Cytosolic Acidification as an Early Transductory Signal of Human Neutrophil Chemotaxis

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The inflammatory reaction of human neutrophils consists of two successive phases. In the first, designated chemotaxis, the cells home in on a foreign intruder. In the second, the cells attempt to eliminate the intruder by secreting lysosomal enzymes and superoxide anions. The initiation of chemotaxis involves prompt morphological changes that are manifested by a sharp biphasic drop in light scattering, accompanied by a transient cytosolic acidification. In a search for a causal relation between these two events, the neutrophil cytoplasm was abruptly acidified by the application of sodium propionate. This evoked a pulse of decreasing light-scattering, the time course and amplitude of which were practically identical to the rapid response induced by chemoattractants such as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Both fMLP- and sodium propionate–induced responses were unaffected by amiloride, but were inhibited with a similar dose-dependence by a series of proton uncouplers. The initial phase of the cytosolic acidification seems, therefore, to fulfill the criteria for a second messenger for the initiation of chemotaxis.

THE NEUTROPHIL INFLAMMATORY reaction consists of sequential series of cellular and biochemical activities (*1*). The encounter of neutrophils with a minute chemoattractant concentration results in a marked cytoskeletal rearrangement leading to whole-cell polarization (*2*) and initiates a vectorial migration up the chemoattractant gradient (chemotaxis). Upon reaching a certain higher chemoattractant concentration, the neutrophils shift the signal transduction toward cytotoxic activities (lysosomal enzyme secretion and superoxide-anion production) while arresting their motility (*3*) so as to maintain proximity to their targets. The coordination of these activities seems to require fine regulation of several signal-transduction pathways.

In a study of chemotactic stimulus-response coupling, we monitored the neutrophil light-scattering response (LSR) (*4*). We observed that the intensity of the perpendicularly scattered light undergoes a biphasic drop immediately after the application of the chemoattractant. The first phase, designated as the rapid LSR, peaks sharply at 10 ± 1 seconds (mean ± SD reported throughout) and decays symmetrically into the successive slow LSR (Fig. 1A). The latter exhibits a less synchronized pattern, peaks at 40 ± 10 seconds, and decays gradually over several minutes. The rapid and the slow LSR are stimulated by different doses of the chemoattractant that are one to two orders of magnitude apart (*4*), corresponding to the doses that induce optimal chemotactic and cytotoxic responses (*3*), respectively. This correlation is strengthened by the divergent susceptibility of these responses to a series of pharmacological agents, one of which is the family of short aliphatic alcohols. In accord with their membrane fluidization effect, these alcohols accelerate neutrophil chemotaxis but inhibit their cytotoxic capacity (*3*) concomitant with the abrogation of the slow LSR, which leaves a fully disclosed rapid LSR (*5*). The rapid LSR thus became a convenient measure for the neutrophil chemotactic responsiveness.

Because chemotaxis is associated with cell motility, and because the rapid LSR has already been correlated without spatial rearrangements of the microfilament system (*4, 6*), effectors of the state of the filamentous-actin superstructure are anticipated to play an important role in transducing the chemoattractant signal. Modulation of the cytosolic free calcium, though capable of stimulating neutrophil cytotoxic activities (*7*), is presumably inadequate as a chemotaxis signal transducer. This notion is based on our failure to alter the LSR induced by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) by eliminating the extracellular calcium with excess EDTA (up to 2 m*M*) or to induce such a response by the application of calcium together with its specific ionophores. These observations support a recent report that suggests that the rise of intracellular concentration of free calcium is neither necessary nor sufficient for stimulating an increase in neutrophil cytoskeletal-associated actin (*8*). Therefore, the transient cytosolic acidification, which has also been observed after chemoattractant stimulation (*9*), was considered. We chose to simulate the chemoattractant-induced acidification by using the sodium salt of the weak *n*-propionic acid (NaPr) (*10*). This approach proved to be advantageous owing to the simple nonspecific characteristics of this process, each phase of which could be fully monitored.

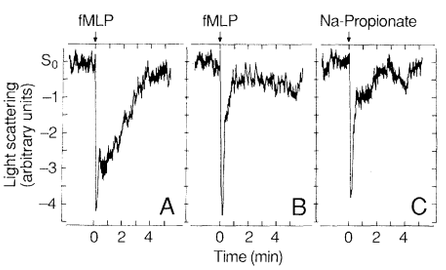

Fig. 1. Perpendicular LSR of neutrophil suspensions to either fMLP or NaPr. Suspensions of 4×10^6 cells in 0.4 ml of Hanks balanced salt solution supplemented with 10 m*M* Hepes (*p*H 7.2) were stabilized by regulating their speed of stirring at 37°C until they yielded steady red (670 ± 20 nm) light-scattering signals for at least 4 minutes. Perpendicular and incident light intensities were monitored by a modified aggregation meter (Sienco, Inc.), at a ±10° averaging because of the instrument's geometrical constraints. The medium of the experimental system depicted in (**B**) contained also 0.2% *n*-butyl alcohol. The neutrophil suspensions were stimulated at the indicated time points (arrows) by a rapid injection of 4 μl of either 1 μ*M* fMLP (**A** and **B**) or 3*M* NaPr (**C**).

