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Protein Kinase C-ζ and Protein Kinase B Regulate Distinct Steps of Insulin Endocytosis and Intracellular Sorting*

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We have investigated the molecular mechanisms regulating insulin internalization and intracellular sorting. Insulin internalization was decreased by 50% upon incubation of the cells with the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002. PI3K inhibition also reduced insulin degradation and intact insulin release by 50 and 75%, respectively. Insulin internalization was reduced by antisense inhibition of protein kinase C-ζ (PKCζ) expression and by overexpression of a dominant negative PKCζ mutant (DN-PKCζ). Conversely, overexpression of PKCζ increased insulin internalization as a function of the PKCζ levels achieved in the cells. Expression of wild-type protein kinase B (PKB)-α or of a constitutively active form (myr-PKB) did not significantly alter insulin internalization and degradation but produced a 100% increase of intact insulin release. Inhibition of PKB by a dominant negative mutant (DN-PKB) or by the pharmacological inhibitor ML-9 reduced intact insulin release by 75% with no effect on internalization and degradation. In addition, overexpression of Rab5 completely rescued the effect of PKCζ inhibition on insulin internalization but not that of PKB inhibition on intact insulin recycling. Indeed, PKCζ bound to and activated Rab5. Thus, PI3K controls different steps within the insulin endocytic itinerary. PKCζ appears to mediate the PI3K effect on insulin internalization in a Rab5-dependent manner, whereas PKB directs intracellular sorting toward intact insulin release.

Insulin binding is followed by tyrosine phosphorylation of insulin receptor (IR)[1] and of its intracellular substrates (1). Thereafter, insulin signaling impinges on at least three major enzymatic systems, the Ras/extracellular signal-regulated kinases, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB), and the protein kinase C (PKC) signal transduction pathways. These systems contribute to insulin regulation of cell metabolism, differentiation, and growth (2).

In addition, hormone-bound IR is rapidly removed from the cell surface and internalized within clathrin-coated vesicles (3). Internalized insulin is then either degraded or released intact in the extracellular medium (4, 5). IR endocytosis is necessary for insulin clearance from the circulation (6, 7), down-regulation of surface binding sites (8), and transduction of specific biological effects (9–11). Defective insulin internalization may also cause insulin resistance in animal models (7). It is now clear that endocytosis of insulin-IR complexes is triggered by receptor tyrosine kinase activation (12, 13). Receptor autophosphorylation on tyrosine residues allows the migration of IR toward coated pits (14), but it may not be sufficient for inducing internalization (15, 16). Nevertheless, the molecular mechanisms, downstream receptor kinase activation, which control the internalization and the subsequent intracellular itinerary, have not been elucidated.

PI3K is necessary for the internalization of several growth factor receptors (17–19). PI3K and its downstream effectors have been implicated in the trafficking of endocytic and exocytic vesicles in yeast and mammalian cells (20, 21). Atypical PKC isoforms, such as PKCζ and PKCλ, which need phosphoinositides generated by the PI3K for their catalytic functions, may regulate vesicle trafficking by modulating cytoskeletal re-organization via Rho and Rac small GTPases (22, 23). Other small GTPases controlling vesicle traffic, such as Rab proteins, can be modulated by growth factors (24) and redistributed intracellularly following receptor tyrosine kinase activation (25). It has also been described recently (26) that Rab5 regulates epidermal growth factor receptor internalization upon engagement by specific intracellular substrates.

In this paper, we have attempted to elucidate the role of major signaling systems in regulating the insulin endocytic itinerary. We show that PI3K inhibitors reduce insulin internalization and alter intracellular sorting. We also present evidence, for the first time, that the PI3K effect on internalization occurs via PKCζ activation, whereas PKB facilitates the release of intact insulin outside the cell. Finally, we show that Rab5 is involved in early events of insulin endocytosis, possibly downstream of PKCζ, but it does not interfere with insulin intracellular sorting.

EXPERIMENTAL PROCEDURES

Materials—Media, sera, antibiotics for cell culture, the LipofectAMINE reagent, and rabbit polyclonal antibodies toward the specific PKC isoforms were from Invitrogen. The PKC assay system was from Promega (Madison, WI). Phosphorothioate PKCδ and PKCζ antisense and scrambled control oligonucleotides have been reported pre-

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[1] The abbreviations used are: IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKB, protein kinase B; DN, dominant negative; wt, wild type; EGF, epidermal growth factor; EGFR, EGF receptor.

