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Am J Physiol Cell Physiol 295:1602-1609, 2008. First published Oct 8, 2008;
doi:10.1152/ajpcell.00090.2008

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Signaling cascade of insulin-induced stimulation of L-dopa uptake in renal proximal tubule cells

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Submitted 15 February 2008; accepted in final form 2 October 2008

Carranza A, Musolino PL, Villar M, Nowicki S. Signaling cascade of insulin-induced stimulation of L-dopa uptake in renal proximal tubule cells. *Am J Physiol Cell Physiol* 295: C1602–C1609, 2008. First published October 8, 2008; doi:10.1152/ajpcell.00090.2008.—The inward L-dihydroxyphenylalanine (L-dopa) transport supplies renal proximal tubule cells (PTCs) with the precursor for dopamine synthesis. We have previously described insulin-induced stimulation of L-dopa uptake into PTCs. In the present paper we examined insulin-related signaling pathways involved in the increase of L-dopa transport into isolated rat PTCs. Insulin (50–500 μU/ml) increased L-dopa uptake by PTCs, reaching the maximal increment (60% over the control) at 200 μU/ml. At this concentration, insulin also increased insulin receptor tyrosine phosphorylation. Both effects were abrogated by the tyrosine kinase inhibitor genistein (5 μM). In line, inhibition of the protein tyrosine phosphatase by pervanadate (0.2–100 μM) caused a concentration-dependent increase in both the uptake of L-dopa (up to 400%) and protein tyrosine phosphorylation. A synergistic effect between pervanadate and insulin on L-dopa uptake was observed only when threshold (0.2 μM), but not maximal (5 μM), concentrations of pervanadate were assayed. Insulin-induced stimulation of L-dopa uptake was also abolished by inhibition of phosphatidylinositol 3-kinase (PI3K; 100 nM wortmannin, and 25 μM LY-294002) and protein kinase C (PKC; 1 μM RO-318220). Insulin-induced activation of PKC-ζ was confirmed in vitro by its translocation from the cytosol to the membrane fraction, and in vivo by immunohistochemistry studies. Insulin caused a wortmannin-sensitive increase in Akt/protein kinase B (Akt/PKB) phosphorylation and a dose-dependent translocation of Akt/PKB to the membrane fraction. Our findings suggest that insulin activates PKC-ζ, and Akt/PKB downstream of PI3K, and that these pathways contribute to the insulin-induced increase of L-dopa uptake into PTCs.

amino acid transport; second messengers; dopamine

INSULIN PRODUCES A RANGE of pleiotropic effects in target cells that include acute metabolic changes and longer-term growth-promoting effects. Both responses are initiated by the binding of the hormone to its cell surface receptor. The insulin receptor signals through a variety of intracellular cascades; one of the best studied pathways involves the receptor autophosphorylation on multiple tyrosine residues. This results in activation of the receptor kinase and tyrosine phosphorylation of a family of insulin receptor substrate (IRS) proteins. Tyrosine phosphorylation of IRS proteins activates phosphatidylinositol 3-kinase (PI3K) and induces activation of downstream signaling molecules such as Akt/protein kinase B (Akt/PKB) and atypical protein kinase C-ξ (PKC-ξ) (see review in Ref. 30).

Renal proximal tubule cells are endowed with a high density of insulin receptors (31). Insulin-receptor stimulation increases the sodium reabsorptive activity of major renal sodium transporters. Indeed, at the proximal tubule level, insulin stimulates the activity of the apical Na^+/H^+ exchanger (17), and the basolateral Na^+,K^+-ATPase (16). In a recent publication, Tiwari et al. (36) reviewed the regulation of the principal renal sodium transporters by insulin, and the antinatriuretic actions of insulin in humans and in animal models.

A counterregulatory mechanism between insulin and dopamine on tubular sodium reabsorption has been proposed. A considerable amount of evidence suggests that sodium retention associated with hyperinsulinemia could be attributed to a reduced D_1 dopamine receptor expression leading to a diminished D_1 receptor-mediated Na^+,K^+-ATPase inhibition (6). Locally formed dopamine is a physiological regulator of sodium excretion through the inhibition of tubular sodium reabsorption. The major source of the dopamine acting at the tubular level is the renal proximal tubule. Because proximal tubule cells lack the ability to hydroxylate tyrosine, the starting substrate in the nonneuronal renal dopamine synthetic pathway is L-dihydroxyphenylalanine (L-dopa). Proximal tubule cells take up L-dopa, which is freely filtered, via a sodium-dependent transporter located at the apical membrane. Intracellular L-dopa is decarboxylated into dopamine by the aromatic amino acid decarboxylase (2). Considering the huge availability of aromatic amino acid decarboxylase in the proximal tubule, it has been proposed that the rate-limiting step in the synthesis of dopamine in the renal tubules is the uptake of L-dopa (3, 21). A variety of sodium-dependent amino acid transporters have been implicated in the uptake of L-dopa by proximal tubule cells (29, 34). In a recent publication, Pinho et al. (27) demonstrated a parallelism between the expression of sodium-dependent neutral amino acid transporters ASCT2 and B(0)AT1, the uptake of L-dopa by isolated renal tubules, and the excretion of urinary dopamine in spontaneously hypertensive rats on a high-salt intake.

