Cyclic AMP mediates keratinocyte directional migration in an electric field

Christine E. Pullar* and R. Rivkah Isseroff

Department of Dermatology, University of California, Davis, CA 95616, USA

*Author for correspondence (e-mail: cepullar@ucdavis.edu)

Accepted 16 February 2005
Journal of Cell Science 118, 2023-2034 Published by The Company of Biologists 2005
doi:10.1242/jcs.02330

Summary

Re-epithelialization of wounded skin is necessary for wound closure and restoration of barrier function and requires directional keratinocyte migration towards the center of the wound. The electric field (EF) generated immediately upon wounding could be the earliest signal keratinocytes receive to initiate directional migration and healing. Keratinocytes express many β2-adrenergic receptors (β2-ARs), but their role in the epidermis is unknown. We have previously shown that β-AR agonists decrease keratinocyte migration in a cyclic AMP (cAMP) independent mechanism involving the activation of protein phosphatase 2A (PP2A). Here, we ask whether β2-ARs play a role in keratinocyte galvanotaxis.

We report a bimodal response. When keratinocytes were exposed to higher concentrations of β-AR agonist (0.1 μM), their tracked migratory speed was inhibited, in both the presence (directional migration) and the absence (random migration) of a 100 mV mm^{-1} EF, as expected. At lower agonist concentrations (0.1 pM to 0.1 nM), there was no effect on migratory speed; however, all directionality was lost – essentially, cells were 'blinded' to the directional cue. Preincubating the cells with β-antagonist restored directional migration, demonstrating that the 'blindness' was β2-AR mediated. Incubation of keratinocytes with agents known to increase intracellular cAMP levels, such as sp-cAMP, pertussis toxin and forskolin, resulted in similar 'blinding' to the EF, whereas random migration was unaffected. The inactive cAMP analog rp-cAMP had no effect on keratinocyte migration, whether directional or random. However, rp-cAMP pretreatment before β-agonist addition fully restored galvanotaxis, demonstrating the complete cAMP dependence of the attenuation of keratinocyte directional migration. This is the first report that cAMP is capable of mediating keratinocyte galvanotaxis. β-AR agonists and antagonists could be valuable tools for modulating re-epithelialization, an essential step in the wound-healing process. Thus, β-ARs regulate the two distinct components of keratinocyte directional migration differently: migration speed via a cAMP-independent mechanism and galvanotaxis by a cAMP-dependent one.

Key words: Wound healing, β-Adrenergic receptor, Electrotaxis, Galvanotaxis, Adenylyl cyclase

Introduction

When skin is wounded, keratinocytes migrate directionally into the wound bed to initiate re-epithelialization, which is necessary for wound closure and restoration of barrier function. Many factors are involved in orchestrating the complex process of wound healing (Martin, 1997). We believe that one of the earliest signals to initiate directed cell migration could be the lateral electric field (EF) generated immediately upon wounding. The lateral EF is driven by the transepithelial potential through the low-resistance wound pathway, creating a negative pole (cathode) at the center of the wound (Ojingwa and Isseroff, 2003). Endogenous direct current (DC) EFs of 40-200 mV mm^{-1} have been measured near wounds in mammalian epithelia (Barker et al., 1982; Chiang et al., 1992; McGinnis and Vanable, 1986; Sta Iglesia et al., 1996; Sta Iglesia and Vanable, 1998). Human neonatal keratinocytes do indeed sense the EF within minutes, re-form their lamellipodia facing the cathode and migrate directionally in physiological DC EFs of 100 mV mm^{-1} in vitro, a process known as galvanotaxis (Nishimura et al., 1996). The mechanism for keratinocyte sensing of the EF is largely unknown, but a role for Ca^{2+} influx (Fang et al., 1998; Trollinger et al., 2002), epidermal-growth-factor receptor (EGFR) phosphorylation (Fang et al., 1999) and protein kinase A (PKA) (Pullar, 2001) have been implicated in previous work from this laboratory. Studying EF-induced directional migration will improve our understanding of directional sensing.

Human keratinocytes express high levels of the β2-adrenergic receptor (β2-AR), no β1-ARs (Orenberg et al., 1983; Schallreuter et al., 1993; Steinkraus et al., 1992; Steinkraus et al., 1996) and no α-ARs (Cavey et al., 1986; Orenberg et al., 1983). They also have the capacity to synthesize the catecholamines epinephrine and norepinephrine (Schallreuter, 1997), but the functional role of catecholamines in the epidermis is unknown. The β2-AR is a G-protein-coupled receptor (GPCR) capable of coupling to at least two different G-proteins, $G\alpha_s$ and $G\alpha_i$ (Daaka et al., 1997). $G\alpha_s$ can activate adenylyl cyclase to increase intracellular cAMP levels and $G\alpha_i$ can decrease intracellular cAMP levels by inhibiting adenylyl cyclase (Hurley, 1999). Noting the role of GPCRs and cAMP in another form of directional migration, chemotaxis (Merlot and Firtel, 2003),

we hypothesized that these mediators might also be involved in the directional migration observed in galvanotaxis. They are known to regulate several aspects of chemotaxis: the chemoattractant signal is perceived by GPCRs (Merlot and Firtel, 2003), G-proteins play a role in chemotaxis (Arai et al., 1997; Jin et al., 2000; Neptune and Bourne, 1997; Neptune et al., 1999; Sun and Firtel, 2003), locally produced cAMP regulates *Dictyostelium discoideum* streaming during chemotaxis (Kriebel et al., 2003; Meili and Firtel, 2003), Gβ-null *D. discoideum* mutants show reduced galvanotaxis (Zhao et al., 2002) and increasing intracellular cAMP inhibits fibroblast chemotaxis (Kohyama et al., 2001; Kohyama et al., 2002) and neutrophil chemotaxis (Armstrong, 1995). For these reasons, we proposed to determine whether intracellular cAMP played a role in EF-mediated directional migration in keratinocytes.

We have previously shown that β2-AR activation (10 nM to 1 μM β-adrenergic agonist) increases cAMP but inhibits keratinocyte migration in a cAMP-independent manner (Chen et al., 2002) via protein phosphatase 2A (PP2A) activation, resulting in reduced extracellular regulated kinase (ERK) signaling (Pullar et al., 2003). Here, we investigate the role of β2-ARs and intracellular cAMP in the EF-mediated directional migration of keratinocytes that might play an important part in initiating re-epithelialization for optimal wound healing.

Materials and Methods

Materials

Isoproterenol (β-adrenergic agonist), forskolin, rp-cAMP (an inactive cAMP analog) and sp-cAMP (an active cAMP analog) were purchased from Calbiochem (San Diego, CA). Timolol (β-adrenergic antagonist) and pertussis toxin (PTX) were purchased from Sigma (St Louis, MO).

Cell culture

Normal human epidermal keratinocytes, derived from neonatal foreskin epidermis, were cultured using a modification (Isseroff et al., 1987) of the method outlined by Rheinwald and Green (Rheinwald and Green, 1975). Cells were stored in liquid nitrogen and subcultured as needed in Epilife medium, supplemented with human keratinocyte growth supplement (HKGS) (Cascade Biologics, Portland, OR), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin (Gibco, Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO_2. Cells isolated from at least two different foreskins were used and experiments were performed with subcultured cells between passages four and seven. The cells were grown for 2-3 days to ~50% confluence. Cells, released by trypsinization, were plated in Epilife medium at 3×10^4 cells cm^{-2} on 60 μg ml^{-1} collagen-I-coated (Vitrogen 100, Collagen Corporation, Palo Alto, CA) glass on the bottom of galvanotaxis chambers (Pullar, 2001). Cells were incubated for an additional 3-6 hours at 37°C to allow attachment. Cells were pretreated with timolol (20 μM), forskolin (0.1 μM), sp-cAMP (50 μM) or rp-cAMP (50 μM) for 30 minutes and PTX (100 ng ml^{-1}) overnight at 37°C before β-agonist addition. In all other experiments, treatments (isoproterenol, 0.1 pM to 0.1 μM; timolol, 20 μM; sp-cAMP, 50 μM; rp-cAMP, 50 μM) were added at time 0.

