Dynamic Chemical Instabilities in Living Cells May Provide a Novel Route in Drug Development

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Chemical Biology and Biophysical Dynamics

One of the chief concerns in Chemical Biology is the identification of new pharmaceuticals. Unfortunately, the advent of high-throughput screening assays, robotics, extensive compound libraries, etc. have not yielded a dramatic improvement in the rate of drug discovery. This approach is essentially a scaled-up version of traditional drug development methods that often rely upon the equilibrium binding properties of organic molecules to isolated biopolymers (i.e., receptors and enzymes). There are three important reductionist shortcomings with this approach: 1) living cells are *never* at equilibrium,[1] 2) cell processes are *dynamic*, often irreversible,[2,3] and 3) isolated proteins are not cells and therefore do not exhibit chemical feedback circuitry. Consequently, paradoxes may arise wherein a compound that binds more tightly to a target is less effective in influencing biological pathways. The constant flux of matter and energy, for example in the form of glucose, necessarily drives cells away from equilibrium.[1–3] Thus, in some ways cells resemble continuously fed chemical reactors. As a consequence of being constrained far from equilibrium, intracellular chemistry may lose stability and spontaneously form dynamic time-dependent chemical waves and oscillations, just as the Belousov–Zhabotinsky reaction does when fed reactants.[4] These spatial patterns and temporal rhythms, which are examples of nonequilibrium self-organization in living cells, can only be maintained by the dissipation of energy and are therefore often referred to as dissipative structures.[3] However, an individual receptor or enzyme does not oscillate. Chemical oscillations require networks of enzymes and receptors with feedback loops; in other words, we need to understand how all of the parts of a cell work together. Therefore, the properties of isolated components can never model the dynamic chemical processes that underlie many dynamic cell functions. This may explain, in part, why the extensive database generated by the Human Genome Project, coupled with modern screening approaches, has not yielded the anticipated cornucopia of new drugs. Obviously, revisions to the existing drug-discovery paradigm are needed. Inclusion of dynamic physicochemical processes within cells and their attendant emergent behaviors are a new area of research in cellular biocomplexity/systems biology, which will likely impact the development of new pharmaceuticals.

I propose that dynamic chemical instabilities are one means by which proteins cooperate to yield biological signals and functions. In other words, dynamic emergent chemical structures link discreet proteins to biological outcomes. For example, proteins constitute a conduit for signal transduction, but are not signals: a drug blocking a specific protein might inhibit signaling or might simply shunt the chemical disturbance through a different protein with no effect on signaling. I further propose that some pharmaceuticals may be understood and developed based upon their ability to perturb dissipative cellular chemical patterns. In this Minireview, I will focus on recent research, largely from my laboratory, concerning biological subsystems of inflammatory cells and tumor cells and the ability of this approach to yield new strategies in patient care.

Emergent Properties of Complex Cellular Subsystems

Humans possess roughly 25 000 genes. Many of these are proteins that are directly or indirectly linked to metabolism. Understanding such a complex and interactive system is presently impossible. However, it is not necessary to know how the entire system operates. It is possible to evaluate discreet subsystems within individual cells or groups of cells. Cells that function semi-autonomously in vivo, such as leukocytes and tumor cells, are particularly good candidates for dynamic evaluation of their chemical behavior in vitro. As an example of a cellular subsystem, the glycolytic apparatus is made up of 10 enzymes characterized by multiple feedback loops. This cellular subsystem has been shown to display temporal oscillations and traveling metabolic waves.[5,6] One could study, for example, how drug-mediated chemical perturbations influence the behavior of a relatively self-contained subsystem such as glycolysis.

