Insulin regulates alveolar epithelial function by
inducing Na⁺/K⁺-ATPase translocation to the plasma
membrane in a process mediated by the action of Akt

Alejandro P. Comellas*, Aileen M. Kelly, Humberto E. Trejo, Arturo Briva‡, Joyce Lee§, Jacob I. Sznajder and
Laura A. Dada¶

Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
*Present address: Division of Pulmonary, Critical Care and Occupational Medicine, University of Iowa, Iowa City, IA 52242, USA
†Present address: Departamento de Fisiopatología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay
§Present address: Departments of Medicine and Anesthesia, University of California, San Francisco, CA 94143, USA
¶Author for correspondence (lauradada@northwestern.edu)

Accepted 3 February 2010
Journal of Cell Science 123, 1343-1351
© 2010. Published by The Company of Biologists Ltd
doi:10.1242/jcs.066464

Summary
Stimulation of Na⁺/K⁺-ATPase translocation to the cell surface increases active Na⁺ transport, which is the driving force of alveolar
fluid reabsorption, a process necessary to keep the lungs free of edema and to allow normal gas exchange. Here, we provide evidence that
insulin increases alveolar fluid reabsorption and Na⁺/K⁺-ATPase activity by increasing its translocation to the plasma membrane
in alveolar epithelial cells. Insulin-induced Akt activation is necessary and sufficient to promote Na⁺/K⁺-ATPase translocation to the
plasma membrane. Phosphorylation of AS160 by Akt is also required in this process, whereas inactivation of the Rab GTPase-activating
protein domain of AS160 promotes partial Na⁺/K⁺-ATPase translocation in the absence of insulin. We found that Rab10 functions as
a downstream target of AS160 in insulin-induced Na⁺/K⁺-ATPase translocation. Collectively, these results suggest that Akt plays a
major role in Na⁺/K⁺-ATPase intracellular translocation and thus in alveolar fluid reabsorption.

Key words: Akt, Na⁺/K⁺-ATPase, Alveolar epithelium

Introduction
The Na⁺/K⁺-ATPase is an integral basolateral membrane protein that participates in the maintenance of cell volume and
electrochemical gradients (Lingrel and Kuntzweiler, 1994). It is composed of an α and β subunit. The α subunit contains the binding
site for Na⁺, K⁺, ATP and the inhibitor ouabain. The β subunit is a glycoprotein and is associated with the insertion of the heterodimer
at the plasma membrane (McDonough et al., 1990). In the alveolar epithelium, the Na⁺/K⁺-ATPase, working in concert with apical Na⁺
channels, produces an osmotic gradient, which constitutes the
primary force driving alveolar fluid reabsorption (AFR) across the
alveolar epithelium (Matthay et al., 1996; Sznajder et al., 2002).
AFR is responsible for the clearance of lung edema from the alveolar
spaces and thus allows optimal gas exchange (Sznajder et al., 2002).
A variety of hormones stimulate Na⁺/K⁺-ATPase activity by
promoting the insertion of the Na⁺/K⁺-ATPase dimer into the plasma
membrane in a process referred to as Na⁺/K⁺-ATPase translocation
or recruitment (Bertorello et al., 1999; Ewart and Klip, 1995; Ridge
et al., 2002; Vinciguerra et al., 2005). This translocation involves the
movement of vesicles containing Na⁺/K⁺-ATPase from intracellular pools to the plasma membrane (Ridge et al., 2002). It
has been reported that the dephosphorylation of the Na⁺/K⁺-ATPase
α; subunit at the serine 18 residue is a major regulator of this process
(Lecuona et al., 2006). However, how the signals generated after
receptor activation converge to regulate the trafficking steps
necessary to translocate vesicles containing Na⁺/K⁺-ATPase to the
plasma membrane has not yet been elucidated.
Insulin-receptor-induced activation of phosphatidylinositol 3-
kinase (PI3K) generates the membrane-associated second
messenger phosphatidylinositol (3,4,5)-trisphosphate
[PtdIns(3,4,5)P₃], which promotes the activation of Akt and
atypical protein kinases C (aPKCs). Akt is a key signaling protein
that regulates glycogen synthesis, protein synthesis and GLUT4
translocation to the cell surface (Tanguchi et al., 2006; Whiteman
et al., 2002). Upon insulin stimulation, the pleckstrin homology
(PH) domain of Akt binds to lipid messengers and Akt is recruited
to the plasma membrane, where it is phosphorylated at T308 and
S473 by upstream kinases (Shiojima and Walsh, 2002). It has been
described that Akt mediates insulin-induced GLUT4 exocytosis
by phosphorylating the Akt substrate protein of 160 kDa (AS160),
which contains a Rab GTPase-activating protein (GAP) domain
(Howe et al., 1996; Sano et al., 2003; Thong et al., 2007). Rab
proteins switch between a GTP-bound active state and GDP-bound
inactive state; the phosphorylation of AS160 by Akt inhibits GAP
activity and stabilizes the Rab proteins in the GTP-bound form,
which has been described to promote vesicle trafficking (Eguez
et al., 2005; Ishikura et al., 2007).