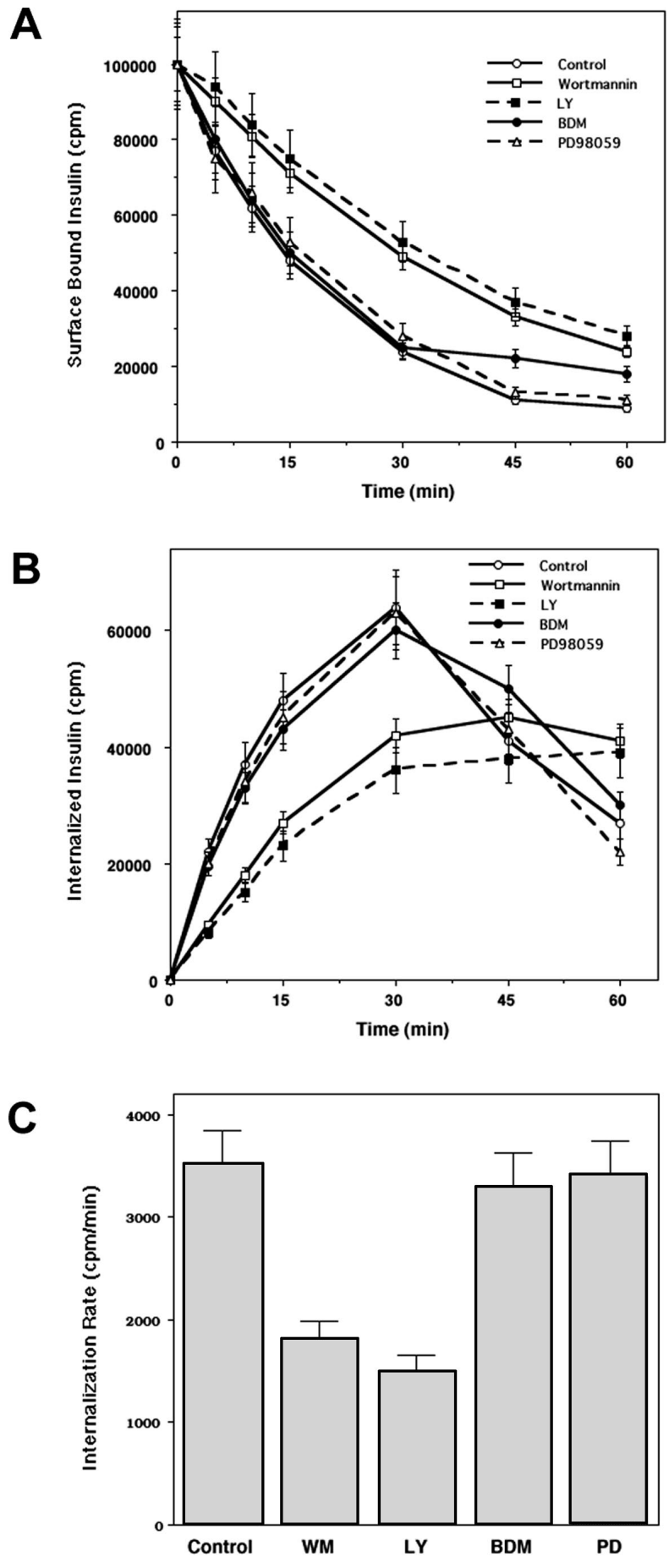

FIG. 1. **Effect of kinase inhibitors on insulin binding and internalization.** 3T3-hIR cells were treated 30 min with 50 nM wortmannin, 15 min with 100 μM LY294002 (*LY*), 30 min with 100 nM bisindolylmaleimide (*BDM*), and 60 min with 50 μM PD98059 and then incubated for 16 h at 4 °C with labeled insulin (^{125}I-insulin). Cells were washed with ice-cold saline solution and further incubated at 37 °C for the indicated times in the absence or in presence of the above-mentioned inhibitors. Surface insulin binding (*A*) and internalization (*B*) were measured as described under "Experimental Procedures." Each *data point* represents the mean ± S.D. of triplicate determinations of at least four independent experiments. Differences of the values measured in presence of wortmannin, LY294002, and bisindolylmaleimide at 5, 10, 15, 30, 45, and 60 min were significantly different ($p < 0.001$), compared with those in control cells. Internalization rate (*C*) was determined as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations of at least four independent experiments. Internalization rates measured in wortmannin (*WM*)- and LY294002 (*LY*)-treated cells were different from those in control cells. *PD*, PD98059. Based on *t* test analysis, differences were significant at $p < 0.001$.

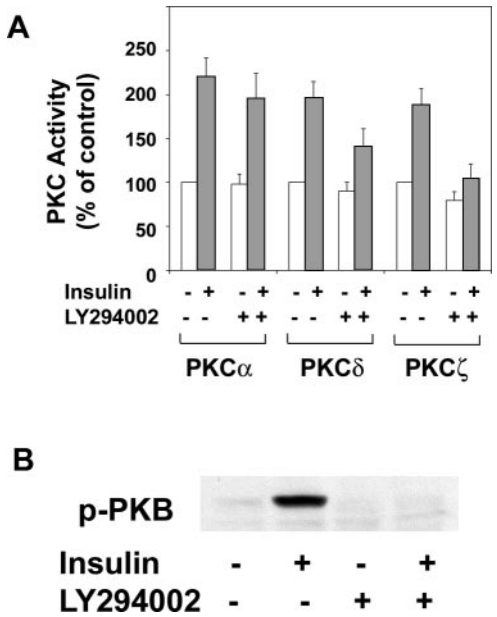

FIG. 2. **Effect of PI3K inhibitors on PKC and PKB activation.** 3T3-hIR cells were preincubated for 15 min with 100 μM LY294002 and further stimulated with 100 nM insulin for 30, 15, and 5 min, respectively, for determination of PKCα, PKCδ, and PKCζ activities, as indicated. Cell lysates were immunoprecipitated with anti-PKCα, anti-PKCδ, or anti-PKCζ antibodies, and PKC activity was measured as phosphorylation of specific substrates as indicated under "Experimental Procedures" (*A*). *Bars* represent the mean ± S.D. of triplicate determinations of at least four independent experiments. Based on *t* test analysis, values of PKCδ and PKCζ activity obtained in LY294002-treated cells were significantly different compared with those in control cells ($p < 0.005$ and $p < 0.001$, respectively). Alternatively, cell lysates were separated by SDS-PAGE and analyzed by Western blot with anti-phospho-PKB antibodies (*B*). The autoradiograph shown is representative of three independent experiments.

viously (27, 28) and were synthesized by PRIMM (Milan, Italy). PKCδ and PKCζ wild-type and dominant negative constructs were the generous gifts of Dr. M. S. Marber (St. Thomas Hospital, London, UK) and Dr. S. Gutkind (NCI, National Institutes of Health, Bethesda, MD), respectively; PKBα/Akt1 and myr-PKB/Akt constructs were provided by Dr. A. Bellacosa (29). Rab5-containing plasmids and Rab5 antibodies have been provided by Dr. C. Bucci (30). NIH-3T3 cells overexpressing EGFR have been kindly provided by Dr. L. Beguinot. Protein electrophoresis reagents were purchased from Bio-Rad and Western blotting, ECL reagent, and ^{125}I-insulin from Amersham Biosciences. All other chemicals were from Sigma.

Cell Culture and Transfection—3T3-hIR and 3T3-EGFR cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 2% L-glutamine in a humidified CO_2 incubator as described previously (31). The SV40-immortalized mouse hepatocytes have been described previously (32). Transient transfection of the PKC isoforms plasmid constructs, the phosphorothioate oligonucleotides, and the PKB and Rab5 constructs was performed by the LipofectAMINE (Invitrogen) method according to the manufacturer's instructions. For these studies, 50–80% confluent cells were washed twice with Opti-MEM (Invitrogen) and incubated for 8 h with 5 μg of plasmid constructs or with 12 μg of oligonucleotides and 45–60 μl of LipofectAMINE reagent. The medium was then replaced with Dulbecco's modified Eagle's medium with 10% fetal calf serum, and cells were further incubated for 15 h before being assayed.

Western Blot Analysis and Immunoprecipitation Procedure—Cells were solubilized in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) for 2 h at 4 °C. Lysates were centrifuged at 5,000 × *g* for 20 min and assayed as described in Miele *et al.* (33). Briefly, solubilized proteins were separated by SDS-PAGE and transferred to 0.45-μm Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with the primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions. Immunoprecipitation experiments were performed as described previously (34).