As mentioned above, dissipative structures can take several forms, such as temporal chemical oscillations and traveling chemical waves. Oscillations are well described in many chemical and biochemical settings.[4,5] Two of the most thoroughly studied chemical oscillators in living cells are metabolism and calcium signaling.[5] Both of these subsystems are comprised of

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a relatively manageable number of genes contributing to crucial widespread clinical problems such as inflammation and metastasis. Figure 1 shows examples of these oscillators, which may be observed for extended periods of time (1–60 min) in

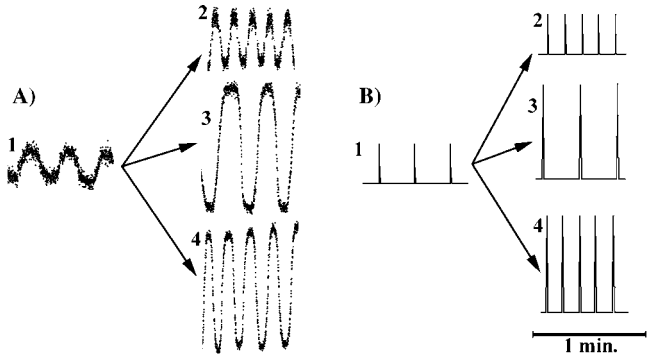

Figure 1. *Oscillations in human neutrophils. Panel A: Metabolic oscillations of neutrophils. Trace 1 shows untreated adherent neutrophils. Traces 2–4 show neutrophils treated with FMLP, interferon-γ, and FMLP plus interferon-γ, respectively. This shows that the frequency (trace 2) and amplitude (trace 3) can be independently controlled or, depending upon the prevailing conditions, simultaneously modulated. Panel B: Calcium oscillations of neutrophils. Trace 1 shows untreated adherent neutrophils. Traces 2–4 show neutrophils treated with FMLP, interferon-γ, and FMLP plus interferon-γ, respectively. The frequency and amplitude responses of calcium parallel those found for NAD(P)H oscillations. For clarity, only brief oscillatory periods are shown; while, adherent to substrates, these oscillations may be observed for extended periods of time.*

the presence of exogenous glucose at concentrations found in blood (~1 mM). Panel A shows the sinusoidal NAD(P)H oscillations of adherent neutrophils. NAD(P)H autofluorescence has been widely used as an indicator of metabolic activity.[5] In neutrophils, these oscillators are sensitive to activators and inhibitors of metabolism and have been confirmed by using another indicator of metabolism, the autofluorescence of flavoproteins.[7–12] Several factors influence neutrophil priming and activation by affecting metabolic oscillations. The increase in frequency illustrated in Figure 1A, trace 2, has been linked to activation of the hexose monophosphate shunt and its associated respiratory burst. This change is stimulated by a variety of biological factors including the chemotactic factor f-Met-Leu-Phe (FMLP), lipopolysaccharide (LPS), IL-8, TNF-α, immune complexes, etc.[7–12] On the other hand, this change is blocked by reagents, such as 6-aminonicotinamide, that inhibit early steps of the hexose monophosphate shunt.[12] Moreover, several other factors, such as interferon-γ, IL-10, phorbol myristate acetate, and phase-matched electric field application, cause the formation of high-amplitude metabolic oscillations (Figure 1A, trace 3). One attribute shared by these latter stimuli is their ability to act upon calcium signaling pathways. Recent computational and experimental findings show that the mobilization of intracellular myeloperoxidase, through fusion with NADPH oxidase-containing structures or release to the plasma membrane where some NADPH oxidase molecules reside, significantly increases the amplitude of NAD(P)H oscillations.[12] In addition, metabolic oscillations exhibiting both high frequency and high amplitude can be observed by combining factors that independently affect these parameters (Figure 1A, trace 4). Thus, biological factors influence the coherent behavior of the metabolic apparatus.

In addition to metabolic oscillations, a variety of other oscillations have been noted for human neutrophils.[9] For example, repetitive calcium spikes are known to occur during cell adherence, locomotion, and phagocytosis. Figure 1B illustrates these calcium spikes. Importantly, the changes in frequency and amplitude noted above for metabolic oscillations are also observed for calcium signals.

