Phosphatidylinositol 3,4,5-trisphosphate: an early mediator of insulin-stimulated sodium transport in A6 cells

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Insulin stimulates sodium transport across A6 epithelial cell monolayers. Activation of phosphatidylinositol 3-kinase (PI 3-kinase) was suggested as an early step in the insulin-stimulated sodium reabsorption (Ref. 35). To establish that the stimulation of the PI 3-kinase signaling cascade is causing stimulation of apical epithelial Na channel, we used permeant forms of phosphatidylinositol (PI) phosphate (P) derivatives complexed with a histone carrier to A6 epithelium. Only PIP3 and PI(3,4)P2 but not PI(4,5)P2 stimulated sodium transport, although each of them penetrated into A6 cell monolayers as assessed using fluorescent permeant phosphoinositides derivatives. By Western blot analysis of A6 cell extracts, the inositol 3-phosphatase PTEN and the protein kinase B PKB were both detected. To further establish that the stimulation of sodium transport induced by insulin is related to PIP3 levels, we transfected A6 cells with human PTEN cDNA and observed a 30% decrease in the natriuretic effect of insulin. Similarly, the increase in sodium transport observed by addition of permeant PIP3 was also reduced by 30% in PTEN-overexpressing cells. PKB, a main downstream effector of PI 3-kinase, was phosphorylated at both Thr 308 and Ser 473 residues upon insulin stimulation of the A6 cell monolayer. PKB phosphorylation in response to insulin stimulation was reduced in PTEN-overexpressing cells. Permeant PIP3 also increased PKB phosphorylation. Taken together, the present results establish that the d-3-phosphorylated phosphoinositides PIP3 and PI(3,4)P2 mediate the effect of insulin on sodium transport across A6 cell monolayers.

epithelial Na+ channel; protein kinase B; phosphatase and TENsin homolog deleted on chromosome 10; kidney

BY REGULATING TOTAL body sodium, the kidney controls extracellular fluid volume and blood pressure. The fine tuning of this homeostasis is performed by the distal nephron and, more particularly, the cortical collecting duct where the principal cells express the amiloride-sensitive epithelial Na+ channels (ENaC) at the apical side, allowing entry of Na+ and Na+-K+-ATPase, extruding Na+ at the basolateral side (34, 36). The concerted action of these two membrane proteins results in transepithelial sodium absorption from the urinary to the extracellular space. The activity of ENaC is usually the rate-limiting step of the sodium transport and is under the control of various hormones, mainly aldosterone (2, 4, 7, 10, 13, 16, 41), insulin (3, 13, 23, 35, 40), and antidiuretic hormone (22, 46). The signal transduction pathways linking each of the hormone receptors to ENaC are still under investigation. Insulin stimulates the sodium transport by upregulating the number of apical ENaCs, or by increasing the open probability, or by a combination of both mechanisms (1, 3, 19, 30). The A6 cell line derived from the kidney of Xenopus laevis oocytes is currently used as a model to investigate sodium transport. When grown on a permeable support, A6 cells form a polarized tight epithelium that displays a high epithelial electrical resistance. After binding to its receptor at the basolateral membrane (5), insulin appears to stimulate phosphatidylinositol 3-kinase (PI 3-kinase) activity in A6 cells (35). The PI 3-kinase inhibitors LY-294002 and wortmannin block the insulin-stimulated sodium transport in a dose-dependent manner (35). The present study aims to further establish the importance of phosphorylated lipid products of PI 3-kinase by adding their permeant forms directly to cells. Our results support the importance of PIP3 and PI(3,4)P2 in mediating the increase in sodium transport.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylinositol phosphate derivatives [diC16-PIP3 sodium salt, diC16-PI(3,4)P2 ammonium salt and sodium salt, diC16-PI(3,5)P2 sodium salt, diC16-PI(4,5)P2 ammonium salt, and diC16-PI3P sodium salt], fluorescent NBD-conjugated phosphatidylinositol phosphate derivatives [NBD-diC16-PIP3, NBD-diC16-PI(3,4)P2, and NBD-diC16-PI(3,4,5)P3], and histone polyamine carrier were purchased from Echelon Biosciences (Salt Lake City, UT). Porcine insulin and DABCO reagent were purchased from Sigma (St. Louis, MO). Gelvatol was purchased from Monsanto. Protease inhibitor tablets were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Millicell inserts were purchased from Millipore (Bedford, MA). Transwell inserts were purchased from Corning Costar (Cambridge, MA). Tissue culture flasks were purchased from Sarstedt (Nümbrecht, Germany). Media was purchased from Invitrogen Life Technologies (Carlsbad, CA). Electrophysiological measurements were realized by use of an epithelial voltohmmeter (EVom) from World Precision Instruments (Sarasota, PA). The current evaluated by this technique is called an epithelial open circuit Na+ current. A polyclonal antibody to phosphatase and TENsin homolog deleted on chromosome 10 (PTEN; used at a 1,000-fold dilution) was purchased from Alexix Biochemicals (San Diego, CA). PhosphoPKB Thr 308 and Ser 473 (at a 250-fold dilution) and total PKB (at a 500-fold dilution) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies to phosphory-rosine at a 1,000-fold dilution) and to PI 3-kinase p85 subunit (at a 250-fold dilution) were purchased from Upstate (Lake Placid, NY). Protein G Sepharose was purchased from Amersham (Arlington Heights, IL). Peroxidase-labeled secondary antibody was purchased by 10.220.32.247 on June 10, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.physiology.org/ by 10.220.32.247 on June 10, 2017
from DAKO (Glostrup, Denmark). Geneticin, Lipofectamine 2000 was used as a transfection reagent, and the cDNA encoding human PTEN in pcDNA3 were purchased from Invitrogen Life Technologies. EGFP-C1 vector was purchased from Clontech (Palo Alto, CA). X. laevis oocytes were a generous gift from Prof. W. Van Driessche (Department of Physiology, Katholieke Universiteit te Leuven, Belgium).

