PKCδ Activation

A Divergence Point in the Signaling of Insulin and IGF-1–Induced Proliferation of Skin Keratinocytes

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Insulin and insulin-like growth factor-1 (IGF-1) are members of the family of the insulin family of growth factors, which activate similar cellular downstream pathways. In this study, we analyzed the effects of insulin and IGF-1 on the proliferation of murine skin keratinocytes in an attempt to determine whether these hormones trigger the same signaling pathways. Increasing doses of insulin and IGF-1 promote keratinocyte proliferation in an additive manner. We identified downstream pathways specifically involved in insulin signaling that are known to play a role in skin physiology; these include activation of the Na^+/K^+ pump and protein kinase C (PKC). Insulin, but not IGF-1, stimulated Na^+/K^+ pump activity. Furthermore, ouabain, a specific Na^+/K^+ pump inhibitor, abolished the proliferative effect of insulin but not that of IGF-1. Insulin and IGF-1 also differentially regulated PKC activation. Insulin, but not IGF-1, specifically activated and translocated the PKCδ isoform to the membrane fraction. There was no effect on PKC isoforms α, η, ε, and ζ, which are expressed in skin. PKCδ overexpression increased keratinocyte proliferation and Na^+/K^+ pump activity to a degree similar to that induced by insulin but had no affect on IGF-1–induced proliferation. Furthermore, a dominant negative form of PKCδ abolished the effects of insulin on both proliferation and Na^+/K^+ pump activity but did not abrogate induction of keratinocyte proliferation induced by other growth factors. These data indicate that though insulin or IGF-1 stimulation induce keratinocyte proliferation, only insulin action is specifically mediated via PKCδ and involves activation of the Na^+/K^+ pump. *Diabetes* 50:255–264, 2001

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DNPKCδ, dominant-negative PKCδ; DTT, dithiothreitol; EcGF, endothelial cell growth factor; EGF, epidermal growth factor; IGFR, IGF-1 receptor; IR, insulin receptor; KGF, keratinocyte growth factor; MEM, minimum essential medium; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3 kinase, PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmunoprecipitation assay; WTPKCδ; wild-type PKCδ

Insulin and IGF-1 are members of the insulin family of growth factors and exert their mitogenic and metabolic effects in different tissues via distinct receptors (1–4). Both of these growth factors are implicated in cellular growth and differentiation and are essential components of the growth medium of cells in vitro (5–9). However, although the mitogenic effects of IGF-1 are well documented, insulin-induced proliferation has been mainly attributed to its transactivation of the IGF-1 receptor (IGFR) (2,8).

Despite the extensive evidence showing remarkable homology between insulin and IGF-1 receptors and similarities in their signaling pathways, these two hormones are known to have distinct physiological functions. The insulin receptor (IR) and the IGFR differentially affect cell growth, apoptosis, differentiation, and transformation (2–4). However, to date, efforts to identify the selective downstream effectors of these two closely related receptors indicate more similarities than differences. When activated, both receptors use IRS-1, IRS-2, and Shc as immediate downstream adapter molecules leading to the activation of the Ras, Raf, extracellular signal-regulated kinase, and the phosphatidylinositol 3 kinase (PI3K) pathways (3,10). This indicates that points of divergence in signaling are likely to be downstream of these pathways.

In the present study, we have focused on the signaling pathways of insulin and IGF-1 in skin keratinocyte proliferation. Keratinocytes are the major cellular component of the epidermis, the stratified squamous epithelia forming the outermost layer of skin. Keratinocytes lie on the basement membrane and are organized into distinct cell layers, which differ morphologically and biochemically (11,12). Cellular proliferation is restricted to the basal layer. Upon division, keratinocytes give rise to either replacement progenitor cells or to cells that are committed to undergo terminal differentiation. The latter cells leave the basal layer and gradually migrate upward, simultaneously progressing along the differentiation pathway and reaching the outer surface of the epidermis in the form of fully mature corneocytes (13).

Several endogenous substances regulate proliferation and growth of keratinocytes. Among these regulators are insulin and IGF-1(9). Indeed, skin keratinocytes express IR and IGFR (14–16). Furthermore, it was shown that human keratinocytes are dependent on insulin for their growth (9) and IGF-1 is mitogenic to both mouse and human keratinocytes (5,6).

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Of the various downstream elements of the insulin and IGF-1 signaling pathways, we have focused on two major downstream elements, the Na^+/K^+ pump and the protein kinase C (PKC) family of serine threonine protein kinases. Both of these protein families are known to be involved in the insulin and IGF-1 signaling pathway and are implicated in cellular proliferation processes (1).

The Na^+/K^+ pump, known to be regulated by insulin, is an intrinsic plasma membrane enzyme, which hydrolyzes ATP to maintain transmembrane gradients of Na^+ and K^+ in mammalian cells (17). The enzyme consists of two catalytic α subunits and two regulatory β subunits. At present, as many as four α subunits (α_1, α_2, α_3, and α_4) and three β subunits (β_1, β_2, and β_3) have been identified in mammalian cells. The multiple isoforms are known to be differentially expressed and regulated in different tissues. Regulation of Na^+/K^+ pump activity by insulin has been suggested to occur by increasing the number of pump sites in the membrane or by increasing the activity of existing pump units in the membrane (18,19).

PKCs are a family of serine-threonine kinases, which play key functions in cellular signal transduction (20,21). Three categories of PKC have been described depending on their mechanisms of activation: conventional PKC (α, β, and γ), nonconventional PKC (δ, ε, and η) and atypical PKC (ι, λ, and ζ). In skin PKC isoforms α, δ, ε, η, and ζ have been detected (22,23). However, their role in mediating the nonmetabolic effects of insulin in keratinocytes has not been studied.

In our studies we have used a model system of murine keratinocytes in culture. Cells are maintained in the proliferative state with a high growth rate by culturing murine keratinocytes in medium containing low Ca^{2+} concentrations (0.05 mmol/l) (24). In the present study, we identified a unique divergence point between insulin and IGF-1 mitogenic signaling pathways. Insulin-induced proliferation was found to involve specific activation of PKCδ and stimulation of the Na^+/K^+ pump, whereas IGF-1–induced proliferation did not.

METHODS

Materials. Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemiluminescence was performed with a kit purchased from Bio-Rad (Israel). Polyclonal antibodies to Na^+/K^+ pump isoforms and monoclonal anti–p-tyr antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal and monoclonal antibodies to PKC isoforms were purchased from Santa Cruz (California, USA) and Transduction laboratories (Lexington, KY). Horseradish peroxidase–anti-rabbit and anti-mouse IgG were obtained from Bio-Rad (Israel). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Na-orthovanadate, and pepstatin were purchased from Sigma Chemicals (St. Louis, MO). Insulin (recombinant human insulin [humulinR]) was purchased from Eli Lilly France SA (Fergersheim, France). IGF-1 was a gift from Cytolab (Israel).

Isolation and culture of murine keratinocytes. Primary keratinocytes were isolated from newborn BALB/C mice as described (25). Keratinocytes were cultured in Eagle's minimal essential medium containing 8% Chelex-(Chelex-100, Bio-Rad) treated fetal calf serum. To maintain a proliferative basal cell phenotype, the final Ca^{2+} concentration was adjusted to 0.05 mmol/l. Experiments were performed 5–7 days after plating.

