Dopamine regulates Na-K-ATPase in alveolar epithelial cells via MAPK-ERK-dependent mechanisms

CARMEN GUERRERO,1,2 EMILIA LECUONA,1 LIUSKA PESCE,1 KAREN M. RIDGE,1 AND JACOB I. SZNAJDER1
1Division of Pulmonary and Critical Care Medicine, Northwestern University, Chicago, Illinois 60611; and 2Centro de Investigación del Cáncer, Universidad de Salamanca, 37007 Salamanca, Spain

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Guerrero, Carmen, Emilia Lecuona, Liuska Pesce, Karen M. Ridge, and Jacob I. Sznaider. Dopamine regulates Na-K-ATPase in alveolar epithelial cells via MAPK-ERK-dependent mechanisms. Am J Physiol Lung Cell Mol Physiol 281: L79–L85, 2001.—Dopamine (DA) increases lung edema clearance by regulating vectorial Na⁺ transport and Na-K-ATPase in the pulmonary epithelium. We studied the role of the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) pathway in the DA regulation of Na-K-ATPase in alveolar epithelial cells (AEC). Incubation of AEC with DA resulted in a rapid stimulation of ERK activity via dopaminergic type 2 receptors. Analysis of total RNA and protein showed a 1.5-fold increase in the Na-K-ATPase β₁-subunit mRNA levels and up to a fivefold increase in β₁-subunit protein abundance after DA stimulation, which was blocked by the MAPK kinase (MEK) inhibitors PD-98059 and U-0126. Also, the DA-ERK pathway stimulated the synthesis of a green fluorescent protein reporter gene driven by the β₁-subunit promoter, which indicates that DA regulates the Na-K-ATPase β₁-subunit at the transcriptional level. The DA-mediated increase in β₁-subunit mRNA protein resulted in an increase in functional Na pumps in the basolateral membranes of alveolar type II cells. These results suggest that the MAPK-ERK pathway is an important mechanism in the regulation of Na-K-ATPase by DA in the alveolar epithelium.

alveolar type II cells; green fluorescent protein; transcriptional regulation; basolateral membrane

Na-K-ATPase is a ubiquitous enzyme essential for the maintenance of membrane potential and control of cellular volume (17). It also has been shown to have an important role in lung edema clearance by increasing active Na⁺ transport (4, 23). A functional Na-K-ATPase is a transmembrane heterodimer protein composed of two subunits (α and β) (29). The catalytic α-subunit contains the binding sites for Na, K, and ATP. The glycosylated β-subunit appears to be important in the insertion of the Na pump to the plasma membrane (20). In alveolar type II (ATII) cells, Na-K-ATPase is predominantly composed of α₁- and β₁-isofoms (25).

Na-K-ATPase can be regulated by short-term or long-term mechanisms. The short-term regulation of the Na pump involves changes in its turnover, its affinity for substrates, and/or its abundance at the cell surface (7, 8, 12). The long-term regulation of Na-K-ATPase includes transcriptional activation, translation, and protein stability (12). Aldosterone, insulin, thyroid hormone, epidermal growth factor, and keratinocyte growth factor are some of the agonists known to regulate the transcriptional and/or translational rate of the Na pump, although the molecular mechanisms involved have not yet been elucidated.

Dopamine (DA) inhibits Na-K-ATPase activity in most tissues, including brain (6), vascular bed (24), and kidney (5). In contrast, recent studies have suggested that DA increases the ability of the lungs to clear edema, probably by the regulation of the alveolar epithelial Na-K-ATPase function (1, 27).

The mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) cascade is a major signaling system by which cells transduce extracellular signals into intracellular responses. ERK proteins (ERK1/2) have many substrates, including the ternary complex factor Elk-1, a member of the Ets family of transcription factors, which is recruited by serum-response factors to bind serum-response elements, located in the promoters of many early genes (31) and also in the 5'-flanking region of the Na-K-ATPase β₁-subunit gene (18).

DA has an inhibitory role on the ERK pathway in most cell types (22, 30), although recent reports suggested that DA activates ERK proteins in Chinese hamster ovary cells (32) and neurons (14). However, there are no studies exploring whether the activation of ERK by DA regulates Na-K-ATPase.

We have investigated whether DA activates the ERK signaling pathway in alveolar epithelial cells (AEC) and whether this activation regulates Na-K-ATPase. Our results demonstrate that DA regulates β₁-subunit mRNA and protein abundance via an ERK-dependent pathway that involves dopaminergic type 2 receptors

Address for reprint requests and other correspondence: J. I. Sznaider, Pulmonary and Critical Care Medicine, Northwestern Univ., 300 E. Superior St. Tarry 14–707, Chicago, IL 60611 (E-mail: j-sznaider@northwestern.edu).

