Phosphatidylinositol 3-kinase (PI 3-kinase) is a cytoplasmic signaling molecule that is recruited to activated growth factor receptors and has been shown to be involved in regulation of stimulated exocytosis and endocytosis. One of the downstream signaling molecules activated by PI 3-kinase is the protein kinase Akt. Previous studies have indicated that PI 3-kinase is necessary for basal Na⁺/H⁺ exchanger 3 (NHE3) transport and for fibroblast growth factor-stimulated NHE3 activity in PS120 fibroblasts. However, it is not known whether activation of PI 3-kinase is sufficient to stimulate NHE3 activity or whether Akt is involved in this PI 3-kinase effect. We used an adenoviral infection system to test the possibility that activation of PI 3-kinase or Akt alone is sufficient to stimulate NHE3 activity. This hypothesis was investigated in PS120 fibroblasts stably expressing NHE3 after somatic gene transfer using a replication-deficient recombinant adenovirus containing constitutively active catalytic subunit of PI 3-kinase or constitutively active Akt. The adenovirus construct used was engineered with an upstream edcsyone promoter to allow time-regulated expression. Adenoviral infection was nearly 100% at 48 h after infection. Forty-eight hours after infection (24 h after activation of the edcsyone promoter), PI 3-kinase and Akt amount and activity were increased. Increases in both PI 3-kinase activity and Akt activity stimulated NHE3 transport. In addition, a membrane-permeant synthetic 10-mer peptide that binds polyphosphoinositides and increases PI 3-kinase activity similarly enhanced NHE3 transport activity and also increased the percentage of NHE3 on the plasma membrane. The magnitudes of stimulation of NHE3 by constitutively active PI 3-kinase, PI 3-kinase peptide, and constitutively active Akt were similar to each other. These results demonstrate that activation of PI 3-kinase or Akt is sufficient to stimulate NHE3 transport activity in PS120/NHE3 cells.

This process explains basal ileal NaCl absorption and the increase in ileal sodium absorption that occurs after meals, and it is also the sodium absorptive process in ileal sodium absorbing cells that is inhibited in most diarrheal diseases. NHE3 is the component of neutral NaCl absorption that has been shown to be acutely stimulated and inhibited under these conditions. NHE3 activity is regulated by multiple growth factors and protein kinases, which mimic the changes associated with the digestive process (1). The mechanisms of inhibition and stimulation are only partially defined. For instance, EGF and clonidine stimulate ileal sodium absorbing cells by increasing the percentage of total NHE3 in the brush border (2). In PS120 fibroblasts stably expressing NHE3, FGF exposure increases NHE3 transport activity and the percentage of total NHE3 in the plasma membrane (3, 4). The percent increase in Na⁺/H⁺ exchange is quantitatively similar to the increase in percentage of NHE3 on the plasma membrane. In contrast, protein kinase C inhibition of NHE3 in Caco-2 cells is associated with a decrease in percentage of NHE3 on the plasma membrane, but in this case, the change in transport exceeds the change in percentage of surface NHE3, indicating change in turnover number in addition to change in NHE3 trafficking (5).

Insights are just beginning to be achieved in identifying the signal transduction processes involved in short-term regulation of NHE3. For instance, phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to be necessary for 1) the basal level of plasma membrane NHE3 amount and NHE3 transport activity (3, 6, 7) and 2) EGF/FGF stimulation of NHE3 activity in PS120 fibroblasts and Caco-2 cells (3). PI 3-kinase is a cytoplasmic signaling molecule that is recruited to activated growth factor receptors (8–10). Activation of PI 3-kinase results in increased intracellular levels of 3’-phosphorylated inositol phospholipids and induction of signaling responses, including the activation of the protein kinase Akt, which is also known as protein kinase B (11, 12). Activation of PI 3-kinase plays a role in growth factor signaling cascades, leading to metabolic and mitogenic cellular responses. In addition, PI 3-kinase activity has been implicated in regulated exocytosis and endocytosis. For instance, its activation is sufficient to stimulate glucose transporter 4 (GLUT4) translocation to the plasma membrane in 3T3-L1 adipocytes in the absence of insulin (13).

