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1,25-Dihydroxyvitamin D Enhances Alveolar Fluid Clearance by Upregulating the Expression of Epithelial Sodium Channels

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ABSTRACT

Vitamin D is implicated in the pathogenesis of asthma, acute lung injury, and other respiratory diseases. 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$), the hormonal form of vitamin D, has been shown to reduce vascular permeability and ameliorate lung edema. Therefore, we speculate that $1,25(OH)_2D_3$ may regulate alveolar Na^+ transport *via* targeting epithelial Na^+ channels (ENaC), a crucial pathway for alveolar fluid clearance. *In vivo* total alveolar fluid clearance was $39.4 \pm 3.8\%$ in $1,25(OH)_2D_3$-treated mice, significantly greater than vehicle-treated controls (24.7 ± 1.9 %, $n = 10$, $p < 0.05$). $1,25(OH)_2D_3$ increased amiloride-sensitive short-circuit currents in H441 monolayers, and whole-cell patch-clamp data confirmed that ENaC currents in single H441 cell were enhanced in $1,25(OH)_2D_3$-treated cells. Western blot showed that the expression of α-ENaC was significantly elevated in $1,25(OH)_2D_3$-treated mouse lungs and $1,25(OH)_2D_3$-treated H441 cells. These observations suggest that vitamin D augments trans-alveolar fluid clearance, and vitamin D therapy may potentially be used to ameliorate pulmonary edema.

Introduction

In order for gas exchange to occur in an optimum fashion, the alveolar space must remain free of fluid. Pulmonary edema is the abnormal fluid accumulation in the interstitial or alveolar spaces of the lung. Besides its involvement in the well-known calcium and phosphorus metabolism, vitamin D is implicated in the pathogenesis of asthma, acute lung injury, and other respiratory diseases.[1] 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$), the hormonal form of vitamin D, has been shown to reduce vascular permeability and ameliorate pulmonary edema,[2-4] but the underlying mechanisms are incompletely understood.

Alveolar salt and fluid reabsorption has been extensively investigated during the last few years. Alveolar fluid clearance (AFC) has been widely used in understanding lung fluid balance, not only under normal but also pathologic conditions, for example, in acute lung injury and acute respiratory distress syndrome. Stimulation of AFC accelerates the resolution of pulmonary edema, which benefits gas exchange across the alveolar epithelium.[5] In contrast, impairment of AFC is often related with worsened survival in acute lung injury and acute respiratory distress syndrome patients,[6,7] which leads to the development of pulmonary edema.[8] Much effort has therefore been focused on identifying the pathogenic mechanisms underlying the relationship of vitamin D and AFC.

Convincing evidence indicates that active sodium transport across the alveolar epithelium *in vivo* contributes to the reabsorption of the fetal fluid and to the maintenance of fluid-free alveolar spaces in adult lungs. Active Na^+ reabsorption across lung epithelia requires the sequential coordination of the entry of Na^+ ions through Na^+-selective, amiloride-sensitive (AS) epithelial Na^+ channels (ENaCs) located at the apical membranes, followed by the extrusion across the basolateral membranes by the energy-consuming Na^+-K^+-ATPase. The entry of Na^+ ions through apical membranes is thought to be the critical step in this process.[9-12] Therefore, we speculate that $1,25(OH)_2D_3$ may regulate alveolar Na^+ transport *via* targeting ENaC, a crucial pathway for AFC.

In this article, we evaluated whether AFC of fluid-instilled mouse lungs is strengthened when BALB/c male mice have been treated with $1,25(OH)_2D_3$ for 2 weeks and measured the ENaC activity in H441 cells, aiming to find the role of strengthened alveolar fluid transport in lung epithelium by vitamin D.

