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Three-dimensional scroll waves organize *Dictyostelium* slugs

(morphogenesis/oscillations/chemotaxis/digital image processing)

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ABSTRACT **To test the hypothesis that periodic signals and chemotaxis direct later morphogenesis in *Dictyostelium discoideum*, we investigated cell behavior and cell movement in slugs. Trails of neutral red-stained prestalk and anterior-like cells were recorded by high-resolution digital image processing. Neutral red-stained anterior-like cells in the prespore zone of slugs move straight forward in the direction of slug migration and, furthermore, show coherent periodic cell movement. In contrast, cells in the prestalk zone move along completely different trajectories. Prestalk cells move perpendicular to the direction of slug migration; that is, they rotate around the tip. The cell movement data show that the chemotactic signal in the slug propagates as a three-dimensional scroll wave in the prestalk zone and as a planar wave in the prespore zone. The different behavior of prestalk and prespore cells is most likely caused by a difference in the oscillatory properties of the two cell types. We provide evidence that the slug stage of *Dictyostelium* behaves like an excitable system and that a (twisted) scroll wave organizes slug formation and migration.**

The cellular slime mold *Dictyostelium discoideum* is well suited to the study of cellular communication and the role of biological oscillations in spatiotemporal pattern formation. During the life cycle, solitary amoebae collect to form a multicellular organism. This aggregation process is well characterized and occurs by chemotaxis to periodic cAMP signals initiated by the aggregation center (1). The cAMP pulses, generated and secreted by cells in the aggregation center, are detected and amplified by the monolayer of surrounding cells. This leads to the outward propagation of cAMP waves. The signal propagates as expanding concentric rings or, more often, as spirals (2). These waves direct the cells toward the aggregation center via chemotaxis toward increasing cAMP concentrations. Signal propagation can be detected by direct autoradiographic determination of the spatial variation in cAMP concentration (3) or as an optical density wave caused by periodic cell shape changes associated with chemotactic cell movement (4, 5). After 20–30 waves, cells collect in bifurcating aggregation streams, in which optical density waves are no longer visible. Therefore little is known about signal propagation during multicellular development.

Up to 10^5 cells collect into a compact aggregate (mound), where they begin to differentiate into three major cell types, prestalk, prespore, and anterior-like cells. The cells differentiate in random positions and then undergo chemotactic cell sorting (6, 7). The prestalk cells collect on top of the mound and form a distinct morphological structure (the tip). As soon as a tip is formed, the mound elongates and forms the slug, which then falls over and starts to migrate. The foremost 20% of the slug including the tip consists of prestalk cells, while the remainder is formed by prespore cells. Anterior-like cells are found scattered throughout the prespore zone. The tip guides slug movement and further morphogenesis (8).

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To test the hypothesis that periodic signals and chemotaxis direct slug formation and migration, we investigated cell behavior and cell movement in slugs. Previously, we investigated the long-term behavior of fluorescently labeled single cells in the prespore zone of slugs. Those experiments showed that cells go through periodic velocity and shape changes, which indicated that the cells move in a chemotactic fashion (9). Since only a few cells could be observed at the same time, it was not possible to deduce the spatial pattern of signal propagation unambiguously. We now can determine this pattern, since we have developed methods to track the movement of many prestalk and anterior-like cells in a slug simultaneously.

MATERIALS AND METHODS

Labeling of the Cells. All experiments were performed with axenic AX2 cells grown according to standard culture conditions (10). Axenically grown cells were washed twice in KK2 (20 mM potassium phosphate buffer, pH 6.8) and resuspended in KK2 at 10^7 cells per ml. The washed cells were stained with the vital dye neutral red by incubation for 5 min in KK2 containing 0.06% neutral red. The cells were washed once in KK2 and pelleted by centrifugation, and drops (10^8 cells per ml) were deposited on 1% water agar plates and incubated in the dark for 24–48 hr at 18°C.