The addition of NaPr to a standard neutrophil suspension (*4*) yielded a rapid and transient decrease in the system's perpendicular light-scattering, which peaked after 10 ± 1 seconds and decayed sharply to the prestimulation baseline level within 20 to 30 additional seconds (Fig. 1C). This light-scattering pattern appears to overlap with the rapid response induced by fMLP (Fig. 1A), which is better resolved in *n*-butyl alcohol–treated systems (*5*) (Fig. 1B). We found that 10 μ*M* of either cytochalasin B or cytochalasin D abolished this NaPr-induced LSR in accordance with the inhibitory effect of each on the fMLP-induced LSR (*4*). A statistically significant LSR could be observed with already 0.3 m*M* NaPr, as the transient decrease in light intensity exceeded twice the noise range of the light-scattering baseline. One half of the maximal response was obtained with approximately 1 m*M* NaPr, and the full effect was reached with >10 m*M* NaPr.

Reacting a neutrophil suspension with up to 30 m*M* NaPr yielded a linearly increasing abrupt cytosolic acidification of up to 0.45 ± 0.05 ($n = 3$) *p*H units; the *p*H was monitored by whole-cell averaged 2,7-biscarboxy-ethyl-5(6)-carboxy-fluorescein (BCECF) fluorescence (*11*) according to, and in agreement with, Grinstein and Furuya (*10*). In comparison, stimulation of the cell suspension by 10 n*M* fMLP, known to induce optimal chemotaxis in vitro (*3*) and the already maximal rapid LSR (*4*), yielded a cytosolic acidification of 0.14 ± 0.02 *p*H units by 40 ± 10 seconds ($n = 5$) after

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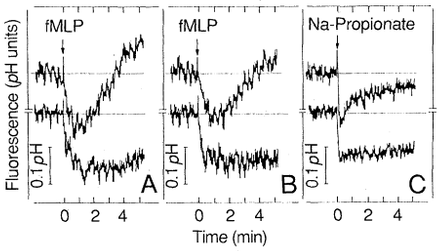

Fig. 2. The modulation of neutrophil cytosolic *p*H by either fMLP or NaPr. Standard suspensions of BCECF-loaded and thoroughly washed neutrophils at 10^7 cells per milliter were stimulated with either 10 n*M* fMLP (**A** and **B**) or 10 m*M* NaPr (**C**) at the indicated time points (arrows). The medium of the experimental system depicted in (B) contained also 0.2% *n*-butyl alcohol. The *p*H modulation in the presence of 0.25 m*M* amiloride is represented by the lower time-course in each panel. The fluorescence change with *p*H was calibrated by monitoring the effect of direct alkalinization in parallel systems of disrupted cells.

stimulation (*10*). This extent of acidification corresponds to the effect of approximately 10 m*M* NaPr (Fig. 2). Both fMLP- and NaPr-induced acidifications were followed by an amiloride (0.25 m*M*)-inhibitable alkalinization. However, while the alkalinization following the fMLP-induced acidification assumed a sigmoidal kinetic pattern, the alkalinization following the NaPr acidification was initiated instantaneously and could better be described by a hyperbolic function (*10*) (Fig. 2).

As expected, 30 m*M* NaPr alkalinized the extracellular medium by 0.040 ± 0.001 *p*H units. By adding sodium hydroxide to the neutrophil suspension to a final concentration of 0.4 m*M*, the extracellular medium was equally alkalinized while the intracellular *p*H remained unchanged for at least several minutes. However, this amount of sodium hydroxide induced no LSR and had no effect on the neutrophil responsiveness to fMLP, whatsoever. Neither could the increase in the extracellular sodium concentration, associated with the application of NaPr, account for the induction of the LSR, since substituting NaPr with an equivalent concentration of sodium chloride did not induce any response by itself nor did it interfere with the fMLP-induced one.

The addition of 30 m*M* NaPr also yielded an appreciable fluidization of the whole-cell averaged membrane lipid domains. This effect was monitored by the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (*12*) that decreased from a value of 0.218 ± 0.001 ($n = 3$), in untreated cells, to a value of 0.212 ± 0.002 ($n = 3$). Very similar membrane fluidization was observed when the cells were exposed to 0.2% *n*-butyl alcohol. However, this treatment did not yield any change in the light-scattering of otherwise unstimulated neutrophil suspensions.