**Cell culture.** A6 cells were received from Prof. W. Van Driessche (Department of Physiology, Katholieke Universiteit te Leuven, Belgium) and originated from Prof. J. P. Johnson (University of Pittsburgh, PA). A6 cells were grown in 28°C in a humidified incubator gassed with 1% CO₂ in O₂. Cells were cultured in 34% Ham’s F-12, 34% Leibovitz’s L-15, 20% water, 10% FBS serum, 3.8 mM L-glutamine, 87 IU penicillin, 87 μg/ml streptomycin, and 8 mM NaHCO₃ medium. The osmolarity of the growth medium was 260 mosmol/kgH₂O. Cells were plated twice a week. For biochemistry experiments, cells were cultured in 75-cm² plastic culture flasks, in 60-cm² plastic culture dishes, or subcultured onto 100-mm Costar Transwell inserts. Cells were used 7–14 days after seeding. For electrophysiological experiments, A6 cells were subcultured onto 24-mm Millicell inserts and incubated overnight in serum-free medium followed by a 4-h incubation in amphibian Ringer solution before electrical measurements. Amphibian Ringer solution had the following composition (in mM): 115 NaCl, 10 glucose, 5 Tris-HEPES, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, pH 7.7, osmolarity 260 mosmol/kgH₂O.

**Cell delivery of phosphorylated phosphoinositides.** Delivery of anionic phosphatidylinositol phosphate derivatives across the cell membrane by complexing with positive lysine-rich histone followed the procedure described by the manufacturer (Echelon Biosciences). Cell monolayers grown to confluence on Millicell insert and incubated overnight in serum-free medium that was replaced on the following day by amphibian Ringer solution, and 4 h later phosphoinositides were added together with histone as a carrier. Di-C16 phosphoinositides were chosen as long carbon chain derivatives appear more abundant membrane constituents than similar derivatives with a shorter carbon chain (42). The phosphoinositide derivatives were complexed with a lysine-rich histone (10-min incubation with vortexing). The concentration ratio of phosphoinositides (20 μM) to histone (5 μM) used in most experiments (32, 39) was in the range suggested by the manufacturer (Echelon Biosciences), i.e., 1:0.1 to 1:1 (phosphoinositides:histone). The concentrations of histone used never exceeded 5 μM as preliminary experiments showed that a higher concentration slightly increased sodium transport.

**Electrophysiology.** Confluent monolayers of A6 cells grown on Millicell and Transwell inserts were used only if their electrical resistance was >4,200 Ω·cm² and a mean transepithelial potential difference >20 mV. Resistance and potential difference were measured using an EVom with chopstick electrodes made of Ag–AgCl pellets. Sodium transport (Iₛ,N) was calculated from the transepithelial potential difference and resistance. Amiloride added to the apical bathing medium completely inhibited this current validating such computation of Iₛ,N. Insulin (100 nM) was always added to the basolateral bathing medium.