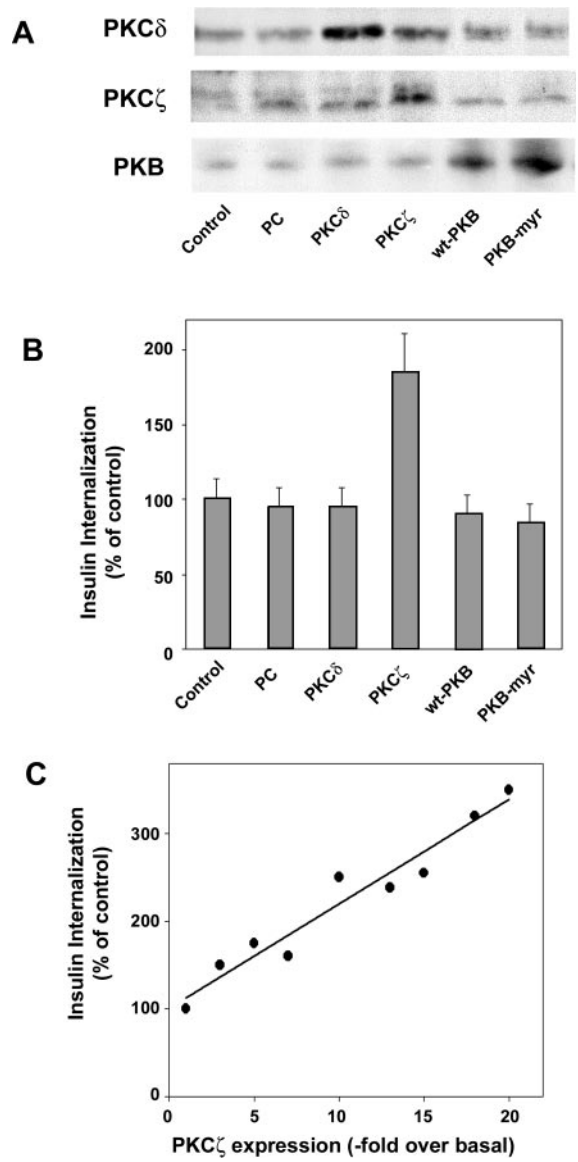

FIG. 3. **Effect of PKCζ overexpression on insulin internalization.** 3T3-hIR cells were transiently transfected with a control plasmid (*PC*), PKCδ, PKCζ, PKB/Akt, or with a constitutively active myr-PKB/Akt mutant. Cells were then incubated for 16 h at 4 °C with ^{125}I-insulin, washed with ice-cold saline solution, and further incubated at 37 °C for 5 min to allow insulin internalization. Aliquots of the cell lysates were separated by SDS-PAGE and analyzed by Western blot with specific antibodies, as indicated (*A*). The autoradiographs shown are representative of four independent experiments. Insulin internalization was measured as acid-resistant radioactivity (*B*) as described in legend to Fig. 1 and under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations of four independent experiments. Based on *t* test analysis, insulin internalization measured in cells overexpressing PKCζ was significantly different compared with that in control cells ($p < 0.001$). *C,* insulin internalization was measured in 3T3-hIR cells transiently transfected with 0.5 μg/ml up to 5 μg/ml of PKCζ cDNA. Aliquots of the cell lysates were probed by immunoblot with PKCζ antibodies. Quantification of PKCζ expression levels was achieved by laser densitometer analysis.

Protein Kinase C Activity—Individual PKC isoforms were immunoprecipitated using specific antibodies and assayed as described previously (34). Cells were solubilized in 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin (extraction buffer), and then clarified by centrifugation at 5,000 × *g* for 20 min. Supernatants were further centrifuged at 60,000 × *g* for 2 h, and pellets were solubilized with extraction buffer containing 0.5% Triton X-100. Proteins were then immunoprecipitated with isoform-specific antibodies and supplemented with lipid activators (10 mM phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidylserine, and 4 mg/ml diolein, final

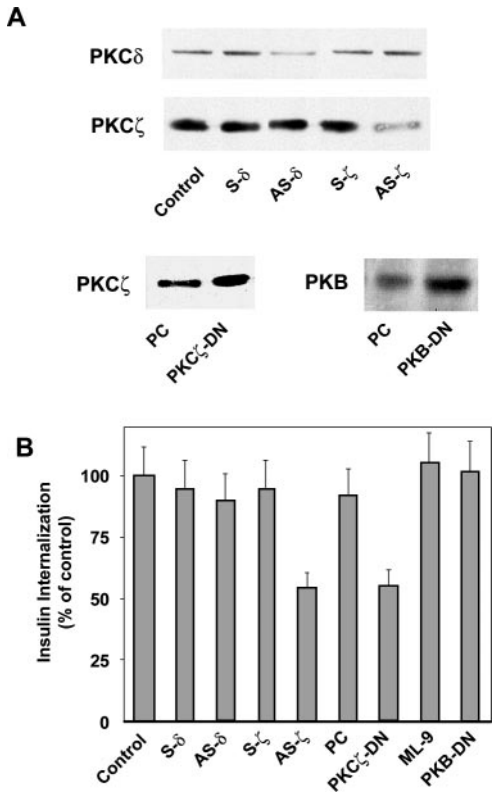

FIG. 4. **Effect of PKCζ inhibition on insulin internalization.** 3T3-hIR cells were transiently transfected with PKCδ antisense oligonucleotides (*AS-δ*), PKCζ antisense oligonucleotides (*AS-ζ*), with control oligonucleotides (*S-δ*; *S-ζ*), with an empty vector (*PC*) and with dominant negative PKCζ (*PKCζ-DN*) or PKB (*PKB-DN*). Cell lysates were separated by SDS-PAGE and analyzed by Western blot with specific antibodies (*A*). The autoradiographs shown are representative of five (for antisense and PKB-DN) and four (for PKCζ-DN) independent experiments. 100 μM ML-9 was added where indicated for 10 min at 37 °C before binding and for a further 15 min at 37 °C during the internalization period. Insulin internalization (*B*) has been measured as described previously. *Bars* represent the mean ± S.D. of triplicate determinations of at least three independent experiments. Insulin internalization levels were significantly different in AS-ζ and PKCζ-DN cells compared with those in control cells ($p < 0.001$).

concentrations). Phosphorylation reactions were initiated by addition of the substrate solution (20 μM ATP, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 4 mM Tris, pH 7.5, and 10 μCi/ml (3,000 Ci/mmol) [γ-^{32}P]ATP, final concentrations) and of specific substrates. An acetylated peptide comprising amino acids 4–14 of myelin basic protein was used as PKCα substrate, the H-Arg-Phe-Ala-Val-Arg-Asp-Met-Arg-Gln-Thr-Val-Ala-Val-Gly-Val-Ile-Lys-Ala-Val-Asp-Lys-Lys-OH peptide as PKCδ substrate, and the pseudosubstrate region of PKCε for PKCζ. The reaction mixtures were further incubated for 10 min at room temperature, rapidly cooled on ice, and spotted on phosphocellulose discs. Disk bound radioactivity was quantified by liquid scintillation counting.

