Perpendicular orientation and directional migration of amphibian neural crest cells in dc electrical fields

galvanotaxis/confluence disruption/membrane potential/Ca2+ fluxes

M. S. Cooper* and R. E. Keller†

*Biophysics and Medical Physics Group and †Department of Zoology, University of California, Berkeley, CA 94720

Communicated by Harry Rubin, August 26, 1983

ABSTRACT The behavior of cultured neural crest cells of Ambystoma mexicanum and Xenopus laevis in dc electrical fields was studied. In fields of 1–5 V/cm, isolated or confluent cells retract both their anode- and cathode-facing margins. Subsequently, the cells elongate, with protrusive activity confined to their narrow ends. In larger fields (≥5 V/cm), protrusions form on the cathode-facing sides of the perpendicularly oriented cells. The cells then begin migrating laterally, perpendicular to their long axes, towards the cathode. We suggest that the perpendicular alignment and cathode-directed migrations result from cytoskeletal changes mediated by modified ion fluxes through the anode-facing (hyperpolarized) and cathode-facing (depolarized) cell membranes. The breaking of cellular confluence in response to dc electric fields is also discussed.

Several findings suggest that the embryonic environment of the neural crest pathways provide factors that support and perhaps guide the movements of neural crest cells and of motile cells in general (1). Melanocytes (2), clumps of normal fibroblasts, and individual transformed fibroblasts (sarcoma 180) (3) migrate through the ventral (perineural) pathway when they are grafted into the neural crest region of avian embryos during neural crest cell migration. Determination of the factors that control the movements of neural crest cells during embryogenesis may be important, therefore, in understanding the behavior of motile and invasive cells in adult tissues (3).

It has been shown that the shape and orientation of cultured amphibian neurons and myoblasts can be changed by constant electrical fields (4). Neurons extend towards the cathode in dc fields, whereas myoblasts extend protrusions at right angles to the fields and become perpendicularly aligned. We report here that dc electrical fields induce both perpendicular orientation and directed migration of cultured amphibian neural crest cells. Furthermore, monolayers of these cells break confluence in response to the applied fields as they become perpendicularly aligned. Preliminary accounts of these findings have been reported elsewhere (5, 6).

MATERIALS AND METHODS

Embryos of the mexican axolotl Ambystoma mexicanum were acquired from the Indiana University Axolotl Colony (Bloomington, IN). Xenopus laevis frog embryos were obtained from mating adults by standard methods. To obtain neural crest cells, cuts were made through the trunk epidermis, parallel and lateral to the neural tube of demembranated stage 28 larvae. The embryos were placed in a solution of collagenase (1 mg/ml; 107 units/ml, Worthington) in 100% Steinberg’s solution (7). When loosened, the epidermis overlying the neural tube was peeled off with forceps. The neural crest was then teased from the top of the neural tube with a hair loop. The crest was washed in culture medium, cut into explants of 50–200 cells, and cultured in 50% (vol/vol) L-15 medium/10% (vol/vol) fetal calf serum. These procedures produce cultures of pure crest cells whose motile behavior, contact, and differentiation has been characterized in detail (8). The cells were cultured in cross-shaped chambers patterned after a design used by Hinkle et al. (4). These chambers were formed by supporting a 15 × 15 mm no. 1 coverslip above the surface of a 35-mm plastic culture dish with 5 × 5 mm supports of the no. 1 coverslip at each corner. All surfaces were sealed together with minimal amounts of dental wax by using a warm spatula. Wax dams were extended from the corners of the chamber to the wall of the culture dish, forming four wells. These wells were connected to each other only through the cross-shaped space of the culture chamber beneath the coverslip roof. Agar/saline (100% Steinberg’s solution gelled with 2% agar) bridges were placed at the ends of one of the chamber’s channels and connected to wells containing 100% Steinberg’s solution and platinum electrodes. The platinum electrodes were connected to a constant current source. Applied electric field strengths were determined by the method of Jaffe and Poo (9).

Cell behavior was recorded through a Zeiss inverted microscope with phase-contrast optics on 35-mm film and on video tape (Panasonic time-lapse video recorder and camera). All experiments were performed at ambient temperature, ~20°C.

