Motility of Cultured Fish Epidermal Cells in the Presence and Absence of Direct Current Electric Fields

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Abstract. The motile behavior and cytoskeletal structures of fish epidermal cells (keratocytes) in the presence and absence of direct current (DC) electric fields were examined. These cells spontaneously show highly directional locomotion in culture, migrating at rates of up to 1 μm/s. When DC electric fields between 0.5 and 15 V/cm are applied, single epidermal cells as well as cell clusters and cell sheets migrate towards the cathode. Cell clusters and sheets break apart into single migratory cells in the upper range of these field strengths. Cell shape and morphology are unaltered when the keratocytes are guided by an electric field. Neither the spontaneous locomotion nor the electrically guided motility were found to be microtubule dependent. 1 mM La^{3+}, 10 mM Co^{2+}, 50 μM verapamil, and 50 μM nitrendipine (calcium channel antagonists) reversibly inhibited lamellipod formation and cell locomotion in both spontaneously migrating and electrically guided cells. Ciba-Geigy Product 28392, which stimulates the opening of calcium channels, and is a competitive inhibitor of nitrendipine, has no effect on the locomotion of keratocytes. Cell motility was also unaffected by hyperpolarizing and depolarizing (low and high K^+) media. It is argued that while a tissue cell may accommodate changes in resting membrane potential without becoming more or less motile, the cell may not be able to counterbalance the effects of depolarization and hyperpolarization simultaneously. In this context, a gradient of membrane potential, which is induced by an external DC electric field, will serve as a persistent stimulus for cell locomotion.

DIRECTED cell locomotion in an electric field is known historically as galvanotaxis (80) (reviewed by Koehler [40], Jahn [35], and Rose [70]). It has been observed in a broad range of cell types, including amoebae (8, 31, 39, 57, 82), ciliates (4–6, 8, 49, 67, 80, 83, 84), flagellates (56, 67, 81), leukocytes (22–24, 60), macrophages (65), and a variety of tissue cells (10, 19, 50, 77). A related phenomenon, galvanotropism (directed growth or extension in an electric field), has also been extensively studied. Neurons (32, 33, 38, 55, 66, 74) and slime molds (3, 85) both extend processes towards the cathode in an applied field.

In addition to biasing spontaneous cell locomotion, it is known that applied electric fields will strongly stimulate stationary cells (4–6, 22–24, 31, 44, 49, 57, 80–82) to become motile. Striking examples of this effect have recently been observed in metazoan tissue cells. Epithelial cells, fibroblasts, and neural crest cells, either isolated or in confluent monolayers, are induced to retract their anode- and cathode-facing margins, becoming perpendicularly aligned to the field (10, 19, 50, 64, 77). These cells then produce lateral protrusions and begin migrating, perpendicular to their long axis, towards the cathode. During the directed migrations there is no habituation to the electric field.

The ability to rapidly and persistently mobilize such a diverse spectrum of cells in the same manner suggests that the electric field induces a physiological state that is common to all migrating tissue cells. Determining how the applied field stimulates motility could therefore be important in elucidating the electrical (e.g., ionic fluxes) and biochemical events that are involved in the onset of tissue cell motility in other situations.

To further understand the mechanical and electrical controls of tissue cell galvanotaxis, and directional tissue cell movements in general, we have examined the motile behavior and cytoskeletal structures of fish epidermal keratocytes in the presence and absence of direct current (DC)[1] electric fields. These cells spontaneously show rapid and highly directional locomotion in vitro. When exposed to external DC fields, keratocytes locomote directionally towards the cathode without any change in their overall migratory morphology. Both electrically controlled and spontaneous locomotion are quickly paralyzed by a variety of calcium channel antagonists, but are insensitive to major shifts ($\geq$50 mM) in K^+, Na^+, and Cl^- in the external medium, which should hyperpolarize or depolarize the cell membrane. Based on these results, we propose that an influx of extracellular Ca^{2+} is required to generate the motile activity of fish keratocytes. Using this premise, we discuss how a DC electric field could promote

[1] *Abbreviations used in this paper:* CGP, Ciba-Geigy Product; DC, direct current.

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and direct the motile activity of keratocytes in terms of asymmetric transmembrane Ca^{2+} fluxes that are induced by the field. We argue, from principles of chemotaxis, that a gradient of membrane potential should be a major stimulus for directed tissue cell motility, in the analogous way that a gradient of chemoattractant or repellent stimulates and directs motility in bacteria and leukocytes.

Preliminary accounts of this work have been reported elsewhere (11–13).

Materials and Methods

Cell Isolation and Culture

Epidermal cells were obtained from either angel fish (*Pterophyllum scalare*) or goldfish (*Carassius auratus*) by removing several scales from the flanks of the animals with forceps. The scales were incubated in Fish Ringer's solution that contained 0.5 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) for 15–30 min to remove the attached epidermis. Once dissociated, epidermal tissue fragments were placed on 18 × 18-mm No. 1 coverslips with a single drop of Fish Ringer's solution supplemented with 2 mM glucose and 20% amphibian culture medium (Gibco, Grand Island, NY). The drop was spread out to the corner of the coverslip to hold the tissue fragments to the glass by surface tension. Within 1 h in a humidified atmosphere, the tissue fragments attach as cell sheets to the coverslips. Migratory single cells and cell clusters frequently break loose from the edges of these sheets and distribute over the coverslip. All cell isolations and experiments were done at ambient temperature.

Photomicroscopy and Cytoskeletal Staining

Cell movements were followed by phase contrast microscopy using a Zeiss standard microscope (Carl Zeiss, Inc., Thornwood, NY), a Dage video camera (Dage-MTI Inc., Michigan City, IN) equipped with a Newvicon tube, and a ¾" NEC (Elk Grove Village, IL) time-lapse video recorder. For fluorescence microscopy, cells on coverslips were fixed with 1% glutaraldehyde in PHEM buffer (72) supplemented with 0.1% Triton X-100 and incubated with rhodamine phalloidin (87) and an antibody against tubulin (kindly provided by Marc Kirschner, University of California, San Francisco) using standard double labeling procedures. Nomarski and fluorescence micrographs were taken on a Zeiss Photomicroscope III equipped with epi-excitation.

Electric Field Application

Chambers for the application of electric fields were constructed by attaching two 0.5 × 1.8-cm parallel strips of No. 1 coverslips to a microscope slide or plastic culture dish with Vaseline. Coverslips with attached cells were supported on these spacers, thus enclosing the cells in a chamber with the dimensions 0.02 × 0.5 × 1.8 cm. The edges of the coverslip were sealed with a mixture of Vaseline, lanolin, and paraffin (1:1:1). A ring of this sealant was also added to each end of the chamber to accommodate pools of culture medium. Steady DC fields were applied via 20-cm agar-saline bridges (Ringer's gelled with 2% agar) connecting the chamber to Pt electrodes in wells of Fish Ringer's solution. Electric field strengths were determined by directly measuring the voltage drop across the length of the chamber with Ag/AgCl electrodes (see reference 32). For safety, the microscope was grounded to the power supply ground.

Ion Substitutions and Drug Applications

The basic Fish Ringer's solution used in most experiments was composed of 112 mM NaCl, 2 mM KCl, 2.4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM Tris buffer, pH 7.0–7.8. When studying the effects of La^{3+} and Co^{2+}, we eliminated $NaHCO_3$ from the media to prevent carbonate precipitates. Sodium gluconate and potassium gluconate were used to replace K^+, Na^+, and Cl^- for various hyperpolarizing and depolarizing media. All of these solutions contained 1 mM $CaCl_2$ but no $NaHCO_3$.

Verapamil was obtained from Knoll Pharmaceutical Co. (Whippany, NJ). Nitrendipine and Ciba-Geigy Product (CGP) 28392 were obtained from Ciba-Geigy (Basel, Switzerland). Nocodazole and cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, MO).

Each experiment described in the Results section was repeated at least three times with separate keratocyte cultures. Descriptions of motile behavior for a specific experimental condition represent summed observations of 30–100 individual cells.

Assay for Directional Cell Locomotion

An index devised by Dixon and McCutcheon (16, 69) to analyze directionality of cell movements during chemotaxis was used to quantify the galvanotactic locomotion of keratocytes. The Dixon-McCutcheon index is defined as the ratio of the distance traversed by a cell in a given direction to the total path length traveled by the cell in the same time interval. In our experiments, maximal positive galvanotaxis towards the cathode is indicated by a value of +1. A value of −1 would indicate maximal movement directed away from the cathode. Random movements of a number of cells in a given culture are expected to produce an average index of 0.

Results

Spontaneous and Electrically Guided Locomotion

Single migratory epidermal cells adopt a distinctive morphology when they move over the substratum (Fig. 1 *A*). The cells, which are ~20 × 40 μm, migrate perpendicular to their long axis and change their direction of movement by making smooth, gradual turns. Migratory cells often persist in their general direction of movement for several hundred micrometers. Their average speed of locomotion is exceedingly high, often averaging 10–30 μm/min and occasionally reaching 1 μm/s.

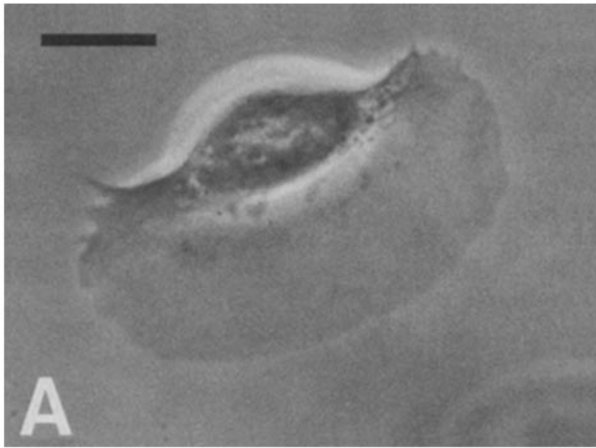
The leading edge of these cells is a broad, canoe-shaped lamellipodium, which is continually extending during cell migration. A globular cell body, which contains the nucleus and all other organelles, is located at the rear of the cell. The trailing edge of the migrating keratocyte usually has no large retraction fibers. The lamellipodium, however, is actively pulled off the substratum in the region where it extends behind the cell body (Fig. 2). Upon detachment, the cell membrane and lamellar cytoplasm of this region are rapidly pulled towards the perinuclear area, which suggests that this material is under tension. The membrane material of these detached lamellar regions, either by flowing forward or by being stretched taut (along with cortical microfilaments), may provide the surface area for further extension of the broad leading lamellipodium. This possibility was suggested earlier by DiPasquale (15) for the extension of epithelial cell lamellipodia.

