Resetting Wave Forms in *Dictyostelium* Territories

Kyoung J. Lee,[1] Raymond E. Goldstein,[2] and Edward C. Cox[3]

[1]*National Creative Research Initiative Center for Neurodynamics and Department of Physics, Korea University, Seoul 136-701 Korea*

[2]*Department of Physics and Program in Applied Mathematics, University of Arizona, Tucson, Arizona 85721*

[3]*Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544*

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The mechanism by which spiral wave patterns appear in populations of *Dictyostelium* was probed experimentally by external chemical perturbation. Spiral waves, which often arise from the breakup of circular waves driven by pacemakers, typically entrain those pacemakers. We studied these processes by resetting the waves with a spatially uniform pulse of extrinsic cyclic AMP. A pattern of spirals reappeared if resetting was early in the signaling stage, but only targets emerged following late resetting, in a manner analogous to cardiac defibrillation. This supports recent hypotheses that wave pattern selection naturally occurs by slow temporal variation of the excitability of the cells.

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Starving populations of the amoebae *Dictyostelium discoideum* form a classical excitable medium able to support propagating chemical waves of various symmetries [1–3]. Through the coupled dynamics of $3'5'$-cyclic adenosine monophosphate (cAMP) production and release, membrane receptor desensitization, and cAMP degradation by the enzyme phosphodiesterase, individual cells may be spontaneously oscillatory, thereby acting as pacemakers for concentric circular waves, or simply excitable, relaying those waves as expanding circles or rotating spirals. A central issue in the development of large-scale coherent wave patterns in spatially extended populations is that of selection between targets and spirals [4–11].

In a system with uniform excitability, spiral waves cannot form from small-amplitude fluctuations. Instead, they typically appear via a roll-up phenomenon at the free ends of wave segments [12,13]. Their appearance in the signaling stage of *Dictyostelium* development thus requires mechanisms for the creation of wave segments. Earlier work demonstrated that cell population density plays a crucial role in determining which of the two patterns dominates in the late signaling stage, with low population density favoring circular waves, and high density favoring spirals [6]. Moreover, the spirals that eventually dominate emerge from broken wave segments created early during signaling, and ultimately entrain the pacemakers responsible for circular waves.

The question we address here is how spiral wave forms evolve in *Dictyostelium* populations. In purely chemical systems, such as the Belouzov-Zhabotinski reaction, oscillators form around catalytic centers, usually specks of dirt or dust or silver electrodes [14,15]. Spirals then arise either directly or indirectly from colliding wave fronts. In biological systems, however, there is growing evidence that spiral wave formation is under direct genetic control [10], or actively suppressed, so that spirals do not normally form [16–18]. Thus, in living systems wave form choice is probably not determined by external noise. An emerging hypothesis in *Dictyostelium* is that signaling is characterized by a gradual change in the underlying kinetics of the cells, which transforms them from excitable to oscillatory and back over time [4,5,8,9]. The initiation of circular waves in the early signaling stage then arises from the fact that individual members of the population are not in complete synchrony following starvation, and, consequently, some cells make the excitable-oscillatory transition before others. Aspects of spiral-wave dynamics in the later stages, including circular symmetry breaking, have been suggested to arise from the slow evolution of excitability [19]. It follows that the appearance of the spiral wave state is a consequence of the particular path in parameter space through which the cells evolve after starvation.

The experimental results reported here bear directly on the question of how spirals are initiated and evolve, and supply new evidence for the evolution of excitability with time. We probed spiral nucleation and pacemaker entrainment by resetting cAMP waves with a spatially uniform mist of cAMP in a manner analogous to cardiac defibrillation. This treatment briefly abolished signaling. The signaling system quickly recovered from this treatment, and fully developed spirals reappeared if resetting was early in the signaling stage. Only targets formed, however, when cells recovered from late resetting.

