Understanding the mechanisms that regulate cell migration is important for devising novel therapies to control metastasis or enhance wound healing. Previously, we demonstrated that β2-adrenergic receptor (β2-AR) activation in keratinocytes inhibited their migration by decreasing the phosphorylation of a critical promigratory signaling component, the extracellular signal-related kinase (ERK). Here we demonstrate that β2-AR-induced inhibition of migration is mediated by the activation of the serine/threonine phosphatase PP2A. Pretreating human keratinocytes with the PP2A inhibitor, okadaic acid, prevented the β2-AR-induced inhibition of migration, either as isolated cells or as a confluent sheet of cells repairing an in vitro “wound” and also prevented the β2-AR-induced reduction in ERK phosphorylation. Similar results were obtained with human corneal epithelial cells. In keratinocytes, immunoprecipitation studies revealed that β2-AR activation resulted in the rapid association of β2-AR with PP2A as well as a 37% increase in association of PP2A with ERK2. Finally, β2-AR activation resulted in a rapid and transient 2-fold increase in PP2A activity. Thus, we provide the first evidence that β2-AR activation in keratinocytes modulates migration via a novel pathway utilizing PP2A to alter the promigratory signaling cascade. Exploiting this pathway may result in novel therapeutic approaches for control of epithelial cell migration.

When skin is wounded, keratinocytes within the epidermis must migrate from the wound edges to re-epithelialize the wound surface (1). One of the best characterized mediators of keratinocyte migration is the epidermal growth factor receptor (EGFR) (2–4). The mitogen-activated protein (MAP) kinases, extracellular signal-related kinase 1 (ERK1) and ERK2, are activated upon EGF binding to its cognate receptor (reviewed in Ref. 5). They are activated by phosphorylation on both conserved threonine and tyrosine residues and inactivated upon dephosphorylation by dual specificity phosphatases, tyrosine phosphatases, and serine/threonine-specific phosphatases (5–9). The activation of ERK is tightly controlled. The MAP kinase pathway is usually only transiently activated, and its constitutive activation is sufficient to cause the oncogenic transformation of some cell types (10). Inhibiting ERK phosphorylation and activation prevents growth factor-induced keratinocyte migration (11), demonstrating the pivotal role of ERK in the keratinocyte promigratory signaling pathways (11–13).

In addition to the EGFR, keratinocytes also express high levels of the G-protein-coupled receptor, the β2-adrenergic receptor, β2-AR (14, 15), whose functional role in the epidermis has not yet been elucidated. Keratinocytes do not express the β1-AR (16). Earlier studies have investigated the role of β2-ARs in epithelial wound healing and migration, but the results have been paradoxical. β-Antagonists have been reported to either delay (17, 18) or enhance (19) corneal epithelial wound healing, and β-agonists can stimulate proliferation and migration of transformed corneal epithelial cells (20), SW 480 colon carcinoma cells (21), and bovine bronchial epithelial cells (22). Conversely, β-AR agonists delay wound healing in the hind limbs of adult newts (23) and inhibit neutrophil chemotaxis to the lungs (24). Since keratinocytes have the capacity to synthesize β2-AR agonists and express a high level of the receptor (25), we initiated studies to examine the role of β2-ARs in keratinocyte migration. Surprisingly, we found that unlike other cell types studied, where β2-AR agonist binding activates ERK (26–32), in keratinocytes the β2-AR agonist isoproterenol (ISO) reduced ERK phosphorylation. Keratinocyte migration was also reduced in a dose-dependent and cAMP-independent manner (33).

The mechanisms by which a β2-AR agonist reduced MAP kinase signaling and keratinocyte migration, however, remained enigmatic. We reasoned that the serine/threonine phosphatase, PP2A, known to play a role in a multitude of cellular functions (reviewed in Ref. 34), could be a good candidate for the mediation of the observed β2-AR agonist effects. A number of studies have linked β2-AR activation to either PP2A function, ERK activation, or migration. ISO can increase the activation of PP2A in the rat heart (35), PP2A can dephosphorylate mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MEK1) and ERK family kinases in vitro (6, 7, 9, 36, 37), and decreased PP2A activity promotes migration in a number of transformed cells and cancer cells lines (38–40). We therefore evaluated the possibility that the observed β2-AR-induced decrease in ERK phosphorylation and migration in human keratinocytes could be regulated by the β2-AR agonist-mediated activation of PP2A.