Preparation of cell extracts and Western blot analysis. For crude membrane fractions, whole-cell lysates were prepared by scraping cells into phosphate-buffered saline (PBS) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mmol/l PMSF, 10 mmol/l EDTA, 200 μmol/l $NaVO_4$, and 10 mmol/l NaF. After homogenization and four freeze/thaw cycles, lysates were spun down at 4°C for 20 min in a microcentrifuge at maximal speed. The supernatant containing the soluble cytosol protein fraction was transferred to another tube. The pellet was resuspended in 250 μl PBS containing 1% Triton X-100 with protease and phosphatase inhibitors, incubated for 30 min at 4°C, and spun down in a microcentrifuge at maximal speed at 4°C. The supernatant contains the membrane fraction. Protein concentrations were measured using a modified Lowry assay (Protein Assay Kit; Bio-Rad). Western blot analysis of cellular protein fractions was carried out as described (26).

Preparation of cell lysates for immunoprecipitation. Culture dishes containing keratinocytes were washed with Ca^{2+}/Mg^{2+}–free PBS. Cells were mechanically detached in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l NaF, 1% Triton X-100, 0.1% SDS, and 1% Na deoxycholate) containing a cocktail of protease and phosphatase inhibitors (20 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mmol/l PMSF, 1 mmol/l DTT, 200 μmol/l orthovanadate; and 2 μg/ml pepstatin). The preparation was centrifuged in a microcentrifuge at maximal speed for 20 min at 4°C. The supernatant was used for immunoprecipitation.

Immunoprecipitation. The lysate was precleared by mixing 0.3 ml of cell lysate with 25 μl of Protein A/G Sepharose (Santa Cruz, CA), and the suspension was rotated continuously for 30 min at 4°C. The preparation was then centrifuged at maximal speed at 4°C for 10 min, and 30 μl of A/G Sepharose was added to the supernatant along with specific polyclonal or monoclonal antibodies to the individual PKC isoforms (dilution 1:100). The samples were rotated overnight at 4°C. The suspension was then centrifuged at maximal speed for 10 min at 4°C, and the pellet was washed with RIPA buffer. The suspension was again centrifuged at 15,000*g* (4°C for 10 min) and washed four times in TBST. Sample buffer (0.5M Tris HCl, pH 6.8, 10% SDS, 10% glycerol, 4% 2 β-mercaptoethanol, and 0.05% bromophenol blue) was added and the samples were boiled for 5 min and then subjected to SDS-PAGE.

Adenovirus constructs. The recombinant adenovirus vectors were constructed as described (27). The dominant negative mutant of mouse PKCδ was generated by the substitution of the lysine residue at the ATP-binding site with alanine (28). The mutant delta cDNA was cut from SRD expression vector with EcoR I and ligated into the pAxCA1w cosmid cassette to construct the Ax vector. The dominant negative activity of this gene was demonstrated by the abrogation of its autophosphorylation activity (29).

Transduction of keratinocytes with PKC isoform genes. The culture medium was aspirated and keratinocyte cultures were infected with the viral supernatant (29) containing PKCδ recombinant adenoviruses for 1 h. The cultures were then washed twice with low Ca^{2+}-containing minimum essential medium (MEM) and refed. Cells were transferred 10-h postinfection to serum-free low Ca^{2+}-containing MEM for 24 h. Keratinocytes from control and insulin-treated cultures were used for proliferation assays, ^{86}Rb uptake, or extracted and fractionated into cytosol and membrane fractions for immunoprecipitation and Western blotting.

PKC activity. Specific PKC activity was determined in freshly prepared immunoprecipitates from keratinocyte cultures after appropriate treatments. These lysates were prepared in RIPA buffer without NaF. Activity was measured with the use of the SignaTECT PKC assay system (Promega, Madison, WI) according to the manufacturer's instructions. PKCα pseudosubstrate was used as the substrate in these studies.

Cell proliferation. Cell proliferation was measured by [^{3}H]thymidine incorporation in 24-well plates. Cells were pulsed with [^{3}H]thymidine (1 μCi/ml) overnight. After incubation, cells were washed five times with PBS and 5% thrichloracetic acid was added to each well for 30 min. The solution was removed and cells were solubilized in 1% Triton X-100. The labeled thymidine incorporated into cells was counted in a ^{3}H-window of a Tricarb liquid scintillation counter.

Na^+/K^+ pump activity. Na^+/K^+ pump activity was determined by the measurements of ouabain-sensitive uptake of ^{86}Rb by whole cells in 1 ml of K^+-free PBS containing 2 mmol/l RbCl and 2.5 μCi of ^{86}Rb (30). Rb uptake was terminated after 15 min by aspiration of the medium, after which the cells were rinsed rapidly four times in cold 4°C K^+-free PBS and solubilized in 1% Triton X-100. The cells from the dish were added to 3 ml H_2O in a scintillation vial. Samples were counted in a ^{3}H-window of a Tricarb liquid scintillation counter. Rb-uptake specifically related to Na^+/K^+ pump activity was determined by subtraction of the counts per minute accumulated in the presence of 10^{-4} mol/l ouabain from the uptake determined in the absence of the inhibitor.

RESULTS

Effects of insulin and IGF-1 on keratinocyte proliferation. Initially we wanted to characterize the mitogenic effects of both insulin and IGF-1 on skin keratinocytes. The ability of the hormones to induce keratinocyte proliferation was evaluated by measuring thymidine incorporation. As shown in Fig. 1*A*, both insulin and IGF-1 stimulated thymidine incorporation in a dose-dependent manner with maximal induction achieved at 10^{-7} and 10^{-8} mol/l, respectively. At each con-

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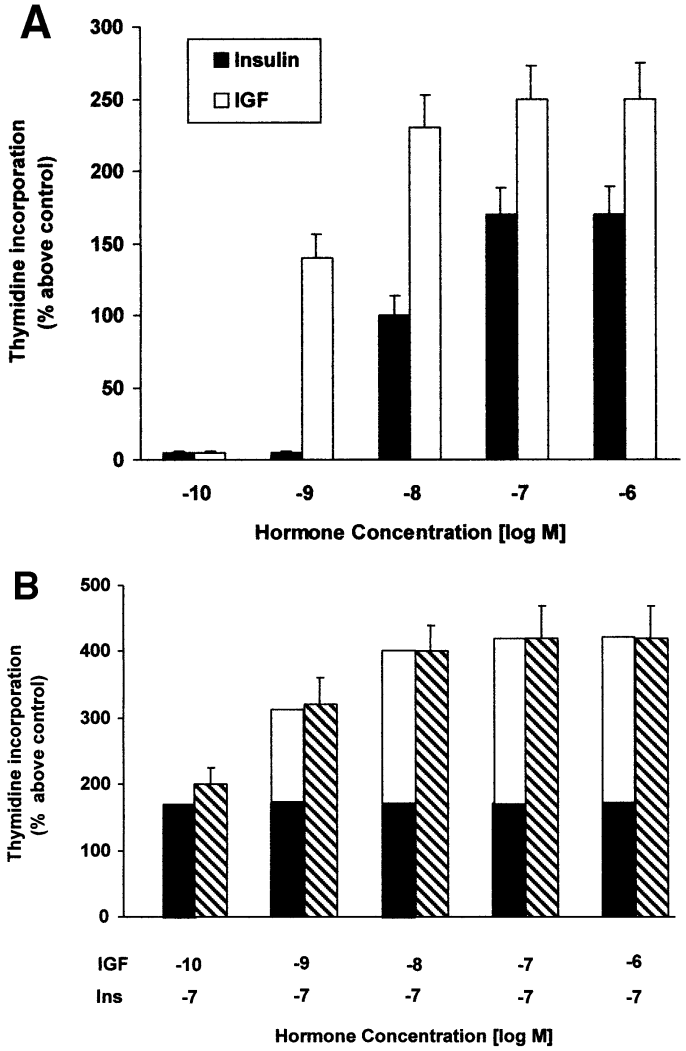