The conclusions of the above previous studies of PI 3-kinase and NHE3 were largely based on pharmacological approaches; inhibition of PI 3-kinase by wortmannin and LY294002 were
Akt is sufficient to stimulate NHE3 in a fibroblast cell model. This study demonstrates that activation of PI 3-kinase and stimulates PI 3-kinase activity similarly stimulated NHE3 (14, adenoviral expression system can induce significant increases
3-kinase or Akt. These studies demonstrate that an inducible
ation-deficient adenovirus containing constitutively active PI
cells after somatic gene transfer using recombinant, replica-
E3V cells (generously provided by J. Noel, University of Montreal)
were maintained in Dulbecco's modified Eagle's medium supplemented
with 25 mM NaHCO3, 10 mM HEPES, penicillin (50 IU/ml), streptomycin
and 10% fetal bovine serum in a 5% CO2/95% O2 humidified incubator at 37 °C. For all experiments, cells were grown on glass coverslips and studied after being serum-starved for 3 days after reaching
confluence. Both the OK/E3V and PS120/E3V cell lines were selected for Na+ exchange activity (every other passage) by exposing
cells to an acid load consisting of 50 mM NH4Cl/94 mM NaCl solution for
1 h, followed by an isotonic 2 mM Na+ solution as described (20). CRE8
cells were grown in Dulbecco's modified Eagle's medium supplemented
with 2 mM GlutaMAX (Invitrogen), 15 mM HEPES (pH 7.4), 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO2/95% O2 humidified incubator at 37 °C.

**Fig. 1.** Linear representation of the ecdysone-inducible adenovirus constructs described in this study. Schematic representations of the adenovirus vector AdVgRXR (receptor virus) and the viral constructs AdEp110*-Myc, AdE myr-Akt-HA, and AdEGI are shown. φ, packing signal; Ecd promoter, ecdysone-inducible promoter; ITR, inverted terminal repeat; RSV, Rous sarcoma virus; VgEcR, modified ecdysone receptor; pA, SV40 polyadenylation signal; MCS, multiple cloning site.

**MATERIALS AND METHODS**

The synthetic rhodamine-linked 10-mer peptide, to be referred to as PI 3-kinase peptide, that is based on the phosphoinositide binding sequence of gelsolin was a gift from P. Janmey (14, 15). Nigericin, Hoechst dye 33342, and 2',7'-bis(2-carboxy-ethyl)-5(6)-carboxyfluorescein acetoxyethyl ester were obtained from Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence reagents were from PerkinElmer Life Sciences. Thin-layer chromatography (TLC) plates were from Merck. γ-32P]ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences. XAR-5 film was obtained from Amersham Pharmacia Biotech. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Life Technologies, Inc. Monoclonal mouse antibodies to the influenza virus HA (HA1) and Myc epitopes (9E10) were from Babco (Berkeley, CA). Phosphospecific-Akt antibody was from New England BioLabs, Inc. (Beverly, MA). A polyclonal anti-p110 PI 3-kinase antibody and protein A-Sepharose were from Upstate Biotechnology Inc. (Lake Placid, NY). Peroxidase-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. The general anti-Akt antibody was previously reported by Tsichlis (16, 17). The NHS-SS-biotin was from Pierce. Other agents were from Sigma.

