Proximity Oscillations of Complement Type 4 ($\alpha_X\beta_2$) and Urokinase Receptors on Migrating Neutrophils

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ABSTRACT Migrating neutrophils utilize β_2 integrins for substrate attachment and urokinase receptors (uPAR) to focus pericellular proteolysis. Our studies show that CR3 associates with uPAR on resting cells, whereas uPAR associates with CR4 at lamellipodia of migrating cells. Using resonance energy transfer (RET) microscopy, we show that the molecular proximity between CR4 and uPAR oscillates on migrating cells, thus suggesting that CR4 molecules periodically bind/release uPAR. Cell contact with fibrinogen, endothelial cells, chemotactic factors and indomethacin, and treatment with sub-optimal doses of signal transduction inhibitors, affect the oscillations' period, amplitude, and/or waveform. The oscillations were indistinguishable in period and 180° out-of-phase with cytosolic NAD(P)H autofluorescence oscillations. Thus, CR4 and CR3 identify a neutrophil's axis of migration and CR4 may restrain uPAR at lamellipodia. Oscillations in signal transduction and energy metabolism may coordinate cell adherence, local proteolysis, oxidant release, actin assembly, and cell extension.

INTRODUCTION

Inasmuch as the immune system is distributed throughout a host, long-range communication and cellular trafficking/recirculation are among its essential elements. Regular cycles of cell attachment and release are an integral feature of leukocyte motility (Springer, 1990). Another aspect of cell motility, which may allow leukocytes to penetrate biological barriers, is the ability to focus pericellular proteolysis at a cell's leading edge (Kindzelskii et al., 1996). β_2 integrins participate in leukocyte adherence to other cells, extracellular matrices, and other surfaces, while urokinase receptors (uPAR) promote local proteolysis. In addition to recognition events, β_2 integrins also mediate chemical and mechanical signal transduction (e.g., Fallman et al., 1989; Wang et al., 1993). Recent resonance energy transfer (RET) and immunoprecipitation studies suggest that CR3 ($\alpha_M\beta_2$) physically associates with the GPI-linked membrane proteins uPAR, FcγRIIIB, and lipopolysaccharide-ligated CD14 (Kindzelskii et al., 1996; Zhou et al., 1993; Xue et al., 1994, 1997; Zarewych et al., 1996; Poo et al., 1995; Bohuslav et al., 1995; Stockl et al., 1995) and shares its pro-inflammatory signaling abilities with these partners (Sehgal et al., 1993; Krauss et al., 1994; Zhou and Brown, 1994; Cao et al., 1995; Sitrin et al., 1996; Petty and Todd, 1996; Galon et al., 1996). Thus, GPI-anchored membrane proteins act as rapidly diffusing expendable scouts for β_2 integrins, which communicate ligand binding status to the cytosol. Furthermore, uPAR dissociates from CR3 as neutrophils polarize for locomotion followed by its accumulation at lamellipodia (Kindzelskii et al., 1996). We now report that CR4 ($\alpha_X\beta_2$) and uPAR are in close physical proximity on migrating cells. We also show that these interactions are oscillatory in nature and that they are likely associated with the metabolic apparatus, thus suggesting novel features of neutrophil motility and signal transduction.

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MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), and 7-amino-4-methylcoumarin-3-acetic acid (AMCA) were obtained from Molecular Probes (Eugene, OR). Fab or $F(ab')_2$ fragments of IgG mAb directed against CR3 (anti-Mo1, clone 44), CR4 (clone CBRp150/2E1), and uPAR (anti-Mo3, clone 3B10) were prepared and conjugated to fluorochromes as described (Kindzelskii et al., 1996; Luk et al., 1995).

Neutrophil preparation and labeling

Neutrophils were isolated from the peripheral blood of normal healthy adults (American Red Cross, Detroit, MI). Neutrophils were labeled with AMCA-conjugated anti-CR3 $F(ab')_2$ then capped with a second-step $F(ab')_2$ fragment of an anti-$F(ab')_2$ antibody that localized CR3 to one region of a membrane (Zhou et al., 1993; Xue et al., 1994). Cells were then labeled with TRITC-anti-uPAR $F(ab')_2$s and (FITC)-anti-CR4 Fab for 20 min at 4°C followed by thorough washing.

