Thyroid hormone stimulates Na-K-ATPase activity and its plasma membrane insertion in rat alveolar epithelial cells

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Lei, Jianxun, Sogol Nowbar, Cary N. Mariash, and David H. Ingbar. Thyroid hormone stimulates Na-K-ATPase activity and its plasma membrane insertion in rat alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 285: L762–L772, 2003. First published May 9, 2003; 10.1152/ajplung.00376.2002.—Na-K-ATPase protein is critical for maintaining cellular ion gradients and volume and for trans epithelial ion transport in kidney and lung. Thyroid hormone, 3,3′,5-triiodo-L-thyronine (T3), given for 2 days to adult rats, increases alveolar fluid resorption by 65%, but the mechanism is undefined. We tested the hypothesis that T3 stimulates Na-K-ATPase in adult rat alveolar epithelial cells (AEC), including primary rat alveolar type II (ATII) cells, and determined mechanisms of the T3 effect on the Na-K-ATPase enzyme using two adult rat AEC cell lines (MP48 and RLE-6TN). T3 at 10^{-8} and 10^{-5} M increased significantly hydrolytic activity of Na-K-ATPase in primary ATII cells and both AEC cell lines. The increased activity was dose dependent in the cell lines (10^{-8} to 10^{-4} M) and was detected within 30 min and peaked at 6 h. Maximal increases in Na-K-ATPase activity were twofold in MP48 and RLE-6TN cells at pharmacological T3 of 10^{-8} and 10^{-5} M, respectively, but increases were statistically significant at physiological T3 as low as 10^{-9} M. This effect was T3 specific, because reverse T3 (3,3′,5′-triiodo-L-thyronine) at 10^{-8} to 10^{-4} M had no effect. The T3-induced increase in Na-K-ATPase hydrolytic activity was not blocked by actinomycin D. No significant change in mRNA and total cell protein levels of Na-K-ATPase were detected with 10^{-9} to 10^{-5} M T3 at 6 h. However, T3 increased cell surface expression of Na-K-ATPase α1- or β1-subunit proteins by 1.7- and 2-fold, respectively, and increases in Na-K-ATPase activity and cell surface expression were abolished by brefeldin A. These data indicate that T3 specifically stimulates Na-K-ATPase activity in adult rat AEC. The up-regulation involves translocation of Na-K-ATPase to plasma membrane, not increased gene transcription. These results suggest a novel nontranscriptional mechanism for regulation of Na-K-ATPase by thyroid hormone.

The electrochemical gradient across the membranes of most animal cells is maintained by Na-K-ATPase (52). It is an integral membrane protein consisting of at least two subunits, α and β, each of which exists as one of several isoforms (α1, α2, α3, α4, β1, β2, and β3) (21). The α-subunit contains the catalytic and regulatory phosphorylation sites and the binding sites for cation substrates and some protein kinases such as protein kinase C and phosphoinositide 3-kinase (21, 56). The role of the β-subunit is less clear, but it is required for normal processing and plasma membrane expression of the enzyme and may have a role in regulating the interaction of cations with the α-subunit (3, 12). The γ-subunit peptide, which can modify Na-K-ATPase function, was at first thought to represent a third component of Na-K-ATPase, but recent evidence suggests that it is not an integral part of the enzyme complex (41, 52, 53). The pump isoforms are expressed in a tissue- and development-specific fashion and are believed to be distinct in function and modes of regulation (5, 30). The α1-isoform is predominant in most epithelia, including rat lung (30), particularly in alveolar type II (ATII) cells.

Although much information about Na-K-ATPase is available, regulation of Na-K-ATPase and the underlying mechanisms are not completely understood (52). The many mechanisms for regulation of Na-K-ATPase activity in a tissue-specific manner have been reviewed (52). Long-term regulation of Na-K-ATPase activity usually involves changes in mRNA/protein synthesis or degradation of Na-K-ATPase subunit isoforms (52). Short-term rapid alternations in Na-K-ATPase activity may be mediated by changes in subcellular distribution of pump units (52), by reversible noncatalytic-site phosphorylation of the α1-subunit (21), or by changes in intracellular Na^+ concentration. Many hormones regulate Na-K-ATPase. Insulin, β-adrenergic agonists, and dopamine can rapidly modulate Na-K-ATPase activity (51, 52). In some studies, dopamine (46) or β-adrenergic receptor stimulation by isoproterenol (4) or cAMP analogs (24) rapidly increased cell surface expression of Na-K-ATPase α-subunits in rat lung epithelia, whereas in rat proximal tubule cells, dopamine stimulated endocytosis of this enzyme from the plasma membrane (11, 56).