**Cell Culture**—Stably transfected full-length rabbit NHE3V (NHE3 with epitope of vesicular stomatitis virus G protein on the C terminus, as previously described (18)) in Chinese hamster lung-derived fibroblasts, the PS120/E3V cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 25 mM NaHCO3, 10 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO2/95% O2 humidified incubator at 32 °C, as described (19). OK/E3V cells (generously provided by J. Noel, University of Montreal) were maintained in Dulbecco's modified Eagle's medium supplemented with 25 mM NaHCO3, 10 mM HEPES, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum in a 5% CO2/95% O2 humidified incubator at 37 °C. For all experiments, cells were grown on glass coverslips and studied after being serum-starved for 3 days after reaching
confluence. Both the OK/E3V and PS120/E3V cell lines were selected for Na+ exchange activity (every other passage) by exposing
cells to an acid load consisting of 50 mM NH4Cl/94 mM NaCl solution for
1 h, followed by an isotonic 2 mM Na+ solution as described (20). CRE8
cells were grown in Dulbecco's modified Eagle's medium supplemented
with 2 mM GlutaMAX (Invitrogen), 15 mM HEPES (pH 7.4), 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO2/95% O2 humidified incubator at 37 °C. **Plasmid Vector Construction**—Mammalian expression vectors direct-
ing the expression of Myc-tagged p110* (PI 3-kinase) and HA-tagged
myr-Akt constructs, as well as these inserted constructs, were described
(21–24). The adenovirus shuttle vector used which contains an eda-
sys-inducible promoter (pAdEcd), and pAdVgRXR encoding the edc-
sysy and RXR receptors were as described (Fig. 1) (25). The p110*
encodes for a constitutively active form of PI 3-kinase in which the inter-SH2 domain of the p55 regulatory subunit was ligated to the NH2
terminus of the p110 catalytic subunit of PI 3-kinase. The p110* protein
was tagged at the COOH terminus with the Myc epitope (22). In order
to subclone the epitope-tagged p110*-Myc from a mammalian expres-
sion vector to pAdEcd, BamHI-digested p110*-Myc DNA was ligated into the multiple cloning site of pAdEcd. The plasmid containing the
BamHI fragment in pAdEcd, the pAdEcd-BamHI plasmid, was digested with Xhol and NheI, and then the Xhol fragment from the p110*-Myc construct was subcloned into pAdEcd-BamHI. The Akt construct con-
tains the HA epitope-tagged to its C terminus and is catalytically active
due to its myristylation-related membrane location. The myr-Akt-HA plasmid was digested with HindIII and EcoRI, and the DNA fragment
was subcloned into the multiple cloning site of pAdEcd for construction
of pAdEmy-yr-Akt-HA (Fig. 1). The expression cassette of eGFP from pEGFP was subcloned into the multiple cloning site of pAdEcd, making
pAdEcd (Fig. 1), which expressed enhanced green fluorescence
protein and was used to assess adenovirus infection efficiency. **Recombinant Adenoviral Vectors**—The recombinant adenoviruses containing the cDNA encoding either the C-terminal Myc epitope-
tagged constitutively active form of PI 3-kinase (AdEp110*-Myc) or
myristoylated Akt-HA (AdEmyr-Akt-HA) were generated by Cre lox
recombination (25, 26) using CRE8 cells that stably express Cre recom-
binase. Construction of recombinant adenovirus AdEp110*-Myc and
AdEmyrrAkt-HA was as described (25) as was determination of multiplicity of infection ( moi ) (27).

Adenoviral Infections—PS120/E3V cells were co-infected with AdVgXRK plus (ii) AdEp110*-Myc, (ii) AdEmyrrAkt-HA, (iii) AdEGI, or (iv) empty virus (Fig. 1) in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and the appropriate amounts of sodium chloride, NaCl, and 20 mM Na3EDTA. Cells were washed three times with ice-cold sodium chloride-buffered saline (150 mM NaCl and 20 mM Na3EDTA, pH 7.4) and once in borate buffer (154 mM NaCl, 1.0 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl2, pH 9.0). Plasma membrane surface was then exposed to 0.5 mg/ml sulfo-NHS-S-S-biotin in borate buffer for 40 min with horizontal shaking. After labeling, cells were washed with the quenching buffer (20 mM Tris and 120 mM NaCl, pH 7.4) to scavenge the unbound biotin. Cells were washed three times with ice-cold sodium chloride-buffered saline and lysed in 1 ml of N+ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na3EDTA, 3 mM EGTA, and 1% Triton X-100). Cells were sonicated for 20 s and agitated on arotating rocker for 30 min at 4 °C. Insoluble cell debris was removed by centrifugation for 30 min at 12,000 ⋅ g. Supernatant representing the total fraction was incubated with avidin-agarose for 2 h. After avidin precipitation, the supernatant was retained as the intracellular fraction. The avidin-agarosebeads were washed five times in N+ buffer to remove all of the nonspecifically bound proteins. The avidin-agarose-bound proteins, representing plasma membrane NHE3, were solubilized in equivalent volumes of loading buffer (5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol), boiled for 5 min, size-fractionated by SDS-polyacrylamide gel electrophoresis, and then electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk, the blots were probed with a monoclonal anti-VSV-G antibody (PS14 hybridoma supernatant) as the primary antibody and horseradish peroxidase-conjugated anti-mouse as the secondary antibody. Bands were visualized by enhanced chemiluminescence.