Microscopy

Cells were examined using an axiovert fluorescence microscope with quartz optics and a 100W mercury lamp (Carl Zeiss, New York, NY) interfaced to a Perceptics Biovision system (Knoxville, TN) (Kindzelskii et al., 1996). All experiments were conducted using a Zeiss temperature stage set to 37°C. RET and NAD(P)H autofluorescence microscopy were performed as described (Liang and Petty, 1992). NAD(P)H was detected using 365DF20 excitation and 405DF35 emission filters and a 405 long-pass dichroic mirror. RET emission intensity was detected using a 485DF22 excitation filter, a 590DF30 emission filter, and a 510 long-pass dichroic mirror (Uster and Pagano, 1986). RET, tetramethylrosamine, and NAD(P)H autofluorescence levels were quantitated using a photomultiplier

tube system (Photochemical Research Associates, Inc.; London, Ont.) coupled to the microscope (Kindzelskii et al., 1996).

RESULTS

To assess the time-dependent cell surface distribution of CR3, CR4, and uPAR, neutrophils were labeled with reagents directed against these molecules. Fig. 1 shows a time-lapse micrograph sequence of a labeled neutrophil at 37°C. Cells were labeled with AMCA-conjugated anti-CR3 $F(ab')_2$, capped with a second-step $F(ab')_2$ fragment of an anti-$F(ab')_2$ antibody (2) then labeled with TRITC-anti-uPAR $F(ab')_2$s and FITC-anti-CR4 Fab for 20 min. at 4°C. CR3 capping was employed as a convenience to segregate CR3 molecules. Controls for co-capping specificity and the second-step reagent have been reported (Zhou et al., 1993; Xue et al., 1994). CR3 and uPAR co-cap on resting cells (*panel b*) (Xue et al., 1994). As the cell polarizes for locomotion (*panels c–e*), uPAR dissociates from CR3 (Kindzelskii et al., 1996). CR4 and uPAR then co-localize at the cell's lamellipodium (*panels e, f*). To better observe the co-localization of CR4 and uPAR, the three labels shown in the pseudocolor image of Fig. 1 *f* are individually printed in *panels g–i*; the high level of overlap between CR4 and uPAR is apparent. These micrographs also suggest that CR4 accumulates at the nascent lamellipodium before uPAR (*panels b, c*). Similar results were obtained in the absence of capping stimuli and when TRITC-conjugated uPA was used in place of anti-uPAR $F(ab')_2$s. Thus, β_2 integrins may contribute to a neutrophil's axis of polarity.

Our imaging experiments show that CR4 and uPAR colocalize to the same membrane domain on migrating neutrophils. To demonstrate physical proximity of CR4 and uPAR at a molecular level ($\leq$7 nm), we used RET microscopy (Uster and Pagano, 1986). Experiments were performed using a photomultiplier tube to detect RET (Kindzelskii et al., 1996). Cells were labeled with anti-CR4 Fab and anti-uPAR $F(ab')_2$ fragments or the intact ligand uPA conjugated to donor (FITC) or acceptor (TRITC) chromophores. Controls indicated that RET emission was specific for both donors and acceptors, i.e., the RET imaging set-up did not detect an RET signal when the donor or the acceptor was omitted from the experiment. Furthermore, cells labeled with FITC only could not be detected with the TRITC filter set, thus indicating that the RET signal was not due to cross talk between the filter sets (see Kindzelskii and Petty, 1996 for additional examples). Further controls showed that RET emission was not observed on cells labeled with $F(ab')_2$s of a mAb against a uniformly distributed neutrophil surface glycoprotein (Mo5) and anti-uPAR or anti-CR4 fragments, although FITC and TRITC intensities were similar. RET emission channel intensity on cells labeled with CR4 and uPAR probes was greatly increased on polarized cells (~15-fold higher), thus indicating biological specificity of the heightened RET signal. However, the RET intensity (acceptor emission) oscillated rather than reaching a stable plateau, as we had expected based on CR3-uPAR interactions (Kindzelskii et al., 1996). Fig. 2 *A* shows a representative gallery of oscilloscope recordings of RET acceptor emission intensity versus time for migrating neutrophils. The oscillating RET level of cells undergoing spontaneous locomotion shows rhythmic coupling approximating a sine wave (Fig. 2 *A*, *trace a*) with a 22-s period, which is highly reproducible (Table 1). The amplitude of these oscillations was 40 $\pm$ 10% (n = 3) of the peak count rate minus the shuttered photomultiplier tube count rate, which constitutes a lower amplitude estimate. As a cell stops, the oscillations' amplitude and period rapidly fall to background levels (data not shown). These infrequent ob-

