Electric Field-directed Fibroblast Locomotion Involves Cell Surface Molecular Reorganization and Is Calcium Independent

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Abstract. Directional cellular locomotion is thought to involve localized intracellular calcium changes and the lateral transport of cell surface molecules. We have examined the roles of both calcium and cell surface glycoprotein redistribution in the directional migration of two murine fibroblastic cell lines, NIH 3T3 and SV101. These cell types exhibit persistent, cathode directed motility when exposed to direct current electric fields. Using time lapse phase contrast microscopy and image analysis, we have determined that electric field-directed locomotion in each cell type is a calcium independent process. Both exhibit cathode directed motility in the absence of extracellular calcium, and electric fields cause no detectable elevations or gradients of cytosolic free calcium. We find evidence suggesting that galvanotaxis in these cells involves the lateral redistribution of plasma membrane glycoproteins. Electric fields cause the lateral migration of plasma membrane concanavalin A receptors toward the cathode in both NIH 3T3 and SV101 fibroblasts. Exposure of directionally migrating cells to Con A inhibits the normal change of cell direction following a reversal of electric field polarity. Additionally, when cells are plated on Con A-coated substrata so that Con A receptors mediate cell-substratum adhesion, cathode-directed locomotion and a cathodal accumulation of Con A receptors are observed. Immunofluorescent labeling of the fibronectin receptor in NIH 3T3 fibroblasts suggests the recruitment of integrins from large clusters to form a more diffuse distribution toward the cathode in field-treated cells. Our results indicate that the mechanism of electric field directed locomotion in NIH 3T3 and SV101 fibroblasts involves the lateral redistribution of plasma membrane glycoproteins involved in cell-substratum adhesion.

DIRECT current (DC)[1] electric fields induce directional locomotion (galvanotaxis) in a wide variety of cultured cells (for review see Nuccitelli, 1988). Several cell types respond to applied electric fields in vitro at field strengths comparable to the endogenous electric fields thought to exist in vivo. It has therefore been suggested that endogenous electric fields may serve as guidance cues for migrating cells during processes such as embryogenesis and the repair of injury (Jaffe and Nuccitelli, 1977; Borgens, 1982). In addition to any potential physiologic significance, in vitro electric fields are a convenient and reliable means of inducing predictable morphological polarization and directional migration. Thus, applied electric fields are a valuable tool in the study of the factors regulating cell shape and motility.

Despite numerous studies on the mechanism(s) of galvanotaxis in recent years, it remains unclear how cells sense and respond to DC electric fields. Due to the high resistivity of the plasma membrane to current flow, electric fields are unlikely to significantly affect the cytosol directly (Jaffe and Nuccitelli, 1977). In contrast, electric fields are known to have distinct physical effects on the plasma membrane. It has been demonstrated that cells experience an asymmetric disturbance of plasma membrane potential toward the poles of an applied electric field. Cathode-facing membrane surfaces are depolarized while anode facing surfaces experience a hyperpolarization (Gross et al., 1986). Additionally, electric fields act on plasma membrane surfaces parallel to the field, causing the electrophoretic (Jaffe, 1977) and/or the electroosmotic (McLaughlin and Poo, 1981) lateral redistribution of several classes of plasma membrane glycoproteins.

Calcium is thought to be an important regulatory element in the process of cellular locomotion, and the directional migration of several cell types has been shown to involve calcium gradients (Hahn et al., 1992) or localized cytosolic calcium increases (Taylor et al., 1980; Brundage et al., 1991). A number of studies have examined the role of intracellular calcium in electric field-directed locomotion, as the perturbation of resting membrane potential by applied electric

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1. *Abbreviations used in this paper*: BSS, balanced salt solution; s-Con A, succinylated Con A; DC, direct current; DMI, directional migration index.

fields may lead to asymmetric calcium fluxes. It has been suggested that calcium influx might occur due to either the increased open probability of voltage-gated calcium channels at the depolarized cathodal membrane (Bedlack et al., 1992) or the increased electrical driving force for calcium entry across hyperpolarized, anode facing membranes (Chen and Jaffe, 1978). In agreement with these predictions, several reports have provided compelling evidence linking galvanic responses to electric field induced calcium fluxes. For example, the cathode-directed migration exhibited by avian neural crest cells, fish keratocytes, and mouse C3H/10T1/2 fibroblasts, is inhibited by calcium channel antagonists and by the removal of extracellular calcium (Cooper and Schliwa, 1985, 1986; Onuma and Hui, 1985; Nuccitelli et al., 1993). Electric fields have been shown to increase intracellular calcium concentrations in galvanotactic C3H/10T1/2 fibroblasts (Onuma and Hui, 1988) and to cause cathode-localized calcium elevations in galvanotropic N1E115 neuroblastoma cells (Bedlack et al., 1992).

DC electric fields cause the lateral redistribution of several types of plasma membrane glycoproteins, including receptors for plant lectins (for review see Poo, 1981), low-density lipoprotein (Tank et al., 1985), and EGF (Giugni et al., 1987). Perhaps the most studied class of electro-mobile glycoproteins are the receptors for the lectin Con A, which undergo electrophoretic or electro-osmotic redistribution in a wide variety of cells. It has been shown that concanavalin A alters the morphology and locomotory behavior of several cell types. Con A causes the rearrangement of cytoskeletal components in fibroblasts (Arena et al., 1990) and inhibits the migration of corneal epithelial cells (Gipson and Anderson, 1980), neural crest cells (Moran, 1974), and macrophages (Kumagai and Arai, 1973). While it is clear that Con A receptors may be involved in the control of cellular architecture and migration, it has yet to be determined whether or not electric field–induced asymmetries of plasma membrane glycoproteins can cause structural polarization and directional migration.

In light of these issues, we sought to examine the roles of calcium and the lateral redistribution of plasma membrane Con A receptors in the galvanotactic response of NIH 3T3 fibroblasts and the SV-40 transformed NIH 3T3 line SV101. The NIH 3T3 cell line has been used extensively in studies of cellular locomotion, adhesion, and stimulus-induced intracellular calcium dynamics, but this is the first report on the galvanotaxis of these fibroblasts. Using various microscopic and fluorometric techniques, we found no evidence for the involvement of calcium in the electric-field directed locomotion of either NIH 3T3 or SV101 fibroblasts. We observed a cathodal accumulation of receptors for the lectin Con A in fibroblasts migrating on both serum and Con A–coated substrata. Additionally, Con A was found to inhibit the turning of cells following reversal of electric field polarity. Immunofluorescent examination of the fibronectin receptor, a presumed subset of the Con A receptors and important element of fibroblast adhesion, indicates a redistribution toward the cathode facing, leading edge in field treated cells. We propose that the directed locomotion of these fibroblasts may involve electric field induced gradients of plasma membrane glycoproteins involved in cell substratum adhesion.

Materials and Methods

Cell Culture and Reagents

NIH 3T3 (CRL 1658; American Type Culture Collection, Rockville, MD) were obtained at passage number 127. SV101 (kindly provided by Dr. Joel Pachter, University of Connecticut Health Center, Farmington, CT) have been previously described (Pollack et al., 1968). Both cell lines were grown in DME supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD) and maintained at 37°C with 5% CO_2. Cultures of NIH 3T3 fibroblasts were not allowed to reach confluence and were discarded after a maximum of 10 serial passages. Subconfluent cultures were harvested by trypsinization (0.05% trypsin with 0.53 mM EDTA; GIBCO BRL) and plated at low density onto sterile, acid washed number one glass coverslips in growth medium 1–3 d before use in experiments. Unless otherwise indicated, all experiments were done with a balanced salt solution (BSS) consisting of 145 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.5 mM $MgCl_2$, 6 mM D-glucose, 5 mM Trizma base, and 10 mM Hepes buffer, adjusted to pH 7.4. Calcium-free BSS was made by omitting $CaCl_2$ and adding EGTA and $MgCl_2$ to yield final free concentrations of 1 mM and 3 mM, respectively, calculated as previously described (Tsien and Pozzan, 1989).

