3,3′,5-Triiodo-L-thyronine Up-regulation of Na,K-ATPase Activity and Cell Surface Expression in Alveolar Epithelial Cells Is Src Kinase- and Phosphoinositide 3-Kinase-dependent*

Received for publication, May 17, 2004, and in revised form, August 26, 2004
Published, JBC Papers in Press, August 31, 2004, DOI 10.1074/jbc.M405497200

Jianxun Lei, Cary N. Mariash, and David H. Ingbar‡

From the Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

We previously reported that thyroid hormone, 3,3′,5-triiodo-L-thyronine (T3), increased Na,K-ATPase activity of adult rat alveolar epithelial cells in a transcription-independent manner via increased cell surface expression of the α1 and β1 subunits of Na,K-ATPase. Now we sought to identify signaling molecules necessary for T3 stimulation of Na,K-ATPase activity in alveolar epithelial cells. Whereas protein kinase A inhibitor H-8 and protein kinase C inhibitor bisindolylmaleimide did not block the T3-induced increase in Na,K-ATPase activity, two inhibitors of phosphoinositide 3-kinase (PI3K), wortmannin and Ly294002, and two Src kinase inhibitors, PP1 and PP2, blocked the T3-induced Na,K-ATPase activity. T3 stimulated the activity of PI3K as measured by phosphatidylinositol 3-phosphate. T3 also stimulated the serine 473 phosphorylation of the PI3K downstream molecule PKB/Akt in a dose-dependent manner. Transient expression of a constitutively active mutant of the PI3K catalytic subunit p110 augmented Na,K-ATPase activity and increased the amount of cell surface Na,K-ATPase α subunit protein. T3 also stimulated Src family kinase activity. Transient expression of a constitutively active Src kinase increased Na,K-ATPase activity, PI3K activity, and phosphorylation of PKB/Akt at serine 473. PP1 or PP2 blocked T3-stimulated PKB/Akt phosphorylation at serine 473 and PI3K activity that was activated by an active mutant of Src; however, wortmannin did not inhibit the T3-stimulated Src kinase activity. Although PP1 and wortmannin abolished the increase in Na,K-ATPase activity induced by the active mutant of Src, PP1 did not inhibit the active mutant of PI3K-up-regulated Na,K-ATPase activity. In summary, T3 stimulates the PI3K/PKB pathway via the Src family of tyrosine kinases, and activation of both the Src family kinases and PI3K is required for the T3-induced stimulation of Na,K-ATPase activity and its cell surface expression in adult rat alveolar epithelial cells.

The integral membrane protein Na,K-ATPase is critical for maintaining cellular ion gradients across the plasma membrane and the cell volume of most animal cells (1). In lung, the Na,K-ATPase in alveolar epithelial cells (AECs) is essential for active transepithelial resorption of Na⁺ ions and fluid clearance from alveolar space (2). The accumulation of fluid in alveolar space, as occurs in the acute respiratory distress syndrome, is a life-threatening condition because the fluid impairs alveolar oxygen action.

The Na,K-ATPase consists of at least two subunits (α and β), each with several isoforms. The Na,K-ATPase isoforms are expressed in a tissue- and development-specific fashion and are believed to be distinct in both function and modes of regulation (3). The α1 isoform is predominant in most epithelia, including rat lung (4). The Na,K-ATPase activity is differentially regulated in a hormone- and tissue-specific fashion (1). In lung epithelial cells, some investigators have found that β-adrenergic agonists up-regulate the Na,K-ATPase activity via PKA (protein kinase A) and MAPK/ERK (mitogen-activated protein kinases/extracellular signaling related kinase) (5). In AECs, Dopamine activates Na,K-ATPase and stimulates translocation of Na,K-ATPase molecules from intracellular compartments to plasma membrane via activation of novel PKC isoforms PKC-δ and PKC-ε (6). In contrast, in renal epithelial cells, dopamine inhibits Na,K-ATPase activity via removal of active Na,K-ATPase molecules from the plasma membrane into early and late endosomes (7). The PKC-ζ-dependent phosphorylation of the Na,K-ATPase α subunit and activation of phosphoinositide 3-kinase are necessary for the dopamine-induced endocytosis of Na,K-ATPase in renal cells (7, 8). Much information about the Na,K-ATPase regulation is available in several systems (1). However, due to the hormone- and tissue-specific regulation of Na,K-ATPase, the mechanisms regulating Na,K-ATPase enzyme activity in many tissues, including the lung, are not completely understood. Specifically, the signal transduction pathway(s) involved in short time regulation of Na,K-ATPase function are incompletely defined.

Thyroid hormones regulate normal cell function and differentiation by interacting with intracellular thyroid hormone receptors, and transcriptional regulatory factors (coactivators and corepressors) (9). It has recently been recognized that thyroid hormones also may rapidly generate biological responses by nongenomic mechanisms that are independent of nuclear receptors for T3 (9). The signal transduction pathways of the nongenomic effects of thyroid hormones are just beginning to be defined. Thyroid hormone (T4) recently was reported to activate the mitogen-activated protein kinase in HeLa and...
CV-1 cells (10). The T3-induced activity of Na\(^+/\)H\(^-\) exchanger and system A amino acid transport in chick embryo hepatocytes was blocked by inhibitors of protein kinase C and PI3K (11). Thyroid hormone differentially regulates Na,K-ATPase isoforms in both tissue- and cell type-specific manners (1). Recently, we reported that in AECs T3 also stimulates rapidly the enzyme activity and cell surface expression of Na,K-ATPase in a transcription-independent manner (12); however, the signaling transduction pathway(s) is not defined. Identifying the signal transduction molecule(s) involved in T3 nontranscriptional stimulation of Na,K-ATPase would provide insight into the molecular mechanism(s) of thyroid hormone function.