Insulin Internalization and Degradation—Insulin internalization was determined as described previously (15). Cells were washed three times with ice-cold KRP solution (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 100 mM Na_2HPO_4), pH 7.8, and incubated at 4 °C for 16 h with 30 pM ^{125}I-insulin diluted in binding buffer (KRP, pH 7.8, containing 2% bovine serum albumin and 1.3 mM $CaCl_2$). Cells were then rinsed with KRP, pH 7.8, in order to remove the unbound ligand and further incubated at 37 °C in binding buffer to allow internalization. After the indicated times, the cells were washed twice with either KRP buffer, pH 7.8 (for determination of total cell associated radioactivity), or with KRP buffer, pH 4.0 (acid buffer, for enabling dissociation of surface-bound insulin). The cells were then lysed with 1 N NaOH. The fraction of total cell radioactivity dissociated by the acid washes was taken as representative of surface-bound insulin. The acid-resistant radioactivity in the lysates was taken as representative of internalized insulin. Degraded and intact ^{125}I-insulin in the incubation media was determined by 10% trichloroacetic acid precipitation as described previously (15).

Rab5 GTP Loading—For the GTP overlay, the cells were solubilized in lysis buffer, precipitated with Rab5 antibodies, and separated by SDS-PAGE. The gel was then soaked in 50 mM Tris-HCl, pH 7.5, 20% glycerol for 1 h and transferred onto nitrocellulose filters as in Formisano *et al.* (35). After the transfer, the filters were rinsed twice for 10 min in 50 mM NaH_2PO_4, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 0.3% Tween 20, pH 7.5, and incubated with 2 μCi/ml [α-^{32}P]GTP for 2 h. After six 5-min washes, the filters were autoradiographed. Quantitation was achieved by laser densitometry.

RESULTS

Role of PI3K in Insulin Internalization—Prior to studying ^{125}I-insulin binding and internalization, 3T3-hIR cells were exposed for 30 min to wortmannin (50 nM) or LY294002 (100 μM), to inhibit PI3K activity, and to PD98059 (50 μM) and bisindolylmaleimide (100 nM), to inhibit mitogen-activated protein kinase and PKC, respectively (Fig. 1). When evaluated at 4 °C, insulin binding levels were not affected by any of these treatments. The incubation of the cells at 37 °C led to a time-dependent reduction of surface binding. However, wortmannin- or LY294002-treated cells displayed a less marked loss of surface ^{125}I-insulin compared with either control cells or to cells exposed to PD98059 and bisindolylmaleimide (Fig. 1*A*). Both untreated cells and cells exposed to PD98059 and bisindolylmaleimide internalized up to 60% of bound insulin in 30 min. Moreover, the intracellular radioactivity decreased upon further incubation (Fig. 1*B*). Wortmannin and LY294002 treatment of the cells caused a reduction of insulin internalization (only 35–40% of bound insulin was internalized in 30 min). This reduction was no longer detectable after 45 min, and after 60 min, a larger amount of labeled insulin accumulated intracellularly, compared with the control and PD98059- or bisindolylmaleimide-treated cells. Consistent with slower kinetics, the insulin internalization rate was reduced by 50% upon preexposure of the cells to either wortmannin or LY294002 but was not affected by PD98059 and bisindolylmaleimide (Fig. 1*C*). Thus, inhibitors of PI3K impaired insulin internalization in 3T3-hIR cells.

Role of PI3K in Activation of Downstream Kinases—In order to investigate PI3K-dependent mechanisms involved in insulin internalization, the activities of protein kinase B (PKB)/Akt and protein kinase C (PKC)-α, -δ, and -ζ were assessed upon the incubation of 3T3-hIR cells with LY294002. Treatment of the cells with LY294002 reduced insulin-stimulated activation of PKCα, and -δ by only 15% (not statistically significant) and 60%, respectively. In contrast, a very marked decrease was observed with PKCζ activation (90% inhibition of insulin-stimulated activity) (Fig. 2*A*). Moreover, PKB/Akt phosphorylation was strongly induced by insulin in control cells and almost completely abolished in the presence of LY294002 (Fig. 2*B*). Similarly, increases in PKB/Akt activity were prevented when PI3K was inhibited (data not shown). Thus, it appears that PI3K inhibition causes a marked impairment of the activation of PKCζ and PKB/Akt by insulin.

Role of PKCζ in Insulin Internalization—Next, we addressed the role of PKCs and PKB/Akt in insulin endocytosis. Overexpression of PKCδ did not significantly alter insulin internalization (Fig. 3). In addition, transient transfection either of wt-PKB/Akt1 or of a constitutively active mutant (myr-PKB) showed no effect on insulin internalization. However, PKCζ overexpression in 3T3-hIR cells, at levels comparable with those obtained for PKCδ (Fig. 3*A*), increased insulin internalization by 100% over control (Fig. 3*B*). The increased internalization positively correlated to the amount of transfected PKCζ cDNA. 50, 150, and 230% increases were achieved by 3-, 10-, and 20-fold overexpression of PKCζ, respectively (Fig. 3*C*).

To analyze whether endogenous PKCζ was relevant for insulin internalization, 3T3-hIR cells were transfected with ei-