RESULTS

Standardization of Adenoviral Infection in PS120/E3V Cells—The adenoviral infection efficiency in PS120/E3V cells was initially standardized to achieve nearly 100% infection 48 h after viral exposure and after activation of the ecdysone promoter with ponasterone A. The percentage of infection was evaluated with different moieties of adenosivirus expressing eGFP as a reporter protein. To estimate the percentage of infected cells, PS120/E3V cells were co-infected with various moieties of edysyne-driven receptors encoding eGFP (AdEGI) plus fixed edysyne-retinoid X receptor virus (AdVgXRK). After 24 h, ponasterone A (3 μm) was added to the media; 24 h later (48 h after infection), cells were fixed, and nuclei were stained with Hoechst 33342. As shown in Fig. 2, nuclear staining and cytosolic eGFP fluorescence were visible in PS120/E3V cells. Comparison was made between the number of cells with cytosolic eGFP fluorescence and the total number of PS120/E3V cells stained with Hoechst 33342 in the same field. Low basal levels of expression of eGFP occurred in the absence of ponasterone A at the different adenosivirus moieties indicating low level of “leakiness.” eGFP expression increased in a moi-dependent manner after addition of ponasterone A (3 μm) (Fig. 2), indicating that the infection percentages increased in a viral dose-dependent manner in the presence of ponasterone A. When the cells were co-infected with both AdEGI ( moi = 4.0) plus AdVgXRK ( moi = 0.8) for 48 h and incubated for the last 24 h in the presence of ponasterone A, ~90% of cells were eGFP-positive. These conditions were used for further experiments. PI 3-kinase and Akt activity and amount and NHE3 activity were measured under these conditions in the absence or presence of ponasterone A.

Expression of p110*-Myc and myr-Akt-HA Proteins—PS120/E3V cells were co-infected with recombinant adenosivirus encoding p110*-Myc (AdEp110*-Myc) or myr-Akt-HA (AdEmyrrAkt-HA) plus receptor virus AdVgXRK and were studied in the absence and presence of ponasterone A. Western blot analysis of total lysates were used to identify the presence of fusion proteins of p110*-Myc and myr-Akt-HA, using monoclonal antibodies directed against the Myc or HA epitope tags. Fig. 3 shows the results of an anti-Myc blot for p110*-protein and an

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anti-HA blot for Akt protein. The level of expression of p110*-Myc and myr-Akt-HA were induced 3.6- and 4.0-fold in the presence of ponasterone A and were minimally above baseline in the absence of ponasterone A, indicating minimal but present leakiness of the ecdysone promoter.

Activities of p110* and myr-Akt—To assess the activities of p110* and myr-Akt, cells were infected with either p110* or myr-Akt encoding recombinant adenoviral construct in the absence and presence of ponasterone A. In the p110* studies, cells were lysed, the lysates were immunoprecipitated with anti-p110, and the immunoprecipitate was resolubilized and used to detect the total endogenous plus exogenous PI 3-kinase activity. For Akt activity, lysates were used, and the parameter to determine Akt activity was an antibody that detects phosphorylated Ser473, which is activated Akt (phospho-Akt) (16, 17). Thus, endogenous plus exogenous Akt was detected. Activity levels of PI 3-kinase (Fig. 4, A and B) and phospho-Akt (Fig. 4, C and D) were induced in the presence of ponasterone A compared with the absence of ponasterone A. As shown in Fig. 4, A and B, PI 3-kinase activity increased ~2.3-fold with ponasterone A (increased to 223 ± 16% of control). As shown in Fig. 4, C and D, Akt activity increased ~3-fold with ponasterone A (increased to 310 ± 31% of control).

Effects of Expression of Constitutively Active PI 3-Kinase on Akt Activity—To investigate whether activated PI 3-kinase is sufficient to stimulate Akt activity, the AdEp110*-Myc recombinant adenoviral construct was infected in PS120/NHE3V cells. The amount of phosphorylated Akt was detected by anti-HA blot for Akt protein. The level of expression of p110*-Myc and myr-Akt-HA were induced 3.6- and 4.0-fold in the presence of ponasterone A and were minimally above baseline in the absence of ponasterone A, indicating minimal but present leakiness of the ecdysone promoter.

Activities of p110* and myr-Akt—To assess the activities of p110* and myr-Akt, cells were infected with either p110* or myr-Akt encoding recombinant adenoviral construct in the absence and presence of ponasterone A. In the p110* studies, cells were lysed, the lysates were immunoprecipitated with anti-p110, and the immunoprecipitate was resolubilized and used to detect the total endogenous plus exogenous PI 3-kinase activity. For Akt activity, lysates were used, and the parameter to determine Akt activity was an antibody that detects phosphorylated Ser473, which is activated Akt (phospho-Akt) (16, 17). Thus, endogenous plus exogenous Akt was detected. Activity levels of PI 3-kinase (Fig. 4, A and B) and phospho-Akt (Fig. 4, C and D) were induced in the presence of ponasterone A compared with the absence of ponasterone A. As shown in Fig. 4, A and B, PI 3-kinase activity increased ~2.3-fold with ponasterone A (increased to 223 ± 16% of control). As shown in Fig. 4, C and D, Akt activity increased ~3-fold with ponasterone A (increased to 310 ± 31% of control).