FIG. 1. Insulin and IGF-1 have an additive effect on keratinocyte proliferation. Primary keratinocytes were isolated and plated as described in RESEARCH DESIGN AND METHODS. Proliferating keratinocytes were maintained for 5 days in low Ca^{2+} medium (0.05 mmol/l) until they reached 80% confluency. *A*: 5-day keratinocyte cultures were stimulated for 24 h with insulin or IGF-1 at the designated concentrations. *B*: In parallel, keratinocytes were stimulated with 10^{-7}mol/l insulin (Ins) and increasing doses of IGF-1 (IGF). At each concentration, the right column (▨) represents proliferation observed when both hormones were added together. The left bar demonstrates the separate effect of 10^{-7} mol/l insulin (■) and increasing concentrations of IGF-1 (□). Thymidine incorporation was measured as described in RESEARCH DESIGN AND METHODS. The results shown are representative of six experiments. Each bar represents the mean ± SE of three determinations expressed as percent above control unstimulated keratinocytes.

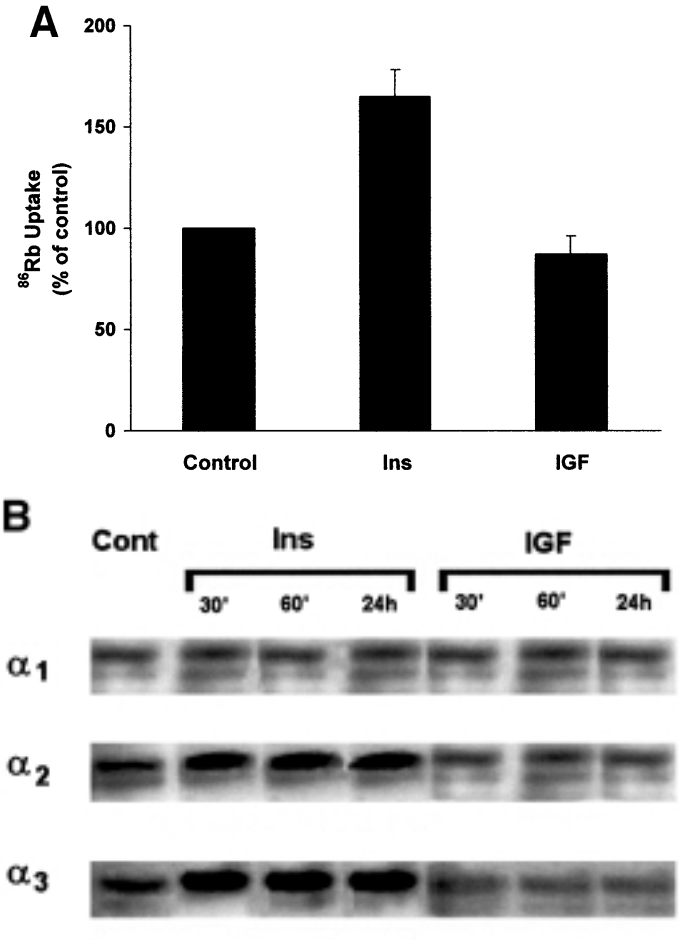

FIG. 2. Insulin but not IGF-1 induces Na^+/K^+ pump activity. Primary keratinocytes were cultured as described in Fig. 1. For the pump activity assay, 5-day-old keratinocytes were stimulated with 10^{-7} mol/l insulin (Ins) or 10^{-8} mol/l IGF-1 (IGF) for the times indicated. *A*: Na^+/K^+ pump activity was evaluated by ^{86}Rb uptake after 30 min stimulation as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three experiments performed on separate cultures. Values are expressed as percent of control unstimulated cells from the same culture in each experiment. *B*: Na^+/K^+ pump isoform expression was analyzed by Western blotting. Cell extracts were prepared from control (Cont) keratinocytes and from cells stimulated with 10^{-7} mol/l insulin (Ins) or 10^{-8} mol/l IGF-1 (IGF) for the times indicated. Whole-cell extracts (20 µg protein) were subjected to SDS-PAGE and transfer. Blots were probed with specific polyclonal antibodies to each isoform. The blots shown are representative of three different experiments.

centration, the maximal stimulation by IGF-1 was greater than that by insulin. Interestingly, when both hormones were given together, their mitogenic effects were additive at all concentrations tested (Fig. 1*B*). These results suggest that insulin and IGF-1 regulate keratinocyte proliferation through distinct pathways.

Effects of insulin and IGF-1 on regulation of the Na^+/K^+ pump. We next attempted to identify the possible downstream elements that could serve as a divergence point in mediating insulin- and IGF-1–induced proliferation. We initially examined the effects of insulin and IGF-1 on Na^+/K^+ pump activity. The Na^+/K^+ pump is an established regulator of proliferation and differentiation of keratinocytes and is known to be regulated by insulin. Figure 2*A* demonstrates the effects of insulin and IGF-1 on Na^+/K^+ pump activity as measured by ouabain-sensitive ^{86}Rb uptake. As seen, insulin but not IGF-1 significantly increased pump activity.

Next, we examined the effects of insulin and IGF-1 on Na^+/K^+ pump protein isoform expression (Fig. 2*B*). Skin keratinocytes express the α_1, α_2, α_3, β_1, and β_2 isoforms of the Na^+/K^+ pump. After insulin stimulation, expression of the α_2 and α_3 but not the α_1 isoforms was increased as early as 30 min after stimulation (Fig. 2*B*). The elevated expression was maintained for up to 24 h (Fig. 2*B*). No change was observed in the protein expression of the β_1 or β_2 subunits (results not shown). Consistent with the lack of effect of IGF-1 on Na^+/K^+

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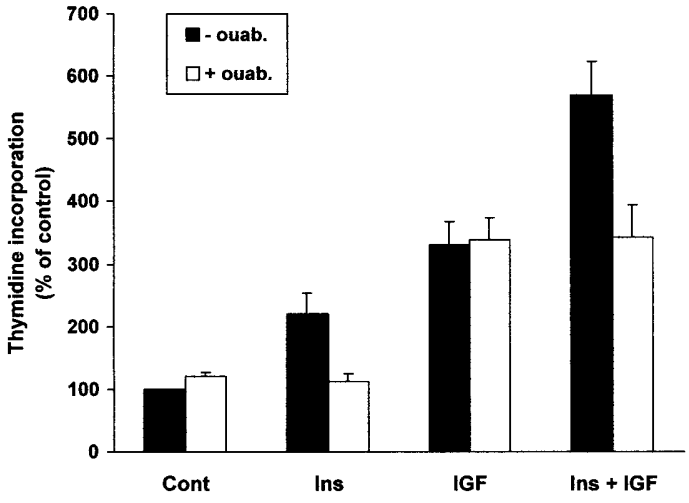