Insulin increases Na⁺/K⁺-ATPase activity and plasma-membrane
content in kidney-epithelial cells and skeletal-muscle cells through
mechanisms that are tissue and cell specific (Al-Khalili et al., 2003;
Feraille et al., 1999; Sweeney et al., 2001). This study examines
the role of insulin in AFR and Na⁺/K⁺-ATPase activity in alveolar
epithelial cells. Specifically, we describe, first, that insulin increases
Na⁺/K⁺-ATPase abundance at the plasma membrane, second, that
the activation of Akt is both necessary and sufficient to recruit
Na⁺/K⁺-ATPase molecules to the cell surface and, third, that the
effects of Akt on Na⁺/K⁺-ATPase are mediated in part by the
phosphorylation of AS160 and the activity of Rab10.
**Results**

**Insulin increases alveolar fluid reabsorption and Na⁺/K⁺-ATPase activity**

In isolated perfused rat lungs, insulin dose dependently increases AFR, with a maximum at 1 unit/kg (Fig. 1A). Because insulin could lead to catecholamine release (which in turn would activate AFR), we carried out studies using the β-adrenergic receptor antagonist propranolol. Propranolol (10⁻⁴ M) alone had no effect on basal AFR and it did not prevent the insulin effects (Fig. 1A). Permeability to sodium or mannitol flux was not modified among experimental animal groups (see supplementary material Fig. S1), indicating that there were no changes in the epithelial barrier. In addition, perfusate flow did not change in any of the experimental protocols (data not shown).

In cultured rat alveolar type II (ATII) cells, insulin up to 100 nM increased the rate of ⁸⁶Rb uptake (Fig. 1B) and the number of Na⁺/K⁺-ATPase molecules at the cell surface by approximately twofold, with maximum translocation achieved at 100 nM (Fig. 1C). The effect of insulin was apparent within minutes and peaked around 15-30 minutes (Fig. 1D). The ability of insulin to stimulate Na⁺/K⁺-ATPase translocation to the plasma membrane is independent of Na⁺ entry into the cell, because it was not altered by co-incubation with 1 µM amiloride, a specific inhibitor of transmembrane Na⁺ entry (Fig. 1E).

**Insulin stimulates Na⁺/K⁺-ATPase translocation through a mechanism mediated by PI3K and Akt**

The insulin receptor tyrosine kinase is the first protein activated in the insulin-signaling network (Deachapunya et al., 1999). Fig. 2A shows that pre-incubation of ATII cells with genistein, a broad tyrosine-kinase inhibitor, completely prevented insulin-induced Na⁺/K⁺-ATPase recruitment without affecting the basal levels.

Previous studies reported the participation of the PI3K-signaling system in the activation of Na⁺/K⁺-ATPase in fibroblast and kidney epithelial cells (Deachapunya et al., 1999; Ragolia et al., 1997; Sweeney et al., 2001). Incubation with 100 nM wortmannin or LY 294002, two PI3K inhibitors, markedly prevented insulin-induced Na⁺/K⁺-ATPase translocation (Fig. 2B,C).