To explore the signaling molecule(s) involved in the T3 effects on Na,K-ATPase, we assessed the role of several kinases...
in regulation of Na,K-ATPase by T3. We found that PI3K inhibitors or Src kinase inhibitors blocked the T3-induced stimulation of Na,K-ATPase activity. Transient overexpression of active mutant of Src or PI3K in the absence of T3 increased Na,K-ATPase activity and plasma membrane protein quantity. These data indicated that the activation of PI3K is required for the T3-stimulated Na,K-ATPase activity and Na,K-ATPase cell surface expression unlike the inhibitory role of PI3K in Na,K-ATPase regulation in renal cells (8). The present study defined the role of Src family kinases and PI3K in the T3 effects on Na,K-ATPase activity and cell surface expression in alveolar epithelial cells.

MATERIALS AND METHODS

3,3',5-triiodo-l-thyronine (T3) and protease inhibitor mixture were purchased from Sigma. The monoclonal anti-p85 subunit of PI3K and anti-phosphotyrosine (PY20) antibodies were purchased from BD Biosciences. Wortmannin, Ly294002, PP2, H-8, bisindolylmaleimide, and Biotin-X-N-hydroxysuccinimide ester (water-soluble, cleavable) were purchased from Calbiochem. PP1 was obtained from Biomol Signal Transduction. Polyclonal anti-PKB/Akt, polyclonal anti-phospho-PKB/Akt (Ser473), and polyclonal anti-phospho-Src (Tyr416) antibodies were obtained from Cell Signaling Technology. Polyclonal anti-Src was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-β of Na,K-ATPase and polyclonal anti-p85 subunit of PI3K were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Cell culture reagents, Waymouth’s MB752/1 medium, DMEM/F-12 medium, HEPES, fetal bovine serum (FBS), and antibiotics were purchased from GIBCOBRL, Invitrogen. The BCA protein assay kit was purchased from Pierce.

Isolation and Culture of Rat Alveolar Epithelial Cells—Primary alveolar type II (AT II) cells were isolated from pathogen-free adult male Sprague-Dawley rats (190–220 g) as described by Jiang et al. (13). Identification of AT II cells was based on the presence of lamellar bodies in cells. AT II cells were grown in DMEM/F-12 medium with 10% FBS and antibiotics (50 units/ml penicillin and 50 units/ml streptomycin) overnight. The cells were cultured in DMEM/F-12 with 5% stripped FBS for 24 h before T3 treatment.

Cell Culture and Transient Transfections—The adult rat ATII-like cell line MP48 (12) was a gift of G. Hunninghake (University of Iowa), and another rat ATII-like cell line RLE-6TN (36) was purchased from ATCC. Both cells exhibit characteristics of alveolar type II cells, such as lipid-containing inclusion bodies. RLE-6TN cells were maintained in DMEM/F-12 medium. MP48 cells were maintained in Waymouth’s MB752/1 medium. Both media were supplemented with 10% FBS, 40 mM HEPES, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B).

To measure the effect of T3 on the hydrolytic activity of Na,K-ATPase, MP48 cells were grown to ~95% confluence in Waymouth’s MB752/1 with 10% FBS. Then cells were cultured in Waymouth’s MB752/1 medium plus stripped 1% FBS in which endogenous T3 and glucocorticoids previously were removed by mixing serum with a resin slurry (5 g of Bio-Rad AG 1-X8 resin/100 ml of serum) as described by Samuels et al. (14). Removal of endogenous T3 by this method was confirmed using tracer amounts of radioisotope-labeled T3.

For inhibition studies, cells were preincubated with inhibitors for 30
ATPase was measured as ouabain-sensitive ATP hydrolysis under conditions by measuring the release of Pi from ATP as previously described (37). The constitutively active Src kinase (Src Y527F) in MP48 cells were transfected with LipofectAMINE PLUS Reagent (Invitrogen) or LipofectAMINE Reagent (Invitrogen) in 6-well plates using 1 or 2 μg of plasmid DNA per well according to the manufacturer’s instructions. Cells were subjected to Na,K-ATPase activity assay, protein phosphorylation, and biotinylation experiments at 48 h after transfection. The constitutively active p110 subunit in vector pSG5 has been previously described (37). The constitutively active Src kinase (Src Y527F) in pUS10amp vector was purchased from Upstate Biotechnology.

Na,K-ATPase Activity Assay—The hydrolytic activity of Na,K-ATPase was measured as ouabain-sensitive ATP hydrolysis under conditions by measuring the release of Pi from ATP as previously described (12). Na,K-ATPase specific activity was calculated as the difference in Pi concentration/mg of protein/min in the absence and presence of 10 mM ouabain. Results were compared with controls and expressed as the percentage increase or inhibition of control Na,K-ATPase activity.

Src Kinase Activity Assay—The Src kinase activity assay kit was purchased from Upstate Cell Signaling Solution. The assay kit is based on phosphorylation of a specific substrate peptide (KVEKIGETGYG-VKY) using the transfer of the [γ-32P]ATP by Src kinase. The phosphotransferase activity of Src kinases was measured in immunoprecipitates according to the manufacturer’s instructions. The immunoprecipitates for kinase activity assay were obtained by incubation of 200 μg of total cellular protein, 3 μg of antibody against Src family kinases, and 20 μl of protein A-agarose beads in 1× immunoprecipitation buffer at 4 °C for 3 h with gentle rocking. The immunoprecipitates were washed three times with 1× immunoprecipitation buffer and were then washed with Src kinase reaction buffer.

PI3K and T3 Regulation of Na,K-ATPase

**Fig. 2.** T3 activated PI3K activity in MP48 cells. MP-48 cells were starved overnight in serum-free Waymouth medium, and cells were then incubated with 10−8 M T3 for 3 h at 37 °C. The radiolabeled PI(3)P produced from PI, and [32P]ATP was analyzed by TLC and autoradiographic densitometry. A, PI3K activity was determined in immunoprecipitates with an antibody against PI3K p85. The Western blots show equal amounts of p85 protein presented in the immunoprecipitates used in the PI3K activity assay. B, PI3K activity was determined in immunoprecipitates with an antibody against phosphotyrosine. C, the densitometric values of radiolabelled PI(3)P are expressed as percentages of control values. **,** p < 0.01. Results are the mean ± S.D. of three independent experiments.