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Activities of p110* and myr-Akt—To assess the activities of p110* and myr-Akt, cells were infected with either p110* or myr-Akt encoding recombinant adenoviral construct in the absence and presence of ponasterone A. In the p110* studies, cells were lysed, the lysates were immunoprecipitated with anti-p110, and the immunoprecipitate was resolubilized and used to detect the total endogenous plus exogenous PI 3-kinase activity. For Akt activity, lysates were used, and the parameter to determine Akt activity was an antibody that detects phosphorylated Ser473, which is activated Akt (phospho-Akt) (16, 17). Thus, endogenous plus exogenous Akt was detected. Activity levels of PI 3-kinase (Fig. 4, A and B) and phospho-Akt (Fig. 4, C and D) were induced in the presence of ponasterone A compared with the absence of ponasterone A. As shown in Fig. 4, A and B, PI 3-kinase activity increased ~2.3-fold with ponasterone A (increased to 223 ± 16% of control). As shown in Fig. 4, C and D, Akt activity increased ~3-fold with ponasterone A (increased to 310 ± 31% of control).
FIG. 4. Effects of overexpression of p110* on PI 3-kinase activity and Myr-Akt on Akt activity. Cells were infected with AdEp110*-expressing or Ad-Emyr-Akt-expressing adenovirus at an moi of 4 in media containing 2% serum for 24 h and then incubated in the absence or presence of ponasterone A (3 μM) for 24 h. A, AdEp110*-myr-infected cells were lysed and subjected to immunoprecipitation with p110 antibodies. The washed immunoprecipitates were assayed for PI 3-kinase activity with phosphatidylinositol as substrate, and the labeled PI 3-phosphate product (PI-3P) was resolved by thin layer chromatography and visualized by autoradiography. B, results of PI 3-kinase activity from three experiments similar to those in A. Results are expressed as the percentages of uninduced ± S.E. PI 3-kinase activity. C, AdEmyr-Akt-HA cells were lysed and assayed for Akt activity. Activity level was measured with phosphospecific Akt antibody. D, results of amount of phospho-Akt from three experiments similar to those in C. Results are expressed as the percentages of uninduced ± S.E. Akt kinase activity.

Stimulatory Effects of Constitutively Active PI 3-Kinase and Akt on NHE3 Activity and Amount—Recently, we and others have reported that activation of PI 3-kinase is a necessary step in FGF stimulation of NHE3 in PS120/E3V and AP-1 cells (3, 4, 6, 7, 32). To investigate whether activation of PI 3-kinase or Akt is sufficient for activation of NHE3 transporter activity, NHE3 activity was determined in AdEp110*-Myc-infected and AdEmyr-Akt-HA-infected cells in the absence and in the presence of ponasterone A. The expression level of total cell NHE3 was very similar among the infected lines and uninfected control (Fig. 6A), despite changing the amount and activity of PI 3-kinase and Akt. The rates of Na+/H+ exchange in acid-loaded cells were measured by the rate of pH recovery with Na+ addition. A comparison of the transport with ponasterone A induction of p110* and Akt is shown in Fig. 6, B and C, respectively. NHE3 activity was stimulated by 21.5 ± 4.0% above control after induction of p110* and 22.0 ± 3.1% after induction of myr-Akt protein (Fig. 6D). There was no significant effect of viral infection with PI 3-kinase or Akt in the absence of ponasterone A compared with viral infection with empty control virus (V5) (Fig. 6, B and C). To further demonstrate the role of constitutively active PI 3-kinase in increasing the NHE3 activity, AdEp110*-Myc-infected PS120/E3V cells were preincubated with the PI 3-kinase inhibitor wortmannin for 30 min prior to study of Na+/H+ exchange rate. Wortmannin (100 nM) pretreatment inhibited the NHE3 activity in both the presence and absence of ponasterone A in PS120/E3V cells (Fig. 6E). These results demonstrate that the expression of either constitutively active PI 3-kinase or Akt is sufficient to stimulate NHE3 transporter activity without changing the total amount of NHE3. Moreover, PI 3-kinase is involved in NHE3 stimulation under basal conditions.