Interestingly, early studies have shown that the amino acid transport system A (an alanine and glycine, sodium-dependent transport) is stimulated by insulin through an increase in the maximal transport capacity (24). In line, we reported in a previous study that insulin enhances L-dopa proximal tubule uptake through the increase in the number of high-affinity transport sites (11). The aim of the present study was to identify the signaling cascade of insulin-

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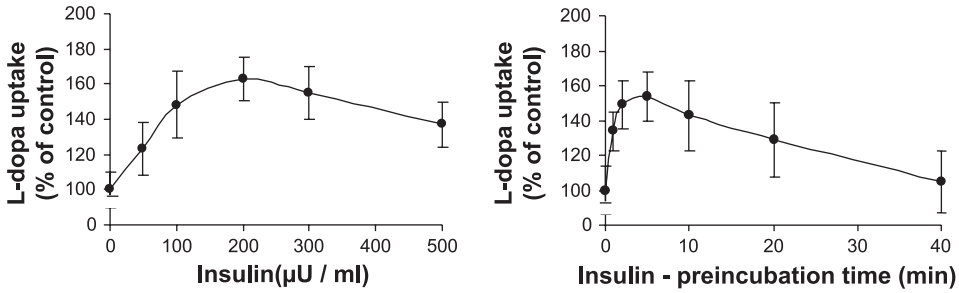

Fig. 1. Concentration- and time-dependent effects of insulin on L-dihydroxyphenylalanine (L-dopa) uptake by proximal tubule cells. Isolated proximal tubule cells were preincubated for 5 min in the presence of increasing concentrations of insulin (*left*) or with insulin (200 μU/ml) for the indicated periods (*right*). L-Dopa (final concentration 200 nM) was added, and incubation was prolonged for 20 min. Results are expressed as the percentage of intracellular L-dopa content in control cells [3.5 ± 0.5 (*left*) and 3.3 ± 0.7 (*right*) pmol L-dopa·mg protein^{-1}·min^{-1}]. Each point represents the mean ± SE of 4 experiments performed in triplicate.

induced stimulation of L-dopa inward transport in isolated renal proximal tubule cells.

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats (Animal Facilities, Veterinary College, Buenos Aires University) weighing 180–230 g were used. Animals were maintained at 22°C on a 12:12-h dark-light cycle and had free access to a commercial standard laboratory chow and tap water. Procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee and were conducted in conformity with institutional guidelines in compliance with international laws (*Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, NIH Publication No. 85-23, Revised 1996).

Preparation of proximal tubule cell suspensions. Proximal tubule cell suspensions were prepared according to the original method described by Seri et al. (33). This preparation is known to consist mainly of proximal tubule cells (33). Briefly, kidney cortex was minced on ice to a pastelike consistency that was digested with 0.7 mg/ml collagenase (Type V, Sigma Chemical, St. Louis, MO) in 10 ml Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES and 24 mM $NaHCO_3$, (pH 7.4). The digested tissue was poured through graded sieves (180, 75, 53, and 38 μm in pore size) to obtain a cell suspension. Proximal tubule cells were washed and resuspended in Krebs buffer (in mM: 120 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.4 $CaCl_2$, 24 $NaHCO_3$, 1.2 KH_2PO_4, 0.5 EDTA, and 11 glucose; pH 7.4). The quality of each preparation was monitored by microscopy, and the viability was assessed by Trypan blue exclusion.

Transport of L-dopa into tubule cells. The transport of L-dopa was determined as previously described (10). Briefly, cells were preincubated for 20 min in Krebs buffer in the presence of an inhibitor of aromatic L-amino acid decarboxylase activity, 3-hydroxybenzylhydrazine (250 μM). Unless specified, inhibitors and insulin were added to the medium 20 min and 5 min before L-dopa, respectively. L-Dopa uptake was started by the addition of L-dopa to the incubation medium (final concentration 200 nM), and incubations were carried out for 20 min at room temperature. After centrifugation (4°C, 60 *g*, 3 min) and rapid removal of the uptake medium, cells were rinsed twice with ice-cold Krebs solution and resuspended in 200 μl of 0.3 N $HClO_4$, disrupted (Sonifier Cell Disruptor, Heat Systems, Ultrasonics), and stored at −20°C until assayed for L-dopa.

L-dopa assays. L-Dopa was determined as reported previously (10). L-dopa was partially purified from cell homogenates by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography using a 4.6 × 150 mm, 5 μm Zorbax C18 column (Agilent Life Sciences and Chemical Analysis, Santa Clara, CA), and quantified amperometrically by the current produced on exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (ESA, Bedford, MA). Recovery through the alumina extraction step averaged 45–55%. L-Dopa concentration in each sample was corrected for recovery of the internal standard dihydroxybenzylamine and was further corrected for differences in the recovery of the internal standard in a mixture of external standards. The limit of detection was ~15 pg/vol assayed.

Immunoblotting and immunoprecipitation. For immunoblotting studies, isolated proximal tubule cells were incubated at 37°C in