Exposure of Cells to DC Electric Fields

The apparatus used to expose cells to a uniform DC electric field is similar to chambers described previously (Bedlack et al., 1992). Cells grown on glass coverslips are attached to the lexan chamber using silicon high vacuum grease and sealed with paraffin. The cells face a thin channel of bathing medium 100 to 200 μm deep. The small cross-sectional area of this region provides high resistance to current flow and minimizes Joule heating. A water bath and pump was used to circulate heated water through a sealed glass compartment adjacent to this channel to maintain the cell bathing medium at 37°C. A DC power source attached to silver chloride electrodes was used to supply current to the electrolyte channel of the chamber via 20 ml BSS reservoirs and 10 cm agar bridges (2% agarose in BSS) to prevent contamination of the chamber by electrolysis products. The voltage drop across the coverslip was measured with two platinum wires embedded in the chamber at either end of the narrow channel.

Motion Analysis of Phase Contrast Images

The motility of populations of fibroblasts with and without electric field exposure was examined using time lapse, phase contrast microscopy, and motion analysis of digitized image sequences. Cells were observed in the electric field chamber mounted on a Nikon Diaphot inverted microscope equipped with phase contrast optics at magnifications of 10–20×. Images were acquired using a cooled CCD camera (Photometrics Inc., Tucson, AZ) and Perceptics (Knoxville, TN) Biovision software and later analyzed using NIH Image version 1.3. Cell movement with time was measured by randomly selecting thirty to forty cells and recording the X-Y coordinates of each cell nucleus at the start and finish of each time lapse image sequence. Directional displacement was analyzed using the "Dixon-McCutcheon" index method as described previously (Dixon and McCutcheon, 1936; Cooper and Schliwa, 1986). Cellular displacement along the axis of the applied field (X coordinate) was divided by the total displacement to yield the directional migration index (DMI). Positive index values indicate cathodal migration and negative values represent migration toward the anode. Completely random migration by a cell population would produce a mean index value of zero. To minimize the effects of variability between cultures, each two part experiment (control and experimental sequence) was performed using the same coverslip.

Calcium Measurements

Fluorescence microscopy and digital image processing were used to examine the intracellular calcium levels of single cells during electric field exposure. Cells grown on glass coverslips were microinjected with 1 mM Fura-2 dextran (10-kD mol wt; Molecular Probes Inc., Eugene, OR) in double-distilled water also containing 120 mM potassium aspartate plus 10 mM Na^+-Hepes, pH 7.0. Microinjection pipettes were made from Omega Dot capillary tubing (FHC, Brunswick, ME) using a Kopf model 720 vertical pipette puller (David Kopf Instruments, Tujunga, CA) and filled with 1 mM Fura-2 dextran stock solution by capillary action. Injected cells were washed in normal BSS and placed on the electric field chamber after a mini-

mum one hour recovery period. Fluorescent image pairs were acquired with excitation at 340 and 380 nm (emission = 510 nm) on a Nikon Diaphot inverted microscope equipped with a 14-bit cooled CCD camera (Photometrics Inc., Tucson, AZ) and a computer-controlled excitation filter wheel (Ludl Electronics Products, Hawthorne, NY). In each experiment, individual cells were imaged before, during, and after electric field exposure. Image processing, including background subtraction, thresholding, ratio calculation, flat field correction, and calculation of mean cellular intensities, was performed using Perceptics Biovision software. Cells were analyzed for calcium gradients by converting digitized images to a spreadsheet format. Spreadsheets were exported to Microsoft Excel where individual cells were divided into 10 equally sized vertical columns perpendicular to the applied field. The average intensity of each region was then calculated. Calcium concentrations were calculated using previously described methods (Grynkiewicz et al., 1985). Calibration values (Rmin, Rmax, Sf2/Sb2) were obtained from slides containing 100 μm Fura-2 Dextran in solution with either 1.8 mM calcium chloride or no added calcium with 1 mM free EGTA. For rapid calcium measurements, the free acid form of Fluo-3 (Molecular Probes, Eugene, OR) was microinjected into cells using the same procedure as above. Single Fluo-3–injected cells were observed on the microscope using a SIT 66 camera (Dage-MTI Inc., Michigan City, IN) and recorded on VHS videotape during electric field exposure.

Intracellular calcium levels of adherent cell populations during plasma membrane depolarization were determined using a SPEX (Edison, NJ) Fluorolog CM dual wavelength fluorometer (excitation = 340 nm and 380 nm; emission = 505 nm). Cells were grown on 11 × 30 mm glass coverslips to near confluence and loaded with Fura-2 AM (2.5 μM, 0.01% Pluronic F-127). Cells were placed at 4°C for 10 min and then loaded at room temperature for 1 h. Coverslips were washed with culture medium and placed at 37°C for 30 min to aid in the complete deesterification of the dye. Dye-loaded coverslips were attached to a special cuvette perfusion assembly as previously described (Manger and Koeppen, 1992). Plasma membrane depolarization was achieved by transiently perfusing the cuvette assembly with BSS containing 145 mM KCl in place of NaCl, followed by the addition of either bombesin (Sigma Chemical Co., St. Louis, MO) or bradykinin (Sigma Chemical Co.) as a positive control. Depolarization of the plasma membrane by potassium was verified using the whole cell patch clamp technique. Calibration points were obtained by perfusion with 5 μm ionomycin (Calbiochem-Behring Corp., San Diego, CA) followed by 2 mM EGTA in calcium-free BSS. Calcium concentrations were calculated using SPEX DM3000CM software.

Preparation of Con A Substrata

Acid washed number one glass coverslips were coated for 3–4 h at room temperature with Con A (Sigma Chemical Co.) dissolved in sterile double-distilled water at a concentration of 10 μg/ml. Coated coverslips were washed several times with double distilled water and soaked for an additional hour at room temperature in BSS with 1% BSA and no glucose. Trypsinized cells were washed and resuspended in BSS, plated on Con A–coated slides, and used in experiments 1–3 h later.

FITC-Con A Labeling and Data Analysis

Cells were post-field labeled with fluorescein isothiocyanate-conjugated Con A (Molecular Probes Inc.) and fixed in the electric field chamber. After 40–60 min of electric field exposure, cells were exposed to FITC-Con A (100 μg/ml in BSS) for 2–3 min and washed several times with BSS. The chamber was then flushed with cold fixative (3% formaldehyde in BSS, pH 7.4), removed from the microscope, and placed at 4°C for 30 min. Coverslips were removed from the chamber, washed several times with BSS containing 1% BSA and 1% glycine, and mounted on glass slides using Slow-Fade (Molecular Probes Inc.) mounting medium. Controls were processed identically with the exception of electric field exposure.

Evaluation of fluorescent gradients was performed by a single-blind analysis. As many cells as possible from each fixed sample were scored as having a clear asymmetry of FITC-Con A label toward the anode (left in controls), the cathode (right in controls), or as showing no apparent gradient. Digital analysis of plasma membrane FITC-Con A fluorescence was done using the gradient analysis technique described above for Fura-2 ratio images. Individual cells from control and electric field-treated coverslips were selected randomly and fluorescent images acquired with a cooled CCD camera (Photometrics, Inc.). Digitized images were converted to spreadsheet format and each cell divided into five vertical columns of equal width. The anode to cathode (or left–right) profile of FITC-Con A fluorescence was created by calculating the mean fluorescent intensity of each of the five regions. Individual profiles were normalized for overall intensity differences by dividing each of the five vertical columns by the region of least intensity.