**PI3K Activity Assay—**PI3K activity was assessed by the incorporation of [32P]ATP into exogenous phosphoinositides, resulting in the production of PI(3)P as described by Ahmad et al. (15). Briefly, MP48 cells were lysed in buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.2 mM Na3VO4, 1× protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM EDTA, and 1% Nonidet P-40). The PI3K enzyme was immunoprecipitated with anti-phosphotyrosine-agarose conjugate (BD Biosciences) and anti-PI3K p85 (Upstate-Cell Signaling Solutions) coupled to protein A-Sepharose beads. The PI3K activity in the immunoprecipitates was assessed directly on the beads by incubating the immunoprecipitates with phosphatidylinositol (Avanti Polar Lipids) and [32P]ATP in kinase buffer for 30 min at 37 °C. The lipids in the reaction mixture were then extracted with chloroform/methanol (1:1, v/v) and were resolved on aluminum-backed Silica Gel 60 TLC plates by chromatography in CHCl3/MeOH/H2O/NH4OH (60:47:11.3:2). The spots corresponding to PI(3)P were visualized by autoradiography and were quantitated by densitometry in arbitrary units using a Bio-Rad Image Analysis system (Molecular Analyst). The PI(3)P product was identified by comparing its relative mobility value with the relative mobility value of standard PI(3)P, which was visualized by exposure of TLC plates to phospholipid-specific molybdenum blue spray reagent (Sigma).

**Cell Lysis and Immunoprecipitation—**The cells were lysed in lysis buffer (Cell Signaling Technology) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, and 10 μg/ml each of aprotinin and leupeptin) and inhibitors of phosphatases (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM Na3VO4). The lysate was drawn 10 times through a 25-gauge needle on ice for further lysis and then was centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected, and the protein concentrations were determined using the BCA protein assay kit. Immediately after this step, equal amounts of protein were subjected to Western blotting.
**FIG. 3. T3 phosphorylated PKB/Akt at Ser^473 in MP48 cells.** Cells were starved for 48 h by serum-free medium before the cells were subjected to T3 stimulation or inhibitor treatment. Starved cells were exposed to various concentrations of T3 for 3 h in serum-free medium. Equal amounts of lysate from each treatment were separated by 10% SDS-PAGE gel and then were immunoblotted with an antibody against phospho-PKB/Akt at Ser^473. The immunoblots for phosphorylation of PKB/Akt were stripped and reprobed with the antibody against PKB/Akt to confirm equal protein loading. All phosphorylation densitometric values were normalized to total protein amount. In each panel, the upper portion shows representative Western blots for phosphorylated PKB/Akt, the next portion shows the Western blots of total PKB/Akt protein, and in A and B the lower portion presents the densitometric values (mean ± S.D.) from three different experiments. The densitometric values of phosphorylation of PKB/Akt are expressed as percentages of control values. *, p < 0.05; **, p < 0.01.

A, cells were exposed to various concentrations of T3 for 3 h, resulting in dose-dependent increases in PKB/Akt phosphorylation. B, cells were exposed to 10^-8 M T3 for different periods. There was a gradual rise in the amount of Akt phosphorylation that persisted for up to 12 h. C, cells were preincubated with either 10 nM wortmannin or 25 μM Ly294002 for 30 min and then were stimulated with 10^-8 M T3 in the presence or absence of 10 nM wortmannin in serum-free medium for 3 h at 37 °C. The inhibitors of PI3K prevented the T3-induced phosphorylation of Ser^473 of Akt.
Immunoprecipitation of p85 was performed as previously described (15, 16) with slight modification. Briefly, the cells were lysed in immunoprecipitation buffer. The immunoprecipitation buffer differs from the above lysis buffer only in the concentration of Tris-HCl (50 mM for the immunoprecipitation buffer). Equal amounts of protein from each treatment were incubated with 5 μg of anti-p85 antibody and 20 μl of protein A-agarose beads in 1× immunoprecipitation buffer at 4 °C overnight with gentle rocking. The immunoprecipitates were washed three times with 1× immunoprecipitation buffer. Proteins were eluted from the beads by boiling for 10 min in Western blot loading buffer and then were subjected to Western blotting.

**Western Blot—**Protein samples were boiled for 10 min in loading buffer containing 2% SDS, 50 mM Tris-HCl (pH 7.5), 10% glycerol, 2% β-mercaptoethanol, and 0.001% bromphenol blue. Proteins were then separated on a 7.5 or 10% denaturing SDS-polyacrylamide gels. Separated proteins were transferred to a polyvinylidene difluoride membrane (catalog no. IPVH00010; Millipore Corp., Bedford, MA), which was then hybridized with primary antibodies. After incubation with a secondary peroxidase-conjugated goat antibody diluted 1:2000 (Cell Signaling Technology) and washing, proteins were detected with the secondary peroxidase-conjugated goat antibody diluted 1:2000 (Cell Signaling Technology) and washing five times in 1× Western blot stripping buffer (Pierce) at 37 °C for 2 h. The chemiluminescence of each lane was quantified by densitometry in arbitrary units using a Bio-Rad Image Analysis system (Molecular Analyst).

For reuse of membranes that had been Western blotted, the antibodies on membrane were removed by incubating the membrane with Restore Western blot stripping buffer (Pierce) at 37 °C for 2 h and washing five times in 1× TBST buffer.