Thyroid hormones play a fundamental role in regulation of normal cell function and differentiation by interacting with intracellular thyroid hormone receptors and transcriptional coregulatory factors (coactivators and corepressors) (7, 54, 55). Thyroid hormones also may generate biological responses by nongenomic mechanisms that are independent of nuclear receptors.

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for 3,3',5-triiodo-L-thyronine (T₃) (13, 18, 55). Thyroid hormone stimulates Na-K-ATPase activity in responsive tissues and differentially regulates Na-K-ATPase isoforms (6, 28). T₃ response elements exist in the 5'-flanking region of Na-K-ATPase α- and β-subunits (20, 29). In nonlung tissues, the T₃-induced increases in Na-K-ATPase activity generally are due to thyroid hormone-induced synthesis of Na-K-ATPase mRNA or protein (36–39). T₃ regulates the activity and gene expression of Na-K-ATPase in tissue- and cell type-specific manners (17, 52). For example, T₃ upregulates Na-K-ATPase activity in rat liver, skeletal muscle, and kidney (39) but inhibits synaptosomal Na-K-ATPase activity in rat cerebral cortex (48). Na-K-ATPase activity is stimulated by T₃ in K562 human erythroleukemia cells (1), but not in human submandibular gland cells (34). T₃ increases significantly Na-K-ATPase activity in liver, skeletal muscle, kidney, small intestine, cardiac muscle, and heart; however, the effect of T₃ on Na-K-ATPase in the lung has not been determined.

The clearance of alveolar fluid is essential for recovery from adult lung injury and respiratory distress syndrome. T₃ pretreatment stimulates alveolar epithelial fluid clearance by 65% in normal adult rat lung (22) and exerts a synergistic effect with dexamethasone in adult rat lung (22) and with hydrocortisone in the developing sheep lung on stimulation of alveolar fluid clearance (2). Although the cellular mechanism of T₃ stimulation in fluid clearance is unknown, reabsorption of fluid out of the distal air space in the lung usually is driven by active Na⁺ transport (16, 40), and increased edema clearance usually requires higher Na-K-ATPase activity in alveolar epithelial cells (AEC) (40, 43). T₃-induced Na-K-ATPase activity may contribute to the alveolar fluid clearance stimulated by T₃ in adult rat lung. Therefore, the first objective of this study was to test the hypothesis that T₃ stimulates Na-K-ATPase activity in adult rat lung epithelium, and if so, the second purpose was to explore possible mechanisms involved in this effect.

**MATERIALS AND METHODS**

**Reagents.** T₃, 3,3',5-triiodo-L-thyronine (reverse T₃), actinomycin D, brefeldin A, and antibody against actin (A-2066) were purchased from Sigma Chemical. A mouse cDNA probe for 18S rRNA was purchased from Ambion. Cell culture reagents, Waymouth's MB752/1 medium, DMEM-Ham's F-12 medium, HEPES, fetal bovine serum (FBS), and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B). Antibiotics, amphotericin, and streptomycin were added to standard culture media for the cell lines and during T₃ exposure but were not present during the Na-K-ATPase activity assay. Removal of amphotericin did not alter Na-K-ATPase activity in MP48 cells.

**Experimental design.** To measure the effect of T₃ on the hydrolytic activity of Na-K-ATPase, the cells were grown to ~80% confluence in the appropriate medium containing 10% FBS. In most, but not all, experiments, the cells were T₃ starved for 24 h; i.e., cells were cultured in appropriate medium supplemented with stripped 10% FBS; then the monolayers were exposed to T₃ or inhibitors for various time intervals in appropriate medium plus 1% stripped FBS. At the indicated times, the cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) to terminate exposure to all agents. In some experiments, cells were not preexposed to stripped serum, but after culture with 10% FBS, T₃ was added to the medium with 1 or 10% FBS, and Na⁺ pump activity was measured. For inhibition studies, cells were preincubated with inhibitors for 30 min at 37°C and then treated with T₃ in the continued presence of inhibitors for various time intervals.