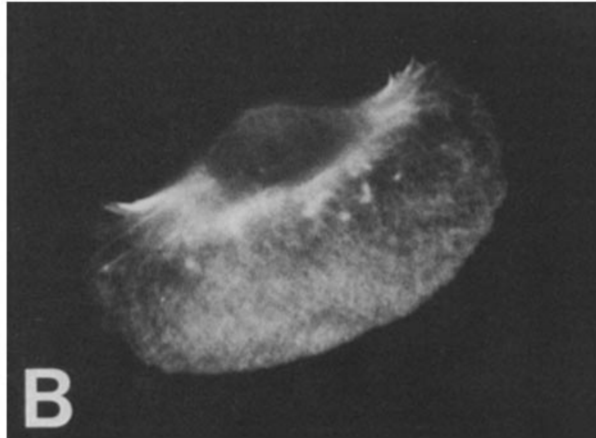
Occasionally, the broad continuous lamellipodium of the canoe-shaped cell bifurcates and forms two competing lamellipodia at opposite ends of the cell (Fig. 3). The membrane surrounding the nucleus and the center of the cell is pulled taut by the extension of these lamellipodia. These cells can migrate perpendicular to their long axes if the lamellar fans are both turned towards one side of the cell (see arrow in Fig. 4).

These fish keratocytes are very likely the "fan" cells Goodrich (26) isolated from *Fundulus* and described many years ago. Their movements also closely resemble those of cultured epidermal cells from the tail fin of frog larvae (43, 76).

Effects of Electric Fields

When DC electric fields of ≳ 2 V/cm are applied to keratocyte cultures, all cells are usually induced to migrate towards the cathode within 1–2 min, maintaining their normal migratory morphology (Figs. 4 and 5). Reversal of the field causes the cells to change their direction of movement within a few minutes (Fig. 5). In most cases, the cells reorient by turning rather than by withdrawing the lamellipodium on the leading edge and extending a new one on the cell posterior. Once the leading lamellipodium fully faces the cathode, the cell follows a directional course, with no changes in overall cell morphol-





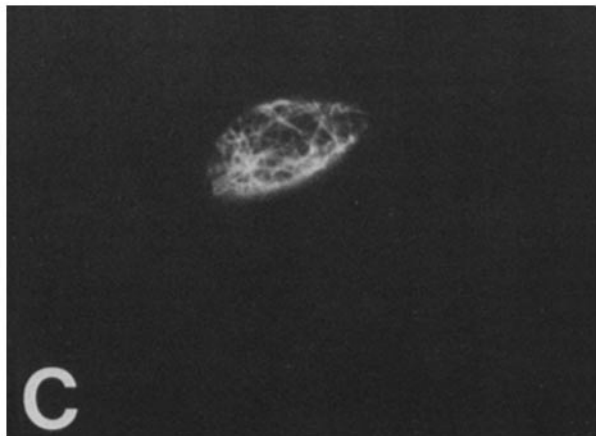


Figure 1. (*A*) Phase micrograph of a migratory fish epidermal cell or keratocyte. A broad lamellipodium extends more than 180° around the cell periphery. The nucleus and all organelles are confined to a globular cell body at the rear of the cell. Bar, 10 μm. (*B*) Actin stain of cell in *A*. A criss-crossing network of actin filaments pervades the lamellipodium. Small fibers are located near the cell body. (*C*) Microtubule antibody stain of same cell. Microtubules are wrapped around the nucleus. None extend into the lamellar region.

ogy. During field application (typically 10–30 min) there is no apparent habituation to the field. Cells are persistently stimulated to migrate towards the cathode. There is usually a smooth transition between strongly cathode-directed movement in the electric field to an unbiased directional movement, once the field is removed (Fig. 5).

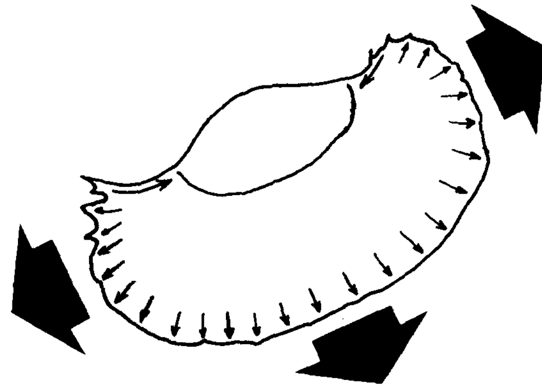


Figure 2. Dynamics of the cell periphery during cell locomotion (small arrows). The lamellipodium margins are in extension everywhere except where the lamellipodium is actively pulled off the substratum at the trailing edge of the cell. This detached material is pulled rapidly towards the nucleus. Tractional forces (large arrows) exerted by the lamellipodium pull the cell into its elongated shape. Since lamellar extensions fail to form in the trailing nuclear region, the cell is continually pulled forward by the leading unopposed margin of the lamellipodium.

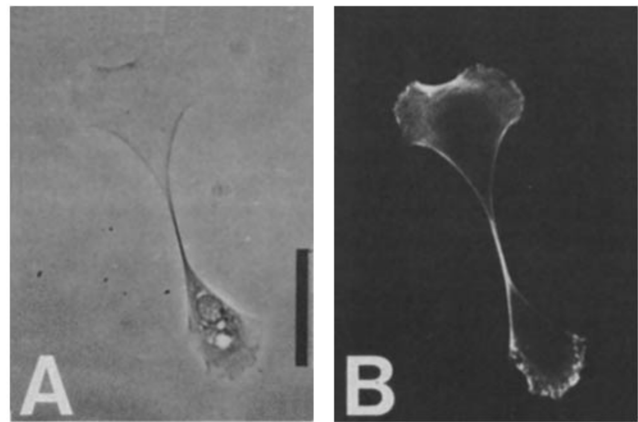
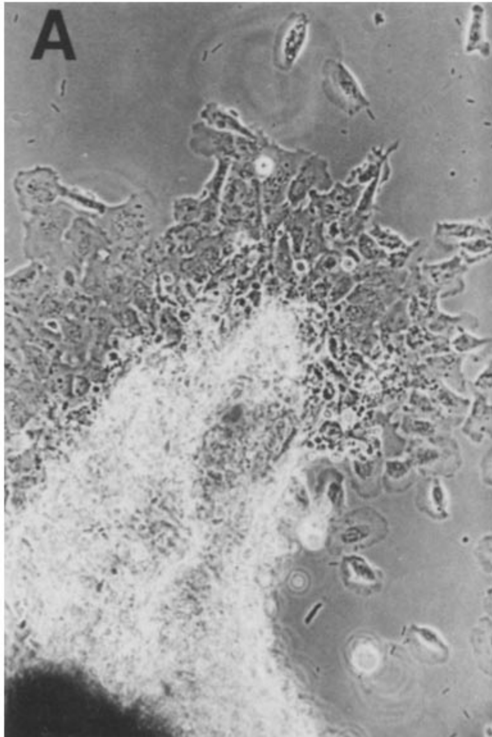


Figure 3. In some keratocytes, the lamellipodium may bifurcate into two competing regions that pull the cell into a bipolar shape (see Goodrich [26]). (*A*) Phase micrograph. (*B*) Phalloidin actin stain. Bar, 20 μm.

To determine an electric field threshold for directional cell locomotion, the Dixon-McCutcheon index (see Materials and Methods) was calculated for a number of cell cultures exposed to various field strengths (Fig. 6 and Table I). A statistically significant bias of cell movement towards the cathode consistently occurred in fields ≥0.5 V/cm.

Perfusion of media through the chamber during electric field application, at rates of 0.1 ml/min (parallel or antiparallel to the field), has no effect on cell locomotion (not shown). Similarly, no noticeable change in cathode-directed movement was observed in experiments where bulk Ringer's solutions within the galvanotaxis chamber were interchanged with a pasteur pipette in less than 30 s. This illustrates that the directed motility is due directly to the field rather than to a chemotactic gradient established by the field. Previous studies of other cell types have not found electrically guided cell movement to be influenced by bulk flow in the external medium (10, 19, 32, 66, 77).

Cell clusters, consisting of several tens of cells, and cell



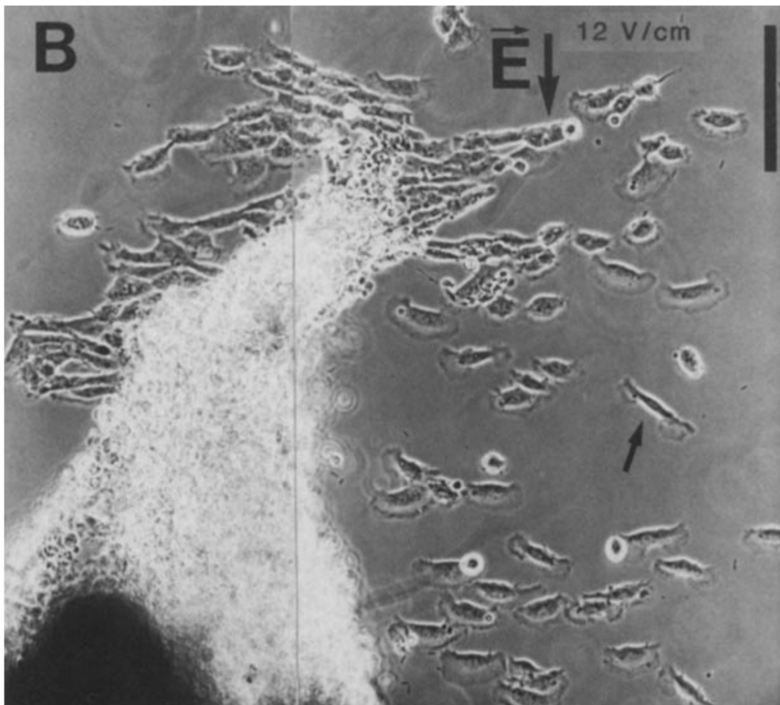


Figure 4. Stimulation of cathode-directed migration of cells in an applied electric field. (*A*) An explanted sheet of fish epidermis before the application of an electric field. Migratory cells frequently detach from the edges of the sheet. The sheet itself is essentially nonmotile except for protrusive activity of cells around its periphery. (*B*) 14 min after an electric field of 12 V/cm is applied. A large onset of motility has occurred throughout the culture. The cells dissociate from each other and begin migrating towards the cathode at a rate of 18 ± 3.5 μm/min (n = 17). The Dixon-McCutcheon index of directional cell movement in the presence of the field is +0.97 ± 0.03 (n = 17). The two lamellipodia of a bipolar cell (arrow) are turned towards the cathode as the cell migrates in that direction. By convention, the electric field vector E points towards the negative pole (cathode). Bar, 100 μm.