**Confocal microscopy.** For the determination of the intracellular uptake of permeant phospholipids, we used fluorescent derivatives complexed with histone. A6 cells were seeded onto 12-mm clear Transwell inserts and treated as described in **Cell culture.** Monolayers were incubated for different times with 20 μM of permeant NBD-diC16PIP₃, NBD-diC16PI(3,4)P₂, and NBD-diC16PI(4,5)P₂. After incubation, inserts were transferred on ice and rinsed 10 times with ice-cold Ringer solution. The A6 cells monolayers were then fixed for 10 min at room temperature in 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. Inserts were rinsed three times in Ringer solution and mounted with a drop of gelvatol solution containing 100 mg/ml DABCO reagent. Cells were observed under a Zeiss Axiovert fluorescence microscope (MRC 1000, Bio-Rad, Hercules, CA) equipped with an argon-krypton laser (excitation wavelength of 488 nm). Confocal images were analyzed using the Laser-Sharp software (Bio-Rad), National Institutes of Health Image 1.62, and Image software program (Adobe Photoshop).

**Western blot analysis.** Immunodetection of phosphoPKB on Thr 328 and Ser 473 residues was performed on extracts of cells grown for 10 days on 100-mm Transwell inserts incubated in the presence or absence of insulin (100 nM) added to the basolateral medium for various periods of time as indicated in each experiment. Cells were harvested in ice-cold lysis buffer (150 mM KCl, 10 mM Tris-HCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM NaF, 10 nM okadaic acid, 0.5% NP-40, 0.1% β-mercaptoethanol, 300 μg/ml pefabloc, and 10 μg/ml leupeptin, pH 7.4) and harvested quickly at 4°C. Immunodetection of PTEN was performed on cells grown at confluence in 75-cm² plastic culture flasks and lysed.

Cell extracts were maintained at 4°C for 1 h, and the pellet was discarded by centrifugation (13,000 rpm; 20 min). Protein concentration was determined using the Bradford method (8). SDS-PAGE was performed as previously described (15). Briefly, the extracts were solubilized in sample buffer (10 mM Tris-HCl, 1.5% SDS, 0.6% DTT, 6% glycerol, 0.1% bromophenol blue, pH 6.8) and denatured by heating at 95°C for 5 min. Proteins were separated on 7.5% acrylamide gels and transferred to nitrocellulose. Membrane blots were blocked and incubated overnight with a primary antibody (2% BSA or 0.1% milk, 150 mM NaCl, 50 mM phosphate buffer, 0.05% Tween 20, 0.1% NaN₃, pH 7.4) at 4°C and then washed and incubated for 1 h at room temperature with the appropriate peroxidase-labeled secondary antibody (DAKO). Detection was performed by exposure to enhanced chemiluminescence (Amersham). Membrane incubated overnight with anti-phosphoPKB antibodies was washed and reprobed with total PKB.

**X. laevis tissues.** Heart, kidney, liver, and brain of X. laevis were homogenized in the following ice-cold buffer (31): 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.01% NaN₃, 1 mM Na₂VO₃, 1 mM okadaic acid, 1 mM EDTA, and a cocktail of proteases inhibitors (Roche tablets). The homogenates were centrifuged and the supernatant was used for Western blot analysis using anti-PTEN antibodies.

**Transfection.** About 10⁶ cells seeded on 60-cm² plastic culture dishes reached 70% confluence after 24 h and were then transfected. Briefly, cells were washed twice in a penicillin-streptomycin serum-free medium and the mixture of human PTEN cDNA (10 μg) or empty vector (10 μg) with 30 μl lipofectamine 2000 reagent was added to this incubation medium and left overnight. Cells were then washed, and the transfection medium was replaced by amphibian serum medium. When cells reached confluence, they were plated at a 1:10 dilution into 2 mg/ml Geneticin amphibian serum medium. Complete selection occurred after ~10 days. In preliminary experiments, GFP in pEGFP-c1 was transfected into A6 cells and treated with a similar Geneticin-selective pressure. It yielded a transfection efficiency of ~40% as checked by fluorescence microscopy. PTEN overexpression was stable only for a few passages. Each physiological experiment was therefore carried out with a new batch of transfected A6 cells. PTEN overexpression was checked by Western blot analysis for each physiological experiment.

**Immunoprecipitation.** To test the transfection of PTEN cDNA and the incubation with permeant PIP₃, the cells were incubated with PI 3-kinase activity, the proteins containing phosphorylated tyrosine residue in A6 cell extracts were immunoprecipitated by anti-phosphotyrosine antibodies and the presence of the p85 regulatory subunit in the precipitate was taken as an index of PI 3-kinase stimulation.