Just as spatial waves can form in chemical media far from equilibrium,[4,5] spatial waves can also form in living cells. The best-studied cellular waves are the calcium waves in oocytes, which have been described in some detail.[13] These experiments were successful because oocytes are very large in size. It is impossible to observe these waves in leukocytes and tumor cells by using traditional imaging methods because the waves traverse an entire cell in a time period that is much shorter than the shutter speed of the camera. We have developed a high-speed microscope system than allows detection of chemical waves within cells such as leukocytes. We employ an efficient microscope set-up and a single photon-sensitive detector with a high-speed gating apparatus and computer interface to study chemical waves in cells.[6] Combining the Einstein equation for diffusional displacement and the Rayleigh equation for microscopic resolution suggests that shutter speeds ~20 to 200 μs, depending upon the diffusion coefficient, optics, etc., are needed to resolve intracellular waves.

Figure 2 illustrates two of the chemical waves formed in human neutrophils, which were obtained by using high speed microscopy. In Figure 2A, NAD(P)H autofluorescence was imaged. NAD(P)H waves were found to propagate from the rear (or uropod) toward the leading edge (or lamellipodium) of the cell.[6,14,15] The NAD(P)H wave is in the shape of a longitudinal wave (or a circular wave with a large radius of curvature). If a compound sensitive to superoxide anions is placed in the extracellular environment, a plume of superoxide release can be seen just as the wave of NAD(P)H reaches the lamellipodium (Figure 2A, arrows).[15] When stimulated by a factor that promotes formation of high-frequency NAD(P)H oscillations, the NAD(P)H wave is found to split, thereby forming two traveling waves.[14,15] If a chemotactic factor is applied to cells from a certain direction, the waves reorient in the direction of the chemotactic factor prior to the directional reorientation of the cell. In contrast, target patterns in metabolism are found during the adherence of spherical cells.[16] Thus, the dynamic, collective properties of cell metabolism are closely linked to cell behavior.

Intracellular calcium waves have also been studied by using high-speed microscopy.[17–21] As calcium signals are observed as brief periodic spikes within cells (Figure 1B), cells were imaged at high speed during the spike to dissect the underlying spatiotemporal organization of the signal. Figure 2B shows a representative experiment of an indo-1 labeled neutrophil. This series of 19 frames, acquired during a single calcium spike, shows the movement of a calcium signal within a cell; each frame was exposed for 150 ns with a 15 ms delay between

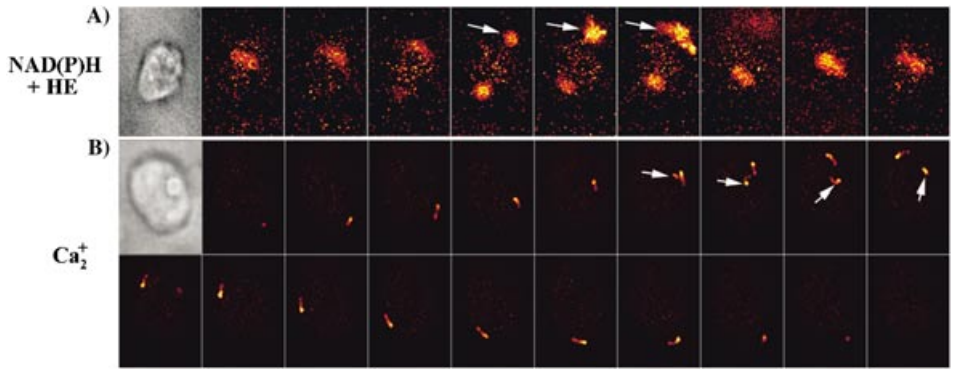

Figure 2. *High-speed microscopy of neutrophil signals. Panel A: Longitudinal NAD(P)H waves are observed in morphologically polarized human neutrophils. When these waves reach the cell's lamellipodium, hydroethidine in the external medium is oxidized to ethidium bromide, which diffuses away from the cell (arrows). The images shown were acquired over 250 ns with a 25 ms interval between each frame. Panel B: Calcium waves inside neutrophil after phagocytosis of a sheep red blood cell. A calcium wave travels around the periphery of the cell. When it reaches an area near the phagosome, the wave splits in two (arrows), with one wave continuing around the cell perimeter while the second propagates about the phagosome. The phagosome-associated wave plays a crucial role in the fusion of phagosomes and lysosomes. Images were collected with an exposure time of 150 ns. The micrographs shown are separated by 15 ms. Magnifications: panel A, ×860; panel B, ×940.*