Stimulation of NHE3 Transport by PI 3-Kinase Peptide-induced Activation of PI 3-Kinase—A second method of increasing PI 3-kinase activity was studied for effects on NHE3 activity without plasma membrane receptor activation. We used a membrane-permeant synthetic peptide (a rhodamine-linked synthetic 10-mer) that is modeled on the polyphosphoinositide binding sequence of gelsolin and has been shown to increase PI 3-kinase activity. Incubation of synthetic peptide (10 μM) for 15 min increased NHE3 transport in PS120 cells and OK cells (Fig. 7, A–D). The latter was studied to expand the findings to a polarized epithelial cell with brush border NHE3 (34). The magnitude of stimulation of NHE3 activity in PS120 cells was similar in magnitude to the stimulation by constitutively active PI 3-kinase (118 ± 8% of control, p < 0.05). PI 3-kinase peptide also stimulated NHE3 activity in OK cells (132 ± 12% of control, p < 0.05). To determine whether this increase in ac-
activity is due to an increase in plasma membrane NHE3 protein, cell surface biotinylation was used. Plasma membrane protein were biotinylated by reaction with sulfo-NHS-SS biotin at 4°C and isolated by precipitation with streptavidin-bound agarose. Plasma membrane NHE3 was then identified by immunoblot.

The addition of PI 3-kinase peptide (10^9/H9262M) to PS120/E3V cells for 15 min increased plasma membrane NHE3 from 10.9% of total (without peptide) to 20.8% (with peptide) (Fig. 8), whereas total NHE3 abundance did not change. These results support the conclusion that the activation of PI 3-kinase is sufficient to stimulate NHE3 activity.

**DISCUSSION**

In this study, the question was asked whether the activation of PI 3-kinase or Akt was sufficient to stimulate NHE3 in PS120 cells. Previous studies demonstrated a role for PI 3-kinase in rapid stimulation of NHE3 (3, 6, 7, 32), but there have been no previous studies implicating Akt in regulation of NHE3. PI 3-kinase activity has previously been shown to be necessary for basal NHE3 activity in the polarized epithelial cell lines Caco-2 and OK, as well as in the fibroblast cell line PS120 and in AP-1 cells, and also in EGF/FGF stimulation of NHE3 in fibroblasts and EGF stimulation of NHE3 in ileal brush border (3, 6, 32). Based on inhibitor studies in PS120, AP-1, OK, and Caco-2 cells, basal NHE3 activity is lowered by the PI 3-kinase inhibitor wortmannin (3, 6, 7). EGF/FGF stimulation of NHE3 is blocked in PS120 cells by the same inhibitors (1, 3, 7). FGF stimulated NHE3 in PS120 cells by 50%. 50% of this stimulation appears to be due to PI 3-kinase because wortmannin caused a 50% reduction of this stimulation (3). Thus, FGF stimulates NHE3 by both PI 3-kinase-dependent and PI 3-kinase-independent mechanisms (1, 3, 7).

Whether increased PI 3-kinase or AKT activity were sufficient to stimulate NHE3 had not been addressed before this study. However, this issue has begun to be studied in regards to the glucose transporter GLUT4 in adipocytes and smooth muscle cells. Insulin and growth factor stimulate glucose uptake and GLUT4 translocation to the plasma membrane by a process associated with activation and movement to the plasma membrane of PI 3-kinase (35–37). Both glucose uptake and GLUT4 translocation are inhibited by the PI 3-kinase inhibitors wortmannin and LY290042 (23, 28). Thus, PI 3-kinase activation is necessary for the insulin stimulation of glucose uptake.