**Measurement of Plasma Membrane Na,K-ATPase Protein—** For determination of plasma membrane Na,K-ATPase, the cell surface proteins were biotinylated as described by Gonin et al. (17) with slight modifications. Cells grown on 6-well plates were subjected to different treatments, detached after being washed twice with ice-cold 1× phosphate-buffered saline and incubated in phosphate-buffered saline containing 2 mM EDTA at 4 °C for 5 min. Then cell surface proteins were biotinylated by incubating cells in biotinylation buffer (10 mM triethanolamine, pH 7.5, 1 mM EDTA, and 150 mM NaCl) containing 1 mg/ml water-soluble, cleavable Sulfo-Biotin-X-N-hydroxysuccinimide ester (Calbiochem) for 30 min at 4 °C. The free unreacted biotin was quenched by washing three times with 1× phosphate-buffered saline containing 100 mM glycine. After lysis, protein content was determined using the BCA protein assay kit. Equal amounts of total cell protein were precipitated with streptavidin-agarose beads (Sigma) diluted in lysis buffer via incubating overnight at 4 °C with gentle rocking. The beads were then washed three times with lysis buffer, twice with high salt buffer (5 mM EDTA, 50 mM Tris-HCl pH 7.4, 500 mM NaCl), and once with 10 mM Tris-HCl, pH 7.4. Proteins were eluted from the beads by incubation of the biotinylated protein-streptavidin-agarose beads for 10 min in 50 μl of SDS-containing buffer (5.6% SDS, 240 mM Tris-HCl, pH 7.5, 6% β-mercaptoethanol, 16% glycerol, and 0.008% bromphenol blue) and analyzed by Western blotting as described above. The amount of proteins was expressed as densitometry in arbitrary units.

**Statistics—** All data are expressed as mean ± S.D. of a minimum of three experiments. Comparisons involving three or more groups were analyzed by analysis of variance and post hoc pairwise comparisons. Differences between means were considered significant at p < 0.05, adjusted for the number of comparisons.

**RESULTS**

Inhibitors of PI3K and Src Family Kinases, but Not PKA and PKC, Abolished the T3-induced Na,K-ATPase Activity in Alveolar Epithelial Cells—We demonstrated previously that T3 at both physiologic and pharmacological concentrations increased the Na,K-ATPase hydrolytic activity in adult rat alveolar epithelial cells, including primary ATII cells and the MP48 and RLE-6TN cell lines in a dose- and time-dependent fashion. The T3-induced increases were detected by 30 min and peaked at 6 h (12). To define the signaling pathways involved, we assessed the inhibitory effects of PI3K, Src family kinases, PKA, and PKC inhibitors on T3-induced Na,K-ATPase activity. Consistent with our previous study, T3 significantly increased the Na,K-ATPase hydrolytic activity in alveolar epithelial cells at both 3 (Fig. 1, C, E, F, and G) and 6 h (Fig. 1, A–E), but the magnitude of the effect was greater at 6 h. The selective PI3K inhibitor wortmannin (10 nm) inhibited completely the T3-induced increase in Na,K-ATPase activity in primary cultures.

![Figure 4](http://www.jbc.org/Downloaded from http://www.jbc.org/)
of adult rat alveolar type II cell (Fig. 1A), cell line RLE-6TN (Fig. 1B), and cell line MP48 (Fig. 1C). The structurally distinct inhibitor of PI3K, LY294002 (25 \( \mu \)M), also inhibited the T3-induced Na,K-ATPase activity in MP48 cells (Fig. 1D). Since activation of PI3K by Src family kinases has been described in some cells other than AECs (41, 42), we assessed the inhibitory effects of two Src kinase inhibitors, PP1 and PP2, on T3-induced Na,K-ATPase activity. Either PP1 (1 \( \mu \)M) or PP2 (10 \( \mu \)M) blocked the T3-induced Na,K-ATPase activity in MP48 cells (Fig. 1E). Neither PI3K inhibitors nor Src kinase inhibitors alone significantly inhibited the Na,K-ATPase activity compared with control. These findings indicated that PI3K and Src family kinases were necessary for the up-regulation of Na,K-ATPase activity by T3.

Activation of PKA by \( \beta \)-adrenergic agonists (5) and of some PKC isoforms by dopamine (6) in alveolar epithelial cells is required for up-regulation of the Na,K-ATPase activity. In addition, the PI3K lipid products, PI(3,4)P2 and PI(3,4,5)P3, are also capable of activation of the PKC (18). Therefore, we sought to test the roles of PKA and PKC in the T3-induced increase in Na,K-ATPase activity using the PKA inhibitor H-8 and PKC inhibitor bisindolylmaleimide. H8 is a highly active inhibitor of PKA and has inhibitory effects at 10 \( \mu \)M in rat fetal AECs (46) and at 30 \( \mu \)M in rat isolated perfused lung (47). Bisindolylmaleimide is a highly selective PKC, \( \alpha \), \( \beta \), \( \beta \)II, \( \gamma \), \( \delta \), and \( \epsilon \) isozyme inhibitor that is active at 1 \( \mu \)M in rat AECs (6). Thus, we assessed the actions of PKA and PKC in T3-stimulated Na,K-ATPase activity using the PKA inhibitor H-8 (30 \( \mu \)M) and PKC inhibitor bisindolylmaleimide (5 \( \mu \)M). T3 significantly stimulated Na,K-ATPase activity at 3 h (Fig. 1, F and G). However, no significant differences were detected between the T3 treatment with and without 30 \( \mu \)M H-8 (\( p = 0.432 \)) (Fig. 1F) or 3 \( \mu \)M bisindolylmaleimide (\( p = 0.197 \)) (Fig. 1G), indicating that neither PKA nor PKC probably were involved in the T3 up-regulation of Na,K-ATPase in adult rat alveolar epithelial cells.

**T3 Stimulated PI3K Activity in MP-48 Cells**—Based on the inhibitory effect of PI3K inhibitors on the T3-induced increase in Na,K-ATPase activity (Fig. 1), we hypothesized that T3 activated PI3K in alveolar epithelial cells. We assessed the activity of PI3K in the presence and absence of T3 in MP48 cells by measuring the \( ^{32} \)P-labeled PI(3)P produced by PI3K. After incubation with T3 for 3 h, a time point at which T3 induced a significant increase in Na,K-ATPase activity (Fig. 1), the PI3K activity in immunoprecipitates with either anti-PI3K p85 or anti-phosphotyrosine was significantly increased by T3 (Fig. 2). We found that neither PKA nor PKC probably were involved in the T3 up-regulation of Na,K-ATPase in adult rat alveolar epithelial cells.

