β-Adrenergic Receptor Activation Inhibits Keratinocyte Migration via a Cyclic Adenosine Monophosphate-independent Mechanism

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There is increasing evidence that G-protein-coupled receptors cross-talk with growth factor receptor-mediated signal transduction in a variety of cell types. We have investigated mechanisms by which the activation of β-adrenergic receptors, classically GTP-binding proteins coupled receptors, influence the migration of cultured human keratinocytes. We found that isoproterenol, a β-adrenergic receptor-selective agonist, inhibited cell migration stimulated by either epidermal growth factor, or extracellular Ca^{2+} in a concentration-dependent manner. This was prevented by pretreatment of the cells with the β-adrenergic receptor-selective antagonist timolol. Interestingly, isoproterenol, at a concentration of 1 nM, did not measurably increase intracellular cyclic adenosine monophosphate concentrations yet inhibited cell migration by 50%. To test further if isoproterenol's actions were mediated via activation of adenylyl cyclase, two inhibitors of its activity, 2′5′-dideoxyadenosine and SQ22536, were used. Both compounds significantly diminished isoproterenol-induced increases in intracellular cyclic adenosine monophosphate concentrations but did not attenuate isoproterenol-induced inhibition of cell migration. Also, forskolin (1 μM) markedly increased intracellular cyclic adenosine monophosphate concentrations but did not significantly inhibit cell migration. As mitogen-activated protein kinases are known to signal growth factor-stimulated cell migration, we examined whether β-adrenergic receptor-mediated inhibition of keratinocyte migration might occur via inactivation of mitogen-activated protein kinases. We found that isoproterenol inhibited phosphorylation of extracellular signal-regulated kinase mitogen-activated protein kinase in a concentration-dependent manner but had no effect on the phosphorylation of the stress mitogen-activated protein kinases c-jun N-terminal kinase and stress-activated protein kinase-2. Neither forskolin nor a membrane permeable cyclic adenosine monophosphate analog inhibited phosphorylation of any of these mitogen-activated protein kinases. These findings suggest that β-adrenergic receptor-induced inhibition of keratinocyte migration is mediated through inhibition of the extracellular signal-regulated kinase mitogen-activated protein kinase signaling in a cyclic adenosine monophosphate-independent manner. ***Key words: cell motility/isoproterenol/wound healing. J Invest Dermatol 119:1261–1268, 2002***

Keratinocyte migration plays an important part in re-epithelialization and skin wound healing (Hunt *et al*, 2000). Cell migration is a multistep cellular process that is regulated by complex extracellular and intracellular signals (Muyderman *et al*, 2001). The regulation of migration by growth factors such as epidermal growth factor (EGF), transforming growth factor-β, keratinocyte growth factor, and fibroblast growth factor has been extensively studied during the last decade in many cell types, including keratinocytes (Hudson and McCawley, 1998; Jaakkola *et al*, 1998). The motogenic signaling pathways for these growth factors have been found to overlap with mitogenic signaling pathways (Klemke *et al*, 1997; Cho and Klemke, 2000; Kain and Klemke, 2001); however, little is known about the role of G-protein-coupled receptors (GPCR) in the regulation of keratinocyte migration and re-epithelialization. Recent studies have shown that G-protein βγ or γ subunits modulate stress fiber formation and focal adhesion assembly in fibroblasts and HeLa cells (Ueda *et al*, 1999, 2000); these findings suggest that GPCR may play an important part in the modulation of cell migration. It is well documented that in many cell types, GPCR cross-talk widely with the growth factor receptor-mediated signaling pathway: migration-related protein kinases such as mitogen-activated protein (MAP) kinases, phosphatidylinositol-3 kinase, and tyrosine kinases, and small GTP-binding proteins, such as Rho family members (Yamauchi

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Abbreviations: DDA, 2′,5′-dideoxyadenosine; GPCR, G-protein-coupled receptor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; phospho-ERK, phosphorylated ERK; phospho-p38, phosphorylated stress-activated protein kinase-2; HKGS, human keratinocyte growth supplement.

et al, 1997; Lopez-Ilasaca, 1998; Luttrell *et al*, 1999a; Grewal *et al*, 2001; Pierce *et al*, 2001) can all be activated by GPCR. These findings suggest that GPCR may also play important parts in motogenesis as they do in mitogenesis.

β-adrenergic receptors activate stimulatory GTP-binding proteins (Gs proteins), which in turn activate adenylyl cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP) concentrations and activation of protein kinase A (PKA). More recently, it has become clear that some of the cellular functions mediated by these receptors are independent of adenylyl cyclase activation and rather involve other mediators. The Gsα and Gsβγ subunits themselves may interact with other effectors, such as other protein kinases and ion channel members of the mitogenic/stress signaling pathways; conversely these receptors may function in a G-protein-independent manner (Bishopric *et al*, 1992; Iwaki *et al*, 1990; Bogoyevitch *et al*, 1996; Hall *et al*, 1998; Lefkowitz, 1998; Brzostowski and Kimmel, 2001).

Expression of β-adrenergic receptors by human keratinocytes was demonstrated many years ago (Harper and Flaxman, 1975; Orenberg *et al*, 1983), and a correlation between increases in intracellular cAMP concentration induced by receptor activation and cellular responses involving proliferation and migration has been proposed (Harper and Flaxman, 1975; Dunlap, 1980; Donaldson and Mahan, 1984). It is not clear, however, that these responses are indeed mediated by changes in intracellular cAMP concentrations.