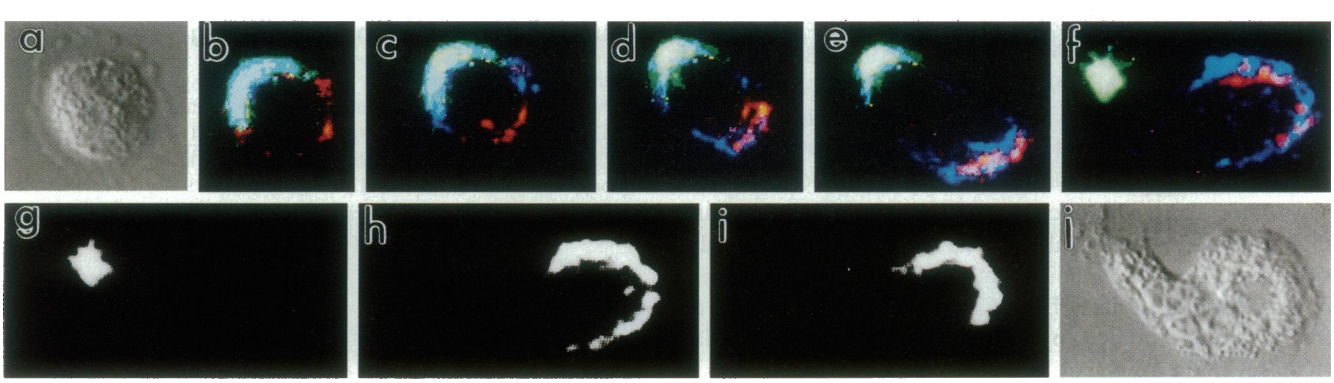

FIGURE 1 A representative series of micrographs showing the kinetic reorganization CR3, CR4, and uPAR on human neutrophils. DIC (*a, j*), pseudocolor fluorescence images of CR3 (*green*), uPAR (*blue*), and CR4 (*red*) (*b–f*), and one set of single color fluorescence images (*g–i*) are shown. The fluorescence of each label was acquired for 3.88 s with ~3 s to cycle between optical filters. Approximately 25 s were required to obtain each three-color panel. CR3 and uPAR co-cap on resting cells (*panel b*) (Zhou et al., 1993); controls for co-capping have been previously published (Zhou et al., 1993; Xue et al., 1994). However, as the cell polarizes, uPAR leaves the co-cap and traffics to the lamellipodium (*c–f*). CR4, which is initially randomly distributed, forms a cluster with uPAR at the lamellipodium (*e*). CR4 apparently arrives at the nascent lamellipodium before uPAR (*c, d*). To better illustrate the overlapping staining patterns of uPAR and CR4, *panels g–i* show the pseudocolor illustration of *panel f* separated into its three-color components. *Panels g–i* show CR3, uPAR, and CR4 staining patterns, respectively. These data suggest that CR3 and CR4 delineate the axis of polarity of neutrophils during spontaneous locomotion and that CR4 co-clusters with uPAR at the lamellipodium of migrating cells. (×1060)