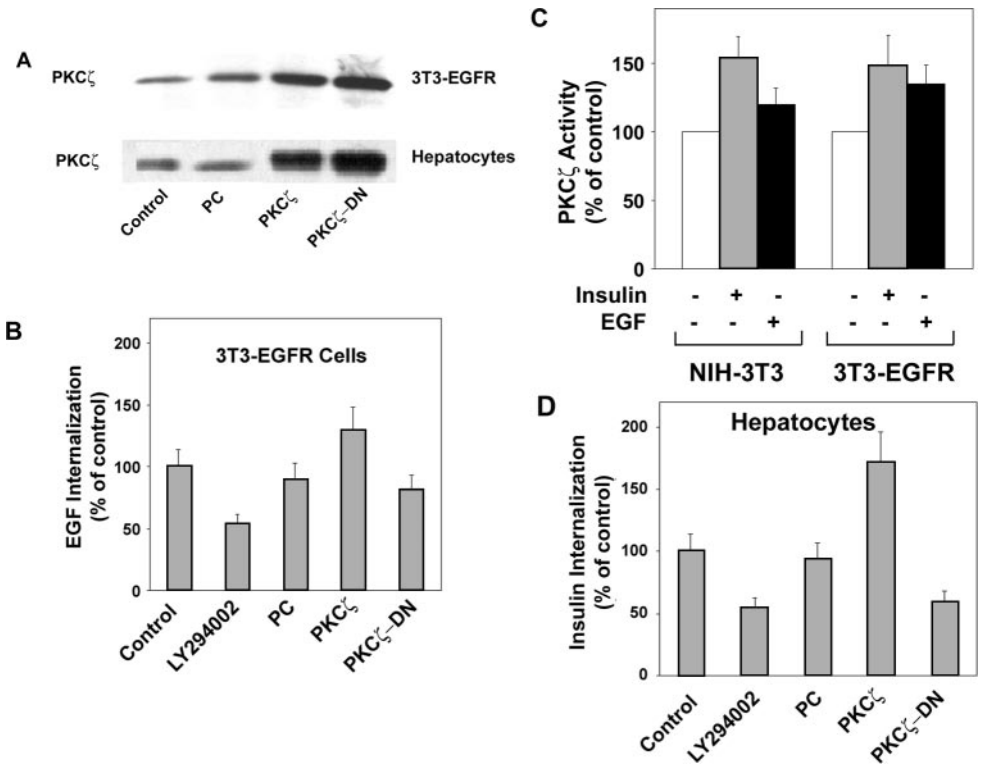

FIG. 5. **Specificity of PKCζ effect on insulin internalization.** 3T3-EGFR cells or SV40 mouse hepatocytes were treated for 15 min with 100 μM LY294002 or transiently transfected with control plasmid (*PC*), wild-type PKCζ, and with PKCζ-DN. Then cell lysates were separated by SDS-PAGE and analyzed by Western blot with anti-PKCζ antibodies (*A*). The autoradiograph shown is representative of four independent experiments for EGFR cells and three experiments for the hepatocytes. Alternatively, cells were incubated for 16 h at 4 °C with ^{125}I-EGF, washed with ice-cold saline solution, and further incubated at 37 °C for 10 min. EGF internalization was measured as acid-resistant radioactivity. Based on *t* test analysis, EGF internalization measured in LY294002-treated cells was significantly different compared with that in control cells ($p < 0.005$). In cells overexpressing PKCζ, EGF internalization was different at $p < 0.01$ compared with that in PC cells (*B*). 3T3-hIR and 3T3-EGFR cells were stimulated for 5 min with 100 nM insulin and with 100 nM EGF, respectively. Cell lysates were immunoprecipitated with anti-PKCζ antibodies, and PKCζ activity was measured as described under "Experimental Procedures" (*C*). Insulin internalization in liver hepatocytes has been measured as described under "Experimental Procedures" (*D*). *Bars* in *B–D* represent the mean ± S.D. of triplicate determinations of at least three independent experiments. Based on *t* test analysis, insulin internalization measured in LY294002-treated cells was significantly different compared with that in control cells ($p < 0.001$). In cells overexpressing PKCζ and PKCζ-DN, internalization was significantly different compared with that in PC cells ($p < 0.001$ and $p < 0.005$, respectively).

ther PKCζ antisense oligonucleotides (PKCζ-AS) or with a dominant negative PKCζ mutant (DN-PKCζ) (Fig. 4*A*). Both procedures caused >70% inhibition of basal and insulin-induced PKCζ activity (data not shown). Insulin internalization was not affected by control oligonucleotides (PKCζ-S) and by transfection of a control vector (Fig. 4*B*). In contrast, in cells treated with PKCζ-AS or DN-PKCζ, insulin internalization was reduced by 50% compared with controls (Fig. 4*B*). PKCδ antisense (PKCδ-AS) and control scrambled oligonucleotides (PKCδ-S) did not significantly alter insulin internalization in 3T3-hIR cells. In addition, inhibition of PKB by DN-PKB or by treatment of the cells with ML9, an agent that blocks PKB with no effect on PI3K activity (36), did not reduce insulin internalization (Fig. 4).

Role of PI3K and PKCζ in EGF Internalization—In order to evaluate the specific role of PKCζ in insulin internalization, epidermal growth factor (EGF) endocytosis was studied in NIH-3T3 cells overexpressing the EGF receptor (3T3-EGFR). Clones of 3T3-EGFR cells were selected for having a number of EGF-binding sites (~72,000 per cell) comparable with those for insulin in the 3T3-hIR cells (~65,000 per cell). Ligand internalization was assessed in the absence or in the presence of LY294002, or following transfection of wild type and DN-PKCζ (Fig. 5*A*). As expected, inhibition of PI3K resulted in 50% decreased EGF internalization (Fig. 5*B*). However, transfection of wt-PKCζ, although robustly increasing insulin internalization in 3T3-hIR, produced only a 30% increase in EGF internalization. Conversely, when DN-PKCζ was overexpressed, EGF internalization was only slightly reduced in 3T3-EGFR cells (20% decrease; not statistically significant).

Consistent with a minor role of PKCζ in EGF internalization, treatment of 3T3-EGFR cells with EGF led to a modest 30% increase of PKCζ activity (Fig. 5*C*). Also, in untransfected NIH-3T3 cells, insulin, but not EGF, elicited a significant PKCζ activation (Fig. 5*C*).

Role of PI3K and PKCζ in Liver Cells—To study the cell-specific role of PKCζ in control of insulin internalization, SV40-transformed murine hepatocytes (32) were incubated with LY294002 or transfected with wild type or DN-PKCζ (Fig. 5*A*). Inhibition of PI3K or PKCζ was accompanied by a 45 and 40% reduction of insulin internalization, respectively (Fig. 5*D*). Conversely, overexpression of PKCζ led to a 70% increase of insulin internalization, indicating that PKCζ was also involved in insulin internalization in liver cells.

Regulation of Insulin Intracellular Sorting by PI3K—After internalization, insulin may be degraded and degradation products released extracellularly. Alternatively, intact insulin may be released into the extracellular medium (4, 5). LY294002 treatment of 3T3-hIR cells caused a 55% inhibition of total extracellular release (Fig. 6*A*). A 35% inhibition of insulin release was also observed when the cells were treated with ML9 (Fig. 6*A*). However, LY294002 inhibited insulin degradation by 50% and intact insulin release by >70% (Fig. 6, *B* and *C*). Most interesting, ML9 inhibited intact insulin release by