Virtually nothing is known about the role of β-adrenergic receptors in modulating human keratinocyte migration and re-epithelialization during skin wound healing. This may be of clinical importance because many patients are treated with β-adrenergic receptor agonists and antagonists, e.g., for asthma and hypertension, respectively. If β-adrenergic receptors alter cell migration, wound healing in patients using these drugs may be influenced in a variety of clinical settings. In this study, we investigated the effects of β-adrenergic receptor activation on growth factor-mediated keratinocyte migration. We made the unexpected observation that migration is markedly inhibited by the β-adrenergic receptor agonist isoproterenol, and that the inhibition is independent of the simultaneous accumulation of cAMP induced by activation of these receptors. Consequently, further experiments were designed to investigate the effector pathways that modulate the inhibition of migration by β-adrenergic receptor agonists in these cells.

MATERIALS AND METHODS

Materials Isoproterenol and timolol were from Sigma (St Louis, MO). 2′,5′-dideoxyadenosine (DDA), SQ22536, and pertussis toxin were purchased from Calbiochem Co. (La Jolla, CA). Antibodies against phosphorylated extracellular signal-regulated kinase (phospho-ERK), ERK2, and phosphorylated stress-activated protein kinase-2 (phospho-p38) were obtained from New England Biolab (Beverly, MA), and phosphorylated c-jun N-terminal kinase (phospho-JNK) from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture Normal human keratinocytes isolated from neonatal foreskin epidermis were prepared and cultured following the methods described previously (Fang *et al*, 1998). Cells were cultured in serum-free Medium-154 (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement (HKGS, containing 0.2 ng EGF per ml, 5 μg insulin per ml, 5 μg transferrin per ml, 0.18 μg hydrocortisone per ml, and 0.2% bovine pituitary extract). The medium contains 0.2 mM Ca^{2+}, 28 mM HEPES, antibiotics, and anti-mycotics (penicillin, streptomycin, and amphotericin). Cultures were kept at 37°C in a humidified atmosphere of 5% CO_2. Passage 4–6 cells were used for all experiments. For some experiments, cells were cultured overnight in the basal Medium-154, free of Ca^{2+} and free of the HKGS supplement (Medium-154CF).

Cell migration assay The migration of normal human keratinocytes was assayed using a computer-assisted videomicroscopy system established previously in our laboratory (Fang *et al*, 1998) with a minor modification. Briefly, 35 mm tissue culture dishes were coated with 30 μg collagen per ml (Vitrogen, Collagen Corp, Palo Alto, CA) in phosphate-buffered saline, and rinsed with Medium-154. Cells were plated in the collagen-coated dishes at a density of 10^5 cells per dish in Medium-154/HKGS. After a 2–6 h incubation, dishes were rinsed once with the medium to remove unattached cells and fresh Medium-154/HKGS or other medium was added, as indicated in *Results*. After continued incubation for 1 h, cells were treated with test agents. The culture dish was placed on a warmed microscope stage set at 37°C and cell migration was monitored using time-lapse computer-controlled videomicroscopy. For each experiment, a field containing 20–30 single cells was selected, and frames were captured every 10 min for total of 1 h. Migration distance (μm) and rate (μm per min) of each individual cell were calculated with an Apple-scripted program using NIH Image 1.60 and FileMaker Pro 3.0, as we have previously described (Fang *et al*, 1998).

cAMP assay Intracellular cAMP levels were determined using the cAMP enzyme immunoassay (EIA) kit (Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, normal human keratinocytes were plated in collagen-coated 24-well culture plates in Medium-154/HKGS at a density of $8–12 \times 10^4$ cells per well. After incubation 3 h or overnight (with a change of medium once at 1.5 h to remove unattached cells), cells were treated with test agents as described in *Results*. The treatment was terminated by the aspiration of the culture medium and the addition of the lysis buffer included in the kit. cAMP was measured using the nonacetylation assay procedure provided by the manufacturer. The mean cell number in each well was determined by trypsinization and counting in four replicate wells. The cAMP level was expressed as fmol per 1000 cells.

Western blot analysis of activated MAP kinases Normal human keratinocytes were plated in collagen-coated 60 mm dishes (using the same coating condition as described in *Migration Assay*) at 5×10^5 cells per dish. After 3 h to overnight incubation in medium-154/HKGS (changed once at 1.5 h with the same medium), cells were pretreated or treated with test agents as described in the figure legends. Reactions were terminated by transferring dishes on to ice, removing medium, and immediately adding lysis buffer [20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 50 mM β-glycerophosphate, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 10 nM okadaic acid, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg per ml of aprotinin and leupeptin]. The lysates were cleared by centrifugation at $12{,}000 \times g$ for 10 min at 4°C. Equal amounts of lysate proteins, typically 10 μg, were fractionated on a 10% sodium dodecyl sulfate–polyacryamide gel and then transferred on to PVDF membrane (Millipore Co., Bedford, MA). Membranes were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. The blots were incubated with anti-phospho-ERK (1 : 1000) or anti-phospho-p38 (1 : 5000), or anti-phospho-JNK (1 : 2000) antibodies at 4°C overnight or at room temperature for 2 h. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, the phospho-MAP kinases on the membranes were detected with ECL Plus chemiluminescence (NEN Life Science Products, Boston, MA) and direct exposure to Hyperfilm (Amersham Pharmacia Biotech). For normalization of protein loading for each sample, the blots were reprobed with antibody against nonphosphorylated kinase to detect total protein.

Statistical analysis The means of control and treatment groups were compared using Student's t test or ANOVA where indicated in the *Results* or figure legends, with significance described at $p < 0.05$.