Using okadaic acid (OA) to inhibit intracellular PP2A (41–44), we demonstrate that OA pretreatment completely prevented the β2-AR agonist-induced decrease in keratinocyte migration in two different in vitro migration assays. Likewise, OA pretreatment also prevented the β2-AR agonist-induced reduction in ERK phosphorylation. Similar results were ob-
In human corneal epithelial cells; OA pretreatment completely prevented the β2-AR agonist-induced decrease in wound healing and also prevented the β2-AR agonist-reduced induction in ERK phosphorylation. Further, treating keratinocytes with ISO increased the intracellular association of PP2A with ERK2 and initiated the association of β2-AR with PP2A. Finally, the β2-AR agonist rapidly and transiently increased intracellular ATP activity. Here we provide the first evidence that PP2A plays a crucial role in the β2-AR agonist-mediated decrease in ERK phosphorylation and provides a novel regulatory mechanism of epithelial cell migration.

EXPERIMENTAL PROCEDURES

Keratinocyte Growth—Human keratinocytes were isolated from primary keratinocyte cultures derived from neonatal and adult foreskins as we have reported previously (45), using a modification of the method of Rheinwald and Green (46). Cells were grown in keratinocyte growth medium (KGM) (Medium 154; 0.2 mM Ca2+), supplemented with human keratinocyte growth supplement (0.2 ng/ml EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 0.18 μg/ml hydrocortisone, and 0.2% bovine pituitary extract) (Cascade Biologics, Inc., Portland, OR) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin). (Gemini Bioproducts, Inc., Calabasas, CA) at 37 °C in a humidified atmosphere of 5% CO2.

Since keratinocyte migration is optimal on a collagen matrix (1, 47), all experiments were performed on plates plated for 2 h at 37 °C on glass coverslips (Eppendorf, Hamburg, Germany) or plastic tissue culture dishes (Corning Glass) that had been coated for 1 h at 37 °C with 0.1% gelatin (100 μg/ml). Plates were coated by incubation with 0.1% gelatin in Hank’s balanced salt solution for 1 h at 37 °C. After each cell type had been plated, they were washed with 0.1% gelatin in Hank’s balanced salt solution and placed in growth medium for at least 24 h. After 24 h, cells were used in all experiments except where noted.

Corneal Epithelial Cell Growth—Human adult corneas that had been donated for research were obtained from the Sierra Eye and Tissue Donation Services (Sacramento, CA; a regional center of Diabetic Diets Inc., Donor Services, Nashville, TN) within 2–10 days of collection. Corneas were stored in Optisol-GS corneal storage medium (Chiron Ophthalmicals, Irvine, CA) at 2–8 °C and were transported to the laboratory on ice. The research followed the tenets of the Declaration of Helsinki, tissue was obtained with appropriate consents from either donor or next of kin, and the research was approved by the University institutional review board. Corneal epithelial cells were isolated as we have described (48) and maintained in a 37 °C incubator with 5% CO2 in EpiLife medium (Cascade Biologics) supplemented with 0.18 μg/ml hydrocortisone, 5 μg/ml transferrin, 0.2% bovine pituitary extract, and 1 μg/ml mouse EGF, calcium (final concentration 0.06 mM), and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B/ml). Passage 3–5 cells were used for all experiments.

Scratch Assay—Cells were grown to confluence on collagen-coated plastic tissue culture dishes. A sterile pipette tip was used to scratch a 1-mm-wide wound along the center of the dish, and a demarcated area of the wound was determined using NIH Image 1.60. After each cell number of cells was tracked, the data were automatically exported to FileMaker Pro 3.0, where they were analyzed and stored. Cell migration was quantitatively analyzed, and the speed and distance of each cell was calculated using a cell tracking program compiled in this laboratory (3). Significance was determined using Student’s t test.

Cell Treatments—1 × 106 cells were preincubated with either KGM alone or RGM containing ISO for 45 min at 37 °C. In some experiments, to avoid the inhibitory effect on PP1, PP2B, or PP2C (42), cells were then stimulated with KGM containing either 10 ng/ml EGF (Biomedical Technology, Stoughton, MA), 1 μM ISO (Calbiochem) or 10 μM ISO plus 10 μM OA, or KGM alone (control) for 15 min at 37 °C unless otherwise noted. Cells were plated immediately on ice, washed twice with ice-cold phosphate-buffered saline containing phos- phatase inhibitors (50 mM NaF and 1 mM NaVO3), and scraped in 50 μl to 1 ml of lysis buffer (phosphate-buffered saline containing 0.5% Triton X-100, 50 mM NaF, 1 mM NaVO3, 10 μg/ml leupeptin, 30 μg/ml apro- tin, 200 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A). The lysates were transferred into 1.5-ml tubes, incubated on ice for 20 min, and then centrifuged at 14,000 × g for 10 min at 4 °C (53). The supernatants were electrophoresed immediately on 10% polyacryl- amide Tris-HCl gels (Bio-Rad) or stored at −80 °C. The protein concentration of the samples was determined using the Bradford assay (Bio-Rad).