**Preparation of crude AEC membranes.** Crude cell membranes were prepared as previously described (51) with slight modification. Briefly, the cells were scraped in ice-cold PBS and centrifuged at 5,000 rpm at 4°C for 5 min. The cell pellets were resuspended in 500 µl of ATPase assay buffer containing 37.5 mM imidazole, 137.5 mM NaCl, 18.75 mM KCl, 6.25 mM sodium azide, 0.825 mM EGTA (pH 7.0), 5 mM MgCl₂, and 0.005% SDS for MP48 cells and primary ATII cells and 0.01% SDS for RLE-6TN cells. The cells were sonicated on ice for 10 s with Vibra Cell (Sonics and Materials, Danbury, CT) at 45% duty cycle and 4.5 output power control on ice and centrifuged at 10,000 rpm at 4°C for 10 min. The sediment was resuspended by sonication for 8 s in 350–500 µl of the ATPase assay buffer described above. Aliquots of crude cell membrane preparations were used for assay of protein contents by using the BCA protein assay kit (Pierce, Rockford, IL) and Na-K-ATPase activity. Na-K-ATPase activity assays were performed immediately after isolation of crude cell membrane preparations.
ferred to a polyvinylidene difluoride membrane (catalog no. IPVH00010, Millipore, Bedford, MA), which was then hybridized with primary mouse antibodies against the Na-K-ATPase subunit proteins, the cells were washed twice with ice-cold PBS, scraped off plates, and collected in ice-cold PBS. The cells were pelleted with centrifugation at 5,000 rpm for 3 min. To lyse the pellets, we drew them 10 times in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 2% -mercaptoethanol, and 1% (vol/vol) Nonidet P-40, with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 10 μg/ml each aprotinin and leupeptin). Protein concentrations were determined using the BCA protein assay kit. Equal amounts of total cell protein were precipitated with streptavidin-agarose beads (Sigma Chemical) diluted in lysis buffer 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% -mercaptoethanol, 16% glycerol, and 0.008% bromphenol blue) and analyzed by Western blotting. The higher SDS concentration disrupts the avidin-biotin complexes. The amount of protein was expressed as densitometry in arbitrary units. 

Statistics. Values are means ± SD of a minimum of three experiments. Comparisons involving three or more groups were analyzed by ANOVA and post hoc pairwise comparisons. Differences between means were considered significant at P < 0.05, adjusted for the number of comparisons. 

RESULTS

T3 augments alveolar epithelial Na-K-ATPase enzyme activity. Na-K-ATPase is required for active Na+ transport across the alveolar epithelia (43). To understand the mechanism of T3 stimulation of alveolar fluid clearance in adult rat lung and identify the effect of T3 on Na-K-ATPase activity in adult lung epithelial cells, we used two adult rat AEC lines, MP48 and RLE-6TN, and adult rat primary ATII cells to investigate the effects of T3 on the ATP hydrolytic activity of Na-K-ATPase from physiological to pharmacological concentrations (10⁻⁹-10⁻⁴ M). We also assessed whether different cell culture conditions affected the response of Na-K-ATPase activity to T3. 

MP48 cells were cultured for 24 h in media with 10% stripped FBS and then exposed to T3. T3 increased the enzyme activity in a dose-dependent fashion when presented in 1% stripped FBS or 1% FBS culture conditions (Fig. 1A); activity increased by >40% (P < 0.05) at concentrations as low as 10⁻⁹ M at 6 h of incubation. The T3-induced maximal increase was ~2.2-fold at 10⁻⁵ M in the presence of stripped 1% FBS medium. The magnitude of the increase in Na-K-ATPase activity at every T3 dose in 1% FBS was somewhat less than that in 1% stripped FBS. Smaller increases in Na-K-
ATPase activity occurred when T3 was presented in 10% FBS (data not shown).

Similarly, in RLE-6TN cells, T3 increased Na-K-ATPase activity at 3 and 6 h and when presented in 1% stripped or 10% FBS (Fig. 1 and data not shown). At 3 h, T3 concentrations as low as 10^{-8} M increased Na^+ pump activity in a dose-dependent fashion. The magnitude of the increase was greater at supraphysiologic T3 levels but was statistically significant (30% increase, P < 0.05) at 10^{-8} M. In contrast to T3, 6 h of exposure to the metabolically inactive analog of T3 (50), reverse T3, at the same concentrations had no effect on Na-K-ATPase activity, indicating that the effect of T3 is specific.