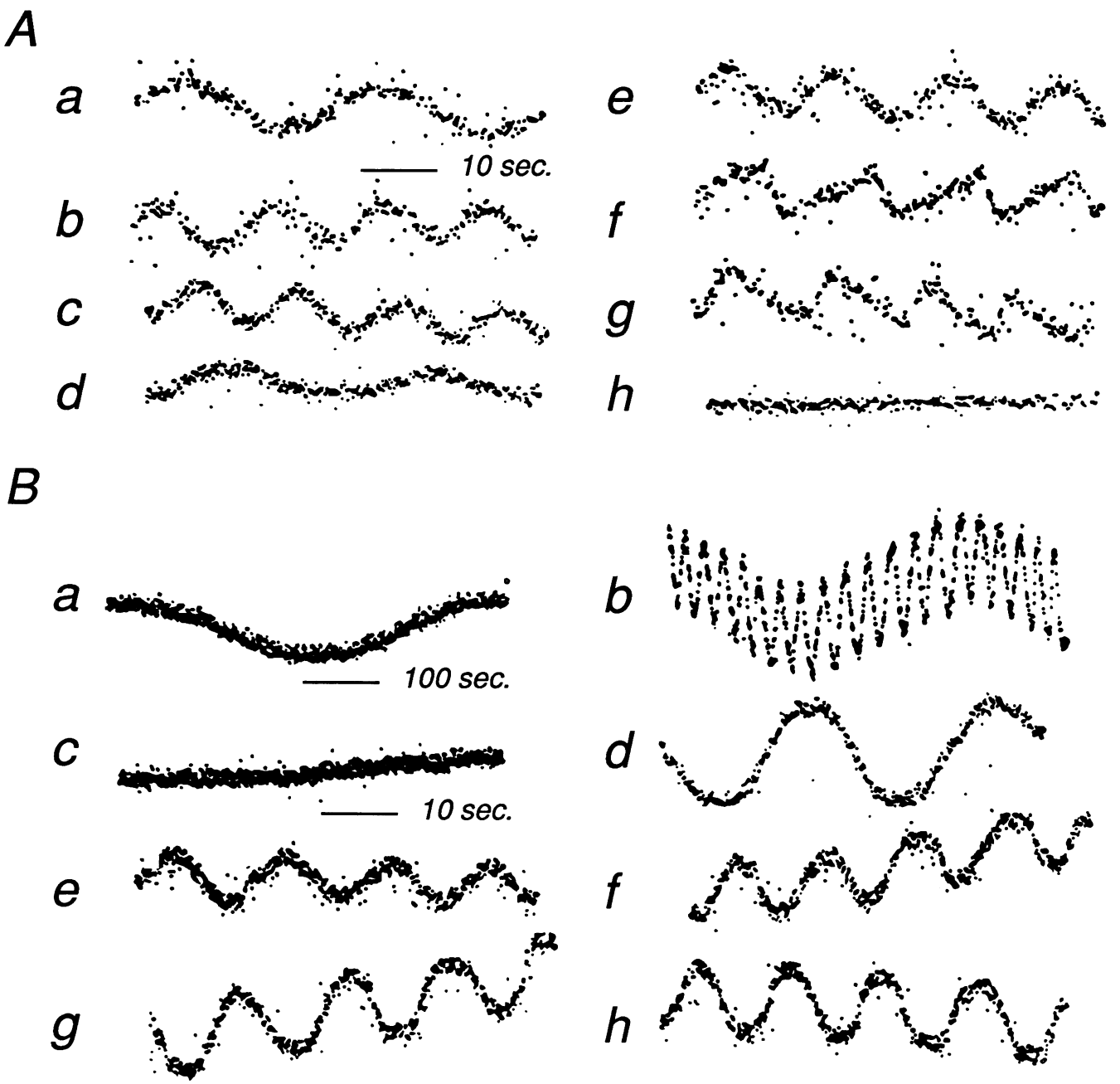

FIGURE 2 Quantitative time-dependent analyses of receptor and NAD(P)H oscillations in migrating neutrophils. Signal intensities (*ordinate*) are plotted versus time (*abscissa*). (*A*) Proximity oscillations of CR4 and uPAR were detected with RET by monitoring acceptor emission. Cells were labeled with anti-CR4 Fabs and anti-uPAR $F(ab')_2$s (or intact uPA with indistinguishable results). RET levels of polarized cells were then measured as a function of time. Polarized/migrating cells exhibit a sine wave of coupling (*a*). RET levels fall to background levels when locomotion stops. During migration on fibrinogen-coated surfaces a sine wave of increased frequency is observed (*b*). Spontaneous neutrophil migration on endothelial cell monolayers (Cell Systems, Inc., Kirkland, WA) led to a sine wave of increased frequency (*c*) compared to glass substrates (*a*). Exposure to indomethacin, however, reduced the amplitude and frequency of the coupling wave (*d*). When chemokinesis is stimulated with FMLP, a wave with an increased frequency is found (*e*). A flyback sawtooth waveform of increased frequency is found when cells are exposed to 0.05 μM staurosporine on glass surfaces (*f*). In contrast, 50 μM pervanadate leads to a reverse sawtooth wave of increased frequency (*g*). Cyclic RET intensities are not observed when irrelevant receptors are labeled (not shown) or when cells are fixed (*h*). In *panel A* each trace is 50 s in length (bar = 10 s). (*B*) Metabolic oscillations of peripheral blood neutrophils. Resting (*a*, *c*) and migrating (*b*, *d*) cells attached to glass coverslips are shown. *Traces a* and *b* are shown at a longer time-scale (bar = 100 s) than *c* and *d* (bar = 10 s). Resting cells show metabolic oscillations of roughly 3 min. In contrast, migrating cells demonstrate two component metabolic oscillations of ~3 min and a superimposed oscillation of 22 s. Cells were also observed while migrating on fibrinogen-coated surfaces (*e*) and on glass surfaces during exposure to the chemotactic factor FMLP (*g*) (*e–h*; bar = 10 s). Both treatments increased the oscillation frequency (Table 1). When neutrophils were exposed to the signal transduction inhibitors staurosporine (*f*) or pervanadate (*h*), as described above, increased oscillation frequencies were observed.