sheets, which maintain a stratified organization of 2–4 layers of cells (similar to epidermis in vivo), were also guided towards the cathode in applied fields of 4–10 V/cm (Fig. 7). Cells located at the cathode-facing edge of a cluster or sheet were stimulated to extend lamellipodia, whereas the anode-facing edges of the ensembles were induced to retract their lamellae. Thus, cell clusters and sheets behave as multicellular units with leading and trailing regions that span several tens of cells. When the direction of the electric field is reversed, directed movements also reverse, on a time scale similar to that of single cells. Reversal of cluster and sheet movement occurs through retraction of protrusions on the anode that faces margins and by the production of new lamellipodia on the side of the cluster that faces the cathode.

At higher field strengths (≳10 V/cm), confluence within the clusters and sheets is disrupted (Figs. 4, 10 C, and 11 *D*). This often occurs by numerous migratory cells detaching from the leading edge. In other instances, the entire cell sheet disaggregates into single migratory cells as the ensemble steadily moves towards the cathode. The electric field apparently

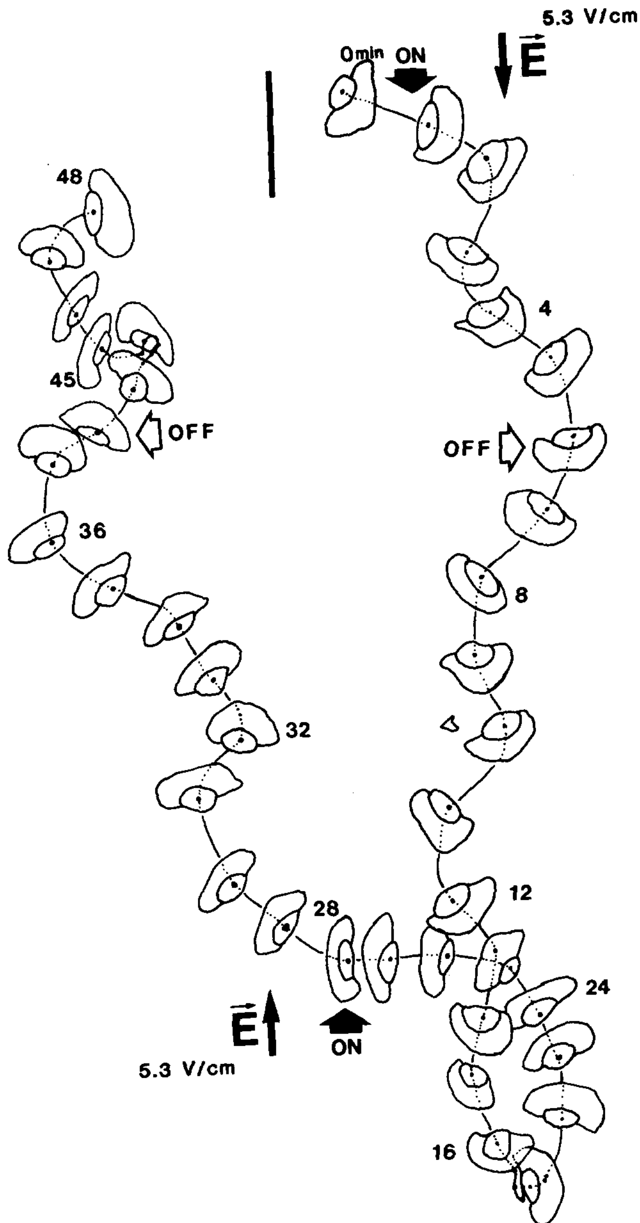


Figure 5. Directional migration of a single migratory cell in a DC electric field. A cell turns towards the cathode within 1 min after application of a 5.3 V/cm electric field. The cell persists in its general direction of movement once the field is removed. Such persistent locomotion is typical of spontaneously migrating cells. After the field is reapplied in the opposite direction, the cell reorients and migrates towards the new cathode. Bar, 100 μm.

induces this confluence disruption by stimulating all cells within the cell sheet to become motile.

Effects of Calcium Channel Antagonists

To determine if transmembrane Ca^{2+} fluxes are required for keratocyte locomotion, La^{3+} or Co^{2+} were added to the external medium. These ions are considered to be antagonists for calcium channels in metazoan cells (29).

Within seconds after application of Fish Ringer's solution that contained 1 mM $LaCl_3$ (Fig. 8, *A* and *B*) or 10 mM $CoCl_2$ (Fig. 8 *C*) there is a cessation of cell locomotion with a concomitant paralysis of the cell periphery. Often, the cells remain frozen in their migratory morphology. However, it is also common for the cells to spread out into circular forms as they become nonmotile (Fig. 8, *A* and *C*). At lower concentrations of the heavy metal ions, 0.1–0.5 mM La^{3+} or 1 mM Co^{2+}, cell movements are not completely blocked but are slowed down to <20 μm/min (not shown).

Cells in the presence of 1 mM La^{3+} or 10 mM Co^{2+} do not respond to electric fields (Fig. 8, *B* and *C*). However, both spontaneous and electrically guided locomotion resume within a few minutes after the heavy metal ions are rinsed away with normal Ringer's solution. The recovery of the cells is slower after prolonged exposure (≳5 min) to these ions (Fig. 8*A*).

Two drugs known to specifically block Ca^{2+}-channels were also tested on keratocytes. 50 μM nitrendipine (Fig. 9*A*) and 50 μM verapamil (Fig. 9*B*) each reversibly blocked the locomotion of the cells. During exposure to the drugs, keratocytes tend to retract portions of their periphery and round up. Taken together, the inhibitory effects of verapamil, nitrendipine, La^{3+}, and Co^{2+} strongly suggest that an influx of extracellular calcium through specific channels is required to generate motile activity in fish keratocytes.

Recently, a novel class of compounds (structural derivatives of dihydropyridine) has been found that stimulates Ca^{2+} entry through specific channels (20). One of these new drugs, CGP

Table I. Dixon-McCutcheon Index of Directional Locomotion for Keratocyte Exposed to Various Electric Field Strengths

Electric field strength	Dixon-McCutcheon index	No. of cells
V/cm		
14.0	0.91 ± 0.08*	20
12.0	0.97 ± 0.03*	19
12.0	0.91 ± 0.07*	17
10.0	0.88 ± 0.13*	19
6.8	0.89 ± 0.13*	23
4.7	0.88 ± 0.12*	19
2.0	0.76 ± 0.18*	25
1.6	0.70 ± 0.40*	38
1.0	0.61 ± 0.38*	32
0.9	0.54 ± 0.24*	24
0.7	0.64 ± 0.32*	38
0.5	0.23 ± 0.38*	19
0.5	0.52 ± 0.54*	20
0.5	0.34 ± 0.52‡	15
0.5	0.43 ± 0.56‡	12
0.5	0.17 ± 0.48	23
0.4	0.37 ± 0.53‡	14
0.3	0.19 ± 0.52	10
0.22	0.11 ± 0.57	15
0.2	0.22 ± 0.59	14
0.15	0.25 ± 0.41‡	15
0.07	0.06 ± 0.54	14
0	0.05 ± 0.54	18
0	0.07 ± 0.59	17
0	0.00 ± 0.72	19
0	0.01 ± 0.41	19
0	0 ± 0.25	23

A positive index indicates directional locomotion towards the cathode (see Materials and Methods and Fig. 6).

* Cultures whose average index is significantly different from zero using the Student's *t*-test at the $P < 0.01$ confidence level.

‡ A significant difference of the mean from zero at $P < 0.05$.

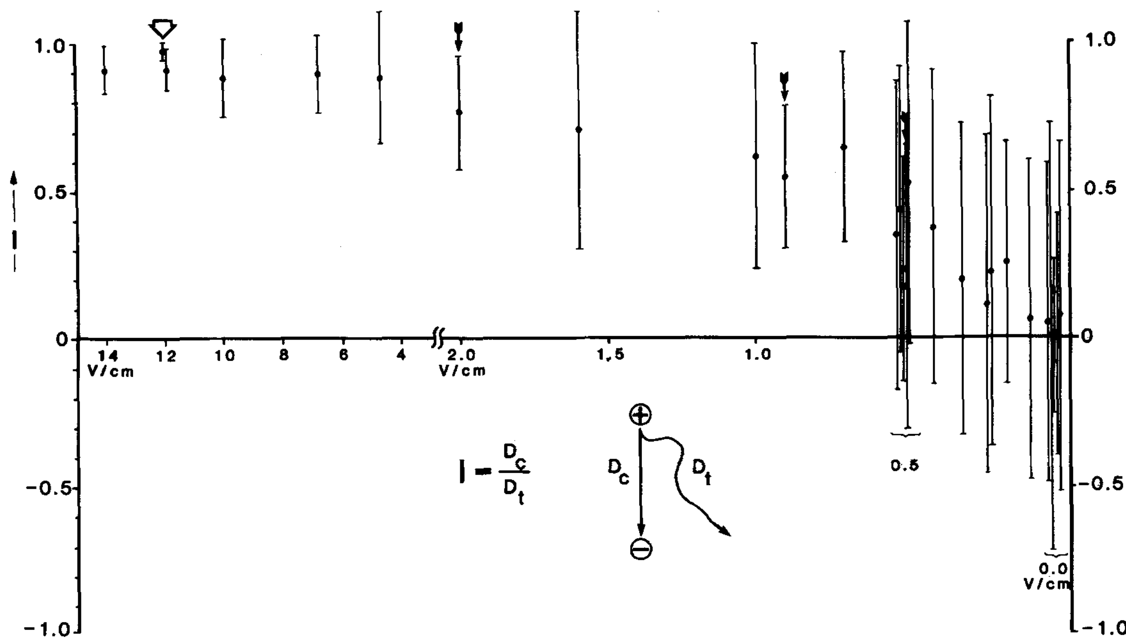


Figure 6. Dixon-McCutcheon index (I) of directional cell locomotion versus electric field strength. I represents the ratio of the distance traveled towards the cathode (D_c) to the total path length (D_t) traversed by the cell in the same time interval (see text). Each data point represents the mean index of a given culture. Bars represent standard deviation associated with the mean. Total observation time averaged 10–20 min. During this time the cells migrated 100–300 μm or ~5–15 cell diameters. A statistically significant ($P < 0.01$, Student's t-test, see Table I) bias of cell movement towards the cathode consistently occurs in fields ≥ 0.5 V/cm. Cells show increasingly biased movement towards the cathode (i.e., I approaches 1) with increasing field strengths. The large open arrow indicates the index of the cells migrating in Fig. 4. The small arrows indicate the index of a keratocyte culture exposed to progressively decreasing field strengths (20-min intervals, reversing polarity).