each frame. In this example, the signal begins at the lamellipodium, which is rich in certain calcium channels,[19] then travels about the cell's periphery. In addition, the calcium signal splits into another wave that encircles the phagosome; this is crucial in the fusion of intracellular lysosomes with phagosomes.[18]

Physiological Relevance of Dissipative Structures

Biomechanisms

Although biochemical oscillators were recognized many years ago (for a review see ref. [5]), their physiological roles were not appreciated until recently. As NADPH is a substrate for the NADPH oxidase, I have proposed that NAD(P)H oscillations are linked to the oscillatory activity of the NADPH oxidase.[7–12] This proposed association is supported by the fact that these oscillations are in phase with one another. Furthermore, the frequency and amplitude of NAD(P)H oscillations vary in parallel with the production rate of superoxide and other reactive oxygen metabolites (ROM) by neutrophils. Factors that perturb metabolism, such as 6-aminonicotinamide and certain drugs, also perturb oxidant production.[11] As the NO synthase requires NADPH and NO production varies in time, the production of several oxidants appears to be linked to the same pathway. Hence, one function of NAD(P)H oscillations appears to be regulation of the extent and timing of oxidant production by leukocytes. Oxidant production, in turn, plays an important role in host resistance to infectious agents and in signaling.

In addition to the temporal relationship between NAD(P)H oscillations and superoxide production, spatial relationships also exist. As Figure 2 A illustrates, superoxide is periodically released from the lamellipodium of polarized neutrophils. Periodic plumes of superoxide diffuse away from the lamellipodium after longitudinal NAD(P)H waves arrive at this site. Thus, the formation of longitudinal NAD(P)H waves promotes the release of oxidative molecules in the direction of a chemotactic gradient or target.

Our work has clearly shown that calcium does not uniformly rise and fall within leukocytes and tumor cells,[17–21] but rather calcium signals are anisotropic waves with specific ignition sites, directions and velocities. One might postulate that asymmetries in signaling are related to asymmetric cell behaviors, such as migration in a specific direction. During cell migration, neutrophils become morphologically polarized and exhibit one calcium wave originating at the leading edge and propagating about its perimeter.[17] Using a micropipet to deliver FMLP to adherent neutrophils, we found that FMLP triggers the formation of a second calcium wave that is initiated at its binding site. Within a short period of time intracellular structures reorganize and the site of FMLP binding becomes the new leading edge of the cell. In this way calcium waves participate in cellular orientation.

Another important function of calcium signals in neutrophils is promoting the fusion of phagosomes and lysosomes. High-speed imaging experiments have shown that calcium signals form at the site of target binding to leukocytes. After phagocytosis, calcium signals travel from the plasma membrane to the phagosome; this suggests that they may play an important role in phagolysosome fusion. Signal routing from the plasma membrane to phagosome following antibody-dependent phagocytosis of sheep erythrocytes is illustrated in Figure 2 B.[17] Similar results were obtained by using transfectants expressing the antibody receptor FcγRIIA.[18] Furthermore, phagolysosome formation was observed in these transfectants. However, site-directed mutagenesis studies showed that a sequence of amino acids (LTL) within the cytoplasmic tail of FcγRIIA was critically important in routing the calcium wave to the phagosome and phagolysosome formation.[17,22] Recent research suggests that FcγRIIA's LTL sequence may interact with components of the endoplasmic reticulum to direct the signal from the cell surface to intracellular membranes (see below). Thus, we have been able to combine previously developed technologies in molecular biology with our new dynamic methods of investigation to reveal new levels of spatiotemporal organization within cells and their molecular signaling motifs.

