficiency of energy utilization, and the ability to drive enzymatic reactions away from equilibrium (Lazar and Ross, 1990; Astumian and Robertson, 1993; Astumian et al., 1989). Our results provide dramatic evidence in support of the physiological relevance of oscillatory intracellular and extracellular biochemical reactions in neutrophil locomotion.

Wymann et al. (1989b) have reported oscillations in luminol-enhanced chemiluminescence from stimulated neutrophils. Since luminol-enhanced chemiluminescence is largely due to hypochlorous acid formed by myeloperoxidase action on hydrogen peroxide and chloride (Arnhold et al., 1993), it is unclear if these oscillations might be accounted for by peroxidase oscillations (e.g., Olson et al., 1995) or oscillations in superoxide production. Thus, we performed experiments with hydroethidine, which is specific for superoxide anions. We have also performed experiments using H_2TMRos, which detects hydrogen peroxide, hydroxyl anions, and potentially other ROMs. To ensure that these reagents were actually detecting ROMs, we employed cells from CGD patients; these cells are known to be completely defective in the ability to produce ROMs via the NADPH oxidase (Orkin, 1989). The inability of CGD neutrophils to affect H_2TMRos and HE labels demonstrate that these labels detect products of the neutrophil's respiratory burst. Our results show that the production of multiple

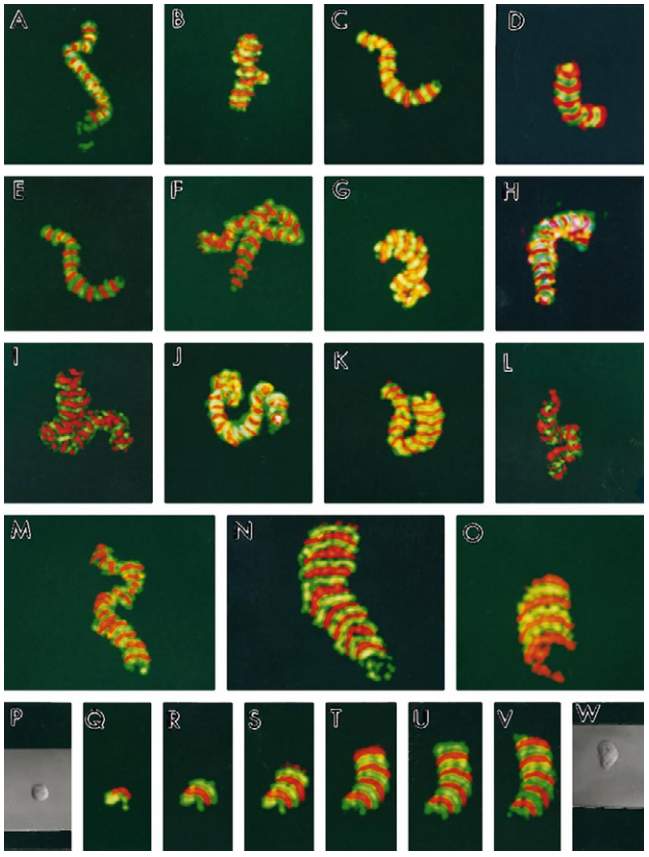

FIGURE 4 Qualitative analysis of relative phase properties of oxidant production and pericellular proteolysis during cell migration. Gelatin matrices containing H_2TMRos (*red*) and Bodipy-BSA (*green*) were prepared as described above. A representative gallery of fluorescence photomicrographs of the alternating pattern of green-red-green-red fluorescence of the gel matrix at a focal plane just above the apical cell surface is shown (*A–O*). These results show that extracellular release of oxidants and proteolytic action oscillate 180° out of phase during cell locomotion. At higher magnification, substructures within the stripes, which are largely spherical, become apparent (*N* and *O*). A kinetic experiment showing the alternating formation of stripe patterns at one focal plane is given in panels *P–W* (the fluorescence images shown in panels *Q–V* were collected over a 2.7 min period of time). The direction of cell migration is approximately toward the top of the page. Thus, intracellular oscillations may propagate through the membrane into the extracellular environment.