Indirect Immunofluorescence

The fibronectin receptor of NIH 3T3 fibroblasts was labeled using rabbit polyclonal antiserum 161 (kindly provided by E. E. Marcantonio, College of Physicians and Surgeons of Columbia University, New York). This antiserum is directed against the cytoplasmic domain of the human integrin α5 subunit (Hynes et al., 1989) and reacts with the major fibronectin receptor in 3T3 cells, α5β1 (Briesewitz et al., 1993). Cells were grown on glass coverslips and exposed to the electric field as described above. Samples were fixed at room temperature for 10 min in 4% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed, and blocked for 30 min with PBS containing 1% nonfat dry milk. Primary antisera, either rabbit anti-α5 or nonimmune rabbit serum, were diluted 1:200 in PBS with 10% normal goat serum. Cells were incubated with primary antibody for 30 min at 37°C and washed with PBS containing 1% BSA. Rhodamine conjugated goat anti-rabbit secondary antibodies (Molecular Probes Inc.) were diluted 1:200 in PBS/10% goat serum. Coverslips were incubated with secondary antibody for 30 min at 37°C, washed with PBS/BSA, and then mounted. Samples were viewed and images acquired on a Nikon Diaphot microscope equipped with a Bio-Rad MRC-600 confocal imaging system (Bio-Rad Laboratories, Melville, NY) and a Nikon 60×, 1.4 NA plano-apochromat objective. Identical gain, neutral density, and aperture settings were used for control and experimental slides. The confocal aperture was adjusted to limit fluorescence to only the ventral cell surface. Negative controls, in which nonimmune rabbit serum was used instead of anti-α5, show only faint, punctate perinuclear staining, and little or no fluorescence toward the cell periphery.

Results

Electric Field–directed Fibroblast Locomotion

Both NIH 3T3 and SV101 fibroblasts exhibit dramatic cathode directed locomotion in the presence of DC electric fields as low as 1 V/cm. For the analysis of cell motility, time lapse digitized phase contrast images were acquired and the directional displacement (see Materials and Methods) of a population of cells analyzed with and without an externally applied electric field. This form of analysis illustrates that the migration of both NIH 3T3 and SV101 fibroblasts is highly directional in the presence of DC electric fields and essentially random in cell populations not exposed to the field. The mean DMI of NIH 3T3 fibroblasts exposed to a 4 V/cm field for 1 h was 0.726 ± 0.052, compared to −0.020 ± 0.109 for the same cells before field treatment (n = 120 cells from four coverslips, ±SEM). For identical experiments with SV101 fibroblasts, the mean DMI during field exposure was 0.761 ± 0.065 compared to −0.014 ± 0.107 in controls (n = 120 cells from three coverslips, SEM).

Cellular displacement along the axis of the applied field, random initially, becomes clearly cathode directed within minutes of electric field application (Fig. 1, *a* and *b*). Polarized extensile activity is visible in both cell lines within 2–3 min of field application. The analysis of cell movement using nuclear tracking would indicate a slower onset of directional movement in NIH 3T3 cells (Fig. 1). However, the apparent difference in the response time of the two cell lines is largely attributable to differences in the locomotory morphologies of the two cell types, as bulk cytosolic and nuclear displacement lags behind leading edge extension in NIH 3T3 cells much more so than in SV101. Although motion analysis via

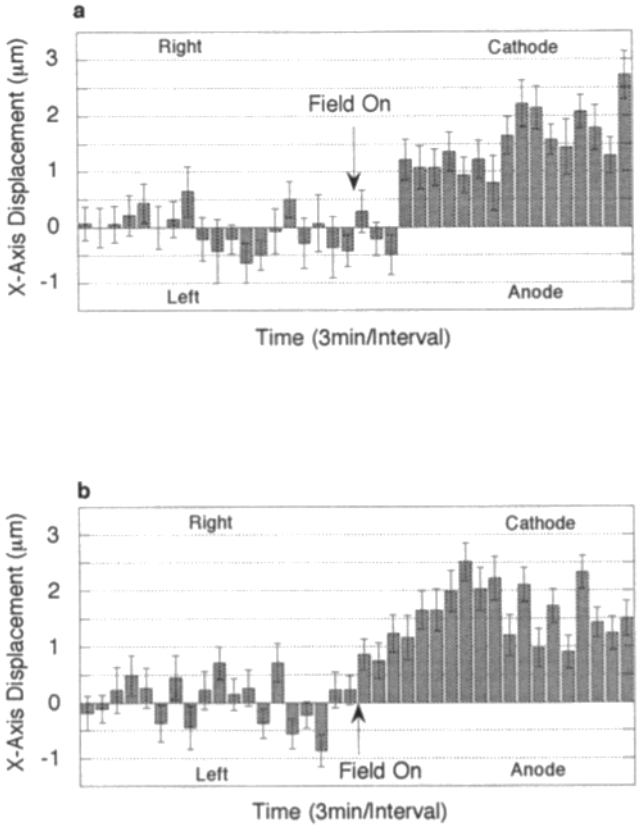

Figure 1. The transition from random to cathode directed migration is apparent within minutes of electric field application. The mean displacement of NIH 3T3 (*a*) and SV101 (*b*) along the axis of the electric field for 1 h before and after field application (4 V/cm). Data is expressed as the mean X-axis displacement ± SEM during 3-min intervals (n = 40 cells from one coverslip for each cell type).

nuclear tracking may not be ideal for describing the kinetics of locomotory responses, the technique is a reliable and convenient means of quantitating the locomotory behavior of large numbers of cells.

The primary response of both cell types appears to be directional locomotion and not the vertical elongation observed in cells such as C3H/10T1/2 mouse fibroblasts (Onuma and Hui, 1988) and chick heart fibroblasts (Harris et al., 1990). SV101 fibroblasts, as viewed with phase contrast optics, extend numerous spikelike projections toward the negative pole, in contrast to the broad lamellipodia seen in the NIH 3T3 cells (Fig. 2). NIH 3T3 fibroblasts generally migrate at rates from 10–20 μm/h, compared to the 20–30 μm/h exhibited by the SV101 line. While the extent of the directional response in both cell lines appears to be very similar, directionally migrating NIH 3T3 fibroblasts are redirected by cell–cell contacts much more so than the SV101 line. The calculated vectorial displacement in an experiment using NIH 3T3 cells can thus be influenced by the cell density of a given sample. Therefore, each two part experiment (control and experimental image sequences) was always performed using a single coverslip.

Galvanotaxis in NIH 3T3 and SV101 Fibroblasts Does Not Involve Voltage-gated Calcium Influx

Electric field directed shape changes and motility have been shown to be inhibited by L type calcium channel antagonists in other cell types, including mouse fibroblasts (Onuma and Hui, 1988). In contrast, we found the calcium channel antagonist verapamil (25 μM) to have no effect on the electric field directed motility in either NIH 3T3 or SV101 fibroblasts. Cells were monitored in a 4 V/cm DC electric field for 1 h in normal BSS, the field turned off, and the chamber flushed several times over 10–15 min with BSS containing 25 μM verapamil. The field was then reapplied with the opposite polarity of the control sequence and the cells monitored for a second hour. The polarity reversal ensures that no previously established directionality contributes to cellular displacement during the second phase of the experiment. NIH 3T3 showed a directional migration index of 0.589 ± 0.076 in the presence of verapamil compared to 0.618 ± 0.063 before verapamil treatment (n = 160 cells from four coverslips, mean index values ± SEM). Comparably, the DMI for SV101 fibroblasts in the presence of verapamil was 0.659 ± .070 versus 0.674 ± 0.068 before verapamil treatment (n = 160 cells from four coverslips). Similar results were obtained with the calcium channel antagonist D600 (15 μM) and Nifedipine (15 μM). Attempts to examine the effects of the inorganic calcium channel blockers La^{3+} and Co^{2+} were unsuccessful, as they proved toxic to the cells within several minutes of their application. In comparison, the response of C3H/10T1/2 fibroblasts to applied electric fields was shown to be inhibited by 1 μM D600 (Onuma and Hui, 1988).