RESULTS

Orientation and Alignment. Fig. 1 shows changes of cell shape in a culture exposed to an electric field of 14.4 V/cm. Initially (Fig. 1a), the cells were multipolar and somewhat elongated but with no preferred axis of orientation (Fig. 2). Seconds after the field was applied, saltatory movements of intracellular particles accelerated. In addition, the cells shortened in the direction parallel to the field by withdrawing protrusions simultaneously from both their anode- and cathode-facing sides. This behavior was observed for isolated cells and for cells in confluent monolayers. As the cells shortened parallel to the direction of the field, they also extended existing protrusions and/or produced new ones at right angles to the field. Thus, the cells became progressively more elongated and bipolar with their long axes aligned perpendicular to the applied field (Fig. 1b and c and Fig. 2). In other experiments, we observed that when the electrical field was removed, elongated cells respread, became less elongate, and returned to multipolar patterns (Figs. 4 and 5).

In fields of 1–15 V/cm, cells lost protrusions at the cathode- and anode-facing sides, and these margins of the cells withdrew. Protrusive activity remained only on the two ends directly perpendicular to the field, which made a major contribution to the further elongation of the cells. Cells that initially had protrusions only facing the anode and cathode lost

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
all major protrusions upon application of the field and, thus, rounded up. These cells subsequently formed protrusions only at the ends lying perpendicular to the field and elongated perpendicular to the field.

**Lateral Directional Migration.** In fields ≥ 5 V/cm, undifferentiated *Xenopus* and axolotl neural crest cells migrated laterally, perpendicular to their long axes, towards the cathode (Fig. 3). During these movements, lateral protrusions were found almost exclusively on the sides of the cells facing the cathode. These cathode-facing protrusions can be seen on axolotl cells in Fig. 1b (arrowheads). Protrusive activity consisted of the same microspike–lamellipodium complex as that seen in the absence of dc electric fields (see ref. 8); thus, the character of motile behavior is not changed in the applied fields.

These lateral protrusions are unusual in that they were small and uniform in size. Large protrusions, capable of dominating the movement of the cell, did not form in the direction of the field. In the absence of an applied field, these cells constantly changed shape and position as first one and then another major protrusion dominated a cell’s movements (8).

The neural crest cells were found to align perpendicularly to the applied electric fields and also to migrate laterally to-
DISCUSSION

Galvanotactic Responses of Animal Cells. A number of cell types have been found to migrate towards the cathode in electrical fields. Cathode-directed galvanotaxis of amoebae has been known since 1896 (10). The growth cones of amphibian and chick neurons move towards the cathode in electrical fields \( \geq 0.7 \text{ V/cm} \) (4, 9, 11, 12). Recent papers have reported field thresholds of 0.1 V/cm for the initiation and bending of neurites towards the cathode (4, 11). Recently, Erickson and Nuccitelli have observed perpendicular orientation and cathode-directed migration of quail fibroblasts and neural crest cells in fields of 1.5–6 V/cm (13, 14). Stump and Robinson have observed independently similar behaviors using cells (probably fibroblasts or neural crest) isolated from tail-bud \textit{Xenopus} embryos (15). Frog epithelial cells align perpendicularly to fields of 5 V/cm and exhibit cathodal galvanotaxis at 15 V/cm (16). Most cell types show directed movement towards the cathode, though macrophages have been reported to be attracted to the anode in dc fields of 4–12 V/cm (17).

In vivo amphibian neural crest cells migrate unidirectionally and parallel to their long axes as they leave the neural tube, with nearly all protrusive activity at their leading edge (8). Electrical fields may control these movements (18), but proof of this attractive hypothesis will require measurement and manipulation of endogenous embryonic fields.

If endogenous fields control neural crest cell movement in vivo, the fields must be of sufficient strength to generate definitive, unidirectional cell migration. In culture, we have found such directed migrations in fields \( \geq 5 \text{ V/cm} \), which may be many times larger than internal bioelectric fields of larval amphibians. However, regardless of their developmental relevance, the electric field-induced perpendicular orientation, elongation, and cathode-directed migrations are phenomena that must be explained in terms of cell motility mechanisms.