Immunoblotting—Five μg of each protein sample was added to an equal volume of 2× reducing sample loading buffer (0.0625% Triton X-100, pH 6.8, 3% SDS, 10% glycerol, 5% β-mercaptoethanol) and electro- phoresed on 10% polyacrylamide Tris-HCl gels. Proteins were trans- ferred to Immobilon membranes and immunoblotted with either an anti-ERK (9102), anti-MEK1 (9122), anti-epidermal growth factor receptor (EGFR) (H-20) (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-phosphotyrosine antibody (Ab-4) (Oncogene, Boston, MA). The immunoblots were developed by ECL according to the manufacturer’s instructions (Amersham Biosciences). Densitometry was performed on scanned images using NIH Image 1.6.

Immunoprecipitation—Five μg of antibody linked to 30 μl of pre- washed protein A/G-Sepharose beads (Amersham Biosciences) was used to immunoprecipitate the desired proteins from 1 ml of keratino- cyte cell lysate prepared from 1–2 × 106 cells. Lysates were initially precleared with 150 μl of prewashed beads for 30 min at room temper- ature and then incubated with the antibody-bound beads at 4 °C over- night on a rotary mixer. The beads were washed five times with lysis buffer, 1× reducing sample loading buffer was added, and the samples were boiled for 3 min and centrifuged to pellet the beads. The super- natants were loaded onto 10% polyacrylamide Tris-HCl gels (Bio-Rad), and the proteins were separated electrophoretically by transfer to Immobilon membrane for immunoblotting as outlined above.

Phosphatase Assay—Phosphatase activity was measured using the nonradioactive serine/threonine phosphatase assay system (Promega, Madison, WI). The system determines the amount of free phosphate released from a phosphopeptide by measuring the absorbance of a molybdate-malachite green-phosphate complex. Cells were either un- treated or stimulated with 10 μM ISO for 30 s at 37 °C. The plates were washed immediately in ice-cold PBS, washed in washing buffer (0.1 M EDTA, pH 8, 0.1 mM EGTA, pH 8), and lysed in a phosphate-free buffer containing no phosphatase inhibitors (20 mM Tris, pH 8, 0.1 mM EDTA, pH 8, 0.1% Triton X-100, protease inhibitor mixture (Roche Diagnostics)). The lys- rates were incubated on ice for 30 min and then cleared by centrifuga- tion at 16,000 × g for 20 min at 4 °C. Control and ISO-treated kerati- nocyte cell lysates were centrifuged at 600 × g for 5 min at 4 °C through a mixture of 50 μl in a 96-well plate to determine PP2A activity. The reaction was terminated at the end of an equal volume of molyb- date dye additive. The plate was incubated at room temperature for 30 min to allow full color development. Absorbance was measured at 630 nm in a plate reader (BioTek Instruments Inc., Winooski, VT). Signif- icance was determined using Student’s t test.
RESULTS

OA Reversed the β2-AR Agonist-induced Inhibition of Keratinocyte Wound Healing—Our previous work demonstrated that activation of β2-AR in human keratinocytes decreased their migratory speed (33). We then reasoned that if β2-AR-mediated activation of PP2A was responsible for the ISO-induced decrease in migration, then preincubating cells with OA should prevent it. We chose to initially investigate the effects of ISO and OA on keratinocyte migration using a “scratch” assay that provided an in vitro model of wound healing (49). This allowed the monitoring of keratinocyte migration at the edge of “wounds” generated in vitro by scraping a cell-free zone in a confluent sheet of cells.

The contribution of cell proliferation to the in vitro wound healing was abrogated by pretreating cultures with mitomycin C. Control wounds, incubated in EGF-containing culture medium, completely healed in 24 h, whereas at that time ISO-C. Control wounds, incubated in EGF-containing culture medium had no effect on the rate of wound closure. Although healing was delayed with ISO treatment, the wounds did heal by 36 h (results not shown), demonstrating that the observed decrease in migratory speed was not due to nonspecific toxicity.

To demonstrate that the ISO-induced reduction in wound healing was the result of its specific receptor activation, the receptor was prebound with a β2-AR antagonist, timolol. A 15-min preincubation with timolol (20 μM) completely prevented the ISO-induced reduction in wound healing (Fig. 1B), demonstrating that the ISO-induced effects on migration were specifically through activating the β2-AR. The ISO-treated cells were only 60% healed by 24 h but did heal completely by 36 h (results not shown).