Electric field induced calcium entry by a voltage-gated mechanism clearly depends on the presence of voltage-gated calcium channels in the plasma membrane. While voltage-gated calcium channels have been described in fibroblasts including the NIH 3T3 cell line (Chen et al., 1988), the density of these channels is relatively low and their functional significance is unknown (Chen et al., 1988; Peres et al., 1989). To test for voltage-gated calcium entry, the cytosolic calcium levels of NIH 3T3 and SV101 fibroblasts were measured during plasma membrane depolarization using the calcium indicator Fura-2. Plasma membrane depolarization was achieved by substantially elevating the extracellular potassium concentration. Subconfluent monolayers on glass coverslips were loaded with Fura-2 AM, mounted in a cuvette, and calcium levels monitored fluorometrically. Perfusion of the cells with BSS containing 145 mM KCl (NaCl omitted) caused no detectable elevations of intracellular calcium levels (Fig. 3, *a* and *b*). The addition of bombesin (50 ng/ml) or bradykinin (100 nM), which trigger calcium influx and/or intracellular release in these cells (Polverino et al., 1991; Woll et al., 1992), caused the expected calcium transients (interestingly, bombesin had no effect on SV101 fibroblasts). These positive controls show that we are indeed able to detect physiologic calcium transients with the techniques used. As it appears that the plasma membranes of our NIH 3T3 and SV101 fibroblasts do not contain significant numbers of voltage-gated calcium channels, our data would argue strongly against any voltage-gated mechanism.

The Galvanotactic Response of NIH 3T3 and SV101 Fibroblasts Does Not Require Extracellular Ca^{2+}

The possibility remains that the applied electric field leads to calcium entry by other, nonvoltage-gated pathways. As stated above, we were unable to examine the effects of

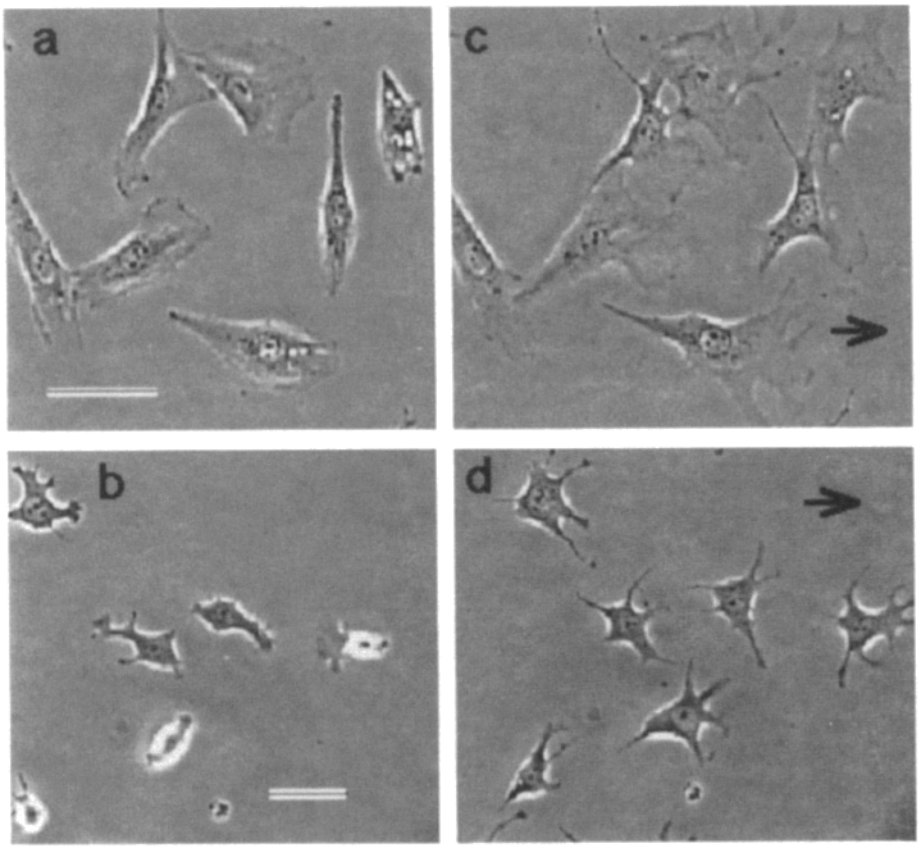

Figure 2. Phase contrast micrographs illustrating the polarization and directional locomotion of NIH 3T3 and SV101 fibroblasts following exposure to a DC electric field. (*a* and *b*) NIH 3T3 and SV101, respectively, before field exposure. (*c* and *d*) NIH 3T3 and SV101, respectively, following 30-min exposure to a 4 V/cm electric field. Arrows in *c* and *d* indicate the direction of the cathode. Bar, 30 μm.

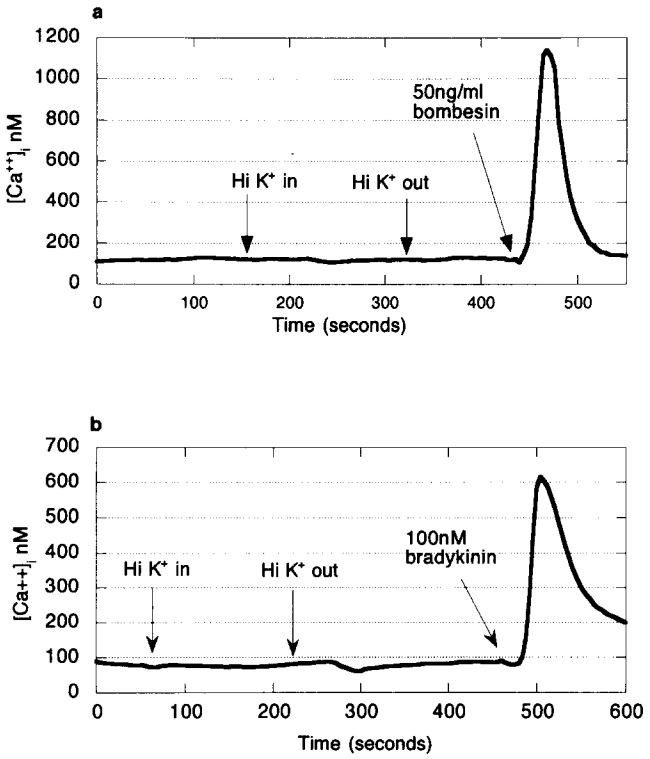

Figure 3. Plasma membrane depolarization does not trigger calcium influx in NIH 3T3 or SV101 fibroblasts. Fluorometric calcium measurements of Fura-2–loaded NIH 3T3 (*a*) and SV101 (*b*) fibroblasts during plasma membrane depolarization by high external potassium. Cells were transiently perfused with BSS containing 145 mM KCl, followed by the addition of bombesin (*a*), or bradykinin (*b*), as positive controls. Scans are representative and were replicated two times each.

nonspecific inorganic calcium channel blockers due to toxicity. To eliminate any possibility of field-induced calcium influx, both cell types were exposed to the electric field in the absence of extracellular calcium. Cells were monitored in a 4 V/cm DC electric field for 1 h in normal BSS, and for a second hour in the presence of calcium free BSS (no $CaCl_2$, 1 mM EGTA, supplemental $MgCl_2$). These experiments were performed in the same manner as those described for verapamil, with a 10–15-min period without an applied field between two field applications of equal magnitude and opposite polarity. In both the NIH 3T3 and SV101 fibroblasts, the removal of extracellular calcium had no effect on the galvanotactic response (NIH 3T3 DMI = 0.563 ± 0.073 with $[Ca^{2+}]_o \approx 0$, vs. 0.570 ± 0.077 before Ca^{2+} removal, n = 200 cells from five coverslips; SV101 DMI = 0.659 ± 0.069 with $[Ca^{2+}]_o \approx 0$, vs. 0.674 ± 0.068 before Ca^{2+} removal, n = 150 cells from four coverslips). Experiments in which calcium was absent during the initial field exposure and replaced before the reversal of field polarity produced the same results. Our data indicate that the electric field directed locomotion of these cells has no extracellular calcium requirement. Taken together, these experiments argue strongly against any field-induced calcium influx in these cells.