Video Microscopy. To track neutral red-stained cells, Petri dishes with slugs migrating on agar were placed on a Zeiss IM 35 inverted microscope equipped with a 40× Neofluar oil objective (n.a., 1.25). Most experiments were performed with slugs submerged in mineral oil (Wacker Chemie AR 20 and 200, 1:20) with a high oxygen solubility. This treatment reduced refraction, and contrast of staining was enhanced by a blue-green filter and diffusion screen in the light path. To the left and right of several migrating slugs were placed two small strips of ACLAR film type g3 c (Allied Chemical, Morristown, NJ) (0.1 mm thick, oxygen-permeable). The resulting cavity was filled with mineral oil and covered with ACLA film. The Petri dish was continuously flushed with oxygen. Under these conditions, slugs migrated with the same speed as on an agar/air surface (data not shown) for more than 24 hr and culmination was inhibited. Without oxygen supply the tip of a slug migrating in mineral oil splits into several tips each of which forms a tiny slug after 15–30 min. We made use of this observation to produce very small slugs. By controlling the onset of the oxygen supply, we obtained slugs that were 1/10th to 1/50th of the normal slug size and that showed no further tip splitting and migrated normally after oxygen was provided. Tiny slugs facilitated cell tracking in the tip. Slugs were filmed with a Hamamatsu C-2400 silicon intensified target (SIT) camera adjusted to maximal sensitivity to reduce illumination intensity.

Digital Image Processing. Digital image processing was performed with a Tandon AT compatible computer equipped with an Imaging Technology FG 100 board (resolution, 512 × 512 pixels). Noise was reduced by averaging 10 digitized video images, and the resulting image was recorded on a computer-controlled Sony EVT-801CE time-lapse video re-

corder. The time-lapse video films gave a good qualitative impression of cell movement. Cell tracking of neutral red-stained cells was performed interactively by identifying the same cells in consecutive still images provided by the video recorder. The video images were digitized and vacuoles of the cells were marked with a screen cursor, using special application programs written by us. This was repeated for 10–50 images and the corresponding x,y coordinates were saved on a hard disc.

RESULTS AND DISCUSSION

Analysis of Global Cell Movement. We analyzed cell movement in time-lapse video films of slugs stained with neutral red. Neutral red stains lysosomal vesicles in prestalk cells