**T3 Stimulated Phosphorylation of PKB/Akt at Ser473 in a Dose-dependent Manner**—Whereas PI3K exerts many downstream effects, PI3K phosphorylates the serine 473 of PKB/Akt activity, and this phosphorylation is a surrogate indicator of PI3K and T3 Regulation of Na,K-ATPase

**Fig. 5.** A constitutively active mutant of p110 stimulated the activity and cell surface expression of Na,K-ATPase in MP48 cells. MP-48 cells were incubated in 10% FBS medium without antibiotics for 48 h after cells were transiently transfected with vector and constitutively active mutant p110. The cells then were starved overnight in serum-free medium for the PKB/Akt phosphorylation assay or 1% stripped FBS medium for Na,K-ATPase activity and biotinylation assay. 10 \( \mu \)M wortmannin or 1 \( \mu \)M PP1 was added during the last 3 h before cells were subjected to various assays. The results are expressed as percentage of controls. * \( p < 0.05 \); ** \( p < 0.01 \). A, constitutively active p110 mutant; constitutively active p110 mutant (1 \( \mu \)g of DNA/well) phosphorylated the PKB/Akt. The bars represent mean \( \pm \) S.D. of three independent experiments. B, constitutively active p110 mutant enhanced the Na,K-ATPase activity. The increased activity was blocked by inhibition of either PI3K (wortmannin) or Src kinase (PP1). The data shown are the mean \( \pm \) S.D. of two different experiments with two different quantities of p110 mutant used for transfection (solid bars, 2 \( \mu \)g of DNA; empty bar, 1 \( \mu \)g of DNA). C, constitutively active p110 (1 \( \mu \)g of DNA/well) also increased the amount of Na,K-ATPase \( \alpha \) subunit protein at the cell surface, and this was prevented by wortmannin. The bars represent mean \( \pm \) S.D. of three different experiments.
FIG. 6. T3 stimulated Akt through the Src family kinase in MP48 cells. A, T3 increased Src family kinase activity, and this increase was inhibited by 10 μM PP2 but not by 100 nM wortmannin. MP-48 cells were starved overnight in serum-free Waymouth medium, and then cells were incubated with 10^{-7} M T3 for 10 min at 37 °C in the presence or absence of 10 μM PP2 or 100 nM wortmannin. The Src family kinase activity was measured as the ^32P incorporated into the substrate peptide by scintillation counter and expressed as percentage of control values. The bars represent mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. B, the time course of T3 stimulation of phosphorylation of Src kinase at Tyr^{416} as demonstrated by Western blots. The upper panel represents one of three independent experiments. The Src phosphorylation at Tyr^{416} increased significantly within 10 min and then decreased within 1 h. MP-48 cells were starved overnight in serum-free Waymouth medium, and cells were then incubated with 10^{-7} M T3 for indicated periods. Equal amounts of lysate from each treatment were separated by 10% SDS-polyacrylamide gel and then were immunoblotted with an antibody against phospho-Src kinase at Tyr^{416}. The immunoblots for phosphorylation of Src kinase were stripped and reprobed with the antibody against Src family kinases to confirm equal protein loading. C and D, these representative Western blots from one each of three different experiments show that either 1 μM PP1 or 10 μM PP2 blocked T3 phosphorylation of PKB/Akt at Ser^{473}. MP48 cells were cultured and treated as described in the legend to Fig. 3.
PI3K and T3 Regulation of Na,K-ATPase

A

![Graph](image)

B

![Graph](image)

C

![Graph](image)

**Fig. 7.** Transient expression of an active mutant of Src kinase increased PI3K activity, PKB/Akt phosphorylation, and Na,K-ATPase activity in MP48 cells. 48 h after transfection with active Src or empty vector, the cells were starved overnight in serum-free medium for PI3K activity and for the PKB/Akt phosphorylation assay or in 1% stripped FBS medium for the Na,K-ATPase activity assay. In some samples, inhibitors (1 μM PPI, 10 μM PP2, and 10 nM wortmannin) were added during the last 1 h before cells were subjected to various assays. The solid bars and the empty bars indicate that 1 μg of DNA/well and 2 μg of DNA/well had been used in the transfection, respectively. *, p < 0.05. A, PI3K activity was measured by an antibody against p85 of PI3K. The upper portion presents the densitometric values (mean ± S.D.) of radio-labeled PI(3)P from three independent experiments. B, representative Western blots of one of three independent experiments show that the active mutant of Src kinase increased the phosphorylation content of PKB/Akt at Ser**473** and that this increase was blocked by either 1 μM PP1 or 10 μM PP2. C, transfection of cells with an active mutant of Src kinase augmented the Na,K-ATPase activity, and this augmentation was blocked by 1 μM PP1, 10 μM PP2, or 10 nM wortmannin. The bars represent mean ± S.D. of three independent experiments.

PI3K activity (21, 24). To assess the dose and time dependence of T3 on PI3K activity in MP48 cells, we assessed the phosphorylation levels of PKB/Akt at Ser**473** (Fig. 3). T3 did not significantly alter the amount of total cell PKB/Akt protein in MP48 cells; however, T3 stimulated the level of phosphorylation of Ser**473** of PKB/Akt in a dose-dependent manner from 10⁻⁸ to 10⁻⁵ M (Fig. 3A), consistent with the dose response effects of T3 on Na,K-ATPase activity (12). T3-induced PKB/Akt phosphorylation at Ser**473** was detectable from 10 min to 12 h of T3 stimulation (Fig. 3B). Moreover, wortmannin and Ly294002 completely abolished the T3-induced PKB/Akt phosphorylation at 3 h (Fig. 3C). These results, taken together with the observations in Fig. 1 and 2, further confirmed that T3 activated the PI3K-Akt pathway in alveolar epithelial cells in a dose-dependent manner.

**Inhibition of PI3K Activity Blocked the T3 Augmentation of Cell Surface Expression of Na,K-ATPase α1 Subunit Protein**—We demonstrated previously that T3 increased the insertion of Na,K-ATPase α1 and β1 subunit proteins at the cell surface and that this accounted for T3-induced Na,K-ATPase activity in alveolar epithelial cells, including MP48 cells. Since wortmannin blocked both the T3-stimulated Na,K-ATPase activity (Fig. 1) and PI3K activity (Fig. 3C), we predicted that the T3-induced increase in cell surface expression of Na,K-ATPase ATPase α1 subunit protein would be blocked by wortmannin.