DC EF application

The galvanotaxis chamber construction and DC application were performed as described previously (Pullar, 2001). Briefly, the galvanotaxis chamber is composed of a rectangular Plexiglass frame with two media reservoirs on opposite sides to which a 45×50 mm piece of No. 1.5 glass coverslip is attached to form the chamber bottom. This allows for continual observation of the plated cells on an inverted microscope. The keratinocytes are plated onto the collagen-coated center of the chamber between two coverslip spacers (25×10 mm). A third 25 mm^2 coverslip is placed on top, straddling the two spacer coverslips and covering the cells plated on the collagen-coated center panel. This third coverslip rests approximately 100-105 μm above the center panel and is sealed on top of the spacer coverslips with silicone high-vacuum grease (Dow Corning, Midland, MI). This small height is chosen to minimize the cross-sectional area through which current flows; a small cross-section creates a high-resistance pathway, resulting in a higher voltage gradient for a fixed current. The aqueduct allows medium and current flow across the cells. The voltage across the coverslip is measured using a voltmeter via silver/sliver-chloride (Ag-AgCl) wire electrodes inserted into both media reservoirs on either side of the center panel. 6-cm-long 2% agar/PBS-filled pieces of polypropylene tubing connect each end of the chamber to a medium-filled well in which the Ag-AgCl electrodes are placed, to separate possible electrode byproducts from the cells themselves. The current is measured with an ammeter in series and we only use chambers for which the current flow is kept below 0.6 mA, to minimize electrical heating. Furthermore, temperature of the medium in the chambers is maintained at 36°C by placing the chamber on a metal plate heated to and maintained at 39°C. Temperature is continuously monitored during the experiment using a YSI 400 analog temperature probe (Yellow Springs Instrument, Yellow Springs, Ohio) directly attached to the metal plate, and does not vary by more than 1°C over the course of the experiment.

Time-course observation and data analysis

The galvanotaxis chambers rest on inverted Nikon microscopes. Time-lapse images of the cell migratory response are digitally captured every 10 minutes over a 1 hour period by a Q-Imaging Retiga-EX cameras (Burnaby, BC, Canada) controlled by a custom automation written in Improvision Open Lab software (Lexington, MA) on a Macintosh G4. Once each cell's center of mass has been tracked using Open Lab, migration speed, distance and directionality are calculated and imported to Excel (Microsoft, Redmond, WA). Cell migration is quantitatively analysed using two criteria: (1) average net cos(ϕ) and (2) average true migration distance (μm). Cos(ϕ) describes the direction of migration and is a measure of the persistence of cathodal directedness, where ϕ is the angle between the field axis and the vector drawn by the cell migration path. The average $\cos(\phi) = \Sigma_i \cos(\phi_i)/N$, where Σ_i is the summation of 60-160 individual cells from at least two different cell strains. An angle of zero (ϕ=0) is assigned to the negative pole (cathode) and increasing angles assigned in a clockwise manner, with ϕ=180° aligned with the positive pole (anode). Therefore, cos(ϕ) will provide a number between –1 and +1 and the average of all of the separate cell events yields an index of directional migration. For example, if a cell were to move directly to the negative pole, ϕ=0° and cos(ϕ)=1. Likewise, if a cell were to move toward the positive pole, ϕ=180° and cos(ϕ)=–1.

'Net cos(ϕ)' therefore refers to the average directional migration index of separate cell migration events at the end of a 1-hour period.

'Average track cosine' refers to the average directional migration index of separate cell migration events at the end of each 10-minute period.

'True distance' refers to the average true distance the cells travel in a 1-hour period.

'True speed' refers to the average true speed the cells travel in a 1-hour period.

Data for each condition were collected from 60-160 cells. Results are given as displacement in μm, speed in μm $minute^{-1}$ or average cos(ϕ) ± the standard error of the mean (s.e.m.). Significance is taken

as $P<0.01$, using Student's t test (unpaired) to compare the means of two cell populations.

Circle graphs are drawn to provide an efficient way of observing both migration rate and direction of migration. Each cell is placed at the origin of the circle graph at time 0, and its final position is plotted as a single point on the graph. The distance of the points from the origin provides an indication of cell migration rate and the distribution of points between the four quadrants indicates the direction of the migration. The radius of each graph is 120 μm unless otherwise noted.

Results

Human keratinocytes move towards the cathode of an applied EF

Human neonatal keratinocytes will attach to collagen within a couple of hours and most of the cells assume a motile phenotype with lamellipodial extensions along the convex side of the cell. In the absence of a directional stimulus, cells migrate randomly. Keratinocyte random migration is represented in Fig. 1A. There are roughly equal numbers of cells in all four quadrants of the circle graph. Cells migrate with a true speed of 0.97±0.04 μm minute^{-1}, a true distance of 58.14±2.16 μm and a net cos(ϕ) of 0.01±0.06. The measurement of track cosine over time demonstrates the random nature of the migration, with cosines averaging to zero throughout the experiment, as would be expected (Fig. 1C). Plotting the individual cell tracks also demonstrates the random nature of the cell migration (Fig. 1E).

In the graphical representation of EF application to the cells, the cathode is set at the top of the graph (0°), with the anode at the bottom (180°) (Fig. 1B). The application of a DC EF of physiological strength (100 mV mm^{-1}) alters the direction of migration dramatically, while having no effect on the migration rate. Keratinocytes sense the EF, reform their lamellipodia and migrate directionally towards the cathode (0°). Most cells are now concentrated in the top two quadrants of the circle graph (Fig. 1B). Cells migrate with a true speed of 0.97±0.03 μm minute^{-1}, a true distance of 57.84±1.78 μm and a net cos(ϕ) of 0.69±0.04. The measurement of track cosine over time demonstrates that cells sense the EF within minutes and increase their directionality steadily throughout the experiment (Fig. 1D). Plotting the cells' individual migratory tracks demonstrates that cells initially traveling in one direction sense the EF, turn and then move persistently towards the cathode (Fig. 1F). Although the application of the EF dramatically increases the net cos(ϕ) from 0.01±0.06 to 0.69±0.04, it is important to realize that the application of physiological EFs has no effect on the migration rate (Fig. 1G).

β-AR agonists attenuate keratinocyte galvanotaxis

The addition of 0.1 pM β-adrenergic agonist decreases the net cos(ϕ) by 52% ($P<0.01$) while having no effect on migration rate. Increasing the concentration of β-agonist tenfold up to 0.1 nM gradually decreases the net cos(ϕ) (results not shown), with a maximum decrease of 75% ($P<0.01$, 0.69±0.04 to 0.22±0.06) observed at 0.1 nM β-adrenergic agonist (Fig. 2A). The random migratory patterns and speeds of β-agonist-treated keratinocytes were practically indistinguishable from untreated keratinocytes monitored in the absence of an EF (Fig. 1A). Increasing the β-adrenergic agonist concentration to 0.1 μM, however, reduces the migration rate by 34%, as expected (Chen et al., 2002; Pullar et al., 2003), as well as decreasing keratinocyte galvanotaxis (Fig. 2A).

An analysis of the track cosine over time in 0.1 nM β-agonist-treated keratinocytes compared with field controls reveals that, although the cells appear to migrate randomly, they are still capable of responding minimally to the EF because they steadily increase their net cos(ϕ) during the experiment (Fig. 2B). This could reflect a decrease in stability of the β-adrenergic agonist over time, however, because the β-adrenergic agonist is added at time 0 and is not refreshed. Isoproterenol is known to be unstable in aqueous solution, especially under alkaline conditions at 37°C (Patel and Vasavada, 1988).

Therefore, the effect of β-adrenergic agonists on directional migration is bimodal. At low doses (0.1 pM to 0.1 nM), there is an agonist-mediated attenuation of directional migration towards the cathode of the applied EF and no adverse effect on migration rate (Fig. 2C). Increasing the β-agonist concentration to 0.1 μM similarly decreases directional migration [net cos(ϕ)] but additionally decreases the migration rate, as previously reported (Chen et al., 2002; Pullar et al., 2003). Therefore β2-ARs are capable of modulating the two distinct components of directional migration: migration rate (high doses: 0.1 μM and above) and directional migration towards the cathode of an applied EF (low doses: 0.1 pM to 0.1 nM).

β-Agonist-induced attenuation of keratinocyte galvanotaxis is mediated by the β2-AR

To determine whether the β-agonist-induced attenuation of keratinocyte galvanotaxis was mediated through the β2-AR, we performed keratinocyte galvanotaxis in the presence and absence of the β-AR antagonist timolol (20 μM). We have previously demonstrated that this concentration of β-AR antagonist can sufficiently block the β2-AR and prevent any β-agonist-mediated effects on migration (Chen et al., 2002; Pullar et al., 2003). The β-adrenergic antagonist has no adverse effect on the migration rate of keratinocytes in an EF but significantly increases the net cos(ϕ) of cell migration by 26% (Fig. 3A). Pretreating keratinocytes with β-adrenergic antagonist before β-agonist addition prevents the β-agonist-mediated attenuation of galvanotaxis (Fig. 3A,B). The migration of β-antagonist-treated cells in the absence of an EF was not significantly different from non-EF controls (data not shown).

