A novel role for protein phosphatase 2A in the dopaminergic regulation of Na,K-ATPase

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Received 3 August 2000; revised 24 August 2000; accepted 24 August 2000

Edited by Felix Wieland

Abstract Stimulation of dopaminergic type 1 (D_1) receptors increases lung edema clearance by regulating Na,K-ATPase function in the alveolar epithelium. We studied the role of serine/threonine protein phosphatases in the Na,K-ATPase regulation by D_1 agonists in A549 cells. We found that low doses of the type 1/2A protein phosphatase inhibitor okadaic acid as well as SV40 small t antigen transiently transfected into A549 cells prevented the D_1 agonist-induced increase in Na,K-ATPase activity and translocation from intracellular pools to the plasma membrane. This was associated with a rapid and transient increase in protein phosphatase 2A activity. We conclude that D_1 stimulation regulates Na,K-ATPase activity by promoting recruitment of Na,K-ATPases from intracellular pools into the basolateral membranes of A549 cells via a type 2A protein phosphatase.

Key words: Na,K-ATPase; D_1 agonist; Protein phosphatase; A549 cell

1. Introduction

The lung epithelium reabsorbs fluid from the alveolar space by generating a sodium gradient with water following isosmotically. Sodium enters the cells via apical sodium channels and it is extruded through the basolaterally located Na,K-ATPases [1–3]. The Na,K-ATPase is a membrane bound protein that establishes and maintains the intracellular high K^+ and low Na^+ concentrations typical of mammalian cells by hydrolyzing ATP. It is an oligomer composed of stoichometric amounts of two major polypeptides, the α- and the β-subunits [4]. Short-term regulation of Na,K-ATPase activity has been demonstrated to increase the clearance of pulmonary edema in rats [5–10]. Na,K-ATPase short-term regulation may be affected by changes in intracellular Na^+ concentrations, increased intracellular Na^+ affinity, translocation of Na,K-ATPases from intracellular compartments to the plasma membrane and/or phosphorylation–dephosphorylation reactions [4,11,12]. Recently, it has been described that Na,K-ATPase translocation to the plasma membrane from intracellular compartments is an important mechanism for Na,K-ATPase regulation in rat alveolar epithelial cells [9,10].

The pathways regulating Na,K-ATPase trafficking from intracellular compartments to the plasma membrane have not been well characterized. Serine/threonine protein phosphatases have been described to be important modulating the secretory process [13] as well as mediating different transporters translocation, such as the Na+/taurocholate cotransport polypeptide [14]. The serine/threonine protein phosphatases are usually classified into type 1 (PP1) or type 2 (PP2), depending on their substrate specificity and sensitivity to inhibitors. Type 2 phosphatases are subdivided into three major groups, including PP2A, calcium-dependent calcineurin (PP2B) and magnesium-dependent PP2C [15]. PP2A, a predominant serine/threonine protein phosphatase in most mammalian tissues, has been implicated in the regulation of many cellular processes including metabolism, transcription and cell division (for recent review see [16]). PP2A is a target for DNA tumor viruses including simian virus 40 (SV40) and binding of SV40 small t antigen (small t) to PP2A results in its specific inhibition [17,18].

In the present study, using okadaic acid (OA) and SV40 small t antigen, we investigated the role of PP2A in the Na,K-ATPase translocation induced by dopaminergic type 1 (D_1) receptors in the human alveolar epithelial cell line A549.

2. Materials and methods

2.1. Materials

Percoll and [γ-^{32}P]ATP were purchased from Amersham Pharmacia (Piscataway, NJ, USA). OA was obtained from Calbiochem (La Jolla, CA, USA). Fenoldopam (FEN) was a generous gift from Neurex Pharmaceutical (Menlo Park, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA). The Na,K-ATPase α_1 antibody (clone 464.6) was a generous gift from Dr. M. Caplan (Yale University). The plasmid containing the SV40 small t antigen has been described elsewhere [19] and the CMV empty vector was a gift from Dr. P. Factor (Northwestern University). The antibody against SV40 small t antigen was purchased from Oncogene Science (Uniondale, NY, USA).

2.2. Cell culture

A549 cells (ATCC CCL 185), a human adenocarcinoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C, and fed twice a week. Cells were seeded in 10 cm plates and grown to confluence. Cells were starved 18–24 h before treatments.