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Materials and Methods

Measurement of In Vivo *Alveolar Fluid Clearance in Mice*

Animals were kept under pathogen-free conditions, and all procedures performed were approved by the Institutional Animal Care and Use Committee of China Medical University. AFC was measured as previously described.[12] In brief, BALB/c male mice (20~30 g) were anesthetized with diazepam (17.5 mg kg^{-1}, intraperitoneally) followed 6 min later by ketamine (450 mg kg^{-1}, intraperitoneally) and were placed on a heating pad. For the 1,25$(OH)_2D_3$-treated group (VD group), mice were treated with vitamin D analogue paricalcitol[13](19-nor-1,25-dihydroxyvitamin D_2, intraperitoneally at 200 ng/kg, dissolved in 70% propylene glycol) or vehicle daily for 2 weeks before the *in vivo* AFC of these mice was measured. The trachea was exposed and cannulated with a trimmed 18-gage intravenous catheter, which was then connected to a mouse respirator (HX-300, Chengdu Taimeng Technology Co. Ltd, Chengdu, China). Mice were ventilated with 100% O_2 with a 200-μL tidal volume (8-10 ml kg^{-1}) at a 160 breaths min^{-1}. Once stable anesthesia was obtained, mice were positioned in the left decubitus position, and 300 μL of isosmolar NaCl containing 5% fatty acid-free bovine serum albumin or 1-mM amiloride (a specific ENaC inhibitor) was instilled *via* the tracheal cannula, followed by 100 μL of room air to clear dead space. After instillation, mice were ventilated for a 30-min period, and then the alveolar fluid was aspirated. All reagents were added to the AFC instillate from stock solutions directly before instillation, in a minimal volume of solvent (1-10 μL mL^{-1}). The protein concentrations in aspirated solutions were measured by Bradford method. We estimated AFC (% AFC_{30}) by the changes of concentration for bovine serum albumin as water was absorbed after 30 min. AFC was calculated as the follow equation: $AFC = [(Vi-Vf)/Vi] \times 100$, where V is the volume of the instilled bovine serum albumin solution (i) and the final alveolar fluid (f). $Vf = Vi \times Pi/Pf$, where P represents the protein concentration in the instilled bovine serum albumin solution (i) and the final alveolar fluid (f).

Cell Culture

H441 cells were obtained from the American Type Culture Collection and grown in Roswell Park Memorial Institute medium (American Type Culture Collection) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). For ussing chamber studies, cells were seeded on permeable support filters (Costar) at a supraconfluent density (~5×10^6 cells/cm^2) and incubated in a humidified atmosphere of 5% CO_2-95% O_2 at 37°C. Cells reached confluency in the Costar Snapwell culture cups 24 h after plating. At this point, media and nonadherent cells in the apical compartment were removed to adapt the cells to air-liquid interface culture. Culture media in the basolateral compartment was replaced every other day; whereas the apical surface was rinsed with phosphate-buffered saline (PBS). An epithelial tissue voltohmmeter (World Precision Instruments) was used to monitor the transepithelial resistance. Highly polarized tight monolayers with resistance >800 Ω·cm^2 were selected for ussing chamber assays. For patch-clamp studies, cells were seeded in 75-cm^2 flasks and were lifted by 0.25% trypsin and 0.53-mM EDTA (Sigma), then seeded at a density of 1×10^6 cells/mL on round coverslips (8 mm, World Precision Instruments) situated in 24-well culture plates. Cells were grown in the previously mentioned medium supplemented with 200-nM dexamethasone to facilitate sodium channel differentiation, the medium was replaced every other day.

Ussing Chamber Assays

Measurements of short-circuit current (Isc) in H441 monolayers were performed as described previously.[14] Briefly, H441 monolayers treated with vitamin D analogue calcitriol 24 h (VD group, 20 nM) or ethanol vehicle (control group) were mounted in vertical ussing chambers (Physiologic Instruments) and bathed on both sides with solutions containing (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 mannitol (apical compartment), and 10 glucose (basolateral compartment). The transepithelial Isc levels were measured with 3-M KCl, 4% agar bridges placed 3 mm on either side of the membrane, which were connected on either side to Ag-AgCl electrodes. The filters were bathed on both sides with the previously mentioned salt solution as designed, bubbled continuously with a 95% O_2-5% CO_2 gas mixture (pH 7.4). The temperature of the bath solution (37°C) was maintained using a waterbath. The transmonolayer potential was short circuited to 0 mV, and Isc level was measured with an epithelial voltage clamp (VCC-MC6, Physiologic Instruments). A 10-mV pulse of 1-s duration was imposed every 10 s to monitor transepithelial resistance. Data were collected using the Acquire and Analyze program, and when Isc level had attained its stable level, 100-μM amiloride was applied to the apical side and AS current component was determined.