Electric Fields Do Not Cause Intracellular Ca^{2+} Elevations or Gradients

Applied electric fields may modulate the disposition and activities of membrane proteins other than voltage-gated ion channels (Blumenthal et al., 1983; Thuren et al., 1987). While the mechanism of galvanotaxis in NIH 3T3 and SV101 fibroblasts does not appear to involve transmembrane calcium fluxes, it is possible that the electric fields indirectly induce calcium release from intracellular stores. To test the possibility of calcium mobilization from intracellular stores during fibroblast galvanotaxis, intracellular free calcium was monitored during electric field exposure.

The effect of applied electric fields on intracellular calcium was examined microscopically in single NIH 3T3 cells microinjected with 10-kD Fura-2 dextran. Dual wavelength fluorescent image pairs of individual NIH 3T3 cells were acquired before electric field exposure. The same cells were then imaged within 10 min of the application of a 4 V/cm field and again 30–40 min after turning off the field. The mean cellular calcium concentration was calculated for each processed image ratio. We found no evidence of electric field induced changes of cellular free calcium concentration by this method. The mean calcium concentration of cells before exposure was 91 ± 4 nM compared with 88 ± 3 nM during the application of the electric field (mean $[Ca^{2+}]_i$ ± SEM; n = 38 cells from seven coverslips). The average calcium levels of the same cells 30–40 min after termination of the field application were slightly elevated to 103 ± 5 nM. NIH 3T3 cells appeared to be very sensitive to photodamage, and we attribute this slight calcium elevation to damage caused to some cells by repeated UV exposure during the process of cell location, focusing, and imaging.

Analysis of the calcium distribution relative to the applied electric field showed no field-induced gradients of intracellular free calcium. Fura-2 ratio images of individual cells were acquired before, during, and after electric field exposure using the same procedure outlined above. Cells were then divided into 10 equal sized vertical regions along the axis of the applied E field and the mean calcium concentration of each region calculated. Our data (Fig. 4) show no significant changes of calcium levels in any cellular region. Our results demonstrate that applied electric fields do not cause any significant, sustained alterations of the levels or distribution of intracellular free calcium.

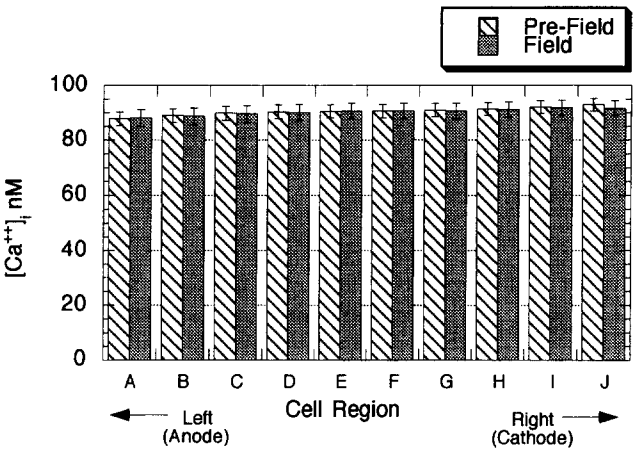

Figure 4. Electric fields do not induce gradients of intracellular-free calcium. Individual NIH 3T3 fibroblasts microinjected with 10-kD mol wt Dextran-conjugated Fura-2-free acid were imaged ratiometrically before and within 10 min of exposure to a 4 V/cm electric field. Calibrated calcium images were converted to spreadsheets, and each cell divided into 10 equal width vertical columns perpendicular to the applied field. The mean calcium concentration ± SEM for each column was then calculated for each cell before and during field exposure (n = 36 cells from seven coverslips).

To determine if electric fields cause rapid transient changes in intracellular calcium, fibroblasts were microinjected with the single wavelength fluorescent calcium indicator Fluo-3 (Minta et al., 1989) and monitored at video rates (30 frames/s) during field application and periodic reversal of field polarity. No calcium transients were observed, but at higher field strengths (10 V/cm), both cell types often showed a gradual decrease of Fluo-3 fluorescence in cell processes facing the cathode. We later determined that this change of Fluo-3 fluorescent intensity was not due to any fluctuations of intracellular calcium concentration, but instead was the result of decreased cell volume toward the cathode. The same phenomenon was observable in cells microinjected with 10-kD mol wt fluorescein-coupled dextran as a nonspecific volume marker. Fura-2 fluorescent ratios of cells acquired during the initial 60 s of field application also showed stable calcium concentrations during field exposure. Comparable results were obtained with SV101 fibroblasts. We therefore conclude that DC electric fields do not induce either rapid or sustained calcium changes in either cell type.

Lateral Redistribution of Con A Receptors on Serum and Con A-coated Substrata

DC electric fields cause the lateral redistribution of Con A receptors in a number of cell types, including fibroblasts (Zagyansky and Jard, 1979). NIH 3T3 and SV101 fibroblasts labeled with FITC-Con A after electric field exposure exhibit a cathodal accumulation of plasma membrane fluorescence (Fig. 5). With or without E field exposure, migrating NIH 3T3 cells typically showed large amounts of fibrous, Con A–positive material (primarily cellular fibronectin) concentrated over the nucleus and trailing edge, as well as in a heavy trail behind the cell. This fibrous, pericellular label was greatly reduced or absent in the SV101 cells, and the cathodal accumulation of a more diffuse, plasma membrane fluorescence was more clearly visible. In addition, cell free regions of the glass coverslip showed a uniform staining, probably due to adsorbed vitronectin, fibronectin, and other glycoproteins present in the serum used in the culture medium.

Trypsinized NIH 3T3 fibroblasts washed and replated in the absence of serum will spread and migrate on Con A–coated glass substrata. The rate of cell locomotion is inversely proportional to the concentration of Con A used in the coating procedure, with little or no cell movement occurring at concentrations of 50 μg/ml or higher. All experiments described below utilized coverslips coated with 10 μg/ml Con A. At this coating concentration, migration rates were comparable to those observed on serum-treated slides (mean displacement = 18.95 ± 1.27 μm/h; n = 120 cells from three coverslips). The cells adhere poorly and fail to spread

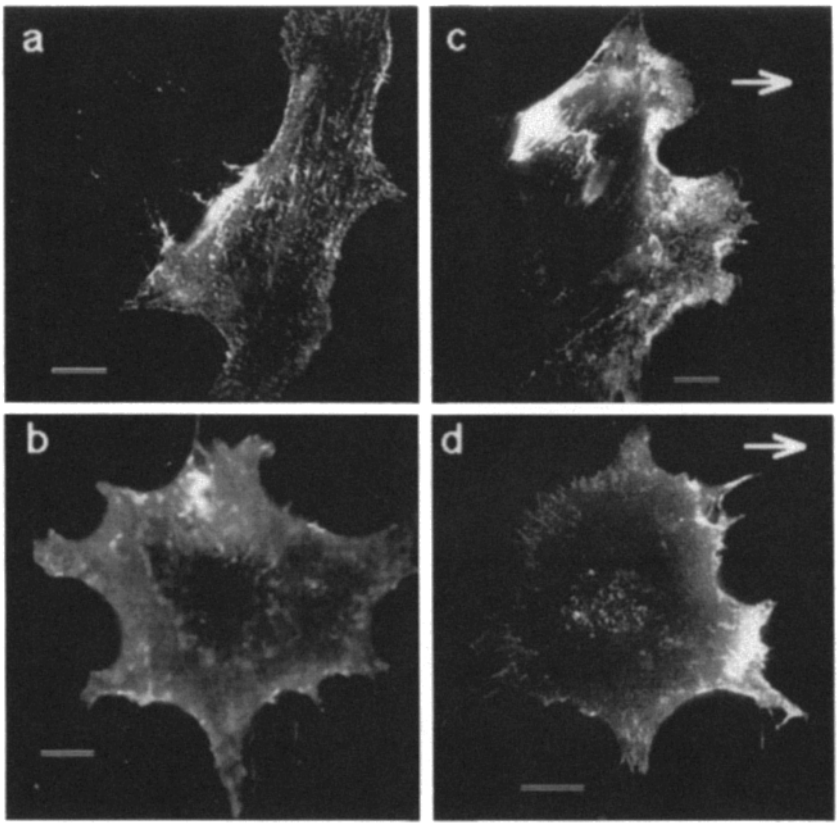

Figure 5. The cathodal accumulation of plasma membrane Con A receptors induced by electric fields in NIH 3T3 and SV101 fibroblasts. Cells were exposed to a 4 V/cm electric field for 40 min, briefly labeled with 100 μg/ml FITC-Con A, and fixed. (*a*) NIH 3T3 and (*b*) SV101 labeled with FITC-Con A without field exposure. (*c*) NIH 3T3 and (*d*) SV101 post-field labeled with FITC-Con A. Arrow in *c* and *d* indicate the direction of the cathode. Bar, 10 μm.