One-hundred microliters of protein G Sepharose, first washed with cold PBS, were incubated with 4 μl of anti-phosphotyrosine antibodies for 2 h at 4°C. The complex was rinsed with ice-cold lysate buffer and incubated overnight at 4°C with 1.2 mg of A6 cell extract. The supernatant was discarded by centrifugation. The pellet was washed and resuspended in 20 μl of sample buffer and boiled for 5 min. The
supernatant was collected and loaded onto a polyacrylamide gel. After immunoblotting with anti-phosphotyrosine antibodies, the membrane was stripped and probed with an anti-p85 subunit followed by incubation with anti-mouse antibodies. The level of p85 recruitment by tyrosine-phosphorylated insulin receptor substrates was compared within different samples.

Statistics. Electrophysiological data are presented as means ± SD. Paired t-tests were used to compare experimental vs. control groups.

RESULTS

Permeant PIP₃ increases sodium transport. The effect of different phosphorylated phosphoinositides on sodium transport across confluent monolayers of A6 cells was investigated by adding permeant forms of phosphatidylinositol phosphate derivatives (20 μM) complexed with histone carrier (5 μM) directly to one of the two compartments bathing the monolayer. Apical addition of permeant (i.e., complexed) PI(4,5)P₂, PI(3,5)P₂, and PIP₃ did not modify sodium transport (Fig. 1A), whereas apical addition of PIP₃ and to a lesser extent PI(3,4)P₂ increased sodium transport with a lag time of 60 min (Fig. 1B). No further increase in sodium transport was observed upon simultaneous addition of PI(3,4)P₂ and PIP₃ compared with the addition of PIP₃ alone (data not shown). Apical addition of PIP₃ in the absence of the histone carrier completely failed to increase the current. Compared with the concentration of 20 μM tested, higher concentration of permeant PIP₃ (50 μM) did not induce a significantly greater increase in sodium transport (Fig. 2). Whatever the histone-phosphoinositide complex tested, their addition to the basolateral medium never increased sodium transport.

Permeant phospholipids penetrate into A6 epithelial cells. To demonstrate that these permeant phospholipid complexes indeed penetrate into A6 epithelial cells, we used three fluorescent permeant derivatives: NBD-diC₁₆-PIP₃, NBD-diC₁₆-PI(3,4)P₂, and NBD-diC₁₆-PI(4,5)P₂ (20 μM) complexed with histone (5 μM). Permeant PIP₃ and PI(3,4)P₂ added in the apical bathing medium labeled the A6 cells after only 10-min incubation (Fig. 3, A and G), but the labeling was further increased after 67 min (Fig. 3, B and H). The localization of

Fig. 1. Sodium transport (Iₙ₆) was evaluated (μA/cm²) as described in EXPERIMENTAL PROCEDURES. Phosphoinositides (20 μM) were made permeant by complexation with a histone carrier (5 μM) and added to the apical bathing medium. A: permeant PI(3,5)P₂, permeant PIP₃, permeant PI(4,5)P₂, and carrier alone did not increase the sodium transport. PI, phosphatidylinositol; P, phosphate. Insulin (100 nM) was added to the basolateral bathing medium. Results are means ± SD; n = 3. β: permeant PIP₃ and permeant PI(3,4)P₂ increased sodium transport after a lag time of 60 min and reached a maximum at ~90 to 120 min. Carrier alone did not stimulate the transport. Insulin (100 nM) was added to the basolateral bathing medium. Results are means ± SD; n = 3.

Fig. 2. Iₙ₆ was evaluated (μA/cm²) as described in EXPERIMENTAL PROCEDURES. Permeant PIP₃ (dotted lines) was added at different concentrations (from top to bottom: 50, 20, 10, 5, 1, and 0.1 μM) to the apical bathing medium; 50 μM did not produce significantly greater effect than 20 μM. The concentrations of 1 and 0.1 μM were not significantly different from the carrier alone. Results are means ± SD; n = 3.
PIP$_3$ in A6 cells appears in cytoplasmic vesicles, mostly concentrated around the nucleus (Fig. 3B). Similar observations were done with permeant PI(4,5)P$_2$ and PI(3,4)P$_2$, although the labeling was more diffuse in the latter case (Fig. 3, E and H). On the contrary, fluorescent permeant PIP$_3$, PI(3,4)P$_2$, and PI(4,5)P$_2$ added to the basolateral side and incubated for 67 min did not enter into the A6 cells (Fig. 3, C, I, F). Furthermore, no uptake of fluorescent PIP$_3$ was observed when it was added to the apical bathing medium without complexing with the histone carrier (Fig. 3D).