**FIG. 6.** Expression of constitutively active PI 3-kinase or Akt is sufficient to stimulate NHE3 activity without changing NHE3 amount and Inhibition of NHE3 Activity by Wortmannin. Cells were infected with AdEp110*-Myc-expressing or AdEmyr-Akt-HA-expressing recombinant adenovirus at an moi of 4 in media containing 2% serum for 24 h and incubated in the absence or presence of ponasterone A for 24 h. Empty adenovirus (pH90235) without the p110*-Myc or Akt-HA coding region was studied as a control. Following infection, PS120/NHE3 cells were acidified by NH4CI and allowed to recover in the presence of sodium medium to a steady-state pH. A, the effect of infection with AdEp110*-Myc-expressing or AdEmyr-Akt-HA-expressing adenovirus in the absence and presence of ponasterone A on amount of the NHE3 transporter was measured. The amount of expression of NHE3 was not altered by expression of constitutively active PI 3-kinase or Akt. The data are representative of three independent experiments. B and C, measurement of NHE3 activity was performed after expression of adenovirus (pH5), AdEp110*-Myc or AdE myr-Akt-HA adenovirus, and results shown refer to in the absence or presence of ponasterone A. A single representative study is shown. D, mean Na+/H+ exchange rates rate ± S.E. are shown normalized to the minus ponasterone condition. NHE3 transporter activity was stimulated 21.5 ± 4.0% above control by the expression of p110*-Myc and 22 ± 3.1% above control by the expression of myr-Akt-HA. E, AdEp110*-Myc adenovirus-infected cells in the absence or presence of ponasterone were incubated with 100 nM of wortmannin for 30 min prior to determination of the Na+/H+ exchange rate. A single experiment is shown that was repeated twice with similar results.
uptake and GLUT4 translocation. Initial studies demonstrated that constitutively active PI 3-kinase expression (p110) increased both glucose uptake and GLUT4 translocation in the absence of insulin, and dominant negative PI 3-kinase (p85) inhibited insulin-stimulated glucose uptake and GLUT4 translocation (38–40). These data were contradictory, however, concerning whether the magnitude of the insulin stimulation of glucose uptake was duplicated by overexpression of constitutively active PI 3-kinase. Recent interpretation is that increasing PI 3-kinase activity alone is quantitatively less than the insulin stimulation of glucose uptake (38, 41). In addition, increasing PI 3-kinase by separate mechanisms, including activation with platelet-derived growth factor and integrin receptor stimulation, did not increase glucose uptake at all (42, 43). Moreover, exposure to membrane-permeant PIP3, which is converted intracellularly to end products of activated PI 3-kinase, failed to alter glucose transport in the absence of insulin (44). These results are currently interpreted as indicating that whereas activated PI 3-kinase is sufficient to induce a partial stimulation of GLUT4-related glucose uptake and membrane translocation, a second (or more) insulin-stimulated non-PI 3-kinase-dependent pathway(s) is necessary to reproduce the full insulin stimulation of glucose uptake (42, 43, 45). A candidate second pathway has been described with the recognition that insulin brings the adapter protein CAP to the insulin receptor, where it recruits Cbl and interacts with flotillin resulting in phosphorylation of Cbl and direction of the Cbl-CAP complex to lipid rafts (or caveolae) in the plasma membrane (47).

Our results demonstrate that stimulation of PI 3-kinase activity either by use of a peptide stimulator of PI 3-kinase activity (14) or transient infection with adenovirus containing constitutively active PI 3-kinase increases NHE3 activity. This stimulation is similar in magnitude to the PI 3-kinase-dependent component of rapid growth factor stimulation of NHE3 (3). The quantitative similarity suggests that an increase in PI 3-kinase activity is not only sufficient to increase NHE3 activity but that this component of the growth factor stimulation is entirely due to the increase in PI 3-kinase activity. The growth factor-stimulated messenger that initiates the PI 3-kinase-dependent growth factor stimulation of NHE3 remains unidentified.

Thus, similarities and differences between insulin stimulation of GLUT4 and growth factor stimulation of NHE3 have been identified. Both involve stimulation by trafficking and increases in exocytosis, with PI 3-kinase being necessary and sufficient for the stimulation. In addition, there is a second component of stimulation in addition to a pathway mediated through PI 3-kinase. The recently recognized pathway of insulin stimulation of GLUT4 that may include CAP-flotillin-Cbl...
has not been studied in regulation of NHE3, and no additional specific factor has been identified for the growth factor stimulation of NHE3. Concerning differences between growth factor stimulation of NHE3 and insulin stimulation of GLUT4, NHE3 trafficking under basal conditions is more than for GLUT4, which is nearly entirely intracellular. This basal stimulation of NHE3 is also PI 3-kinase-dependent. This difference is not surprising in that NHE3 is rapidly regulated by both stimulation and inhibition in the intestine and kidney, whereas regulation of glucose uptake is stimulated by insulin, and no inhibitory mechanism from basal rate has yet been identified. Importantly all GLUT4 mobilization is PI 3-kinase-dependent, while only part of NHE3 depends on PI 3-kinase. Another difference of the stimulatory mechanisms of GLUT4 and NHE3 is the suggestion that insulin mobilizes glucose from both the recycling endosomes and a special storage compartment, whereas growth factor mobilization of NHE3 has only been suggested as coming from the recycling compartment (48), with no specific storage compartment for NHE3 yet identified.