Clinical Mechanisms

Although dissipative structures explain biological events at a more fundamental physiochemical level, their greatest contribution may be their ability to explain long-standing puzzles in

clinical medicine. For example, we all know that fevers are associated with illness. Chemical studies have provided the structures of fever-inducing bacterial components, such as LPS, as well as the structures of molecules such as cytokines that promote changes in body temperature. Yet, the physiological role of the thermal component of fever, in distinction to the changes in cytokine production, etc., was unknown. Thermal changes do not reduce bacterial growth at fever-associated temperatures, but rather alter host defense.[23] As mentioned above, we have shown that the frequency and amplitude of NAD(P)H oscillations are closely tied to the production of ROMs, which are synthesized by the NADPH oxidase of leukocytes. The frequency of human leukocyte NAD(P)H oscillations was found to increase with temperature, with an especially high rate above 37 °C.[24] In parallel, the rate of ROM production also rapidly increased above 37 °C. As ROMs promote the destruction of bacteria, we propose that the function of the thermal component of fever is to increase the rate of ROM production by leukocytes. This, in turn, is driven by temperature-dependent changes in emergent metabolic properties of leukocytes.

Another long-standing, but poorly understood, clinical observation is the reduction of inflammatory autoimmune disease during pregnancy. About 70% of women with multiple sclerosis or arthritis go into remission when they become pregnant, but relapse after delivery.[25] When peripheral blood leukocytes from pregnant women were evaluated, their metabolic oscillations and ability to produce ROMs in response to conventional agonists, such as LPS and FMLP, were altered. Specifically, inflammatory agonists were unable to stimulate formation of the higher frequency (10 s period) metabolic oscillations in adherent human neutrophils.[26] This metabolic change paralleled an inability to fully activate the hexose monophosphate shunt and respiratory burst. A simple spatial model of enzyme distribution may explain these fundamental changes in cell metabolism (Figure 3). We have shown that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the first two enzymes of the hexose monophosphate shunt, are transported to the centrosome (or cell center) of neutrophils from pregnant women, but are found at the periphery of cells from nonpregnant individuals.[26,27] As hexokinase is found at the periphery of activated leukocytes, the translocation of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to the centrosome allows glucose-6-phosphate, the product of hexokinase, to be metabolized through phosphoglucose isomerase and phosphofructokinase, not the hexose monophosphate shunt, thereby reducing NADPH availability and oxidant production (Figure 3). These findings may explain why pregnant women are more susceptible to certain infectious diseases and often experience diminished symptoms of autoimmune disease.

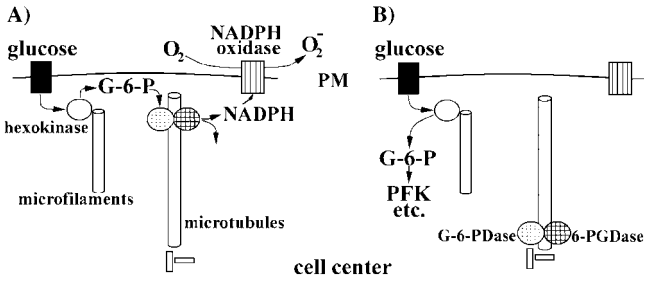

Figure 3. *Spatial model of neutrophil regulation during pregnancy. Panel A: In cells from nonpregnant individuals, both hexokinase and the hexose monophosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are found at the periphery of the cell. This spatial arrangement makes glucose-6-phosphate easily available to the hexose monophosphate shunt, thereby providing more NADPH for the NADPH oxidase, which, in turn, leads to superoxide production. Panel B: However, in the case of pregnant women, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase traffic to the centrosome; this allows glucose-6-phosphate to be metabolized by other enzymes at the cell periphery.*

Pharmacology

Not surprisingly, conventional pharmacological studies have yielded compounds that act on dissipative structures. For example, the anti-inflammatory steroid dexamethasone inhibits the acquisition of the 10 s period oscillations,[11] which have been associated with the hexose monophosphate shunt. Similarly, indomethacin affects the intracellular translocation of hexokinase and cell metabolism.[28] Additionally, several drugs have been found to affect calcium signaling in tumor cells. For example, carboxyamidotriazole (CAI) affects the dynamic events of calcium signaling.[20] Although these drugs have proven to be useful, it is possible that a greater understanding of their underlying dynamic biomechanisms will lead to further clinical improvements.