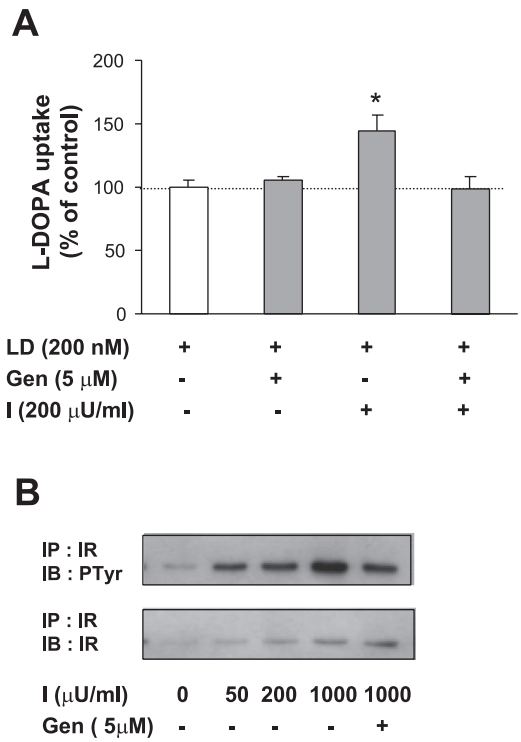

Fig. 2. Involvement of tyrosine kinase in the stimulatory effect of insulin on L-dopa uptake by proximal tubule cells. *A*: isolated proximal tubule cells were preincubated in Krebs buffer in the absence (control, open bar) or presence of genistein (Gen, 20 min) and/or insulin (I, 5 min) and were further incubated with L-dopa (LD, 20 min). Results are expressed as the percentage of intracellular L-dopa content in control cells (2.6 ± 0.22 pmol L-dopa·mg protein^{-1}·min^{-1}). Each point represents the mean ± SE of 3 experiments performed in triplicate. *$P < 0.05$ vs. control. *B*: cells were incubated with the indicated concentrations of insulin (5 min) in the presence or absence of genistein. Cells were lysed, and samples (1 mg protein) were subjected to immunoprecipitation (IP) with an antibody against insulin receptor β-subunit (IR). The immunocomplexes were subjected to SDS-PAGE (7.5%), immunoblotted (IB) with an antiphosphotyrosine antibody (PTyr), stripped, and reblotted with an IR antibody. The blot of one out of two experiments is shown.

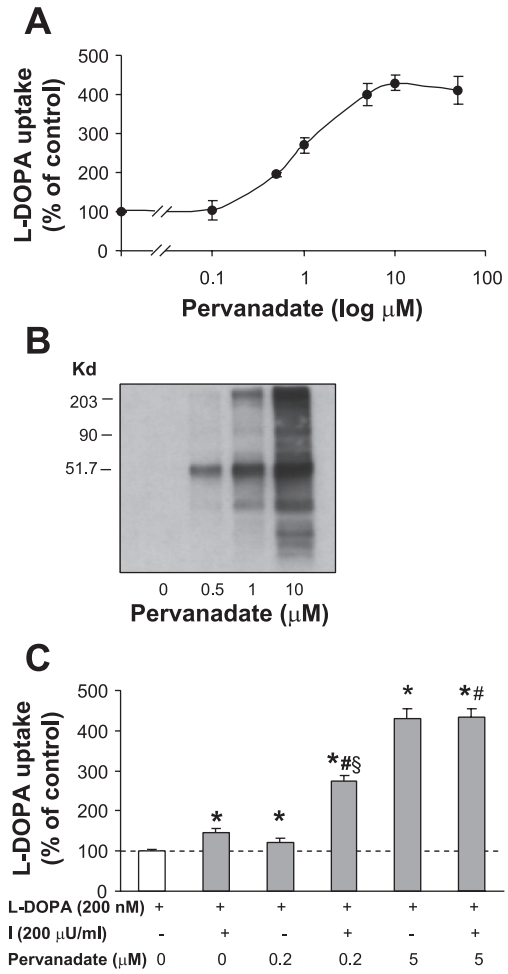

Fig. 3. Involvement of phosphotyrosine phosphatases in the stimulatory effect of insulin on L-dopa uptake by proximal tubule cells. *A*: isolated proximal tubule cells were preincubated in Krebs buffer in the absence (control) or presence of increasing concentrations of sodium pervanadate (20 min) and were further incubated with L-dopa for 20 min. Results are expressed as the percentage of intracellular L-dopa content in control cells (1.95 ± 0.04 pmol L-dopa·mg protein^{-1}·min^{-1}). Each point represents the mean ± SE of 3 experiments performed in triplicate. *B*: cells incubated with increasing concentrations of sodium pervanadate (20 min) were lysed, and samples (40 μg protein/well) were subjected to 7.5% SDS-PAGE and immunoblotted with an antiphosphotyrosine antibody. The blot of one out of three experiments is shown. *C*: incubations were performed as in *A*. Results are expressed as the percentage of intracellular L-dopa content in control cells (2.65 ± 0.03 pmol L-dopa·mg protein^{-1}·min^{-1}, open bar). Each point represents the mean ± SE of 4 experiments performed in triplicate. *$P < 0.05$ vs. control; #$P < 0.01$ vs. LD + I; §$P < 0.001$ vs. LD + the corresponding concentration of pervanadate.

Krebs buffer in the conditions specified for each experiment. Incubations were stopped by centrifugation (4°C, 60 *g*, 3 min) and rapid removal of the medium. Cells were homogenized in ice-cold RIPA buffer [in mM: 20 Tris·HCl, 2.5 EDTA, 50 NaF, 1 Na_3VO_4, 0.57 phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, plus a protease inhibitor cocktail (Sigma), pH 7.4]. Cells were disrupted by sonication, and lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an antiphosphotyrosine antibody (PY20, mouse monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA; 1:750), antiphospho-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA; 1:1,000). As a control of protein loading, the antiphospho-Akt antibody was stripped off in a buffer containing (in mM) 100 2-mecaptoethanol, 62.5 Tris·HCl, 2% SDS, pH 6.7 (50°C, 15 min), and membranes were reblotted with the anti-Akt antibody (Cell Signaling Technology; 1:1,000). Membranes were incubated with peroxidase-conjugated secondary antibodies and examined with an enhanced chemiluminescence method (ECL Plus Western blotting analysis system). The X-ray films were scanned using a HP Scan Jet 5100C and HP Precision Scan software (Hewlett-Packard, Palo Alto, CA). The images obtained were analyzed using NIH Image 1.57 software.