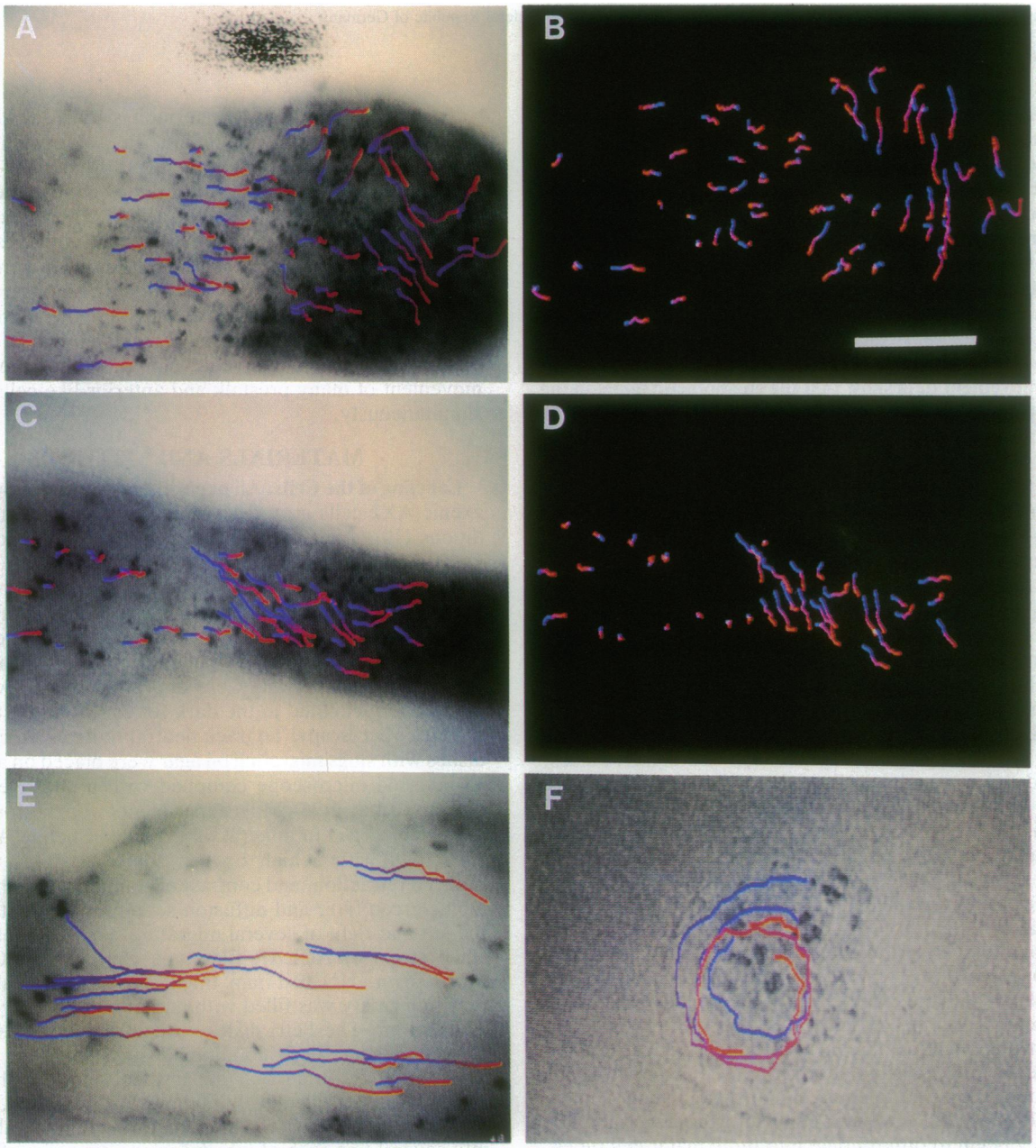

FIG. 1. Tracks of cell movement of neutral red-stained cells in slugs. Direction of cell movement is color-coded: the start of the track is blue and the end of the track is red. Direction of slug movement in *A–E* is from left to right. (*A* and *C*) Photographs showing the front 40% of two different slugs and the tracks of neutral red-stained prestalk and anterior-like cells. The prestalk zone appears dark gray. Anterior-like cells are dispersed in the prespore zone, which appears light gray. Cell tracks represent 50 sec of cell movement with respect to the substratum; measurements were made every 5 sec. Cells in the prespore zone move in the direction of slug movement, and cells in the prestalk zone move at ≈45° angles to the direction of slug movement. In all slugs filmed (>60 in 20 different experiments) we observed cells rotating in the tip. The apparent opposite directions of cell motion in the prestalk zone are caused by the fact that the tip extends away from the substratum and that a plane of focus parallel to the substratum will cut the prestalk zone in a tilted fashion. This then shows cells moving in the upper and lower part of the prestalk zone. (*B* and *D*) Same tracks as in *A* and *C*, but corrected for slug speed. The velocity of slug movement was determined as the displacement of the tip between the beginning and the end of the measurement. From this value the displacement in pixels per 5 sec was calculated and subtracted from the x,y coordinates of each time point. After correction for slug movement, it becomes clear that cells in the prespore zone move at approximately slug speed. Cells in the prestalk zone move perpendicular to the direction of slug movement. (*E*) Rear part of the prespore zone of the slug shown in *A* and *C*. All cells move straight forward in the direction of slug migration. Cell movement was followed for 3 min. (*F*) Cells rotating around a central core in an isolated prestalk piece. Cell movement was followed for 10 min. (Bar = 50 μm.)

and a subset of cells referred to as anterior-like cells in the prespore zone of a slug (6). Fig. 1 *A* and *C* shows cell movement in two typical slugs. Anterior-like cells in the prespore zones of both slugs moved straight forward in the direction of slug movement. Fig. 1*E* shows the rear part of the slug shown in Fig. 1*A*. Cell tracks clearly indicate the forward movement of the anterior-like cells. Cells of the prestalk zone show a completely different movement pattern: they always move at an angle to the anterior–posterior axis of the slug. When cell tracks are corrected for slug speed (Fig. 1 *B* and *D*), tracks of anterior-like cells are reduced to spots, which indicates that these cells move more or less at the same speed as the slug. In contrast, prestalk cells move perpendicular to the direction of slug movement—they rotate in the tip. If prestalk cells are isolated from the tip of a slug and placed on agar they start to rotate almost immediately. Fig. 1*F* shows rotating prestalk cells in an isolated tip piece.

Rotational cell movement in the tip is directly seen in time-lapse video films, especially during long-term observation of fluorescently labeled cells. The periodic appearance and disappearance of single labeled cells can be observed. However, to deduce the exact mode of signal propagation it is necessary to track many neighboring cells simultaneously. As a consequence cells can be tracked only in the short period during which they all stay in the focus plane.