OA Reversed the β2-AR Agonist-induced Reduction of Keratinocyte Migratory Speed—Since OA treatment completely prevented the β2-AR-induced reduction in wound healing, this suggested that PP2A may likewise play a role in the β2-AR agonist-induced decrease in keratinocyte migration. To more precisely measure the β2-AR agonist-induced reduction in keratinocyte speed, we quantitated the locomotory speed of individual cells (47). The addition of ISO to the migration chamber reduced both the mean distance traveled and the speed by half (**p < 0.01) (Fig. 1A). OA pretreatment completely prevented the ISO-induced reduction in both distance and speed traveled by the cells over the 1-h period. Preincubation with OA alone did not significantly affect either distance traveled or speed (Fig. 1A and B). There was no statistically significant difference between the control, OA, and OA/ISO groups. OA pretreatment also prevented the ISO-induced reduction in migratory speed of keratinocytes isolated from adult foreskins (results not shown).

These results, demonstrating OA-mediated restitution of keratinocyte migratory speed in ISO-treated cells, complement those obtained in the scratch assay, providing additional support for the hypothesis that PP2A activation mediates the decrease in migration speed observed after β2-AR agonist treatment.

OA Reversed the β2-AR Agonist-induced Dephosphorylation of ERK in Human Keratinocytes—The ability of OA to restore migration in β2-AR agonist-treated cells prompted us to investigate whether it could also prevent the β2-AR-mediated decrease in ERK phosphorylation observed in our earlier work (33). In order to determine the mechanism by which β2-AR activation resulted in decreased ERK phosphorylation, we examined critical members of the ERK/MAP kinase pathway (Fig. 7) after treating cells with ISO and or OA.

First, the ability of β2-AR ligation to transactivate the EGFR, as previously reported (54–58), was examined. EGF (0.2 ng/ml) was continuously present in the experimental cultivation medium, providing a basal level of EGFR tyrosine phosphorylation (Fig. 3A, lane 1). Increasing the concentration of EGF in the medium markedly increased the tyrosine phosphorylation of the EGFR, as anticipated (lane 2), whereas the addition of either ISO or OA or combinations of these two to the medium had no effect (lanes 3–7). Thus, in human keratinocytes,
OA reversed the β₂-AR agonist-induced reduction of keratinocyte migratory speed. Keratinocytes were pretreated with okadaic acid (10 nM OA or OA/ISO) for 45 min at 37 °C or not. The medium was replaced with KGM (control), KGM containing 10 nM ISO plus 10 nM OA, or both, and the migration of each single cell was monitored over a 1-h period as described. The distance traveled and the speed are represented graphically in panels A and B, respectively (n = 98 (control), 86 (ISO, 97 (OA), and 92 (OA/ISO)). Values plotted are means ± S.E. *, p < 0.01 between ISO and all other conditions.

The phosphorylation of MEK, the immediate upstream activator of ERK, was altered by β₂-AR activation (Fig. 3B). ISO reduced MEK phosphorylation to 51% (10 nM) and 44% (1 μM) (lanes 3 and 4) of the base-line phosphorylation (lane 1), respectively. EGFR signaling was intact in these cells as evidenced by the robust 3-fold increase in MEK phosphorylation in response to added EGF (lane 2). Surprisingly, OA treatment alone had no effect the phosphorylation of MEK (100%) (lane 5), a unique result compared with other cell types where MEK is activated by OA (59, 60). OA pretreatment did not prevent the ISO-induced dephosphorylation of MEK (49% (lane 6) and 40% (lane 7)), suggesting that the ISO-induced dephosphorylation of MEK was not mediated by PP2A.

The phosphorylation of ERK, the immediate downstream substrate of MEK, was also diminished by ISO activation of β₂-AR (Fig. 3C, lanes 3 and 4). ISO-mediated signaling to ERK was intact (lane 2), the addition of ISO reduced ERK phosphorylation to 64% (10 nM) and 42% (1 μM) of baseline values (lanes 3 and 4). OA pretreatment completely reversed the ISO (10 nM)-mediated dephosphorylation of ERK (100%, lane 6) and almost completely reversed it (85%) for the higher (1 μM) ISO dose (lane 7), implying that the activation of PP2A was responsible for the ISO-induced reduction in ERK phosphorylation.

OA Reversed the β₂-AR Agonist-induced Inhibition of Wound Healing and Dephosphorylation of ERK in Human Corneal Epithelial Cells—In order to determine whether the ability of OA to restore migration and ERK phosphorylation in β₂-AR agonist-treated cells was unique to keratinocytes, we extended our investigations to a second epithelial cell type, adult human corneal epithelial (AHCE) cells. When confluent cultures of AHCE were wounded, control wounds, incubated in EGF-containing culture medium, completely healed in 48 h. On the other hand, at 48 h the ISO-treated wounds were only 30% healed (p < 0.01) (Fig. 4A). Pretreatment of cells with OA (10 nM) prior to ISO, however, reversed the ISO-induced defects in wound repair, resulting in complete wound healing at 48 h. OA alone had no effect on the rate of wound closure. Although healing was delayed with ISO treatment, the wounds did heal by 96 h (results not shown), demonstrating that the observed decrease in migratory speed was not due to nonspecific toxicity.