Additionally, an analysis of the average track cosine over time reveals that β-antagonist-treated keratinocytes appear to sense the EF significantly earlier than un-treated keratinocytes (Fig. 3C). Whereas untreated keratinocytes attain a net cos(ϕ) of 0.5 after 30 minutes of EF exposure, β-antagonist-treated keratinocytes attain a net cosine of 0.5 within 20 minutes and steadily increase their net cos(ϕ) throughout the experiment (Fig. 3C). It appears, therefore, that the β-adrenergic antagonist increases the keratinocytes ability to sense the EF and to respond to it by migrating towards the cathode.

cAMP attenuates keratinocyte galvanotaxis

The active cAMP analog sp-cAMP (Van Haastert et al., 1984) attenuates keratinocyte galvanotaxis by 64% while having no

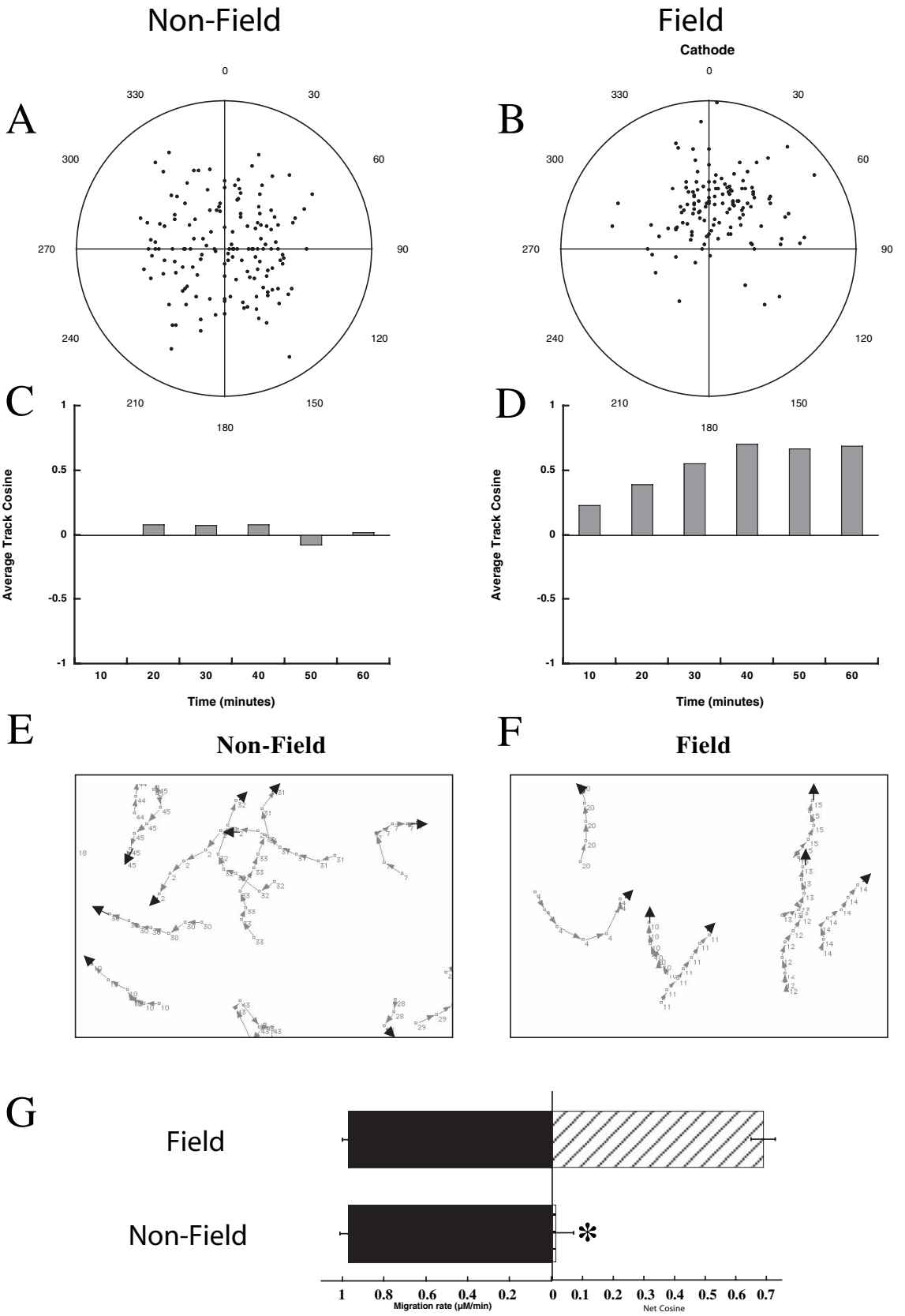

Fig. 1. Human keratinocytes migrate towards the cathode in the presence of 100 mV mm^{-1} DC EFs. Images of the migration paths were captured every 10 minutes and the translocation distance and directionality calculated. For each experiment, the data were plotted using a circle graph. Each cells position 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 EF is plotted as a single point on the graph. The radius of each circle represents 120 μm of translocation distance. The cathode is at the top of each graph (0°) and the anode at the bottom (180°). (A) Keratinocytes in the absence of an EF are the negative control (n=153). (B) Keratinocytes in the presence of an EF are the positive control (n=132). The average track cosine for each 10 minute time period was plotted against time for keratinocytes in the absence of an EF (negative control, C) or the presence of an EF (positive control, D). Individual cell tracks are displayed to show random migration (Non-Field) (E) versus EF-mediated directional migration (Field) (F). Bar, 25 μm. The migration rate and the cosine of the migration angle [cos(ϕ)] for the keratinocytes were measured after 1 hour in the absence (Non-Field) or presence (Field) of an applied DC EF (G). Solid bars (left) represent migration rate, striped bars (right) represent directionality [net cos(ϕ)]. Error bars indicate s.e.m. *P<0.01. The data shown are combined from three independent experiments on two separate cell strains.