TABLE 1 Quantitative analyses of CR4-uPAR proximity oscillations and metabolic oscillations

Experimental Condition	Addition	Substrate	CR4-to-uPAR Proximity Oscillations		Metabolic Oscillations
			Period (s)	Waveform	Period (s)
Spontaneous Migration	None	Glass	22.8 ± 1.5*	sine	21.1 ± 5.6*
	None	Fibrinogen/glass	12.8 ± 2.6	sine	12.0 ± 3.9
	None	Endothelial cells	12.8 ± 2.2	sine	12.5 ± 2.5
Signal transduction reagents	Indomethacin	Endothelial cells	25.8 ± 4.2*#	sine	27.1 ± 2.1*
	FMLP 10^{-7} M	Glass	13.3 ± 2.8	sine	12.5 ± 3.7
	10^{-8} M	Glass	12.7 ± 1.9	sine	12.9 ± 2.1
	Staurosporine	Glass	11.5 ± 4.9	Flyback sawtooth	12.6 ± 1.7
	Pervanadate	Glass	11.3 ± 2.2	Reverse sawtooth	11.4 ± 2.2
Fixed	Paraformaldehyde	Glass	∞	None	∞

The number of separate days of experimental studies, n, was 7 to 21. A minimum of 50 cells were averaged for the mean ± standard deviations listed.
* $p < 0.001$ in comparison to FMLP (10^{-7} M)-activated cells.
# The amplitude of this signal was ~70% of the other items listed in this column.

servations in which locomotion ceases are consistent with ~100% of the RET signal oscillating relative to the nonmigrating RET channel intensity of the same cell. Thus, at least 40% of the RET signal intensity oscillates. Oscillations of FITC quenching with the same period were also observed (data not shown) by monitoring FITC emission in the presence of acceptor. The ratio of FITC emission intensity in the presence and absence of acceptor was ~0.6. These oscillations cannot be due to an instrumental artifact since they disappear when cells return to a spherical morphology. Furthermore, they cannot be explained by an inner filter effect since neutrophils are transparent in this spectral region, and such effects should be greater for spherical cells. In addition, photochemical decomposition cannot account for oscillatory behavior. Thus, a biological event tied to cell polarization must be responsible for sinusoidal interreceptor interactions. To exclude the possibility that these oscillations are due to cell shape changes, we monitored the FITC emission and the TRITC emission of cells during migration. Oscillations were not observed for these measures, thus indicating that the fluorescence was not being diminished in some way by, for example, multiple overlapping lamellipodia during cell shape changes, and that oscillations were specific for the RET channel. However, cell shape oscillations can be observed using spot illumination of the lamellipodium (unpublished observations), but not during whole cell illumination, as performed in the present study. Thus, we interpret the oscillatory signal as being due to differences in local changes in receptor proximity relationships.

To explore the role of signal transduction pathways in RET oscillations, we measured the RET channel output of CR4/uPAR-labeled neutrophils during spontaneous migration on various surfaces and in the presence of exogenous compounds. When neutrophils migrated on fibrinogen (a ligand of CR3 and CR4)-coated surfaces (Fig. 2 *A*, *trace b*) or endothelial cells (*trace c*), a higher frequency sine wave was found (Table 1 lists quantitative data). The RET emission amplitude and frequency associated with neutrophils attached to endothelial cell monolayers were reduced during exposure to 100 μM indomethacin, an antiinflammatory drug that blocks prostaglandin synthesis (Fig. 2 *A*, *trace d*). To determine if chemotactic factors, which affect signaling pathways and cell motility, influence these oscillations, we stimulated chemokinesis by adding 10^{-7} to 10^{-8} M N-formyl-met-leu-phe (FMLP) to labeled neutrophils in contact with glass surfaces. FMLP at both doses was found to increase CR4's oscillatory frequency (Fig. 2 *A*, *trace e*; Table 1). When FMLP was added to cells during RET measurements, an abrupt increase in frequency was observed (data not shown). Since integrins, including CR4 (Roubey et al., 1991; Chatila et al., 1989), are regulated by β chain phosphorylation and since this is known to affect β_2 integrin-cytoskeletal interactions (Peter and O'Toole, 1995), we pharmacologically altered phosphorylation reactions. To avoid complete inhibition of oscillatory interactions, we titered these reagents to deliver sub-optimal doses. When staurosporine, a kinase inhibitor, was added at 0.05 μM, we observed a flyback sawtooth waveform of increased frequency (Fig. 2 *A*, *trace f*). Conversely, 50 μM pervanadate (Bennett et al., 1993), a phosphatase inhibitor, led to a reverse sawtooth (negative-going ramp) of increased frequency (*trace g*). These waveform changes were observed for all migrating cells tested. We suggest that repetitive RET emission is a physical readout of cyclic phosphorylation/dephosphorylation events of the signaling apparatus, as previously conjectured by Hunter (1987).