In an attempt to correlate the acidification with the transduction of the fMLP signal toward the LSR, we subjected neutrophils to increasing doses of the protonophores carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) (Fig. 3), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), or 3,5-di(*t*-butyl)-4-hydroxylbenzylidene-malononitrile (SF-6847) (*13*). All of these agents were found to gradually inhibit the fMLP-induced rapid as well as slow LSR. The latter response was inhibited already by 7.5 μ*M* FCCP (a dose commonly used for dissipating proton gradients across biological membranes), whereas the rapid LSR remained unaffected. This rapid LSR was inhibited only by more than a tenfold higher FCCP dose. The NaPr-induced LSR could also be inhibited by FCCP, and revealed a dose-dependent susceptibility that was practically identical to that of the fMLP-induced rapid LSR (Fig. 3).

Recently, several reports have indicated that the amiloride-susceptible sustained cytosolic alkalinization (either fMLP-induced or directly imposed by varying the extra- or intracellular *p*H or sodium concentrations) has a regulatory capacity in neutrophil chemotaxis (*14*). However, the application of up to 1 m*M* amiloride, which inhibits approximately 90% of the fMLP-induced neutrophil chemotaxis (*14*), had no effect on the LSR induced by either fMLP or NaPr. Independently, it seems unlikely that the fMLP-induced alkalinization, which becomes apparent about 1 to 2 minutes after stimulation and overcomes the initial acidification only after several additional minutes (Fig. 2) (*10*), can account for the induction of the chemotaxis-related rapid LSR that decays to the prestimulatory baseline level within 30 to 40 seconds. Furthermore, the induction of the LSR seems independent of the mobilization or distribution of intracellular sodium. This has been concluded from the lack of any effect of as much as 20 μ*M* of the sodium-channel blocker tetrodotoxin, 100 μ*M* of the Na^+,K^+-ATPase inhibitor ouabain, or 10 μ*M* of the K^+ ionophore valinomycin, on the light-scattering of quiescent neutrophils or their response to either fMLP or NaPr. These data imply that the sodium proton antiport and cytosolic alkalinization may be involved only in transductory phases that succeed those that are necessary for the induction of the LSR.

The results clearly indicate that the NaPr-induced LSR was not mediated by medium alkalinization, membrane fluidization, elevation of cytosolic ionic strength, or activation of amiloride-sensitive cytosolic alkalinization. Thus, we conclude that the induction

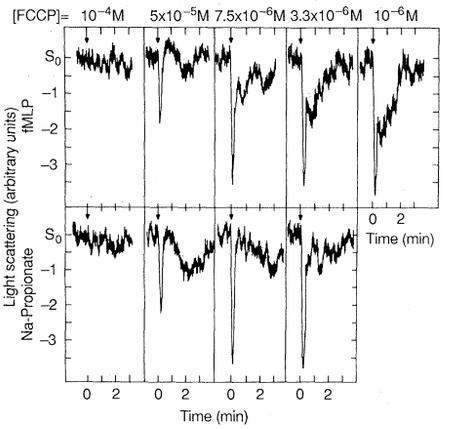

Fig. 3. The FCCP dose-dependent inhibition of the LSR induced by either fMLP (**upper row**) or NaPr (**lower row**). Stirred suspensions of neutrophils at 10^7 cells per milliliter were stabilized in the presence of 100, 50, 7.5, 3.3, and 1 μ*M* FCCP (left to right) for 15 minutes at 37°C. Once a 4-minute light-scattering baseline had been established, the neutrophil suspensions were stimulated by a rapid injection (arrows) of 4 μl of either 1 μ*M* fMLP (upper row) or 3*M* NaPr (lower row).

of the response is associated with the cytosolic acidification; however, it could not be attributed merely to the amplitude of the *p*H decrease. In the first place, fMLP and NaPr, both of which induce instantaneous LSR, have different acidification kinetics. That is, only NaPr (definitely not fMLP) induced maximal acidification within the time domain of the induction of LSR (Fig. 2) (*10*). In the second place, cytosolic acidification that is capable of inducing LSR may need to be properly compartmentalized. This is implied by the phorbol-12-myristate-13-acetate (PMA)–induced acidification (*11*) that is not associated with the induction of either chemotaxis or LSR (*4*) or with the abolishment of the fMLP-induced LSR. Therefore, we suggest that the transduction of a chemoattractant signal is correlated solely with the initial phase of the acidification it evokes.