Patch-Clamp Recordings

Immediately before each experiment, a coverslip bearing human lung H441 epithelial cells treated with vitamin D analogue calcitriol 24 h (VD group, 20 nM) or vehicle (control group) was removed from the culture dish and put into a recording chamber, which was mounted on the stage of an inverted fluorescent microscope (Leica DM IRB). For the whole-cell mode of patch-clamp recording, cells were perfused continuously with extracellular fluid containing (in mM) 140 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.2).[15] After being exposed to the Na^+ channel blocker amiloride (10 μM), transepithelial transport indicated by AS currents were measured. Pipettes were made from capillary glass electrode with a P-97 micropipette puller (Sutter). They were back-filled with internal solution (in mM) 100 K-gluconate, 40 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 K_2ATP, 4 egtazic acid, and 10 HEPES (pH 7.2).[16] The pipette resistance varied from 5 to 10 MΩ when filled with this intracellular fluid. Offset potential was corrected before a gigaohm seal formation. Series resistance and capacitance transients were then compensated with an Axopatch 700B amplifier (Molecular Devices). Currents were digitized with a Digidata 1440A converter (Molecular Devices), filtered through an internal 4-pole Bessel filter at 1 kHz, and sampled at 2 kHz. Inward and outward whole-cell currents were elicited by using a step-pulse protocol from −120 mV to +80 mV in 20 mV increments every 10 s for 500 ms duration at a holding potential of −40 mV. Steady state currents were averaged between 100 and 200 ms, and only the cells with stable baseline currents were included in the results.

Western Blot Analysis

Mouse lungs and H441 cells treated or not with 1,25$(OH)_2D_3$ were washed 2 times with PBS and lysed for 15 min under agitation at 4°C in lysis buffer (1% Triton X-100, 150-mM NaCl, 5-mM EDTA, and 50-mM Tris, pH 7.5) supplemented with protease inhibitor and phosphatase inhibitor cocktails (Life Technologies). The cells were subsequently scraped with a rubber policeman, collected, and centrifuged at 12,000 × *g* for 10 min. Protein concentration of the supernatants was evaluated with the Bradford method (Bio-Rad Laboratories, Mississauga, ON). Total proteins (50 μg) were solubilized in sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 0.2% bromophenol blue, 10% glycerol, and 7.7% β-mercaptoethanol), subjected to SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically onto polyvinylidene difluoride

membranes (Immobilon, Millpore). The membranes were blocked with 5% dried fat-free milk for 1 h at room temperature and then incubated overnight at 4°C with primary antibody α-ENaC (Abcam Technology), in tris-buffered saline with tween plus 5% milk. After being washed with TBST, the membranes were incubated with goat anti-rabbit (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h. After TBST washes, the membranes were incubated with ECL (Thermo, SuperSignal) for 5 min before the luminescent signals were recorded. The intensity of each specific band was quantified with Image Lab software.

Reverse Transcription Polymerase Chain Reaction

Confluent H441 cells treated or not with $1,25(OH)_2D_3$ for 24 h were scraped into ice-cold PBS. Total RNAs were extracted using TRIzol reagent (Invitrogen). First-strand cDNA templates were synthesized using PrimeScript RT reagent kit (TaKaRa, Mountain View, CA). Real-time polymerase chain reaction (PCR) was performed using SYBR Premix Ex kit (TaKaRa) in a Bio-RAD IQ5 real-time system. Relative amount of transcripts was calculated by the $2^{-\Delta\Delta}$Ct formula, using glyceraldehydes-phosphate dehydrogenase as an internal control. The specific oligonucleotide primers for the RT-PCR analysis of α subunits were as follows: 5'-AAC AAA TCG GAC TGC TTC TAC-3' (sense), 5'-AGC CAC CAT CAT CCA TAA A-3' (antisense).