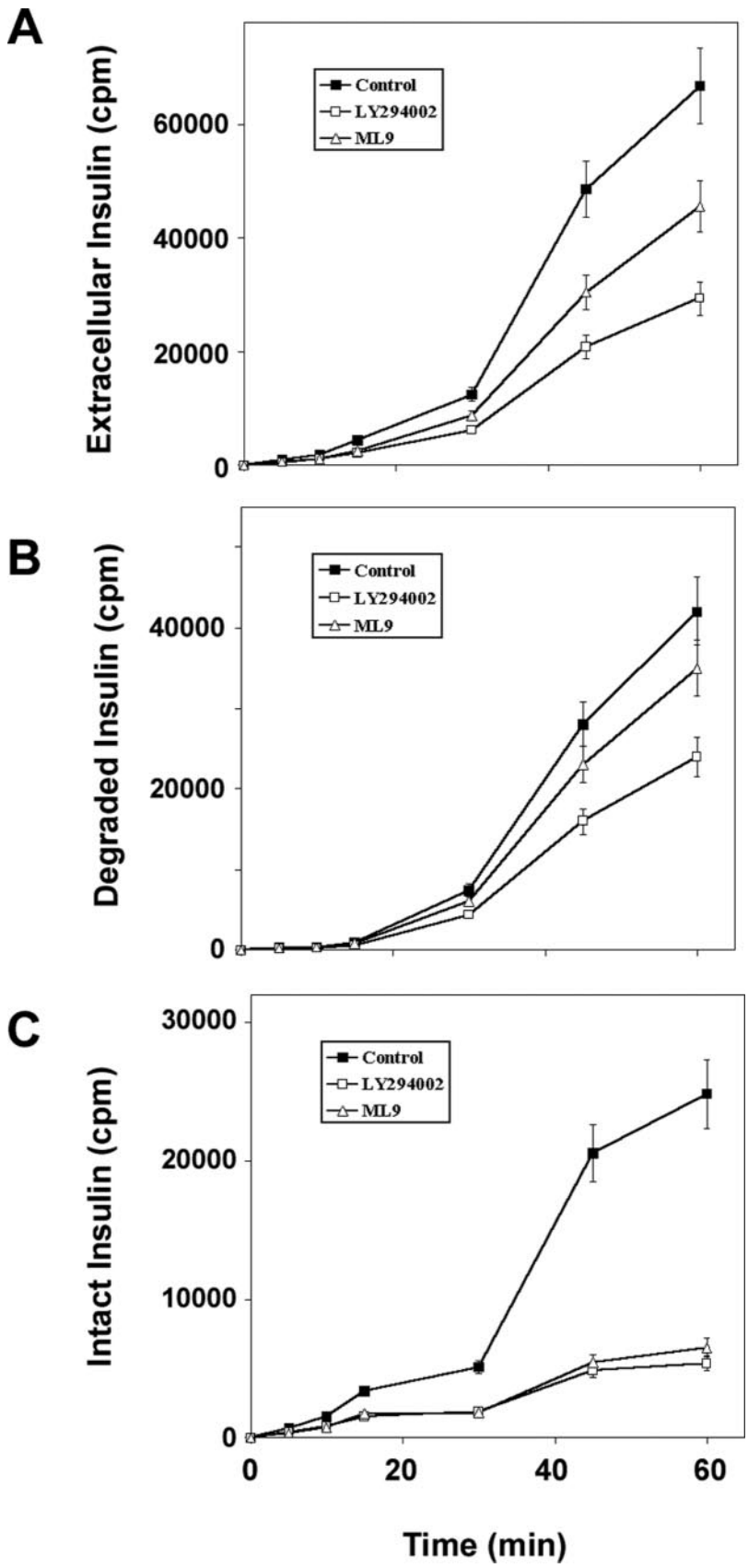

FIG. 6. **Effect of PKB inhibition on insulin release.** 3T3-hIR cells were treated for 15 min with 100 μM LY294002 or with 100 μM ML-9 before insulin binding. Cells were then shifted to 37 °C for the indicated times, in the absence or in the presence of LY294002 or ML-9, to measure total extracellular ^{125}I-insulin (*A*), degraded (*B*), and intact insulin (*C*), as described under "Experimental Procedures." Each *data point* represents the mean ± S.D. of triplicate determinations of five independent experiments. Values of total extracellular insulin (*A*) measured in the presence of LY294002 and ML-9 at 30, 45, and 60 min were significantly different ($p < 0.005$ at 30 min; $p < 0.001$ at 45 and 60 min), compared with those in control cells. Also, the differences of extracellular insulin between LY294002 and ML-9 treatment were significant at 45 and 60 min ($p < 0.005$). For degraded insulin (*B*), in LY294002-treated cells, differences were significant at 45 and 60 min, compared with control and ML-9 treated cells ($p < 0.005$). For intact insulin (*C*) values in LY294002- and ML-9-treated cells were significantly different from those in control cells ($p < 0.001$).

~70% and had no significant effect on degradation (Fig. 7, *B* and *C*). We have therefore investigated whether PKB has a role in regulation of insulin intracellular sorting. To this end, total insulin release, insulin degradation, and intact insulin release were studied in cells transfected with either wt-PKBα, myr-PKB, PKCδ, or PKCζ. Total insulin release was increased by 80, 120, and 150% by PKCζ, wt-PKB, or myr-PKB overexpression, respectively. In addition, total insulin release was inhibited by about 50% when dominant negative mutants of either PKCζ or PKB were transfected in 3T3-hIR cells (Fig. 7*A*).

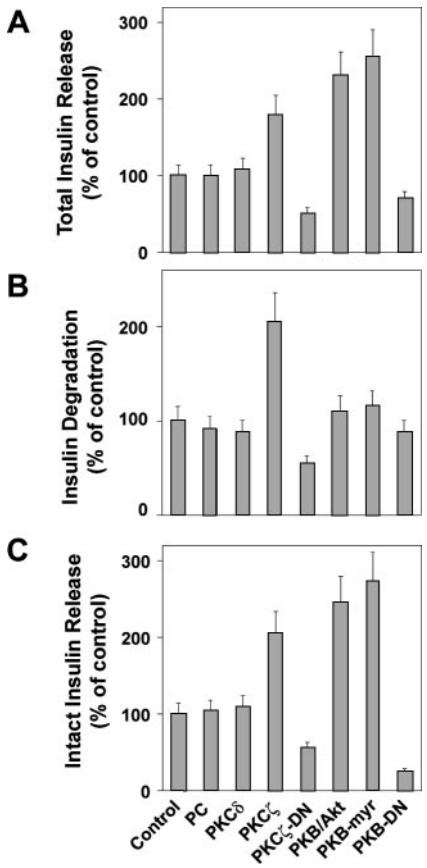

FIG. 7. **Effect of PKB and PKCζ on insulin degradation and release.** 3T3-hIR cells were transiently transfected with control plasmid (*PC*), PKCδ, PKCζ, a dominant negative PKCζ mutant (*PKCζ-DN*), PKB/Akt, a constitutively active (*myr-PKB*) or a dominant negative mutant (*PKB-DN*) of PKB/Akt cDNAs as indicated. Total insulin release (*A*), degraded insulin (*B*), and intact insulin (*C*) were measured upon 1 h at 37 °C, as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations of five independent experiments. Based on *t* test analysis, for total insulin release (*A*) and intact insulin release (*B*), values measured in cells overexpressing PKCζ, PKCζ-DN, PKB/Akt, PKB-myr, and PKB-DN were significantly different compared with those in control and PC cells ($p < 0.001$). For insulin degradation, values measured in cells overexpressing PKCζ and PKCζ-DN were significantly different compared with those in control and PC cells ($p < 0.001$).