on glass treated with BSA only and attachment to the Con A substratum is inhibited by the presence of 50 mM α-methyl-D-mannoside during plating, indicating that the adhesion of the cells to the Con A–coated coverslips is mediated by specific lectin–plasma membrane glycoprotein interactions. Cells plated in this manner exhibit dramatic directional locomotion toward the cathode in the electric fields (mean DMI = 0.78 ± 0.15; n = 120 cells from three coverslips). The trypsinization and serum free plating procedure eliminate the fibrous Con A–positive staining, but not the diffuse plasma membrane fluorescence; much of the background fluorescence seen under normal culture conditions is also reduced, facilitating visual and digital analysis of fluorescent images for gradients of FITC-Con A label.

Post-electric field labeling of cells plated on the Con A substratum reveals a clear cathodal accumulation of fluorescently labeled Con A receptors compared to controls (Fig. 6). After 40 min in a 4 V/cm electric field, cells were labeled with FITC-Con A and fixed. A number of pre-field– and post-field–labeled cells were imaged with a cooled CCD camera (Photometrics Inc.) and the distribution of cell surface FITC-Con A fluorescence relative to the axis of the applied electric field was analyzed digitally. Fig. 7 illustrates the results of this gradient analysis. The frequency with

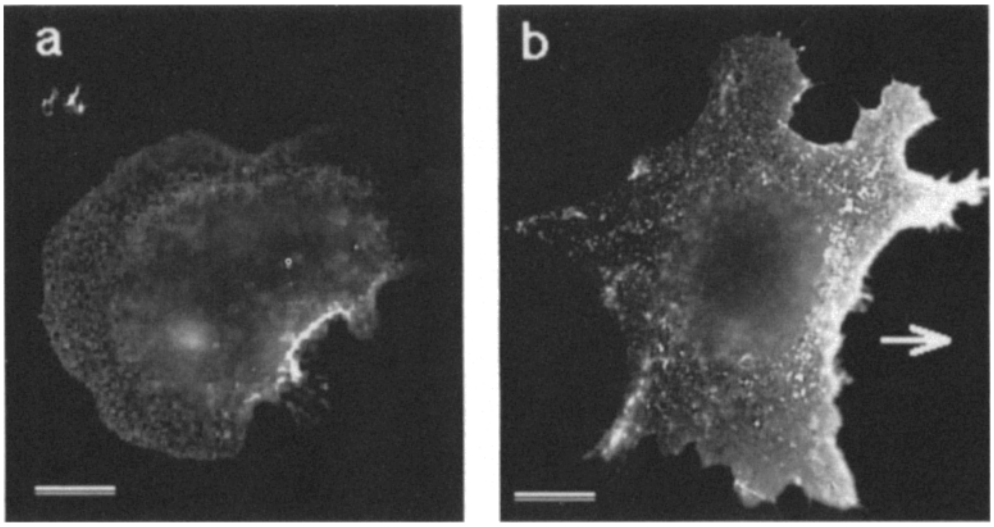

Figure 6. The electric field induced, cathodal accumulation of plasma membrane Con A receptors in NIH 3T3 plated on Con A–coated substratum. (*a*) Pre-field label with 100 μg/ml FITC-Con A. (*b*) FITC-Con A label following 40-min exposure to a 4 V/cm electric field. Arrow in *b* indicates the direction of the cathode. Bar, 10 μm.

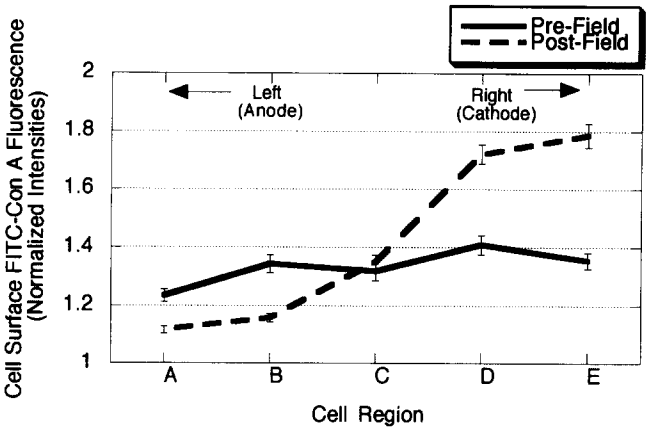

Figure 7. Digital analysis of FITC-Con A gradients in NIH 3T3 fibroblasts on Con A–coated substrata with and without electric field exposure. Images of fixed, labeled cells were converted to spreadsheets, divided into five equal width regions perpendicular to the applied field, and the mean fluorescent intensity of each region calculated. Values were normalized for overall intensity differences by dividing each cell by its region of least intensity. Values are presented as mean regional normalized intensities ± SEM (n = 120 cells without field exposure; n = 110 cells after field exposure.

which such gradients occur was determined by a single-blind analysis of fixed control and E field–treated samples. Numerous cells from each sample were scored as either showing an accumulation of FITC fluorescence toward the cathode (right in controls), toward the anode (left in controls), or as having no difference. A clear accumulation of Con A receptors toward the cathode occurs in a substantial majority of cells after electric field exposure (Fig. 8). The observed asymmetry of FITC-Con A label is due to glycoprotein redistribution and not increased membrane surface area, as post-field labeling with DiI (18 carbon) shows a uniform distribution of DiI fluorescence. Since the cells are utilizing Con A receptors for adhesion, the cathodal accumulation of Con A receptors corresponds to an electric field–induced redistribution of plasma membrane glycoproteins involved in cell–substratum adhesion.

Galvanotaxis Is Inhibited by the Cross-linking of Cell Surface Glycoproteins

Pre-field exposure of NIH 3T3 and SV101 fibroblasts to Con A inhibits the normal galvanotactic response. Cells exhibit filopodial extension and ruffling in all directions but show no polarization and little or no displacement. Additionally, the exposure of directionally migrating NIH 3T3 fibroblasts to Con A prevents the change of direction of locomotion which normally occurs after the reversal of field polarity. Cells were labeled with 100 μg/ml FITC-Con A during the last 5 min of a 65-min exposure to a 4 V/cm electric field. After several washes with BSS, the field polarity was immediately reversed, and the cells monitored for a second hour. Phase contrast image sequences for the first and second hour were then analyzed for directional cellular locomotion. While total cellular displacement is reduced by exposure to the lectin, cells continue to slowly migrate in the original direction, which is now the anode, after the reversal of field polarity (Fig. 9). Cells exposed to divalent, succinylated Con A (s-Con A) are able to turn and migrate toward the new cathode during the second hour, similar to controls. It should be noted that, in contrast to the experiments described in previous sections, electric field exposure is continuous in these studies. As a result, the majority of cells must be redirected

