INTRODUCTION

The epidermal growth factor receptor (EGFR) is one of the most intensely studied growth factor receptors. Much has been learned about its molecular structure, biological functions and signaling pathways (reviewed in Carpenter, 1987; Schultz et al., 1991; Wiley et al., 1995; Yarden and Ullrich, 1988). The EGFR is involved in the stimulation of mitogenesis and in promoting cell motility. Its tyrosine kinase activity is required for both functions (Chen et al., 1987). Kinase-negative mutations abolish EGF-induced cell proliferation and cell motility (Chen et al., 1994a). The EGFR autophosphorylates tyrosine residues at its C terminus and phosphorylates other cellular proteins. While autophosphorylation is not required for EGF-stimulated cell proliferation, it is crucial for EGF-induced cell motility. Mutation of all five tyrosine residues at the C terminus, or deletion of the C-terminal region that contains all the tyrosine residues, does not change the thymidine incorporation rate, nor does it alter the downstream activation of protein kinase C and MAP kinase (Chen et al., 1989; Decker, 1993). However, these mutants fail to exhibit EGF-induced cell movement (Chen et al., 1994a). At least one tyrosine residue is needed at the C terminus of the EGFR for EGF-induced cell motility (Chen et al., 1994a), and therefore the signaling pathways for EGF-induced mitogenesis and cell motility diverge after EGFR kinase activity (Chen et al., 1994b).

The EGFR mediates chemotaxis and migration in a number of cell types, including fibroblasts, keratinocytes and other epithelial cells (Ando and Jensen, 1993; Barrandon and Green, 1987; Blay and Brown, 1985; Matthay et al., 1993; Westermark et al., 1991). Increased expression of the EGFR has been correlated with increased cell motility. A transient fivefold upregulation of the EGFR has been observed at the borders of tape-stripped wounds and has been hypothesized to promote the cell migration associated with wound healing (Stoscheck et al., 1992). Overexpression of the EGFR in cells such as fibroblasts, squamous cell carcinoma and breast cancer cells, enhances EGF-induced cell movement (McCawley et al., 1997; Verbeek et al., 1998). EGFR-mediated cell motility plays an important role in many biological processes, including embryonic development, wound healing, tumor progression and metastasis. Mutant mice lacking EGFR have impaired epithelial development involving skin, eyes, ears, lungs and gastrointestinal tract, generally leading to early death (Miettinen et al., 1995; Sibilia and Wagner, 1995). Increased levels of EGFR correlate with an increased invasiveness and metastasis of several tumors, such as squamous cell carcinoma, melanoma, bladder and prostate cancers, and are associated with a poor clinical prognosis (Grandis et al., 1998;
Kaufmann et al., 1996; Khazaie et al., 1993; Ma and Niederkorn, 1998; Turkeri et al., 1998; Turner et al., 1996; Verbeek et al., 1998). The role of EGF-stimulated cell motility in promoting wound healing has been demonstrated in vitro on cell culture (Matthay et al., 1993) as well as in vivo on gastroduodenal ulcers, corneal and skin wounds (reviewed in Schultz et al., 1991). While the signaling events initiated by EGFR activation continue to be uncovered, a full understanding of the mechanism by which EGFR activation contributes to the migratory response remains to be elucidated.

Several studies have shown that Wounding skin generates a lateral electric field at the wound edges. In mammals, lateral fields of 10-100 mV/mm have been measured at the borders of skin wounds, with the negative pole directed toward the center of the wounded area (Barker, 1981; Barker et al., 1982; Chiang et al., 1992; Olivotto et al., 1996). These wound-created electric fields and currents play a role in promoting cell migration during wound healing (Chang et al., 1996; Lee et al., 1993; Nuccitelli, 1988; Vodovnik and Karba, 1992; Zhao et al., 1996). In vitro, several cell types including keratinocytes, fibroblasts, corneal epithelial cells and neural crest cells, have exhibited directional migration to the cathode in DC electric fields, a phenomenon called galvanotaxis (Brown and Loew, 1994; Chang et al., 1996; Nishimura et al., 1995; Nuccitelli and Erickson, 1983; Nuccitelli et al., 1993; Soong et al., 1990; Zhao et al., 1996). Recently we have shown that normal human keratinocytes (NHK) migrate to the cathode at a rate of about 1 μm/minute in fields of physiological strength (100 mV/mm) (Fang et al., 1998; Nishimura et al., 1995). In the absence of the field, NHKs move at a similar rate but without directionality, indicating that electric fields mainly affect the directionality of cell motility. These data suggest that cell motility and migration directionality are two distinct properties of cell locomotion, subject to different regulatory mechanisms.

Since DC electric fields enable us to influence the directionality of cell motility, observing cells’ motility in the DC electric fields provides an ideal system in which to examine the independent variables of directionality and rate of keratinocyte locomotion. We have previously shown that EGF promotes directional migration of NHK in DC electric fields (Fang et al., 1998). In the absence of exogenous growth factors NHKs still move towards the cathode, probably due to the effect of autocrine secretion of factors such as TGF-α and amphiregulin, which both are natural ligands of EGFRs (van der Geer et al., 1994). In this study, we provide evidence that EGFR kinase activation is required for directional cell migration by using specific protein tyrosine kinase inhibitors. We show the role of EGFR redistribution in keratinocyte galvanotaxis by immunostaining the receptors before and after exposure to the electric field. This study provides the first insights into the signaling mechanism of directional cell migration in electric fields.