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amplification of the Na-K-ATPase activity. U-0126 (Promega, Madison, WI) were used as inhibitors of ERK activity. PD-98059 (New England Biolabs) and a D786-bp fragment of the Na-K-ATPase subunit gene promoter region containing 773 bp upstream and 12 bp downstream from the nucleotide +1 was subcloned into the EcoRV site of plasmid pT7blue(R) (Novagen, Madison, WI) and then subcloned as a HindIII-EcoRI fragment into the promoter reporter vector pEGFP-1 (Clontech, Palo Alto, CA). The final construct was named pEGFP-β1P.

Cloning of the Na-K-ATPase β1-subunit promoter into pEGFP-1 plasmid. A 786-bp fragment of the Na-K-ATPase β1-subunit gene promoter region containing 773 bp upstream and 12 bp downstream from the nucleotide +1 was subcloned into the EcoRV site of plasmid pT7blue(R) (Novagen, Madison, WI) and then subcloned as a HindIII-EcoRI fragment into the promoter reporter vector pEGFP-1 (Clontech, Palo Alto, CA). The final construct was named pEGFP-β1P.

Generation of stable cell lines overexpressing pEGFP-β1P construct. A549 cells derived from human AEC (American Type Culture Collection CCL 185) were transfected with 2–5 μg of plasmid pEGFP-β1P by using SuperFect Reagent (QIAGEN, Valencia, CA) and selected in the presence of 600 μg/ml Geneticin (G418).

RNA isolation and analysis by semiquantitative RT-PCR. Total cellular RNA from ATII cells was isolated by using RNeasy total RNA kit (QIAGEN). The reverse transcription (RT) reaction was performed using the SUPERSCRIPT premix amplification system (GIBCO BRL, Grand Island, NY). The resultant cDNAs were amplified by PCR using specific primers and analyzed by agarose gel electrophoresis. The amplified bands were quantified by densitometric scan and normalized against the internal control gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Oligonucleotides and conditions used in PCR. For the amplification of the Na-K-ATPase β1-subunit, we used the following set of oligonucleotides: 5'-AAT CAT GAA CGA GGA GAG CG-3' and 5'-AGG TGA GGT TGG TGA ACT GC-3' that correspond to the positions 418–437 and 786–805, respectively, of cDNA from the rat gene (considering as number 1 the “A” of the first ATG of the gene). The conditions for the PCR were 94°C, 1 min; 53°C, 1 min 30 s; and 72°C, 2 min for 41 cycles.

For the amplification of the control gene GAPDH, we used the rat GAPDH Control Amplifier Set (Clontech) at 94°C, 45 s; 60°C, 45 s; and 72°C, 2 min for 21 cycles. Primers used for the detection of D2R were 5'-CCT TCA CCA TCT CTT GC-3' and 5'-CCT TCT GGT GGG AGA GC-3', corresponding to positions 488–504 and 1089–1105, respectively, of cDNA from the mouse gene, and the conditions for the PCR were 94°C, 1 min; 53°C, 1 min 30 s; and 72°C, 2 min for 30 cycles.

Quantification of green fluorescent protein fluorescence. pEGFP-1- or pEGFP-β1P-expressing A549 cultures were plated in four-well Permanox chamber slides (Nalge Nunc International, Naperville, IL), starved, and treated with DA or DA plus PD-98059 for 24 h. Cells were fixed by incubation with 4% formaldehyde in PBS for 15 min, washed with PBS, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Photographs were taken using a GFP (green fluorescent protein) filter set in a Nikon Eclipse E800 fluorescence microscope equipped with a 100-W mercury lamp. DA effect was evaluated by visually comparing the regional level of fluorescence in pictures of cultures. Quantitative data were obtained by fluorometric assay and immunoblotting analysis.

A549 clones expressing pEGFP-β1P or transfected with control plasmid pEGFP-1 were plated in 6-cm plates, starved at subconfluence, and treated with DA or DA plus PD-98059 for 24 h. Cell cultures were washed twice in PBS and resuspended in 300 μl of sonication buffer (50 mM NaH2PO4, 10 mM Tris-HCl, and 200 mM NaCl), pH 8.0. Twofold serial dilutions ranging from 50 to 3.125 μg were prepared in sonication buffer. Fluorescence intensity was measured in a Perkin-Elmer fluorescence spectrometer (model LS-3B; Perkin-Elmer, Oakbrook, IL) using an excitation filter of 490 nm and an emission filter of 510 nm.