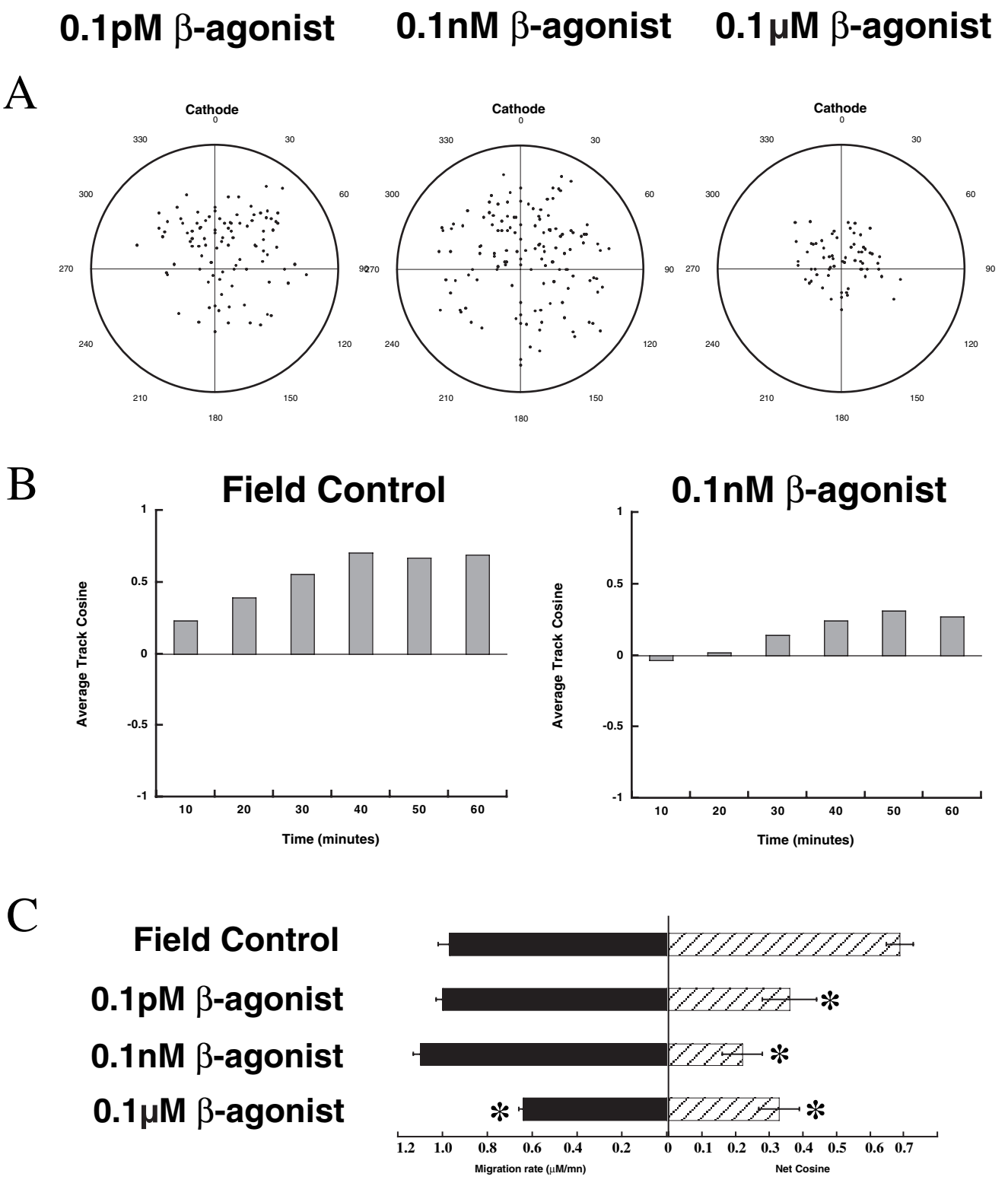

Fig. 2. β-Adrenergic agonists attenuate keratinocyte galvanotaxis at low concentrations but have no effect on migration rate. Circle graphs (radius 120 μm) were plotted to represent the translocation of keratinocytes after 1 hour in an applied DC EF of 100 mV mm^{-1} in the presence of increasing concentrations of β-adrenergic agonist (A). The average track cosine for each 10 minute time period was plotted against time for untreated and 0.1 nM β-adrenergic-agonist-treated cells in the presence of an EF (B). The migration rate and cosine of the migration angle [cos(ϕ)] for control and β-adrenergic-agonist-treated cells (0.1 pM, 0.1 nM, 0.1 μM) were measured after 1 hour in the presence of an applied DC EF. Solid bars (left) represent migration rate, striped bars (right) represent directionality [cos(ϕ)]. Field control, n=132; 0.1 pM β-agonist, n=100; 0.1 nM β-agonist, n=137; 0.1 μM β-agonist, n=66. The data shown are combined from three independent experiments on two separate cell strains. Error bars indicate s.e.m. *P<0.01.

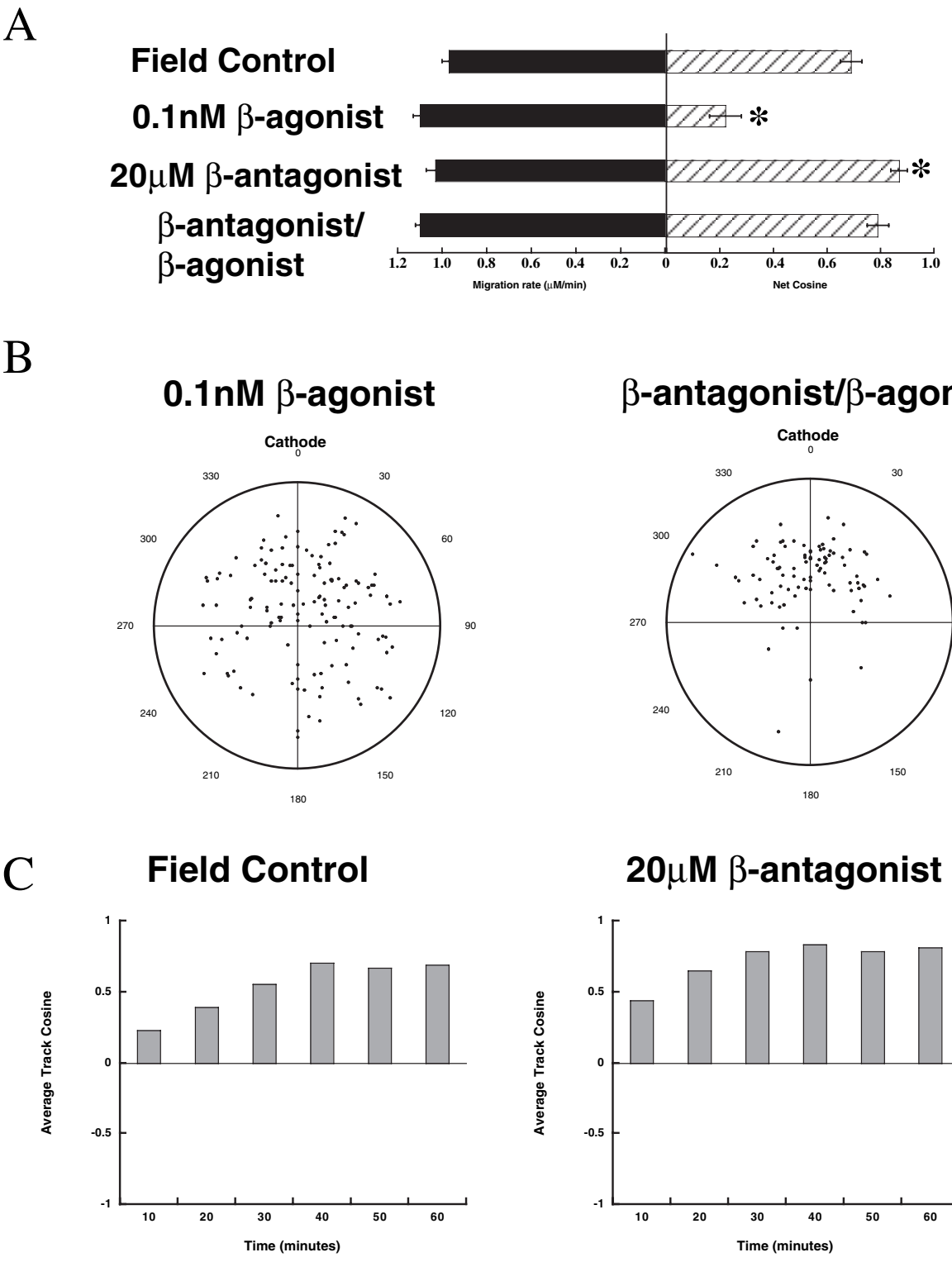

Fig. 3. β-Adrenergic antagonists prevent the β-adrenergic-agonist-mediated attenuation of keratinocyte galvanotaxis. The migration rate and cosine of the migration angle [cos(ϕ)] for untreated, β-adrenergic-agonist-treated and β-adrenergic-antagonist-treated cells, and cells pretreated with antagonist before agonist addition were measured after 1 hour in the presence of an applied DC EF. Solid bars (left) represent migration rate, striped bars (right) represent directionality [cos(ϕ)] (A). Field control, n=132; 0.1 nM β-agonist, n=137; 20 μM timolol, n=69; β-antagonist/β-agonist, n=92. The data shown are combined from three independent experiments on two separate cell strains. Error bars indicate s.e.m. *P<0.01. Circle graphs (radius 120 μm) were plotted to represent the translocation of keratinocytes after 1 hour in an applied DC EF of 100 mV mm^{-1} in the presence of 0.1 nM β-adrenergic agonist alone or cells pretreated with 20 μM antagonist before agonist addition at time 0 (B). The average track cosine for each 10 minute time period was plotted against time for untreated and 20 μM β-adrenergic-antagonist-treated cells in the presence of an EF (C).

effect on the migration rate (Fig. 4A). Cells treated with sp-cAMP migrate randomly and similarly to the migration of keratinocytes in the presence of β-agonist (0.1 nM) in an EF (4B). The migration of sp-cAMP-treated keratinocytes in the absence of an EF is indistinguishable from non-EF controls (data not shown). Pretreating keratinocytes with sp-cAMP before β-agonist addition (0.1 nM) at the time of EF application also attenuates directional migration by 68% while having no adverse effect on migration rate. In fact, sp-cAMP-pretreated keratinocytes appears to move slightly faster than untreated cells (Fig. 4A). The combination of sp-cAMP and β-adrenergic agonist does not further attenuate EF-induced directional migration; in fact, the net cos(ϕ) values for 0.1 nM β-adrenergic-agonist-treated and β-adrenergic-agonist/sp-cAMP-treated keratinocytes are identical (0.22).

PTX can also activate adenylyl cyclase and correspondingly increase intracellular cAMP owing to G_i-protein inhibition in keratinocytes (Choi and Toscano, 1988). In fact, it has a greater effect on cAMP accumulation than cholera toxin activation of G_s protein (Jacquemin et al., 1986). Overnight treatment with 100 ng ml^{-1} PTX attenuates keratinocyte directional migration in an EF in the presence or absence of β-agonist, while having no significant effect on random migration (Fig. 4C). This is suggestive of a cAMP-dependent mechanism, although other cAMP-independent mechanisms could play a role (Kimmel and Parent, 2003).