FIG. 3. Ouabain specifically blocks insulin-induced, but not IGF-1–induced, keratinocyte proliferation. Primary keratinocytes were cultured as in Fig. 1. After 5 days, keratinocyte cultures were either untreated (Cont) or stimulated for 24 h with 10^{-7} mol/l insulin (Ins) or 10^{-8}mol/l IGF-1 (IGF) in the presence or the absence of ouabain (10^{-4} mol/l). Thymidine incorporation was measured as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three separate experiments performed on separate cultures. Values are expressed as percent of control unstimulated cells in the absence of ouabain from the same culture in each experiment.

pump activity, this hormone did not affect protein expression of either the α (Fig. 2*B*) or β (data not shown) subunits. Interestingly, in contrast to the differential effects of insulin and IGF-1 on the Na^+/K^+ pump, both factors similarly activated other immediate downstream elements of the insulin- and IGF-1–signaling pathway. These included the phosphorylation and activation of IRS1, IRS2, MAPK, and PI3K (results not shown). Because the Na^+/K^+ pump activity plays a role in skin proliferation, we next wanted to determine whether the distinct regulation of Na^+/K^+ pump activity by insulin is associated with keratinocyte proliferation. Thus, we studied the effects of insulin and IGF-1 on keratinocyte proliferation in cells that were pretreated with ouabain, a specific inhibitor of the Na^+/K^+ pump. As shown in Fig. 3, ouabain (10^{-4} mol/l) completely blocked insulin-induced thymidine incorporation. In contrast, the proliferative effects of IGF-1 were essentially unaffected by ouabain. Moreover, the addition of ouabain with both insulin and IGF-1 reduced the increase in thymidine incorporation to the level induced by IGF-1 alone. Thus, the ability of ouabain to block only the insulin-associated component of proliferation further suggests that insulin and IGF-1 use different signaling pathways to induce their respective proliferative effects.

Effects of insulin and IGF-1 on PKC isoform translocation and activity. PKC is another major signaling pathway, which mediates keratinocyte proliferation and differentiation (28,31,32) and was shown in other tissues to be regulated by insulin signaling (33–35). In skin, PKC isoforms α, δ, ε, η, and ζ are expressed (36). Because the activation of PKC isoforms is associated with their translocation to membrane fractions, we first examined the effects of insulin and IGF-1 on translocation of the various PKC isoforms from cytosol to the membrane. As seen in Fig. 4*B*, as early as 1 min after stimulation, insulin specifically induced translocation of PKCδ from the cytosol to the membrane fractions. Membrane expression of PKCδ was maintained for several hours after insulin stimulation. In contrast, IGF-1 reduced PKCδ expression in the membrane and increased its relative level of expression in the cytosol fraction. No change in distribution of the other PKC isoforms was seen after stimulation by either insulin or IGF-1 (Fig. 4*A*). Interestingly, whereas stimulation with epidermal growth factor (EGF) and high calcium concentrations induced tyrosine phosphorylation of PKCδ, neither insulin nor IGF-1 induced tyrosine phosphorylation of the PKCδ isoform (Fig. 4*D*). To determine if the differential regulation of PKCδ could be mediated by the Na^+/K^+ pump, we further analyzed the effects of ouabain on the expression and translocation of PKCδ. As seen in Fig.4*C*, ouabain, the Na^+/K^+ pump inhibitor, did not affect PKCδ distribution or expression in nonstimulated cells. Furthermore, ouabain did not interfere with insulin-induced translocation of PKCδ.

To determine whether the translocation of PKCδ is sufficient for its activation, we next measured kinase activity of PKC immunoprecipitates from the cytoplasmic and membrane fractions of insulin- and IGF-1–treated keratinocytes. As shown in Fig. 5, insulin but not IGF-1 increased activity of PKCδ in the membrane fraction. No elevation in PKCδ activity was observed in the cytoplasmic fraction. The insulin-induced activation was specific for PKCδ and no activation of PKCs α, ε, η, or ζ was observed for up to 30 min after insulin stimulation (not shown). Altogether, these results suggest selective PKCδ activation specifically by insulin but not by IGF-1 stimulation.

To specifically link insulin-induced PKCδ activation to insulin-induced keratinocyte proliferation we used rottlerin, a specific inhibitor of PKCδ, and studied its effects on insulin-induced proliferation. As seen in Fig. 6, rottlerin inhibited keratinocyte proliferation induced by insulin. In contrast, wortmanin, a PI3K inhibitor, did not have any effect on insulin induced proliferation. These results suggest that insulin-induced proliferation is independent of PI3K but is specifically linked to PKCδ activation.

To directly study the association between insulin-induced PKCδ activation and insulin-induced keratinocyte proliferation, we used recombinant PKC adenovirus constructs to overexpress both wild-type PKCδ (WTPKCδ) as well as a kinase-inactive dominant-negative PKCδ (DNPKCδ), which abrogates the endogenous PKCδ activity. Both constructs, as well as a PKCα construct, were efficiently expressed in keratinocytes (Fig. 7*A*). Furthermore, overexpressing PKCδ and PKCα induced an increase in isoform-specific PKC activity several fold above control levels (Fig. 7*B*). Next, we followed the effects of overexpressing WTPKCδ and DNPKCδ on insulin-induced keratinocyte proliferation. As can be seen in Fig. 8*A*, overexpression of WTPKCδ without insulin treatment, but not overexpression of PKCα, increased thymidine incorporation. The increase was similar to the increase induced by insulin in control cells. Moreover, insulin could not further increase the upregulated proliferation of the WTPKCδ overexpressing cells. In contrast, stimulation by IGF-1 increased thymidine incorporation in a similar manner in both noninfected cells and in cells overexpressing WTPKCδ and PKCα (Fig. 8*A*). These results indicate that insulin, but not IGF-1, mediates proliferation of keratinocytes through a pathway involving PKCδ.