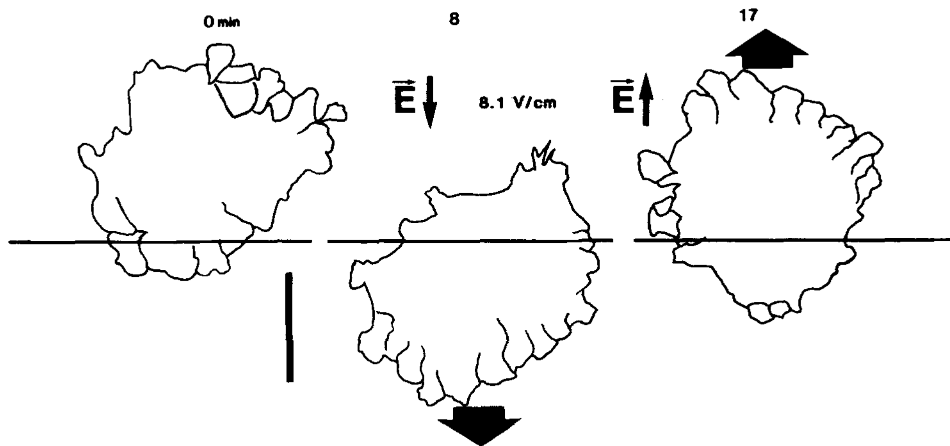


Figure 7. Tracing of a cell cluster's movements in an applied field. 0 min shows the outline of ~40 contiguous cells just before the application of an 8.1 V/cm electric field. As the field is applied, lamellipodia on the anode-facing side of the cluster retract while lamellipodia are extended on the cluster's cathode-facing side. The entire cluster translocates towards the cathode at a rate of ~20 μm/min. After 8 min, the field is reversed in direction. The cluster reverses locomotion and migrates towards the new cathode. Bar, 100 μm.

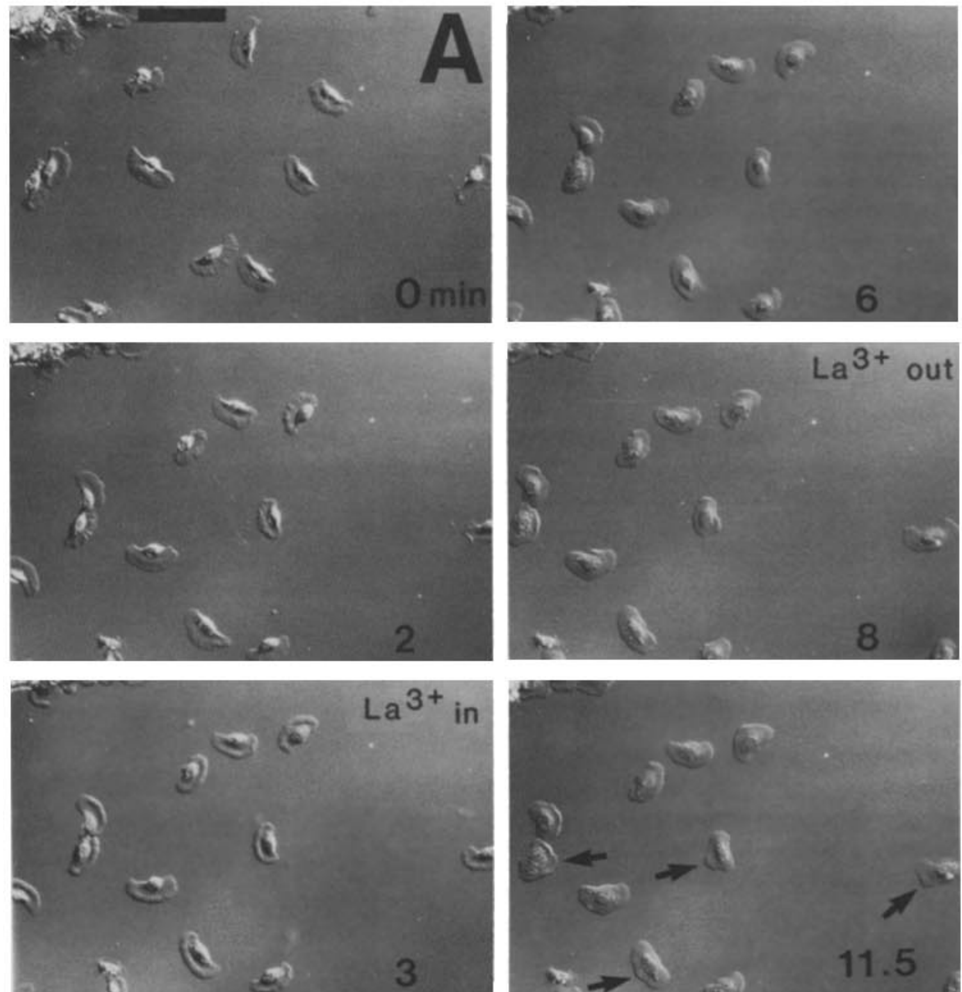
28392, is competitively inhibited by nitrendipine, which suggests a common site of action for the two compounds (20).

The behavior of a fish keratocyte in separate applications of CGP 28392 and nitrendipine is shown in Fig. 9*A*. The Ca^{2+}-channel stimulator does not alter the spontaneous motility of the cell, whereas nitrendipine causes the cell to round up. Although we do not know the activity of CGP 28392 in keratocytes, its lack of effect on cell locomotion is not inconsistent with the hypothesis that the nitrendipine-sensitive Ca^{2+} channels are frequently in their conducting state.

Effects of Hyperpolarizing and Depolarizing Media

With the result that an influx of extracellular Ca^{2+} is probably required to maintain epidermal cell motility, we wished to test whether the Ca^{2+} conductance of the cell membrane is voltage-sensitive. Since the epidermal cells are small and extremely thin (≤1 μm), voltage-clamp measurements were not attempted. Instead, the cells were exposed to various media of different ionic composition to test whether a general depolarization or hyperpolarization of the cell membrane would affect motility. We found that cells will migrate with normal morphology at comparable rates (i.e., within a factor of 2) in a variety of media in which K^+, Na^+, and Cl^- are greatly altered from normal Fish Ringer's media.

Fig. 10*A* shows keratocytes migrating in a medium of 134 mM sodium gluconate, 0 mM KCl, and 1 mM $CaCl_2$. The reduction of potassium and chloride has no effect on the motile behavior of the cells. Fig. 10*B* shows a keratocyte migrating in a medium of 67 mM potassium gluconate, 67 mM sodium gluconate, and 1 mM $CaCl_2$. The motility of the cell is unaffected by the large increase in external potassium over normal Fish Ringer's solution that contains 2 mM K^+.