**MATERIALS AND METHODS**

**Cells and cell culture**

Normal human keratinocytes (NHK) from neonatal foreskin epidermis were prepared and cultured following the methods described previously (Isseroff et al., 1987). Samples of cells derived from each donor were cryopreserved separately. Cells were cultured in the serum-free keratinocyte growth medium (KGM). KGM (Cascade Biologics Inc., Portland, OR) is M154 medium with a calcium concentration of 0.2 mM, 10 mM Hepes (pH 7.4), antibiotics/antimycotics (penicillin, streptomycin, amphotericin) and human keratinocyte growth supplement (HKGS), which contains 0.18 μg/ml hydrocortisone, 5 μg/ml transferrin, 0.2% bovine pituitary extract, 0.2 ng/ml EGF and 5 μg/ml insulin. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂. Cells from two donors were used in this work, and cells from passage 2-5 were used for these experiments.

**Galvanotaxis**

Coverslips and cells were prepared following a procedure described previously (Fang et al., 1998; Sheridan et al., 1996). Briefly, 12 mm-wide glass coverslips were coated with bovine collagen I by soaking in calcium- and magnesium-free phosphate buffer solution (PBS) containing 2% Vitrogen 100 (60 μg/ml) (Collagen Corporation, Palo Alto, CA) for at least 1 hour at 37°C. Coverslips were rinsed three times with PBS and air-dried for 5-10 minutes before cells were plated. NHK were plated on the collagen-coated coverslips at a density of 2-5·10⁵/60 mm plate. HKGS, with or without an inhibitor for 1 hour, followed by addition of 10 ng/ml recombinant human EGF (Gibco Life Technologies, CA). PD158780 is a modified product of PD13530 (Fry et al., 1994) and was kindly provided by Park-Davis. PD158780 is at least as specific and potent inhibitor as PD13530 for EGFR (Fry et al., 1997). Recombinant human EGF was from Gibco Life Technologies (Grand Island, NY).

The galvanotaxis chamber and apparatus for applying a DC electric field has been described previously (Fang et al., 1998; Nishimura et al., 1995). KMM, or modifications as otherwise stated, was used in all experiments. An electric field of 100 mV/mm was used, based on previous studies (Nishimura et al., 1995), with a current of 0.1-0.6 mA, depending on the resistance across the chamber. All experiments were performed at 37±2°C in room air.

**Recording and data analysis for galvanotaxis**

Cells were observed with phase contrast optics on inverted microscopes. Video images of cells were digitally captured every 10 minutes for 1 hour, or as otherwise indicated, to an image analysis program on a Power Macintosh 8500 using a modified version of NIH Image 1.60 and File Maker Pro 3.0. At the end of 1 hour, the center of each cell was identified manually on each image. The translocation distance and directionality of migration of each imaged cell were plotted. The translocation distance covered by each cell was measured and the migration rate was expressed as μm/minute. The directionality of the cell translocation was indicated by an average cosine θ of the angle made by each cell’s migration vector with respect to the direction of the electric field. Using the following formula: <cosθ> = (Σ cosθ/n) (Nishimura et al., 1995), where Σ = summation of cosine values obtained from 40-130 individual cells (n) taken from 3-7 experiments. cosθ = -1 if the cell moves towards the anode; cosθ = +1 if the cell moves directly towards the cathode; and cosθ = 0 if the cell moves perpendicular to the field direction or moves randomly. For any given condition, a compilation of the average rate and cosine from 40-130 cells (collected from 3-7 experiments) is presented. Also for any given condition, the migration characteristics of control cells exposed to the electric fields in the presence of unmodified KMM are included.

**Immunoblotting**

NHK were plated in KMM at a density of 1.5×10⁵ per 60 mm plate. After 2 hours, cells were incubated in 2 ml of the same medium devoid of HKGS, with or without an inhibitor for 1 hour, followed by addition of 10 ng/ml recombinant human EGF (Gibco Life Technologies, Natick, MA), lavendustin A and tyrphostin B46, (LC Lab, San Diego, CA), PD158780 is a modified product of PD13530 (Fry et al., 1994) and was kindly provided by Park-Davis. PD158780 is at least as specific and potent inhibitor as PD13530 for EGFR. The galvanotaxis chamber and apparatus for applying a DC electric field has been described previously (Fang et al., 1998; Nishimura et al., 1995). KMM, or modifications as otherwise stated, was used in all experiments. An electric field of 100 mV/mm was used, based on previous studies (Nishimura et al., 1995), with a current of 0.1-0.6 mA, depending on the resistance across the chamber. All experiments were performed at 37±2°C in room air.
Grand Island, NY) for 10 minutes. Cells were rinsed 3 times with ice-cold PBS and lysed in 0.4 ml sample loading buffer. Samples of cell lysates (40 μl) were subjected to 8% SDS-PAGE, followed by immunoblotting (Fang et al., 1994) with antiphosphotyrosine antibody PY20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or monoclonal anti-EGFR antibody Ab-1 (Oncogene Research, Cambridge, MA). The immunoblots were developed by enhanced chemiluminescence according to the manufacturer’s instructions (ECL, Amershams Life Science).

**Immunostaining of EGFR on the cell surface**

NHKs were plated on collagen-coated coverslips and rinsed with ice-cold KGM. Nonspecific binding sites were blocked by incubation in the same medium supplemented with 10% normal goat serum and 3% BSA for 10 minutes. Cells were then incubated with 10 μg/ml anti-human EGFR monoclonal antibody, Ab-1 (Oncogene Research, Cambridge, MA) for 1 hour at room temperature, and washed 3 times with KGM. Cells were then fixed in 4% paraformaldehyde for 15 minutes, washed 3 times in PBS, and incubated with biotin-conjugated anti-mouse antibody (1:1500) overnight at 4°C. After washing in PBS, cells were incubated with avidin-FITC (1:1500) for 30 minutes, washed 3 times in PBS, and incubated for 5 minutes in equilibrium buffer (Molecular Probe, Eugene, Oregon). Coverslips were mounted with glycerol buffer (Molecular Probe, Eugene, Oregon) to minimize quenching of fluorescence. Cells were viewed with epifluorescence optics at 600x.