Deglycosylation of the Na-K-ATPase β1-subunit protein. Total protein (100 μg) from ATII cells was digested with 1 U of N-glycosidase F as described previously (15).

Western blot analysis. Total deglycosylated protein (100 μg) or 5 μg of PLMs, isolated as described previously (8), were resolved by 12.5% SDS-PAGE and analyzed by immunoblotting using specific Na-K-ATPase anti-β1 polyclonal antibody or anti-α1 monoclonal antibody (a generous gift from Dr. Martin-Vasallo, University of La Laguna, Spain, and Dr. M. Caplan, Yale University, CT, respectively). For the detection of GFP, 25 μg of total protein were resolved by 12.5% PAGE and analyzed by immunoblotting using an anti-GFP monoclonal antibody, clone B34 (BABCO, Richmond, CA).

Transport measurements. Ouabain-sensitive 86Rb⁺ uptake was used to estimate the rate of K⁺ transport by Na-K-ATPase in AEC. Briefly, cells were preincubated for 5 min at 37°C in a gyratory bath at 100 rpm by adding 5 mM ouabain in HEPES-buffered DMEM. This medium was then removed, and otherwise identical fresh medium containing 1 μCi/ml 86Rb⁺ was added. After a 5-min incubation (37°C, 100 rpm), uptake was terminated by aspirating the assay medium and washing the plates in ice-cold MgCl₂. Plates were allowed to dry, and cells were solubilized in 0.2% SDS. 86Rb⁺ influx was quantified in aliquots of the SDS extract with a liquid scintillation counter. Protein was quantified in aliquots by the Lowry method.

Statistical analysis. Data are means ± SE. All statistical analyses were made using one-way ANOVA, except data in Figs. 4 and 6 that were treated with two-way ANOVA (time and DA as the 2 independent variables), followed by a multiple comparison test (Tukey) when the F statistic indicated significance. Results were considered significant when P < 0.05.

RESULTS

DA activates MAPK-ERK in ATII cells. Figure 1 shows that DA induced up to 3.5-fold increase in ERK activity after 5 min of incubation of subconfluent, serum-starved ATII cells. This activation was specific because it was abolished when cells were pretreated with the specific MAPK kinase (MEK) inhibitor PD-98059 2 h before DA stimulation.

DA stimulates ERK activity via D2R. RT-PCR analysis of total RNA showed that D2R (2) and D3R mRNA are both expressed in ATII cells (Fig. 2). To determine...
whether D₁R or D₂R receptors mediate ERK activation by DA, serum-starved ATII cells were treated with DA in the presence and absence of the mitogen-activated protein kinase kinase (MEK) inhibitor PD-98059 (50 μM), and the ERK activity was determined as phosphorylation of the transcription factor Elk-1 (relative values). *Top:* means ± SE; *n* = 4; **P < 0.01 vs. control (CT). *Bottom:* representative ERK assay. P-Elk-1, phosphorylated Elk-1.

β-Adrenergic receptors (β-ARs) activate ERK proteins via a G protein-dependent mechanism (11). DA has been found to activate β-ARs at high doses (9), but pretreatment of ATII or A549 cells with 10 μM propranolol, a specific β-AR antagonist, did not affect the DA-mediated ERK stimulation (data not shown), which indicates that the observed DA effect is not mediated by the β-adrenergic pathway.

DA regulates Na-K-ATPase β₁-subunit mRNA and protein abundance in ATII cells via an ERK-dependent mechanism. To determine whether DA regulates Na-K-ATPase at the mRNA and/or protein level and whether this effect is mediated by ERK activation, serum-starved ATII cells were incubated with DA in the presence and absence of the MEK inhibitor PD-98059. Changes in mRNA and protein levels were examined at different times. As shown in Fig. 4, there was a 1.5-fold increase in the Na-K-ATPase β₁-subunit mRNA levels after ~18 h of DA stimulation. This increase was abolished in cells preincubated with PD-98059. The optimal conditions for the PCR of both the Na-K-ATPase β₁-subunit gene (41 cycles) and the GAPDH gene (21 cycles) were established in preliminary studies (Fig. 5).