The ability of OA to restore migration in β₂-AR agonist-treated AHCE cells prompted us to investigate whether β₂-AR agonists also decreased ERK phosphorylation in AHCE cells and whether OA pretreatment could prevent the β₂-AR-mediated reduction in ERK phosphorylation as observed in keratinocytes.

The phosphorylation of ERK was diminished by ISO activation of β₂-AR in AHCE cells (Fig. 4B, lanes 3 and 4). Whereas EGF-mediated signaling to ERK was intact (lane 2), the addi-
The data shown are representative of three independent experiments with and without OA or ISO. The data plotted are means ± S.E. (n = 3). *, p < 0.01 compared with control (C). Values plotted are means ± S.E. (n = 3).

OA reversed the β2-AR agonist-induced inhibition of wound healing and dephosphorylation of ERK in human corneal epithelial cells. AHCE cells were grown on collagen-coated plastic dishes and pretreated with odakic acid (10 μM OA) or OA/ISO for 45 min at 37 °C or not. The medium was then replaced with KGM (control), KGM containing 10 μM ISO plus 10 μM OA, or both. Cultures were wounded as described, and a demarcated area of the wound was photographed at the time of wounding (time 0) and again at 18, 28, and 48 h. The percentage of wound healing was calculated and is represented graphically in A (control (C), ISO (☐), OA (●), and OA/ISO (×)). The data shown are representative of three independent experiments from two separate cell strains. Values plotted are means ± S.E. (n = 3). *, p < 0.01 between ISO and all other conditions. AHCE cells were either pretreated for 45 min with 10 μM OA at 37 °C (lanes 3–5) or not (lanes 1–4). After pretreatment, cells were stimulated for 15 min at 37 °C with EGF (10 ng/ml) (lane 2), 10 μM ISO (lanes 3 and 6), or 1 μM ISO (lanes 4 and 7) in the presence of 10 μM OA (lanes 5–7) or left as untreated controls (lane 1). After treatment, cell lysates were prepared as described, electrophoresed on 10% polyacrylamide gels, and transferred to membrane. Membranes were immunoblotted with either an anti-PP2A antibody (A), an anti-β2-AR antibody (B), or an anti-ERK antibody (C). The data shown are representative of three independent experiments from two separate cell strains. Three separate anti-ERK immunoblots from three separate experiments (C) were scanned, and densitometry was performed using a gel plotting macro in NIH Image 1.62. Data were averaged, statistically analyzed, and represented graphically (D). Values plotted are means ± S.E. (n = 3). *, p < 0.01 compared with control (C, lane 1).

PP2A Co-immunoprecipitated with Both β2-AR and ERK2 in β2-AR-activated Human Keratinocytes—Macromolecular signaling complexes that provide a platform for specific interaction and rapid signal relay have recently been discovered for a growing number of signaling molecules, including the β2-AR and PP2A (61–63). We hypothesized that β2-AR activation in keratinocytes may likewise initiate complex assembly between the β2-AR and PP2A. Additionally, we examined whether β2-AR activation could induce the physical association of ERK with PP2A. To assess this possibility, PP2A was immunoprecipitated from β2-AR agonist-treated and control cell lysates, and membranes were probed for co-associated proteins.

Immunoblotting with an anti-PP2A antibody as a control revealed that equal amounts of PP2A were immunoprecipitated from both control and ISO-treated cells, as expected (Fig. 5A). Immunoblotting with β2-AR revealed that the phosphatase was associated with the receptor intracellularly only in ISO-treated cells (Fig. 5B). ERK2, however, was already associated with PP2A intracellularly in control cells, but there was a statistically significant 37% increase in association upon β2-AR activation (Fig. 5, C and D). Although there have been many reports that PP2A can regulate ERK activation in cell-free systems (6, 7, 9, 36), this is the first evidence of a physical intracellular association of PP2A with ERK2. Complex formation between PP2A and β2-AR and PP2A and ERK are novel findings in human keratinocytes and provide additional evidence that PP2A mediates the β2-AR agonist-induced decrease in ERK phosphorylation and migration.

