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RESEARCH

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K⁺ channel openers restore verapamil-inhibited lung fluid resolution and transepithelial ion transport

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Abstract

Background: Lung epithelial Na⁺ channels (ENaC) are regulated by cell Ca²⁺ signal, which may contribute to calcium antagonist-induced noncardiogenic lung edema. Although K⁺ channel modulators regulate ENaC activity in normal lungs, the therapeutical relevance and the underlying mechanisms have not been completely explored. We hypothesized that K⁺ channel openers may restore calcium channel blocker-inhibited alveolar fluid clearance (AFC) by up-regulating both apical and basolateral ion transport.

Methods: Verapamil-induced depression of heterologously expressed human $\alpha\beta\gamma$ ENaC in *Xenopus* oocytes, apical and basolateral ion transport in monolayers of human lung epithelial cells (H441), and *in vivo* alveolar fluid clearance were measured, respectively, using the two-electrode voltage clamp, Ussing chamber, and BSA protein assays. Ca²⁺ signal in H441 cells was analyzed using Fluo 4AM.

Results: The rate of *in vivo* AFC was reduced significantly ($40.6 \pm 6.3\%$ of control, $P < 0.05$, $n = 12$) in mice intratracheally administrated verapamil. K_{Ca3.1} (1-EBIO) and K_{ATP} (minoxidil) channel openers significantly recovered AFC. In addition to short-circuit current (I_{sc}) in intact H441 monolayers, both apical and basolateral I_{sc} levels were reduced by verapamil in permeabilized monolayers. Moreover, verapamil significantly altered Ca²⁺ signal evoked by ionomycin in H441 cells. Depletion of cytosolic Ca²⁺ in $\alpha\beta\gamma$ ENaC-expressing oocytes completely abolished verapamil-induced inhibition. Intriguingly, K_v (pyrithione-Na), K_{Ca3.1} (1-EBIO), and K_{ATP} (minoxidil) channel openers almost completely restored the verapamil-induced decrease in I_{sc} levels by diversely up-regulating apical and basolateral Na⁺ and K⁺ transport pathways.

Conclusions: Our observations demonstrate that K⁺ channel openers are capable of rescuing reduced vectorial Na⁺ transport across lung epithelial cells with impaired Ca²⁺ signal.

Background

Drug-induced noncardiogenic lung edema is one of the pulmonary manifestations of the life-threatening side effects resulting from an overdose of medicines. All four subgroups of calcium channel blockers (CCB) have been reported to lead to both cardiogenic and noncardiogenic pulmonary edema [1-8]. CCB-induced noncardiogenic edema appears to be due to diffuse damage and increased

permeability of the alveolocapillary membrane, which results in accumulation of excess fluid in alveolar air spaces [9]. To keep the alveolar space free from flooding, accumulated cytosolic salts are extruded [10-12]. The major determinant pathway for this process is apically located epithelial Na⁺ channels (ENaC). Increasing amounts of etiological evidence suggests that genetic and pathologic ENaC deficiency gives rise to the genesis of flooding airspaces [13,14]. For example, α ENaC knock-out leads to the death of newborn mice due to their inability to resolve amniotic fluid in their lungs [15]. In adult lungs, high altitude pulmonary edema and patho-

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gen-challenged edematous lung injuries have been linked to a reduction of both ENaC expression and activity levels [16,17].