To address the potential origins of signaling oscillations, we studied cellular metabolic oscillations by measuring NAD(P)H autofluorescence (Liang and Petty, 1992). NAD(P)H autofluorescence is a well-established tool to monitor the metabolism of cells in real time (Chance et al., 1973; Hess and Boiteux, 1971). The Embden-Meyerhof pathway of ATP generation is especially important in neutrophil metabolism [2% and 1.3% of consumed glucose is metabolized by the citric acid cycle and hexose monophosphate shunt pathways, respectively; Roos and Balm (1980)], which allows cells to function under low oxygen tension. Stationary neutrophils exhibited sinusoidal NAD(P)H oscillations with a ≈3-min period (Fig. 2 *B*, *trace a*). However, cells undergoing spontaneous polarization demonstrated a

sinusoidal 21-s oscillation superimposed on the 3-min oscillation (Fig. 2 *B*, *trace b* and *d*). The rapid oscillation period decreased to 13 s for cells undergoing chemokinesis (Fig. 2 *B*, *trace g*). The amplitude of these rapid metabolic oscillations is ~29 ± 4% (n = 3) of the peak count rate minus the shuttered photomultiplier tube count rate. Moreover, the periods of receptor proximity and metabolic oscillations were indistinguishable for neutrophils attached to fibrinogen and endothelial cells (Table 1). Parallel changes in proximity and metabolic oscillation periods were also found for cells treated with indomethacin, staurosporine, and pervanadate (Table 1). Since NAD(P)H waveforms were sinusoidal, RET waveform modulation is likely downstream from metabolic oscillations. Thus, the periods of NAD(P)H and interreceptor RET oscillations matched for cells under all conditions (Table 1; Fig. 2).

We next sought to ascertain the oscillations' relative phases. The phase relationship between RET intensity and NAD(P)H autofluorescence was determined by switching between these two optical set-ups during spontaneous migration of cells. These experiments showed that the trough in NAD(P)H autofluorescence corresponds to a peak in RET intensity (Fig. 3, *a* and *b*); thus, these two measures have the same frequency but are 180° out-of-phase. Thus, physical properties of surface receptors and biochemical reactions in metabolism express relative phases.

DISCUSSION

Our results suggest that oscillatory neutrophil functions are linked with metabolic clocks, and that these functions are temporally coordinated by metabolic phase relationships.

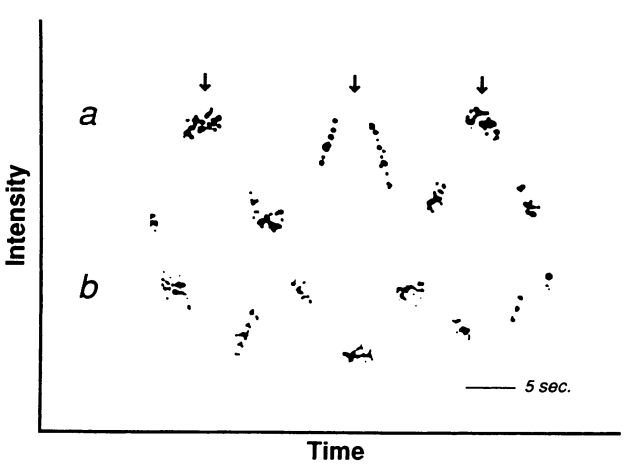

FIGURE 3 Phase relationships among interreceptor RET and metabolic oscillations. Signal intensities (*ordinate*) are plotted versus time (*abscissa*). Cells were analyzed for RET and NAD(P)H autofluorescence oscillations as described in the legend to Fig. 2. Intermittent recordings of NAD(P)H autofluorescence (*trace a*) and RET (*trace b*) were made by rapidly switching the optical filters and dichroic mirrors. By comparing these traces, the peaks of *trace a* (*arrows*) are seen to correspond to the troughs of *trace b*. Thus, interreceptor oscillations are out-of-phase with NAD(P)H oscillations.