Statistics

Ussing chamber data were collected using the Acquire and Analyze program (version 2.3; Physiologic Instruments) and electrophysiological data from patch-clamp studies were primarily analyzed with the Clampfit 10.0 (Molecular Devices) software. Furthermore, the measurements were imported into OriginPro 8.0 software (OriginLab) for statistical computation and graphic plotting. AS currents are defined as the difference between total currents and amiloride-resistant currents. The whole-cell currents among each single H441 cell were divergent due to various cell size (capacitance), passage, and culture reagents. Thus, the whole-cell channel activity was presented as amiloride-sensitive whole-cell current density ($ASI_{density}$, pA/pF).

All results are presented as mean ± SE. One-way ANOVA computations were used to analyze the difference of the means for normally distributed data. Mann-Whitney *U* test was applied for nonparametric data. $p < 0.05$ was considered as significant.

Results and Discussion

$1,25(OH)_2D_3$ is a pleiotropic hormone with a broad range of physiological functions.[13,17-19] It is a member of the lipophile family of ligands that exerts its biological actions by a nuclear hormone receptor.[20,21] The presence of $1,25(OH)_2D_3$ receptors in the alveolar type II cells of the lung and the participation of $1,25(OH)_2D_3$ in maturation and differentiation of these cells and synthesis of surfactant released from these cells indicated that the alveolar type II cells may be principal targets for $1,25(OH)_2D_3$.[22,23] Besides its participation in the well-known calcium and phosphorus metabolism, vitamin D and its analogs have also been shown to be related to therapeutic actions in a number of pathological conditions, including the edema diseases.[3,4,24,25] Sprinchorn et al.[26] found a strong association with transient bone marrow edema in the foot and ankle and low systemic bone mineral density, which appears to be due to a vitamin D deficiency and vitamin D treatment is effective in bone marrow edema.[27] Acute pulmonary edema was also related to severe left ventricular dysfunction caused by vitamin D deficiency—induced hypocalcemia.[24] AFC plays an important role in maintaining alveolar space free of fluid.[28] Little is known about the effects of vitamin D on alveolar fluid transport, and it is very vital to study the relationship of vitamin D and AFC.

Alveolar salt and fluid reabsorption has been extensively investigated during the last 3 decades. Na^+ ions enter both type I and type II alveolar epithelial cells through apically located cation channels and are extruded actively across the basolateral membrane by the Na^+- K^+-ATPases. Importantly, Na^+ entry through apical channels is a rate-limiting step in this process. This vectorial transport of Na^+ ions creates an osmotic gradient favoring the reabsorption of fluid *via* transcellular and paracellular pathways. ENaC contribute to the vectorial Na^+ transport in most species, particularly at birth. Therefore, we speculate that $1,25(OH)_2D_3$ may regulate alveolar Na^+ transport *via* targeting ENaC, a crucial pathway for AFC.

$1,25(OH)_2D_3$ Enhances Mouse AFC In Vivo

To investigate whether $1,25(OH)_2D_3$ could improve alveolar Na^+ transport in the mouse lungs, AFC was measured by a way of fluid instillation and the body surface area integrity after instillation 30 min has been identified by the other group in our laboratory. In this setup (Fig. 1), the rate of *in vivo* total AFC was 39.4 ± 3.8% in $1,25(OH)_2D_3$-treated mice, significantly greater than vehicle-treated controls (24.7 ± 1.9%, n = 10, $p < 0.05$). Intratracheal administration of amiloride to the instillate decreased AFC by about 50%, which is in accordance with our previous studies.[12] One millimolar concentration of amiloride was used in the AFC setup because a large fraction of amiloride becomes protein bound, and a significant fraction rapidly leaves the air spaces due to its low molecular weight, thus the effective alveolar concentration was probably lower. In addition, the same amiloride concentration has been used in other studies of AFC in both developing and adult animals.[12,29]