Additionally, to provide more proof that increasing intracellular cAMP was indeed responsible for 'blinding' keratinocytes to the EF, we performed galvanotaxis experiments in the presence and absence of forskolin, a well-known activator of adenylyl cyclase (Insel and Ostrom, 2003), which results in increased intracellular cAMP levels. Forskolin (0.1 μM) reduces the net cos(ϕ) by 58% from 0.69±0.04 (n=132) to 0.29±0.1 (n=92), while having no effect on migration rate (results not shown). Increasing intracellular cAMP therefore appears to play a major role in the mechanism for the β-adrenergic-agonist-mediated attenuation of keratinocyte galvanotaxis.

Blocking cAMP signaling prevents the β-AR-mediated attenuation of galvanotaxis

To confirm the role of cAMP in the mechanism for the β-agonist-mediated attenuation of keratinocyte galvanotaxis, we treated keratinocytes with an inactive cAMP analog, rp-cAMP, that blocks downstream cAMP signaling (Van Haastert et al., 1984). Treatment with rp-cAMP completely prevents the β-adrenergic-agonist-mediated decrease in cos(ϕ); in fact, it significantly increases the net cos(ϕ) of keratinocyte migration by 19% while migration rates are comparable to those of EF controls (Fig. 5A,B). In *Dictyostelium discoideum*, blocking adenylyl cyclase activation and the subsequent increase in intracellular cAMP has no adverse effect on EF-induced directional migration, as we demonstrate here for keratinocyte galvanotaxis, but does not increase the persistence of cathodal directedness (Zhao et al., 2002).

Pretreating keratinocytes with rp-cAMP (50 μM) before β-agonist addition (0.1 nM) at the time of EF application completely prevents the β-agonist-mediated attenuation of EF-induced directional migration. Cells migrate with a net cos(ϕ) of 0.71±0.05 (EF control: 0.69±0.04) with migration rates comparable to EF controls (Fig. 5A,B).

An analysis of the track cosine over time also reveals that rp-cAMP-treated cells appear to sense and respond to the EF by migrating towards the cathode earlier than untreated keratinocytes. Cells treated with rp-cAMP have net cos(ϕ) values of 0.5 or above within 20 minutes, compared with 30 minutes for EF control cells (Fig. 5C). Remarkably, the addition of β-adrenergic agonist to the rp-cAMP-pretreated keratinocytes does not effect the increased sensitivity of the keratinocytes to the EF. In fact, rp-cAMP-pretreated β-adrenergic-agonist-treated keratinocytes have net cos(ϕ) values above 0.5 as early as 10 minutes after EF application (Fig. 5C). Therefore, the addition of rp-cAMP increases the ability of keratinocytes to sense and respond to an applied EF while having no effect on the migration rate. Pretreating keratinocytes with rp-cAMP before β-agonist addition restores keratinocyte galvanotaxis, demonstrating the cAMP dependence of the attenuation of keratinocyte EF-induced directional migration.

β2-ARs can therefore regulate the two distinct components of directional migration by different mechanisms: migration speed at higher concentrations of β-adrenergic agonist (0.1 μM) via a cAMP-independent mechanism (Chen et al., 2002; Pullar et al., 2003); and EF-induced directional migration at lower β-adrenergic agonist concentrations (0.1 pM to 0.1 nM) by a cAMP-dependent mechanism.

Discussion

Here, we have investigated the effect of β-adrenergic agonists and antagonists on keratinocyte galvanotaxis. Directional migration towards the cathode of the applied DC EF is attenuated at low concentrations (0.1 pM to 0.1 nM) of β-adrenergic agonist. The cells are essentially 'blinded' to the EF while maintaining normal migratory speeds. There is a very gradual decrease in net cos(ϕ) from 0.1 pM to 0.1 nM β-adrenergic agonist. At higher β-adrenergic-agonist concentrations (0.1 μM) migration rate was attenuated, as previously described (Chen et al., 2002; Pullar et al., 2003), as was cathodal directional migration. β-Adrenergic agonists are therefore capable of modulating the two distinct components of directional migration: directional migration and migration rate.

Interestingly, a subpopulation of cells do not appear to sense and respond to the EF. A few cells migrate persistently towards the anode, represented by dots in the bottom two quadrants of cells in the circle graph (Fig. 1B). It will be interesting to isolate this subpopulation and to compare them with cells capable of responding to the field with cathodal migration.

The ability of β-adrenergic antagonists to restore keratinocyte galvanotaxis in the presence of β-adrenergic agonist confirmed that the attenuation of EF-induced directional migration was indeed mediated by the β2-AR. Additionally, both β-adrenergic-agonist- and rp-cAMP-treated keratinocytes appeared to sense and respond to the EF by migrating towards the cathode earlier than untreated cells. One explanation for this could be the ability of keratinocytes to synthesize the catecholamines epinephrine and norepinephrine endogenously (Schallreuter, 1997; Schallreuter et al., 1993; Schallreuter et al., 1995). Any catecholamines synthesized by the keratinocytes during the experiments would be blocked from binding to the β2-AR by the β-adrenergic antagonist or

blocked from signaling downstream by rp-cAMP, preventing any catecholamine-mediated attenuation of keratinocyte galvanotaxis. The question would arise then of why control cells move directionally towards the cathode in the presence of endogenously synthesized catecholamines when the addition of β-adrenergic agonists, at extremely low concentrations, attenuates keratinocyte galvanotaxis. This could be explained by the sensitivity of β2-ARs to naturally occurring catecholamines and β-adrenergic agonists. β2-ARs exhibit a strong response to isoproterenol (β-adrenergic agonist) with much less sensitivity to epinephrine or norepinephrine (Mycek, 1997). We are presently exploring the endogenous production of catecholamines by keratinocytes in the time frame of our experiments.

We have previously demonstrated that the β2-AR-mediated decrease in keratinocyte migration is cAMP independent (Chen et al., 2002), because G proteins and cAMP appear to play an important role in the directional movement of cells towards a