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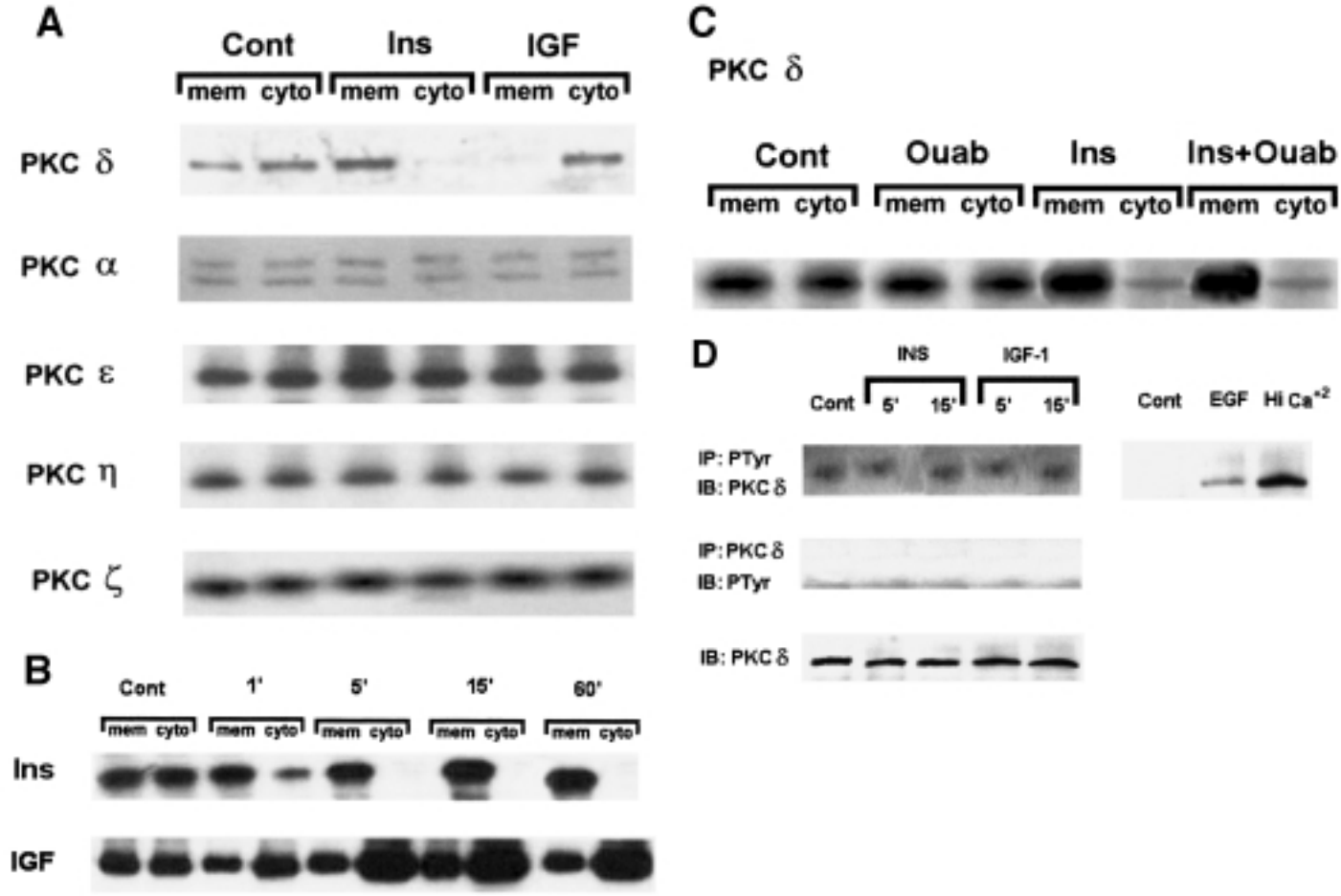

FIG. 4. Insulin, but not IGF-1 specifically, induces translocation of PKCδ in proliferating keratinocytes. Primary keratinocytes were isolated and plated as described in RESEARCH DESIGN AND METHODS. Proliferating keratinocytes were maintained for 5 days in low Ca^{2+} medium (0.05 mmol/l) until they reached 80% confluency. *A*: Cells were stimulated with 10^{-7} mol/l insulin (Ins) or 10^{-8} mol/l IGF-1 (IGF) for 5 min. Cells were lysed, as described, and 20 µg of membrane or cytosol extracts of stimulated and control unstimulated cells were subjected to SDS-PAGE and transfer. Blots were probed with specific polyclonal antibodies to each PKC isoform. *B*: Cells were stimulated with 10^{-7} mol/l insulin (Ins) or 10^{-8} mol/l IGF-1 (IGF) for the times indicated. Cells were lysed, as described, and 20 µg of membrane or cytosol extracts of stimulated and control unstimulated (Cont) cells were subjected to SDS-PAGE and transfer. Blots were probed with PKCδ antibody. *C*: Cells were stimulated for 30 min with 10^{-7} mol/l insulin (Ins) in the presence or absence of ouabain. Cells were lysed, as described, and 20 µg of membrane or cytosol extracts of stimulated and control unstimulated (Cont) cells were subjected to SDS-PAGE and transfer. Blots were probed with specific polyclonal antibodies to PKCδ isoform. *D*: Cells were stimulated with 10^{-7} mol/l insulin (Ins) or with 10 ng/ml EGF for 10 min or maintained in 1 mmol/l Ca^{2+} for 18 h. After treatment, PKCδ or p-tyr immunoprecipitates were subjected to SDS-PAGE and transfer. Blots were probed with monoclonal anti–p-tyr antibody (4G10, UBI) or anti-PKCδ and reblotted with anti-PKCδ. The data presented are representative of three separate experiments.

The direct involvement of PKCδ in insulin-induced proliferation was further proven by abrogating PKCδ activity. As seen in Fig. 8*B*, basal thymidine incorporation in cells overexpressing the DNPKCδ was slightly, but significantly, lower than that in noninfected cells. However, overexpression of DNPKCδ completely eliminated insulin-induced proliferation but did not affect IGF-1–induced proliferation. Moreover, the additive effects of insulin and IGF-1 were reduced to that of IGF-1 alone.

Finally, the specificity of PKCδ activation to the insulin-mediated pathway was analyzed by investigating the effects of DNPKCδ mutant on the mitogenic response to a variety of growth factors including the following: IGF-1, EGF, keratinocyte growth factor (KGF), endothelial cell growth factor (EcGF), and platelet-derived growth factor (PDGF). As seen in Fig. 9, the overexpression of DNPKCδ selectively eliminated the proliferative effects induced by insulin but did not block those of any of the other growth factors tested.

Effects of overexpressed WTPKCδ and DNPKCδ on insulin-induced ^{86}Rb uptake. Our results so far demonstrate that insulin-induced proliferation is selectively mediated by activation of PKCδ and is associated with stimulation of Na^+/K^+ pump activity. To demonstrate that these effects are causally related, we examined effects of WTPKCδ or DNPKCδ on insulin-induced Na^+/K^+ pump activity. As can be seen in Fig. 10*A*, overexpression of WTPKCδ increased resting pump activity to a level similar to that induced by insulin. Insulin did not cause a further increase in pump activity in the cells overexpressing WTPKCδ. Furthermore, DNPKCδ significantly reduced resting pump activity and blocked the insulin-induced stimulation of the pump to a level lower than basal pump activity in control unstimulated cells. Finally, we examined the effects of WTPKCδ and DNPKCδ on the expression of Na^+/K^+ pump isoforms (Fig. 10*B*). Interestingly, whereas insulin induced the expression of α_2 and α_3 iso-

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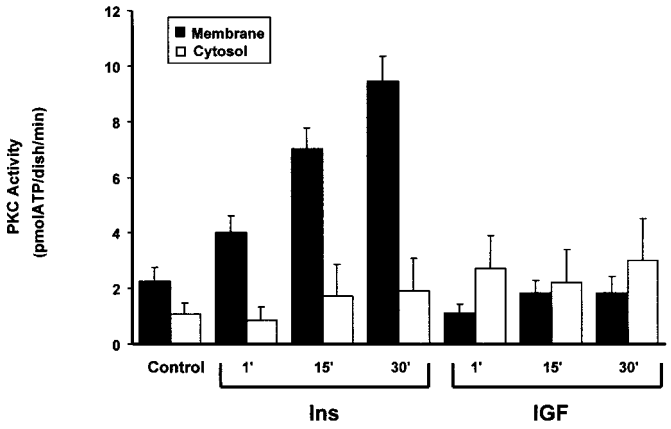