We measured the effect of wortmannin on T3-stimulated cell surface expression of Na,K-ATPase α1 subunit by employing biotinylation experiments. T3 did not change the total cellular amount of α1 subunit protein (Fig. 4A). However, wortmannin blocked the increase in cell surface expression of Na,K-ATPase α1 subunit protein with T3 (Fig. 4B), indicating that activation of PI3K was required for the T3-induced cell surface expression of Na,K-ATPase α1 subunit.

**Constitutively Active PI3K p110 Subunit Increased the Na,K-ATPase Activity and Cell Surface Expression of Na,K-ATPase α1 Subunit Protein**—To directly demonstrate the involvement of PI3K in T3-induced Na,K-ATPase activity and cell surface expression of this enzyme, MP48 cells were transiently transfected with 1 or 2 μg of a constitutively active mutant of the PI3K p110 catalytic subunit. Control cells were transfected with empty vector alone. MP-48 cells were incubated in 10% FBS medium without antibiotics for 48 h after cells were transiently transfected. The cells then were starved overnight in serum-free medium for Akt phosphorylation assay or in 1% stripped FBS medium for Na,K-ATPase activity assay and cell surface expression assay of Na,K-ATPase α1 subunit protein. In some experiments, the cells were incubated with 10 nM wortmannin or 1 μM PP1 for 1 h before cells were lysed for assessment of the inhibitory effects of wortmannin or PP1.

The efficiency of the transfection was assessed by examining...
the stimulation of phosphorylation of PKB/Akt at Ser473 by constitutively active p110. As shown in Fig. 5A, the phosphorylation level of PKB/Akt in cells transfected transiently by 1 μg of active mutant of p110 increased significantly compared with that in cells transfected with empty vector alone. The increase in PKB/Akt phosphorylation was inhibited by wortmannin. Thus, our transfection protocol augmented PI3K activity, presumably due to the constitutively active p110 in MP48 cells.

Using the same transfection protocol, we assessed the effect of constitutively active p110 on Na,K-ATPase activity. The constitutively active p110 mutant significantly enhanced the Na,K-ATPase activity in a dose-dependent fashion (1 and 2 μg), and the augmentation was blocked by wortmannin, but not by Src kinase inhibitor PP1 (Fig. 5B). Moreover, the p110 mutant also increased the cell surface expression of Na,K-ATPase α1 subunit protein (Fig. 5C), and this was similarly inhibited by wortmannin. The data demonstrated that augmented PI3K activity, as occurs with T3, is sufficient to stimulate Na,K-ATPase activity and Na,K-ATPase α1 subunit expression at the cell surface. PP1 did not inhibit the active p110 mutant-increased Na,K-ATPase activity, consistent with Src kinase being upstream of PI3K in AECs.

**T3 Stimulates PI3K Activity through Activation of Src Family Kinases**—In other systems, Src kinase members activated PI3K (38, 39, 41, 42). Since, in our results, the Src kinase inhibitors PP1 and PP2 blocked the T3-induced increase in Na,K-ATPase (Fig. 1E), we predicted that T3 increased PI3K activity and Na,K-ATPase via T3-activation of Src kinase members(s) in adult ATII cells.

First, we assessed the effect of T3 on Src family kinase activity in MP48 cells using immunoprecipitates obtained with an antibody against multiple Src family members (catalog no. sc-18; Santa Cruz Biotechnology). T3 increased significantly the Src family kinase activity by 156.5 ± 16.7% in 10 min (Fig. 6A). The phosphorylation of tyrosine 416 within the catalytic domain of Src kinase is part of the enzyme activation mechanism (40, 43), so we investigated the time course of T3 effect using an antibody specific for phospho-Src at tyrosine 416. T3 increased significantly the phosphorylation content of Src at Tyr416 from 10 to 20 min (Fig. 6B) followed by a gradual decline in phospho-Src content over 1 h. The data indicated that T3 activated Src family kinase activity in MP48 cells.

Second, we investigated the effect of Src kinase inhibitors, PP1 and PP2, on T3 activation of PKB/Akt as an indirect indicator of PI3K activity in MP48 cells. Either PP1 (1 μM) or PP2 (10 μM) completely blocked T3-stimulated phosphorylation of Akt at Ser473 (Fig. 6, C and D). In addition, wortmannin did not inhibit T3-stimulated Src family kinase activity (Fig. 6A). PP1 was not capable of blocking the active PI3K mutant-increased Na,K-ATPase activity (Fig. 5B). Taken together, these data demonstrated that Src kinase family member(s) are upstream of PI3K and are necessary for the T3 activation of PI3K activity in MP48 cells.

**Constitutively Active Src Kinase Increased PI3K and Na,K-ATPase Activity**—To further confirm the role of Src kinase in PI3K activity and Na,K-ATPase activity in adult ATII cells, we transiently expressed a constitutively active mutant of Src kinase (Src Y529F) in MP48 cells and assessed its effect on PI3K and Na,K-ATPase activity. After 48 h of transfection, the active mutant of Src kinase increased significantly the PI3K activity and the phosphorylation content of PKB/Akt at Ser473. The increases in PI3K activity and PKB/Akt phosphorylation were abolished by either PP1 or PP2 (Fig. 7, A and B). The active mutant of Src kinase also increased significantly the Na,K-ATPase activity, and the increases in Na,K-ATPase activity also were blocked by PP1, PP2, and PI3K inhibitor wortmannin (Fig. 7C). Thus, even in the absence of T3, activation of Src kinase is necessary for PI3K activation and stimulation of Na,K-ATPase activity in adult ATII cells. Wortmannin inhibited the active Src mutant up-regulated Na,K-ATPase activity, further confirming that the Src kinase is upstream of the PI3K in the T3-stimulated pathway in AECs.