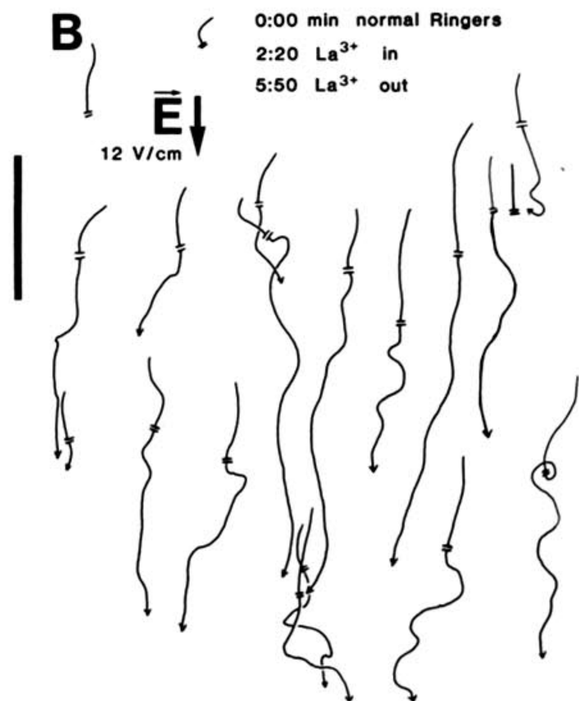
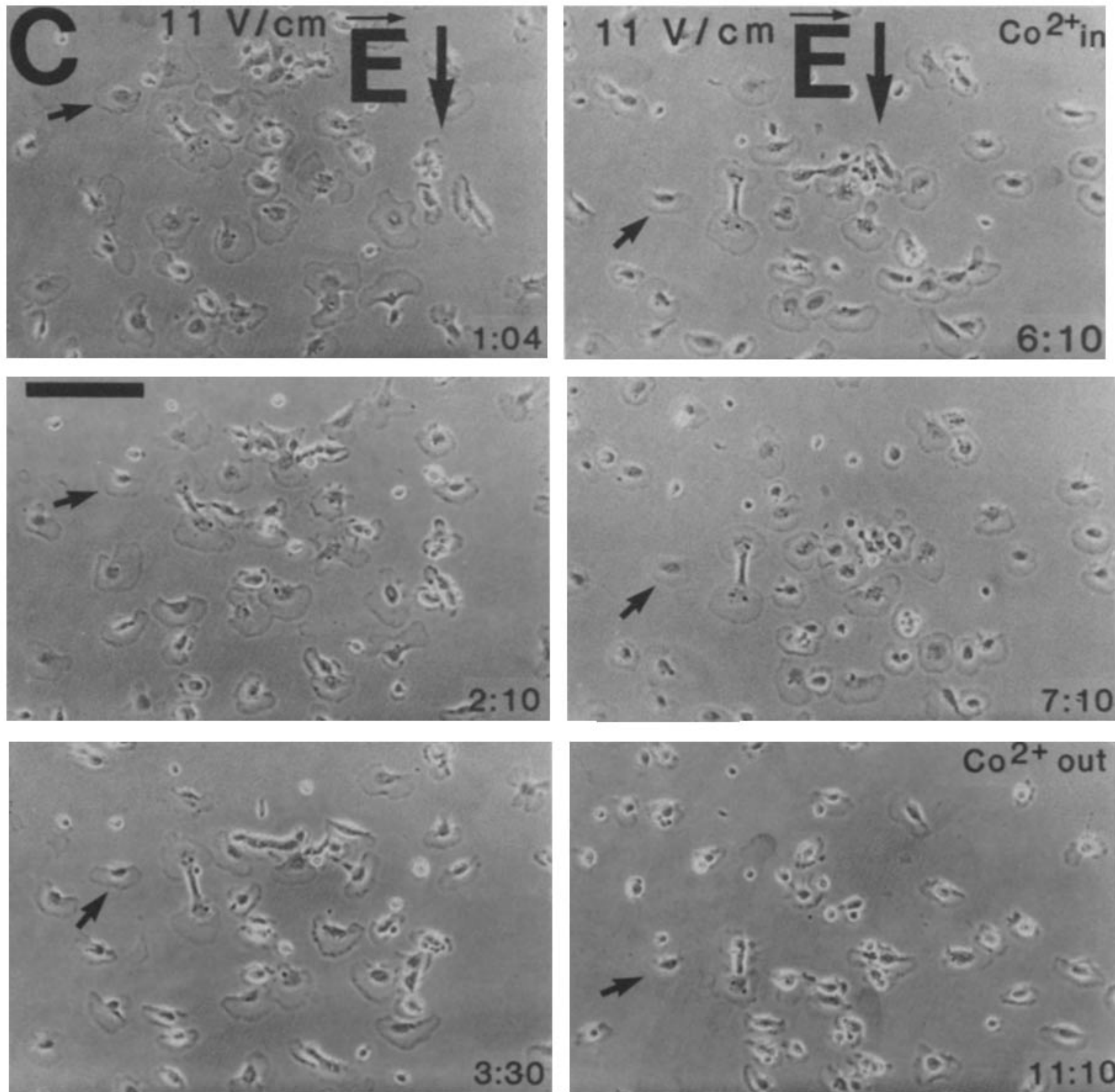


Figure 8. Paralysis of keratocyte locomotion by La^{3+} and Co^{2+}. (*A*) An ensemble of spontaneously migrating keratocytes (0 and 2 min) is exposed to 1 mM $LaCl_3$ 15 s before the photo at 3 min. The cells are paralyzed in their normal migratory morphology and begin to spread into more flattened forms (6 min). Movement in the periphery of the cells (arrows in 11.5 min) resumes a few minutes after La^{3+} is washed away at 6:10 min. Bar, 50 μm. (*B*) Inhibition of electrically-guided locomotion with La^{3+}. A keratocyte culture was exposed to an electric field of 12 V/cm for 10 min to orient the cells towards the cathode before the beginning of the tracing at 0:00 min. At 2:20, 1 mM La^{3+} was perfused through the chamber. Cell movements are paralyzed within seconds even though the field was continuously applied. At 5:50, the La^{3+} solution was replaced with normal Ringer's. Cell migration towards the cathode resumed. Horizontal lines represent position of paralyzed cells between 2:20 and 5:50. End of tracing was at 8:20 min. Bar, 50 μm. (*C*) Inhibition of electrically-guided locomotion with Co^{2+}. Keratocyte culture was exposed to an electric field of 12 V/cm at 0:00 min. Nonmotile, circular cells become active and begin migrating towards the cathode. At 5:50, 10 mM Co^{2+} was perfused through the chamber while the field was continuously applied. Within seconds (6:10), the cells become paralyzed. At 7:10, the cells have spread out into more circular forms. At 7:30 the Co^{2+} medium is replaced with normal Ringer's solution. Within 1–2 minutes, the cells again become motile and migrate towards the cathode (11:10 min). Arrows trace the behavior of a single cell. Bar, 100 μm.



Besides having no noticeable effect on spontaneous motility, these ionic substitutions did not affect the galvanotactic behavior of the cells. Fig. 10, *C* and *D* shows keratocytes migrating towards the cathode in both high and low potassium media.

Although the ionic permeability properties of fish keratocytes have not been measured, it is reasonable to expect that these media alter the resting membrane potential of keratocytes by at least several millivolts to several tens of millivolts. An ideal K^+-selective cell membrane is depolarized (hyperpolarized) by 58 mV by a 10-fold increase (decrease) in extracellular potassium. Overall, the motile behavior of keratocytes appears to be insensitive to major shifts (≥50 mM) of K^+, Na^+, and Cl^- in the extracellular medium, and the hyperpolarization and depolarization of the cell membrane produced by these shifts in external ions.

Cytoskeletal Structures

To determine the involvement of actin- and tubulin-containing cytoskeletal assemblies in galvanotactic and spontaneous movement, cells were exposed to cytochalasin D (2 μg/ml) or nocodazole (4 μg/μl), respectively. In untreated cells, actin staining with rhodamine phalloidin shows an array of criss-crossing fibers in the leading lamellipodium (Fig. 1 *B*). Tubulin immunofluorescence shows a complement of apparently randomly oriented microtubules close to the nucleus, with none extending into the lamellar region (Fig. 1 *C*). Treatment of migratory cells with cytochalasin D (0.5–2 μg/ml) inhibits locomotion within 0.5–2 min and leads to either complete retraction or gross distortion of the lamellipodium (not shown). These cells are incapable of random or directional migration, which indicates that an intact actin network is essential for epidermal cell locomotion.

Since microtubules have been repeatedly proposed to be necessary for directional cell motility (25, 79), and because epidermal cells migrate in a highly directional fashion both in the presence and absence of electric fields, we sought to determine whether microtubules are required for epidermal cell locomotion. Microtubules were disassembled using a combination of cold and nocodazole treatment. Cells were chilled on ice for 30–40 min in the presence of 4 μg/ml nocodazole and then rewarmed to room temperature in the presence of the drug. Fig. 11, *A–C* shows phase contrast, tubulin immunofluorescence, and rhodamine phalloidin flu-

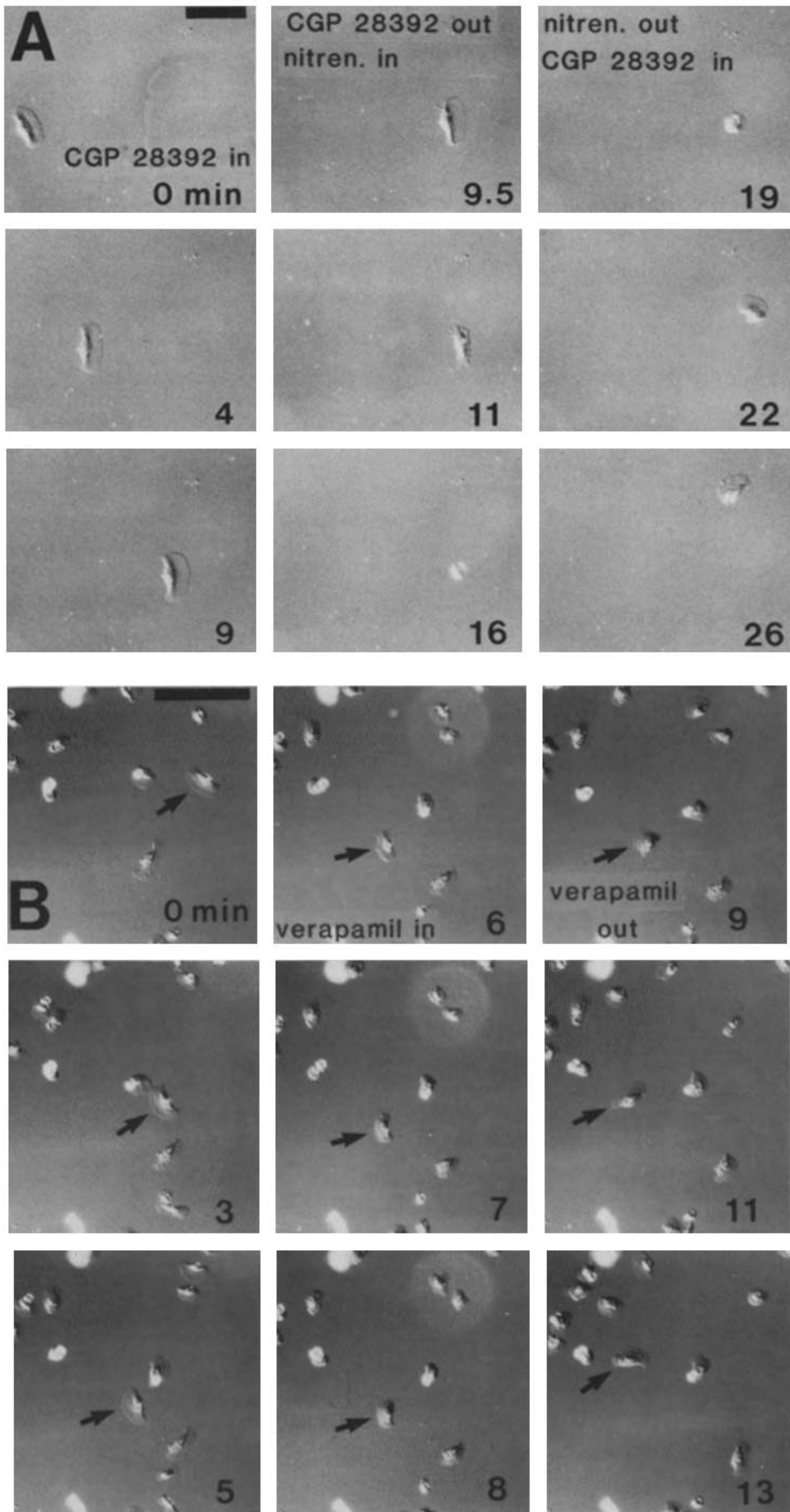


Figure 9. Effects of drugs that open or close calcium channels. (*A*) CGP 28392 and nitrendipine. 0–9 min: A spontaneously migrating keratocyte is unaffected by 22 μM CGP 28392, which stimulates the opening of calcium channels. 9.5–16 min: Medium with CGP 28392 is removed. The same cell is exposed to 44 μM nitrendipine, a calcium channel antagonist and a competitive inhibitor of CGP 28392. The cell rapidly retracts its periphery and rounds up. 17–26 min: Nitrendipine medium is removed and is replaced with 22 μM CGP 28392 medium. The cell respreads and resumes locomotion. Bar, 50 μm. (*B*) Effects of verapamil. Spontaneously migrating cells (0–5 min) are exposed to 50 μM verapamil at 5 min. Cells retract their lamellipodia and cease locomotion (6–8 min). After the verapamil is washed away (8 min), the cells re-extend broad lamellipodia and resume locomotion. Arrows trace the behavior of a single cell. Bar, 100 μm.