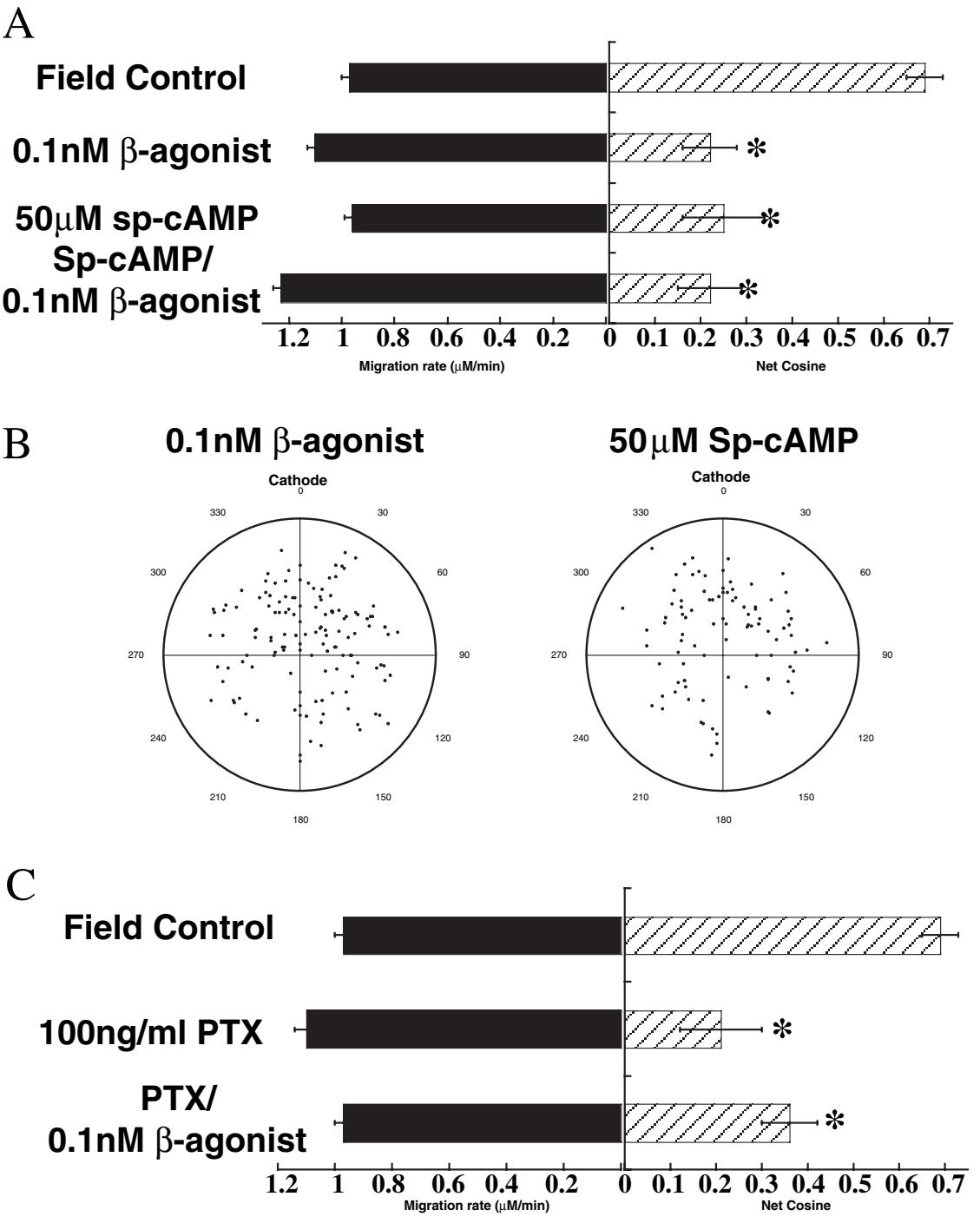

Fig. 4. The active cAMP analog sp-cAMP also attenuates keratinocyte galvanotaxis. The migration rate and cosine of the migration angle [cos(ϕ)] for control, β-adrenergic-agonist-treated (0.1 nM), sp-cAMP-treated cells (50 μM) and cells pretreated with sp-cAMP before agonist addition were measured after 1 hour in the presence of an applied DC EF. Solid bars (left) represent migration rate, striped bars (right) represent directionality [cos(ϕ)]. Field control, n=132; 0.1 nM β-agonist, n=137; 50 μM sp-cAMP, n=67; sp-cAMP/0.1 nM β-agonist, n=93. The data shown are combined from three independent experiments on two separate cell strains. Error bars indicate s.e.m. *P<0.01 (A). Circle graphs (radius 120 μm) were plotted to represent the translocation of keratinocytes after 1 hour in an applied DC EF of 100 mV mm^{-1} in the presence of 0.1 nM β-adrenergic agonist alone or cells treated with 50 μM sp-cAMP before agonist addition at time 0 (B). The migration rate and cosine of the migration angle [cos(ϕ)] for control, PTX-treated cells (100 ng ml^{-1}) and cells pretreated with PTX before agonist addition were measured after 1 hour in the presence of an applied DC EF. Solid bars (left) represent migration rate, striped bars (right) represent directionality [cos(ϕ)]. Field control, n=132; 100 ng ml^{-1} PTX, n=67; 50 μM PTX/0.1 nM β-agonist, n=96. The data shown are combined from three independent experiments on two separate cell strains. Error bars indicate s.e.m. *P<0.01 (C).

chemoattractant (chemotaxis) (Arai et al., 1997; Jin et al., 2000; Kriebel et al., 2003; Meili and Firtel, 2003; Neptune and Bourne, 1997; Neptune et al., 1999; Sun and Firtel, 2003) and increasing intracellular cAMP inhibits fibroblast chemotaxis (Kohyama et al., 2001; Kohyama et al., 2002) and neutrophil chemotaxis (Armstrong, 1995), we were, therefore, interested to determine whether an increase in intracellular cAMP was responsible for the β-agonist-mediated attenuation of EF-

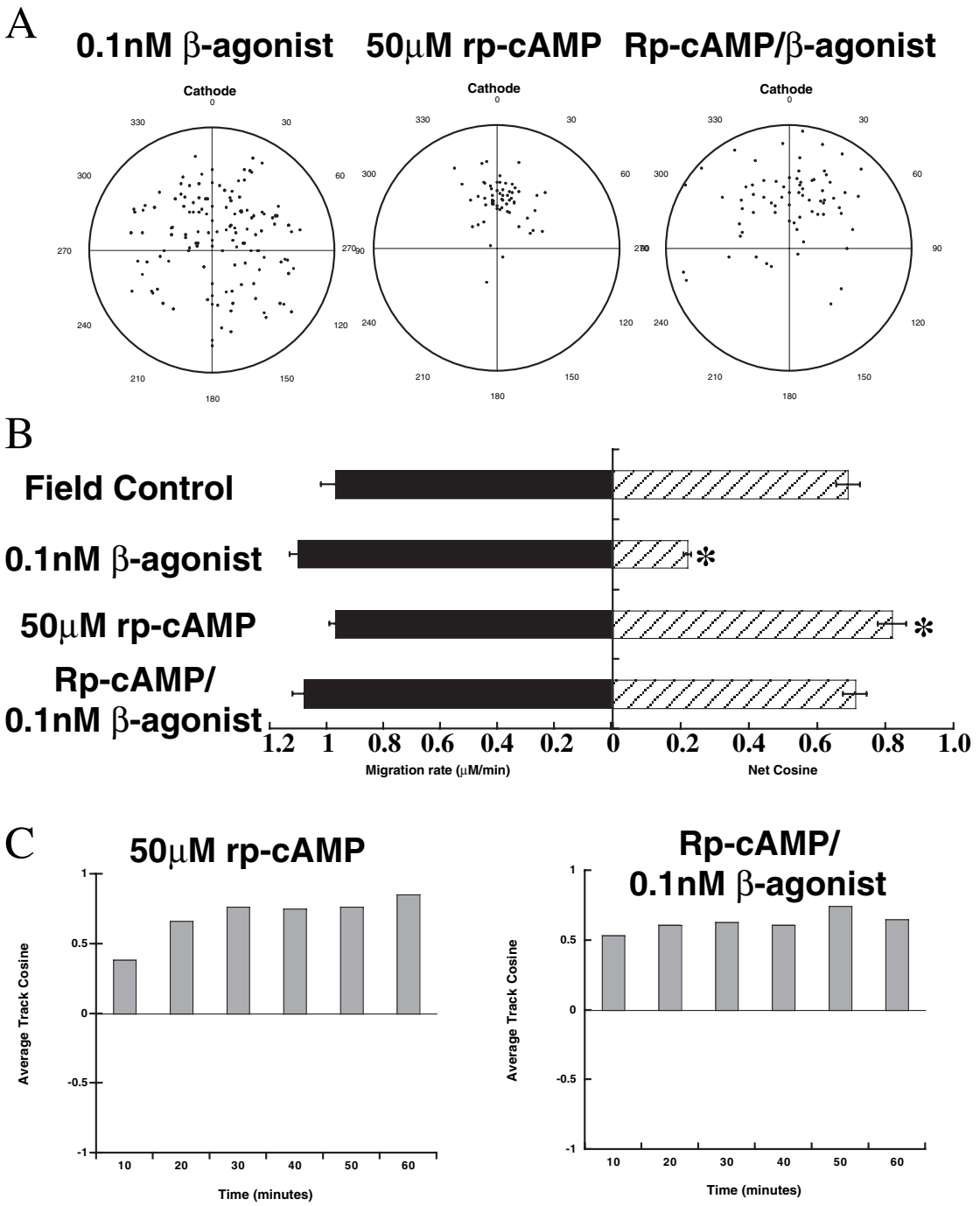

Fig. 5. The inactive cAMP analog rp-cAMP prevents the β-adrenergic-agonist-mediated attenuation of keratinocyte galvanotaxis. Circle graphs (radius 120 μm) were plotted to represent the translocation of keratinocytes after 1 hour in an applied DC EF of 100 mV mm^{-1} in the presence of β-adrenergic agonist alone (0.1 nM), rp-cAMP alone (50 μM) or cells pretreated with rp-cAMP before β-adrenergic-agonist addition (A). The migration rate and cosine of the migration angle [cos(ϕ)] for untreated, β-adrenergic-agonist-treated (0.1 nM), rp-cAMP-treated (50 μM) cells or cells pretreated with rp-cAMP (50 μM) before β-adrenergic-agonist (0.1 nM) addition were measured after 1 hour in the presence of an applied DC EF. Solid bars (left) represent migration rate, striped bars (right) represent directionality [cos(ϕ)]. Field control, n=132; 0.1 nM β-agonist, n=137; 50 μM rp-cAMP, n=61; rp-cAMP/0.1 nM β-agonist, n=72. The data shown are combined from three independent experiments on two separate cell strains. Error bars indicate s.e.m. *P<0.01 (B). The average track cosine for each 10 minute time period was plotted against time for rp-cAMP-treated cells and cells pretreated with rp-cAMP before 0.1 nM β-adrenergic-agonist addition in the presence of an EF (C).

induced directional migration. Increasing intracellular cAMP levels with an active cAMP analog (sp-cAMP), PTX or forskolin did indeed replicate the attenuation of keratinocyte galvanotaxis observed with β-adrenergic agonists, and had no effect on migration rate. Remarkably, we could fully restore keratinocyte galvanotaxis by pretreating with the inactive c-AMP analog rp-cAMP before β-adrenergic-agonist addition, providing compelling evidence that the mechanism for the β-adrenergic-agonist-mediated attenuation of keratinocyte galvanotaxis was cAMP dependent. This is the first report of a role for cAMP in keratinocyte galvanotaxis.