Because the cell lines may not reflect the response of the primary rat ATII cells, we also assessed the effect of T3 on Na-K-ATPase activity in primary ATII isolates at 3 days of culture. The cells were T3 starved by exposure to 5% stripped FBS for 24 h before addition of T3 in 1% stripped FBS. T3 increased the Na-K-ATPase activity at 6 h of 10^{-8} M T3 by 131.7% and at 3 h of 10^{-5} M T3 by 149.3% (Fig. 1C). Because the responses to T3 were similar to those of AEC, complete dose-response curves were not determined.

The time course of the T3 effect on Na-K-ATPase activity in MP48 cells is shown in Fig. 2. Surprisingly, the T3-induced increase in Na-K-ATPase activity occurred rapidly at 10^{-5} or 10^{-6} M T3 in 1% stripped FBS. The increase in Na-K-ATPase activity was significant at 1 h of T3 exposure and peaked at 6 h (Fig. 2A). Pharmacological levels of T3 (10^{-4} M) also rapidly increased Na-K-ATPase activity in RLE-6TN cells in 10% FBS (Fig. 2B), with the maximal effect at 6 h. The rapid T3-induced increase in Na-K-ATPase activity suggested that the T3-induced increase may not require transcription or translation of Na-K-ATPase.

T3 does not alter expression of Na-K-ATPase α1- and β1-subunit mRNA and total cellular protein. The principal mechanism for action of T3 on Na-K-ATPase usually is transcriptional (7, 55), but in our study, the rapid increase in Na-K-ATPase activity observed within 0.5–1 h of exposure suggested that T3 stimulation was not transcriptional. To test this hypothesis, the change of Na-K-ATPase mRNA and protein levels was determined after cells were subjected to T3 treatments for various periods. Although the ATII cells expressed some quantities of the Na-K-ATPase α1-, α2-, α3-, β1-, and β3-isoforms, the predominant Na-K-ATPase...
In RLE-6TN cells, Northern blot quantification of the steady-state mRNA levels of the α1-, α2-, α3-, β1-, and β3-subunits was performed to determine whether T3 induced a shift in Na-K-ATPase isoform expression. At 6 h of T3 exposure, there was no significant change in α1- and β1-subunit levels in each of three experiments (data not shown). At 24 h, 10^{-8}–10^{-6} M T3 did not increase the levels of any of the subunits (n = 3, mean change <10% for each subunit at 10^{-4} M T3). High T3 concentrations (10^{-5} M) led to small increases in the mRNA α1-subunit (mean 30% increase, n = 3) but not of other mRNA subunit levels during 24 h of exposure. Thus T3 did not increase the total cellular mRNA and protein levels of Na-K-ATPase subunits during the period with significantly increased Na-K-ATPase activity. These data suggested that the T3-induced increase in Na-K-ATPase activity was independent of transcription.

**Fig. 2.** Time course of effect of T3 on Na-K-ATPase hydrolytic activity in MP48 and RLE-6TN cells. Values are means ± SD of 4 independent experiments. *P < 0.05; **P < 0.01 vs. control. A: MP48 cells were exposed to 10^{-6} or 10^{-5} M T3 in medium with 1% stripped FBS after cells were T3 starved for 24 h by culture in 10% stripped FBS. B: RLE-6TN cells that were cultured in 10% FBS were incubated with 10^{-6} M T3 in medium supplied with 10% FBS.

ATPase isoforms that we detected in adult rat primary ATII cells and in the cell lines are α1, β1, and β3 (data not shown). Therefore, we investigated the effect of T3 on α1- and β1-subunit mRNA and proteins to identify whether T3 affects the Na-K-ATPase activity in a fast manner by regulating the gene expression of α1- and β1-subunits in adult alveolar cells.

In MP48 cells, T3 did not significantly alter the steady-state contents of Na-K-ATPase α1- and β1-subunit mRNA at 6 h of exposure (P > 0.05; Fig. 3). The Northern blot for the β1-subunit mRNA revealed two bands just below the 18S rRNA marker, with the signal much stronger for the lower band.

T3 also did not significantly change the protein levels of α1- and β1-subunits at 10^{-9}–10^{-5} M T3 at 6 h of exposure (Fig. 4, A and B) and at 10^{-5} M at 1–6 h of exposure (Fig. 4, C and D).