*Insulin-stimulated sodium transport is decreased in PTEN-overexpressing cells.* The transient nature of the increase in sodium transport brought about by permeant PIP$_3$ could be explained by the action of PIP$_3$ 5- or 3-phosphatase. Western blot analysis shows the presence of the inositol 3-phosphatase PTEN in A6 cells and as well in several *X. laevis* tissues (heart, kidney, liver, brain) (Fig. 4A). Overexpression of PTEN in A6 cells was achieved by transfecting human PTEN cDNA (Fig. 4B). PTEN overexpression was lost after passages 4 and 5 of the cells, and therefore each experiment was carried out with a new batch of transfected A6 cells. The rate of PTEN overexpression was assessed independently for each experiment (Fig. 4B). In vivo PTEN converts mainly PIP$_3$ into PI(4,5)P$_2$, whose addition (as its permeant form) did not raise sodium transport (Fig. 1A). Basal (unstimulated) sodium transport was not significantly different in PTEN-overexpressing monolayers vs. control monolayers. The stimulation of sodium transport induced by insulin was also reduced in PTEN-overexpressing monolayers compared with those transfected with empty vector (Fig. 5A). The difference between stimulated and basal current was estimated at the peak of the response (Peak $I_{Na^+}$ = peak $I_{Na^+}$ - basal $I_{Na^+}$), i.e., 60 min after addition of insulin and was found 30% higher in empty vector cells compared with PTEN-transfected cells (Fig. 5B). Thus overexpression of PTEN was sufficient to decrease insulin-induced stimulation of sodium transport.
transport, most probably by decreasing the levels of PIP₃ in response to insulin.

The effect of permeant PIP₃ added to the apical medium was also tested in PTEN-overexpressing cells. The increase in sodium transport induced by permeant PIP₃ was also reduced in A6 monolayers overexpressing PTEN compared with control monolayers (Fig. 5A). The difference between basal current and maximal current stimulated by exogenous PIP₃ (Peak/INa), evaluated at the peak of the response, was also reduced by ~30% in PIP₃-stimulated PTEN-transfected cells compared with the PIP₃-stimulated control cells (Fig. 5B). Thus overexpression of PTEN in A6 cells reduces similarly the stimulation of sodium transport induced either by insulin or by exogenous PIP₃. Altogether, these results strongly support that PIP₃ plays a major role in insulin-stimulated sodium transport.

**PI 3-kinase activity.** Stimulation of PI 3-kinase by insulin results from the recruitment of the p85 regulatory subunit by tyrosine-phosphorylated insulin receptor substrate. We therefore immunoprecipitated tyrosine-phosphorylated proteins in extracts from A6 epithelial cells stimulated with insulin for various times and detected by Western blotting whether the p85 subunit was coprecipitated as an indirect assessment of PI 3-kinase activity as performed by others (11, 21, 45). A6 cell monolayers were stimulated with insulin (100 nM) for 0, 2, and 5 min and extracts were immunoprecipitated by anti-phospho-tyrosine antibodies (Fig. 6A). The level of recruitment of the p85 subunit was weak in unstimulated A6 cells but increased after 2- and 5-min stimulation with insulin (Fig. 6B). Crude extracts not submitted to immunoprecipitation showed no difference in the level of PI 3-kinase regulatory subunit (Fig. 6B). Also PTEN transfection did not affect the activity of PI 3-kinase. The recruitment of the p85 subunit was comparable in PTEN-overexpressing cells vs. empty vector-transfected cells (Fig. 6C). Furthermore, incubation with permeant PIP₃
The major observation of the present study can be summarized as follows: both PIP3 and to a lesser extent P(3,4)P2 reproduced the insulin-induced increase in sodium transport when added with a carrier directly to the apical side of the A6 epithelium. However, when these phosphoinositides were added together, there was no greater effect than with PIP3 alone, suggesting that the molecules are interacting at the same