RESULTS

Inhibition of human keratinocyte migration by isoproterenol The migration rate of human keratinocytes was measured in cells cultivated at 37°C in Medium-154/HKGS containing various concentrations of isoproterenol, a β-adrenergic receptor agonist. Isoproterenol treatment significantly decreased the migration rate in a concentration-dependent manner (**Fig 1**). Isoproterenol at 10 nM resulted in a maximal inhibition of migration, demonstrating an 80% decrease in migration rate as compared with untreated cells. Statistically significant inhibition of migration was also observed when the cells were exposed to isoproterenol at concentrations as low as 0.01 nM. Pretreatment of the human keratinocytes with timolol (20 μM for 15 min at 37°C),

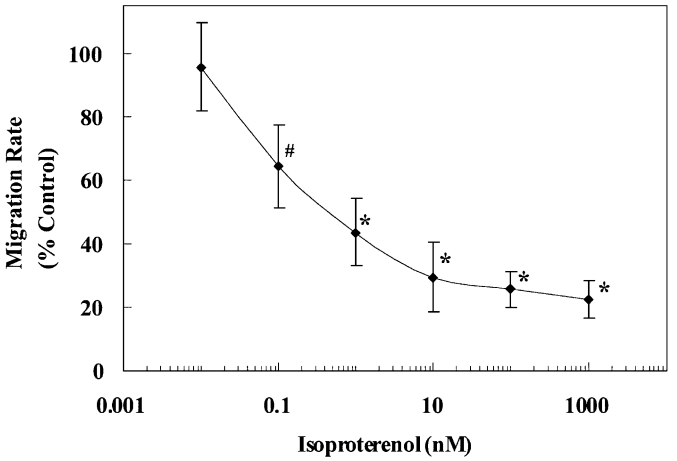

Figure 1. The β-adrenergic receptor agonist isoproterenol inhibits human keratinocyte migration in a concentration-dependent manner. Human keratinocytes were plated on collagen-coated dishes in serum-free Medium-154/HKGS 2–4 h prior to treatment with 0–1000 nM of isoproterenol for 15 min. Cell migration was monitored using a time-lapse computer-assisted videomicroscopy at 37°C, as described in *Materials and Methods*. The migration rate of the control, untreated cells was 0.85 ± 0.19 μm per min, which was taken as 100% (baseline). The data, expressed as percentage of control (untreated cells), are mean ± SD of 60–100 cells from two to four separate experiments, #$p < 0.05$ and *$p < 0.01$ with Student's t test as compared with control (untreated) cells.

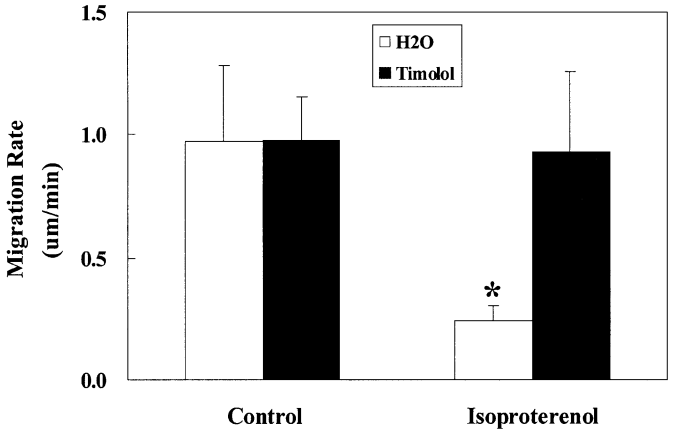

Figure 2. The β-adrenergic receptor antagonist timolol reverses isoproterenol-induced inhibition of human keratinocyte migration. Human keratinocytes were cultured in serum-free Medium-154/HKGS as described in **Fig 1**. The cells were pretreated with 20 μM timolol or its solvent H_2O for 15 min, followed by stimulation with 100 nM isoproterenol for 15 min. The migration was monitored with computer-assisted time-lapse videomicroscopy as described in **Fig 1**. The cell migration rate was analyzed and expressed as μm per min. The data are mean ± SD of 60–100 cells from three to four separate experiments. *$p < 0.01$, compared with either control or timolol plus isoproterenol groups.

a β-adrenergic receptor antagonist, completely prevented the isoproterenol-induced inhibition of migration (**Fig 2**). This suggests that isoproterenol-induced inhibition of keratinocyte migration is mediated by activation of β-adrenergic receptors.

The HKGS supplement added to the Medium-154 routinely used in our migration assays contains several components, including EGF and Ca^{2+}, both of which can stimulate keratinocyte migration (Fang *et al*, 1998; Hudson and McCawley, 1998). In order to determine if β-adrenergic receptor-mediated inhibition of keratinocyte migration was specific for either EGF or Ca^{2+}-stimulated migration, we assayed cell migration in basal

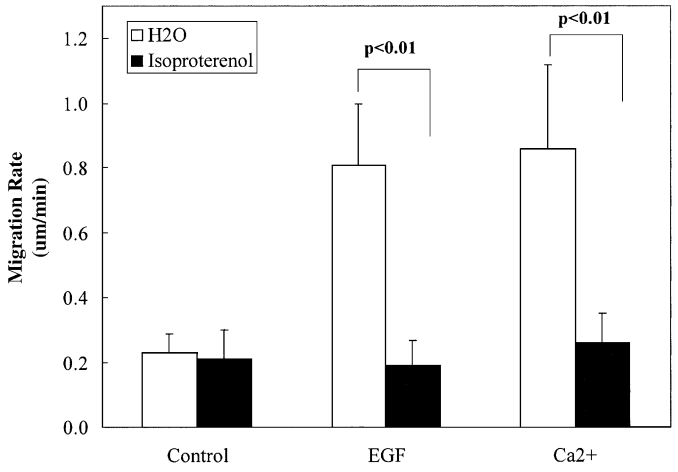

Figure 3. Isoproterenol inhibits both EGF- and Ca^{2+}-induced human keratinocyte migration. Human keratinocytes were cultured as shown by **Figs 1 and 2**, followed by rinsing three times with Ca^{2+} free and HKGS-free medium (Medium-154CF). The cells were quiescent overnight (about 20 h) in Medium-154CF and then treated with EGF (10 ng per ml) or Ca^{2+} (1.8 mM) or untreated (Control) for 10 min followed by treatment with 100 nM isoproterenol or H_2O (vehicle) for 15 min. The cell migration was monitored as described in **Fig 1**. The data are mean ± SD of 60–70 cells from two separate experiments.