Such phase information is necessarily lost when large numbers of cells are analyzed or when cells are disrupted for biochemical analysis. We show that many CR4 molecules are simultaneously engaging/disengaging (or undergoing conformational changes with) uPAR molecules. This "phase-locked" behavior can extend over long periods of time and coincides with cell motility. The association of membrane receptor proximity oscillations with the signal transduction apparatus is suggested by neutrophil interactions with fibrinogen, chemotactic factors, endothelial cells, and indomethacin. A linkage is also suggested by the ability of staurosporine and pervanadate to retard the risetime and falltime, respectively, of waveforms. A phase-locked signaling apparatus is a radical departure from current random diffusion-reaction models of signaling. We suggest that during neutrophil migration CR4 or associated molecules are simultaneously phosphorylated by the oscillatory signaling machinery; this phosphorylation wave leads to CR4-to-uPAR coupling (as suggested by staurosporine data). CR4-uPAR coupling focuses proteolysis next to the region where cell extension is to occur. Cell extension and actin assembly then proceed through the adherence/proteolysis site. Nonrandom cell motility (Hartman et al., 1994) may arise from phase-locked signaling; it is difficult to envision a mechanism for nonrandom motility based upon random enzyme-substrate reactions.

We hypothesize that ATP oscillations may entrain signaling oscillations. Previous workers have found that ATP oscillates 180° out-of-phase with NAD(P)H (Hess and Boiteux, 1971), although to our knowledge this phase relationship has not been demonstrated for human neutrophils. Our results show that CR4-uPAR proximity oscillates 180° out-of-phase with NAD(P)H. Thus, we tentatively infer that CR4-uPAR interreceptor interactions oscillate in-phase with cellular ATP levels. This is consistent with fact that the mean level of free cytosolic ATP ($\sim 10^{-5}$ M, assuming a cell radius of 5 μm) (Kay et al., 1980) is roughly similar to the equilibrium constant of ATP binding to protein kinase C ($\sim 10^{-6}$ M; Petty, 1993).

The oscillation periods reported above are similar or identical to previously reported oscillations in neutrophil shape change, actin assembly, and respiratory burst (Wymann et al., 1989a, b; Hartman et al., 1994; Omann et al., 1989, 1995; Ehrengruber et al., 1995). FMLP-induced frequency changes reported here parallel previous reports (Wymann et al., 1989a). Cytosolic calcium oscillations at this frequency within neutrophils have been observed (e.g., Kruskal and Maxfield, 1987; Marks and Maxfield, 1990). Furthermore, an 8- to 10-s period has been found for neutrophil signal processing in chemotaxis and galvanotaxis (Gerish and Keller, 1981; Franke and Gruler, 1990; Albrecht and Petty, unpublished). Thus, metabolic clocks may be broadly important in cell signaling and migration.

Although others have speculated regarding the presence of intracellular phosphorylation/dephosphorylation pathways (e.g., Hunter, 1987), these have not been observed in real time. One reason for this difficulty is that conventional

cell extraction/SDS-PAGE experiments have a resolution of ~1 min, which is much longer than the 10- to 20-s periods reported here. Another factor is the relative phases of the intracellular biochemical reactions. When the phases are aligned or locked, the oscillatory metabolic/signaling machinery is apparent. In the absence of phase-locking, such signals would simply be "averaged-out." Thus, the temporal coherence provided by physiologically induced phase-locking is a key in observing this aspect of the signaling apparatus. We suggest that these metabolic/signaling oscillations and their phase locking are important in cell functions including migration, adherence, and phagocytosis, but have gone unrecognized because of their phase properties. Prior biological examples of regulatory phosphorylation/dephosphorylation waves are the cyclic phosphorylation of lamins during the cell cycle and in the phosphorylation/dephosphorylation of light harvesting complex II of thylakoids, although their periods are much longer (e.g., Petty, 1993).