Then, does $1,25(OH)_2D_3$ act directly on transepithelial sodium transport? It is well known that AFC is majorly determined by amiloride-sensitive sodium transport pathway.[28] During this study, we applied ENaC-specific inhibitor to identify the contribution

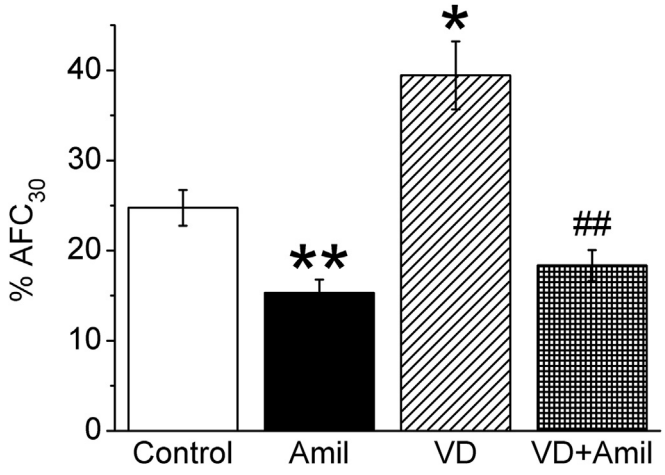

Figure 1. Upregulation of mouse alveolar fluid clearance *in vivo* by $1,25(OH)_2D_3$. Mouse lungs were treated with 5% bovine serum albumin dissolved in physiologic saline solution in vehicle-treated control group (control), and in the presence of amiloride (1 mM, Amil). The same treatment was also with vitamin D analogue paricalcitol-treated group (VD and VD + Amil, respectively). Reabsorption rate of instillate was computed as the percentage of instilled volume after 30 min (% AFC_{30}). Average AFC values are presented as mean ± SE, One-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$, compared with control group; $^{\#\#}p < 0.01$, compared with VD group. n = 10.

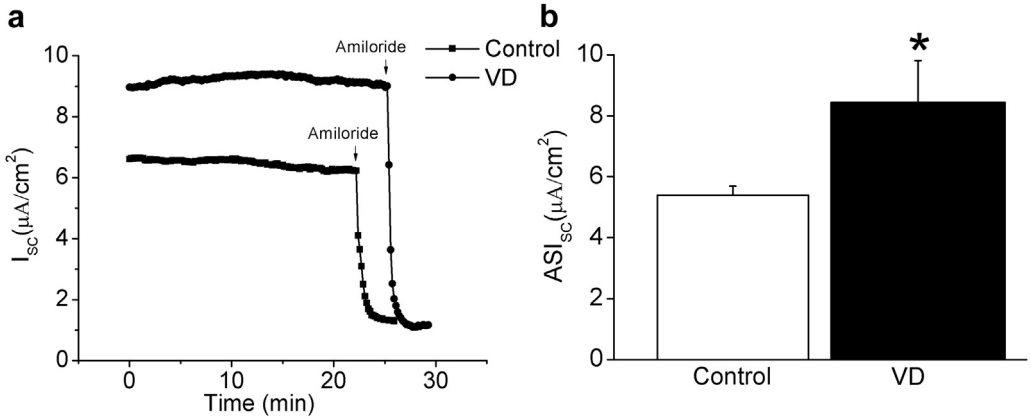

Figure 2. Enhancement of transapical short-circuit current levels by $1,25(OH)_2D_3$ in H441 monolayers. (a) Representative Isc traces obtained in H441 monolayers treated with vitamin D analogue calcitriol 24 h (VD) or vehicle (control). Amiloride (100 μM) was applied at the end of recording to calculate amiloride-sensitive (AS) Isc level. (b) AS Isc level comparation between VD and control group. $^*p < 0.05$. n = 10.