Dictyostelium discoideum AX2 cells were grown, harvested, and imaged essentially as described [6]. Briefly, cells in a phosphate buffer were allowed to settle at $2 \times 10^6/\mathrm{cm}^2$ on a 2% agar surface. Dark field images were gathered at 0.5 min intervals over an 8 h period. Successive images were enhanced by frame subtraction. Wave propagation was interrupted by spraying 20 μL of cAMP as a fine mist onto the surface of the agar. A 20 μL mist of water was used as a control. At high cell densities and without external perturbation, the development of the spiral wave state shown in Fig. 1 involves first the appearance of targets and broken wave segments in the early

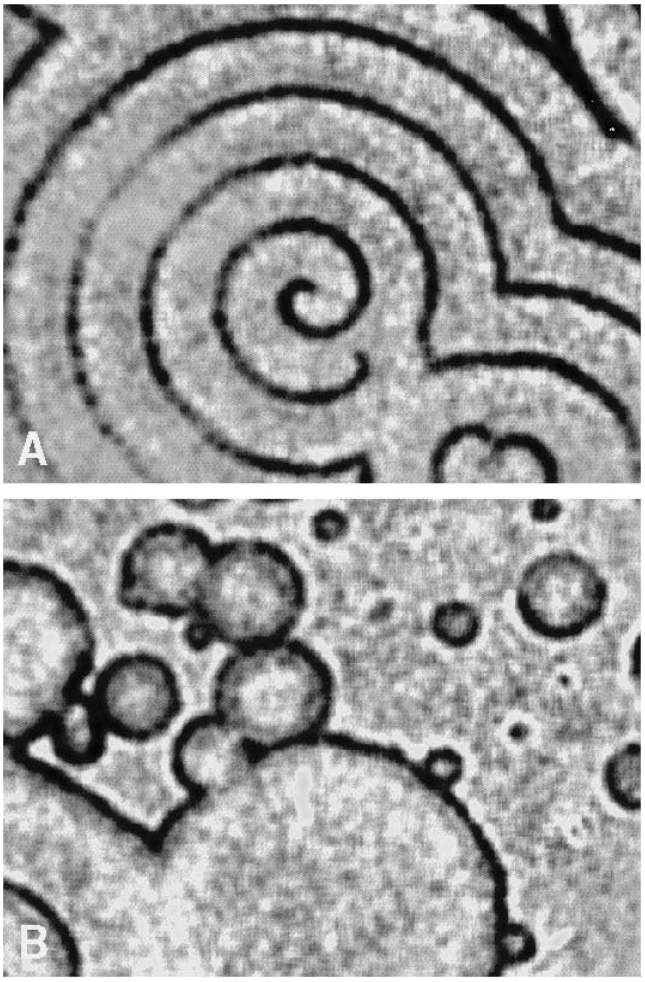

FIG. 1. Two different signaling patterns in an identical monolayer of *Dictyostelium discoideum*: (A) The spiral state selected naturally by the population; (B) pattern consisting only of targets following a homogeneous perturbation with cAMP. The fields are 1.6 cm wide. Increasing gray-scale values correspond to increasing concentrations of cAMP.

signaling state, and then the disappearance of the few pacemaker cells as they are entrained by spirals, a process that arises from the faster propagation of spirals relative to targets (see, for example, [20]).

In the presence of spiral waves, the suppressed pacemakers never reappear to generate circular waves. We hypothesized that, if it were possible to extinguish spirals, then any remaining pacemakers could signal autonomously (this would be analogous to cardiac defibrillation, in which an external voltage pulse allows autonomous pacemakers to regain control of wave symmetry [18]). We found that the application of a fine mist of cAMP accomplished the desired resetting with minimal disturbance to the cells. A 20 μL of $10^{-5}M$ cAMP solution was used for each perturbation. The particular concentrations and volumes of cAMP solution were chosen to provide minimum disruption of the cell monolayer. Signaling ceased for approximately two wave periods after the perturbation, and then began anew. During this quiescent period, cells presumably produced phosphodiesterase that degraded the extra cAMP delivered by the perturbation, and cAMP also diffused away, allowing the cells to recover from high cAMP levels.

A sequence of wave patterns before and after such a perturbation is summarized in Fig. 2, in which Figs. 2A, 2B, and 2C show the natural evolution from the initial nonsignaling state to spiral domination. Figure 2D, taken 4 min after the perturbation, illustrates the homogeneity and effectiveness of the perturbation in arresting wave propagation. Within 20 min of the cAMP mist, pacemakers reappeared and initiated circular waves (Fig. 2E). The most unexpected observation following the perturbation is shown in Fig. 2F, for the newly formed circular waves persisted without forming wave segments or spirals. Note that, while the outgoing circular waves collided and merged, no broken ends appeared. There was thus no indication of spiral formation until the next developmental stage of chemotactic cell aggregation, when well-developed aggregation streams emerged (results not shown). It is important to note that targets never persist naturally in populations at the high cell density used here [6].