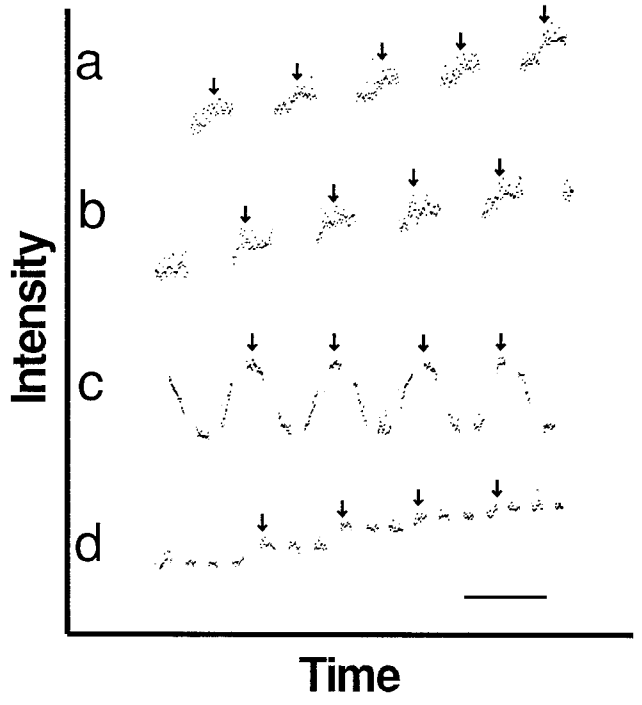

FIGURE 5 Quantitative analyses of relative phase properties of NAD(P)H autofluorescence and oxidant production and of oxidant production and pericellular proteolysis during cell migration. The fluorescence intensity (arbitrary units) is plotted versus time. (*a, b*) Quantitative analysis of the phase relationship between oxidant release and pericellular proteolysis. Gelatin matrices containing H_2TMRos and Bodipy-BSA were prepared as described above. Neutrophil migration through these matrices was studied using quantitative microfluorometry. To temporally coordinate these observations, we switched between the optical set-ups for these two chromophores. The fluorescence of TMRos and Bodipy increased in a stepwise fashion. However, the incremental steps in TMRos fluorescence occur ~10 s after Bodipy increases ($n = 3$). Thus, oxidant release is out of phase with pericellular proteolysis. (*c, d*) The phase relationship between NAD(P)H oscillations (*c*) and oxidant release (*d*) was determined by switching between optical set-ups during migration of individual cells. Briefly, neutrophil migration in a gelatin matrix containing 50 ng/ml H_2TMRos was quantitatively observed. Oscillations in NAD(P)H autofluorescence intensity with a period of 20 s were observed as previously described (Kindzelskii et al., 1997). The TMRos signal (*d*) increases in a stepwise fashion, as shown above. The peaks in NAD(P)H concentration (*c, arrows*) correspond to periods of maximal oxidant delivery into the matrix (*d*). Thus, oxidant release is in-phase with NAD(P)H oscillations. ($n = 7$) (Bar = 20 s).

ROMs oscillate in time, thus suggesting that these oscillations can be traced to the NADPH oxidase.

Our results also highlight the fact that cell activation with exogenous agents such as bacteria, chemotactic factors, signal transduction modifiers, etc. is not a prerequisite for oxidant release from neutrophils. That is, cell migration is a sufficient stimulus to trigger extracellular release of ROMs. ROMs may participate in cell locomotion by inactivating nearby protease inhibitors as cells move through complex environments (e.g., Weiss, 1989).

We have observed oscillations of NAD(P)H matching the oscillation periods of ROM release; thus, the oxidase's substrate, NADPH, and product, superoxide anions, oscillate with the same frequency. Since the oscillation periods for ROM deposition and NAD(P)H oscillate with the same period and are phase-matched, we suggest that metabolic oscillations drive ROM oscillations. Thus, the rising and falling levels of NADPH may drive more or less superoxide production. The mean cytosolic NADPH concentration is 270 μM (Patriarca et al., 1971). The amplitude of the oscillating NAD(P)H pool is 40% of the peak oscillatory amplitude. Since NAD(P)H autofluorescence is linear in this concentration range (Liang and Petty, 1992), we estimate that the cytosolic NADPH concentration varies from ~181 to 358 μM in migrating neutrophils (with a 10% correction for non-NAD(P)H autofluorescence). These concentrations are within an order of magnitude of the K_m of NADPH for the NADPH oxidase (~40 μM), as judged by