It is known that PI3K stimulation leads to the activation of Akt and aPKCs. In ATII cells, 1-minute treatment with insulin leads to Akt phosphorylation (Fig. 3A), which was prevented by pre-treatment of the cells with wortmannin (Fig. 3B). Even though the translocation of Na⁺/K⁺-ATPase to the plasma membrane induced by insulin occurs in parallel with Akt activation, it was not known whether Akt phosphorylation is required for this process. We studied the involvement of Akt in insulin-dependent Na⁺/K⁺-ATPase stimulation by infecting ATII cells with an adenovirus encoding a dominant-negative (DN) form of the kinase (Ad-HA-Akt-T308A, S473A), which prevented the increase in Na⁺/K⁺-ATPase activity.
fused in frame with the N terminus of Akt (CA-Akt). In the absence of insulin, overexpression of CA-Akt elicited an increase in Na+/K+-ATPase activity and recruitment to the cell surface that was comparable to that obtained after insulin treatment (Fig. 3D,E). Pretreatment of ATII cells with Akt1/2 inhibitor completely blocked insulin-induced Na+/K+-ATPase translocation (Fig. 3F). Taken together, these results suggest that Akt is necessary and sufficient for the recruitment of Na+/K+-ATPase to the cell surface.

Effects of AS160 on insulin-induced Na+/K+-ATPase translocation

To study whether insulin promotes AS160 phosphorylation in alveolar epithelial cells, we transfected A549 cells with FLAG-WT-AS160 (FLAG-tagged wild-type AS160). A549 cells are from a human alveolar epithelial cell line that shows many characteristics of ATII cells (Lieber et al., 1976), including the regulation of the Na+/K+-ATPase, and are more easily transfected than primary ATII cells (Bertorello et al., 2003; Dada et al., 2003; Lecuona et al., 2006). AS160 phosphorylation was assessed with a phospho-Akt substrate (pAS) antibody after immunoprecipitation with a FLAG antibody. Insulin promotes AS160 phosphorylation in a time-dependent manner (Fig. 4A). In addition, we assessed the insulin-induced phosphorylation of the endogenous AS160 by immunoprecipitating AS160 from cell lysates with an AS160-specific antibody after 5 minutes of treatment with insulin. AS160 phosphorylation was then evaluated using the pAS antibody. We found that insulin increased endogenous AS160 phosphorylation by approximately fourfold and, as expected, this response was prevented by wortmannin and the Akt1/2 inhibitor (Fig. 4B).

Therefore, we investigated the role of AS160 with regard to the effect of insulin on Na+/K+-ATPase activity and translocation. COS-7 cells (which exhibit a higher transfection efficiency than isolated alveolar epithelial cells) are known to be useful for the study of Na+/K+-ATPase translocation (Dada et al., 2007). COS-7 cells were transfected with a mutant form of AS160 that has four of the Akt phosphorylation sites mutated to alanine (FLAG-4P-AS160). This prevented the increase in Na+/K+-ATPase activity and translocation induced by insulin (Fig. 4C). In cells transfected with FLAG-WT-AS160, neither the basal nor the insulin-stimulated Na+/K+-ATPase activity or translocation were affected (Fig. 4D), suggesting again that insulin-induced phosphorylation of AS160 is necessary for Na+/K+-ATPase translocation.

It has been proposed that, in nonstimulated cells, AS160 acts as a negative regulator of trafficking, with its GAP activity functioning to maintain the Rab proteins in their inactive state (Bruss et al., 2005; Eguez et al., 2005; Thong et al., 2007). We analyzed the effects of mutating arginine 973, which is responsible for GAP activity (Eguez et al., 2005), to a lysine residue on insulin-induced Na+/K+-ATPase activity and translocation induced by insulin (Fig. 4C). In cells transfected with FLAG-WT-AS160, neither the basal nor the insulin-stimulated Na+/K+-ATPase activity or translocation were affected (Fig. 4D), suggesting again that insulin-induced phosphorylation of AS160 is necessary for Na+/K+-ATPase translocation.