As indicated by the fluorescence anisotropy data, the propionate anion presumably partitions into the lipid core of the neutrophil's plasma membrane in the form of the uncharged propionic acid. Later, the propionic acid could proceed into the cytoplasm according to its chemical potential gradient. The relevant acidification is, therefore, assumed to occur at the interface of the plasma membrane with the cytoplasm, where the acid can be readily deprotonated. A certain concentration of propionic acid would always be retained in the plasma membrane, thus fluidizing its lipid core by a mechanism similar to that observed with aliphatic alcohols. This notion is supported by the inhibition of the fMLP-induced slow LSR, but

not the rapid one, observed in neutrophils treated with 30 m*M* NaPr. We assume that membrane fluidization by aliphatic alcohols and NaPr is associated with an impairment of some necessary component of the slow LSR, thus leaving the system with the capability of executing only the chemotaxis-related rapid LSR (*4*, *5*). Since membrane fluidization is an integral and inevitable aspect of the NaPr incorporation into the neutrophils, the NaPr-induced LSR should operationally be referred to as the combined effect of fMLP and an aliphatic alcohol (Fig. 1, B and C).

Thus we have shown that the LSR, which correlates with neutrophil chemotaxis, can be specifically induced and suppressed by defined experimental manipulations of the neutrophil cytosolic proton concentration. The comparison of the LSR time course with the kinetics of the concurrently induced cytosolic acidification implies that only the initial decrease in *p*H is necessary for the intracellular signal. Consequently, we suggest that the perception of a chemoattractant by its specific receptors is translated into an abrupt accumulation of protons at the interface of the plasma membrane and cytoplasm, which can then trigger the chemotactic signal-transduction cascade.

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Chaotic Bursts in Nonlinear Dynamical Systems

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Several elementary nonlinear dynamical systems in the complex plane may provide models for abrupt transitions to chaotic dynamics. In particular, the complex trigonometric and exponential functions explode into chaos as a parameter is varied. Numerical evidence is presented that supports the contention that these explosions occur whenever an elementary bifurcation occurs. This numerical evidence, in the form of computer graphics, is an example of the increasing importance of experimentation in mathematics research.

RESEARCH OF MATHEMATICIANS, physicists, and others over the past 20 years has made it clear that many systems of physical, biological, or chemical interest exhibit highly unstable or chaotic behavior. How does a relatively tame or stable system make the transition to complete irregularity or instability? Several different scenarios for the transition to chaos have been put forth. There is the older Landau–Lifshitz approach of successive superposition of frequencies (*1*) and the relatively new approach of Feigenbaum (*2*) via successive period-doublings. Both of these transitions have been shown to be mathematically feasible and have been observed in physical systems (*3*). But both of these transitions are gradual transitions; the systems involved become increasingly irregular in well-defined stages, which eventually accumulate and terminate in complete chaos. These scenarios are therefore good models for systems such as fluid flows, which gradually make the transition from steady state to turbulence, and ecological systems, wherein the populations change slowly over time until a chaotic regime is reached. But they do not serve well as models for systems that become chaotic rapidly. A number of systems in nature exhibit this type of burst or explosion into chaos. For example, combustion often involves rapid transitions between stable and chaotic states. The phenomenon in meteorology called microbursts also exhibits rapid changes of state.

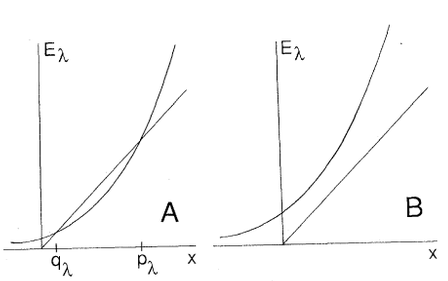

Fig. 1. Graphs of E_λ for real $z = x$. Straight line shows $E_\lambda = x$. (**A**) $\lambda < 1/e$. (**B**) $\lambda > 1/e$.

There are a number of mathematical techniques for studying such abrupt changes in physical systems. For example, the theory of shock waves in partial differential equations is well developed and can be used to construct mathematical models that accurately describe a rapid change of state. Also, catastrophe theory developed by Zeeman (*4*) and others has been applied to a number of systems that undergo such rapid changes. Both of these approaches, however, usually deal with an apparently discontinuous jump in the system between one stable equilibrium and another. These transitions, albeit abrupt, do not in general occur between stable and completely chaotic states.

My goal in this report is to suggest some simple mathematical models that do exhibit this type of transition. These models have the advantage of being simple—they are all iterated mappings of the plane—and effectively computable—they involve only complex sines, cosines, or exponentials. In each case, it can be proved rigorously that the systems undergo a burst into complete irregularity as a parameter is varied. Admittedly, these dynamical systems are approximate models of real physical systems, but it is my feeling that the bursts illustrated with these simple models hold the key to understanding similar phenomena in more complicated settings. These sudden chaotic bursts have

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