For immunoprecipitation studies, isolated proximal tubule cells were preincubated for 20 min in Krebs buffer alone or in the presence of genistein (5 μM) and incubated for 5 min with increasing concentrations of insulin. Cell lysates were prepared in RIPA buffer as above, spun (7,000 *g*, 10 min, 4°C) and supernatants were precleared by mixing 1.5 mg protein/sample with 10 μl protein A/G Plus-Agarose (Santa Cruz Biotechnology) plus anti-rabbit IgG (1/10,000, Santa Cruz Biotechnology) (30 min, 4°C). Samples were centrifuged, and 20 μl protein A/G Plus-Agarose were added to the supernatant along with an antibody against the insulin receptor β-subunit (1/400) (rabbit polyclonal IgG, Santa Cruz Biotechnology) and incubated overnight at 4°C. The immune complex was washed three times with buffer (in mM: 100 NaCl, 1 Na_3VO_4, and 20 HEPES, pH 7.5), and the pellet was resuspended in 40 μl of sample buffer, subjected to SDS-PAGE, and immunoblotted with the antiphosphotyrosine antibody. As a control of protein loading, the antibodies were stripped off and membranes were reblotted with the antibody against the insulin receptor β-subunit.

Cell fractionation. For protein translocation studies, proximal tubule cells were incubated for 5 min with increasing concentrations of insulin, resuspended in ice-cold homogenization buffer (20 mM Tris·HCl, pH 7.4) containing a protease inhibitor cocktail, disrupted by three cycles of freezing (dry ice, 5 min) and thawing (37°C water bath, 5 min), and briefly sonicated. Cell lysates were first centrifuged at 8,000 *g*, and the supernatants were further centrifuged at 100,000 *g* (4°C, 60 min). The supernatant was designated as the cytosolic fraction. The pellets were resuspended in homogenization buffer containing 0.1% Triton X-100 (30 min at room temperature) and centrifuged at 100,000 *g* (4°C, 60 min). The supernatant was designated as the membrane fraction. Cytosol and membrane samples (5 μg) were subjected to SDS-PAGE and immunoblotted with the antiphospho-Akt (Ser473) antibody or an anti PKC-ζ (rabbit poly-

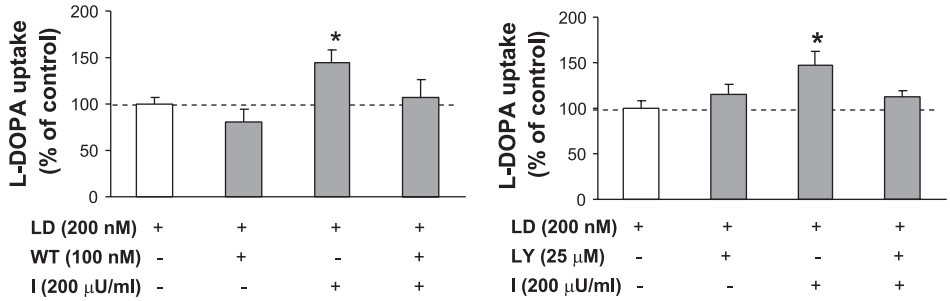

Fig. 4. Involvement of phosphatidylinositol 3-kinase in the stimulatory effect of insulin on L-dopa uptake by proximal tubule cells. Isolated proximal tubule cells were preincubated in Krebs buffer in the absence (control, open bars) or presence of wortmannin (WT, 20 min; *left*) or LY-294002 (LY, 20 min; *right*) and/or insulin (5 min) and were further incubated with L-dopa (20 min). Results are expressed as the percentage of intracellular L-dopa content in control cells (2.6 ± 0.22 pmol L-dopa·mg protein^{-1}·min^{-1}). Each point represents the mean ± SE of 3 experiments performed in triplicate. *$P < 0.05$ vs. control.

clonal IgG, Santa Cruz Biotechnology; 1:1,000). The antiphospho-Akt antibody was stripped off, and membranes were reblotted with the anti-Akt antibody. For protein loading control, after PKC immunodetection, the polyvinylidene difluoride membranes were stained with amido black (amido black-10B 0.1%, methanol 10%, acetic acid 2%, 5 min) and washed in destaining solution (methanol 45%, acetic acid 7%).

Immunohistochemistry. Rats were anesthetized intraperitoneally with chloral hydrate (350 mg/kg), and a polyethylene catheter (T4) was placed in the femoral vein for the intravenous injection of insulin (40 mU/kg) or normal saline (control animals). Rat tissues were fixed by the retrograde perfusion through the abdominal aorta with 60 ml of 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer at 37°C plus insulin (200 μU/ml) and 200 ml of the same solution at 4°C. Control animals were infused with the same fixative solution but without insulin. Following perfusion, the kidneys from control and treated animals were dissected out and immersed in the same fixative for 90 min and stored in 20% sucrose for 48 h. The tissue was then frozen and cut into 14-μm-thick sections with a cryostat (Microm, Zeiss). The sections were mounted onto gelatin-precoated glass slides, allowed to dry for at least 1 h, rinsed twice in PBS, dehydrated, and processed for the indirect immunofluorescence technique (13).

Briefly, sections were incubated overnight in a humid chamber at 4°C with an anti PKC-ζ antibody (rabbit polyclonal IgG, Santa Cruz Biotechnology; 1:500), diluted in PBS containing 0.2% (wt/vol) bovine serum albumin, 0.03% Triton X-100, and 0.1% (wt/vol) sodium azide. After rinsing in PBS, sections were incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:80). The sections were rinsed and mounted in a mixture of glycerol and PBS (3:1) containing para-phenylenediamine (28). Controls were done by incubating sections with a preimmune serum and by incubation without the primary or secondary antibodies.