**DISCUSSION**

Despite ample evidence that sodium transport is regulated by insulin, the intracellular signaling cascade triggered on insulin binding to its receptor is largely unknown in the kidney. Dysregulation may lead to enhanced sodium reabsorption and hypertension. A6 cells cultured on a permeable support have been used as a model epithelium to demonstrate this natrifieric effect of insulin (19), and subsequently this effect has been attributed to an increased number of active ENaC within the apical membrane and/or their open probability. More recently, Blazer-Yost’s group (35) implicated the stimulation of PI3-kinase as one of the early events triggered by insulin receptor occupancy. This was based on the detection of PIP3 1 min after insulin stimulation by HPLC analysis of lipid extracts. This increase in PIP3 as well as the sodium current were both prevented by LY-294002, an inhibitor of PI 3-kinase (47). However, the basal current was also sensitive to this drug, questioning its specificity in insulin stimulation (33). Indeed, such an inhibition was also observed in the early rise in the current elicited by the mineralocorticoid hormone aldosterone (4) and by antidiuretic hormone (17). Thus the suggestion has been made that LY-294002 might inhibit the maintenance of sodium transport by affecting the insertion of Na+ within the apical membrane, i.e., the fusion or trafficking of Na+ containing vesicles regardless of hormonal stimulation (14). More recently, however, Blazer-Yost et al. (1) showed that insulin induces a redistribution of Na+ to both apical and lateral membranes. At the latter site, there was a colocalization of Na+ and PI 3-kinase regulatory subunits, suggesting that this could represent a storage pool of Na+ that can be recruited and targeted to the apical membrane.

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did not affect the recruitment of p85 in anti-phosphotyrosine immunoprecipitates (Fig. 6C).

**PKB phosphorylation by insulin in A6 cells.** Three major pathways have been shown to be activated by insulin: PI 3-kinase, MAP kinase, and CAP/Cbl/TC10 cascades as least in adipocytes (38, 50). The PI 3-kinase pathway is constantly associated with an increase in PKB activity (9) that can be detected by its phosphorylation on Thr 308 and Ser 473.

Confluent A6 cell monolayers grown on 60-cm2 Transwell inserts were incubated for 18 h in serum-free medium and then stimulated with insulin (100 nM) for 0, 5, 7, 10, 15, and 30 min. Insulin-stimulated and -unstimulated Chinese hamster ovary cells overexpressing the insulin receptor (CHO-IR) cell extracts were used, respectively, as positive and negative control for phosphorylated-PKB (6). In the absence of insulin, no phosphorylated band was detectable. Five minutes after addition of insulin, PKB was already phosphorylated on Thr 308 and Ser 473 residues. After 7 min, the intensity of the phosphorylated bands was maximal and decreased slightly thereafter (Fig. 7, A and B), whereas the amount of total PKB reprobed on the same membranes was identical at the various times examined.

The level of PKB phosphorylation after 7-min insulin stimulation was compared in PTEN-overexpressing cells vs. empty vector-transfected cells. PKB phosphorylation was reduced in PTEN-overexpressing cells, the difference being stronger in phosphorylation of the Thr 308 residue than in the phosphorylation of the Ser 473 residue (Fig. 8A).

A6 cells grown on 60-cm2 Transwell inserts were incubated with permeant PIP3 added to the apical solution for 67 min, a time sufficient to raise sodium transport. Permeant PIP3 induced the phosphorylation of PKB on the Thr 308 residue but no phosphorylation could be detected on Ser 473 residue (Fig. 8B).

**Fig. 5. I\textsubscript{Na} was evaluated (\mu A/cm\textsuperscript{2}) as described in experiment PROCEDURES. A6 monolayers either overexpressing PTEN or transfected with an empty vector. A: sodium transport was stimulated by insulin (100 nM) or by addition of permeant PIP3 (20 \mu M). The increase in sodium transport following insulin stimulation was lower in PTEN-overexpressing cells vs. control A6 cells. PTEN-overexpressing cells also show a lower stimulation of sodium transport than the control A6 cells when stimulated with permeant PIP3. Results are representative of 3 PTEN transfections. Results are means \pm SD; n = 3. B: maximal stimulation of I\textsubscript{Na} by insulin and by PIP3. The difference in sodium transport between unstimulated and insulin-stimulated monolayers (\Delta I\textsubscript{Na}) was evaluated 60 min (Peak \Delta I\textsubscript{Na}) after insulin addition and shows a 30% decrease in the PTEN-transfected cells compared with the empty vector cells. Similarly, the difference in sodium transport between unstimulated (carrier alone) and permeant PIP3-stimulated monolayers (\Delta I\textsubscript{Na}) was evaluated 120 min (Peak \Delta I\textsubscript{Na}) after its addition and shows a 30% decrease in the PIP3-stimulated PTEN-transfected cells compared with the PIP3-stimulated control cells. Results are means \pm SD; n = 3, P < 0.001.
site. On the other hand, PI(4,5)P₂ does not seem to play a major role in this mechanism. This is at variance with some results obtained by patch-clamp analysis, which suggested a role for PI(4,5)P₂, although only in the presence of GTP (52). In this particular setting of excised patches, PI(4,5)P₂ is important to alleviate a spontaneous rundown effect, i.e., spontaneous disappearance of ENaC activity (29). Yue et al. (52) found no effect of PIP₃ but in this inside-out patch-clamp setting, critical intermediate signaling molecules might have been lost. However, consistent with the present results, Tong et al. (44) very recently demonstrated that PIP₃ and PI(3,4)P₂ both activate ENaC in excised patches from CHO cells cotransfected with the three human ENaC subunits. The lag time observed in the present study between PIP₃ addition and the final effect on sodium transport is most probably explained by the slow diffusion of PIP₃ into the cell. Although a 10-min incubation time was already sufficient to detect NBD-PIP₃ inside the cells (Fig. 3A), higher intracellular labeling was observed after 67 min (Fig. 3B) and is probably required to build up sufficient PIP₃ to trigger the increase in sodium transport. Our confocal studies on A6 cells show an apparent inhomogeneity of the fluorescent probe from cell to cell within the same monolayer. The reason for this is not understood, but such a variability from cell to cell was also observed in the confocal studies of Blazer-Yost et al. (1). The critical role of PIP₃ was further underlined by transfection of the inositol 3-phosphatase PTEN, which led to decreased stimulation of sodium transport induced by insulin as well as by permeant PIP₃. The results provide an independent set of data suggesting a role of PIP₃ in mediating insulin’s effect on sodium transport. However, unlike LY-294002, PTEN transfection did not change the rate of basal (unstimulated) sodium transport.