Medium-154, without HKGS supplementation, to which either EGF or Ca^{2+} were separately added. In the absence of both EGF and Ca^{2+} from the assay medium (the cells were made quiescent overnight), keratinocytes migrated at a rate of about 0.2 μm per min (**Fig 3**). Addition of either Ca^{2+} (1.8 mM) or EGF (10 ng per ml) to the culture medium increased the migration rate by 4-fold. Isoproterenol (100 nM) completely inhibited either EGF or Ca^{2+}-stimulated cell migration. Isoproterenol did not alter the minimal migration observed in cells maintained in the basal medium that did not contain growth factors or Ca^{2+}.

β-adrenergic receptor-mediated inhibition of migration is independent of increased intracellular cAMP concentrations In human keratinocytes, β-adrenergic receptors activate adenylyl cyclase resulting in increased intracellular cAMP concentrations (Orenberg *et al*, 1983). We examined whether the adenylyl cyclase/cAMP signaling pathway accounted for β-adrenergic receptor-induced inhibition of human keratinocyte migration. The cells were pretreated with either of two adenylyl cyclase inhibitors, namely DDA and SQ22536, before cell migration was assayed in the presence of isoproterenol. Pretreatment with either inhibitor alone did not alter keratinocyte migration under basal conditions nor did either of these compounds prevent the inhibition of migration induced by isoproterenol (**Fig 4*A***). As expected, however, both of these adenylyl cyclase inhibitors significantly decreased intracellular cAMP accumulation stimulated by isoproterenol (**Fig 4*B***). These results suggest that isoproterenol-mediated inhibition of cell migration may involve cAMP-independent mechanisms.

The inhibition of isoproterenol-stimulated cAMP accumulation by DDA and SQ22536 was partial. As there can be a large redundancy in cAMP signaling pathways, for example as has been demonstrated in adipocytes (Hoffman *et al*, 1989), it is possible that even the small cAMP response to isoproterenol in the presence of adenylyl cyclase inhibitors, could be sufficient to inhibit cell migration. Consequently, to test further the hypothesis that β-adrenergic receptor-mediated inhibition of migration of human keratinocytes was independent of increased cAMP concentrations, we asked whether forskolin, a well-known activator of adenylyl cyclase activity, could inhibit migration of

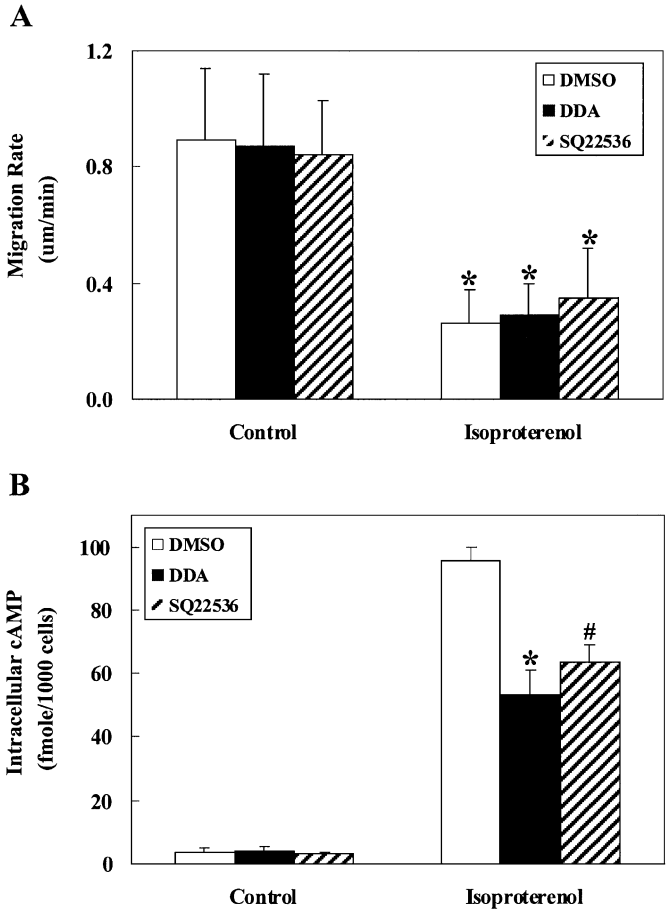

Figure 4. Isoproterenol-mediated inhibition of human keratinocyte migration is independent of adenylate cyclase activity. The cells were cultured as described in **Fig 1**, and pretreated with 100 μM DDA, or 100 μM SQ22536 or dimethyl sulfoxide for 30 min at 37°C, followed by 10 nM isoproterenol for 15 min. (*A*) The cell migration was measured as described in **Fig 1**. The data of migration rate (μm per min) are mean ± SD of 50–100 cells from three to four separate experiments. $^{*}p < 0.01$ comparing isoproterenol-treated cells with corresponding control cells. (*B*) Intracellular cAMP levels, measured with a cAMP EIA system, as described in *Materials and Methods*, expressed as fmol cAMP per 1000 cells. The data are mean ± SD of two to four separate experiments. $^{*}p < 0.01$ and $\#p < 0.05$, comparing DDA- or SQ22536-pretreated cells with dimethyl sulfoxide control in isoproterenol groups.