**Fig. 3.** Time course of effect of T3 on Na-K-ATPase steady-state content of subunit α1- and β1-mRNA in MP48 and RLE-6TN cells. The mean change (± SD) of α1-subunit (A) and β1-subunit (B) mRNA was determined by Northern blot analysis. The steady-state contents of Na-K-ATPase α1- and β1-subunits were not increased by T3 stimulation. MP48 cells were T3 starved for 24 h in 10% stripped FBS and then T3 was added to the medium for 6 h in 10% FBS. T3 stimulation of Na-K-ATPase activity was not diminished by actinomycin D (Fig. 5A). Similarly, actinomycin D did not inhibit T3 stimulation in MP48 cells at 3 or 6 h in the presence of 1% FBS (data not shown). Similarly, in RLE-6TN cells, actinomycin D did not inhibit T3 stimulation of Na-K-ATPase activity at 3 or 6 h, even at very high T3 concentrations (10^{-4} M; Fig. 5B). Actinomycin D alone had no significant effect on Na-K-ATPase activity in MP48 and RLE-6TN cells. It is not surprising that actinomycin D also had little inhibitory effect on Na-K-ATPase, because the half-life of the Na^+ pump protein is long in primary ATII cells and Madin-Darby canine kidney cells (unpublished data). Thus these data strongly support the concept that T3 stimulation of Na-K-ATPase activity is principally through a non-transcriptional mechanism.

**Fig. 4.** Effect of T3 on Na-K-ATPase subunit protein levels in plasma membrane of MP48 cells. The short-term post-transcriptional regulation of Na-K-ATPase activity by hormones may involve direct effects on the kinetic behavior of the enzyme or translocation of the enzyme between the plasma membrane and intracellular stores (52). On the basis of the minimal effect of T3 on mRNA and protein expression of Na-K-ATPase subunits in MP48 and RLE-6TN cells, we hypothesized that T3 stimulates rapid recruitment of Na-K-ATPase proteins to the plasma membrane in MP48 and RLE-6TN cells. To investigate the effect of T3 on cell surface expression of Na-K-ATPase, intact cells treated with T3 were reacted with a membrane-impermeable biotinylation reagent (sulfobiotin-X-NHS, water soluble, 1-subunits in adult alveolar cells. In MP48 cells, T3 did not signifi- cantly change the protein levels of α1- and β1-subunits at 10^{-9}–10^{-5} M T3 at 6 h of exposure (Fig. 4, A and B) and at 10^{-5} M at 1–6 h of exposure (Fig. 4, C and D).
Fig. 3. T3 does not change steady-state mRNA levels of Na-K-ATPase α1- and β1-subunits in MP48 cells. MP48 cells were cultured as described in Fig. 1. Cells were incubated with T3 for 6 h in 1% stripped FBS medium. Total RNA was isolated, and Northern blot analysis was performed with 10–20 μg of total RNA and 32P-labeled full-length rat cDNA probes for Na-K-ATPase α1- and β1-subunit mRNA and mouse cDNA probes for 18S rRNA. RNA bands were visualized with standard autoradiography. Integrated optical density of RNA bands was determined with Densitometry Image software (Molecular Analyst). All RNA densitometric values were normalized to 18S rRNA.

A and B: representative Northern blots for Na-K-ATPase α1- and β1-subunit mRNA. Lane 1, control (untreated sample); lanes 2, 3, 4, 5, and 6, treatment with 10–9, 10–8, 10–7, 10–6, and 10–5 M T3, respectively. β1 Northern blot revealed two bands, with the lower band having sufficiently greater intensity; the 2 bands are difficult to distinguish in this image.

C: quantitation of effect of T3 on mRNA levels of Na-K-ATPase α1- and β1-subunits at 6 h. Values are means ± SD of 4 independent experiments.