Thus, as shown by Blazer-Yost et al. (1), insulin receptor occupancy induces the recruitment of PI 3-kinase to the plasma membrane, which activates its catalytic activity and produces 3-phosphorylated inositide lipids such as PIP₃ and PI(3,4)P₂ that are the principal mediators of PI 3-kinase stimulation. In
adipocytes as well as in many other tissues, PIP₃ activates PDK1 and possibly PDK2, leading to phosphorylation of PKB on both Thr 308 and Ser 473 residues. Those phosphorylation steps that activate PKB are necessary but not sufficient to target GLUT4 to the adipocyte plasma membrane (49). The GLUT4 translocation in adipocytes and muscle cells required not only activation of PKB (49) but also the stimulation of CAP/Cbl/TC10 cascade, a PI 3-kinase-independent pathway (18, 24). Yet, the presence and role of PKB in A6 epithelial cells or in the collecting duct have not been questioned so far, whereas emphasis has been placed on another protein kinase, namely, serum and glucocorticoid-inducible kinase (sgk). This kinase

Fig. 7. Immunodetection of phospho-protein kinase B (PKB: top) and PKB (bottom). A: anti-phosphoPKB-Thr 308 (top) and anti-PKB (bottom) antibodies. Top: from left to right (in μg protein loaded/lane): lane 1: CHO-IR (45 μg) stimulated with 100 nM insulin for 5 min used as a positive control. Lane 2: unstimulated CHO-IR (45 μg) used as a negative control. Lane 3: unstimulated A6 cells (60 μg). Lanes 4-8: A6 cells (60 μg) stimulated with 100 nM insulin, respectively, for 5, 7, 10, 15, and 30 min (overnight incubation with primary antibody and 10-min film exposure; n = 3). Bottom: same membrane reprobed for total PKB antibody (overnight incubation with primary antibody and 1-min film exposure; n = 3). B: anti-phosphoPKB-Ser 473 (top) and anti-PKB antibodies. Top: from left to right (in μg protein loaded/lane): lane 1: CHO-IR (45 μg) stimulated with 100 nM insulin for 5 min used as a positive control. Lane 2: unstimulated CHO-IR (45 μg) used as a negative control. Lane 3: unstimulated A6 cells (60 μg). Lanes 4-8: A6 cells (60 μg) stimulated with 100 nM insulin, respectively, for 5, 7, 10, 15, and 30 min (overnight incubation with primary antibody and 10-min film exposure; n = 3). Bottom: same membrane reprobed for total PKB antibody (overnight incubation with primary antibody and 1-min film exposure; n = 3).