Rab10 is involved in insulin-induced Na+/K+-ATPase recruitment to the plasma membrane

The GAP domain of AS160 has been described to have activity towards Rab2A, Rab8A, Rab10 and Rab14 (Mîinea et al., 2005; Roach et al., 2007). We have previously shown that the vesicles containing Na+/K+-ATPase are located in the perinuclear area
(Bertorello et al., 2003; Dada et al., 2003), and that different stimuli promote translocation from late endosomes to the plasma membrane (Bertorello et al., 1999; Ridge et al., 2002). It has been reported that Rab2 is involved in the trafficking of proteins from the endoplasmic reticulum to the Golgi (Roach et al., 2007), and that Rab10 (adipose tissue), Rab8A and Rab14 (L6 cells) are implicated in insulin-induced GLUT4 trafficking (Ishikura and Klip, 2008; Sano et al., 2008). To study which of the Rab proteins are involved in insulin-induced Na+/K+-ATPase translocation in alveolar epithelial cells, we isolated total membranes and further subfractionated them on a sucrose gradient, as described in Materials and Methods. Fractions were collected from the gradient and analyzed by immunoblotting. Fig. 5A shows that, in alveolar epithelial cells, there is overlap in the subcellular distribution of Rab8, Rab10, Rab14 and the Na+/K+-ATPase \( \alpha_1 \) subunit. We further characterized the subcellular localization of the different Rab proteins and the Na+/K+-ATPase in V5-\( \alpha_1 \) A549 cells by confocal microscopy. In these cells, the GFP-tagged Rab proteins were preferentially located in the perinuclear area, whereas the Na+/K+-ATPase was located at both the perinuclear area and the plasma membrane (Fig. 5B). We observed that Rab10 colocalized with the Na+/K+-ATPase at the perinuclear region, but not at the plasma membrane (Fig. 5B, upper panels). There was very little or no colocalization between Rab8 and Rab14 and the Na+/K+-ATPase \( \alpha_1 \) subunit (Fig. 5B, middle and lower panels).

We further investigated the role of Rab10 by transfecting COS-7 cells with GFP-T23N-Rab10; this mutation has been shown to decrease the affinity of Rab for GTP, resulting in an increase of the GDP-bound form (Babbey et al., 2006). Overexpression of this mutant prevented insulin-induced Na+/K+-ATPase translocation to the plasma membrane (Fig. 6A). A second mutant, in which the glutamine at position 68 was changed to leucine (Rab10-QL), increases the amount of the GTP-bound form of the protein and has been described to have dominant-active effects on Rab10 function (Babbey et al., 2006). Overexpression of GFP-Rab10-QL resulted in increased Na+/K+-ATPase abundance at the plasma membrane in the absence of insulin, whereas overexpression of wild-type GFP-Rab10 had no effect (Fig. 6B). Consistent with the data shown in Fig. 6B, transfection of GFP-Rab10-QL rescued the inhibition of insulin-induced Na+/K+-ATPase translocation to the plasma membrane caused by AS160-4P (Fig. 6C).

**Discussion**

This study shows that insulin increases alveolar fluid reabsorption and, through the action of Akt and its substrate, AS160, stimulates Na+/K+-ATPase activity by increasing the number of functioning Na+/K+-ATPase molecules at the plasma membrane of alveolar epithelial cells.

![Fig. 3. Insulin-stimulated Na+/K+-ATPase recruitment requires Akt.](image-url)

(A) Serum-starved ATII cells were incubated with 100 nM insulin (INS) for 1 or 5 minutes. Then, phosphorylated Akt (p-Akt) or the total amount of Akt was measured by western blot analysis of aliquots of cell lysate containing equal amounts of protein. A representative blot is shown (n=3). (B) Serum-starved ATII cells were incubated with 100 nM insulin in the absence or presence of wortmannin (WM; 100 nM), and pAkt and total Akt were determined as described in A (n=3). (C) ATII cells were infected with a null adenovirus (Sham) or dominant-negative Akt adenovirus (DN-Akt). Na+/K+-ATPase activity, measured by \( ^{86} \text{Rb} \) uptake, was determined in serum-starved cells with or without insulin treatment (100 nM, 30 minutes) 24 hours post-infection (n=6). Representative immunoblot of Akt expression level are shown. (D) ATII cells were infected with a null adenovirus (Sham) or constitutively active HA-tagged Akt adenovirus (CA-Akt). Na+/K+-ATPase activity was measured as described in Fig. 1B (n=6). Representative immunoblots of Akt expression levels are shown. (E) ATII cells were infected with a null adenovirus (Sham) or CA-Akt. 24 hours post-infection, Na+/K+-ATPase abundance at the plasma membrane was measured as described in Fig. 1C (n=3). Representative western blots of \( \alpha_1 \)-subunit abundance and Akt expression levels are shown. (F) Serum-starved ATII cells were incubated in the presence or absence of insulin (100 nM) for 30 minutes in the presence or absence of Akt 1/2 inhibitor (1 \( \mu \)M, 60 minutes preincubation). Na+/K+-ATPase recruitment to the plasma membrane was determined as described in Fig. 1C. A representative Na+/K+-ATPase \( \alpha_1 \) subunit immunoblot is shown. Values are expressed as mean ± s.e.m. **P<0.01. CT, control.
Akt in Na⁺/K⁺-ATPase translocation