A β2-AR Agonist Directly Increased Intracellular PP2A Activity in Human Keratinocytes—The ability of OA to prevent the β2-AR agonist-induced decrease in migration and ERK phosphorylation together with the association of PP2A with both β2-AR and ERK2 in β2-AR agonist-treated cells suggests that β2-AR agonist binding could activate PP2A. This prompted us to measure specific PP2A activity in ISO-treated cells. Levels of intracellular PP2A activity increased rapidly and transiently after ISO treatment. Incubation of keratinocytes with 10 μM ISO for 30 s resulted in a statistically significant (p < 0.01) 2-fold increase in their PP2A activity (Fig. 6). Phosphatase activity remained significantly elevated (p < 0.01) above control levels up to 15 min, at which time activity was no longer significantly different than the control levels. The addition of a β2-AR agonist therefore increased PP2A activity in human
keratinocytes within the time frame that we observed the β2-AR-mediated reduction in ERK phosphorylation, providing a strong evidentiary link that PP2A mediates the β2-AR-induced effects.

Since PP2A is reported to be activated by cAMP (64) and β2-AR activation results in increased intracellular levels of cAMP in keratinocytes (33), we asked whether increasing keratinocyte cAMP levels alone was sufficient to activate PP2A. The addition of forskolin, an activator of adenylyl cyclase, however, did not significantly increase PP2A activity (Fig. 6). These observations support our previous conclusions that the ISO-induced reduction in ERK phosphorylation and migration are independent of cAMP (33).

**DISCUSSION**

Cell migration is a critical component not only of wound healing but of cancer metastasis and numerous physiologic processes, including embryogenesis, the inflammatory response, and tissue remodeling. In the skin, wound healing is completed by the migration of keratinocytes from the wound edges to re-epithelialize the open wound bed (1). Our laboratory recently made the surprising observation that activation of the β2-AR in human keratinocytes reduces phosphorylation of the promigratory ERK MAP kinase. The decrease in phosphorylation was accompanied by a decrease in the migratory ability of the cultured keratinocytes. These findings are in marked contrast to the responses of other previously examined cell types. Upon β2-AR activation in cardiomyocytes (26, 30), HEK293 cells (29, 32), COS-7 cells (28, 31), and Chinese hamster ovary cells (27), β2-AR activation results in increased ERK phosphorylation. Here we elucidate the mechanism by which β2-AR activation decreases ERK phosphorylation and migration. We find that the effect is mediated by the β2-AR-induced activation of PP2A. β2-AR activation initiates the intracellular association of PP2A with the β2-AR and increases the association of PP2A with ERK2, placing PP2A in close proximity to its target for rapid signal relay. Immediately following β2-AR activation with the receptor agonist ISO, there was a 2-fold increase in the measurable activity of the enzyme. The β2-AR-mediated transient activation of PP2A results in decreased ERK phosphorylation, and since ERK phosphorylation and activation are required for keratinocyte migration (11), the downstream effect is decreased migration (Fig. 7). Our elucidation of this novel mechanism for regulating keratinocyte migration suggests that this signaling pathway may be promising as a drug therapy target in wound healing.

It was important to determine whether a similar mechanism was operational in epithelial cells other than keratinocytes. Corneal epithelial cells were chosen for investigation, since β-adrenergic antagonists are the most frequently prescribed ophthalmic drugs (19); indeed, norepinephrine, a naturally occurring β-agonist, is normally found in the cornea (20). Additionally, enhancement or inhibition of wound healing by β2-AR activation in corneal epithelium has been previously reported (17–19). Here we demonstrate that β2-agonists both delay wound healing and reduce ERK phosphorylation in primary AHCE cells. OA reversed the effects, suggesting that PP2A was also responsible for the β2-AR-mediated reduction in migration of ACHC cells. Thus, it is likely that elucidation of this novel mechanism for regulating corneal epithelial cell migration will also have clinical applications in ocular biology.

PP2A, a dual specificity phosphatase capable of dephosphorylating tyrosyl residues as well as serine/threonine residues (65, 66), plays a role in a multitude of cellular functions (reviewed in Ref. 34). This enzyme can be selectively inhibited by the phosphatase inhibitor OA with a half-maximal concentration of 1 nm in cell-free systems (51, 52). Selectivity toward intracellular PP2A can be achieved when OA concentrations are held below 1 μM (42). We have shown that the addition of OA at concentrations that selectively inhibit PP2A (10 nm) to an in vitro keratinocyte wound healing model completely prevents the β2-AR-induced delay in wound repair. Further, when the migratory paths of individual keratinocytes are measured, OA effectively reverses the β2-AR agonist-induced retardation of locomotory speed. These findings suggest that inhibition of migration induced by β2-AR activation is attributable to its modulation of PP2A activity.