Fig. 4. T3 does not alter the quantity of total cell Na-K-ATPase α1- and β1-subunit proteins in MP48 cells. MP48 cells were cultured as described in Fig. 1. Cells were exposed to T3 for 1 or 6 h in medium with 1% stripped FBS at 37°C. Total cell protein was collected and analyzed by Western blotting. Na-K-ATPase α1- and β1-subunits and actin were detected by specific monoclonal antibodies against α1- and β1-subunits and actin. All α1- and β1-subunit protein densitometric values were normalized to actin protein. A: representative Western blot. MP48 cells were exposed to T3 for 6 h. B: densitometric values. Values are means ± SD from 4 different experiments. C: representative Western blot of Na-K-ATPase proteins from MP48 cells exposed to 10–5 M T3 for 1 and 6 h. D: densitometric values. Values are means ± SD from 6 and 9 different experiments for 1 and 6 h of exposure, respectively.
cleavable) that labels proteins exposed on the cell surface but not proteins in intracellular membranes. T3 increased the amount of Na-K-ATPase α1- and β1-subunit proteins at the cell surface in MP48 cells (Fig. 6). T3 at 10^{-6} M stimulated a significant increase in α1- and β1-subunit proteins at the cell surface after 6 h of T3 exposure; the maximal increase was 1.7-fold for the α1-subunit and 2-fold for the β1-subunit. Significant augmentation (P < 0.05) of α1-subunit (133.9% of control, n = 4) and β1-subunit (131.5% of control, n = 3) proteins at the plasma membrane also was induced at 1 h of T3 exposure (Fig. 6). T3 did not significantly change the total cell protein level of the Na-K-ATPase α1-subunit at 6 h of T3 treatment (Fig. 4); however, it markedly increased the amount of Na-K-ATPase α1- and β1-subunit protein at the cell surface. Taken together with the prior findings, these data suggest that the T3-induced increase in Na-K-ATPase activity and protein at the membrane is due to increased plasma membrane recruitment of this enzyme and not increased de novo protein synthesis.

Brefeldin A decreases T3-induced plasma membrane expression and activity of Na-K-ATPase. Brefeldin A disassembles and redistributes the Golgi complex into

Fig. 5. Actinomycin D (ActD) does not block T3-induced Na-K-ATPase activity in MP48 (A) or RLE-6TN (B) cells. Values are means ± SD of 4 independent experiments. *P < 0.05; **P < 0.01 vs. control. A: MP48 cells were incubated for 6 h in the presence or absence of 10^{-6} M T3 and 10 μg/ml actinomycin D in medium with 1% stripped FBS. B: RLE-6TN cells were maintained for 3 or 6 h in the presence or absence of 10^{-6} M T3 and 10 μg/ml actinomycin D in medium with 10% FBS.

Fig. 6. T3 increases protein amount of Na-K-ATPase α1- and β1-subunits at plasma membrane in MP48 cells. Cells were cultured as described in Fig. 1. Cells were exposed to 10^{-6} M T3 for 1 and 6 h in medium with 1% stripped FBS at 37°C, respectively. Cell surface proteins were biotinylated and lysed. Labeled proteins were immunoprecipitated by streptavidin-agarose beads. Na-K-ATPase α1- and β1-subunits were detected by Western blotting using specific monoclonal antibodies against α1- and β1-subunits. Values are means ± SD of 4 independent experiments. *P < 0.05; **P < 0.01 vs. control. A and C: representative Western blots for Na-K-ATPase α1- and β1-subunits, respectively. B and D: densitometric values (means ± SD) from 4 (α1, 1 h), 8 (α1, 6 h), 3 (β1, 1 h), and 6 (β1, 6 h) different experiments.
the endoplasmic reticulum within minutes of application (8) to disrupt cellular protein trafficking. Brefeldin A inhibits protein transport at various concentrations. Brefeldin A at 1–10 μg/ml completely inhibited protein secretion in cultured rat hepatocytes (42), and at 20 μg/ml, it prevented the dibutyryl-cAMP-dependent increase in cell surface expression and activity of Na-K-ATPase in renal epithelia (24). The effect of 10 μg/ml brefeldin A on the T3-induced increase in protein content of Na-K-ATPase in the plasma membrane was determined. Figure 7, A and B, shows that brefeldin A alone did not significantly modify the basal quantity of cell surface Na-K-ATPase α1- and β1-subunit proteins but completely abolished the T3-induced increment in plasma membrane expression of Na-K-ATPase α1- and β1-subunits in MP48 cells during 6 h of exposure. Thus T3 increased cell surface expression through a brefeldin A-dependent process in MP48 cells, suggesting that T3 stimulation of Na-K-ATPase trafficking is related to the Golgi complex. As expected, 10 μg/ml brefeldin A also completely blocked the T3-induced increment in Na-K-ATPase activity but did not alter basal Na-K-ATPase activity in MP48 cells (Fig. 7C). Abolition of the T3-induced plasma membrane expression and activity of Na-K-ATPase by brefeldin A established that T3-dependent translocation of Na-K-ATPase is responsible for upregulation of the enzyme activity.