The observed rotational cell movement requires external signals or forces. The signal might be a gradient of a substance that modulates cell movement, thereby causing a convective flow as a temperature gradient can induce circular convection in a fluid. A model using convective cell flow has been proposed to explain slug movement (11). Alternatively, the signal might be a propagating wave of a chemotactic substance as is the case during aggregation. Our observation that the cell movement is periodic (see below) has led us to consider the latter possibility the most likely.

During chemotaxis the direction of cell movement is opposite to the direction of signal propagation (1, 3). Therefore the rotation implies the existence of a three-dimensional scroll wave as the chemotactic signal (see Fig. 4). The straight forward movement of anterior-like cells implies planar waves traveling anterior to posterior in the prespore zone. In >70% of cases ($n > 60$) prestalk cells moved perpendicular to the anterior-posterior axis after correction for slug speed. In some cases prestalk cells moved along the path of a twisted scroll wave (Fig. 1*C*). In the remaining cases prestalk cells showed more complicated rotational movement patterns. We never observed that the cells in the tip moved straight forward in the direction of slug migration.

Cells in Slugs Move Periodically in a Chemotactic Fashion. We have shown previously that fluorescently labeled cells in the prespore zone of slugs clearly move periodically and simultaneously go through cycles of pseudopod extension—i.e., they extend pseudopods during the phase in which they move slowly (9). This behavior is characteristic for chemotactically moving cells (12). Anterior-like cells also move periodically (13). We now show that anterior-like cells in the back of a slug move synchronously in a periodic fashion implying a planar wave front. Fig. 2*A* shows the coherent movement of four anterior-like cells in the back of a slug. They all move forward in a periodic fashion (Fig. 2*B*) and at slightly different speeds. Prestalk cells in the tip cannot be followed long enough to show periodic movement, since they move out of focus in <2 min. However, if cells are isolated from the tip they can be followed for a longer time, since they rotate around a central core in the plane of focus (Fig. 3*A*). Their movement is also periodic (Fig. 3*B*).

For both prespore and anterior-like cells we measured a mean period length of 3 min. Given a signal propagation velocity of 200 μm/min [the lowest value measured for darkfield waves (5)] and an average slug length of 1 mm, there should be only one or two wavefronts in the prespore zone of a slug at one time.

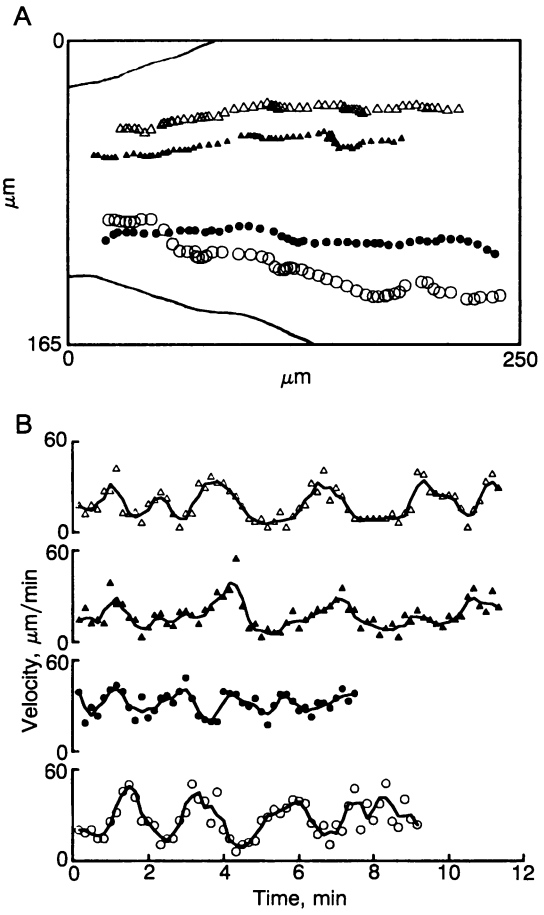

FIG. 2. Periodic cell movement in the prespore zone shown in Fig. 1*E*. (*A*) Tracks of four anterior-like cells. Solid lines show the position of the slug at the beginning of the measurement. The slug moves from left to right. (*B*) Velocity changes of these same cells. The solid line is derived from the data points by moving average smoothing over three neighboring points. Symbols in *B* correspond to symbols in *A*.