Microscopy. All sections were examined with a Nikon Eclipse E-800 photomicroscope either under bright field or fluorescence illumination using proper filter combinations for FITC. Photographs were taken with a Nikon DXM 1200 digital camera (Tokyo, Japan) connected to the microscope optics. Brightness and contrast of the digital images were optimized using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Data analysis. All data are presented as means ± SE. The criterion for significant difference was set at $P < 0.05$. Comparisons have been done by one-way ANOVA followed by Newman-Keuls post hoc test.

RESULTS

As shown in Fig. 1, the facilitatory effect of insulin on the uptake of L-dopa by isolated proximal tubule cells was found to be dependent on the concentration of the hormone and the incubation time. The maximal response to insulin (~60% increase in the uptake of L-dopa over the control) was observed after 5-min preincubation with 200 μU/ml insulin.

Insulin-induced increase in the uptake of L-dopa by isolated proximal tubule cells was abolished by the tyrosine kinase inhibitor genistein (5 μM) (22), which per se did not modify the uptake of L-dopa (Fig. 2*A*). In line, immunoblotting studies showed a dose-dependent increase in phosphotyrosine signal in insulin receptor immunoprecipitates prepared from isolated tubule cells incubated with insulin (50–1,000 μU/ml). The increase in phosphotyrosine signal in response to insulin 1,000 μU/ml was abolished by genistein (Fig. 2*B*).

To further explore the role of protein tyrosine-phosphorylation on the effect of insulin on L-dopa uptake, insulin-induced response was analyzed in cells preincubated with sodium pervanadate, a nonselective inhibitor of protein-tyrosine phosphatases. Functional experiments showed that pervanadate (0.2–100 μM) per se elicited a dose-dependent increase in L-dopa uptake (Fig. 3*A*). In line, a concentration-dependent increase in tyrosine phosphorylation level of different proteins was evident within a similar range of pervanadate concentrations (Fig. 3*B*). To assess whether pervanadate may affect insulin-induced increase in L-dopa uptake by tubule cells, this response was studied in the presence of threshold (0.2 μM) or maximal (5 μM) pervanadate concentrations. Figure 3*C* shows that a synergistic effect between insulin and pervanadate was only observed when submaximal concentrations of pervana-

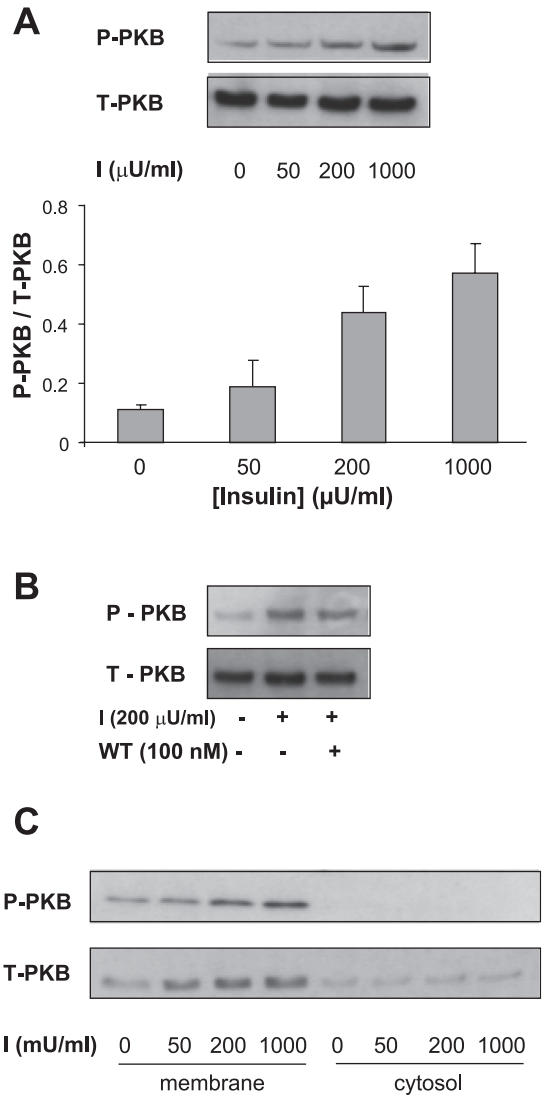

Fig. 5. Activation of Akt/PKB by insulin in proximal tubule cells. *A* and *B*: isolated proximal tubule cells were incubated in Krebs buffer in the presence of the indicated concentrations of insulin (5 min) and wortmannin (20 min). Cells were lysed, and samples (20 μg protein/well) were subjected to SDS-PAGE and transfer. Blots were probed with a specific anti-phospho-PKB (P-PKB) antibody, stripped, and reprobed with anti-total-PKB (T-PKB) antibody. The densitometric analysis of the immunoreactivity of PKB expressed as the ratio P-PKB/T-PKB of three independent experiments is also shown. *C*: isolated proximal tubule cells were incubated with the indicated concentrations of insulin (5 min). Cells were lysed, and membrane and cytosolic fractions were separated by ultracentrifugation. P-PKB and T-PKB were analyzed by Western blot as in *A*. The blot of one out of two experiments is shown.

date were assayed, whereas no further increase over the maximal stimulation of L-dopa uptake by pervanadate (5 μM) was elicited by insulin (Fig. 3*C*).

To examine the involvement of PI3K in insulin-induced increase of L-dopa uptake, this effect was studied in the presence of two structurally different inhibitors of PI3K, wortmannin (100 nM) (41) and LY-294002 (25 μM) (12). Both inhibitors abolished the insulin-induced increase in L-dopa uptake, without modifying per se the uptake of L-dopa (Fig. 4).