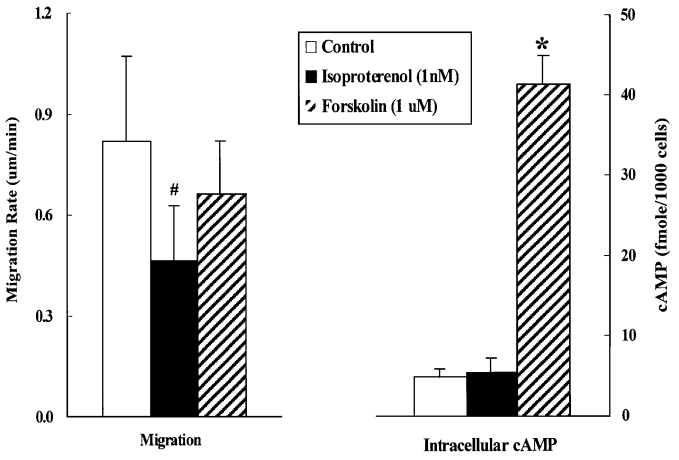

Figure 5. Dissociation of isoproterenol-induced inhibition of human keratinocyte migration from intracellular cAMP level. The human keratinocytes, as described in **Fig 1**, were treated with 1 nM isoproterenol, or 1 μM forskolin, or untreated for 15 min. *Left panel*: cell migration was assayed and analyzed as described in **Fig 1**. The data of migration rate (μm per min) are mean ± SD of 32–100 cells from three to four separate experiments. $\#p < 0.05$, compared with control cells, and no statistically significant difference between control and forskolin-treated cells. *Right panel*: intracellular cAMP levels, measured with cAMP EIA system, expressed as fmol cAMP per 1000 cells. The data are mean ± SD of two to four separate experiments. $^{*}p < 0.01$ compared with control cells, and no statistically significant difference between isoproterenol-treated and control cells.

these cells. As shown in **Fig 5**, forskolin (1 μM) markedly increased the intracellular concentration of cAMP, whereas a low concentration of isoproterenol (1 nM) did not measurably increase intracellular cAMP concentrations, compared with untreated control cells. Interestingly, forskolin did not significantly inhibit cell migration, whereas there was a readily measured effect of isoproterenol on the migration of these cells. This apparent dissociation between inhibition of keratinocyte migration and intracellular cAMP concentrations supports the hypothesis that the cAMP signaling pathway does not play an important part in the inhibition of keratinocyte migration induced by the activation of β-adrenergic receptors.

As indicated above, we found that extracellular Ca^{2+} increased the migration rate of human keratinocytes. We also found that extracellular Ca^{2+} increased intracellular cAMP accumulation (**Fig 6**). Preincubation of the cells with DDA or SQ22536 completely inhibited the extracellular Ca^{2+} induced increase in cAMP accumulation but had no significant effects on the migration rate (**Fig 6**). These results lend further support to the hypothesis that cAMP is not an important regulator of migration rate in human keratinocytes.

β-adrenergic receptor activation selectively inhibits MAP kinase independent of intracellular cAMP concentrations Members of MAP kinase family, ERK, JNK, and p38, are part of the signal transduction pathway initiated by growth factors, cytokines, and adhesion molecules (Rousseau *et al*, 1997; Force and Bonventre, 1998; Hudson and McCawley, 1998; Leng *et al*, 1999; Glading *et al*, 2000). Activation of MAP kinases may lead not only to mitogenesis but also to motogenesis (Kundra *et al*, 1994; Yenush *et al*, 1994; Klemke *et al*, 1997; Nguyen *et al*, 1999). To investigate whether inhibition of migration induced by β-adrenergic receptor stimulation is associated with the alteration of MAP kinase activity, we measured activities of ERK, JNK, and p38 MAP kinases in human keratinocytes as indicated by phosphorylation of each of these kinases (Pearson *et al*, 2001). In the presence of EGF and Ca^{2+}, isoproterenol inhibited phosphorylation of ERK in a concentration-dependent manner (**Fig 7**), but had no significant effect on JNK and p38 phosphorylation. The inhibition of ERK phosphorylation by isoproterenol was prevented by pretreatment of the cells with timolol (**Fig 8*A***). Treatment of the cells with forskolin increased cAMP accumulation comparably with isoproterenol (**Fig 8*B***). Nonetheless, neither forskolin nor CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorylkated, RP-isomer a cell-permeable cAMP analog, inhibited ERK phosphorylation: indeed, these compounds may have slightly stimulated ERK phosphorylation (**Fig 8*A***).