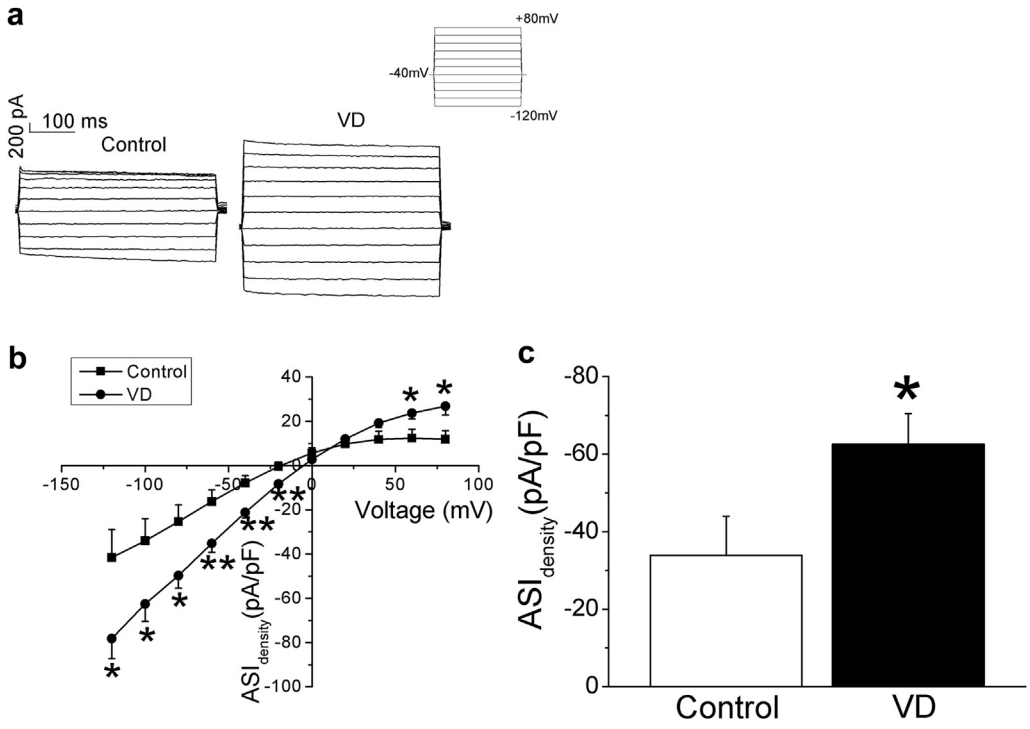

Figure 3. Patch-clamp recordings of amiloride-sensitive currents in H441 cells. (a) Representative recordings of whole-cell current traces in a cell treated with calcitriol 24 h (VD) or vehicle (control) to the bath. (b) I-V curves for VD and control groups. For each cell, inward currents recorded at −40 mV were measured during perfusion with external solutions. Amiloride-sensitive (AS) currents were normalized and the whole-cell channel activity was presented as AS whole-cell current density ($ASI_{density}$, pA/pF). (c) Comparison of $ASI_{density}$ at −100 mV. Average values are presented as the mean ± SE. $^*p < 0.05$. n = 7.

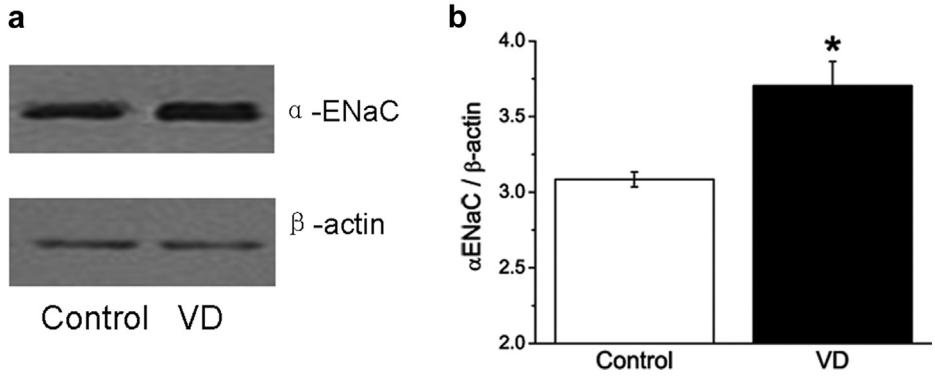

Figure 4. Effects of $1,25(OH)_2D_3$ on protein levels of α-ENaC in mice. BALB/c male mice were pretreated with paricalcitol (VD) or vehicle (control) for 2 weeks. (a) The protein bands of α-ENaC in VD and control groups. (b) Analysis of the protein expression levels for 3 times. $^*p < 0.05$.