We have also calculated the average velocities of cell movement of the three major cell types from the cell tracks: 23.7 ± 6.4 μm/min for prestalk cells ($n = 118$), 16.0 ± 6.3 μm/min for anterior-like cells ($n = 119$), and 17.3 ± 5.2 μm/min for prespore cells ($n = 64$). The velocity of prespore cells was determined by tracking fluorescently labeled cells in the prespore zone. The average velocities of prespore and anterior-like cell movement are similar to average slug speeds reported previously (14).

The movement trajectories of the fast-moving prestalk cells can be decomposed in two approximately equal, large velocity vectors, one in the direction of slug movement and one perpendicular to the slug's long axis. This could imply that prestalk cells move actively perpendicular to the slug long axis and are pushed in the direction of slug migration and, therefore, that prespore and prestalk cells move at approximately similar speeds; alternatively, the prestalk cells could generate both velocity components themselves, suggesting that they do move faster than prespore cells.

Isolated prestalk pieces generate a motive force per unit volume that exceeds the motive force of a slug per unit volume, when migrating in an agar tunnel (15). Our experiments did not allow us to estimate the contribution of the prestalk cells to slug migration. However, since the prestalk cells move as an independent entity more or less perpendic-

ular to the direction of slug migration and the tip is lifted from the substratum most of the time, it is difficult to envisage how they could contribute motive force to the migration of the slug. In our opinion the role of the prestalk zone is to organize the coherent movement of cells in the prespore region.

Models for Slug Migration. Two different models have been proposed to explain slug migration. The fountain flow model postulates differential cell movement of cells in the middle and periphery of the slug (11). Our results indicate that all cells in the prespore zone move forward at similar speed, while cells in the tip rotate around the slug axis. The squeeze–pull model (16) postulates that the cells in the slug move forward by contraction of a ring of cells in the back of the slug and pulling of cells which are stationary to the substratum in the front of the slug. We never observed stationary cells in the anterior part of the slug as claimed by the squeeze–pull model.

Our results show that slug movement and morphogenesis can be simply explained by the same principles that govern aggregation—i.e., wave propagation and chemotaxis. The prespore region consists of individual cells all moving chemotactically in the same direction, coordinated by signals coming from the tip. The tip consists of an aggregation center stuck on the front end of a prespore zone. The cells get traction from an extracellular matrix secreted by all cells. This matrix forms a slime sheath that surrounds the slug and is stationary with respect to the substratum. It is left behind as a slime trail when the slug moves.

The rotation of a scroll wave around a central core gives a simple explanation for the formation of the stalk–tube. There are two classes of prestalk cells in the tip, which express two different extracellular matrix genes, *ecmA* and *ecmB*. Expression of *ecmA* is restricted to the front 10% of the slug, and *ecmB* is expressed in a central core that occupies 10–20% of the length of the slug (7). Each of the cell types requires a specific combination of morphogens: *ecmA* expression is stimulated by cAMP and differentiation-inducing factor (DIF-1); *ecmB* expression is stimulated by DIF-1 and inhibited by cAMP (17). According to our model, cells in the center of the scroll wave should be adapted most of the time and produce little cAMP. This would lead to a low cAMP concentration in the core and a higher cAMP concentration in the periphery. The low cAMP concentration in the core will favor *ecmB* expression, while the higher cAMP concentration in the periphery will favor expression of *ecmA* (Fig. 4).

Scroll wave propagation is a general property of excitable media (18). Two-dimensional propagating scroll and concentric waves as seen during aggregation of *Dictyostelium* are also found in chemical oscillators and have been well studied both experimentally and theoretically in the Belousov–Zabotinsky (BZ) reaction, an oscillating redox reaction (18). Recently, three-dimensional scroll waves have been analyzed in the BZ reaction and shown to decompose to twisted scroll waves and then into planar waves when propagating into a medium of graded excitability (19). Such a description