**DISCUSSION**

Our studies focused on regulation of Na,K-ATPase in alveolar epithelial cells because of the critical role of this enzyme in alveolar fluid resorption. The major and novel finding is that T3 stimulates Na,K-ATPase activity through a nongenomic pathway that requires PI3K and Src family kinases.

**T3 Stimulates the PI3K/Akt Pathway in an Src Family Kinase-dependent Manner**—T3 regulates many cell functions, but the nongenomic effects of T3 and the signaling pathways involved are not well defined. Recently, Incerpi et al. (11) reported that T3 stimulated Na+/H+ exchanger in chick embryo hepatocytes. This effect was sensitive to PI3K inhibitor wortmannin (50 nM), but direct activation of PI3K by T3 was not demonstrated. In our system, T3 not only increased the PI3K activity (Fig. 2) but also increased the phosphorylation levels of PKB/Akt at Ser473 in a dose-dependent manner (Fig. 3). Moreover, the increase in PKB/Akt at Ser473 was sensitive to two structurally distinct inhibitors of PI3K, wortmannin (10 nM) and LY294002 (25 μM) (Fig. 3). Taken together, these data strongly suggested that T3 activates the PI3K/PKB pathway in alveolar epithelial cells and that this pathway is required for T3 stimulation of Na,K-ATPase.

Src family kinases activates PI3K activity in various types of cells, such as in B cell lymphoma (38), Nb2 cells (39), androgen-positive cells (41), and endothelial cells (42). In the present study, we demonstrated in alveolar epithelial cells that T3 stimulated Src family kinase activity (Fig. 6A). The active mutant of Src kinase enhanced PI3K activity and phosphorylation content of Akt at Ser473 (Fig. 7, A and B). Both T3-stimulated and active Src mutant-increased PI3K activity as measured by PI(3)P or Akt phosphorylation at Ser473 were sensitive to Src kinase inhibitors PP1 and PP2 (Fig. 6, C and D, and Fig. 7, A and B). Whereas we did not directly demonstrate that PP1 or PP2 blocks the T3-induced increase in PI(3)P quantity, we did find that PP1 and PP2 block T3-stimulated phosphorylation of Akt (Fig. 6, C and D). We cannot completely exclude an effect of PP1 or PP2 on Akt phosphates, but Src activation did increase PI(3)P quantity (Fig. 7A). Thus, in AECs, the T3-stimulated PI3K/Akt pathway is Src family kinase(s)-dependent. We confirmed that Src kinase activity is upstream of PI3K in AEC in several ways. T3-stimulated Src kinase activity was not blocked by PI3K inhibitor wortmannin (Fig. 6A), whereas, conversely, the PI3K activity up-regulated by an active mutant of Src kinase was blocked by Src kinase inhibitors PP1 and PP2 (Fig. 7A). In addition, the increase in Na,K-ATPase activity enhanced by the active mutant of Src was sensitive to wortmannin (Fig. 7C), but the active PI3K mutant-increased Na,K-ATPase activity was not inhibited by PP1 (Fig. 5B). These observations also supported the view that T3 activated PI3K via stimulation of Src family kinase(s) in AECs.

**PI3K Is Necessary for T3-induced Na,K-ATPase Activity and Cell Surface Expression**—PI3Ks are a subfamily of lipid kinases that catalyze the addition of a phosphate molecule specifically to the 3-position of inositol ring of phosphoinositides. Activation of PI3K phosphorylates phosphoinositides at the D3 position to rapidly generate phospholipids PI(3)P, PI(3,4)P2, PI(3,4,5)P3, and PI(3,5)P2 (18). Although multiple forms of PI3K exist in higher eukaryotes, the class Ia isoform is primarily...
responsible for production of D3 phosphoinositides in response to various stimuli (45). Class IA PI3K is composed of a regulatory subunit (p85) and a catalytic subunit (p110). The p85 subunit contains an Src homology 3 domain capable of binding to proline-rich sequences, a p110 binding domain, and two Src homology 2 domains. PI3K is involved in the regulation of many diverse cell processes, including protein trafficking (7, 18, 26, 27). This occurs through protein-protein interaction of PI3K with other proteins, such as receptors of hormones and growth factors (18, 26, 28), and through the phospholipid products that selectively bind to specific protein domains (18).

In vascular smooth muscle cells, the PI3K inhibitors, wortmannin and Ly294002, block the stimulation of Na,K-ATPase activity by insulin-like growth factor I (30). Wortmannin also abolishes insulin-induced Na,K-ATPase activity in skeletal muscle cells (31) and insulin-induced plasma membrane insertion of Na,K-ATPase α1 proteins in skeletal muscle (32) and human embryonic kidney-293 cells (33). These effects of PI3K inhibitors on Na,K-ATPase activity or translocation indicate that PI3K is involved in the Na,K-ATPase regulation of activity and translocation to the cell surface in several cell types. However, wortmannin also inhibits the activity of several phospholipases (phospholipases C, D, and A2) (44), and, in high concentration, it inhibits other kinases such as phosphoinositide 4-kinase and myosin light chain kinase. Ly294002 also inhibits the related protein kinases TOR (25). Thus, direct evidence is necessary and important to verify the involvement of the PI3K pathway in Na,K-ATPase stimulation by hormones. In the present study, we not only identified that two structurally distinct inhibitors of PI3K, wortmannin and Ly294002, blocked T3-induced Na,K-ATPase activity (Fig. 1) and cell surface expression (Fig. 4), we also verified that PI3K is necessary and sufficient for T3-induced Na,K-ATPase activity and cell surface expression using a constitutively active mutant of PI3K p110 subunit (Fig. 5). This established a direct association between activation of PI3K and the activity and cell surface expression of Na,K-ATPase in alveolar epithelial cells.

In addition, T3 phosphorylated the PKB/Akt at Ser473 (Fig. 3A) and stimulated Na,K-ATPase activity in a dose-dependent manner (12). The parallel increases in Na,K-ATPase and PI3K activity induced by T3 supported the likelihood that T3 induced Na,K-ATPase activity through PI3K. Wortmannin or Ly294002 abolished the T3-induced increases in Na,K-ATPase activity (Fig. 1), in cell surface protein of Na,K-ATPase (Fig. 4), confirming that T3-induced Na,K-ATPase activity required the activation of PI3K.