2.3. Cell lysate and basolateral membrane isolation

Cells were treated with the different agonists/antagonists at 37°C,

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Abbreviations: BLM, basolateral plasma membrane; D_1, dopaminergic type 1 receptor; FEN, fenoldopam; OA, okadaic acid

0014-5793/00/$20.00
PII: S0014-5793(00)02009-3

placed on ice and washed twice with ice-cold phosphate-buffered saline (PBS). For cell lysates, cells were scraped in 1 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF; New England Biolabs, Inc.) and centrifuged at $14\,000\times g$ to eliminate all the insoluble material. Basolateral membranes were obtained as described before [5,9] using the technique described by Hammond et al. [20]. Cells were scraped in PBS, centrifuged, resuspended in homogenization buffer (300 mM mannitol in 12 mM Tris–HEPES, pH 7.6), homogenized, and centrifuged twice to discard the nuclear and mitochondrial pellet. Supernatant was centrifuged at $48\,000\times g$ for 30 min and the basolateral membrane fraction was recovered after the membrane pellet was centrifuged in a 16% Percoll gradient at $48\,000\times g$ for 30 min.

2.4. Na,K-ATPase activity

Na,K-ATPase activity was determined in the basolateral plasma membrane (BLM) fraction as described before [9,21]. Briefly, 5–10 μg protein were transferred to the Na,K-ATPase assay medium (final volume 100 μl) containing in mM: NaCl 50, KCl 5, $MgCl_2$ 10, EGTA 1, Tris–HCl 50, Na_2ATP 10 and [γ-^{32}P]ATP in trace amounts. Cells were transiently exposed to a cold shock (10 min at −20°C) to render membranes permeable to ATP. The samples were then incubated at 37°C for 15 min, and the reaction was terminated by addition of TCA/charcoal (5%/10% v/w) suspension and rapid cooling to 4°C. After separating the charcoal phase ($12\,000\times g$ for 5 min) containing the unhydrolyzed nucleotide, the liberated ^{32}P was counted in an aliquot (200 μl) from the supernatant. Na,K-ATPase activity was calculated as the difference between test samples (total ATPase activity) and samples assayed in the same medium, but devoid of Na^+ and K^+ and in the presence of 2.5 mM ouabain. Non-specific ATP hydrolysis was determined in samples in the absence of enzyme. The specific activity of the enzyme is expressed in nmol Pi/mg protein/h.

2.5. Western blot analysis

Protein was quantified by Bradford assay (Bio-Rad, Hercules, CA, USA) and resolved in a 10% polyacrylamide gel. Thereafter, proteins were transferred onto nitrocellulose membranes (Optitran, Schleicher and Schuell, Keene, NH, USA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). Incubation with specific antibodies was performed overnight at 4°C. Blots were developed with an enhanced chemiluminescence detection kit (ECL+, Amersham, Buckinghamshire, UK) used as recommended by the manufacturer. The bands were quantified by densitometric scan (Eagle Eye II, Stratagene, La Jolla, CA, USA).

2.6. Transient transfection

A549 cells were plated at a density of 5×10^5 cells in 6 cm plates, and transient transfection was carried out with 5 μg of the different plasmids using lipofectin reagent (Life Technologies, Gaithersburg, MD, USA). After 48 h, treatment was added, cells scraped and basolateral membranes and cell lysates were obtained as described above.

2.7. PP2A assay

PP2A assay kits were purchased from Promega (Madison, WI, USA). The assay is based on determining the amount of free phosphate generated in the reaction by measuring the absorbance of a molybdate malachite green–phosphate complex. The assay was performed on the membrane fraction obtained as the Triton X-100 soluble fraction. Briefly, 5 μg of total membranes were incubated on a 96-well plate together with a peptide substrate RRA(pT)VA and buffer (50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin) for 30 min at 30°C. After incubation, the molybdate complex dye was added and incubated for an additional 20 min at room temperature for color development. The level of molybdate malachite green–phosphate complex formed was monitored at 630 nm.

2.8. Statistical analysis

Data are represented as means ± S.E.M. When comparisons were made between two experimental groups an unpaired Student's *t*-test was used. When multiple comparisons were made a one-way analysis of variance was used, followed by a multiple comparison test (Tukey) when the *F* statistic indicated significance. Results were considered significant when $P<0.05$.