FIG. 5. Insulin but not IGF-1 induces PKCδ activity. To determine PKCδ activity, 5-day keratinocyte cultures were stimulated with 10^{-7} mol/l insulin (Ins) or 10^{-8}M IGF-1 (IGF) for the designated times (1, 15, or 30 min). PKCδ was immunoprecipitated from membrane (■) and cytosol (□) fractions using specific anti-PKCδ antibody. PKCδ immunoprecipitates were analyzed for PKC activity using an in vitro kinase assay as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three separate experiments. Values are expressed as picomoles of ATP per dish per minute.

forms, overexpression of PKCδ increased expression of α_2 similarly to insulin stimulation, and insulin could not further increase α_2 expression. In contrast, no change in α_3 isoform expression was observed and WTPKCδ did not interfere with insulin-induced expression of α_3. Furthermore, abrogating PKCδ activation by overexpressing DNPKCδ completely inhibited insulin-induced α_2 expression but had no effect on insulin-induced expression of the α_3 isoform (Fig. 10*B*).

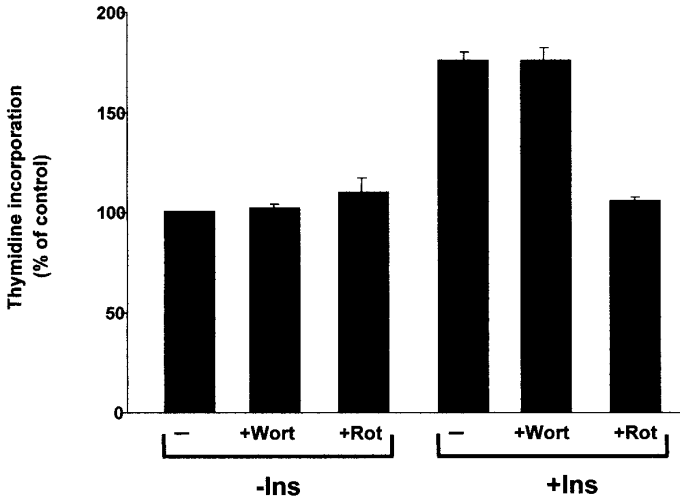

FIG. 6. Rottlerin specifically blocks insulin-induced proliferation. Primary keratinocytes were cultured as in Fig. 1. After 5 days, keratinocyte cultures were either untreated or stimulated for 24 h with 10^{-7} mol/l insulin (Ins) in the presence or the absence of rottlerin (Rot) (5 µmol/l) or wortmanin (Wort) (10^{-8} mol/l). Thymidine incorporation was measured as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three separate experiments performed on separate cultures. Values are expressed as percent of control unstimulated cells in the absence of inhibitors from the same culture in each experiment.

DISCUSSION

Insulin and IGF-1 exert their mitogenic and metabolic effects in different tissues via distinct receptors (4,8). Both insulin and IGF-1 are essential for the growth and maintenance of several cell types including keratinocytes in culture and are essential components of the growth medium of these cells (9). However, skin is not considered to be a classic insulin responsive tissue, because glucose transport is not induced in response to acute insulin stimulation. Therefore, the effects of insulin in skin were mostly attributed to its ability to activate the closely related IGFR (1). We have previously shown that in keratinocytes, insulin and IGF-1 can both stimulate receptors and activate similar downstream effectors (37). However,

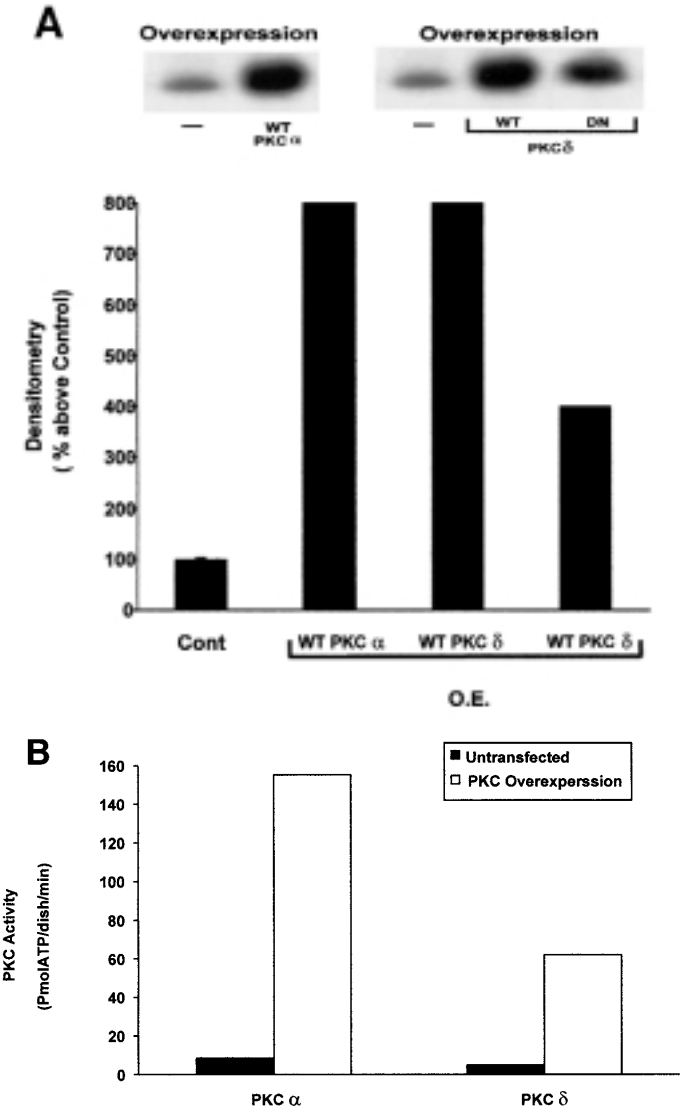

FIG. 7. Overexpression of recombinant PKC adenovirus constructs. Keratinocyte cultures were infected using recombinant adenovirus constructs containing WTPKCδ, WTPKCα, or a dominant DNPKCδ. *A*: After infection, cells were cultured 24 h, harvested, and 20 µg of protein extracts were analyzed by Western blotting using specific anti-PKCα and anti-PKCδ antibodies. The blots presented are representative of five separate experiments. The relative increase in expression of PKCs in overexpressing keratinocytes was analyzed by densitometry. *B*: 24 h after infection, cells were harvested and PKCα and PKCδ immunoprecipitates were evaluated by in vitro kinase assay as described in RESEARCH DESIGN AND METHODS.