In a control experiment, we applied a mist of an equal volume of water to a spiral wave state and found only a temporary broadening of propagating waves, but no change

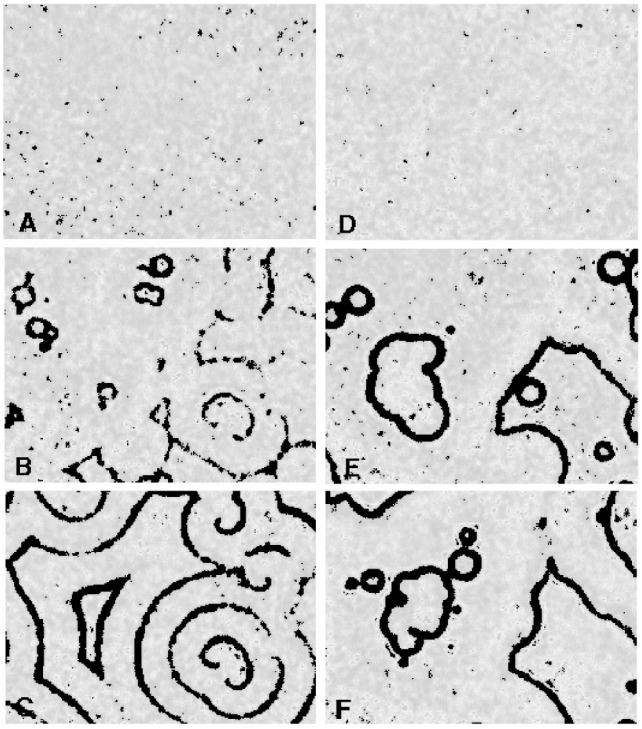

FIG. 2. A sequence of images showing signaling activity before and after cAMP perturbation: (A) The homogeneous state 281 min after nutrient deprivation; (B) pacemaker (target) and spiral waves coexist at 352 min; (C) fully developed spiral state at 408 min; (D) homogeneous state 4 min after a cAMP perturbation at 413 min; (E) pacemaker waves reestablished 16 min and (F) 46 min later.

in the morphology of the pattern. Thus, we deduce that cAMP, rather than the physical act of spraying, is responsible for the resetting phenomenon.

We determined the locations of all pacemakers occurring during the sequence shown in Fig. 2, and found a strong correlation between their locations before and after cAMP perturbation. This strongly suggests that the pacemakers that disappeared before the perturbation were overcome by spirals, but not permanently altered. This extends the analogy between defibrillation of cAMP waves and defibrillation of heart arrhythmias. In both cases, shutting down spiral wave forms can restore oscillators. One noticeable difference between the pacemakers that appeared before perturbation and those seen later is that the former oscillated at a higher frequency. This is perhaps due to a gradual shift in the pacemaker values of aging cells.

In order to quantify the strength of the cAMP signals, we show in Fig. 3 a time series analysis of the average optical intensity within a 10×10 pixel box located at the center of the frames shown in Fig. 2. The time series extends from the early to the late signaling stage, just before extensive cell movement begins. As Fig. 3A and the images in Fig. 2 show, the amplitude of the propagating waves gradually increases in time while the noise level does not change significantly. This gradual increase in signal amplitude appears to be independent of spatial location within the population: When we acquired independent time series at ten equally spaced locations over the whole sample frame, we found the same trend. The signal-to-noise ratio (SNR) as a function of elapsed time after starvation is shown in Fig. 3B. It increases monotonically with time. Furthermore, we find that only for a signaling stage with a SNR value beyond a critical value (shown hatched in Fig. 3B) does the perturbation result in a circular wave state at late times.