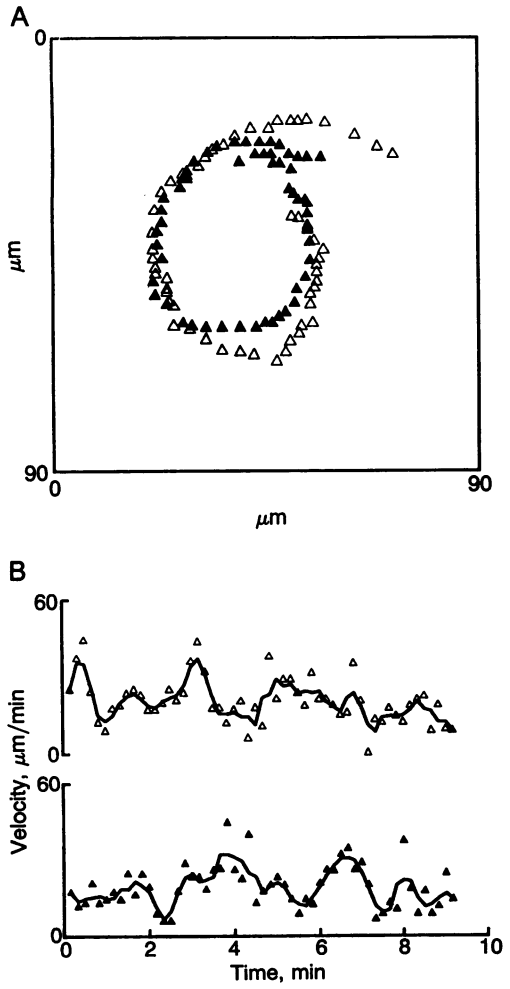

FIG. 3. Periodic cell movement in isolated tip cells shown in Fig. 1*F*. (*A*) Movement of two cells in a group of prestalk cells isolated from the tip and placed on agar. Groups of cells isolated from a tip always rotate. The measurement extends over 10 min starting within 10 min after isolation. (*B*) Velocity changes of these same cells. Solid line is derived from the data points by moving average smoothing over three neighboring points. Symbols in *B* correspond to symbols in *A*.

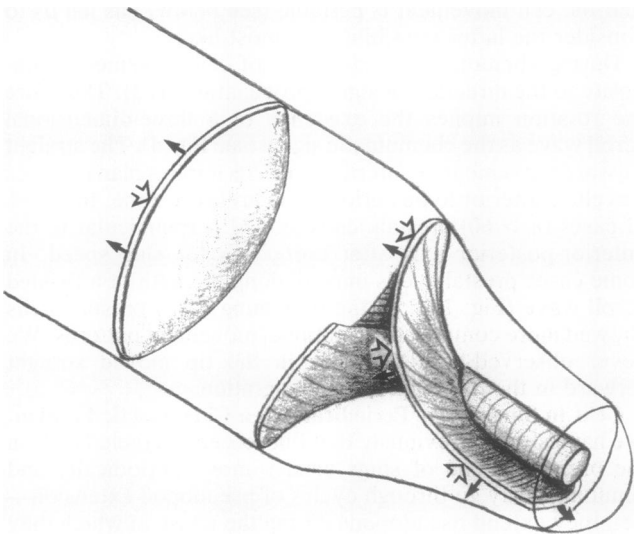

FIG. 4. Model of signal propagation and cell movement in slugs. The observed cell movement is explained by chemotaxis to a scroll wave propagating in the prestalk zone, which unwinds to planar waves in the prespore zone. The intersection of the wave front with the visible surface of the slug is shown as a double line. Black arrows indicate the direction of signal propagation. Open arrows indicate the direction of cell movement. The direction of cell movement is opposite to the direction of signal propagation. The scroll wave in the tip rotates around a central core, in which the cells are adapted. The exact nature of the transition of the scroll wave in the tip to planar waves in the prespore zone is unknown. However, for better visualization we have drawn an imaginary transition. The strong curvature of the wave front at the prestalk/prespore boundary is in agreement with the observed cell movement. Understanding the exact nature of the transition (what happens in the core) will be crucial to complete understanding of three-dimensional wave propagation. This will require detailed mathematical modeling and further experimentation.

agrees well with our observations of cell movement in slugs. A graded excitability is also probable in *Dictyostelium* slugs. Prestalk cells were shown to have higher intrinsic oscillation frequencies than prespore cells, since it is possible to separate aggregation stage cells into populations that will sort to the tip and those that will sort to the back of the slug. The cells sorting to the front have higher intrinsic oscillation frequencies than those sorting to the back (20).