Thus, β2-ARs can regulate the two distinct components of keratinocyte directional migration differently: migration speed via a cAMP-independent PP2A-dependent mechanism involving the mitogen-activated-protein-kinase pathway (Chen et al., 2002; Pullar et al., 2003); and EF-induced directional migration or galvanotaxis by a cAMP-dependent one.

Globally increasing intracellular cAMP levels might mask a small, highly localized EF-induced change in intracellular cAMP that might play an essential role in keratinocyte EF directional sensing. Highly localized β2-AR signaling has been demonstrated in hippocampal neurons, in which β2-ARs complex with L-type calcium channels, adenylyl cyclase and PKA (Davare et al., 2001). However the mechanism requires further elucidation. We have previously demonstrated that the PKA inhibitor KT5720 (50 nM) (Kase et al., 1987) decreases the net cos(ϕ) of keratinocyte migration by 53%, suggesting a role for cAMP-dependent PKA in keratinocyte galvanotaxis (Pullar, 2001). By contrast, we here demonstrate that rp-cAMP, also a PKA inhibitor, does not adversely affect galvanotaxis; indeed, it enhances it. KT5720 (Kase et al., 1987) and rp-cAMP (Van Haastert et al., 1984) have completely different structures and modes of action (Kelly et al., 1999), which could partly explain their different effects on keratinocyte galvanotaxis. It has also recently been shown that many selective and specific kinase inhibitors actually have several cellular targets as well as the target they have traditionally been used to inhibit. KT5720 appears also to inhibit phosphorylase kinase at the concentrations used in our initial experiments (Davies et al., 2000). Whether PKA plays a role in keratinocyte galvanotaxis requires further experimentation.

How would an EF-induced change in intracellular cAMP fit with current theories about EF-induced galvanotaxis? A change in intracellular cAMP levels would suggest the activation or inhibition of adenylyl cyclase (Hurley, 1999). It is well known that changes in intracellular Ca^{2+} can directly activate or inhibit specific isoforms of adenylyl cyclase (Chabardes et al., 1999; Hurley, 1999). Adenylyl cyclase isoforms I and VIII are activated by Ca^{2+} (Defer et al., 2000) and adenylyl cyclase isoforms V and VI are inhibited by Ca^{2+} (Chabardes et al., 1999). The isoforms of adenylyl cyclase expressed by human keratinocytes are presently unknown but fetal rat keratinocytes do express isoforms VI and VIII (Takahashi et al., 1998), meriting further investigation of the adenylyl-cyclase isoforms expressed in human keratinocytes. The mechanism used by keratinocytes to sense and respond to an applied EF does have a very specific requirement for Ca^{2+} influx that is not blocked by either amiloride or verapamil (Trollinger et al., 2002) and is, therefore, independent of the non-specific cation channel (Mauro et al., 1995) previously identified.

How could the EF initiate a change in intracellular Ca^{2+} levels? The EF could hyperpolarize the plasma membrane at the anodal face of the cell, which might cause ion channels to open and will alter the driving force of ions through channels (for a review, see Nuccitelli, 2003). Ca^{2+} waves have been identified in prostate cells (Perret et al., 1999), and were found to be capable of triggering focal-adhesion disassembly (Giannone et al., 2004). A Ca^{2+} wave could be initiated from the anode to the cathode by passive electrochemical diffusion (Mycielska and Djamgoz, 2004) and could then activate or inhibit adenylyl cyclase, leading to localized changes in intracellular cAMP levels. Alternatively, keratinocytes could express a member of the family of hyperpolarization-activated cAMP-gated mammalian cation channels (Distler et al., 1994) that can be activated by hyperpolarization and modulated by hormone- and neurotransmitter-induced rises in cAMP (Ludwig et al., 1998). Elucidation of the precise mechanism of proposed EF-induced changes in keratinocytes awaits further exploration.

In conclusion, we have demonstrated that β-adrenergic agonists are capable of modulating the two distinct components of keratinocyte directional migration via divergent signaling pathways: migration rate via a cAMP-independent, mitogen-activated-protein-kinase-dependent pathway (Chen et al., 2002; Pullar et al., 2003) and galvanotaxis by a cAMP-dependent one. This is the first report that cAMP can modulate keratinocyte galvanotaxis and could place cAMP in an important upstream position in the signaling cascade responsible for keratinocyte sensing and responding to an applied EF. Additionally, because β-adrenergic agonists and antagonists modulate both keratinocyte migration and galvanotaxis, they could be valuable tools for controlling re-epithelialization and restoration of barrier function, an essential component of the wound healing process.

This work was supported in part by National Institutes of Health grants AR 44518 (RRI) and AR 48827 (CEP).

References

Arai, H., Tsou, C. L. and Charo, I. F. (1997). Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: evidence that directed migration is mediated by betagamma dimers released by activation of Galphai-coupled receptors. *Proc. Natl. Acad. Sci. USA* **94**, 14495-14499.

Armstrong, R. A. (1995). Investigation of the inhibitory effects of PGE2 and selective EP agonists on chemotaxis of human neutrophils. *Br. J. Pharmacol.* **116**, 2903-2908.

Barker, A. T., Jaffe, L. F. and Vanable, J. W., Jr (1982). The glabrous epidermis of cavies contains a powerful battery. *Am. J. Physiol.* **242**, R358-R366.

Cavey, M. T., Cavey, D., Shroot, B., Reichert, U. and Gazith, J. (1986). Receptor-linked adenylate cyclase in the membranes of cultured human epidermal keratinocytes. *Arch. Dermatol. Res.* **278**, 293-297.

Chabardes, D., Imbert-Teboul, M. and Elalouf, J. M. (1999). Functional properties of Ca^{2+}-inhibitable type 5 and type 6 adenylyl cyclases and role of Ca^{2+} increase in the inhibition of intracellular cAMP content. *Cell. Signal.* **11**, 651-663.

Chen, J., Hoffman, B. B. and Isseroff, R. R. (2002). Beta-adrenergic receptor activation inhibits keratinocyte migration via a cyclic adenosine monophosphate-independent mechanism. *J. Invest. Dermatol.* **119**, 1261-1268.

Chiang, M., Robinson, K. R. and Vanable, J. W., Jr (1992). Electrical fields in the vicinity of epithelial wounds in the isolated bovine eye. *Exp. Eye Res.* **54**, 999-1003.

Choi, E. J. and Toscano, W. A., Jr (1988). Modulation of adenylate cyclase

in human keratinocytes by protein kinase C. *J. Biol. Chem.* **263**, 17167-17172.

Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**, 88-91.

Davare, M. A., Avdonin, V., Hall, D. D., Peden, E. M., Burette, A., Weinberg, R. J., Horne, M. C., Hoshi, T. and Hell, J. W. (2001). A beta2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel Cav1.2. *Science* **293**, 98-101.

Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *J. Biochem.* **351**, 95-105.

Defer, N., Best-Belpomme, M. and Hanoune, J. (2000). Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am. J. Physiol. Renal Physiol.* **279**, F400-F416.

Distler, M., Biel, M., Flockerzi, V. and Hofmann, F. (1994). Expression of cyclic nucleotide-gated cation channels in non-sensory tissues and cells. *Neuropharmacology* **33**, 1275-1282.

Fang, K. S., Farboud, B., Nuccitelli, R. and Isseroff, R. R. (1998). Migration of human keratinocytes in electric fields requires growth factors and extracellular calcium. *J. Invest. Dermatol.* **111**, 751-756.

Fang, K. S., Ionides, E., Oster, G., Nuccitelli, R. and Isseroff, R. R. (1999). Epidermal growth factor receptor relocalization and kinase activity are necessary for directional migration of keratinocytes in DC electric fields. *J. Cell Sci.* **112**, 1967-1978.

Giannone, G., Ronde, P., Gaire, M., Beaudouin, J., Haiech, J., Ellenberg, J. and Takeda, K. (2004). Calcium rises locally trigger focal adhesion disassembly and enhance residency of focal adhesion kinase at focal adhesions. *J. Biol. Chem.* **279**, 28715-28723.

Hurley, J. H. (1999). Structure, mechanism, and regulation of mammalian adenylyl cyclase. *J. Biol. Chem.* **274**, 7599-7602.