One mechanism for modulating the local activation of PP2A is its recruitment into intracellular macromolecular signaling complexes that provide a platform for specific interaction (reviewed in Ref. 34). For example, PP2A is associated in a macromolecular complex along with a calcium channel, protein

![Diagram](https://example.com/diagram.png)

**Fig. 7.** A diagrammatic representation of the role of PP2A in the β2-AR agonist-induced reduction of keratinocyte migration. A simplified EGFR-activated promigratory ERK cascade is shown outlining the proposed role of PP2A in the β2-AR-induced modulation of keratinocyte migration.
kinase A, and $\beta_2$-AR in HEK293 cells and hippocampal neurons (61). In HEK293 cells, it is the activation of the $\beta_2$-AR that initiates receptor desensitization, resulting in the co-association of PP2A with c-Src and $\beta$-arrestin in the clathrin-coated vesicles (60, 63). It was therefore logical to propose that, likewise, $\beta_2$-AR activation could facilitate the association of PP2A with the $\beta_2$-AR in human keratinocytes, providing a plausible mechanism for $\beta_2$-AR agonist modulation of PP2A activity. In keratinocytes, $\beta_2$-AR activation does indeed initiate the association of PP2A with the receptor. The association may play a role in the $\beta_2$-AR-induced reduction in keratinocyte migration by facilitating PP2A activation, or alternatively, the association may mediate resensitization of the $\beta_2$-AR receptor. PP2A accomplishes this function in A431 (67) and HEK293 cells (63) by dephosphorylating the receptor in clathrin-coated pits, thus restoring its function. This could represent a generalized method for G-protein-coupled receptor resensitization, since PP2A has also recently been discovered associated with the chemokine receptor CXCR2, upon internalization, resulting in its dephosphorylation (68). At present, the association of PP2A and the $\beta_2$-AR is unknown.

$\beta_2$-AR activation in keratinocytes also increases the association of PP2A with ERK2. Whereas PP2A was associated with ERK2 in the absence of $\beta_2$-AR activation, the association is increased by 37% upon $\beta_2$-AR agonist binding. The $\beta_2$-AR-mediated increase in the physical intracellular association of PP2A and ERK2 probably provides an organizing platform by which $\beta_2$-AR activation results in the observed reduction in ERK phosphorylation. Activation of membrane receptor tyrosine kinases by their cognate growth factors generally results in a transient activation of the ERK/MAP signaling cascade. The duration and magnitude of the signaling response are determined by the relative contributions of both kinases and phosphatases (5, 8, 10). Thus, the finding of an association of PP2A with ERK in control, nonstimulated cells suggests that PP2A may play a regulatory role in ERK phosphorylation, providing a basal rate of dephosphorylation and allowing only its transient phosphorylation and activation. Recently, Meves et al. (69) have shown that incubating human keratinocytes with much higher concentrations of OA (900 nM) for longer time periods (60–90 min) results in increased phosphorylation of ERK, providing pharmacological support for the proposed role of PP2A as a regulator of ERK activity.

ERK plays a pivotal role in the keratinocyte promigratory signaling cascade (11–13). Its activation occurs as a downstream event in the promigratory EGF/ERK MAP kinase signaling cascade (Fig. 7). We find that $\beta_2$-AR activation reduces not only ERK phosphorylation but also the phosphorylation of its immediate upstream activator MEK. MEK is serine-phosphorylated and activated by Raf-1 (reviewed in Ref. 5). Although PP2A is reported to dephosphorylate MEK in vitro (6, 36, 70), in this study, OA (at 10 nM) did not reverse the ISO-induced reduction in MEK phosphorylation in keratinocytes. This suggests that an OA-insensitive phosphatase is activated by the $\beta_2$-AR agonist. Recent evidence suggests that PP1 is not involved in the dephosphorylation of MEK or ERK (71). Thus, the identity of the phosphatase or phosphatases responsible for MEK deactivation is presently unknown.

Our finding that the $\beta_2$-AR agonist, ISO, rapidly (30 s) and transiently (returned to base-line range by 15 min) increases the intracellular activity of PP2A by 2-fold perhaps presents the strongest evidence that the $\beta_2$-AR-induced decrease in ERK phosphorylation is mediated by PP2A. Multiple overlapping mechanisms for regulating PP2A activity have been reported. They include two post-translational modifications of amino acids in its catalytic subunit, phosphorylation of tyrosine residue 307 that inhibits its activity (72), and methylation of leucine that increases its activity (73–75). Ceramide can also activate PP2A in T9 rat glioma cells (79), and there are two specific inhibitors of PP2A, I$_1$PP2A (PHAP-1) and I$_2$PP2A (SET) purified from bovine kidney (80–83). The rapid activation of PP2A suggests that the most likely mechanism could be an ISO-induced post-translational modification of the catalytic subunit of PP2A. A number of cytokines and growth factors can phosphorylate PP2A on tyrosine 307, resulting in deactivation (84), including interleukin-2 (85), colony-stimulating factor 1 (43), tumor necrosis factor, interleukin-1 (86), insulin (87, 88), and EGF (89). Whether ISO decreases the phosphorylation of tyrosine 307 or increases the methylation of leucine 309 to increase the activity of the catalytic subunit of PP2A is currently under investigation in our laboratory.