Two main kinases have been identified as insulin effectors downstream PI3K, Akt/PKB and PKC-ζ. Incubation of proximal tubule cells with insulin resulted in a dose-dependent phosphorylation of Akt/PKB at Ser473 (Fig. 5*A*). Preincubation with the PI3K inhibitor wortmannin precluded insulin-induced phosphorylation of Akt/PKB (Fig. 5*B*). Cell fractionation studies revealed the presence of total Akt/PKB in both, the cytosolic and membrane fractions in the absence of insulin. Insulin induced a dose-dependent augmentation of total Akt/PKB in the membrane fractions. Additionally, a dose-dependent increase in phospho-Akt/PKB signal was observed only in the membrane fractions from insulin-incubated cells (Fig. 5*C*).

The PKC inhibitor RO-318220 (1 μM) (35) abrogated insulin-induced increase in L-dopa uptake without changes in basal L-dopa incorporation (Fig. 6*A*). Cell fractionation studies evidenced the presence of PKC-ζ isoform in both cytosolic and membrane fractions in the absence of insulin. A dose-dependent translocation of PKC-ζ from the cytosol to the membrane was observed in insulin-incubated cells (50–1,000 μU/ml) (Fig. 6*B*). Time course studies indicated that insulin (200 μU/ml) induced a rapid translocation of PKC-ζ to the membrane fraction, which was evident after 2 min insulin-incubation and persisted during the 60-min study period (Fig. 6*C*).

The distribution of PKC-ζ in rat renal cortex was studied in vivo by immunohistochemistry (Fig. 7). A strong staining for PKC-ζ was observed on blood vessels in kidney slices from control and insulin-treated animals (Fig. 7, asterisks). PKC-ζ immunoreactivity signals were weak and evenly distributed within the cells in renal tubules from control rats. In response to insulin, a shift in PKC-ζ distribution toward the apical brush border microvilli was clearly seen (Fig. 7, arrows). No signals were observed when the primary antibody had been pread-

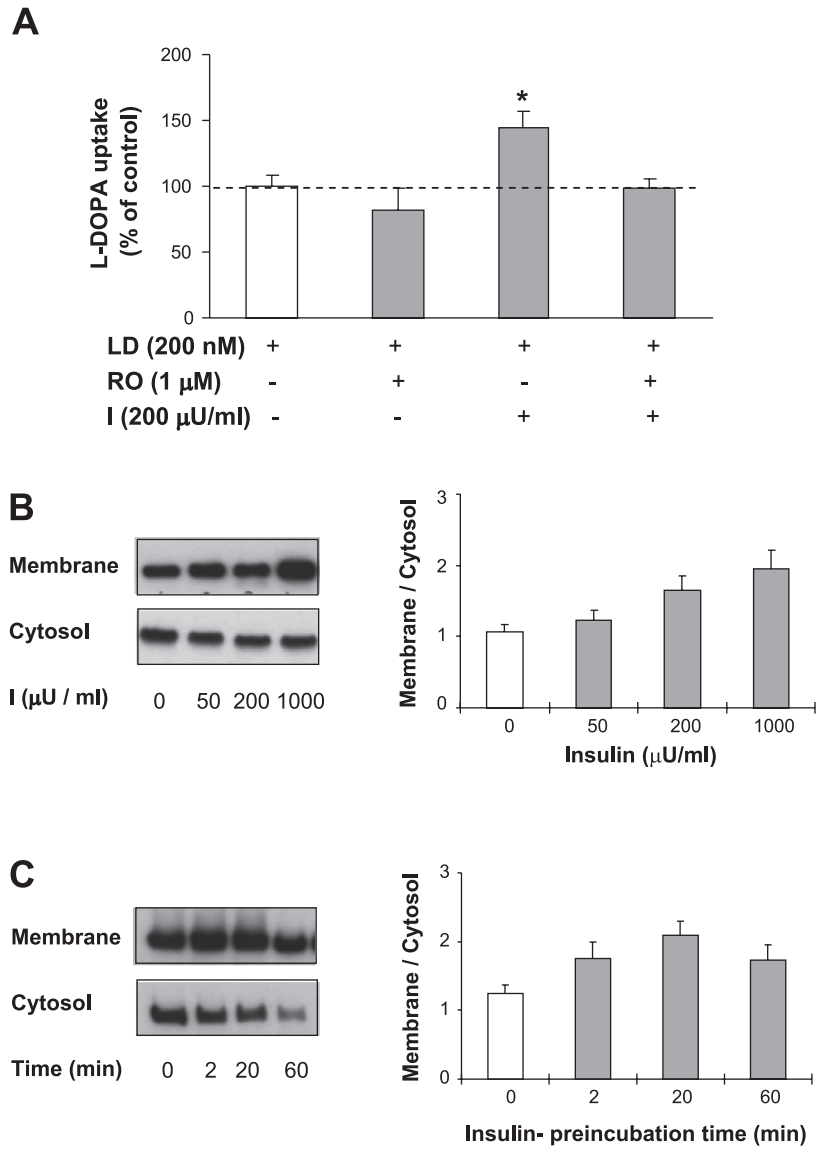

Fig. 6. Involvement of PKC-ζ in the stimulatory effect of insulin on L-dopa uptake by proximal tubule cells. *A*: isolated proximal tubule cells were preincubated in Krebs buffer in the absence (control, open bars) or presence of RO-318220 (RO, 20 min) and/or insulin (5 min). Intracellular L-dopa content was measured after 20-min incubation with L-dopa. Results are expressed as the percentage of intracellular L-dopa content in control cells (2.9 ± 0.34 pmol L-dopa·mg protein^{-1}·min^{-1}). Each point represents the mean ± SE of 3 experiments performed in triplicate. *$P < 0.05$ vs. control. *B* and *C*: proximal tubule cells were incubated with insulin (*B*, indicated concentrations, 5 min; *C*, 200 μU/ml, indicated period). Membrane and cytosolic fractions were separated by ultracentrifugation and subjected to Western blot analysis using an anti PKC-ζ antibody. The blot of one out of three experiments is shown. The densitometric analysis of the immunoreactivity, expressed as the ratio between membrane-bound and cytosol PKC-ζ, is also presented.