epithelial cells. Moreover, we described Rab10 as the downstream target of AS160 involved in the translocation of Na⁺/K⁺-ATPase.

Pulmonary edema accumulates as a consequence of changes in hydrostatic pressure gradients across the pulmonary circulation or increased alveolar capillary permeability. It is cleared by the concerted actions of epithelial Na⁺ channels and Na⁺/K⁺-ATPase (Mutlu and Sznajder, 2005). Treatment of rats with insulin increases AFR to a level similar to that we have previously reported for β-adrenergic receptor agonists (Saldias et al., 1998; Saldias et al., 2002; Saldias et al., 2000; Sznajder et al., 2002). In some experiments, rats were pretreated with the β-receptor antagonist propranolol to prevent a potential increase in AFR due to hypoglycemia-induced catecholamine release. Propranolol had no effect on baseline AFR or on the insulin-induced increase in AFR. The effects of insulin on AFR are in agreement with previous reports that, in a mouse model of type 2 diabetes, a lower AFR rate was due in part to decreased active Na⁺ transport and decreased Na⁺/K⁺-ATPase activity (Bellmeyer et al., 2007).

Rat alveolar epithelial cells express specific high-affinity insulin receptors that share biochemical and immunological similarities with insulin receptors characterized from other cell types (Sugahara et al., 1984). Here, we show that short-term (15-30 minutes) incubation with 100 nM insulin stimulated Na⁺/K⁺-ATPase activity in parallel with an increase in the number of functioning Na⁺ pumps at the plasma membrane, as assessed by biotin cell-surface labeling. Moreover, this effect occurs independently of apical Na⁺ transport. Because the increase in the number of Na⁺ pumps at the plasma membrane is observed within 5 minutes, we reasoned that this is not due to increased synthesis, but the result of Na⁺/K⁺-ATPase molecules being stored in intracellular compartments and then translocated to the basolateral membrane, as we have previously reported for incubation with catecholamines and dopamine (Bertorello et al., 1999; Lecuona et al., 2000; Ridge et al., 2002).

The first step in the insulin-signaling cascade involves insulin binding to its plasma-membrane receptor followed by stimulation of receptor-mediated tyrosine kinase activity. We demonstrated that a tyrosine kinase inhibitor blocked insulin-stimulated Na⁺/K⁺-ATPase translocation. Also, selective inhibitors of PI3K activity abolished the stimulatory effects of insulin on Na⁺/K⁺-ATPase translocation. PI3K is a central signaling molecule in insulin action.
and the phosphoinositide signal is mostly transmitted through its downstream Akt and aPKC targets (Cong et al., 1997; Taniguchi et al., 2006). It has been already described that the activation of PKCζ regulates insulin-induced Na⁺/K⁺-ATPase translocation in renal cells (Sweeney et al., 2001); here, we focused on the role of insulin-induced Akt activation. In alveolar epithelial cells, insulin increased the phosphorylation of Akt after 1 minute and inhibition of Akt using DN-Akt blocked insulin stimulation in 86Rb uptake. We have previously reported that the adenoviral infection efficiency of isolated rat ATII or A549 cells is ~90%; infection of these cells with the Akt constructs resulted in levels of expression similar to that we have previously observed with adenoviral delivery of other constructs (Vadasz et al., 2008). In the case of CA-Akt, we were able to demonstrate that overexpression of this protein resulted in increased Na⁺/K⁺-ATPase activity and translocation to the plasma membrane, independently of insulin. To rule out the possibility that the observed effects were a product of the overexpression of Akt,
we used a selective Akt inhibitor, which blocked Na⁺/K⁺-ATPase translocation to the plasma membrane, suggesting that the endogenous Akt is likely to play a role in this process. These results are the first evidence of a physiological role for Akt in insulin-induced Na⁺/K⁺-ATPase translocation. Our results suggest that Akt activation is sufficient for Na⁺/K⁺-ATPase translocation. In this sense, the recruitment of Na⁺ pumps to the plasma membrane is different from GLUT4 translocation, in which Akt-dependent and -independent signals have been described (Gonzalez and McGraw, 2006).