orescence micrographs of cells treated in this way. The cells shown in Fig. 11, *A–C* detached from the leading edge of a cell sheet that was migrating towards the cathode in an electric field of 13.3 V/cm (Fig. 11*D*). No microtubular structures within the entire cell culture are detectable by immunofluorescence microscopy. The complete absence of microtubules in cells subjected to cold/nocodazole treatment was further confirmed by electron microscopy (not shown). Fig. 11 illustrates that both normal migratory morphology and directional motility of the cells were maintained in the complete absence of microtubules. The ability of the cells to be guided by a DC electric field was also unaffected.

Discussion

Mechanics of Directional Locomotion

Several hypotheses have been proposed to explain persistent directional locomotion of tissue cells in culture. It has been suggested that polarized cytoskeletal elements in migratory cells, such as microtubule bundles or stress fibers, might determine the directionality of future cell movements (1). Other hypotheses are based on the localization of certain organelles within the cell such as the Golgi apparatus and the centrosome (2, 27, 48, 54). Microtubule organizing centers and the Golgi apparatus shift to the leading edge of the nucleus in migrating endothelial cells and fibroblasts (27, 48). It has been proposed that these organelles might direct cell locomotion by causing new membrane to be added to the existing cell membrane in nearest proximity to the Golgi apparatus (48). While these hypotheses may be applicable to certain cell types, they do not explain several aspects of fish epidermal cell locomotion.

The directional migration and maintenance of morphology of epidermal cells in the absence of microtubules (Fig. 11) suggest that these structures do not generate cell polarity. This conclusion is further supported by the observation that anucleate, centrosome-, and microtubule-free lamellar fragments from keratocytes are capable of directional motility (21). This is in contrast to cultured neurons, fibroblasts, and endothelial cells, in which microtubules are required to maintain an elongated cell shape and directional movements (25, 27, 75, 79).

The canoe shape and polarity of fish epidermal cells can be described simply and concisely in terms of concepts proposed by Kolega (41, 42) to explain the shape and persistent locomotion of fish melanoma cell clusters. Kolega emphasizes that whenever a cell or cell cluster elongates, cell margins with little protrusive activity will tend to be pulled taut by the outward pull of more active regions. This induced tension tends to further reduce lamellar activity in the less active regions. A distribution of lamellar activity therefore becomes self-reinforcing. Cells will persist in their direction of movement if a relatively constant distribution of lamellar activity is generated (42).

A migrating fish keratocyte is pulled into an elongated shape by the two regions of the lamellipodium on either side of the nucleus, whose tractional forces are oriented in opposite directions (see Fig. 2). The region of the lamellipodium in front of the nucleus, however, has no opposition due to the lack of a lamellar region behind the nucleus. Thus, the cell is displaced forward due to the force generated by this unbalanced edge. Persistent locomotion in this direction occurs because a competing lamellar extension fails to form in the trailing nuclear region.

According to Goodrich (26), the trailing nuclear region of keratocytes is easily displaced from the substratum with a microneedle, whereas the lamellipodium is tightly attached. There may be multiple reasons for why the nuclear region of the cell membrane is loosely associated with the substratum, and consequently does not support lamellar spreading. For example, it may be less adhesive than other areas of the membrane. Alternatively, the bulging nuclear region, with its greater curvature, may create vertical tension at local membrane–substrate contact zones when the entire cell membrane is stretched taut during lamellipodial extension. In either case, overall cell movement is dominated by a mechanical instability between the front and the rear of the cell.

Stimulus Accommodation and the Mechanism of Galvanotaxis

Two major hypotheses have been proposed to explain how an applied electric field could interact with a cell and direct its locomotion. First, an applied electric field will impose a gradient of membrane potential upon a cell, causing the anode- and cathode-facing halves of the cell to become hyperpolarized and depolarized, respectively. This imposed membrane potential gradient will asymmetrically alter transmembrane ion fluxes (e.g., Ca^{2+}) across the cell, which may bias cytoskeletal and motile activity (34, 37). Second, electric fields $\gtrsim 0.1$–1 V/cm can redistribute mobile charged components in the plane of the cell membrane through lateral electrophoresis or electroosmosis (36, 58, 68). It has been proposed that the accumulation of ion channels or adhesive molecules at specific ends of a cell might bias protrusive activity, and thereby direct cell locomotion (38, 66).

While either of these mechanisms could conceivably bias the movements of a spontaneously migrating cell, it is important to ask first whether such interactions should strongly stimulate motile activity. The issue is central to understanding why cells do not rapidly accommodate and ignore the electric field. To address why DC electric fields stimulate and persistently bias the motility of keratocytes, it is useful to examine briefly how other cells adapt to external stimuli. As specific examples, we will discuss chemotaxis in bacteria and leukocytes, and electrical stimulation of motility in ciliates.

Bacteria and leukocytes transiently change their motile behavior when the absolute concentration of a chemotactic factor is rapidly shifted in their external medium (53, 90). Bacteria alter their tumbling frequency, whereas leukocytes change the lamellipodial and ruffling activity of their periphery. In both cases, the cells adapt to the new concentration and return to their baseline of activity (53, 90).

In bacteria, it is known that enzymes in the biochemical pathway that regulate flagellar beating are covalently modified (methylated or demethylated) as the cells adapt to new concentrations of chemotactic agents (28, 45). The level of methylation, which alters enzymatic activity and effectively acts as an antagonist against chemotactic stimulation, is roughly proportional to the average concentration of the chemotactic agent (46). In this manner, the bacterium can desensitize itself to absolute levels of stimuli, while increasing its ability to discriminate small changes in stimuli in a large background level of the same stimulus (47).

Leukocytes use a somewhat different system of accommo-

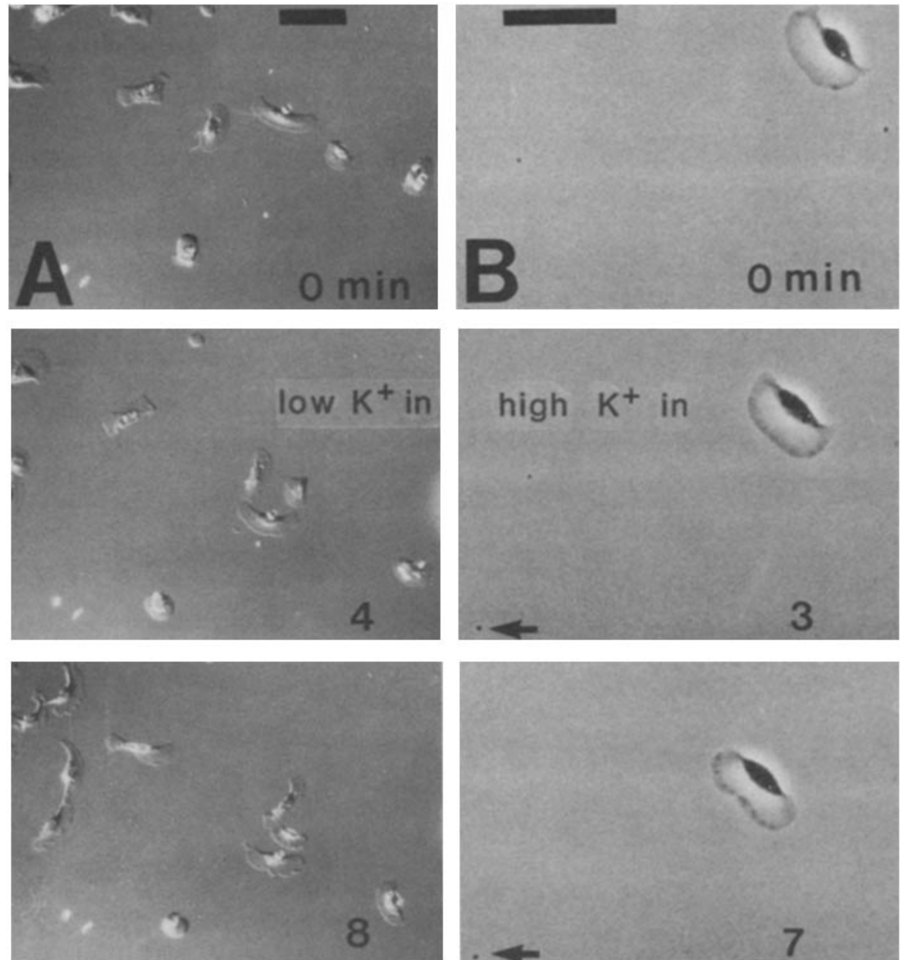


Figure 10. Effects of low and high K^+ media. Bars, 50 μm. (*A*) Cells migrating spontaneously in normal Fish Ringer's solution (0 min) are exposed to a solution of 1 mM $CaCl_2$ and 134 mM sodium gluconate at 3:30 min. Cell motility is unaffected (4 and 8 min) by a large reduction of Cl^- and total removal of K^+ from normal Fish Ringer's solution. (*B*) A cell migrating in normal Fish Ringer's (0 min) is exposed to a solution of 67 mM potassium gluconate, 67 mM sodium gluconate, and 1 mM $CaCl_2$ at 1 min. The cell continues to migrate normally in this media (3 and 7 min) which should tend to depolarize the cell membrane. Arrows point to a reference particle. (*C*) Keratocytes exposed to a 14 V/cm electric field in 1 mM $CaCl_2$, 67 mM sodium gluconate, and 67 mM potassium gluconate. Cells migrate towards the cathode with normal morphology in this high K^+ media. A cluster of cells is dissociating into single migratory cells in the center of the photo. (*D*) A keratocyte cluster exposed to a 11 V/cm electric field migrates towards the cathode in a medium composed solely of 1 mM $CaCl_2$ and 134 mM sodium gluconate.