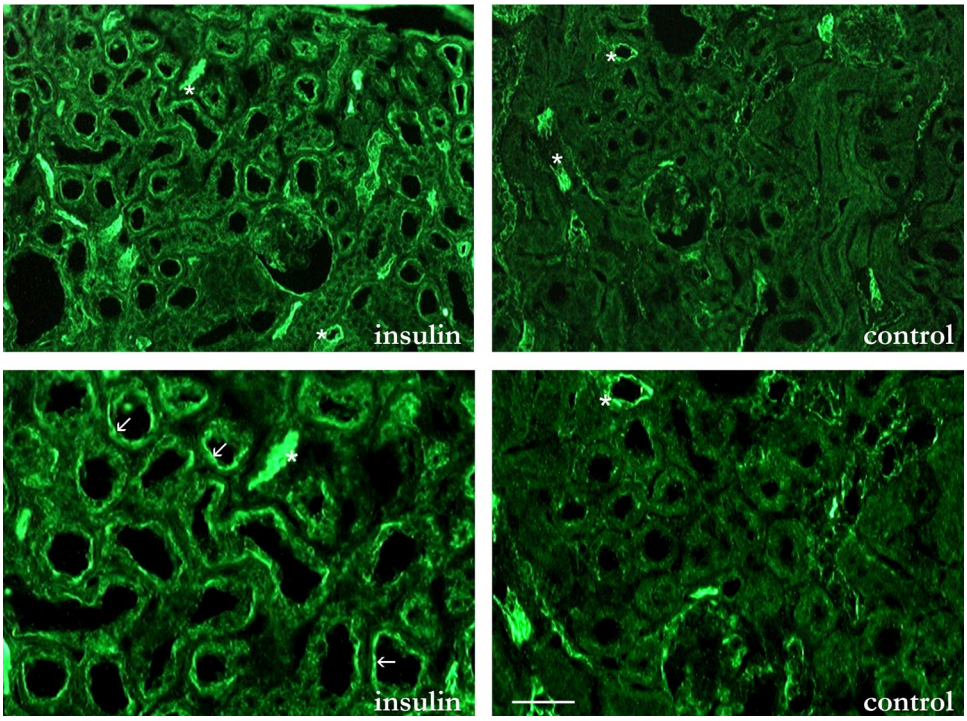

Fig. 7. Immunofluorescent micrographs of transverse sections of the kidney showing the expression of PKC-ζ in animals injected with 40 mU/kg followed by infusion with 200 μU/ml insulin (insulin) or normal saline (control) (*top*). Magnifications are shown at *bottom*. Immunoreactivity of blood vessels is indicated by asterisks. PKC-ζ immunoreactivity in the brush border microvilli is indicated by arrows. Scale bar: 100 μm (*top*) and 60 μm (*bottom*).

sorbed with the specific peptide, or tissue incubations were performed with the secondary antibody only (data not shown).

DISCUSSION

Pioneer studies suggested that insulin-induced increase in amino acid and glucose transport correlates with the recruitment of preformed carriers to the plasma membrane (18, 37). Furthermore, in a previous paper, we reported an increase in the expression of high-affinity L-dopa transport sites in isolated proximal tubule cells in the presence of insulin (11). Moreover, the translocation of both, glucose and L-dopa carriers, to the plasma membrane in response to insulin depends on the integrity of actin filaments (11, 40). Thus, because of these similarities, we analyzed the hypothesis that insulin may stimulate similar intracellular signaling pathways for increasing L-dopa transport in proximal tubules as it does for the regulation of glucose uptake in other cell types. For this purpose we used proximal tubule cell suspensions, a preparation that is known to consist mainly of proximal tubule cells (33).

Insulin action is initiated by the stimulation of the insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity (30). In our experimental conditions, insulin enhanced the uptake of L-dopa by ~60%, and this effect was completely abolished by inhibition of the activity of tyrosine kinase, thus confirming the role of tyrosine kinase in this effect. Stimulation of tyrosine kinase activity by insulin in proximal tubule cells was further confirmed: immunoprecipitation of the β-subunit of the insulin receptor showed a clear increase in tyrosine phosphorylation signal in the presence of 200 and 1,000 μU/ml insulin. The fact that the effective concentrations of insulin for increasing tyrosine phosphorylation of the β-subunit of the insulin receptor were the same as found to be effective for increasing L-dopa uptake in functional studies strongly supports the involvement of tyrosine kinase in insulin-stimulated L-dopa uptake.