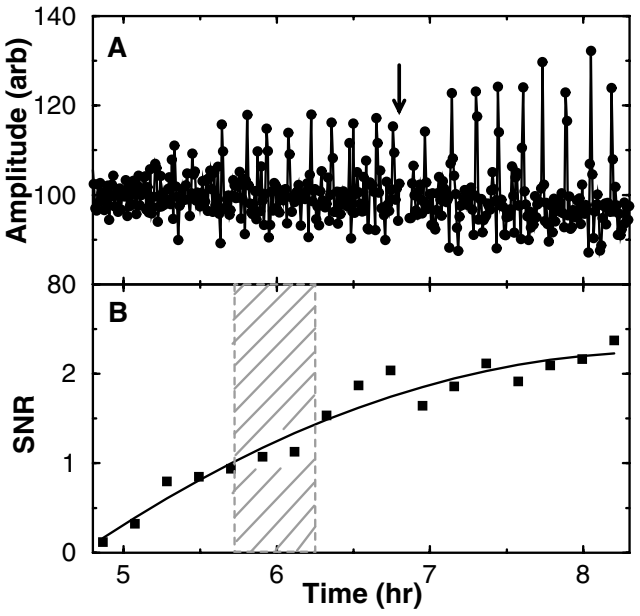

FIG. 3. Signal amplitude and signal-to-noise ratio as a function of time elapsed since nutrients were removed from the cells. (A) The signal amplitude gathered by signal averaging as a function of time in a 10×10 pixel box in the center of the image from Fig. 2. The arrow in (A) indicates the time of perturbation, i.e., 4 min before the image in Fig. 2D was taken. (B) The signal to noise ratio obtained from ten equally spaced locations over the entire 1.6×2.4 cm^2 image frame. The width of the noise amplitude was computed by taking the half width of the Gaussian distribution of the signal amplitude. The hatched area indicates the approximate time below which resetting with cAMP leads to spiral domination and above which resetting produces the target state.

In summary, our chief finding is that, when spiral waves are extinguished with a mist of cAMP, only circular waves reemerge as the cells recover. This result depends on starvation time. Prior to 6 h, spirals and circles reemerge. After this time only circular waves form.

In seeking an explanation for these results we can distinguish between two general ideas, those that invoke local and those that invoke global changes in the excitability of the system. An example of the former is the Belouzov-Zhabotinski reaction, where local inhomogeneities act catalytically to break circular wave fronts, and the broken ends roll up to form double-ended spirals (reviewed in [1]). Local symmetry breaking events also initiate spiral Ca^{++} waves when Xenopus oocytes are injected with inositol 1,4,5-triphosphate [16,21], and reentrant spiral waves that arise during heart arrhythmias are also thought to be triggered locally [22]. A local model has also been used to explain the circle to spiral transition in *Dictyostelium* [9]. Here, external levels of cAMP are regulated by secretion, degradation by a phosphodiesterase, and inhibition of the phosphodiesterase by a secreted protein inhibitor. In this model, local random pulses of the inhibitor are postulated to cause premature wave initiation by increasing the local cAMP concentration, and premature firing behind a propagating circular wave causes wave breakup and spiral initiation. This model is supported by genetic studies in which the inhibitor was deleted. In these strains spiral waves cannot form [10].

The results presented here are in broad agreement with the expectations of a purely local model under control of the phosphodiesterase inhibitor, because the inhibitor is made and secreted early during starvation, just as the cells become excitable. A few hours later, however, synthesis stops, and secreted inhibitor decays by diffusion in the supporting medium and by binding irreversibly to the phosphodiesterase [23]. Thus, one interpretation of our results is that, when cells recover from a cAMP mist prior to 6 h, the inhibitor can fire pulses locally, restoring spiral and circular waves; whereas after this period, only circular waves can arise because the inhibitor is no longer available.

It has also been shown that global changes in excitability may explain the circle to spiral transition [5,7,8,19]. It has been recognized for some time that excitability evolves in starving populations of *Dictyostelium,* and that cells

progress through a series of stages, from weakly to fully excitable, accompanied by an increase in wave frequency [2]. Moreover, cAMP is both the signaling molecule and an inducer of developmental genes, one consequence being that the number of cAMP receptors on the cell surface continuously changes. This progression along the "developmental path" [24] can work on a global scale to break the symmetry of circular waves, either by competition with a postulated third wave [19], tuning of the various forcing parameters as gene regulation changes with time [7,8], or by the appropriate transitions in space and time between coexisting excitable states [25]. The results presented here do not allow a clear choice between local and global models, because, although mutant strains of the phosphodiesterase inhibitor form spirals rarely, it is possible that in wild-type strains the inhibitor is secreted from all cells, thereby altering global excitability, and, consequently, the initiation of spirals by a nonlocal mechanism.

In previous experiments, we showed that spirals entrain oscillators [6]. Here we show for the first time that oscillators appear to be a persistent feature of *Dictyostelium* signaling, since they reappear soon after all wave activity has been extinguished by a mist of cAMP. These results also suggest quite strongly that, whatever the mechanism underlying the generation of spiral waves, it is unlikely to be physical or cellular spatial inhomogeneities in the surrounding surface or cell population, since these either do not change during the course of these experiments or increase with time as cells begin to sort and move. This is expected on theoretical grounds [11], and distinguishes this system again from the Belouzov-Zhabotinski reaction.

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