**DISCUSSION**

Alveolar fluid clearance is critical for oxygenation and for recovery from alveolar flooding. Active lung fluid resorption requires alveolar epithelial Na-K-ATPase. In a recent study, T3 administered parenterally for 48 h stimulated alveolar fluid clearance in rat lungs in vivo (22). The present study establishes that T3 acts directly on AEC using specific and rapid pathways to increase Na-K-ATPase activity, primarily through stimulation of translocation of Na+ pump α-subunit and β1-subunit protein to the plasma membrane, rather than through increased gene transcription.

T3 specifically stimulates Na-K-ATPase activity in primary ATII cells and two alveolar cell lines. This finding is important for several reasons. Although Na-K-ATPase is a ubiquitous and essential cell enzyme that has been studied intensively, tissue-specific regulation of Na-K-ATPase and the underlying mechanisms are not completely understood (52). Specifically, little is known about the T3 effect on Na-K-ATPase function in the lung. Because Na-K-ATPase is necessary for active Na+ and fluid transport across the alveolar epithelia (43) and T3 stimulates the alveolar fluid in adult rat lung (22), we hypothesized that T3 would stimulate AEC Na-K-ATPase. However, no prior studies have investigated whether the thyroid hormone activates AEC Na-K-ATPase. In the present study, we investigated T3 effects on Na-K-ATPase in primary ATII cells and two adult rat AEC lines, MP48 and RLE-6TN. We demonstrated that T3 stimulated alveolar epithelial Na-K-ATPase activity in a dose- and time-dependent fashion, as T3 does in rat liver cells (27). The inactive isomer reverse T3 had no significant effect on Na-K-ATPase activity in RLE-6TN cells, indicating that the T3 effect is specific.

In contrast to many other tissues or cell types, the stimulatory effect of T3 on Na-K-ATPase activity in AEC is not derived from transcriptional augmentation. Our data show that T3 stimulation of Na-K-ATPase activity was detected within 60 min in MP48 cells and within 30 min in RLE-6TN cells, with maximal stimulation at 6 h. Similarly, T3 rapidly activates Na-K-ATPase in liver of the Anabas fish (49). The mechanism underlying the short-term effect of T3 on Na-K-ATPase remains to be clarified. T3 is a well-known transcriptional regulator for Na-K-ATPase genes in an isoform-specific fashion (17, 29). There are positive thyroid hormone response elements in the 5′-flanking region of the Na-K-ATPase β1-subunit (20) and negative thyroid hormone response elements in the 5′-flanking region of Na-K-ATPase α2- and α3-subunits (29). T3 stimulates Na-K-ATPase activity and isoform mRNA levels in various mammalian tissues (17, 23, 29). However, in

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**Fig. 7.** Brefeldin A eliminates T3-induced cell surface expression of Na-K-ATPase α1-subunit protein (A and B) and T3-induced activity (C) of Na-K-ATPase in MP48 cells. MP48 cells were incubated for 6 h in the presence or absence of 10^{-5} M T3 and 10 μg/μl brefeldin A in 1% stripped FBS medium. Protein on cell surface was measured as described in Fig. 6 legend, and activity of Na-K-ATPase was detected as described in Fig. 1 legend. **P < 0.01 vs. control. A: representative Western blots. B: densitometric values (means ± SD) from 3 different experiments. C: Na-K-ATPase activity data (means ± SD) from 5 different experiments.**
our AEC, T₃ did not change the steady-state levels of α₁- and β₁-subunit mRNAs (Fig. 3). Moreover, the T₃-induced Na-K-ATPase activity was not sensitive to a general inhibitor of gene transcription, actinomycin D. The failure of actinomycin D to block the T₃-induced Na-K-ATPase activity directly demonstrated that T₃ stimulated the Na-K-ATPase activity in a transcription-independent manner. Virtually all thyroid hormone effects previously were believed to be transcriptional; however, nongenomic effects of thyroid hormone are gaining some recognition (13, 18, 55).