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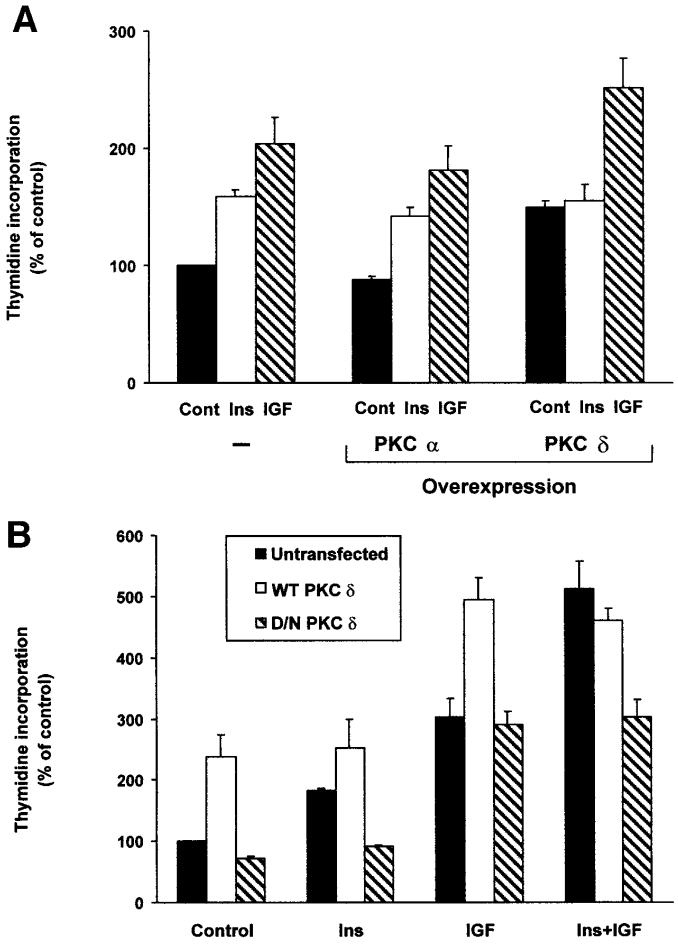

FIG. 8. Effects of PKC overexpression on insulin or IGF-1–induced proliferation. Primary keratinocytes were cultured as described in Fig.1. *A*: Mock-infected cells or keratinocytes infected with WTPKCα or WTPKCδ were either untreated (Cont) or were treated for 24 h with 10^{-7} mol/l insulin (Ins) or 10^{-8} mol/l IGF-1 (IGF). *B*: Noninfected (■) cells overexpressing WTPKCδ (□) or DNPKCδ (▨) were treated for 24 h with 10^{-7} mol/l insulin (Ins), 10^{-8} mol/l IGF-1 (IGF), or both (Ins+IGF). Thymidine incorporation was measured as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three experiments done on separate cultures. Values are expressed as percent of control unstimulated cells from the same culture in each experiment.

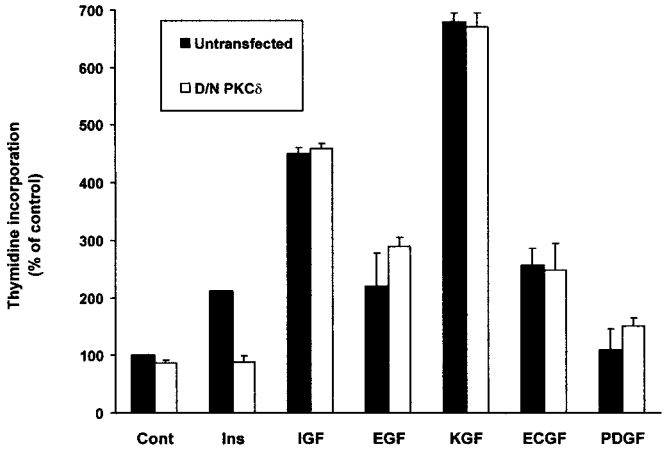

FIG. 9. Inhibition of PKCδ activity specifically abrogates insulin-induced keratinocyte proliferation. Primary keratinocytes were cultured as described in Fig.1. Noninfected cells or keratinocytes infected with DNPKCδ were stimulated for 24 h with the following growth factor concentrations: 10^{-7} mol/l insulin (Ins), 10^{-8} mol/l IGF-1 (IGF), 10 ng/ml EGF, 10 ng/ml PDGF, 1ng/ml KGF, or 5ng/ml EcGF. Thymidine incorporation was measured as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three experiments done on separate cultures. Values are expressed as percent of control unstimulated cells from the same culture in each experiment.

the current study demonstrates that whereas both growth factors induce keratinocyte proliferation in a dose-dependent manner, each hormone exert its effects through distinct signaling pathways. Our initial indication for differential regulation of keratinocyte proliferation by insulin and IGF-1 was confirmed by our finding that these hormones had additive effects on keratinocyte proliferation when given together, at maximal proliferation-inducing concentrations for each hormone (Fig. 1). To identify the divergence point in insulin- and IGF-1–signaling pathway in regulation of keratinocyte proliferation, we investigated elements known to both regulate keratinocyte proliferation and to act as downstream effectors of insulin signaling. These studies revealed that insulin but not IGF-1 signaling is mediated by PKCδ and involves the stimulation of the Na^+/K^+ pump.

In this study, we determined that the Na^+/K^+ pump actively participates in transmitting insulin but not IGF-1 signals, leading to keratinocyte proliferation. Insulin-induced Na^+/K^+ pump activation was associated with selective increases in expression of the α_2 and α_3 Na^+/K^+ pump subunit isoforms. The significant role of the Na^+/K^+ pump in the insulin-signaling pathway was also confirmed pharmacologically by treatment of the cells with ouabain, a selective inhibitor of the Na^+/K^+ pump. Pretreatment of keratinocytes with ouabain completely blocked insulin-induced proliferation of keratinocytes but did not affect proliferation induced by IGF-1. Furthermore, in studies in which additive effects of insulin and IGF-1 were examined, ouabain inhibited only the insulin component and reduced proliferation to a level induced by stimulation with IGF-1 alone. These findings demonstrate the involvement of the Na^+/K^+ pump in mitogenic effects of insulin and further strengthen the idea that insulin and IGF-1 act via separate signaling pathways to induce keratinocyte proliferation.

Na^+/K^+ pump activity has been demonstrated to be regulated by a variety of hormones in different tissues (17). After pump activation, the Na^+/K^+ gradient provides the force for active transport of amino acids, phosphate, and glucose. Several studies have suggested the involvement of the Na^+/K^+ pump in regulation of cellular proliferation in variety of cell types (18,38–41). However, whereas the activation of the Na^+/K^+ pump was known to be an important target of insulin action (42,43), this is the first study that directly implicates specific regulation of the Na^+/K^+ pump in insulin-induced keratinocyte proliferation. The modulation of Na^+/K^+ pump activity is thought to be regulated by direct phosphorylation and dephosphorylation of Na^+/K^+ pump isoforms by protein kinases and protein phosphatases (44,45). Specifically, PKC phosphorylation of the α subunits of the Na^+/K^+ pump was shown to affect the activation state of the Na^+/K^+ pump in vitro and in vivo (19,46–48). However, the functional significance of the PKC-mediated changes in the phosphorylation state of the Na^+/K^+ pump has not been conclusively demonstrated.