Mechanism of the Electric Field Effect. A dc electrical field may act directly on a cell in several ways. Through lateral electrophoresis and electroosmosis, charged membrane components can accumulate at either the anode- or cathode-facing ends of a cell (19, 20). Acetylcholine receptors, concanavalin A receptors, and charged lipids move to specific ends of cultured embryonic cells in electric fields (20, 21).

In addition to these effects, applied electric fields of 1–15 V/cm can alter significantly the cell's transmembrane potential (22). For a flattened cell on a culture dish, a linear voltage drop will occur over the surface of the cell in the extracellular medium if the current is uniform and parallel to the substratum. Because very little current penetrates the cell membrane, the cytoplasm remains essentially an equipotential region. The transmembrane potential of the cell thus becomes a function of position, which is given by

\[
\Delta \psi(r, \theta) = \Delta \psi_0 - E \bar{f} \cos \theta
\]

where \( \Delta \psi_0 \) is the initial transmembrane potential, \( E \) is the applied electric field strength, and \( \bar{f} \) and \( \theta \) are positional coordinates from the cell's electrical center as defined in Fig. 6. For a cell of 100-\( \mu \)m length in an electrical field of 2 V/cm, the anode-facing membrane will be hyperpolarized by 10 mV over its initial transmembrane potential, whereas the catho-
ode-facing membrane will be depolarized by 10 mV. Note, however, that the transmembrane potential along the perpendicular midline of the cell ($\theta = 90^\circ, 270^\circ$) remains at $\Delta \psi$.

The initial response of neural crest cells in electric fields is the withdrawal of their outlying protrusions or margins in the direction of the applied field, while maintaining protrusions at right angles to the field. Moreover, further elongation is connected with presumptive activity localized at these ends of the cells. Thus, protrusions are withdrawn in regions of extreme transmembrane potential perturbation (hyperpolarization or depolarization), while protrusions are extended at the margins of the cell, which remain at their initial transmembrane potential.

We suggest that the initial selective withdrawal of protrusions from the anode- and cathode-facing sides of the cells is in response to transmembrane potential perturbation. We believe that this membrane potential mechanism is the basis for the common perpendicular orientations of avian and amphibian neural crest cells, fibroblasts, epithelial cells, and myoblasts in dc electrical fields (4–6, 13–16, 20).

The presumptive behavior involved in the perpendicular orientation and galvanotaxis of neural crest cells in dc electrical fields is likely connected with contractile activity of the cytoskeleton. There is considerable evidence that solution and contraction of the cytoskeleton at a given site in the cell is controlled by the local ionic environment and that Ca$^{2+}$ levels play a major role in regulating these events (23). Free Ca$^{2+}$ is normally present at low levels in cells (<0.1 $\mu$M) (24). When free Ca$^{2+}$ exceeds micromolar concentrations, solution of the cytoel and contraction of the actin–myosin system occurs. Raising cytoplasmic free Ca$^{2+}$ by exposure of cultured neural crest cells to calcium ionophore A23187 results in rapid retraction of cellular protrusions, presumably by contraction (25). Likewise, the increase of free Ca$^{2+}$ is a likely mechanism to explain how transmembrane potential perturbations resulting from applied electric fields could selectively induce retraction of the anode- and cathode-facing protrusions of the cells in our experiments.

Hyperpolarization of the cell membrane results in a larger electromotive force to drive cations into the cell. Thus, influx of free extracellular Ca$^{2+}$ will be enhanced on the anode-facing membrane of a cell in an applied electrical field. Such influx might be involved in the retraction of the anode-facing protrusions of the cells in our study. Electrically enhanced influx of extracellular Ca$^{2+}$ has been invoked to explain the accelerated utricle retraction of amoebae during cathode-directed galvanotaxis in dc electrical fields (26).

Voltage-sensitive Ca$^{2+}$ channels are present in embryonic neurons (27). If these channels are also present in neural crest cells, they may have a role in the withdrawal of depolarized protrusions. However, the ionic fluxes postulated to underlie the selective retraction of anode and cathode protrusions remain to be determined experimentally.

Patel and Poo have shown that concanavalin A receptors are electrophoresed to the tips of amphibian neuron growth cones growing towards the cathode in dc electrical fields (11). They have suggested that cathodal receptor accumulation may be causally related to cathode-directed outgrowth of neurites. Concanavalin A receptors have been found in adhesion plaques (28), a fact consistent with the argument that their localization might stimulate adhesion and migration.