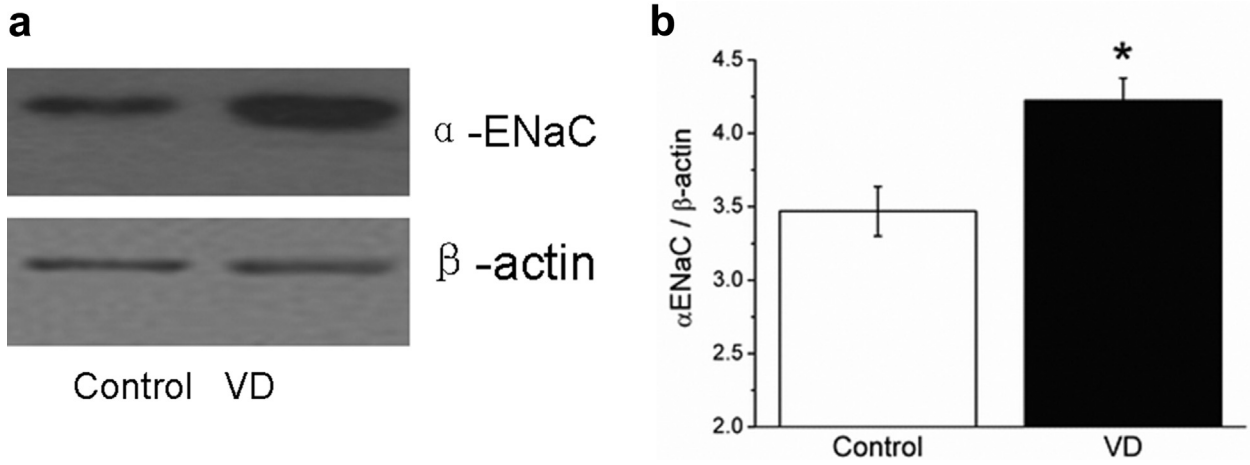

Figure 5. Effects of $1,25(OH)_2D_3$ on (a) protein levels of α-ENaC in H441 cells. H441 cells were pretreated with calcitriol (VD) or vehicle (control) for 24 h. (b) Analysis of the protein expression levels in VD and control groups for 3 times. $^*p < 0.05$.

of ENaC to the total AFC. When $1,25(OH)_2D_3$ pretreated mice were instilled with amiloride, further inhibition effects occurred. Because the effect of this combination showed obviously statistic differences from the effect of $1,25(OH)_2D_3$ pretreated alone, these findings demonstrate that $1,25(OH)_2D_3$ indeed increased the amiloride-sensitive component of AFC. $1,25(OH)_2D_3$ might maintain the airspace fluid balance by improving both alveolar fluid resolution and turnover in mouse lungs.

$1,25(OH)_2D_3$ may also regulate potassium channels, cystic fibrosis transmembrane conductance regulator, Na^+-K^+-ATPase, and other transport pathways that coordinately regulate alveolar fluid balance with ENaC and contribute to alveolar fluid absorption. The effects of $1,25(OH)_2D_3$ on the other channels mentioned previously still await for future studies.

$1,25(OH)_2D_3$ Increases Alveolar Ion Transport In H441 Cells

It has been reported that the total Na^+ Isc level in lung epithelial monolayers is predominately determined by apical and basolateral vectorial Na^+ movement.[30] We asked whether $1,25(OH)_2D_3$ might regulate electrogenic pathways across apical membrane. To examine the effects of $1,25(OH)_2D_3$ on apical Na^+ influx, we treated human lung epithelial cell (H441 cell) monolayers with $1,25(OH)_2D_3$ for 24 h. $1,25(OH)_2D_3$ enhanced transepithelial amiloride-sensitive Isc levels from 5.4 ± 0.3 to 8.4 ± 1.4 μA/cm^2 (paired t-test, $p < 0.05$, n = 10, Figs. 2a and 2b). Ussing chamber studies suggest that transepithelial Na^+ transport is enhanced by $1,25(OH)_2D_3$ in H441 cells monolayers.