Potential Therapeutic Strategies Aimed at Emergent Chemical Properties

Several lines of evidence suggest that the biophysical dynamics of intracellular chemical processes are important, yet unattended, variables in drug development. These include, for example, the ability of dissipative structures to explain, at a more fundamental level, biological mechanisms including cell direction finding and clinical mechanisms such as neutrophil regulation in pregnancy. If chemical dissipative structures are central in cell functions, it should be possible to design new drugs or develop combinations of existing drugs to perturb these dissipative structures, thereby improving patient care. This possibility is strengthened by the fact that several useful drugs affect dissipative structures. Table 1 lists several approaches under study in this laboratory that utilize this strategy.

The analysis of metabolic oscillations has suggested several potential therapeutic approaches and identified biomechanisms that might lead to new drugs and clinical protocols. Computational studies of cell metabolism, NADPH oxidase activity, and the peroxidase cycle suggested that melatonin increases the amplitude of metabolic oscillations and oxidant production.[11] These findings were confirmed experimentally and account for the ability of melatonin to prime leukocytes. This suggests that melatonin, or similar compounds, might be useful in augmenting a depressed inflammatory response. Im-

Table 1. Potential contributions of biophysical dynamics in medicine.

Dynamic Measure	Approach	In vitro activity	Potential application
Calcium wave	LTL peptide	phagolysosome inhibition	keritits
Calcium wave	multiple drug interactions	inhibits tumor cell motility/invasion	cancer chemotherapy
Calcium wave	multiple drug interactions interactions	inhibits neutrophil function	arthritis, uveitis, multiple sclerosis, etc.
Metabolism	pulsed magnetic field	inhibits neutrophil function	arthritis
Metabolism	leukocyte changes in pregnancy	depresses neutrophil function	arthritis, uveitis, multiple sclerosis, etc.
Metabolism	melatonin	pro-inflammatory	immunocompromised patients, neonates

munocompromised individuals, such as neonates, might benefit from such an approach.

Dynamic studies have found that the brief application of weak electric fields at the trough of NAD(P)H oscillations increased their amplitude (frequency- and phase-matched application), whereas frequency-matched but out-of-phase application (i.e., peak) abolished the oscillations.[29–32] Extensive studies indicate that ion channel clustering on morphologically polarized neutrophils (ref. [18] and unpublished) accounted for weak-field detection according to the model of Galvanovskis and Sandblom.[33] These effects can be duplicated by the application of brief weak magnetic fields, which induce local electric fields.[34] When weak electric or magnetic field pulses are applied out-of-phase with respect to the intracellular chemical oscillators, metabolic oscillations and ROM production are dramatically reduced. This raises the possibility that the local application of external weak magnetic fields (which penetrate tissues) at the same frequency, but periodically advancing or retarding the relative phase of field application, might help in the control of chronic inflammatory diseases, such as arthritis, by reducing the total number of oscillatory inflammatory cells in the tissue.

Another ongoing area of research at the basic/translational interface in my laboratory concerns the regulation of the hexose monophosphate shunt during pregnancy. As mentioned above, we are actively studying the retrograde trafficking of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase within leukocytes of pregnant women.[26,27] Dynamic metabolic assays allow rapid evaluation of cells from pregnant women. Thus, in addition to identifying the underlying mechanism of leukocyte regulation in pregnancy, which could not be detected by using conventional screening assays, our biophysical studies might lead to the identification of pregnancy-associated factors controlling inflammation. Drugs aimed at regulating metabolic dynamics may be applicable in the treatment of many acute and chronic inflammatory diseases.