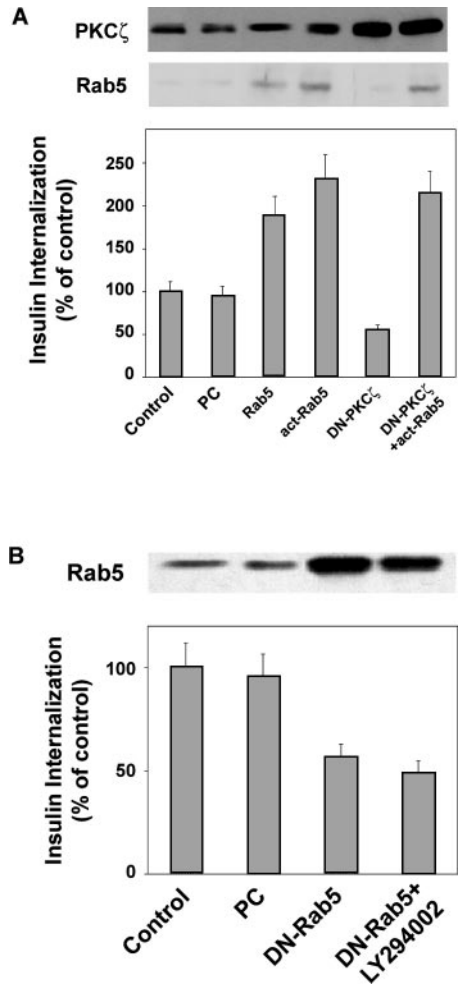

FIG. 8. **Effect of Rab5 on insulin internalization.** 3T3-hIR cells were transiently transfected with control plasmid (*PC*), wild-type Rab5 (*Rab5*), a constitutively active mutant of Rab5 (*act-Rab5*), dominant negative PKCζ (*PKCζ-DN*), or with both act-Rab5 and PKCζ-DN (*A*). Alternatively, a control plasmid (*PC*) or a dominant negative Rab5 construct were transfected in 3T3-hIR cells (*B*). Then insulin internalization was measured as described under "Experimental Procedures." The autoradiographs shown are representative of four independent experiments. *Bars* represent the mean ± S.D. of triplicate determinations of four independent experiments. Based on *t* test analysis, insulin internalization measured in cells overexpressing Rab5, act-Rab5, and DN-PKCζ and co-transfected with DN-PKCζ and act-Rab5 was significantly different compared with that in control and PC cells ($p < 0.001$). Also, the values obtained in cells overexpressing DN-Rab5 were significantly different from those in control and PC cells ($p < 0.005$).

However, PKCζ induced parallel increases in both insulin degradation and intact insulin release, whereas overexpression of either wt- or myr-PKB selectively increased the release of intact insulin by >100%, with a minor effect on degradation (Fig. 7, *B* and *C*). Again, no change was detected when PKCδ was overexpressed. Thus, when normalized for the amount of internalized insulin, only PKB, but not PKCζ, increased the release of intact insulin. Similar results were also obtained in SV40 immortalized hepatocytes (data not shown).

Role of Rab5 in Insulin Internalization and Sorting—To further investigate the molecular mechanisms regulating insulin internalization and release, 3T3-hIR cells were transiently transfected with Rab5 cDNAs, either alone or in combination with dominant negative PKCζ. Insulin internalization was about 100 and 150% increased upon overexpression of Rab5 and of a constitutively active Rab5 mutant (act-Rab5), respectively (Fig. 8*A*). In addition, a dominant negative Rab5 (DN-Rab5) construct reduced insulin internalization by 50% in the 3T3-hIR cells (Fig. 8*B*). Treatment of DN-Rab5 transfected cells with LY294002 did not induce any further decrease. Also, both insulin degradation and intact insulin release were increased in Rab5 and act-Rab5 transfected cells compared with the control cells (Fig. 9). Most interesting, in cells co-transfected with act-Rab5 and DN-PKCζ, insulin internalization (Fig. 8*A*), degradation, and intact insulin release (Fig. 9) were also increased, indicating that Rab5 activation bypassed the effect of inhibiting PKCζ. In contrast there was no Rab5 rescue of intact insulin release when PKB was inhibited (Fig. 9*B*).

Next, we performed co-precipitation experiments in 3T3-hIR cells transiently co-transfected with Rab5 and PKCζ, or with the dominant negative Rab5 and PKCζ mutants. Most interesting, wt-PKCζ co-precipitated with either wt- or DN-Rab5, whereas DN-PKCζ failed to interact with Rab5, indicating that PKCζ activity was necessary for this interaction (Fig. 10*A*).

To evaluate the effect of PKCζ on Rab5 function, we measured Rab5 GTP loading in cells co-transfected with Rab5 and either wt- or DN-PKCζ. PKCζ overexpression caused a 50% increase of GTP binding to Rab5, whereas the dominant negative mutant caused a 30% decrease when compared with control cells (Fig. 10*B*).

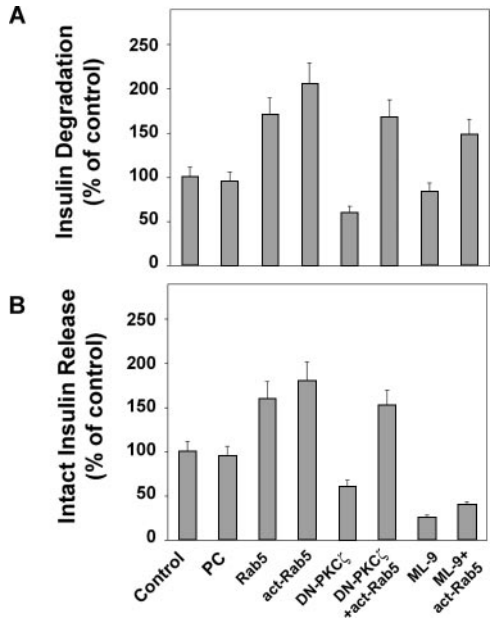

FIG. 9. **Effect of Rab5 on insulin degradation and release.** 3T3-hIR cells were transiently transfected with control plasmid (*PC*), wild-type Rab5 (*Rab5*), a constitutively active mutant of Rab5 (*act-Rab5*), dominant negative PKCζ (*PKCζ-DN*) or with both act-Rab5 and PKCζ-DN. 100 μM ML-9 was added 15 min before insulin binding and for further incubation at 37 °C for measuring degradation and intact insulin release, where indicated. Then insulin degradation (*A*) and intact insulin release (*B*) were measured as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations of four independent experiments. Based on *t* test analysis, insulin degradation and intact insulin release measured in cells overexpressing Rab5, act-Rab5, and DN-PKCζ and co-transfected with DN-PKCζ and act-Rab5 were significantly different compared with those in control and PC cells ($p < 0.005$). Insulin degradation in act-Rab5-overexpressing cells, treated with ML-9, was significantly different from that in control and PC cells ($p < 0.005$). Also intact insulin release was significantly different in both untransfected and act-Rab5-transfected cells treated with ML-9 compared with that in untreated control and PC cells ($p < 0.005$).