**Analysis of staining intensities on plasma membrane**

Fluorescence micrographs of stained cells were captured by NIH Image 1.60 and stored in a Power Macintosh 8500. A computer program was written in Matlab to calculate the intensity of the staining on the cell surface. The algorithm contains the following steps, and an example in action is given in Fig. 6. The image of a cell was thresholded to get a black and white image where the black area approximates the shape of the cell (Fig. 6A,B). The closing operation from the mathematical morphology described previously by Serra (1982) and Stoyan et al. (1995) was employed to clean up the threshold, followed by filling in the white areas in the cell and removing the black areas in the background unattached to the cell (Fig. 6C). The boundary of the stained cell was defined by a width of several pixels and the intensity of the boundary was measured (Fig. 6D). The threshold and boundary width were chosen for each set of cells to achieve the best approximation to the original cell images. To make statistical inference about the staining location on the cell surface, the correlation between the intensity curve $I(θ)$ and a function $f(θ)$ is presented in Fig. 6E for a single cell, according to the following formula:

$$\text{Asymmetry Index (AI)} = \frac{\langle I(θ) \times f(θ) \rangle}{\sqrt{\langle I(θ) \rangle^2 - \langle I(θ) \rangle^2 \langle f(θ) \rangle}} ,$$

where $f(θ)$ is a function with zero average, i.e. $\langle f(θ) \rangle = 0$. We take that $f(θ) = \cos θ$, with the result that when the stain is stronger on the upper boundary, i.e. the cathodal side, AI is positive. When the stain is stronger on the lower boundary, i.e. the anodal side, AI is negative. When the stain is close to uniform, or random, or concentrated around $θ = ±90°$, AI is close to zero. Data from many cells can be compiled to derive the average AI of a group of test cells (Fig. 6F).

**RESULTS**

**Effects of protein kinase inhibitors on keratinocyte galvanotaxis**

In DC electric fields of physiological strength (100 mV/mm) in environments that include EGF, human keratinocytes migrate directionally toward the cathode at a rate of about 1.0 μm/minute and exhibit a net $\cos θ$ of 0.5±0.1 in 1 hour (Fang et al., 1998; Nishimura et al., 1995) (Fig. 1A). Based on this information, we first examined the role of the EGFR kinase in

Fig. 1. The net translocation of human keratinocytes in 100 mV/mm DC electric fields. Images of the migration paths were captured every 10 minutes and the translocation distance and directionality calculated as described in Materials and methods. For each experiment, the data were plotted using a circle graph. Each cell’s position at time (t) = 0 minutes is at the origin (0, 0), and its final position at the end of the 1 hour exposure to the DC field is plotted as a single point on the graph. The radius of each circle represents 75 μm of translocation distance. The cathode is at the top of each graph (0°), and the anode at the bottom (180°). In the upper left corner of each circle plot is indicated the average rate of migration, the average cosine and the total number of cells studied for a given condition (n). (A) control cells in the absence of any inhibitor, (B) cells incubated with 0.5 μM PD158780, (C) with 1 μM PD158780 or (D) with 50 μM tyrphostin B46, each for 1 hour prior to exposure to the DC field. X, the coordinate along the field direction; Y, the coordinate transverse to the field.
keratinocyte migration by applying specific inhibitors. One of the most specific and the strongest inhibitors for the EGFR kinase is PD158780 (Fry et al., 1997). NHKs were preincubated in the presence of PD158780 at various concentrations for 1 hour then exposed to DC electric fields, 100 mV/mm. The control cells were incubated in medium containing DMSO at the same concentrations as the test cells. A DMSO level equal or lower than 0.5% in medium for several hours had no effect on cell survival, morphology and motility (data not shown). As shown in Fig. 2A and Table 1, at 0.1 μM, PD158780 did not change either the rate or the directionality of NHK locomotion. At 0.25 μM, it decreased NHK migration directionality by 44%, to \( \cos \phi = 0.28 \pm 0.08 \) (\( P<0.001 \)), but the migration rate remained the same as observed in control cells. At 0.5 μM, PD158780 abolished NHK migration directionality to an average \( \cos \phi = 0.014 \) (\( P<0.0001 \)), but only mildly reduced NHK migration rate to 0.8±0.04 μm/minute, which was 89% of that exhibited in the control cells (NS with \( P>0.05 \)) (Fig. 1B). As the concentration increased to 1 or 5 μM, PD158780 continued to inhibit NHK migration directionality (average \( \cos \phi \) near zero), and further reduced the NHK migration rate to 0.5-0.6 μm/minute, 62% and 60% of the control rate, respectively (\( P<0.0001 \)) (Figs 1C, 2A). Therefore, at low concentrations (0.25-0.5 μM) PD158780 inhibits only NHK migration directionality, while at higher concentrations (1.0 μM and above) it inhibits both cell migration directionality and rate.