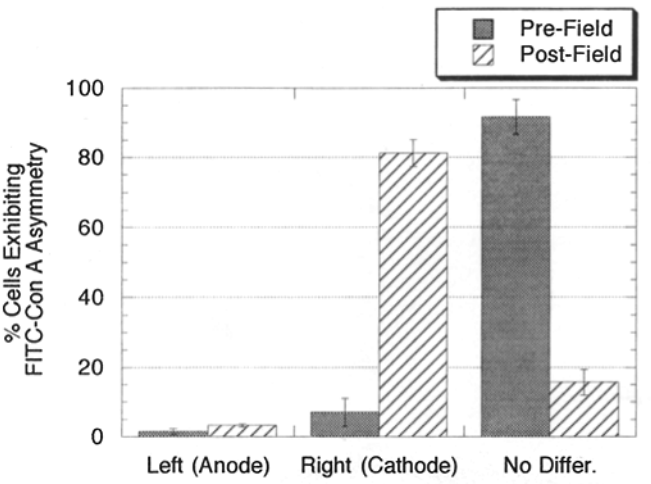

Figure 8. Cathodal accumulation of Con A receptors occurs in the majority of cells exposed to electric fields on Con A–coated substrata. A single-blind analysis was done on NIH 3T3 cells labeled with FITC-Con A and fixed either before or after field exposure (40 mins at 4 V/cm). Cells were scored as either showing a clear asymmetry of FITC fluorescence toward the cathode (right in controls), anode (left in controls), or as having no obvious difference, and the percentage in each category calculated for each sample (n = four pre-field coverslips, 618 cells total; n = four post-field coverslips, 681 cells total).

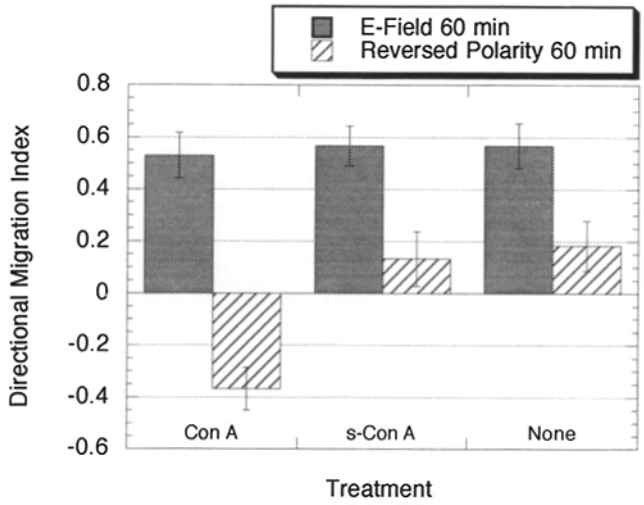

Figure 9. Con A inhibits the change of direction of cell migration following the reversal of electric field polarity. Cells were exposed to a 4 V/cm electric field for 1 h and then transiently exposed to 100 μg/ml Con A or 100 μg/ml s-Con A (simple BSS exchange in controls). Immediately following these treatments the electric field polarity was reversed (field was never turned off) and the cells monitored for a second hour. Data is presented as the mean directional migration index ± SEM (n = 200 cells from five coverslips for Con A; n = 80 cells from two coverslips for both s-Con A and control experiments).

a full 180° after the reversal of field polarity, increasing the apparent response-lag described earlier. While controls and s-Con A-treated cells show cathode-directed ruffling and extension within minutes of the polarity reversal, the redirection of nuclear movement is delayed. The apparent decrease in directional locomotion in controls during the hour following polarity reversal is the result of this delay. Our results indicate that the lateral mobility of Con A receptors is required for NIH 3T3 galvanotaxis. More importantly, these data would suggest a link between the lateral redistribution of cell surface molecules and directional locomotion in NIH 3T3 fibroblasts.

Electric Field Induced Redistribution of the Fibronectin Receptor

To link our results with Con A receptors to the idea that galvanotaxis involves the lateral redistribution of physiologically relevant adhesion receptors, we examined the effects of electric field exposure on the distribution of the integrin fibronectin receptor ($\alpha5\beta1$) in NIH 3T3 cells. The fibronectin receptor was visualized via indirect immunofluorescence using a polyclonal antiserum directed against the cytoplasmic domain of the $\alpha5$ subunit (Hynes et al., 1989; Briesewitz et al., 1993). Fibroblasts were exposed to a 4 V/cm electric field for 50 min, fixed, permeabilized, and stained for $\alpha5$. Controls were processed identically with the exception of electric field exposure. The ventral surfaces of cells were examined by confocal microscopy to eliminate the perinuclear fluorescence, which, in standard microscope images, caused a broad haze in all focal planes that precluded the distinction between clusterred and unclustered $\alpha5$. Fibroblasts not exposed to the electric field show discrete patterns of focal staining ventrally and large aggregates of $\alpha5$ on the perinuclear, dorsal surface. In cells having a locomotory phenotype, the fibrillar streaks of $\alpha5$ staining are typically larger and more concentrated toward the trailing edge relative to the lamellipodium (Fig. 10 *a*), but diffuse fluorescence is low across the cell. After electric field exposure, the majority of cathode-facing lamellipodia appear devoid of extensive fibrillar staining, and instead show a concentration of smaller streaks and diffuse fluorescence, while large fibrillar streaks of $\alpha5$ label remain prominent at the rear of the cell (Fig. 10 *b*). These results suggest that the field may disrupt highly aggregated fibronectin receptor complexes and draw the smaller receptor clusters into newly forming extensions toward the cathode.

Discussion

We have examined the roles of both calcium and cell surface molecular redistribution in the electric field-directed locomotion of murine fibroblasts. The transformation of NIH 3T3 and other murine fibroblastic cell lines with the SV-40 virus is known to cause alterations in adhesion (Willingham et al., 1977), cytoskeleton (Pollack et al., 1975; Nigg et al., 1986), and calcium homeostasis (Klug and Steinhardt, 1991; Newcomb et al., 1993). We studied both cell types with the expectation that differences in the galvanotaxis of the two cell lines might provide additional insight to the mechanism of directional locomotion in response to electric fields. Other than differences in morphology, rates of locomotion, and sensitivity to contact inhibition of movement, the galvanotactic responses of the two cell lines are surprisingly similar. While directional migration in a variety of cell types has been found to involve transmembrane calcium fluxes and localized intracellular calcium elevations, we have concluded that the directional locomotion of both NIH 3T3 and SV101 fibroblasts in response to electric fields is a calcium-independent process. Our data support an alternative mechanism involving the lateral electro-migration of cell surface glycoproteins involved in cell-substratum adhesion.

Our results show that applied electric fields do not cause voltage gated calcium influx in either NIH 3T3 or SV101 fibroblasts. Additionally, the galvanotaxis of the NIH 3T3 and SV101 cell lines has no extracellular calcium requirement, as both exhibit cathode-directed migration despite the removal of extracellular calcium and the presence of EGTA. Electric fields do not cause elevations or gradients of intracellular free calcium in NIH 3T3 fibroblasts. With fluorescent indicators such as Fura-2, negative findings such as these are difficult to prove because of potential artifacts which may limit the sensitivity of the method to small changes. Therefore, in studies on single cells, we chose microinjection of the 10-kD mol wt dextran-conjugated Fura-2 free acid to minimize dye leakage, and to avoid the problems of sequestration and incomplete de-esterification commonly encountered with Fura-2 AM. The use of positive controls also ensured that we were indeed able to detect