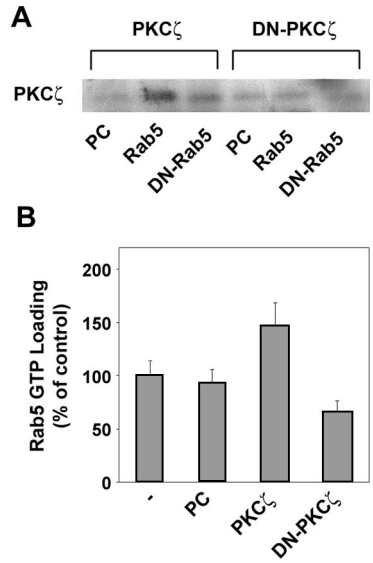

FIG. 10. **PKCζ effect on Rab5 function.** 3T3-hIR cells were co-transfected with the wild-type or dominant negative mutants of PKCζ and Rab5 as indicated. The cell lysates were immunoprecipitated with hemagglutinin peptide epitope antibodies and blotted with PKCζ antibodies (*A*). The Rab GTP loading was estimated by the GTP overlay assay as described under "Experimental Procedures." Overlay filters were autoradiographed, and GTP loading was quantitated by densitometry (*B*). *Bars* represent the means ± S.D. of duplicate determinations in three independent experiments. Based on *t* test analysis, values in cells overexpressing PKCζ and DN-PKCζ were different compared with those in control and PC cells ($p < 0.005$).

DISCUSSION

In this paper, we have investigated the involvement of major signaling pathways in insulin endocytosis. Although IR activation is required for internalization of insulin-IR complexes (12–14), the role of the molecules downstream from the receptor kinase in the control of the endocytic process is largely unknown. We have addressed this issue by studying NIH-3T3 cells overexpressing human insulin receptor at relatively low levels in order to preserve the normal intracellular routing. We have also investigated the regulation of insulin endocytosis in a liver-derived murine cell line. Insulin signal transduction pathways and endocytosis have been extensively studied in these cellular systems (11, 15, 32–34, 37), which therefore represent attractive models to clarify the regulatory processes.

We show that PI3K is required for insulin internalization and further intracellular processing. The role of PI3K in the endocytosis of other tyrosine kinase receptors has been shown previously (17–19), although the mechanisms remain unclear. Overexpression and antisense studies have now defined a specific role for PKCζ in insulin internalization. In contrast, EGF internalization, which is also blunted by PI3K inhibitors, is not affected by modulation of PKCζ expression and/or function. Similarly, transferrin internalization is unchanged whether PKCζ is overexpressed or inhibited (data not shown), indicating that PKCζ may play a selective role in regulating the trafficking of insulin vesicles.

Thus, common routes used by tyrosine kinase receptors might diverge at critical steps. Indeed, in IR overexpressing NIH-3T3 cells, insulin robustly elicits PKCζ activation and receptor co-precipitation (34), whereas PKCζ activation was barely detectable in the NIH-3T3 cells overexpressing the EGFR. Also, in untransfected cells insulin, and not EGF, stimulated PKCζ activation. PI3K activation represents a pivotal common step in insulin and EGF signaling systems, whereas PKCζ appears to play a specific role in insulin signaling and endocytic itinerary.

PKCζ is involved in insulin regulation of glucose uptake (38–40), but the mechanisms responsible for the translocation of vesicles containing glucose transporters to the plasma membrane have not been completely elucidated. Recently, Braiman *et al.* (40) have presented evidence that PKCζ phosphorylates VAMP-2, an important component of trafficking vesicles. We and others (34, 40) have shown previously that insulin receptor recruits and activates PKCζ in a biphasic manner. It is possible that distinct subsets of PKCζ molecules control distinct signals (*i.e.* insulin internalization and GLUT translocation) in a time- and/or location-dependent fashion. Alternatively, the effect of PKCζ on IR internalization might generate signals which, in turn, regulate glucose metabolism. In support of the latter hypothesis, Ceresa and co-workers (9) have shown that inhibition of IR internalization suppresses insulin-stimulated glucose uptake. We have also reported that ligand-independent internalization of a mutant insulin receptor correlated with an increase of insulin-independent glucose uptake in NIH-3T3 and L6 cells (11, 15, 36). PKCδ has also been described to regulate both glucose uptake and IR internalization in primary cultured skeletal muscle cells (41, 42, 43), indicating that distinct PKC isoforms might play similar roles in different cell types. Here we show that PKCζ regulates insulin internalization and degradation in mouse hepatocytes, suggesting it may be involved in the control of insulin clearance from the circulation.

Several lines of evidence indicate that PKCζ regulation of insulin internalization is exerted, at least in part, through

Rab5. First, as shown in this paper, overexpression of Rab5 increases insulin internalization, in a similar manner to PKCζ. Second, a constitutively active Rab5 mutant bypasses the blockade of insulin internalization upon PKCζ inhibition. Third, activated PKCζ can interact with Rab5, suggesting a direct regulation of Rab5 function. For instance, Rab5 GTP loading can be modulated by PI3K (44) and is substantially increased in PKCζ-overexpressing cells.

Once internalized, insulin is sorted to either the degradative or the recycling compartment, in a cell type-specific fashion (45). It appears that the intracellular sorting is not affected by PKCζ. Previous studies indicated that cPKCs could be involved (35). Here we show that overexpression of PKBα selectively increased intact insulin release, independently of internalization and degradation in fibroblasts (Figs. 6 and 7) and in liver cells (data not shown). One might argue that, beside exerting parallel functions in regulation of glucose uptake and glycogen synthesis (46, 47), PKCζ and PKBα control different steps of the insulin endocytic itinerary. Distinct subsets of trafficking vesicles are likely involved in PKCζ and PKBα control of insulin endocytosis. Indeed, intact insulin release appears to be only indirectly regulated by Rab5. In this regard, overexpression of Rab5 is unable to rescue intact insulin release when PKB is inhibited, while completely bypassing the blockade on the release of both intact and degraded insulin when PKCζ is inhibited. In addition, PKB co-localizes with Rab4-containing vesicles (48, 49), suggesting a direct involvement in trafficking from the cell interior toward the plasma membrane.

Thus, PI3K controls different steps of insulin endocytic itinerary in murine fibroblasts and hepatocytes. PI3K-mediated effects on early internalization events are mediated by PKCζ in a Rab5-dependent manner, whereas subsequent targeting toward intact insulin release is controlled by PKB in a Rab5-independent manner.

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Protein Kinase C-ζ and Protein Kinase B Regulate Distinct Steps of Insulin Endocytosis and Intracellular Sorting

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