Previous workers have shown that FMLP-triggered neutrophil chemiluminescence oscillates with a period of ~10 s (Wymann et al., 1989a). We hypothesize that metabolic oscillations may contribute to oscillations in oxidant release. Since oxidant production is in-phase with NAD(P)H oscillations (they switch on and off together), NADPH oscillations may entrain the NADPH oxidase's production of oxidants during cell migration. The magnitude of NAD(P)H oscillations can be estimated as follows. First, the amplitude of NAD(P)H oscillations is estimated at ~30% of the NAD(P)H pool, although amplitudes of 40% of the NAD(P)H pool have also been measured (Kindzelskii and Petty, submitted). Second, the mean cytosolic concentration of NADPH is 270 μM (Patriarca et al., 1971). Third, NADPH autofluorescence is linear in this low concentration range (Liang and Petty, 1992). With these factors and a 10% correction for non-NAD(P)H-specific autofluorescence, the concentration oscillates between estimated values of ~209 to ~331 μM. The potential role of NAD(P)H oscillations in regulating the production of oxidants is also supported by the finding that indomethacin, an antiinflammatory drug known to reduce oxidant release from neutrophils (Smolen and Weissmann, 1980), decreased the frequency of NAD(P)H oscillations. This, of course, raises the intriguing possibility that metabolic waveform modulation mechanistically links indomethacin action with oxidant release. [A parallel argument can be made linking RET oscillations with the indomethacin-mediated reduction in cell locomotion (e.g., Spisani et al., 1979)]. The suggested role of NAD(P)H oscillations in NADPH oxidase activity is consistent with the observation that heightened NAD(P)H levels during metabolic resonance in electric fields is accompanied by increased levels of oxidant release (unpublished observations). One biological function of oxidants is to inactivate protease inhibitors (Weiss, 1989). The phase difference in oxidant production and receptor coupling may allow inactivation of protease inhibitors before focusing/activation of proteases, thus coordinating cell migration. Thus, the frequency, amplitude, and phase properties of NAD(P)H oscillations may be biologically relevant.

We suggest that cyclic signal transduction is coupled to metabolic events (outside-in signaling) and that metabolic events are tied to signal transduction (inside-out signaling). For example, metabolic/signaling oscillations may provide the molecular logic participating in the binary switch model of actin assembly (Stossel, 1993). Others have speculated regarding the presence of an "internal clock" in cell locomotion (e.g., Jager et al., 1988; Vicker, 1994); the oscillatory signaling/metabolic machinery may be this clock, thus providing cellular memory. Oscillatory ATP levels are a likely coupling and feedback mechanism. Oscillatory receptor/signaling/metabolic events vary in amplitude and period in different environments, suggesting sensory input. We speculate that cell motility decision-making may involve concepts analogous to electric circuit theory. For example, the system may act as a filter in discriminating among different stimuli. Oscillatory receptor and signal transduction capabilities may offer systems gain advantages and error reduction by periodic sensory input. Oscillatory energy/signaling input is consistent with the idea of "alternating current chemistry" (Lazar and Ross, 1990) in living cells to optimize thermodynamic efficiency and drive enzymatic reactions away from equilibrium (Richter and Ross, 1981; Lazar and Ross, 1990). In addition to the role of coherent protein phosphorylation and NAD(P)H oscillations in signaling during cell locomotion, coherent ADP production may also be important since this will affect metabolism, which in turn affects signaling. This loop may account for increased NAD(P)H amplitudes during metabolic resonance and exaggerated cell functions. Concerted phosphorylation/dephosphorylation events predict extremely low frequency AC electrical fields at lamellipodia, which have been reported for neutrophils undergoing shape changes (Jager et al., 1988). The potential role of such electric fields is unknown, but may be related to the reported effects of electromagnetic fields on inflammation (Zecca et al., 1985; Mizushima et al., 1975). In another related paper we demonstrate that correct phase application of extremely low frequency electric fields triggers metabolic resonance and exaggerated cell extension for locomotion. We have also identified a pyoderma gangrenosum patient who displays defective neutrophil locomotion in vivo and aberrant interreceptor interactions and metabolic oscillations in vitro (unpublished observations). Thus, several aspects of the model discussed above have proven successful. The role of metabolic oscillations in signaling and cell function may extend to other cell types. For example, oscillations at this same frequency have been observed in pancreatic β cells (e.g., Larsson et al., 1996; Smolen and Keizer, 1992). Recently, O'Rourke et al. (1994) have shown that metabolic oscillations are associated with ion fluxes and excitation contraction coupling in myocytes. To summarize the findings of the present study, complex cellular processes such as locomotion, which involves cell adherence, focused proteolysis, actin assembly, shape change, oxidant release, etc., may

be coordinated by the cadence of the signaling/metabolic apparatus.

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