AS160 is an Akt substrate protein that has been implicated in the intracellular retention of GLUT4 under basal conditions (Eguez et al., 2005; Sano et al., 2003). We have previously described that, under basal conditions, Na⁺/K⁺-ATPase is mostly retained in intracellular compartments (Lecuona et al., 2009b; Riddle et al., 2002). In alveolar epithelial cells, insulin stimulates AS160 phosphorylation within 5 minutes and, in agreement with the fact that Na⁺/K⁺-ATPase recruitment to the plasma membrane is dependent on PI3K and Akt activation, AS160 phosphorylation was prevented by wortmannin and the Akt inhibitor. Insulin-induced Na⁺/K⁺-ATPase translocation to the plasma membrane depends on AS160 phosphorylation, because overexpression of AS160-4P showed a strong inhibitory effect, whereas expression of the wild-type protein had no effect. The expression of AS160 with a mutation of the GAP domain increases Na⁺/K⁺-ATPase translocation even in the absence of insulin, suggesting that, by maintaining the Rab(s) in the inactive GDP-bound state, AS160 is involved in the intracellular retention of the Na⁺/K⁺-ATPase. Insulin- and Akt-induced phosphorylation of AS160 somehow inactivates the GAP activity of the Rab(s), allowing the translocation of the Na⁺/K⁺-ATPase to the plasma membrane. These results are in agreement with the described role of AS160 in insulin-induced GLUT4 translocation (Miinea et al., 2005; Sakamoto and Holman, 2008; Sano et al., 2003; Thong et al., 2007). Moreover, AS160 has been proposed to be a molecule that integrates the activation of kinases such as Akt, PKCζ and the AMP-activated protein kinase (Sakamoto and Holman, 2008; Thong et al., 2007).

Subcellular fractionation of alveolar epithelial cells revealed that Rab10, Rab8 and Rab14 (which have been previously described as substrates of AS160) are distributed in the same fractions as the Na⁺/K⁺-ATPase (2) subunit. Immunofluorescence analysis showed that, even though the three Rab proteins studied have a mostly perinuclear localization, only Rab10 colocalizes with the Na⁺/K⁺-ATPase. Our data also suggested that Rab10 is involved in the recruitment of the Na⁺/K⁺-ATPase to the plasma membrane, because a constitutively active form of Rab10 stimulates the translocation of Na⁺/K⁺-ATPase in the absence of insulin, whereas a mutation rendering it inactive prevented insulin-induced Na⁺/K⁺-ATPase translocation. Importantly, overexpressing constitutively active Rab10 overcomes the inhibition of AS160-4P, reinforcing the concept that Rab10 is a substrate of AS160. Even though constitutively active Rab10 stimulates Na⁺/K⁺-ATPase recruitment to the plasma membrane in the absence of insulin, the levels of translocation achieved are lower than those obtained by insulin treatment, suggesting that other Rab proteins might also be involved and/or that other insulin-induced steps necessary to achieve complete Na⁺/K⁺-ATPase translocation are independent of AS160 and Rab. Interestingly, in polarized epithelial cells, Rab10 is specifically associated with basolateral trafficking, regulating transport from basolateral sorting endosomes to common endosomes (Bab bey et al., 2006). Furthermore, it has been described that Rab proteins control motor-protein recruitment to their specific target membranes (Jordens et al., 2005). Accordingly, in muscle cells, Rab8A, which shares high sequence identity with Rab10, binds to myosin Va and Vb, facilitating the transport of GLUT4 (Ishikura and Klip, 2008; Klip, 2009). Interestingly, our group has described that myosin Va plays a role in the intracellular retention of Na⁺/K⁺-ATPase under basal conditions (Lecuona et al., 2009a).