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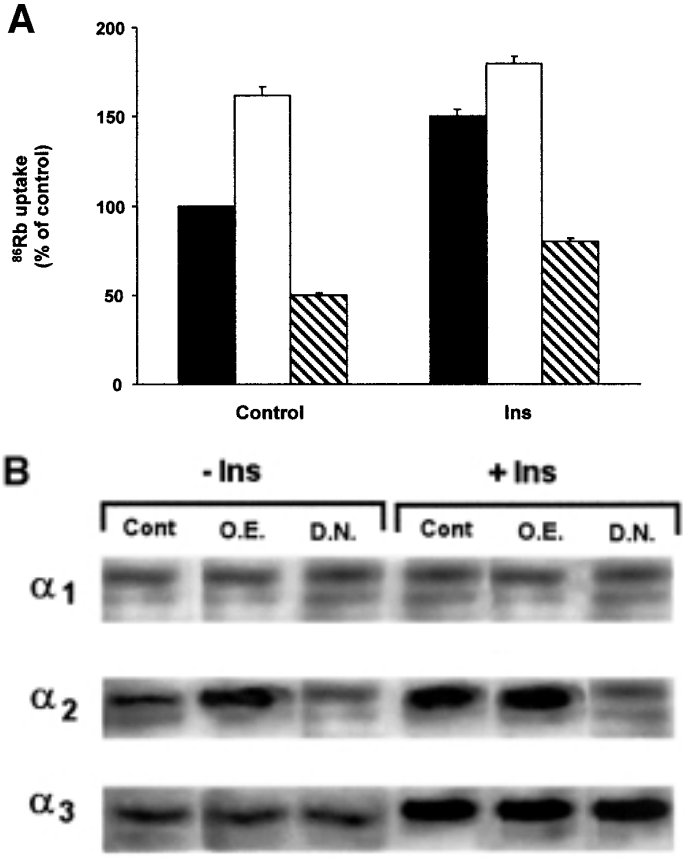

FIG. 10. Effects of overexpressed WTPKCδ and DNPKCδ on basal and insulin-stimulated Na^+/K^+ pump. Primary keratinocytes were cultured as in Fig. 1. Mock-infected keratinocytes (■), keratinocytes infected with WTPKCδ (□), or DNPKCδ (▨) were either untreated or stimulated for 15 min with 10^{-7} mol/l insulin (Ins) 24 h after infection. Na^+/K^+ pump activity was evaluated by ^{86}Rb uptake as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean ± SE of three determinations in three separate experiments. Values are expressed as percent of control unstimulated cells from the same culture in each experiment. *B*: Mock-infected keratinocytes or keratinocytes infected with WTPKCδ or DNPKCδ were either untreated or stimulated for 15 min with 10^{-7} mol/l insulin (Ins) 24 h after infection. Whole-cell extracts (20 µg protein) were subjected to SDS-PAGE and transfer. Blots were probed with specific polyclonal antibodies to each isoform. The blots shown are representative of three different experiments.

Furthermore, as the majority of the studies used nonspecific pharmacological activators and inhibitors of PKC, a specific PKC isoform or a distinct function for PKC could not be identified. In this study, we directly linked hormonal stimulation of the Na^+/K^+ pump to specific activation of PKCδ leading to the induction of cellular proliferation. Activation of the Na^+/K^+ pump by overexpression of PKCδ and the fact that insulin could not further increase this effect indicate that a common pathway is involved. Moreover, the blockade of insulin-induced Na^+/K^+ pump activity by overexpression of a DNPKCδ mutant and the ability of ouabain, a specific pump inhibitor, to abolish the effects of insulin on proliferation without abrogating insulin-induced activation of PKCδ places the Na^+/K^+ pump downstream of insulin-mediated PKC activation. However, whereas insulin stimulation–induced expression of both α_2 and α_3 isoforms, insulin-induced PKCδ activation was only associated with changes in α_2 expression. Induction of Na^+/K^+ pump activity and isoform expression has been linked to cell proliferation in different cell systems (49–51). However, this is the first report linking the insulin-induced proliferation with PKCδ- mediated induction of the Na^+/K^+ pump in keratinocytes. These results are in accordance with the existence of an ion gradient in skin in vivo and with the well-documented effects of Ca^{2+}, K^+, and Na^+ ions on keratinocyte proliferation and differentiation (52–54). These observations are consistent with a role for both insulin and the Na^+/K^+ pump in keratinocyte proliferation and may explain the significance of insulin as an essential component of growth medium of cultured keratinocytes.

Several isoforms of PKC, including α, δ, and η, have been shown to regulate growth and differentiation of skin keratinocytes (28,31,55). Our results provide further evidence for the role of PKCδ in keratinocyte proliferation. PKCδ is a unique isoform among the PKC family of proteins involved specifically in growth and maturation of various cell types (56). This isoform was shown to participate in apoptosis (57,58) differentiation (59,60) and cell-cycle retardation or arrest (61,62). However, PKCδ was also shown to be specifically regulated by stimulation of several growth factors including EGF, PDGF, and neurotransmitters, as well as by the mitogenic signal by v-src and the oncogenic form of c-Ha-ras (59,63–66). Changes in PKCδ regulation are usually associated with its translocation to membranal fractions, tyrosine phosphorylation of the enzyme, and activation or deactivation of its intrinsic kinase activity (60,64). In several of these studies, PKCδ tyrosine phosphorylation was associated with inhibition of PKCδ activity or degradation of the enzyme (64,65,67). In the current study, we found that insulin-induced PKCδ activity was not associated with induction of tyrosine phosphorylation. Rather, PKC activation was associated with translocation of the enzyme and stable expression of PKCδ in the membrane fraction for several hours. Because the phosphorylation level of PKCδ is thought to regulate its activity, enzyme stability, and/or substrate specificity (59,63–67), the functional significance of the unphosphorylated state of PKCδ in this study could be related to its effect on keratinocyte growth. In contrast to the effects of insulin, IGF-1 translocated PKCδ from the membrane to the cytosol but had no appreciable effect on PKC activity. The importance of this effect to the mitogenic action of IGF-1 is currently unclear. However, because mitogenic stimulation by EGF, KGF, PDGF, EcGF, or IGF-1 was not abrogated by the dominant negative mutant of PKCδ, insulin appears to be the primary activator of this PKC isoform in the regulation of keratinocyte proliferation.

The link between PKCδ and insulin signaling has also been established in several other systems. For example, we have recently shown that in muscle cultures, PKCδ mediates insulin-induced glucose transport (33,34). Similarly, in cells overexpressing the IR, insulin stimulation was shown to be associated with activation of PKCδ (68,69). Furthermore, the insulin stimulation was found to be specifically associated with activation of PKCδ (33–35,69). In addition, we have shown in this study that whereas insulin-induced proliferation of kertinocytes is mediated by PKCδ, this pathway was independent of PI3K, an important mediator of both insulin and IGF-1. Similar to the findings in this study, in a previous report we have found that in another model system of muscle myotubes, insulin-induced PKCδ activation was independent of PI3K activity (33,34). However, whereas in these studies

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insulin-mediated PKCδ activation has been linked to the metabolic effects of insulin, this is the first report linking PKCδ to insulin-mediated cell proliferation. In conclusion, this study shows for the first time that PKCδ, a multifunctional serine kinase, serves as a divergence point in transmitting insulin but not IGF-1 mitogenic signals. Future studies will be aimed at elucidating the role of insulin-induced PKCδ-mediated proliferation and its effects on the transmission of mitogenic signals by a variety of growth factors in skin keratinocytes.

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