Fig. 8. Immunodetection of phosphoPKB (top) and PKB (bottom) in PTEN-overexpressed cells vs. empty vector control cells and in unstimulated vs. PIP₃-stimulated A6 cells. A: anti-phosphoPKB-Thr 308 (top) and anti-PKB (bottom) antibodies. Top: from left to right (in μg protein loaded/lane): lane 1: CHO-IR (45 μg) stimulated with 100 nM insulin for 5 min used as a positive control. Lane 2: unstimulated CHO-IR (45 μg) used as a negative control. Lane 3: unstimulated empty vector cells (60 μg). Lane 4: empty vector cells (60 μg) stimulated with 100 nM insulin for 7 min. Lane 5: unstimulated PTEN-overexpressed cells (60 μg). Lane 6: PTEN-overexpressed cells (60 μg) stimulated with 100 nM insulin for 7 min. Lane 7: unstimulated A6 cells (60 μg). Lane 8: A6 cells stimulated with permeant PIP₃ for 67 min (60 μg) (overnight incubation with primary antibody and 10-min film exposure; n = 3). Bottom: same membrane reprobed for total PKB antibody (overnight incubation with primary antibody and 1-min film exposure; n = 3). B: anti-phosphoPKB-Ser 473 (top) and anti-PKB (bottom) antibodies. Top: from left to right (in μg protein loaded/lane): lane 1: CHO-IR (45 μg) stimulated with 100 nM insulin for 5 min used as a positive control. Lane 2: unstimulated CHO-IR (45 μg) used as a negative control. Lane 3: unstimulated empty vector cells (60 μg). Lane 4: empty vector cells (60 μg) stimulated with 100 nM insulin for 7 min. Lane 5: unstimulated PTEN-overexpressed cells (60 μg). Lane 6: PTEN-overexpressed cells (60 μg) stimulated with 100 nM insulin for 7 min. Lane 7: unstimulated A6 cells (60 μg). Lane 8: A6 cells stimulated with permeant PIP₃ for 67 min (60 μg) (overnight incubation with primary antibody and 10-min film exposure; n = 3). Bottom: same membrane reprobed for total PKB antibody (overnight incubation with primary antibody and 1-min film exposure; n = 3).
plays a major role in the aldosterone stimulation of sodium reabsorption, and its expression is indeed induced by aldosterone and by hypotonic shock in A6 cells (37). Although transfection of a dominant kinase dead sgk inhibited basal and insulin-stimulated sodium transport, overexpression of sgk did not increase insulin-stimulated sodium transport nor did transfection of a mutant form of sgk that cannot be phosphorylated by PKB-2 decrease insulin-stimulated sodium transport (13, 20). Using Western blot analysis, we were able to demonstrate phosphorylation of PKB under the control of insulin addition and that the observed time course is compatible with a physiological mediator role in the enhancement of sodium transport. Furthermore, the inositol 3-phosphatase PTEN (25, 48) was also detected in the A6 cells. In vivo, this 3-phosphatase has for major substrate PIP3 which is degraded into PI(4,5)P2 (27, 28). PTEN knockout mice have greatly elevated basal levels of PIP3 and phosphorylated basal PKB activity (43). To investigate the role of PI 3-kinase without perturbing the system with an inhibitor, we used permeant derivatives of different phosphatidylinositol phosphates, using the procedure developed by Echelon Biosciences. The intracellular delivery of the membrane-impermeable anionic inositol phosphate lipids is greatly improved by complexation with cationic polyanamines. Preincubation of phosphoinositides with carrier polyanymes produces complexes that enter into living eukaryotic cells such as mammalian, plant, yeast, bacterial, and protozoal cells (32). This phosphoinositide delivery method has been proven efficient to investigate the signaling role of PI 3-kinase (26) and PI kinases (39, 51). Delivery of PIP3 to its intracellular target into A6 cells is attested by the intracellular uptake of fluorescent NBD-PIP3, by the phosphorylation of PKB, and by the increase in sodium transport, which, at variance with GLUT4 translocation (24), was induced by addition of permeant PIP3 alone. When A6 cells were incubated with permeant PIP3, we detected phosphorylation on the Thr 308 residue but not on the Ser 473 residue. Furthermore, the reduction of PKB phosphorylation observed in PTEN-overexpressing cells stimulated with insulin was more pronounced on the Thr 308 residue than on the Ser 473 residue. This agrees with the reported greater sensitivity to PIP3 of the Thr 308 residue (39) than the Ser 473 residue.

In conclusion, the present study demonstrates that PIP3 and to a lesser extent PI(3,4)P2 mediate the increase in sodium transport induced by insulin in A6 epithelium. Furthermore, insulin also phosphorylates PKB. This downstream effector of PIP3 could represent an important intermediate step leading to the increase in sodium transport. Increased sodium reabsorption by the distal nephron under the influence of hyperinsulinemia could then constitute the pathological factor responsible for arterial hypertension in the metabolic X syndrome.

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