There are remarkable similarities between two-dimensional wave propagation in the BZ reaction and slime mold aggregation (9, 21–24). Complex modes of wave propagation have been observed in three-dimensional BZ reaction systems (25). It will be exciting to see whether a similar variety of wave propagation modes exists during pattern formation in *Dictyostelium*—i.e., whether all morphogenetic movements are controlled by modulation of the oscillator and cell movement speed. In addition to their complicated morphogenetic movements, slugs show a series of behavioral responses such as photo- and thermotaxis. Both morphogenetic and photo- or thermotactic movements require local differences in cell movement speed. The differences in cell movement can be caused by modulation of the signal leading to anisotropic wave propagation or by modulation of the chemotactic response. Substances such as ammonia that control slug migration, culmination, phototaxis, and thermotaxis (26, 27) act by modulating both cAMP relay (28) and cell movement speed (29).

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1. Devreotes, P. N. (1989) *Science* **245,** 1054–1058.
2. Durston, A. J. (1974) *Dev. Biol.* **37,** 225–235.
3. Tomchik, K. & Devreotes, P. N. (1981) *Science* **212,** 443–446.
4. Alcantara, F. & Monk, M. (1974) *J. Gen. Microbiol.* **85,** 321–334.
5. Siegert, F. & Weijer, C. J. (1989) *J. Cell Sci.* **93,** 325–335.
6. Sternfeld, J. & David, C. N. (1981) *Differentiation* **20,** 10–21.
7. Jermyn, K. A. & Williams, J. G. (1991) *Development* **111,** 779–787.
8. MacWilliams, H. K. (1982) in *Developmental Order: Its Origin and Regulation* (Liss, New York), pp. 463–483.
9. Siegert, F. & Weijer, C. J. (1991) *Physica D (Amsterdam)* **49,** 224–232.
10. Sussmann, M. M. (1987) in *Methods in Cell Biology*, ed. Spudich, J. A. (Academic, New York), pp. 9–29.
11. Odell, G. M. & Bonner, J. T. (1986) *Philos. Trans. R. Soc. London B* **312,** 487–525.
12. Condeelis, J., Bresnick, A., Demma, M., Dharmawardhane, S., Eddy, R., Hall, A. L., Sauterer, R. & Warren, V. (1990) *Dev. Genet.* **11,** 333–340.
13. Durston, A. J. & Vork, F. (1979) *J. Cell Sci.* **36,** 261–279.
14. Bonner, J. T., Koontz, P. G. & Paton, D. (1953) *Mycologia* **45,** 235–240.
15. Inouye, K. & Takeuchi, I. (1980) *J. Cell Sci.* **41,** 53–58.
16. Williams, K. L., Vardy, P. H. & Segel, L. A. (1986) *Bioessays* **5,** 148–151.
17. Berks, M. & Kay, R. R. (1990) *Development* **110,** 977–984.
18. Winfree, A. T. (1987) *When Time Breaks Down* (Princeton Univ. Press, Princeton, NJ).
19. Yamaguchi, T. & Müller, S. C. (1991) *Physica D (Amsterdam)* **49,** 40–46.
20. Weijer, C. J., McDonald, S. A. & Durston, A. J. (1984) *Differentiation* **28,** 13–23.
21. Foerster, P., Müller, S. C. & Hess, B. (1990) *Development* **109,** 11–16.
22. Dockery, J. D., Keener, J. P. & Tyson, J. J. (1988) *Physica D (Amsterdam)* **30,** 177–191.
23. Foerster, P., Müller, S. C. & Hess, B. (1988) *Science* **241,** 685–687.
24. Tyson, J. J., Alexander, K. A., Manoranjan, V. S. & Murray, J. D. (1989) *Physica D (Amsterdam)* **34,** 193–207.
25. Pertsov, A. M., Aliev, R. R. & Krinsky, V. I. (1990) *Nature (London)* **345,** 419–421.
26. Bonner, J. T., Suthers, H. B. & Odell, G. M. (1986) *Nature (London)* **323,** 630–632.
27. Bonner, J. T., Har, D. & Suthers, H. B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2733–2736.
28. Williams, G. B., Elder, E. M. & Sussman, M. (1984) *Dev. Biol.* **105,** 377–388.
29. Van Duijn, B. & Inouye, K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4951–4955.