Whereas a role for PP2A in the regulation of cell motility has been suggested by pharmacological approaches using okadaic acid in transformed cells (90) and cancer cell lines (38, 40, 91, 92), the investigations reported here demonstrate that intracellular PP2A activity can be manipulated in keratinocytes by a novel, receptor-mediated mechanism with resultant effects on the migratory ability of the cells. Our findings open up the possibility of exploring the use of $\beta_2$-adrenergic agonists as pharmaceutical agents to inhibit cell migration. Additionally, this work is the first to implicate PP2A as an important mediator of cell motility in keratinocytes and corneal epithelial cells, providing pertinent insights that could be harnessed to control this complex and highly regulated promigratory ERK signaling pathway. Exploring the regulation of this promigratory cascade will hopefully lead to the development of novel therapeutic approaches relevant to cancer, immune defense, and wound healing.

Acknowledgments—We thank Jennifer Grahn for helpful discussions and Dr. Fu-Tong Liu for critical review of the manuscript.

REFERENCES

1. Woodley, D., O’Toole, E., Nadelman, C. M., and Li, W. (1999) Dermatol. Found. 33, 1–12.
2. Ando, Y., and Jensen, P. J. (1993) J. Invest. Dermatol. 100, 633–639.
3. Fang, K. S., Farboud, B., Nucetillici, R., and Isseroff, R. R. (1998) J. Invest. Dermatol. 111, 751–756.
4. Fang, K. S., Ionides, E., Oster, G., Nucetillici, R., and Isseroff, R. R. (1999) J. Cell Sci. 112, 1967–1978.
5. Cobl, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846.
6. Gomez, N., and Cohen, P. (1991) Nature 353, 170–173.
7. Alessi, D. R., Gomez, N., Morehead, G., Lewis, T., Keynes, S. R., and Cohen, P. (1995) Curr. Biol. 5, 283–295.
8. Keynes, S. M. (2000) Curr. Opin. Cell Biol. 12, 186–192.
9. Zhou, B., Wang, Z. X., Zhao, Y., Bruttagan, D. L., and Zhang, Y. Z. (2002) J. Biol. Chem. 277, 31818–31825.
10. Cowley, S., Paterson, H., Kemp, P., and Marshall, J. C. (1994) Cell 77, 841–852.
11. Zeiger, M. E., Chi, Y., Schmidt, T., and Varani, J. (1999) J. Cell. Physiol. 180, 271–284.
12. Leng, J., Klemke, R. L., Reddy, A. C., and Cheresh, D. A. (1999) J. Biol. Chem. 274, 37855–37861.
13. Glading, A., Chang, P., Lauffenburger, D. A., and Wells, A. (2000) J. Biol. Chem. 275, 2890–2898.
14. Schallreuter, K. U., Wood, J. M., Petticrew, M. R., Swanson, N. N., and Steintraus, V. (1999) Arch. Dermatol. Res. 285, 216–220.
15. Steintraus, V., Steintraus, M., Kornier, C., and Mensing, H. (1992) J. Invest. Dermatol. 98, 475–480.
16. Steintraus, V., Mak, J. C., Pichlmieier, U., Mensing, H., Ring, J., and Barnes, P. J. (1996) Arch. Dermatol. Res. 288, 549–553.
17. Haruta, Y., Ohashi, Y., and Matsuda, S. (1997) Eur. J. Ophthalmol. 7, 334–339.
18. Liu, G. S., Trope, G. E., and Basu, P. K. (1990) J. Ocul. Pharmacol. 6, 101–112.
19. Bely, J. J., Zawarz, J., Thompson, H. W., and Beuerman, R. W. (1994) Br. J. Ophthalmol. 78, 377–380.
20. Murphy, C. J., Campbell, S., Araki-Sasaki, K., and Marfurt, C. F. (1998) Cancers 17, 529–536.
21. Masur, K., Niggemann, B., Zanker, K. S., and Entschladen, F. (2001) Cancer Res. 61, 2866–2869.
22. Schlessner, J. R., Gupta, J., Veyts, T., Kneifl, R. K., Benudd, S. I., and Wyatt, T. A. (2002) Am. J. Physiol. 282, L1108–L1116.
23. Donaldson, D. J., and Mahan, T. J. (1984) Comp. Biochem. Physiol. C 78, 267–270.
24. Silvestri, M., Oddera, S., Lantero, S., and Rossi, G. A. (1999) Respir. Med. 93, 416–423.
25. Schallreuter, K. U. (1997) J. Invest. Dermatol. Symp. Proc. 2, 37–40.