DISCUSSION

Whereas the capacity of growth factor and adhesion molecules to mediate cell migration has been extensively studied, hormonally or drug-mediated inhibitory effects on cell migration and their potential signaling mechanisms are much less understood. In this study, we have demonstrated that activation of β-adrenergic receptors significantly inhibited keratinocyte migration in cells that were stimulated to migrate either by growth factors or extracellular Ca^{2+}. Furthermore, the data suggest that these prominent inhibitory effects are independent of increased intracellular cAMP accumulation promoted by the activation of these

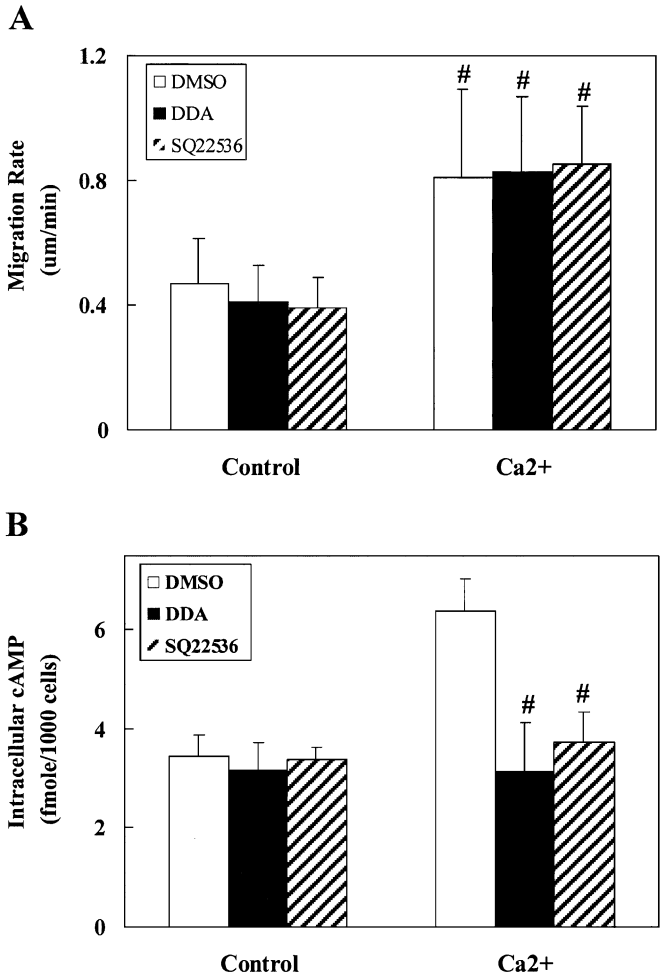

Figure 6. Ca^{2+}-stimulated migration is independent of adenylate cyclase activity in human keratinocytes. Human keratinocytes were cultured in Medium-154/HKGS for 2 h, followed by rinsing three times with Ca^{2+}-free medium and HKGS-free medium (Medium-154CF), as described at **Fig 3**. After incubation in Medium-154CF overnight, the cells were pretreated with 100 μM DDA or SQ22536 or dimethyl sulfoxide for 30 min, then treated with Ca^{2+} (1.8 mM) for 15 min. (*A*) Cell migration rates, expressed as μm per min, are mean ± SD of 50–60 cells from two separate experiments. #$p < 0.05$ between control and Ca^{2+} treatment. (*B*) Intracellular cAMP levels were measured with cAMP Direct EIA kit, expressed as fmol cAMP per 1000 cells. The data are mean ± SD of two separate experiments. #$p < 0.05$, between DDA- or SQ22536-, and dimethyl sulfoxide-pretreated cells in Ca^{2+} groups.

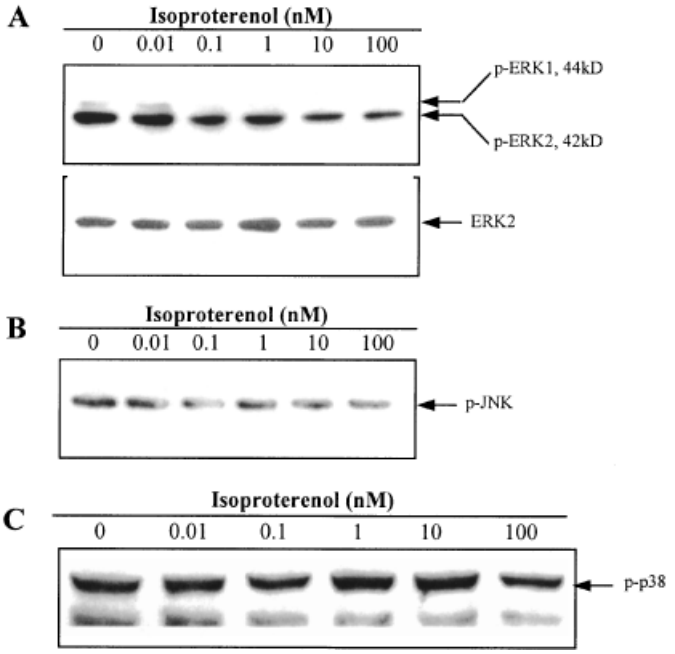

Figure 7. The β-adrenergic receptor agonist isoproterenol inhibits ERK MAP kinase, but not p38 and JNK MAP kinases in human keratinocytes. Human keratinocytes were cultured in Medium-154/HKGS in collagen-coated dishes, as described in **Fig 1**. Cells were stimulated with various concentrations of isoproterenol (0–100 nM) for 15 min. Reactions were terminated by aspiration of medium and rinsed with ice-cold phosphate-buffered saline, followed by the addition of lysis buffer. Ten micrograms of lysate protein from each sample were subjected to polyacrylamide gel electrophoresis and immunoblotting using specific antibodies against phospho-ERK (p-ERK) or unphosphorylated ERK2 (*A*), phospho-JNK (p-JNK) (*B*), and phospho-p38 (p-p38) (*C*).

receptors. The mechanism underlying the negative regulation of migration by β-adrenergic receptors in keratinocytes is presently unknown but appears to be associated with selective attenuation of ERK MAP kinase phosphorylation.