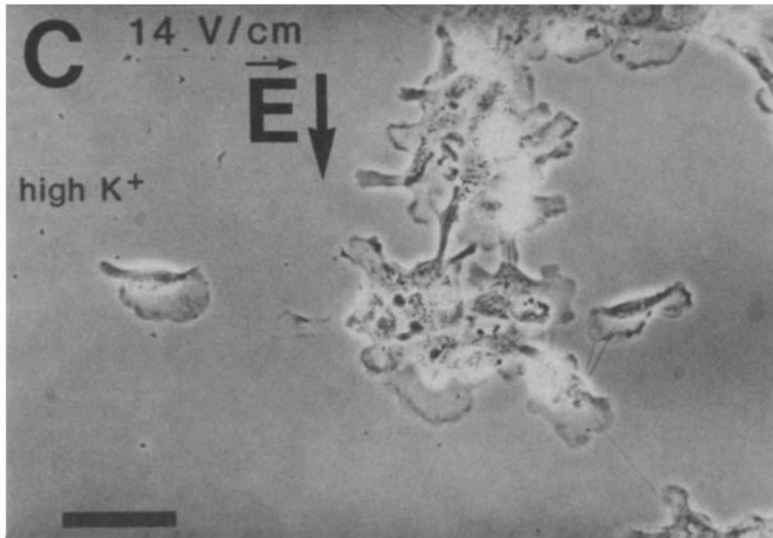
dation to chemotactic stimuli. Chemoreceptors are removed from or inserted into the leukocyte cell membrane to accommodate changes in background levels of chemotactic stimuli (90). This change in chemoreceptor density in leukocyte membranes is, however, functionally equivalent to the modification of enzymatic activity in bacteria, since both systems essentially reset the basal rate of motile activity to the average level of chemotactic stimulus in the external medium. In both leukocytes and bacteria, a chemotactic gradient continuously stimulates motile activity since the mean value of the chemotatic stimulus changes as the cell moves up or down the gradient.

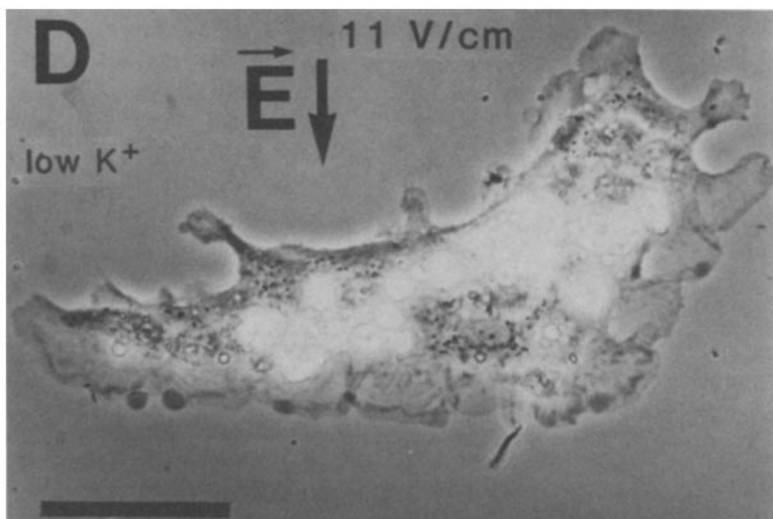
Bacteria need not have a specific receptor for detecting an environmental stimulus. Agents that suddenly alter membrane potential initiate transient changes in bacterial tumbling rates, in the same manner that concentration shifts of chemoattractants and repellents elicit motile reactions (59). Thus, a bacterium will accommodate changes in absolute membrane potential, and exhibit motile reactions to perturbations from a resting membrane potential to which it was previously accommodated (59).

Similar behavior is seen in ciliates. When *Paramecia* are transferred to solutions that induce a depolarization of the cell membrane (e.g., high KCl), voltage-sensitive Ca^{2+} channels open, which results in an influx of extracellular Ca^{2+} that triggers ciliary reversal and backwards swimming (61, 62). This response is, however, transient. Eventually, the cells adapt to the new medium and swim forward with a normal ciliary stroke. Adaptation may arise from permeability changes of the cell membrane which tend to restore the potential drop across the cell membrane to its initial value (17). Accommodation to depolarization and associated Ca^{2+} influx may also occur by increased Ca^{2+} pumping or Ca^{2+} binding, or by a reduction of the Ca-conductance of the ciliary membrane (51). The same processes shifted in the opposite direction may explain the accommodation of ciliates to hyperpolarizing stimuli (51).

Careful studies of ciliary motor responses under voltage-clamp control have shown that sequential hyperpolarization and depolarization stimuli act as mutual antagonists (51). It is believed that membrane hyperpolarization antagonizes effects of depolarization in that it acts to reduce intracellular Ca^{2+} concentration (e.g., inducing closure of voltage-sensitive Ca-channels), and that depolarization antagonizes effects of hyperpolarization through the raising of intracellular Ca^{2+}. In this context, internal Ca^{2+} serves as a second messenger for triggering accommodation to membrane potential perturbations.

While it is clear that many cells can accommodate changes in absolute membrane potential when the ionic composition of their medium is changed (17, 62, 73, 86, 88), we wish to raise the issue of whether the same cells can accommodate a gradient of membrane potential induced by a DC electric field. Analogous to the motor responses of ciliates (51), we suggest that field-induced hyperpolarization and depolarization act as mutual antagonists for generating motile activity





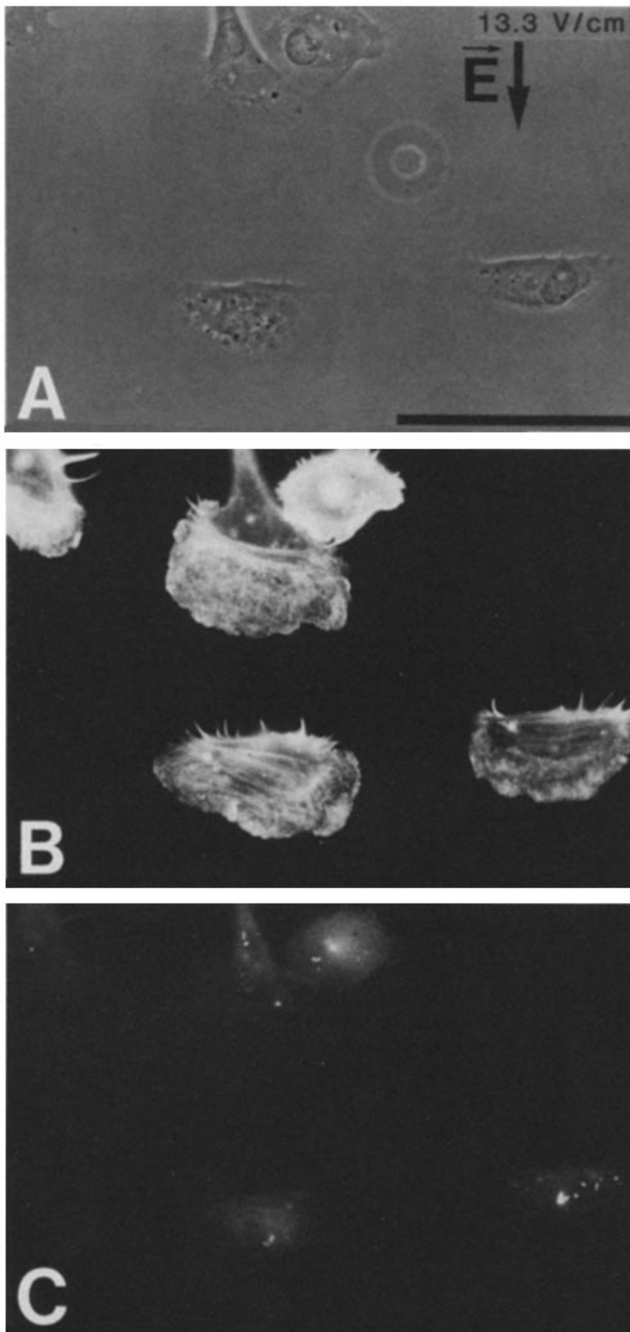
in keratocytes exposed to DC electric fields. Furthermore, like in ciliates, we suggest that this antagonism arises because hyperpolarization and depolarization shift the metabolism of the cell, and possibly the level of intracellular Ca^{2+}, in opposite directions.

Both hyperpolarization and depolarization of a cell membrane can be expected to initiate accommodative processes that tend to counteract excitation and restore the local concentration of cytoplasmic Ca^{2+} to its initial level. However, if regulatory molecules and enzymes involved in these pathways are free to diffuse through the cytoplasm, the cell may not be able to counterbalance the effects of hyperpolarization and depolarization simultaneously (i.e., when the cell is placed in a DC electric field). Cytoplasmic molecules produced in the accommodative process for the hyperpolarized side of the cell may diffuse into the depolarized region of the cell and antagonize the ability of this region to accommodate the local depolarization. Conversely, diffusing molecules involved in the depolarization accommodative processes may antagonize the hyperpolarized region of the cell to accommodate its local perturbation. As a consequence, the hyperpolarized and depolarized regions of the cell should be expected to show strong motile reactions, since deviations from the average membrane potential are chronically induced by the external electric field. A similar model for gradient sensing and background accommodation, based upon the diffusion of internal regulatory molecules, was proposed for leukocyte chemotaxis some time ago (89).