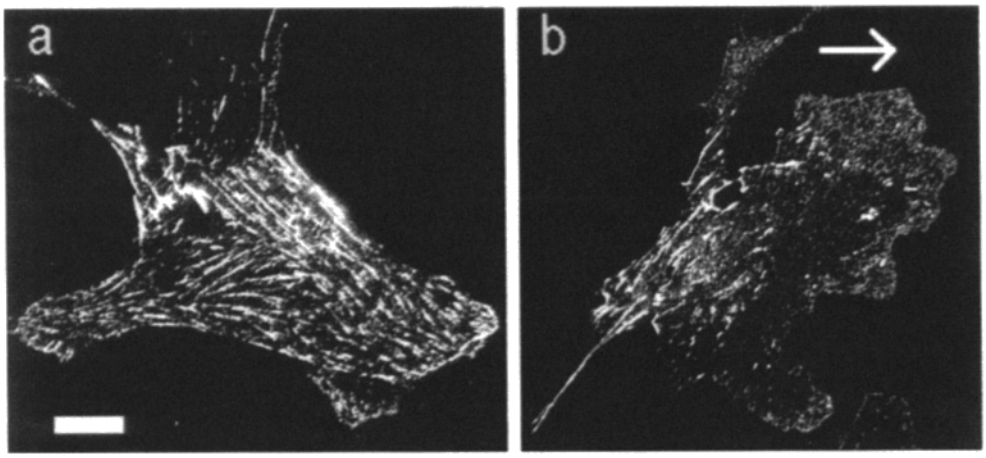

Figure 10. Confocal fluorescent micrographs showing the distribution of the fibronectin receptor $\alpha5$ subunit on the ventral surface of NIH 3T3 fibroblasts without (*a*) or with electric field exposure (*b*). Arrow in *b* indicates direction of the cathode. Bar, 30 μm.

physiologic calcium transients by these techniques. From these studies we conclude that the mechanism of galvanotaxis in these fibroblasts is, in apparent contrast to other systems, a calcium-independent process.

We have observed an accumulation of Con A receptors toward the cathode in NIH 3T3 and SV101 fibroblasts after electric field exposure. The electro-migration of glycoproteins toward the negative pole in the applied electric field is presumably an electro-osmotic rather than an electrophoretic process, as the glycoproteins are most likely negatively charged (McLaughlin and Poo, 1981; Poo, 1981). We have presented evidence suggesting that the redistribution of Con A receptors toward the cathode is linked to the directional migration of NIH 3T3 fibroblasts. When fibroblasts migrating directionally toward the cathode are exposed to Con A, the cellular reorientation normally observed after the reversal of electric field polarity is inhibited. Although the lectin causes a reduction of the rate of migration, cells continue to slowly migrate along their original trajectory after the polarity reversal. The cross-linking of plasma membrane glycoproteins with Con A is known to restrict their lateral mobility, and post-field cross-linking of redistributed plasma membrane glycoproteins with Con A inhibits the relaxation of Con A receptors to their pre-field distribution (Poo, 1981). The hysteresis caused by post-field Con A exposure would suggest that the cathodal accumulation of plasma membrane glycoproteins is involved in the establishment of cell polarity and directional migration in the presence of DC electric fields.

The electromigration of Con A receptors has been linked to electric field–directed growth in cultured *Xenopus* neurons (Patel and Poo, 1982). This is the only previous report supporting a causal relationship between Con A receptor electromigration and galvanic responses. The lateral transport of Con A receptors has been correlated to the direction of locomotion in other cell types. The active forward transport of Con A receptors was shown in migrating fish epidermal keratocytes, and it has been suggested that this transport process delivers proteins involved in motility, such as adhesive receptors, to the leading edge of the cell (Kucik et al., 1989). Similarly, cell surface molecules were found to be transported toward the leading edge of growth cones in cultured neurons (Sheetz et al., 1990). We have found that NIH 3T3 cells plated on Con A–coated substrata exhibit cathode directed locomotion as well as a cathodal accumulation of Con A receptors when exposed to electric fields. As cell adhesion to the Con A substratum is necessarily mediated by lectin–glycoprotein interactions, the asymmetry of Con A receptors corresponds to a cathodal accumulation of adhesion receptors. Therefore, the lateral migration of Con A receptors toward the cathode would imply the passive forward transport of adhesive receptors toward the leading edge of the cell.

The suggestion that the directional migration NIH 3T3 fibroblasts is due to electric field directed, lateral redistribution of plasma membrane receptors for extracellular matrix might be compared to the process of haptotaxis (Carter, 1967), in which the migration of a variety of cultured cells can be directed by gradients of substratum-bound adhesive molecules. Nonuniform distributions of cellular adhesion molecules should elicit the equivalent response, with directional locomotion resulting from gradients of cell surface adhesiveness rather than gradients of substratum adhesiveness. Morphological reorientation and cathodal displacement become apparent in NIH 3T3 and SV101 fibroblasts within minutes of electric field application. It may be argued that the process of glycoprotein electromigration is too slow to account for the rapid response of the cells to the electric field. However, if cellular adhesion receptors are among the redistributed Con A receptors, sufficient gradients of cellular adhesiveness might be created very rapidly. It has been found that the migration of B16F10 murine melanoma cells may be directed by an estimated 0.1% difference in the density of substratum-bound adhesive ligand per cell diameter (Brandley and Schnaar, 1989). Therefore, as cell locomotion may be sensitive to even small asymmetries of substratum adhesiveness, so might it be directed by very slight gradients of plasma membrane adhesion receptors. The short period of time required to create such an immeasurably small gradient of cellular adhesiveness would be consistent with the kinetics of the galvanotactic response of NIH 3T3 and SV101 fibroblasts.

The migration of NIH 3T3 fibroblasts grown on serum-coated glass is most likely mediated by the fibronectin receptor ($\alpha5\beta1$ integrin), as these cells express little vitronectin receptor (Briesewitz et al., 1993). We have shown that exposure to DC electric fields changes the distribution of the fibronectin receptor in NIH 3T3 fibroblasts. The localization of the $\alpha5$ subunit of the fibronectin receptor to focal contacts and fibrillar streaks is reduced following field exposure. The more diffuse, less striated distribution of $\alpha5$ integrin subunit toward the cathode suggests the separation of laterally mobile and immobile pools of the receptor. The increased lateral mobility of integrin receptors has been correlated to increased cellular motility (Duband et al., 1988), and it has been reported that the migration of fibroblasts involves the forward transport of integrins toward the leading edge of the cell (Regen and Horwitz, 1992). The creation of a larger pool of less aggregated, laterally mobile fibronectin receptors toward the cathode might therefore facilitate the remodeling of adhesion and cytoskeletal architecture toward the field-defined leading edge. The observed redistribution of $\alpha5$ may be the result of fibronectin receptor electromigration. Alternatively, receptor redistribution might occur in response to a field-induced reorganization of pericellular and extracellular fibronectin.

We feel that the concept of gradients of cell surface adhesiveness may be useful toward developing a general understanding of directed cell motility. We have presented evidence that the mechanism of electric field–directed locomotion in NIH 3T3 and SV101 fibroblasts involves the electromigration of cell surface glycoproteins. In contrast to several reports using other cell types, calcium plays no apparent role in the galvanotaxis of these cells. We have observed the lateral redistribution of Con A receptors toward the cathode, and our data suggest that this event may indeed be involved in the electric field–directed locomotion. The fibronectin receptor, a presumed subset of Con A receptors, shows a cathode-directed redistribution away from large, focal aggregates. We propose a signal transduction mechanism in which electric field–induced asymmetries of plasma membrane receptors for extracellular matrix direct cellular

migration. The polarized redistribution of extracellular matrix receptors might directly influence cell adhesion and cytoskeletal assembly. In comparison, a calcium-based mechanism for directed cell migration (Hahn et al., 1992; Taylor et al., 1980; Onuma and Hui, 1988; Brundage et al., 1991), may use the same cellular machinery but in a different sequence, with intracellular calcium gradients directly or indirectly leading to polarized cytoskeletal assembly followed by the reorganization of cell–substratum contacts. Still a third related mechanism could involve intracellular modulation of integrin–extracellular ligand adhesion via cytoplasmic integrin regulatory domains (Diamond and Springer, 1994); thus, an adhesion gradient could be initiated without a reorganization of adhesion molecules. In light of the immense complexity of the mechanics and regulation of cellular locomotion, it is probable that the direction of cell migration can be influenced at many levels, but a gradient in cell surface adhesiveness may be a common unifying feature.

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