To determine if this inhibitory effect of PD158780 was common to all protein tyrosine kinase inhibitors, we examined several widely used inhibitors, including genistein, lavendustin A and tyrphostin B46. All three have been reported to influence cell motility (Basson et al., 1994; Hu and Fan, 1995; Klemke et al., 1994; Linossier et al., 1990; Nakamura et al., 1995; Ridley and Hall, 1994; Williams et al., 1994). When NHKs were placed in electric fields in the presence of these inhibitors, cells migrated more slowly than control cells (Fig. 2B, Table 1). Cell migration rates were further reduced when the concentration of inhibitors increased. However, under all these conditions, NHKs continued to exhibit directional cathodal migration, with average \( \cos \phi \) equivalent to those of each experiment’s control cells (Table 1, Fig. 1D). When the concentration of genistein reached 50 μM, NHK stopped moving without any alteration of cell morphology. The migration rate of 0.2 μm/minute observed under this condition (Table 1) was too small to be reliably measured by this image analysis system (see Materials and methods), so there was no valid measurement of directionality, i.e. the average \( \cos \phi \) value. These data indicate that other less EGFR-specific tyrosine kinase inhibitors, including genistein, lavendustin A and tyrphostin B46, inhibit only the keratinocyte migration rate without affecting migration directionality.

### Effects of tyrosine kinase inhibitors on protein tyrosine phosphorylation levels

Inhibitors used in our studies are known to be specific for protein tyrosine kinases. Some, such as PD 158780, have exhibited higher specificity for the EGFR kinase (Fry et al., 1997), and others, such as lavendustin A, were known for their

#### Table 1. Effects of protein tyrosine kinase inhibitors on keratinocyte galvanotaxis

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Rate§ (μm/min) (%)</th>
<th>Average ( \cos \phi )</th>
<th>Cell number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.7±0.08 (100)</td>
<td>0.55±0.07</td>
<td>67</td>
</tr>
<tr>
<td>PD158780 (0.1 mM)</td>
<td>0.7±0.05 (100)</td>
<td>0.58±0.06</td>
<td>35</td>
</tr>
<tr>
<td>PD158780 (0.25 mM)</td>
<td>0.8±0.07 (114)</td>
<td>0.28±0.08</td>
<td>80</td>
</tr>
<tr>
<td>Control</td>
<td>0.9±0.04 (100)</td>
<td>0.44±0.06</td>
<td>126</td>
</tr>
<tr>
<td>PD158780 (0.5 mM)</td>
<td>0.8±0.04 (89)</td>
<td>0.00±0.08</td>
<td>114</td>
</tr>
<tr>
<td>Control</td>
<td>0.8±0.05 (100)</td>
<td>0.51±0.07</td>
<td>80</td>
</tr>
<tr>
<td>PD158780 (1.0 mM)</td>
<td>0.5±0.02 (62)</td>
<td>0.02±0.08</td>
<td>132</td>
</tr>
<tr>
<td>Control</td>
<td>1.0±0.06 (100)</td>
<td>0.48±0.07</td>
<td>57</td>
</tr>
<tr>
<td>PD158780 (5.0 mM)</td>
<td>0.6±0.04 (60)</td>
<td>0.05±0.09</td>
<td>61</td>
</tr>
<tr>
<td>Control</td>
<td>1.0±0.06 (100)</td>
<td>0.31±0.11</td>
<td>47</td>
</tr>
<tr>
<td>Genistein (10 mM)</td>
<td>0.6±0.04 (60)</td>
<td>0.32±0.10</td>
<td>43</td>
</tr>
<tr>
<td>Genistein (50 mM)</td>
<td>0.2±0.02 (16)</td>
<td>NV</td>
<td>34</td>
</tr>
<tr>
<td>Control</td>
<td>0.9±0.05 (100)</td>
<td>0.42±0.08</td>
<td>155</td>
</tr>
<tr>
<td>Lavendustin A (10 mM)</td>
<td>0.6±0.04 (67)</td>
<td>0.32±0.09</td>
<td>74</td>
</tr>
<tr>
<td>Lavendustin A (20 mM)</td>
<td>0.5±0.03 (56)</td>
<td>0.26±0.13</td>
<td>62</td>
</tr>
<tr>
<td>Control</td>
<td>1.0±0.05 (100)</td>
<td>0.24±0.12</td>
<td>30</td>
</tr>
<tr>
<td>Tyrphostin B46 (50 mM)</td>
<td>0.4±0.03 (40)</td>
<td>0.50±0.11</td>
<td>39</td>
</tr>
</tbody>
</table>

§Cell migration rate under the indicated conditions is compared in percentage with the rate of control cells.

*\( P<0.001 \) compared to control of each set.

‡\( P<0.05 \) compared to control of each set.

NS, not significant at all compared to control of each set.

NV, not valid. Cells that moved at 0.3 μm/min or less are considered as no movement.

![Fig. 2. Protein tyrosine kinase inhibitors inhibit keratinocyte galvanotaxis](image-url)
EGF receptor directs keratinocyte galvanotaxis (Hsu et al., 1991; Onoda et al., 1989). To confirm their biochemical activities in vitro, we examined the cellular protein tyrosine phosphorylation levels after EGF stimulation of cells incubated in the presence of these inhibitors. NHKs were plated at a density similar to that used for the galvanotaxis studies, where most cells were not in contact with another cell. Following stimulation by EGF, cell lysates were immunoblotted with the anti-phosphotyrosine antibody, PY20. Fig. 3A shows that NHKs have a low basal level of tyrosine phosphorylation on cellular proteins and EGFR (lane 1). The position of EGFR on the western blot was confirmed by immunoprecipitation of the EGFR, followed by western blot using anti-EGFR antibody (data not shown). EGF stimulation greatly increased tyrosine phosphorylation levels (lane 2). All four inhibitors used in our study effectively reduced EGF-induced tyrosine phosphorylation in cells, including the EGFR itself (lanes 3-6). Inhibitors were employed at higher concentrations, at which they exhibited unequivocally strong effects on cell migration (Table 1). Among them, 5 μM PD158780 showed the strongest and the most complete inhibition on EGF-induced tyrosine phosphorylation (lane 3), while genistein (50 μM), lavendustin A (20 μM) and tyrphostin B46 (50 μM) offered partial but similar inhibition (lanes 4-6). The inhibitory effect of PD158780 was further examined to correlate its biological effect on cell locomotion (Fig. 3B). As the PD158780 level increased from 0.1 to 5 μM, less EGF-induced protein phosphorylation was observed. Once its concentration reached 5 μM or above, both cell migration directionality and motility were inhibited, and the tyrosine phosphorylation level dropped below the basal level. These immunoblotting assays confirm the biochemical activities of inhibitors and are consistent with previous reports (Fry et al., 1994; Levitzki, 1992; Onoda et al., 1989; Williams et al., 1994). Our data indicate that a partial inhibition of EGF-induced phosphorylation correlated with a reduced migration rate, while the complete blockage of EGF-induced phosphorylation reduced both migration rate and directionality.