Insel, P. A. and Ostrom, R. S. (2003). Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling. *Cell. Mol. Neurobiol.* **23**, 305-314.

Isseroff, R. R., Ziboh, V. A., Chapkin, R. S. and Martinez, D. T. (1987). Conversion of linoleic acid into arachidonic acid by cultured murine and human keratinocytes. *J. Lipid Res.* **28**, 1342-1349.

Jacquemin, C., Thibout, H., Lambert, B. and Correze, C. (1986). Endogenous ADP-ribosylation of Gs subunit and autonomous regulation of adenylate cyclase. *Nature* **323**, 182-184.

Jin, T., Zhang, N., Long, Y., Parent, C. A. and Devreotes, P. N. (2000). Localization of the G protein betagamma complex in living cells during chemotaxis. *Science* **287**, 1034-1036.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. and Kaneko, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* **142**, 436-440.

Kelly, M. J., Lagrange, A. H., Wagner, E. J. and Ronnekleiv, O. K. (1999). Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* **64**, 64-75.

Kimmel, A. R. and Parent, C. A. (2003). The signal to move: *D. discoideum* go orienteering. *Science* **300**, 1525-1527.

Kohyama, T., Ertl, R. F., Valenti, V., Spurzem, J., Kawamoto, M., Nakamura, Y., Veys, T., Allegra, L., Romberger, D. and Rennard, S. I. (2001). Prostaglandin E(2) inhibits fibroblast chemotaxis. *Am. J. Physiol. Lung. Cell Mol. Physiol.* **281**, L1257-L1263.

Kohyama, T., Liu, X., Wen, F. Q., Zhu, Y. K., Wang, H., Kim, H. J., Takizawa, H., Cieslinski, L. B., Barnette, M. S. and Rennard, S. I. (2002). PDE4 inhibitors attenuate fibroblast chemotaxis and contraction of native collagen gels. *Am. J. Respir. Cell. Mol. Biol.* **26**, 694-701.

Kriebel, P. W., Barr, V. A. and Parent, C. A. (2003). Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell* **112**, 549-560.

Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F. and Biel, M. (1998). A family of hyperpolarization-activated mammalian cation channels. *Nature* **393**, 587-591.

Martin, P. (1997). Wound healing – aiming for perfect skin regeneration. *Science* **276**, 75-81.

Mauro, T., Dixon, D. B., Hanley, K., Isseroff, R. R. and Pappone, P. A. (1995). Amiloride blocks a keratinocyte nonspecific cation channel and inhibits Ca(++)-induced keratinocyte differentiation. *J. Invest. Dermatol.* **105**, 203-208.

McGinnis, M. E. and Vanable, J. W., Jr (1986). Voltage gradients in newt limb stumps. *Prog. Clin. Biol. Res.* **210**, 231-238.

Meili, R. and Firtel, R. A. (2003). Follow the leader. *Dev. Cell.* **4**, 291-293.

Merlot, S. and Firtel, R. A. (2003). Leading the way: directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J. Cell Sci.* **116**, 3471-3478.

Mycek, M. J., Harvey, R. A. and Champe, P. C. (1997). *Lippincott's Illustrated Reviews: Pharmacology* (Philadelphia: Lippincott-Raven Publishers).

Mycielska, M. E. and Djamgoz, M. B. (2004). Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease. *J. Cell Sci.* **117**, 1631-1639.

Neptune, E. R. and Bourne, H. R. (1997). Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. *Proc. Natl. Acad. Sci. USA* **94**, 14489-14494.

Neptune, E. R., Iiri, T. and Bourne, H. R. (1999). Galphai is not required for chemotaxis mediated by Gi-coupled receptors. *J. Biol. Chem.* **274**, 2824-2828.

Nishimura, K. Y., Isseroff, R. R. and Nuccitelli, R. (1996). Human keratinocytes migrate to the negative pole in direct current electric fields comparable to those measured in mammalian wounds. *J. Cell Sci.* **109**, 199-207.

Nuccitelli, R. (2003). A role for endogenous electric fields in wound healing. *Curr. Top. Dev. Biol.* **58**, 1-26.

Ojingwa, J. C. and Isseroff, R. R. (2003). Electrical stimulation of wound healing. *J. Invest. Dermatol.* **121**, 1-12.

Orenberg, E. K., Pfendt, E. A. and Wilkinson, D. I. (1983). Characterization of alpha- and beta-adrenergic agonist stimulation of adenylate cyclase activity in human epidermal keratinocytes in vitro. *J. Invest. Dermatol.* **80**, 503-507.

Patel, R. A. and Vasavada, R. C. (1988). Transdermal delivery of isoproterenol HCl: an investigation of stability, solubility, partition coefficient, and vehicle effects. *Pharm. Res.* **5**, 116-119.

Perret, S., Cantereau, A., Audin, J., Dufy, B. and Georgescauld, D. (1999). Interplay between Ca^{2+} release and Ca^{2+} influx underlies localized hyperpolarization-induced $[Ca^{2+}]_i$ waves in prostatic cells. *Cell Calcium* **25**, 297-311.

Pullar, C. E., Isseroff, R. R. and Nuccitelli, R. (2001). Cyclic AMP-dependent protein kinase A plays a role in the directed migration of human keratinocytes in a DC electric field. *Cell Motil. Cytoskeleton* **50**, 207-217.

Pullar, C. E., Chen, J. and Isseroff, R. R. (2003). PP2A activation by beta2-adrenergic receptor agonists: novel regulatory mechanism of keratinocyte migration. *J. Biol. Chem.* **278**, 22555-22562.

Rheinwald, J. G. and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**, 331-343.

Schallreuter, K. U. (1997). Epidermal adrenergic signal transduction as part of the neuronal network in the human epidermis. *J. Investig. Dermatol. Symp. Proc.* **2**, 37-40.

Schallreuter, K. U., Wood, J. M., Pittelkow, M. R., Swanson, N. N. and Steinkraus, V. (1993). Increased in vitro expression of beta 2-adrenoceptors in differentiating lesional keratinocytes of vitiligo patients. *Arch. Dermatol. Res.* **285**, 216-220.

Schallreuter, K. U., Lemke, K. R., Pittelkow, M. R., Wood, J. M., Korner, C. and Malik, R. (1995). Catecholamines in human keratinocyte differentiation. *J. Invest. Dermatol.* **104**, 953-957.

Sta Iglesia, D. D. and Vanable, J. W., Jr (1998). Endogenous lateral electric fields around bovine corneal lesions are necessary for and can enhance normal rates of wound healing. *Wound Repair Regen.* **6**, 531-542.

Sta Iglesia, D. D., Cragoe, E. J., Jr and Vanable, J. W., Jr (1996). Electric field strength and epithelization in the newt (*Notophthalmus viridescens*). *J. Exp. Zool.* **274**, 56-62.

Steinkraus, V., Steinfath, M., Korner, C. and Mensing, H. (1992). Binding of beta-adrenergic receptors in human skin. *J. Invest. Dermatol.* **98**, 475-480.

Steinkraus, V., Mak, J. C., Pichlmeier, U., Mensing, H., Ring, J. and Barnes, P. J. (1996). Autoradiographic mapping of beta-adrenoceptors in human skin. *Arch. Dermatol. Res.* **288**, 549-553.

Sun, B. and Firtel, R. A. (2003). A regulator of G protein signaling-containing kinase is important for chemotaxis and multicellular development in *Dictyostelium*. *Mol. Biol. Cell.* **14**, 1727-1743.

Takahashi, H., Kinouchi, M., Tamura, T., Ishida-Yamamoto, A. and Iizuka, H. (1998). Adenylate cyclases in keratinocytes: FRSK cells express

types I, II, III, IV, VI and VIII, and 1,25(OH)2D3, retinoic acid and TPA augment forskolin-induced cyclic AMP accumulation in the absence of altered isozyme expression. *Arch. Dermatol. Res.* **290**, 407-412.

Trollinger, D. R., Isseroff, R. R. and Nuccitelli, R. (2002). Calcium channel blockers inhibit galvanotaxis in human keratinocytes. *J. Cell. Physiol.* **193**, 1-9.

Van Haastert, P. J., van Driel, R., Jastorff, B., Baraniak, J., Stec, W. J. and de Wit, R. J. (1984). Competitive cAMP antagonists for cAMP-receptor proteins. *J. Biol. Chem.* **259**, 10020-10024.

Zhao, M., Jin, T., McCaig, C. D., Forrester, J. V. and Devreotes, P. N. (2002). Genetic analysis of the role of G protein-coupled receptor signaling in electrotaxis. *J. Cell. Biol.* **157**, 921-927.