Time-course studies demonstrated that DA increases Na-K-ATPase β₁-subunit protein abundance in whole cell homogenates with a maximum (~5-fold).
effect at 24 h (Fig. 6), which correlates with the maximum increase in mRNA abundance observed at 18 h (Fig. 4). The DA-mediated increased in $\beta_1$-subunit protein abundance was dependent on ERK proteins because it was inhibited by PD-98059 (Fig. 6) and also by the newly described MEK inhibitor U-0126 (Fig. 7A). Additionally and according to results in Fig. 3, treatment with quinpirole for 24 h also increased total $\beta_1$-subunit protein abundance in an ERK-dependent manner (Fig. 7B). No significant changes were observed in the Na-K-ATPase $\alpha_1$-subunit mRNA or protein levels in whole cell homogenates (data not shown).

DA regulates the transcription of the Na-K-ATPase $\beta_1$-subunit. To determine whether DA regulates the Na-K-ATPase $\beta_1$-subunit at the transcriptional level, permanent transfectants of A549 cells expressing pEGFP-$\beta_1$P or vector alone were serum starved and treated with DA in the presence and absence of PD-98059 or U-0126. DA activated ERK in A549 cells to the same extent as in ATII cells (Fig. 8A). Cells were analyzed by 1) fluorescent microscopy, 2) fluorescent spectrophotometry, and 3) Western blot with anti-GFP antibodies. The three different approaches showed that DA increased GFP expression under the control of the Na-K-ATPase $\beta_1$-subunit promoter (Fig. 8B). The increase observed in both the fluorometric analysis (Fig. 8B, a) and the immunoblotting (Fig. 8B, c) was $\sim$1.5-fold, which agrees with the increase in mRNA detected by PCR (Fig. 4). The DA-induced increase in GFP expression was inhibited by PD-98059, as demonstrated by Western blot (Fig. 8B, c). We found that

Fig. 4. DA increased the Na-K-ATPase $\beta_1$-subunit mRNA via ERK proteins. Serum-starved ATII cells were incubated with 10 $\mu$M DA in the presence and absence of the MEK inhibitor PD-98059 (50 $\mu$M; I) for the indicated times. mRNA from stimulated cells was analyzed by semiquantitative RT-PCR using specific $\beta_1$ primers. Amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as internal control. Top: means $\pm$ SE; $n$ = 3; **P < 0.05 vs. control. Bottom: representative PCR amplification of $\beta_1$ and GAPDH genes.

Fig. 5. Linear range of Na-K-ATPase $\beta_1$-subunit amplification by PCR. Same amount of an RT reaction from ATII cells of total RNA was used to amplify the Na-K-ATPase $\beta_1$-subunit gene and the GAPDH gene in a PCR. Top: aliquots from the reaction were taken at different cycles and fractionated by electrophoresis in a 1.5% agarose gel. Bottom: log of the DNA band density was plotted as a function of the number of cycles.

Fig. 6. DA increased Na-K-ATPase $\beta_1$-subunit protein abundance via an ERK-dependent mechanism. ATII cells were serum starved and treated with 10 $\mu$M DA for the indicated times in the presence and absence of the MEK inhibitor PD-98059 (I). Western blot of whole cell homogenates of total deglycosylated protein was performed using anti-$\beta_1$ polyclonal antibody. Top: means $\pm$ SE; $n$ = 3; *P < 0.05, **P < 0.01 vs. CT. Bottom: representative Western blot.
PD-98059 is autofluorescent, precluding the ability to evaluate quantitatively its inhibitory effect on DA-induced GFP expression by fluorometric analysis. Additionally, in accordance with results in Fig. 3, quinpirole also induced GFP expression, which was inhibited by U-0126 (Fig. 7C). Taken together, these results demonstrate that DA regulates Na-K-ATPase β1-subunit at the transcriptional level by an ERK-dependent mechanism.

DA increases Na-K-ATPase protein abundance and activity in BLMs. BLMs were isolated from serum-starved ATII cells treated with DA for 24 h in the presence or absence of PD-98059. Immunoblotting with anti-α and anti-β1 antibodies showed an increase in both Na-K-ATPase subunits in BLMs of cells treated with DA compared with control cells and cells pretreated with PD-98059 (Fig. 9A). This increase in Na pump abundance at BLMs resulted in an increase in functional pumps; as shown in Fig. 9B, Na-K-ATPase activity (measured as 86Rb+ uptake) increased in serum-starved ATII cells 24 h after treatment with DA, an effect prevented by PD-98059.

DISCUSSION

The major findings of the present study are that DA regulates Na-K-ATPase β1-subunit via a long-term mechanism that results in a functional Na-K-ATPase dimer in the BLMs of AEC and that these events involve D2R but not D1R.