Immunostaining of EGFRs on NHK plasma membrane

In addition to the signaling role of the EGFR kinase, we also investigated whether the electric field-induced redistribution of EGFRs on the cell surface could contribute to the mechanism for the directional galvanotaxis response of NHK. Due to the low density of EGFR on normal keratinocytes (estimated at about 10^2-10^4 EGFRs on the plasma membrane per cell; Kaufmann et al., 1996; Matthay et al., 1993; Zendegui et al., 1996) chemical instability, NHK were incubated in the absence (−) or presence of different inhibitors (A) or various concentrations of PD158780 (B) for 1 hour. Cells were then stimulated with (+) or without (−) 10 ng/ml EGF for 10 minutes. Whole cell lysates were harvested and subjected to western blots by antiphosphotyrosine antibody PY20 and stained bands were visualized by enhanced chemiluminescence. In A the concentrations of inhibitors used were as follows: PD, 5 μM PD158780; Gen, 50 μM genistein; Lav, 20 μM lavendustin A; TyB46, 50 μM tyrphostin B46. Molecular mass values (kDa) are shown on the left. Arrowheads indicate the position of EGFR.

### Table 2. Distribution of EGFRs on keratinocyte plasma membrane after exposure to DC electric fields

<table>
<thead>
<tr>
<th>Time in electric field (minutes)</th>
<th>Asymmetry Index‡ for sides of cell facing poles of the field</th>
<th>P value*</th>
<th>Asymmetry Index for sides parallel to field</th>
<th>P value*</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.05</td>
<td>0.3690</td>
<td>0.034</td>
<td>0.472</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>0.0001</td>
<td>0.186</td>
<td>0.029</td>
<td>23</td>
</tr>
<tr>
<td>15</td>
<td>0.26</td>
<td>0.0000</td>
<td>0.080</td>
<td>0.119</td>
<td>36</td>
</tr>
<tr>
<td>60</td>
<td>0.14</td>
<td>0.0020</td>
<td>-0.101</td>
<td>0.172</td>
<td>38</td>
</tr>
<tr>
<td>PD158780</td>
<td>0.01</td>
<td>0.861</td>
<td>0.063</td>
<td>0.100</td>
<td>47</td>
</tr>
</tbody>
</table>

‡When this number is positive, more receptors are found on the side of the cell facing the cathode than on that facing the anode. When it is negative, more receptors are located on the anode-facing side.

*Probability of obtaining the indicated asymmetry index if there were no asymmetry. A two-tailed Student’s t-test was used to determine significance.

Fig. 3. Protein tyrosine kinase inhibitors prevent EGF-induced protein tyrosine phosphorylation. NHK were incubated in the absence (−) or presence of different inhibitors (A) or various concentrations of PD158780 (B) for 1 hour. Cells were then stimulated with (+) or without (−) 10 ng/ml EGF for 10 minutes. Whole cell lysates were harvested and subjected to western blots by antiphosphotyrosine antibody PY20 and stained bands were visualized by enhanced chemiluminescence. In A the concentrations of inhibitors used were as follows: PD, 5 μM PD158780; Gen, 50 μM genistein; Lav, 20 μM lavendustin A; TyB46, 50 μM tyrphostin B46. Molecular mass values (kDa) are shown on the left. Arrowheads indicate the position of EGFR.
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1988) and the stronger intracellular EGFR signal observed on immunostaining, it was difficult to visualize the EGFR on the plasma membrane of individual keratinocytes. We modified the immunostaining protocol by incubating live cells with monoclonal antibody, Ab-1, which recognizes an extracellular domain of human EGFR, followed by fixation. The staining signal was amplified by biotin-avidin bridging of the fluorescein indicator. By these modifications, we were able to minimize the intracellular signal and to visualize EGFRs on NHK plasma membrane (Fig. 4A). Without Ab-1 there was no signal visible (Fig. 4B). We used the human epithelial carcinoma cell A431 as a positive control because A431 cells possess a larger number of EGFRs on cell surface (up to $10^6$ per cell; Good et al., 1992; Wiley, 1988). Indeed A431 cells were stained strongly by Ab-1 (Fig. 4C). Ab-1 is specific for the human EGFR and does not recognize the rodent counterpart, thus a mouse keratinocyte line PVD (Fig. 4D) and mouse fibroblast NIH3T3 (data not shown) exhibited no labeling.