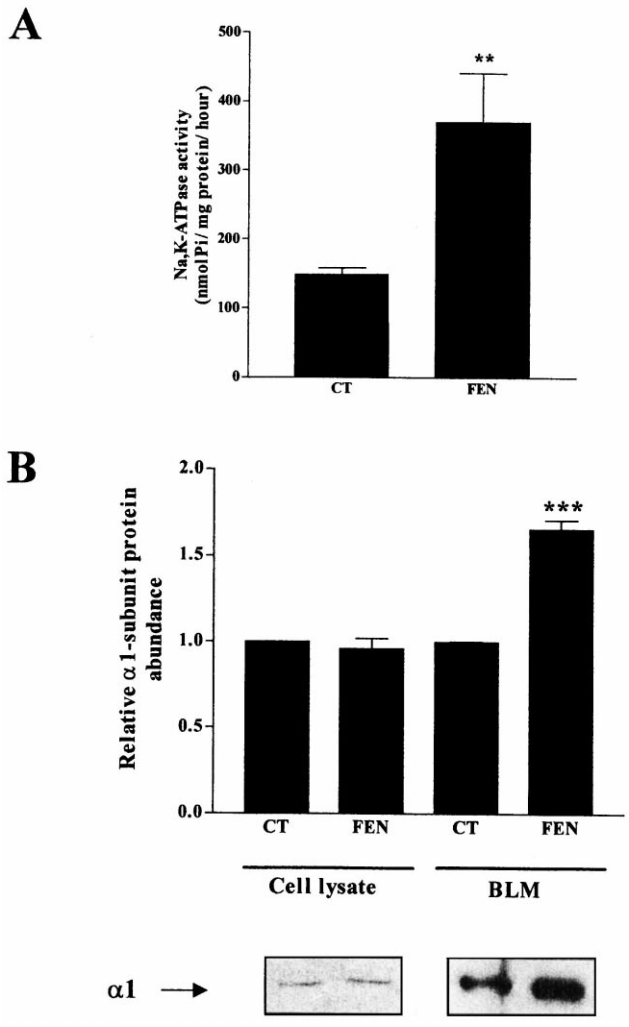

Fig. 1. Effect of D_1 agonists in Na,K-ATPase activity and translocation from intracellular pools. A549 cells were treated with 10 μM FEN for 15 min and cell lysate and BLM isolation were carried out as described in Section 2. A: Na,K-ATPase activity in the basolateral membrane. B: Western blots of cell lysates and BLM using an antibody against the Na,K-ATPase α1-subunit. Graph represents the mean ± S.E.M. of four different experiments. $^{**}P<0.01$, $^{***}P<0.001$.

3. Results and discussion

Recently it has been described that dopamine (via D_1 type receptors) stimulates sodium transport and edema clearance in rat lung epithelium [7,8] via the regulation of Na,K-ATPase activity [10]. The present study was designed to study the role of serine/threonine protein phosphatases in the short-term effect of D_1 agonists on Na,K-ATPase. We used A549 cells, a cell line frequently employed in the study of alveolar epithelial cell biology [22], which we have previously used to study Na,K-ATPase function [23]. As shown in Fig. 1A, stimulation with 10 μM of the D_1 agonist FEN for 15 min increased Na,K-ATPase activity in A549 cells. As depicted in Fig. 1B, Na,K-ATPase translocated to the BLM from intracellular compartments without changing the Na,K-ATPase α1-subunit protein abundance in total cell lysates. These data suggest that no de novo synthesis of protein occurred within the 15 min of treatment with FEN, but rather recruitment of

preformed Na,K-ATPases from intracellular compartments into the BLM, resulting in increased Na,K-ATPase activity.

Serine/threonine protein phosphatases have been shown to play an important role in the regulation of secretory processes [13] as well as in the translocation of transporters from intracellular compartments to the plasma membrane [14]. PP1 and PP2A are specifically inhibited by a variety of naturally occurring toxins such as OA [24]. OA potently inhibits both PP1 and PP2A, although with different sensitivities. PP1 has an IC_{50}: 100–200 nM OA whereas PP2A has an IC_{50}: 0.3–1 nM OA [25]. OA has little or no effect on PP2B or PP2C. To determine whether PP1 or PP2A plays a role in Na,K-ATPase stimulation induced by D_1 agonists, we preincubated A549 cells for 30 min with 500 nM OA before adding 10 μM FEN. As shown in Fig. 2, preincubation with 500 nM OA abolished the increase in Na,K-ATPase activity induced by FEN (Fig. 2A), as well as the Na,K-ATPase translocation to the BLM (Fig. 2B), suggesting a role for protein phosphatases in the regulation of Na,K-ATPase by D_1 agonists.