Our *in vivo* data imply that $1,25(OH)_2D_3$ may enhance ENaC activity. To corroborate these observations, we measured ENaC activity next in single H441 cell by patch-clamp technique. Our whole-cell current recordings showed that amiloride-sensitive Na^+ currents (ENaC activity) were greater (184%) in $1,25(OH)_2D_3$-treated H441 cells compared to vehicle-treated cells (Fig. 3). Our electrophysiology data in H441 cells are in good agreement with the above *in vivo* AFC results. Considering the crucial role of ENaC in the alveolar fluid transport, these observations suggest that vitamin D could enhance transalveolar fluid clearance by increasing ENaC activity.

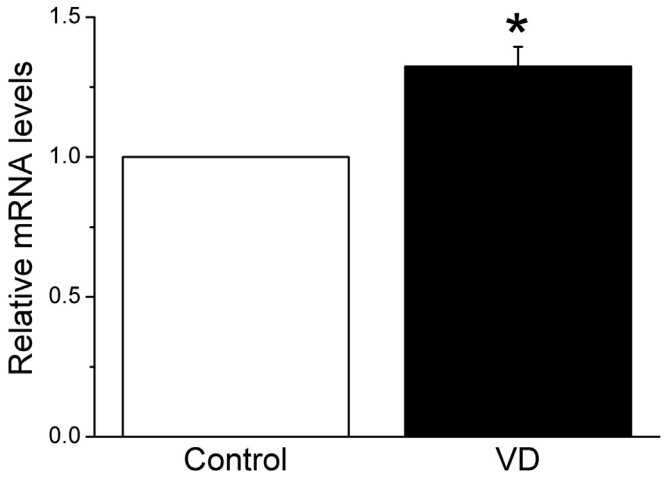

Figure 6. Effects of $1,25(OH)_2D_3$ on mRNA expression of α-ENaC in H441 cells. H441 cells were pretreated with calcitriol (VD) or vehicle (Control) for 24 h. Analysis of the mRNA expression levels in VD and control groups for 4 times. $^*p < 0.05$.

$1,25(OH)_2D_3$ Increases α-ENaC Protein and mRNA Expression

ENaC constitutes the rate-limiting step in transepithelial sodium transport and is a key target for hormone regulation.[31] Four subunits of ENaC (α, β, γ, and δ subunits) have been cloned in mammals to date.[32] The widely accepted concept is that the functional ENaC channels must be composed of at least one α or α-like subunit. The β and γ subunits are only required to amplify channel activity up to 2 orders of magnitude. We then studied the relationship of $1,25(OH)_2D_3$ and α-ENaC and our Western blot results showed that the expression of α-ENaC was significantly elevated in $1,25(OH)_2D_3$-treated mouse lungs and $1,25(OH)_2D_3$-treated H441 cells (Figs. 4 and 5), compared with vehicle-treated models. We also tested the protein expression regulation of γ-ENaC by $1,25(OH)_2D_3$, which showed little but no significant increase compared with control group (data not shown). Furthermore, we compared the mRNA expression level in H441 cells pretreated with $1,25(OH)_2D_3$ for 24 h. As shown in Figure 6, $1,25(OH)_2D_3$ could increase the mRNA expression level of α-ENaC ($p < 0.05$, n = 4). Thus, the $1,25(OH)_2D_3$ enhanced ENaC activity may be related to the higher protein and mRNA expression of α-ENaC.

Conclusion

Taken together, these observations suggest that vitamin D augments transalveolar fluid clearance and ENaC activity, and the mechanism may be related to the enhanced protein and mRNA

expression of α-ENaC, which provides a substantial theory foundation for vitamin D in potential therapy to ameliorate pulmonary edema.

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