Distribution of EGFRs on NHK plasma membrane after exposure to electric fields

We investigated the effect of DC electric fields on the distribution of EGFRs on NHK after exposure to physiological electric fields for various lengths of time. Fig. 5B-D shows that keratinocytes exhibit stronger staining at the cathodal side of the plasma membrane after exposure to the electric fields, compared to cells without exposure (Fig. 5A). This field-induced cathodal staining was observed as early as 5 minutes after exposure to DC electric fields (Fig. 5B), and remained so at 15 minutes and 60 minutes after exposure to the fields (Fig. 5C,D). Fig. 5G,H presents an example of the EGFR-staining pattern and the transmitted light image of a keratinocyte after exposure to DC electric field (100 mV/mm) for 15 minutes. The EGFR was stained more strongly not only at the leading edge but also on the cathodal side of the motile cells. We noticed that, to preserve the asymmetrical staining on cells, it was necessary to rinse cells with the ice-cold medium immediately after termination of electric fields, followed by incubation of cells with Ab-1 at room temperature. These treatments probably minimize the receptor internalization and lateral diffusion in plasma membrane; the latter is about $2.4 \times 10^{-10}$ cm$^2$/second (Giugni et al., 1987).

In order to quantitate the asymmetry in the EGFR distribution on the cell surface, the fluorescence micrographs were analyzed using a Matlab program (see Materials and methods). For each time point, 23-47 cells exposed to the electric field were analyzed to achieve statistical significance, and experiments were repeated 2-3 times. Quantitation of the EGFR distribution is exemplified in Fig. 6E as the staining profile for a single cell and in Fig. 6F for a group of cells ($n=23$; these cells were exposed to DC electric fields for 5 minutes), where 0 degree is designated to the cell surface directly facing to the cathode and 180 degree facing to the anode. The statistical values of the EGFR asymmetries at various time points are summarized in Table 2.

When cells are not exposed to electric fields, they exhibit a relatively uniform distribution of EGFRs on the plasma membrane and have an average cathodal-to-anodal EGFR asymmetry index near zero ($n=46$, Table 2). DC electric fields induced a redistribution of EGFRs on the NHK cell surface, as indicated by an average cathodal-to-anodal staining asymmetry index of 0.25, 0.26 and 0.14 after 5, 15 and 60 minutes exposure to the fields, respectively (Table 2). Cells continued to exhibit the cathodal staining as long as they were exposed to the fields up to 2 hours (data not shown). The staining asymmetry index for two sides of the cells parallel to the electric field lines is presented as internal controls to demonstrate that there was either no difference (as 0.03 and 0.08 at 0 minutes and 15 minutes, respectively, Table 2) or

*Fig. 4. Immunostaining of EGFRs on keratinocyte plasma membrane. NHK (A,B), A431 (C) and PDV (D) were plated on collagen-coated coverslips. After rinsing in PBS, cells were incubated with (A,C,D) or without (B) anti-EGFR antibody Ab-1, followed by 4% paraformaldehyde. Stainings were visualized by avidin-FITC (see Materials and methods). Bar, 30 μm.*
some difference but of less statistical significance ($P=0.03$ and 0.17 at 5 minutes and 60 minutes, Table 2) in the distribution of the EGFR on the sides of the cells parallel to the field. These data suggest that DC electric fields induced EGFR receptor relocalization to the cathodal side of the plasma membrane, which in turn guided NHK directional migration. Since the half time of the keratinocyte galvanotaxis response is 10-15 minutes (Fang et al., 1998; Nishimura et al., 1995), EGFR lateralization on the plasma membrane occurs earlier than the detectable directional cell movement. Interestingly, 1 μM PD158780 abolished this electric field-induced EGFR relocalization on NHK plasma membrane (Fig. 5E) with an average cathodal-to-anodal staining asymmetry index of 0.01 (Table 2), suggesting another possible mechanism of PD158780 in inhibiting cell migration directionality.

During galvanotaxis, we noticed that if two cells were less than one-cell diameter apart, they tended to attract and move towards each other first, and then moved together towards the cathode (Nishimura et al., 1995). This two-cell attraction is most likely chemotactical, mediated by autocrine factors such as TGF-α and amphiregulin, both of which act through binding to EGFRs. On these cells, stronger EGFR staining was indeed observed on the sides of the plasma membrane facing each other (Fig. 5F), rather than on the cathodal face of the cell. This is yet another indicator for the role of the EGFR in directing cell movement.

**DISCUSSION**

Several lines of evidence presented in this study demonstrate that the EGFR is involved in regulating the direction of
keratinocyte migration. In DC electric fields, EGFR concentrates on the cathodal side of the plasma membrane. This receptor relocalization occurs before cell movement to the cathode can be detected. The loss of directional migration in the presence of the EGFR kinase inhibitor PD158780 was correlated with greatly diminished EGF-stimulated tyrosine phosphorylation of EGFR and other cellular proteins, and with the disappearance of EGFR lateralization. When two cells approached each other, EGFRs aggregated at the leading edges of the plasma membranes (Fig. 5F). These findings provide the first insights into the role of EGFR signaling in the mechanism of galvanotaxis.

The directional migration seen in galvanotaxis is similar to chemotaxis, and may also involve growth factors and their receptors (Anand-Apte and Zetter, 1997; Foxman et al., 1997; Mackay, 1996). Directed cell movement requires two essential events: a localized stimulus and an asymmetrically activated intracellular signaling pathway. The localized stimulus creates a spatial difference between a cell’s leading edge compared to its trailing edge. The asymmetrically activated signaling pathway leads to reorganization of the cytoskeletal architecture at the leading edge and initiates the motility machinery.

In chemotaxis, the localized stimulus is provided by a gradient of soluble factors, while in galvanotaxis it is provided by a gradient in voltage. Unlike chemotaxis, where the stimulation of a chemotactic agonist is hard to maintain (Foxman et al., 1997), the electric field provides a sustained and constant stimulus during galvanotaxis, so that keratinocytes continue to move towards the cathode as long as they are exposed to the field (Nishimura et al., 1995). When the field is discontinued, cells gradually lose their directionality and eventually return to random migratory motion (data not shown).