We did not directly demonstrate the intracellular compartments from which T3 shifts Na,K-ATPase to the plasma membrane; nor did we directly prove whether T3 decreased the rate of internalization of the sodium pump or increased the rate of delivery to the plasma membrane. In our previous study (12), T3 enhanced the cell surface expression of both the Na,K-ATPase α1 and β1 subunits, whereas the total cell amount of those subunit proteins was not altered, but the T3-induced increase in Na,K-ATPase α1 protein at plasma membrane was sensitive to a protein-trafficking inhibitor, brefeldin A, that disrupts the protein trafficking from Golgi complex to plasma membrane. Those observations indicated that T3-induced cell surface expression of Na,K-ATPase may be derived from translocation of this enzyme. Those data all supported the view that T3-induced Na,K-ATPase cell surface expression via PI3K may be due to increased delivery of Na,K-ATPase to the plasma membrane.

Src Family Kinase(s) Is Necessary for T3-stimulated Na,K-ATPase Activity in AECs—T3 increased Src kinase activity in AECs (Fig. 6A). To our knowledge, this pathway of Na,K-ATPase activation has not previously been reported. The Src kinase inhibitors PP1 or PP2 abolished T3-induced Na,K-ATPase activity (Fig. 1E). Also, an active mutant of Src enhanced Na,K-ATPase activity (Fig. 7C). These data revealed that Src activation is necessary for T3-induced stimulation of the PI3K/Akt pathway and Na,K-ATPase activity in AECs. There are several members in the Src kinase family. Although we verified that the T3 activated Src kinase activity in AECs using a polyclonal antibody that recognizes several Src kinase family members, such as Src p60, Yes p62, Fyn, p59, and c-Fgr, p55, the specific Src family member(s) involved in the T3 effect on PI3K and Na,K-ATPase activity remain to be identified.

Other Signaling Molecules in T3-stimulated Na,K-ATPase Activity—Our data cannot prove that the increases in Src kinase and PI3K activity result directly from T3, and we cannot exclude an indirect activating mechanism. At present, we do not know if the receptor(s) of thyroid hormone is required for the Na,K-ATPase response.

Activation of PKA and PKC isoforms (PKC-δ, PKC-ε, and PKC-ζ) also has been reported to affect Na,K-ATPase activity (1, 5–7). In alveolar epithelial cells, activation of PKA confirmed by H89 is required for up-regulation of the Na,K-ATPase activity by β-adrenergic agonists (5), and the PKC inhibitor, bisindolylmaleimide, prevents the PKC-δ and PKC-ε-dependent increases in Na,K-ATPase activity and exocytosis of Na,K-ATPase to the plasma membrane induced by dopamine (6). In our present study, neither the PKA inhibitor H-8 nor the PKC inhibitor bisindolylmaleimide inhibited the T3-induced increase of Na,K-ATPase in adult rat alveolar epithelial cells (Fig. 1, F and G). This indicated that the T3 effect on Na,K-ATPase activity and cell surface expression may not be related to PKA and some PKC isoforms in our system, although PKC-ε and PKC-ζ are also known as downstream targets of PI3K (18). Thus, the T3 signaling pathway(s) in AECs that augments Na,K-ATPase function differs from that used by dopamine and β-adrenergic agonists.

Activation of PKB/Akt also is linked to protein trafficking. For example, constitutively active PKB/Akt stimulated translocation of GLUT4 (35) and Na/H+ exchanger 3 (29) to the plasma membrane. In the present observations, T3 stimulated the PKB/Akt activity (Fig. 3). However, the association of PKB/Akt activation with the T3-induced activity and translocation to plasma membrane of Na,K-ATPase remain to be determined.

In summary, our investigations demonstrated a critical role of activation of Src family kinases and PI3K in the T3 stimulation of cell surface expression of an integral membrane protein Na,K-ATPase and Na,K-ATPase activity in adult alveolar epithelial cells.

Acknowledgments—We thank Dr. Craig Henke and Dr. Bin Tian for generous assistance in the use of the constitutively active mutant of PI3K p110 subunit and Dr. Shana Ahmad and Dr. Carl W. White for advice about the PI3K activity assay. We also appreciate Dr. Peter Bitterman’s critical review and constructive suggestions about this research.

REFERENCES
1. Therien, A. G., and Blostein, R. (2000) Am. J. Physiol. 279, C541–C566
2. Szajdaj, J. I., Factor, P., and Inghar, D. H. (2002) J. Appl. Physiol. 93, 1860–1866
3. Blanc, G., and Mercer, R. W. (1998) Am. J. Physiol. 275, F633–650
4. Inghar, D. H., Weeks, C. B., Gilmore-Herbert, M., Jacobsen, E., Duvick, S., Dowin, R., Savik, S. K., and Jamieson, J. D. (1996) Am. J. Physiol. 270, L619–L629
5. Pesce, L., Guerrero, C., Comellas, A., Ridge, K. M., and Szajdaj, J. I. (2000) FEBS Lett. 486, 310–314
6. Ridge, K. M., Dada, L., Lecuona, E., Bortorello, A. M., Katz, A. I., Mochly-Rosen, D., and Smajer, J. I. (2002) Mol. Biol. Cell 13, 1381–1389
7. Ogiomo, G., Wudowski, G. A., Barker, C. J., Kohler, M., Katz, A. I., Feraile, E., Pedemonte, C. H., Berggren, P. O., and Bortorello, A. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3242–3247
3,3′,5-Triiodo-l-thyronine Up-regulation of Na,K-ATPase Activity and Cell Surface Expression in Alveolar Epithelial Cells Is Src Kinase- and Phosphoinositide 3-Kinase-dependent
Jianxun Lei, Cary N. Mariash and David H. Ingbar

J. Biol. Chem. 2004, 279:47589-47600.
doi: 10.1074/jbc.M405497200 originally published online August 31, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405497200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 15 of which can be accessed free at http://www.jbc.org/content/279/46/47589.full.html#ref-list-1