In animal models, the fluid is reabsorbed from the lungs predominantly by active Na+ transport via the apical Na+ channels and basolateral Na-K-ATPases located in the AEC. There is a large body of information regarding regulation of Na-K-ATPase in the kidney but much less is known about its regulation in the alveolar epithelium. Catecholamines, in particular β-adrenergic agonists, have been shown to regulate Na+ channels and Na-K-ATPase activity in lung alveolar epithelium (8, 19). Recent studies have demonstrated that DA stimulates lung edema clearance, possibly via modulation of Na-K-ATPase in the pulmonary epithelium (1, 27).

DA-mediated regulation of Na-K-ATPase by short-term mechanisms has been reported in kidney (5, 7) and brain (6). We are reporting for the first time that DA stimulates ERK activity in ATII cells and that this
Activation results in the transcriptional regulation and translation of the Na-K-ATPase \( \beta_1 \)-subunit via a MAPK-ERK-dependent mechanism. In most systems, DA inhibits Na-K-ATPase activity by short-term mechanisms via the dopaminergic receptors \( D_1 \)R and \( D_2 \)R (5, 6, 14). In the kidney, stimulation of \( D_1 \)R inhibits Na-K-ATPase via the cAMP-protein kinase A pathway (12), a negative regulator of ERK activation (10, 28). On the contrary, DA-mediated \( D_1 \)R activation appears to regulate Na-K-ATPase within 15 min by short-term regulatory mechanisms (26). However, as shown in Fig. 3, fenoldopam, a \( D_1 \)R agonist, did not alter basal ERK activation, and preincubation of ATII cells with the \( D_2 \)R antagonist SCH-23390 did not affect the stimulation of ERK by DA. In contrast, inhibition of \( D_2 \)R by \( S \)-sulpiride, a specific \( D_2 \)R antagonist, resulted in inhibition of DA-stimulated ERK activity. In agreement with these results, stimulation of ATII cells with the \( D_2 \)R agonists quinpirole and R-NPA activated ERK to similar levels as DA. Activation of ERK proteins by \( D_2 \)R stimulation also has been recently described in neuronal tissue (33), but this is the first study showing that activation of ERK via \( D_2 \)R results in transcriptional regulation of Na-K-ATPase.

Our results showing that DA stimulation is associated with augmented Na-K-ATPase mRNA and protein abundance are similar to a recent report where \( \beta \)-adrenergic agonists increased Na-K-ATPase \( \alpha_1 \)-subunits in AEC (21). However, that study did not identify the mechanisms regulating this event. In the present study, the Na-K-ATPase \( \beta_1 \)-subunit mRNA levels increased by 1.5-fold after ~18 h of incubation with DA (Fig. 4) without change in the \( \alpha_1 \)-subunit mRNA levels. This increase is due to transcriptional activation because it is similar to the increase observed in GFP synthesis under the control of the \( \beta_1 \)-subunit promoter (Figs. 7C and 8B). We also found that Na-K-ATPase \( \beta_1 \)-subunit protein increased by up to fivefold after incubation with DA (Fig. 6). The larger increase in protein abundance compared with the mRNA levels may suggest that DA probably has additional effects at posttranscriptional or translational levels. In this line, parallel studies indicate a role of a posttranscriptional rapamycin-sensitive mechanisms involved in the regulation of the Na-K-ATPase by \( \beta \)-adrenergic agonists (Pesce L, unpublished data). The existence of posttranscriptional regulatory mechanisms has been reported previously in the regulation of the Na pump (12). Our results are consistent with previous studies suggesting that upregulation of the \( \beta_1 \)-subunit mRNA can occur without modification of the Na-K-ATPase \( \alpha_1 \)-subunit mRNA levels (3, 13, 16). Also, the fact that both \( \alpha_1 \) and \( \beta_1 \)-subunits increase in the BLMs of ATII cells treated with DA, resulting in increased Na-K-ATPase activity (see Fig. 9), suggests that the DA-mediated regulation of the Na pump is physiologically relevant. We reason that the increase in \( \alpha_1 \)-subunit in ATII cell BLMs can be due to recruitment of proteins from intracellular pools as described previously (8).

In summary, these results demonstrate that in AEC, DA regulates Na-K-ATPase by ERK proteins via the \( D_2 \)R by a mechanism that involves de novo synthesis of \( \beta \)-subunits and possibly recruitment of preexisting \( \alpha \)-subunits. This is the first report that links ERK activation to Na-K-ATPase regulation. Further studies are needed to elucidate the posttranscriptional regulatory mechanisms involved in the DA-mediated Na-K-ATPase regulation.

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