The accumulation of the EGFR at the cathodal face of the cell provides the opportunity for triggering localized signaling. EGFR kinase activation and autophosphorylation initiate intracellular signaling pathways. It has been reported that addition of EGF triggers reorganization of actin filaments (Rijken et al., 1991; Schlessinger and Geiger, 1981; Van Bergen en Henegouwen et al., 1989), and causes association and colocalization of EGFR with its downstream effector proteins, such as phospholipase C-γ1 (PLC-γ1), and with actin filaments at membrane ruffles (Diakonova et al., 1995). The activated EGFR binds and activates PLC-γ1, resulting in hydrolysis of phosphatidylinositol-4,5-bisphosphate with production of inositol phosphates and diacylglycerol (Chen et al., 1994b). Each of these mediators contributes to cell motility. Diacylglycerol has the potential to nucleate actin assembly, inositol triphosphates release calcium from intracellular stores, and phosphatidylinositol-4,5-bisphosphate associates with several proteins implicated in actin reorganization and motility responses (e.g. profilin, gelsolin, myosin type I, actin-binding proteins and MARCKS) (Chen et al., 1996; Giuliano and Taylor, 1994; Janney et al., 1998; Myat et al., 1997; Shariff and Luna, 1992). Autophosphorylated EGFR also binds to phosphotyidylinositol-3 kinase (Payrastre et al., 1992); the latter has been implicated in PDGF-induced actin reorganization and cell motility (Hooshmand-Rad et al., 1997; Wennstrom et al., 1994). EGFR also associates with cytoskeletal elements in vivo (Van Bergen en Henegouwen et al., 1992) and binds to actin filaments directly in vitro (den Hartigh et al., 1992). Reorganization of actin filaments accompanies changes in cell morphology, membrane ruffling, lamellipodium formation and cell movement (Anderson et al., 1996; Lauffenburger, 1996; Lewis and Bridgman, 1992; Nobes and Hall, 1995). EGFR also interacts directly or indirectly with Src, Grb2 and SHC in cells, all of which are implicated in membrane ruffling and cell motility (Hall, 1994; van der Geer et al., 1994). Recently, Xie and his colleagues have demonstrated an EGF-induced disassembly of focal adhesions in fibroblasts, which correlates with EGF-induced cell motility but may be regulated by different signaling pathways (Xie et al., 1998). Thus, there are potentially multiple downstream mechanisms which can be asymmetrically activated near the cathodal face of the cell following electric field-induced EGFR relocation. Collectively, these may contribute to the directional migratory response.

Other cell types also exhibit galvanotaxis, including...
fibroblasts, epithelial cells, neural crest cells and osteoclasts (Brown and Loew, 1994; Chang et al., 1996; Nishimura et al., 1995; Nuccitelli and Erickson, 1983; Nuccitelli et al., 1993; Soong et al., 1990; Zhao et al., 1996). At the molecular level, the electric field has been shown to induce microfilament reorganization in fibroblasts and hepatoma cells (Cho et al., 1996; Luther et al., 1983; Onuma and Hui, 1988), and to induce redistribution of proteins on the cell surface such as receptors for transferrin, low density lipoprotein, acetylcholine, concanavalin A, immunoglobulin E, as well as EGF (Cho et al., 1994; Jaffe, 1977; McLoughlin and Poo, 1981; Ryan et al., 1988; Tank et al., 1985; Peng et al., 1993). The mechanism of galvanotaxis is unknown. The field-induced redistribution of these cell surface proteins could be one of the ways for cells to sense and respond to electric fields. Several theories have been proposed for the mechanism by which proteins redistribute in response to electric fields: (1) lateral electrophoresis and/or electroosmosis (Jaffe, 1977; McLoughlin and Poo, 1981; Poo, 1981); (2) field-induced asymmetric depolarization of cells at the cathodal versus the anodal face (Gross et al., 1986) (reviewed in Nuccitelli, 1988); (3) asymmetric protein distribution may involve interaction(s) between cell surface proteins and other cellular or extracellular components (Arena et al., 1990; Giungi et al., 1987; Stollberg and Fraser, 1990). Lastly, it may also involve differential exocytosis/endocytosis of receptors on the cathodal versus the anodal face of cells. Recently Peng and his colleagues found that activation of protein tyrosine kinase(s) is required for accumulation and possibly retention (‘trapping’) of acetylcholine receptor (AChR) at the cathodal side of Xenopus muscle cells (Peng et al., 1993). An inhibitor specific for tyrosine kinase abolishes the cathodal accumulation of AChR on cells. In any event, redistribution of these membrane proteins alone has not been shown to associate with any altered biological function. Our data on keratinocyte galvanotaxis and its modification in the presence of PD158780, an inhibitor highly specific for EGFR, have demonstrated for the first time a direct correlation between tyrosine kinase activity, receptor relocation and directional migration of human keratinocytes in DC electric fields.