The current studies also represent the initial demonstration of involvement of Akt in growth factor stimulation of NHE3. Akt represents a major downstream signaling molecule in the PI 3-kinase cascade. In unpublished studies, we have shown that EGF stimulation of ileal NaCl absorption and brush border NHE3 activity is associated with a rapid increase in brush border Akt activity. Due to lack of available Akt inhibitors, the functional significance of this stimulation could not be determined. These studies show that increasing Akt activity and amount stimulates NHE3. In addition, the similarity in magnitude of stimulation of NHE3 with transient transfection with constitutively active PI 3-kinase and Akt mutants and the fact that increasing PI 3-kinase activity stimulates Akt activity without altering Akt amount suggests that most, if not all, of the PI 3-kinase stimulation of NHE3 may be due to activation of Akt in PS120 cells.

Results assessing the contribution of AKT to insulin/PI 3-kinase stimulation of glucose uptake in adipocytes and muscle cells indicates that AKT activation is necessary and appears sufficient to stimulate glucose uptake. Constitutively active AKT (using several different constitutively active constructs different than that used in our studies consisting of Myr/–129 Akt (in which the Akt plextin homology domain is deleted) and a v-Akt analogue (called Gag-protein kinase B) stimulated glucose uptake and GLUT4 plasma membrane translocation. The magnitude of the effects was between 69 and 100% of the insulin stimulation (49–51). However, disagreement exists in that studies performed with several dominant negative AKT constructs either inhibited most of the insulin-stimulated glucose uptake or had inhibitory effects of only 0–20% (52–54).

Other systems, in which the downstream signaling of PI 3-kinase involves Akt, have had additional downstream signals identified. In addition to Akt, atypical forms of protein kinase C (especially ζ and λ), but also the conventional isoforms β-2 and the novel isoform δ have been shown to represent downstream signaling molecules following PI 3-kinase activation in some cells (46). The current studies have not considered involvement of protein kinase C in the PI 3-kinase stimulation of NHE3. However, the similarity in magnitude of FGF stimulation of NHE3 and effects of constitutively active PI 3-kinase and Akt, as well as similarity in the stimulation of NHE3 with the
increase in Akt activation when constitutively active PI 3-kinase is transfected, make it likely that the contribution of atypical forms of protein kinase C to PI 3-kinase stimulation of NHE3 is small, if it is present at all, in these cells.

Attempts were made to examine the contribution of PI 3-kinase and Akt in regulation of NHE3 by expressing dominant negative forms using the same adenoviral expression system used for these activation studies. The constructs used were described previously and consisted of p110α mutated in the kinase domain and Akt-ΔA (T308A/S473A) (12, 53). In both cases, there was significant loss of cell viability, which we presumed, but did not demonstrate, was due to induction of apoptosis.

The adenovirus infection system was used for transient infection in order to develop a method of obtaining transient expression in a high percentage of cells to allow correlation of biochemistry and functional data, in this case, NHE3 transport. The motivation for developing an inducible system was that in studying molecules that alter cell division and state of differentiation, we wanted to be able to select conditions in which changes in the differentiation status of cells studied could be minimized by controlling the time of expression of selected signaling molecules. Thus, after use of eGFp as a marker to determine conditions to infect nearly all cells, we selected a time window of expression of the signaling molecule of interest. The level of expression of PI 3-kinase due to the inducible expression was not compared with the endogenous level; however, the increase in activity due to ponasterone A was similar to that which occurred with growth factor exposure (17, 32). In addition, our studies documented the low level of leakage in this expression system in the absence of activation of the edcsyme receptor with ponasterone A (Fig. 3). Thus, we suggest the usefulness of this inducible adenovirus system for expressing signaling molecules that are to be activated at a relatively fixed time and studied over a limited period. In addition, the relatively small percentage increase in amount of expressed protein may prove to be an advantage compared with other methods that lead to problems from large amounts of protein expression.

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Constitutively Active Phosphatidylinositol 3-Kinase and AKT Are Sufficient to Stimulate the Epithelial Na\(^+\)/H\(^+\) Exchanger 3
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