The mechanisms by which thyroid hormone regulates Na-K-ATPase vary among tissues. T₃ increases the protein level of Na-K-ATPase in kidney, heart, and skeletal muscle tissues (17); however, the effects of T₃ on lung Na-K-ATPase subunit protein have not been reported. Our data indicate that the T₃-induced increase in Na-K-ATPase activity was not accompanied by an increase in total cell Na-K-ATPase α₁- and β₁-subunit protein, suggesting that T₃ also does not affect translation of Na-K-ATPase mRNA at 6 h. However, the detailed effects of T₃ on translation of Na-K-ATPase subunits and rate of translation need further investigation.

T₃ increased plasma membrane Na-K-ATPase enzyme in AEC, likely because of stimulation of translocation. T₃ previously has been reported to affect sorting and trafficking of proteins. T₃ stimulates the translocation of Trip230, a coactivator of thyroid hormone receptor, from the Golgi complex to the nucleus (10), of choline phosphotransferase from cytosol to mitochondria (9), and of type II iodothyronine 5'-deiodinase from plasma membrane to endosomes (19). In our study, T₃ did not change the total cell content of Na-K-ATPase α₁- and β₁-subunit proteins but increased the plasma membrane expression. The T₃-induced increase of Na-K-ATPase protein at the cell surface was abolished by brefeldin A, a potent inhibitor of translocation that disassembles the Golgi complex and redistributes it into the endoplasmic reticulum (10). Because brefeldin A also may alter recycling of plasma membrane proteins, these data strongly suggest, but do not prove, that T₃ stimulates delivery of Na-K-ATPase to the cell surface via the endoplasmic reticulum-Golgi complex constitutive pathway in AEC. It is somewhat surprising that brefeldin A did not affect basal Na-K-ATPase activity or membrane protein quantity. The effects of T₃ on internalization of Na-K-ATPase and the mechanism(s) underlying the control of T₃ on trafficking of Na-K-ATPase remain to be determined.

The T₃-stimulated translocation of Na-K-ATPase enzyme to the cell surface is necessary for T₃-induced Na-K-ATPase activity in both AEC lines. Lo and Edelman (38) reported that the T₃-induced increase in Na-K-ATPase activity in rat kidney in vivo appeared to occur through stimulation of enzyme synthesis, as they measured incorporation of labeled methionine into the protein in a membrane-rich fraction. However, because their assayed proteins came from the membrane-rich fraction, the observed increase in enzyme protein may have resulted from translocation of Na-K-ATPase enzyme units to the membrane, rather than increased synthesis. Indeed, their subsequent study also verified that T₃ augmented the number of membrane-bound Na-K-ATPase units in rat kidney (39). Thus we propose that the T₃-induced rapid increase of Na-K-ATPase activity depends on translocation of Na-K-ATPase to the cell plasma membrane but cannot exclude effects on recycling.

This rapid increase in plasma membrane Na-K-ATPase is similar to the augmentation of ATII cell Na-K-ATPase activity by dopamine and β-adrenergic agonists reported by Sznajder and colleagues (4, 26, 45, 46). In their studies, increased Na⁺ pump activity resulted from a rapid translocation of Na-K-ATPase to the plasma membrane from a late endosomal compartment and from the slower effects of a mitogen-activated protein kinase pathway. Protein kinase C and phosphoinositide 3-kinase also regulate Na-K-ATPase protein trafficking, as occurs with dopamine in the kidney (56). In our studies of T₃, it is likely that other processes, such as phosphorylation of Na-K-ATPase, and other T₃-activated signals are required for this short-term stimulation by T₃ along with translocation to the plasma membrane. For example, thyroid hormone stimulates the mitogen-activated protein kinase in 293T cells, CV-1 cells, and HeLa cells (14, 35). Therefore, T₃ stimulation of Na-K-ATPase function may also be linked to T₃ activation of other kinases, such as phosphoinositide 3-kinase or mitogen-activated protein kinase.

In summary, T₃ directly stimulates ATII cell and AEC Na⁺ pump activity through posttranscriptional activation, likely by increasing translocation of Na⁺ pump molecules to plasma membrane. Because T₃ augments alveolar fluid clearance in vivo (22), we hypothesize that increased activity of Na-K-ATPase is at least part of the in vivo stimulatory mechanism. However, the effects of T₃ on apical proteins involved in Na⁺ transport, such as the epithelial Na⁺ channel, have not been defined.

DISCLOSURES

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