Such electrophoretic accumulation of concanavalin A receptors or other surface proteins might be involved in the cathode-directed presumptive activity and migration that we have observed during galvanotaxis of neural crest cells. It is also plausible that this presumptive activity is stimulated by modified ion fluxes connected with the depolarization of the cathode-facing plasma membrane.

**Maintenance of Orientation During Galvanotaxis.** Cell shape is continually controlled by the field throughout galvanotaxis because the perpendicular cells never elongate in the direction of migration, even though virtually all lateral presumptive activity occurs on one side of the cell. It is clear from Eq. 1 that, as any lateral protrusion elongates in the direction of the cathode, its membrane will become progressively more depolarized while, simultaneously, the trailing margin of the cell will become increasingly hyperpolarized. This is because protrusion extension towards the cathode not only increases the voltage drop across the cell but also shifts the electrical midline of the cell forward. We suggest that this increasing hyperpolarization results in the retraction of the anode-facing margin, possibly because of an enhanced influx of extracellular free Ca$^{2+}$ (Fig. 7). Thus, individual lateral protrusions would be prevented from elongating, enlarging, and dominating the shape and movement of the cell. This provides an explanation for why the cells remain perpendicular to the field as they move laterally under the influence of many small protrusions.

Patel and Poo (11) have observed that amphibian neurons in 5-V/cm fields retract their trailing cell bodies as growth cones are extended towards the cathode, maintaining relatively constant lengths as the neurons galvanotactically migrate. Because growth cones exert only small tractive forces (29) and do not displace cell bodies under normal culture conditions, it seems likely that the retraction of the trailing cell bodies during galvanotaxis is related to a disassembly of cytoskeletal elements in the anode-facing region of the neuron followed by detachment.

We suggest that the retraction of the trailing neuron cell bodies is analogous to the retraction of the anode-facing margin of migrating neural crest cells. As a neuron extends an axon in the direction of the field, the trailing portion of the cell becomes progressively more hyperpolarized. An enhanced influx of extracellular free calcium because of this
FIG. 7. Transmembrane potential of a perpendicular cell migrating in a dc electric field. The electrical midline of the cell in shifts forward (a) as protrusions are formed on the cathode-facing side of the cell (b). Simultaneously, the anode- and cathode-facing sides of the cell become more hyperpolarized and depolarized, respectively. Enhanced Ca\(^{2+}\) influx because of the increasing hyperpolarization (vertical arrow) may be the cause of the retraction of the anode-facing cell margin. (c) Displacement of the cell forward as the anode-facing side is retracted and as new protrusions form towards the cathode.

hyperpolarization might explain the unusual retraction of the cell body.

Guidance of Cell Movement and Confluence Disruption in vivo. It has been proposed that endogenous bioelectrical currents may guide cell movements involved in wound closure, limb development, and morphallactric remodeling during limb regeneration (30, 31). Our results and those of others (13–16) may lend support to this proposal, in the sense that the motility of tissue cells in culture can be directed by dc electric field strengths on the order of the fields (2 V/cm) found at epidermal lesions in mammals (30). Further work is needed to determine whether or not endogenous electric fields control the migration of embryonic or adult tissue cells in vivo.

Externally applied dc electrical fields have been shown in several instances to stimulate limb and skin regeneration (32–36). In our experiments, monolayers of cells were disrupted as contacts with neighboring cells and the substratum were broken under the influence of the electric field. A cell type that exhibits density-dependent contact inhibition of cell division might be expected to renew proliferation in response to the electrically induced confluence disruption. We suggest that cellular proliferation, in response to electrically induced confluence disruption, might play a role in the hypertrophy of tissues exposed to either externally applied or endogenous bioelectric fields.

We thank Richard Borgens for several useful discussions and suggestions and Carol Erickson and Richard Nuccitelli for sharing unpublished results in the course of this study. We also thank John Shih for technical assistance. This work was supported by National

Research Service Award 1T32-GM07379 to M.S.C. and National Science Foundation Grant PCM 81-10985 to R.E.K.