There are five lines of evidence in this study to support these major conclusions: (i) isoproterenol-induced inhibition of keratinocyte migration was completely prevented by timolol, a β-adrenergic receptor antagonist; (ii) adenylyl cyclase inhibitors inhibited isoproterenol-induced increase in intracellular cAMP concentrations but had no effect on migration; (iii) the adenylyl cyclase activator forskolin dramatically increased intracellular cAMP concentration, without, however, inhibiting cell migration; (iv) extracellular Ca^{2+} stimulated an increase in intracellular cAMP concentrations; when this was prevented by adenylyl cyclase inhibitors, the Ca^{2+} induced migration rate of the cells was unchanged; and (v) β-adrenergic receptor activation-induced decrease in ERK MAP kinase phosphorylation was not mimicked by the adenylyl cyclase activator forskolin nor by a membrane permeable cAMP analog. Thus, it is likely that β-adrenergic receptors utilize a novel signaling pathway to regulate keratinocyte migration.

β-adrenergic receptors were identified in human keratinocytes about two decades ago (Harper and Flaxman, 1975; Orenberg *et al*, 1983). Early studies suggested that β-adrenergic receptor agonists, such as isoproterenol, inhibited migration of newt keratinocytes (Dunlap, 1980; Donaldson and Mahan, 1984) and mitosis of human keratinocytes (Harper and Flaxman, 1975), while also increasing intracellular cAMP concentrations in these cells. Subsequent studies, however, failed to confirm a causal relationship between the increase in intracellular cAMP and inhibition of cell migration in either human (Iwasaki *et al*, 1994) or newt keratinocytes (Dunlap and Donaldson, 1980; Dunlap, 1980; Donaldson *et al*, 1984). The complexity of this question is illustrated by the fact that in neutrophils, increased cAMP accumulation stimulates migration in response to endothelin (Elferink and de Koster, 1998) and N-formyl-methonyl-leucyl-phenylalanine (Spisani *et al*, 1996) or else has no effect on cell migration (Neptune and Bourne, 1997).

We found that activation of the β-adrenergic receptor by isoproterenol both increased intracellular cAMP concentrations and inhibited keratinocyte migration in a concentration-dependent manner. Adenylyl cyclase inhibitors significantly decreased intracellular cAMP concentrations but did not prevent the inhibition of keratinocyte migration induced by isoproterenol. That isoproterenol inhibited migration independently of cAMP was further supported by the fact that forskolin-stimulated an increase in intracellular concentrations of cAMP yet had no significant inhibitory effects on migration. Further, isoproterenol at a concentration of 1 nM did not measurably increase intracellular concentrations of cAMP, yet induced a 50% inhibition of migration. Recent evidence indicates that cAMP signaling can be localized to intracellular microdomains (Rich *et al*, 2000), and

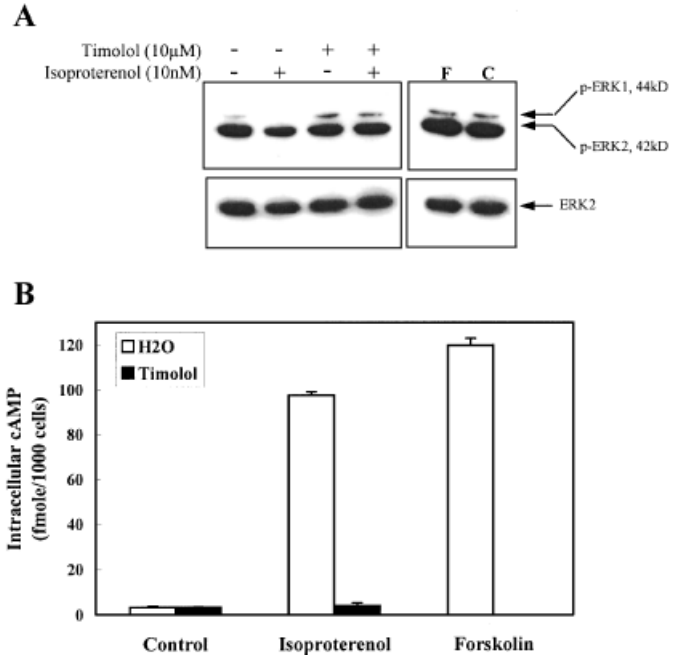

Figure 8. Isoproterenol-induced inhibition of ERK MAP kinase is completely reversed by timolol, but is independent of intracellular cAMP level. Human keratinocytes were cultured in Medium-154/HKGS in collagen-coated dishes, as described at **Fig 1**. Cell were pretreated with or without timolol (10 μM, 15 min) followed by treatment with isoproterenol (10 nM, 15 min) at 37°C. In separate experiments, cells were treated for 15 min at 37°C with 10 μM forskolin (F) or 1 mM CPT-cAMP (C), a plasma membrane permeable cAMP analog. (*A*) Western blot analysis of phospho-ERK, as described in **Fig 7**. The data shown are representative of two independent experiments. (*B*) Intracellular cAMP measurement, as described in **Fig 4**. The data are mean ± SD from two experiments performed in duplicate.

therefore measurements of global changes in cAMP may not accurately reflect microdomain changes that are important in signaling; however, as forskolin effectively activates all adenylyl cyclase isoforms and increases both total and local microdomain levels of cAMP (Taussig and Gilman, 1995; Rich *et al*, 2001; Schwartz, 2001), yet does not inhibit migration, microdomain changes in cAMP do not appear to mediate the observed effect. Taken together, these data suggest that it is very unlikely that the β-adrenergic receptor-mediated inhibitory effects on human keratinocyte migration are mediated through the Gsα/cAMP pathway.