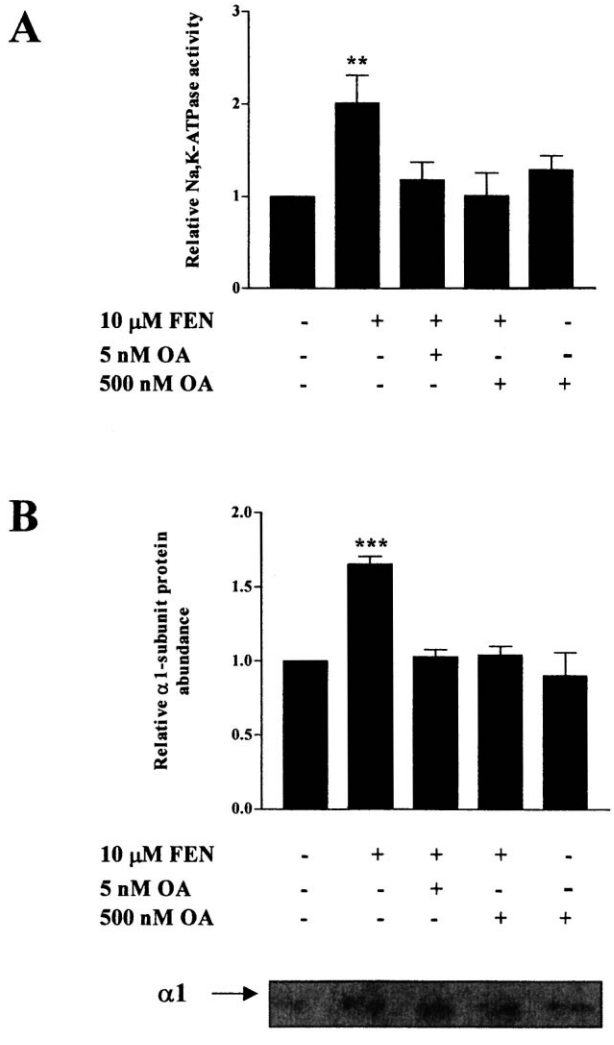

Fig. 2. Effect of OA in Na,K-ATPase activity and translocation induced by D_1 agonists. A549 cells were preincubated with 5 nM and 500 nM OA for 30 min before adding FEN for 15 min, and BLMs were isolated as described in Section 2. A: Na,K-ATPase activity. B: Western blot of BLMs using an antibody against the Na,K-ATPase α1-subunit. Graphs represent the means ± S.E.M. of three different experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

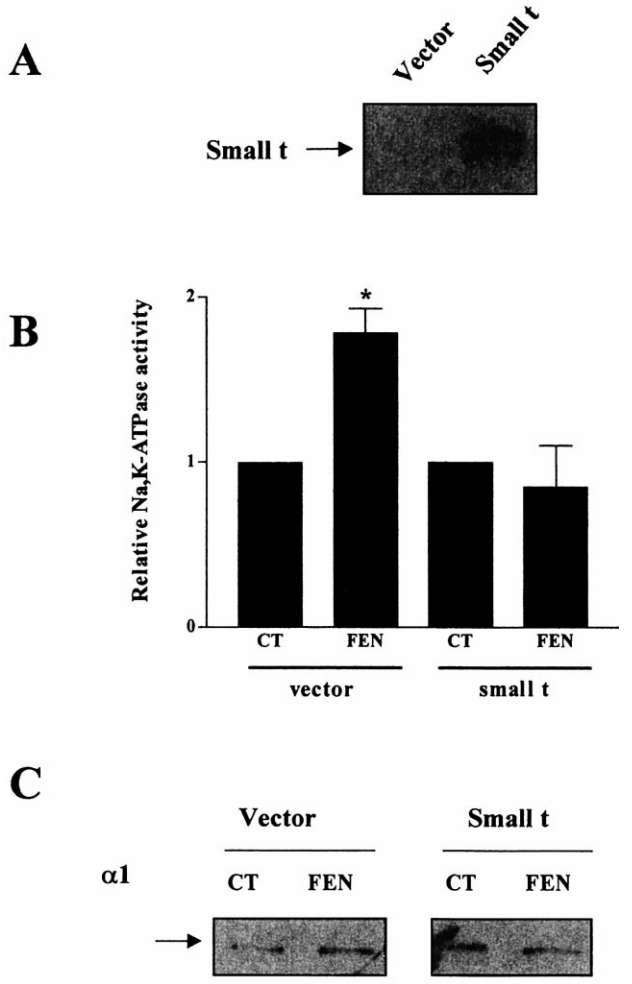

Fig. 3. Small t prevents Na,K-ATPase activity and translocation induced by D_1 agonists. A549 cells were transiently transfected with the SV40 small t antigen as described in Section 2 and the effect of FEN on Na,K-ATPase stimulation was studied. A: Representative Western blot of A549 cell lysate using an antibody against small t. B: Na,K-ATPase activity; graph represents the mean ± S.E.M. of three different experiments. C: Representative Western blot of the Na,K-ATPase α1-subunit in BLMs. $^{*}P < 0.05$.