in vitro biochemistry experiments (Babior, 1987), although equilibrium constants are not directly applicable in these conditions. If we artificially enhance the amplitude of NAD(P)H oscillations using applied electric fields, we also enhance the magnitude for ROM deposition. Furthermore, both the NAD(P)H and ROM oscillation frequencies are doubled in parallel by addition of chemotactic factors (Petty et al., 1996). Thus, the proposed relationship between metabolic and ROM oscillations is both simple and consistent with cellular perturbation by electric fields and exogenous compounds. ROM deposition was found to be essentially a square-wave pattern, whereas metabolic oscillations were sinusoidal. Although the origin of this is unknown, it may well be due to nonlinearities in fluorescence emission kinetics of the probes at short times (<10 s), which have been previously observed (Bass et al., 1983; Ryan et al., 1990). Alternatively, nonlinearities could be introduced by oxidase or regulatory components in situ.

Urokinase-type plasminogen activator and its receptor focus at the lamellipodium of migrating neutrophils (Estreicher et al., 1990; Kirchheimer and Remold, 1989; Kindzelskii et al., 1996a). The accumulation of urokinase-type plasminogen activator at the lamellopodium may contribute to the stripes of extracellular proteolysis found near the lamellipodium. We have previously demonstrated proximity oscillations between CR4 and the urokinase receptor during neutrophil migration (Kindzelskii et al., 1997) which match the frequency and phase properties of extracellular proteolysis reported here. Thus, we hypothesize that integrin-urokinase receptor interactions (Kindzelskii et al., 1997) may also regulate proteolysis of extracellular matrices. However, neutrophil degranulation could also contribute to the observed stripes of proteolytic action. For example, degranulation could be controlled by cytoplasmic oscillations in ATP levels. Thus, the observed proteolytic phase properties may be linked with degranulation, which has recently been found to become activated in discrete packets (Liou and Campbell, 1996). Thus, our results showing oscillatory proteolytic function may be accounted for by cell surface proteolytic activities and/or cell degranulation. In either case, oscillatory extracellular matrix degradation is a newly defined oscillatory property of cells.

Our findings are consistent with the proposed role of metabolic clocks in coordinating neutrophil function (Kindzelskii et al., 1997). We have observed that oscillations in ROM deposition and local proteolysis are 180° out of phase. This suggests that oxidant and proteolytic oscillations are driven by separate reactants likely coupled with the glycolytic oscillator. We hypothesize that NADPH and ATP oscillations, which are 180° out of phase in lower eukaryotes (Hess and Boiteux, 1971), are responsible for functional oscillations. Thus, cytoplasmic NADPH oscillations may drive NADPH oxidase-mediated superoxide oscillations, while ATP oscillations, which may be responsible for signaling (Kindzelskii et al., 1997), could account for proteolytic oscillations. Thus, extracellular functions display oscillatory and phase properties. The phase relationship between oxidation and proteolysis may be relevant to cell locomotion in vivo. One function of ROMs is to inactivate protease inhibitors (Weiss, 1989). Thus, during locomotion, neutrophils release pulses of oxidants that inactivate nearby protease inhibitors, thus clearing the way for local proteolytic activation.

We have previously defined a relationship between metabolic phase-matched electric fields and the intracellular responses of metabolic resonance and cell extension (Kindzelskii and Petty, 1997). We have now extended these analyses to include the relationships between phase-matched electric fields and extracellular responses of pericellular proteolysis and ROM deposition. All of these processes are tied to cell locomotion and may be driven by the cadence of the oscillatory metabolic/signaling machinery. Previous workers have reported small, but significant, increases in the respiratory burst of activated neutrophils in the presence of pulsed electric fields or low frequency magnetic fields (Bobanovic et al., 1992; Roy et al., 1995). In the present study, activation with mediators such as phorbol esters was not required. Our ability to detect oxidant production in the absence of activators may be due to neutrophil adherence, which potentiates oxidant production (Nathan, 1987) and the high sensitivity of the techniques employed. The enhanced level of oxidant production reported by others is consistent with our imaging studies. Furthermore, perturbation of cells by external electric fields affects intracellular metabolic oscillatory pathways and, in turn, these oscillatory pathways affect extracellular functions.

We thank Nataliya Panchuk-Voloshina for technical assistance.

This work was supported by the American Heart Association of Michigan and National Institutes of Health Grants AI/CA 27409 (to H.R.P.) and AI20065 (to L.A.B.).

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