A number of motogenic signaling components that converge on the cytoskeletal migratory system and consequently regulate cell locomotion in response to growth factors, cytokines, and adhesion molecules have been identified. Two opposing motogenic signaling pathways have recently been characterized (Cho and Klemke, 2000; Klemke *et al*, 1997; Nguyen *et al*, 1999; Kain and Klemke, 2001): namely, a stimulatory pathway involving the ERK/MLCK cascade and an inhibitory pathway that utilizes Abl tyrosine kinase-mediated phosphorylation to uncouple CAS-Crk. Appropriate migration rate and direction are physiologically balanced by these positive and negative controls. In pathologic conditions, however, this balance can be disturbed and thus impair migration. The β-adrenergic receptor-mediated inhibition of keratinocyte migration observed in this study could be due to inactivation of the positive control signaling and/or activation of the negative control pathway.

We found that growth factor-induced phosphorylation of ERK but not JNK and p38 MAP kinases was inhibited by activation of β-adrenergic receptors. This suggests that β-adrenergic receptors may likely impact the positive control pathway. Elements signaling upstream of ERK MAP kinase activation could be inactivated by β-adrenergic receptor-mediated transducers. These include tyrosine kinases (receptor and nonreceptor), MEK, Ras/Raf, protein kinase C, phosphatidylinositol-3 kinase, and others (Lopez-Ilasaca *et al*, 1997; Force and Bonventre, 1998; Lopez-Ilasaca, 1998; Cobb, 1999; Pearson *et al*, 2001; Qi *et al*, 2001). On the other hand, it is also possible that β-adrenergic receptor signaling dephosphorylates and inactivates phospho-ERK MAP kinases in human keratinocytes through the activation of protein phosphatases. Phosphorylation of tyrosine and subsequent threonine residues in the active site of ERK MAP kinases is required for ERK activation (for review see Pearson *et al*, 2001). The removal of one or both these phosphates by tyrosine, serine/threonine, or dual-specificity MAP kinase phosphatases can significantly reduce ERK MAP kinase activity (Keyse, 2000; Pearson *et al*, 2001). Increasing evidence suggests that protein phosphatases, particularly protein tyrosine phosphatases, play the same important part as protein kinases in the regulation of cell adhesion and migration (Angers-Loustau *et al*, 1999a,b). The identities of the protein phosphatases that are downstream of β-adrenergic receptors are presently unknown. A previous study has demonstrated, however, that cantharidin, an inhibitor of protein phosphatase types 1 and 2A, significantly attenuated isoproterenol-induced cAMP-independent vasorelaxation of bovine coronary artery rings *in vitro* (Knapp *et al*, 1997). Recently, another study showed that inhibition of protein tyrosine phosphatases by vanadate compounds significantly inhibited isoproterenol-induced L-type Ca^{2+} channel activity in guinea pig ventricular myocytes through a cAMP-independent pathway (Sims *et al*, 2000). These observations suggest that protein phosphatases are likely to be involved in the intracellular signal transduction stimulated by β-adrenergic receptors.

In many other cell types, stimulation of β-adrenergic receptors increases ERK MAP kinase activity in either a cAMP/PKA-dependent or cAMP/PKA-independent manner (Crespo *et al*, 1995; Daaka *et al*, 1997; Wan and Huang, 1998), in part involving tyrosine kinases (Luttrell *et al*, 1999b; Cao *et al*, 2000). For example, in transfected COS-7 cells ERK activation is mediated by βγ subunits of G-protein and is independent of cAMP (Crespo *et al*, 1995), whereas PKA-dependent phosphorylation of the β-adrenergic receptor is required for ERK MAP kinase activation in HEK293 cells (Daaka *et al*, 1997). On the other hand, Gsα is responsible for transducing the β-adrenergic receptor signal to MAP kinase in a cAMP/PKA-dependent manner in S49 mouse lymphoma cells (Wan and Huang, 1998). Thus, the finding of β-adrenergic receptor agonist-mediated inhibition of ERK MAP kinase activity is unique for keratinocytes.

There is increasing evidence that β-adrenergic receptors play important physiologic and pathologic roles in many cell types using pathways other than the initially identified Gsα/adenylate cyclase pathway. Signaling may be through either Gsβγ subunits, different G-proteins or effectors other than G-proteins, such as β-arrestin (for review see Lefkowitz, 1998; Brzostowski and Kimmel, 2001). For example, the β-adrenergic receptor regulation of intracellular pH is independent of G-protein activation, and is mediated through agonist-promoted association of the receptors with the Na^{+}/H^{+} exchanger regulatory factor (Hall *et al*, 1998). In our study, pertussis toxin pretreatment did not alter effects of isoproterenol on keratinocyte migration (data not shown) suggesting that pertussis toxin-sensitive G-protein is unlikely involved in β-adrenergic receptors-induced effects on keratinocyte migration. Certainly, further detailed pharmacologic and molecular characterization of β-adrenergic receptors will help us better understand the mechanisms that underlie β-adrenergic receptor-regulated motogenesis as well mitogenesis in human keratinocytes.

In summary, these data demonstrate that β-adrenergic receptors negatively regulate keratinocyte migration stimulated by growth factors or Ca^{2+} through a cAMP-independent pathway that may involve inhibition of ERK MAP kinase phosphorylation. As β-adrenergic receptor agonists and antagonists are widely used to treat asthma and hypertension, these drugs may be altering the

wound healing ability of a large group of patients. Understanding the checkpoints responsible for the positive and negative control of motogenesis will help us better understand the signaling mechanisms of migration required for wound healing, as well as offer sound rationales for developing target-specific drugs to enhance this process.

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