PD158780 inhibits the directional migration of keratinocytes in electric fields, possibly by affecting both of the events that are essential to directed cell movement. It abolishes the field-induced relocalization of EGFRs, overriding the effect of the voltage gradient. Similar to AChR (Peng et al., 1993), EGFR relocalization and accumulation to the cathodal face of keratinocytes may require activation of protein tyrosine kinase(s), including EGFR itself. Receptor accumulation and retention may also rely on interactions between the phosphorylated EGFR with other cellular proteins. Such interactions restrain protein diffusion in the plasma membrane. By inhibiting tyrosine kinase activity and autophosphorylation of EGFR, PD158780 can prevent EGFR aggregation and retention to the cathodal face of the cell. By the same inhibitory effect, PD158780 can also block the activation of intracellular signaling pathways downstream from EGFR. As discussed above, these pathways involve several known proteins, such as PLC-γ1, phosphotidylinositol-3 kinase, Src, Grb2 and SHC. These proteins are associated with cell motility (Diakonova et al., 1995; Hall, 1994; van der Geer et al., 1994). We would like to bear in mind that PD158780 may exert its action on keratinocyte galvanotaxis via some unknown mechanism other than inhibiting EGFR tyrosine kinase activity. Interestingly, at lower concentrations PD158780 selectively inhibits EGFR kinase, which prevents EGFR relocalization and abolishes the directionality of keratinocytes migration. At higher concentrations PD158780 may extend its inhibitory action to the downstream signaling pathways or to other protein tyrosine kinases that reduce cell migration rate.

Another possible mechanism involved in directing cell migration is the site-specific phosphorylation on EGFR. Autophosphorylation of the EGFR is crucial for EGF-induced cell motility. By introducing mutated EGFRs to receptor-deficient NR6 cells, Chen and his colleagues have demonstrated that autophosphorylation of at least one tyrosine residue at the C terminus of the EGFR is required for EGF-induced cell movement (Chen et al., 1994a). However, the full motility may require autophosphorylation on several EGFR tyrosine residues. Since there are at least five tyrosine residues at the C terminus, partial blocking of autophosphorylation by low levels of PD158780 may not affect overall cell migration rate (Fig. 3B, Table 1). The cell migration rate decreases only when the phosphorylation of most tyrosine residues is prevented, or when other protein tyrosine kinases are inhibited by high levels of PD158780 or by other, less specific, inhibitors used in this study. This scenario can be further examined by galvanotaxis of cells containing EGFR mutants on different tyrosine residues. Using these mutants, one may also obtain information on which tyrosine residue of EGFR may be involved in directional cell migration. Such studies are currently underway in our laboratory.

The relative staining intensity of EGFRs on the cathodal as compared to the anodal sides of cells can be affected by the rate of receptor internalization. This has been demonstrated by Tank and his colleagues on low-density lipoprotein-receptors (Tank et al., 1985). We have also found that the asymmetrical EGFR staining can be only reliably detected when the post-field cells are rinsed immediately by cold medium and incubated with antibodies at room temperature, presumably due to the fact that the lower temperature slows down the rate of receptor internalization and lateral diffusion in plasma membrane (Azevedo and Johnson, 1990; Carpenter and Zendegui, 1986; Gadella and Jovin, 1995). The staining intensity of EGFRs can also be influenced by the strength of applied electric fields, as demonstrated for IgE receptors (Ryan et al., 1988). Previous work from this laboratory has also shown that keratinocyte galvanotaxis begins earlier in fields of 400 mV/mm than in those of 100 mV/mm (Nishimura et al., 1995). Presumably EGFR relocalization also begins earlier at these higher field strengths. Indeed, we observed a stronger asymmetry in EGFR staining in fields of 400 mV/mm than in 100 mV/mm (data not shown). Nevertheless, we chose to perform this investigation with the field strength of 100 mV/mm because it matches the physiological values measured during wounding, development and neurite induction (Barker et al., 1982; Chiang et al., 1991; Erickson and Nuccitelli, 1984; Iglesia et al., 1996; Illingworth and Barker, 1980; Jaffe and Poo, 1979; Nuccitelli, 1988; Nuccitelli and Erickson, 1983; Patel and Poo, 1984; Stump and Robinson, 1986). We also noted that, although EGFRs are generally stained more strongly on the cathodal side of cell surface, receptors do not always redistribute directly towards the cathode. Instead, the
staining frequently favors one side or the other, even occasionally on the anodal side. This phenomenon may explain the zigzag movement of keratinocytes towards the cathode in DC electric fields (Nishimura et al., 1995; data not shown). This ‘tacking behavior’ may result from stochastic cell rotation (Alt, 1995; Anderson et al., 1996), although more restrained by the electric field, or to cyclic membrane ruffles, hydrostatic pressure or mechanical tension (Alt et al., 1995; Bray and Hollenbeck, 1988; Kolega, 1986; Oliver et al., 1994).

In light of our results, we propose the following scenario. When an extracellular electric field is applied, the EGFR asymmetrically redistributes to the cathodal face of the cell. The localized EGFRs are activated, either by ligand binding or by the electric field itself, phosphorylated and trapped. This is followed by localized recruitment and activation of downstream signaling proteins, such as PLC-γ1, phosphotyrosininositol-3 kinase and actin binding proteins. These proteins induce focal adhesion disassembly, stress fibers to disappear, actin filaments to reorganize and lamellipodia to appear (Xie et al., 1998; Anderson et al., 1996; Divecha and Irvine, 1995; Laufenberger, 1996; Payrastre et al., 1992; van der Geer et al., 1994). Because of the higher concentration of EGFRs on the cathodal side of the cell surface, asymmetric membrane activity on the cathodal side prevails and directional cell migration towards the cathode ensues. Of course, other cellular events such as activation of calcium channels, formation of integrin gradients, and localized secretion of extracellular matrix and/or proteases may also take place in DC electric fields (Brown and Loew, 1996; Clark and Brugge, 1995; Matrisian and Hogan, 1990; Onuma and Hui, 1985; Rosen and Greenberg, 1996; Yoshida et al., 1990). The relationships between EGFR, calcium concentration, integrin distribution and microfilament reorganization are central questions for our future studies.

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