Our combined high-speed microscopy and site-directed mutagenesis studies have shown that the Fc receptor's LTL sequence plays a crucial role in directing calcium signals to phagosomes. We have recently prepared a "Trojan peptide" that delivers an LTL sequence to the cell cytoplasm. Trojan-LTL, but not Trojan-AAA, peptides decorate the neutrophil's endoplasmic reticulum and block calcium-signal routing from the plasma membrane to phagosome (Clark, Kindzelskii, and Petty, unpublished). This raises the interesting possibility that Trojan-LTL peptides might be useful in controlling topical inflammatory responses, such as keritis, in which inflammatory cells damage the eye's cornea.

Migrating cells exhibit intracellular calcium waves. As cell migration is required for tumor cell invasiveness and metastasis, it might be possible to diminish the aggressive phenotype of certain tumors by perturbing calcium waves. Calcium-active drugs have been used in chemotherapy. For example, CAI, which blocks non-voltage-gated cation channels and certain calcium signals, is presently in phase II clinical trials.[35] Recently, we have found that CAI affects the propagation of the calcium waves of tumor cells. As this calcium signal is a cooperative property of many proteins, we hypothesized that other calcium channels might participate in forming this signal. We found that T-type calcium channels participate in calcium-wave propagation.[20] Furthermore, the combination of CAI and mibefradil, which blocks T-type channels, inhibited tumor-cell motility and invasiveness more than either drug alone.[20] Therefore, it may be possible to improve clinical care by using multiple drugs that influence different proteins participating in the formation of the same dissipative structure.

Conclusion

Cells are more than the sum of their parts. Cellular components exhibit numerous collective properties that give rise to behaviors such as cell motility and nonrandom ROM release. Such spatiotemporal properties are explained by the coherence and synchrony of underlying biochemical processes, which may be manifested as chemical oscillations and waves. These dissipative structures emerge in living cells, as well as purely chemical systems, that are far from equilibrium and characterized by nonlinear kinetics and feedback loops. As described above, the importance of chemical waves and oscillations in living cells is just beginning to be appreciated; relevance to certain heritable leukocyte diseases and toxicology has also been suggested.[36–38] Although I have simplified the problem by focusing on semiautonomous cells such as leukocytes and tumor cells, these same principles might apply equally well to tissues. The power of dynamic biophysical approaches to explain complex biological phenomena is hard to miss. High-speed microscopy is capable of dissecting signals and signal transduction in cells at an unprecedented level; indeed, chemical wave propagation within cells is a novel level of physicochemical self-organization in living cells. The coherence of chemical reactions, the direction of cell orientation, the routing of cell signals, as well as organelle–organelle and

cell–cell communication are better understood with this approach. On the basis of high-speed experiments,[6,12,15–21] I postulate that chemical waves travel well-defined pathways within cells at specific times to mediate information transduction, processing, and distribution—much like a computer chip. Furthermore, the mechanisms underlying long-standing clinical observations involving fever and disease susceptibilities and remissions during pregnancy can be understood within the framework of this paradigm. The dynamic evaluation of systems behavior is a next logical step in chemical biology, which at its roots is simply the inclusion of well-established principles of physical chemistry in drug development. Our work does not anticipate an end to reductionism, but rather heralds its betrothal to biocomplexity/systems biology.

As biophysical dynamics is just entering the arena of drug development, one has a fairly free hand to speculate about the future. Two possibilities seem particularly noteworthy. As illustrated by our recent paper,[20] one approach to improving patient care is to use dynamic assays to search for combinations of existing drugs that will improve overall clinical performance. The second and more ambitious possibility is to combine dynamic measures with high-volume screening. The dynamic evaluation of cellular subsystems as a screening approach may identify novel lead compounds that act at unexpected sites to regulate dissipative structures and cell behavior. The concepts described in this paper may provide useful alternatives and promote an integrative agenda in drug development.

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