Ca^{2+} Influx in Keratocytes and Motile Activity

The inhibition of fish keratocyte motility with 1 mM La^{3+}, 10 mM Co^{2+}, 50 μM verapamil, and 50 μM nitrendipine are strong evidence that an influx of extracellular calcium through specific channels in the cell membrane is required to generate motile activity. The concentration of heavy metal ions required for inhibition are comparable to the levels used to block Ca^{2+}-dependent action potentials, contractility, and cell locomotion in other cell systems (7, 30, 65, 71, 78). Strohmeier and Bereiter-Hahn have recently shown the inhibition of amphibian keratocyte locomotion with 2 mM La^{3+} or 4 mM Tb^{3+} (76). Furthermore, they have reported that 1–4 mM Sr^{3+} in the external medium can replace Ca^{2+} for triggering cell locomotion. These results, as well as ours, suggest that a frequently opening Ca^{2+}-channel transduces the cytoskeletal and motile activity in both fish and amphibian keratocytes.

Since Ca^{2+} influx appears to generate motile activity in keratocytes, it is reasonable to expect that an electric field could bias cell locomotion by altering the Ca^{2+} fluxes through



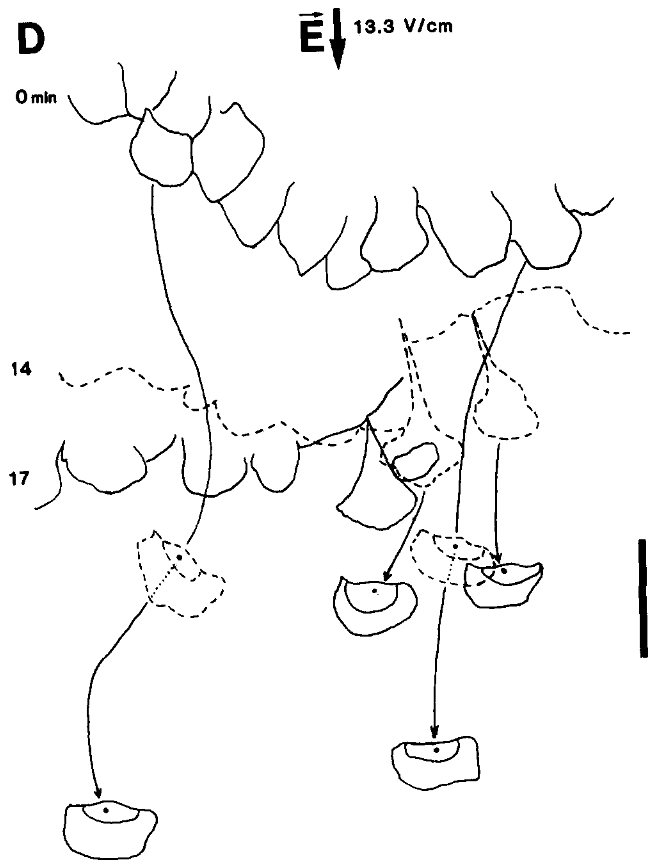


Figure 11. Galvanotaxis of keratocytes with no microtubules. (*A*) Phase micrograph of fixed cells that were previously migrating in an electric field of 13.3 V/cm and treated with nocodazole/cold to diassemble microtubules. The cells correspond to cells at the right side of *D* at 17 min. Bar, 50 μm. (*B*) Actin stain of cell in *A*. (*C*) Microtubule antibody stain of same cells shows no extended microtubules. A few microtubule fragments are located near cell nuclei. (*D*) A culture was treated with nocodazole and cold (4°C) to disassemble microtubules. The culture was warmed to room temperature in the presence of nocodazole and exposed to a 13.3 V/cm electric field. The tracing of the cell sheet at three time points (in minutes) are shown. The cell advances towards the cathode. Cells frequently detach from the leading edge of the sheet, migrating more rapidly as they are freed from mechanical constraint. The cluster was fixed seconds after the 17-min timepoint and stained for microtubules and actin (*A–C*). Bar, 50 μm.

the cell membrane. The degree by which transmembrane Ca^{2+} fluxes of a cell are perturbed by an electric field can be directly estimated from membrane potential perturbations that are induced by the field. A uniform electric field of magnitude E will impose a linear gradient of membrane potential on a flat cell (which is smaller than the electrical length constant of its membrane) given by

$$\Delta\Psi(x) = \Delta\Psi_0 - Ex, \quad (1)$$

in which x is the distance from the geometric center of the cell in the direction of the field, and $\Delta\Psi_0$ is the resting membrane potential of the cell before the application of the electric field (9). The perturbations to the membrane potential expressed in Eq. 1 will alter the driving force for Ca^{2+} influx through the cell membrane. At a specific region of the cell membrane, the transmembrane Ca^{2+} current density I is determined by the local Ca^{2+} conductance of the cell membrane, G_{Ca} (possibly voltage-sensitive), the reversal potential for Ca^{2+}, $\Psi_{rev_{Ca}}$, and the transmembrane potential $\Delta\Psi$:

$$I_{Ca} = G_{Ca} \cdot (\Delta\Psi - \Psi_{rev_{Ca}}). \quad (2)$$

With 10^{-7} M intracellular free calcium, 1 mM external free calcium, and a given resting membrane potential of −50 mV, the total driving force $(\Delta\Psi - \Psi_{rev_{Ca}})$ fo calcium entry will be ~170 mV (17).

We have found that the fish keratocytes can be directed by electric fields as low as 0.5–1 V/cm. These fields will induce a 1–2 mV potential drop across a cell that is 20-μm wide.

From Eq. 1, it can be seen that these potential drops produce a 0.5–1 mV hyperpolarization and depolarization, respectively, of the extreme anode- and cathode-facing membranes of the cells. These transmembrane potential perturbations change the theoretical driving force for Ca^{2+} of 170 mV by ~ ± 0.3–0.6% across the length of the cell.

Besides altering the driving force for transmembrane ion fluxes, it is also very probable that the electric field–induced depolarizations and hyperpolarizations used in our experiments (1–30 mV) will affect voltage-sensitive Ca^{2+} channels in keratocytes. In this case, Ca^{2+} channels would be expected to open more frequently on the cathode-facing (depolarized) side of the cell and open less frequently on the anode-facing side of the cell. This asymmetry in calcium conductance is likely to be the major calcium stimulus for directional locomotion towards the cathode.

Recently, it has been proposed that Ca^{2+} channel activation/inactivation, in response to depolarizing and hyperpolarizing stimuli, may be regulated by phosphorylation/dephosphorylation of the channel (18). If the activation and inactivation of Ca channels in keratocytes are regulated by such processes, it is likely that the kinases and phosphatases that act on the channel mutually interfere with each other's actions as they diffuse through the cell. As we have previously argued, this type of metabolic interference could prevent a cell, which is exposed to an electric field, from producing the appropriate accommodative reactions (in this case Ca^{2+} channel activation/inactivation) to simultaneously counterbalance the effects of depolarization and hyperpolarization.

The mutual antagonism between depolarization and hyperpolarization accommodative processes, interacting across the cell because of diffusion, may provide the explanation for why epithelial cells, fibroblasts, and neural crest cells selectively retract both their anode- and cathode-facing protrusions when exposed to an electric field (10, 19, 50). If accommodation to a membrane potential perturbation (hyperpolarization or depolarization) was an entirely local process, these cells should be expected to rapidly accommodate to the voltage perturbations (typically 1–30 mV) and not withdraw protrusions in response to the field. Changes of a few millivolts to a few tens of millivolts in membrane potential should easily be produced by a 10-fold change in external potassium, which is unlikely to alter the motile behavior of the cell in a comparable manner (note keratocytes in Fig. 10). Furthermore, it is unlikely that only one of the perturbations induced by the field (hyperpolarization or depolarization) should be solely responsible for a persistent withdrawal of protrusions and an onset of motility.

Several investigators have previously discounted membrane potential perturbations as a means of evoking galvanotatic and galvanotropic responses in low electric fields that produce a 1 mV potential drop across an individual cell (37, 63, 77). They have argued that such changes in membrane potential should not influence membrane ion conductances or significantly alter the driving forces for transmembrane ion fluxes. Changes in ciliary motor responses in the ciliates *Stylonchia* and *Paramecium*, however, have been produced by only 2–3 mV perturbations under voltage-clamp conditions (14, 52). Furthermore, it is known that leukocytes can orient and directionally migrate with only a ~1% concentration difference of a chemotactic agent across their dimensions (89). It therefore seems possible that a ≳1% difference in transmembrane Ca^{2+} fluxes, induced by a 1 mV potential drop across a cell, could direct keratocyte locomotion, as well as the locomotion of other cells.

Although it is possible that the redistribution of membrane components by electrophoresis or electroosmosis could orient a spontaneously migrating cell (36, 38), it is difficult to explain why motile activity should dramatically increase in the center of a confluent sheet of cells, such as the keratocytes in Fig. 4, solely because mobile components in the plane of the cell membrane are redistributed by the field. We are presently designing experiments that will separate the contribution of membrane electrophoresis and membrane potential perturbations to the galvanotaxis of individual keratocytes. However, on the basis of our present experiments, we believe that the major increase in cell motility in keratocytes is due to the effects of an imposed gradient of membrane potential. Since this same onset of motility is seen in a broad spectrum of cells exposed to DC electric fields, we argue that in general, whenever a cell cannot counterbalance the effects of differential stimuli, such as a chemoattractant gradient or an imposed gradient of membrane potential, directed motility will ensue as antagonistic or accommodative controls on motile activity become unbalanced across the cell.

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Note Added in Proof. Recently, we have found that keratocytes that are paralyzed by 10 mM Co^{2+} can be reactivated with calcium ionophore A23187 at 5–20 μM. These cells, however, show a distinct reversal in their direction of galvanotaxis, migrating instead toward the anode. Cells treated with A23187 alone do not reverse their direction of migration. The fact that reversal does not occur until Ca^{2+} channels are blocked by Co^{2+} strongly indicates that the keratocyte's Ca^{2+} channels provide the dominant polarity cue for cathode-directed galvanotaxis (Cooper, M. S., and M. Schliwa, Ionic Currents in Development Conf., UCLA August 1985, Alan R. Liss, Inc., in press).

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