The steady-state level of phosphotyrosine in most cellular proteins is a consequence of the catalytic activity of intracellular tyrosine kinases and protein tyrosine phosphatases (PTPs). PTP-1B is the prototypical PTP, and biochemical studies have implicated this enzyme in the dephosphorylation of several tyrosine kinase substrates (25). Sodium pervanadate is a powerful inhibitor of tyrosine phosphatases, thus increasing the amount of tyrosine-phosphorylated proteins (32). The insulinomimetic activity of sodium pervanadate has been previously reported and might be explained by this mechanism (14, 32). In our experimental conditions, sodium pervanadate had clear insulin-like effect on the uptake of L-dopa by proximal tubule cells. In line with results obtained in functional experiments, sodium pervanadate dose-dependently increased tyrosine phosphorylation signal of different intracellular proteins. Again, the fact that similar pervanadate concentrations increased both protein phosphorylation level and L-dopa uptake supports the relationship between tyrosine phosphorylation state and functional response. Maximal response of sodium pervanadate on L-dopa uptake was higher than that induced by insulin. This is in line with previous observations by other authors and suggests that pervanadate may inhibit other protein tyrosine phosphatases different from those involved in insulin pathway that are compartmentalized within the cell in a nonspecific way (7). Inhibition of all these phosphatases may lead to a response qualitatively similar, but quantitatively higher, to that induced by insulin (19). Thus, the synergism between insulin and submaximal concentrations of sodium pervanadate confirms that insulin signaling cascade is negatively modulated by PTPases. On the other hand, the absence of a sumatory effect between insulin and maximal doses of pervanadate confirms the tyrosine kinase pathway as the unique signal transduction pathway for insulin at this step of the signaling cascade.

It has been previously shown that activation of PI3K by insulin in rat liver cells leads to a rapid translocation of its regulatory subunit (p85) from cytosol to plasma membrane where it interacts with tyrosine-phosphorylated IRS proteins. This is followed by the generation of PI-3,4,5-P3, which in turn creates localized sites for attachment and further activation of downstream signaling molecules (4, 26). In our hands, the increase in the uptake of L-dopa by insulin was inhibited in the presence of two different PI3K inhibitors, thus confirming its involvement in the effect of insulin on L-dopa uptake. This result is in line with previous reports on a lower activity of system A amino acid transporters activated by insulin in the presence of wortmannin (20, 38).

Operating immediately downstream of PI3K is 3-phosphoinositide-dependent protein kinase 1 (PDK1), the activity of which is required for insulin-stimulation of PKC-ζ and Akt/PKB, which, in turn, are thought to control glucose transport and other metabolic processes (15).

The model of Akt/PKB activation involves binding of this protein to products of PI3K generated at the plasma membrane where the activating phosphorylation at Thr308 and Ser473 takes place (1). In our hands, insulin induced a dose-dependent increase in Akt/PKB phosphorylation that requires the activity of PI3K. Moreover, the fact that the dose-dependent increase in the phospho-Akt/PKB signal was only observed in the membrane fraction supports the concept of the requirement of Akt-PKB association to the membrane for its phosphorylation in proximal tubule cells.

The participation of the atypical PKC-ζ in insulin-stimulated glucose transport has been documented in skeletal muscle and adipocytes (8, 9, 23, 35), although this notion is not supported consistently by other studies (39). In our experimental model, involvement of PKC in insulin response was confirmed by the inhibition of the stimulatory effect of insulin on L-dopa uptake by the PKC inhibitor RO-318220. The fact that the inhibitor had no effect per se suggests that PKC did not elicit a tonic regulation on L-dopa transport in our experimental model. It should be noted that the required concentration of this inhibitor to inhibit PKC-ζ exceeds the effective concentration for inhibiting classical and novel PKCs (35). Therefore no further speculations about the identity of PKC isoforms involved in this effect may be drawn from these results.

It has been shown that upon insulin receptor stimulation, PKC-ζ translocates to plasma membrane in muscle cells (23). In our hands, cell fractionation of proximal tubule cells showed that insulin elicited a rapid and dose-dependent translocation of PKC-ζ toward the membrane fraction. These findings were confirmed and further extended by experiments performed in vivo: renal perfusion with insulin induced a clear redistribution of PKC-ζ toward the brush border microvilli area of renal tubules from where most of the reabsorptive processes take place. We could speculate that PKC-ζ may phosphorylate an amino acid transporter located in this membrane that by this way may become activated. Interestingly, in line with this hypothesis, Liu et al. (23) have recently reported that upon insulin stimulation, PKC-ζ in skeletal muscle cells translocates to plasma membrane, where it colocalizes with GLUT-4 transporters, leading to an increase in glucose uptake (23). The identity of the insulin-stimulated amino acid transporter in proximal tubule cells and the molecular mechanism of this putative phenomenon deserve further investigation.

A counterregulatory mechanism between insulin and dopamine at the renal tubules has been previously suggested (5, 6). We have already demonstrated that insulin enhances the tubular uptake of the dopamine precursor L-dopa (11). The present study describes the intracellular pathways triggered by insulin in proximal tubule cells to stimulate the uptake of L-dopa. In summary, our findings demonstrate that insulin activates Akt/PKB and PKC-ζ as downstream effectors of PI3K in proximal tubule cells. Moreover, our results also indicate that the PI3K-Akt/PKB-PKC-ζ pathways mediate the insulin-induced increase of L-dopa uptake in this nephron segment.

ACKNOWLEDGMENTS

The authors express gratitude to Dr. A. J. Montserrat (Departamento de Patología, Facultad de Medicina, Universidad de Buenos Aires) for helpful discussions, to Dr. M. Barontini [Centro de Investigaciones Endocrinológicas-Consejo Nacional de Investigaciones Científicas y Técnicas (CEDIE-CONICET)] for critical reading of the manuscript, and to O. Rodriguez and S. Ruffolo for technical assistance.

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GRANTS

This study was supported by grants PIP 5184 (CONICET, Argentina), and PICT 25428 [Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Argentina].

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