To further examine whether PP2A had a role in the Na,K-ATPase translocation induced by D_1 agonists, A549 cells were preincubated for 30 min with a low dose of OA (5 nM) before adding 10 μM FEN for 15 min. As shown in Fig. 2, even 5 nM OA inhibited the Na,K-ATPase activity (Fig. 2A) and translocation (Fig. 2B) from intracellular pools induced by FEN, suggesting a role for PP2A. Thus, to better establish the involvement of PP2A in the Na,K-ATPase regulation by D_1 agonists, we tested the role of SV40 small t antigen transiently transfected in A549 cells in the FEN-induced Na,K-ATPase stimulation. As depicted in Fig. 3A, the transiently transfected A549 cells expressed the SV40 small t antigen after 48 h, whereas SV40 small t was not detected in cells transfected with the empty vector. A549 cells transfected with the empty vector had an increased Na,K-ATPase activity and α1-subunit protein abundance in the BLM after 15 min of FEN treatment. However, A549 cells transfected with the SV40 small t antigen did not have increased Na,K-ATPase activity and α1-subunit protein abundance in the BLMs in response to FEN (Fig. 3B,C). These data strongly suggest that PP2A regulates

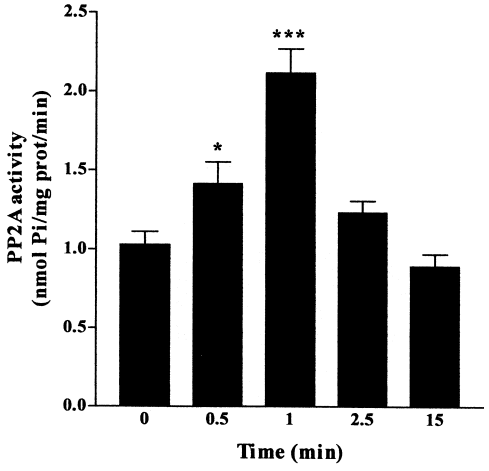

Fig. 4. PP2A activity after FEN treatment. PP2A activity was measured as described in Section 2. A549 cells were treated with 10 μM FEN for the different times represented in the figure, total membranes were isolated and PP2A activity was assessed. Graph represents the mean ± S.E.M. of three different experiments. $*P < 0.05$, $***P < 0.001$.

the dopaminergic-induced Na,K-ATPase translocation to the BLM.

To confirm that FEN-induced Na,K-ATPase stimulation in A549 cells was associated with the activation of PP2A, we performed a time-course of PP2A activity in cells treated with FEN. As shown in Fig. 4, PP2A was transiently activated by FEN with peak activity at 1 min. The A549 membrane fraction was used for measurement of PP2A activity as suggested by a previous study, where a transient translocation of PP2A to the membrane fraction after agonist stimulation was required in the secretion process [13].

Similarly to many ion channels and cotransporters [14,26,27], Na,K-ATPase activity can be modulated by phosphorylation/dephosphorylation reactions. Our findings are in agreement with those of Blot-Chabaud et al. [28], where PP2A regulated the vasopressin-induced Na,K-ATPase trafficking in the cortical collecting duct. However, recent reports suggest PP1-mediated dephosphorylation of the Na,K-ATPase α1-subunit [29,30] as the mechanism regulating the insulin activation of Na,K-ATPase function. The fact that different types of protein phosphatases have been described to play a role in Na,K-ATPase regulation demonstrate the complexity of the signaling pathways that probably are receptor and cell type specific. There is also growing evidence suggesting an important role for PP2A in protein trafficking between cellular compartments such as in the microtubular transport [31,32] as well as in endosome sorting [33].

In conclusion, our results, based on the experiments with OA and small t, strongly suggest that PP2A is critically involved in the dopaminergic-induced Na,K-ATPase translocation from intracellular compartments to the BLMs. Further studies are warranted to elucidate the pathways and targets of PP2A regulating the Na,K-ATPase cellular trafficking.

Acknowledgements: The authors wish to thank Dr. A. Bertorello, Dr. L. Dada, Dr. C. Guerrero, Dr. K. Ridge, and Dr. L. Pesce for valuable discussions, R. Vena for technical support, and Dr. E. Sontag for the small t expression plasmid. This research was supported in part by HL 65161 to J.I.S., the Department of Medicine, Northwestern University, and grants ARC 9449 and 9294 to A.G.

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