Translocation of the Na⁺/K⁺-ATPase to the plasma membrane is a complex process that requires the participation of different signaling pathways. We propose that Akt, acting through AS160 and Rab10, links the PI3K and insulin signaling pathway to the vesicle-trafficking machinery involved in Na⁺/K⁺-ATPase translocation. Moreover, we have shown that Akt is necessary and sufficient for insulin-stimulated Na⁺/K⁺-ATPase recruitment.

Materials and Methods

Materials and constructs
Insulin from bovine pancreas (27 USP units/mg), recombinant human insulin from yeast (27.5 units/mg), amiloride and propranolol were from Sigma-Aldrich. Genistein, Wortmannin, LY 294002 and Akt inhibitor 1/2 were from EMD and ouabain from ICN Biomedicals. Rabbit polyclonal antibodies against phospho-Akt (p-Akt, Ser473), total Akt, phospho-Akt substrate (anti-PAS) and human AS160 were from Cell Signaling Technology and Rab14 was from Abcam. The following mouse monoclonal antibodies were purchased: FLAG (Sigma-Aldrich), Na⁺/K⁺-ATPase α1 subunit (clone 464.6, Millipore), GFP (Santa Cruz Biotechnology), HA ( Covance), V5 (Invitrogen) and Rab8 (Transduction Laboratories).

Plasmids encoding WT-AS160 tagged with a FLAG epitope (FLAG-WT-AS160), AS160 with four of its six predicted Akt phosphorylation sites mutated to alanine (S318A, S388A, T642A and S751A; FLAG-4P-AS160) or AS160 with arginine 973 mutated to lysine (FLAG-R/K-AS160) were kind gifts from Gustav E. Lienhard (Dartmouth Medical School, Hanover, NH, USA) (Kane et al., 2002; Sano et al., 2003). These plasmids were constructed from a cDNA encoding AS160 provided by the Kazusa DNA Research Institute. Plasmids encoding GFP-Rab10-WT, a Rab10 mutant in which glutamine 68 was changed to leucine (GFP-Rab10-Q68L), a Rab10 mutant in which a critical threonine in the Rab10 GTP-binding domain was mutated to alanine (GFP-Rab10-T22N) (Bab bey et al., 2006) or GFP-Rab8 were a kind gift from Kenneth W. Dunn (Division of Nephrology, Indiana University Medical Center, Indianapolis, IN, USA). Rab14-EGFP was a gift from Richard H. Scheller (Genentech, South San Francisco, CA, USA) (Juntutula et al., 2004). All other reagents were commercial products of the highest grade available.

Animals
Pathogen-free male Sprague-Dawley rats weighing 300-325 g were used for the isolated lung model and male Sprague-Dawley rats (200-225 g) were used in epithelial ATII cell isolation. All animals were provided with food and water ad libitum, maintained on a 12:12 hour light-dark cycle, and handled according to National Institutes of Health guidelines and experimental protocols approved by the Northwestern University Animal Care and Use Committee.

Isolated perfused rat lung model
Rats were anesthetized and then injected with insulin (i.v.) 30 minutes before starting the experiment, or pretreated with propranolol (i.p.) and then injected with insulin. The isolated lung preparation has been previously described (Briva et al., 2007; Myrianthefs et al., 2005; Rutschman et al., 1993; Saldias et al., 2001). Briefly, the lungs and heart were removed en bloc. The pulmonary artery and left atrium were catherized and perfused continuously with a solution of 3% BSA in buffered physiological salt solution (135.5 mM Na⁺, 119.1 Cl⁻, 25 mM HCO₃⁻, 4.1 mM K⁺, 2.8 mM Mg²⁺, 2.5 mM Ca²⁺, 0.8 mM SO₄²⁻, 8.3 mM glucose) at constant pressure, 37°C and pH 7.4. Trace amounts of fluorescein isothiocyanate (FITC)-albumin were also added to the perfusate. The fluorescein was measured in a ‘pleural’ bath (100 ml) filled with the same BSA solution. The lungs were then instilled with a total of 5 ml volume of the BSA solution containing 0.1 mg/ml Evans blue dye-albumin, 0.02 μCi/ml of [³²Na] (GE-Healthcare) and 0.12 μCi/ml of [³H]mannitol (Perkin Elmer). Samples were taken from the instillate, perfusate and bath solutions after an equilibration time of 10 minutes following the instillation and again 60 minutes later. Absorbance analysis of the supernatant or Evans blue dye-albumin was performed at 620 nm in a Hitachi model U2000 spectrometer (Hitachi). Analysis of FITC-albumin (excitation 487 nm and emission 520 nm) was performed in a Perkin-Elmer fluorometer (model LS-3B).

Epithelial ATII cell isolation, cell culture and insulin treatment
ATII cells were isolated by the Pulmonary Division Cell Culture and Physiology Core B, as previously described (Ridge et al., 1997), and used two or three days after isolation.
A549 cells (ATCC CCL 185, a human adenocarcinoma cell line) and COS-7 cells (ATCC CRL 1651) were grown over glass coverslips (~5 \times 10^4 cells/coverslip), fixed, permeabilized with 0.1% Triton X-100 in PBS, blocked in 1% normal goat serum, and stained with an antibody against V5 in blocking solution and then incubated with Alexa-Fluor-568 goat anti-mouse IgG. The cellular distribution of Na⁺/K⁺-ATPase α1 and the different Rab proteins was analyzed using a Zeiss LSM 510 laser-scanning confocal microscope (objective Plan Apochromat, \( 	imes 63/1.4 \) oil) (Zeiss, Heidelberg, Germany). Contrast and brightness settings were adjusted so that all pixels were in the linear range.

**Contrast and brightness settings were adjusted so that all pixels were in the linear range.**

**Transfection of A549 or COS-7 cells were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Experiments were carried out 48 hours after transfection. The transfection efficiency of the A549 and COS-7 cells was ~50 and 80%, respectively.**

**Immunofluorescence**

V5 α1-A549 cells transiently transfected with GFP-Rab10, GFP-Rab14 or GFP-Rab8 were grown over glass coverslips (5 \times 10^4 cells/coverslip) and fixed to achieve optimal membrane expression. The cells were permeabilized with 0.1% Triton X-100 in 1% PBS and blocked with 1% normal goat serum. The primary antibodies were used in blocking solution and then incubated with Alexa-Fluor-568 goat anti-mouse IgG. The cellular distribution of Na⁺/K⁺-ATPase α1 and the different Rab proteins was analyzed using a Zeiss LSM 510 laser-scanning confocal microscope (objective Plan Apochromat, \( 	imes 63/1.4 \) oil) (Zeiss, Heidelberg, Germany). Contrast and brightness settings were adjusted so that all pixels were in the linear range.

**Determination of Na⁺/K⁺-ATPase activity**

Na⁺/K⁺-ATPase activity in intact ATII cells was determined by ouabain-sensitive 86Rb⁺ uptake as previously described (Ridge et al., 2002). 86Rb⁺ influx was quantified by a liquid scintillation counter.

**Determination of Na⁺/K⁺-ATPase activity**

Na⁺/K⁺-ATPase activity in intact ATII cells was determined by ouabain-sensitive 86Rb⁺ uptake as previously described (Ridge et al., 2002). 86Rb⁺ influx was quantified by a liquid scintillation counter.

**Supplementary material available online at**

http://jcbiologists.org/cgi/content/full/123/8/1343/DC1

**References**


Supplementary Figure 1. Passive movement of $^{22}$Na$^+$ (Black bars) and $^3$H-Mannitol (Grey bars) were measured in control, propanolol (P) and insulin treated animals as described in detail in Methods. Scintillation counts for $^{22}$Na$^+$ and $^3$H-Mannitol were measured in a Beckman beta counter (Model LS 6500). Graph represents the mean±SEM (n = 5). Differences among groups were not statistically significant.