Basolateral K⁺ channels in epithelia play a major role in maintaining the electrochemical gradient necessary for Na⁺ and Cl⁻ transepithelial transport, and in restoring the resting membrane potential. The potential physiological importance of voltage-gated K⁺ channels (K_V), calcium-activated K⁺ channels (K_{Ca}), and ATP-sensitive K⁺ channels (K_{ATP}) in transepithelial ion transport has been implicated [18-22]. K_V channels constitute a large family (*i.e.*, K_VLQT1-K_V7.1, KNCQ, and KCNQ channels). So far, KCNQ 3 and 5 but not 1 have been identified in H441 cells by a very recent publication [23]. K_{Ca} channels, until recently known as K_{Ca3.1} and BK_{Ca}, are functionally detected in ENaC-expressing primary airway and ATI cells [24-26]. These commonly basolaterally located K_{Ca3.1} channels are blocked by clotrimazole and are activated by 1-ethyl-2-benzimidazolinone (1-EBIO). K_{ATP} channels, which can be inhibited by glibenclamide and activated by minoxidil, have been identified in both fetal and adult alveolar cells [21,27]. These three types of K⁺ channels have been confirmed to functionally modify the ionic and fluid transepithelial transport in cystic fibrosis airway epithelial cells [22] and may have an important role in lung fluid clearance [21,28]. These crucial K⁺ channels together with basolaterally located Na⁺/K⁺-ATPase recycle K⁺ ions across interstitial membrane of alveolar cells. The regulation of transepithelial Na⁺ transport by the K⁺ channel blockers in normal primary alveolar type II cells has recently been reported [21,25]. The underlying mechanisms for the coupling of Na⁺ and K⁺ transport are unknown. More importantly, K⁺ channel openers facilitated alveolar fluid clearance in resected human lungs [29] and transepithelial ion transport in human airway [30]. However, whether K⁺ channel openers are able to restore the CCB-inhibited transepithelial salt and fluid clearance in edematous lungs remains to be elucidated.

Verapamil has been broadly used clinically for combating hypertension, ischemic heart diseases, supraventricular tachyarrhythmias, and tycolysis. In this study, we investigated the effects of verapamil on ENaC activity in confluent H441 monolayers—a human bronchoalveolar epithelial cell line, in *Xenopus* oocytes heterologously expressing human αβ ENaC, and in murine lungs. Our results showed that K⁺ channel openers recovered verapamil-inhibited vectorial Na⁺ transport in H441 cells. Moreover, verapamil-reduced alveolar fluid resolution can be restored by these K⁺ channel openers in murine lungs.

Methods

Cell culture

NCI-H441 (H441) cells were obtained from the American Type Culture Collection (ATCC). H441 cells were grown in RPMI medium (ATCC) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Dexamethasone (250 nM, Sigma) was supplemented to stimulate ENaC expression. Cells were seeded on permeable support filters (Costar) at a supra-confluent density (~5 × 10⁶ cells/cm²), and incubated in a humidified atmosphere of 5% CO₂-95% O₂ at 37°C. Cells reached confluency in the Costar Snapwell culture cups 24 hrs after plating. At this point media and non-adherent 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 PBS. An epithelial tissue voltohmmeter (World Precision Instruments) was used to monitor the transepithelial resistance. Highly polarized tight monolayers with resistance >800 Ω·cm² were selected for Ussing chamber assays.

In vivo alveolar fluid clearance

Animals were kept under pathogen-free conditions, and all procedures performed were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Tyler. Alveolar fluid clearance was examined *in vivo* as previously described by us and other groups [31-34]. Briefly, 8-10 week old, weighting 20-30 g, pathogen-free, male C57/BL/6 mice were used (National Cancer Institute). An isosmotic instillate containing 5% bovine serum albumin (BSA) was prepared with 0.9% NaCl. Anesthetized mice were ventilated with 100% O₂ via a volume-controlled ventilator (model 683, Harvard Apparatus) for a 30-minute period. 5% BSA (0.3 ml), with or without verapamil (100 μM) and amiloride (1 mM) was instilled intratracheally. The instilled alveolar fluid was aspirated by applying gentle suction to the tracheal catheter with a 1-ml syringe. The BSA content of the alveolar fluid was measured with a 96-well microplate reader. Alveolar fluid clearance (AFC) was calculated as follows: AFC = (Vi - Vf)/Vi*100, where Vi and Vf denote the volume of the instilled and recovered alveolar fluid, respectively. Vf was obtained as Vf = (Vi * Pi)/Pf, where Pi and Pf represent protein concentration of instilled and collected fluid.

Ussing chamber assays

Measurements of short-circuit current (I_{sc}) in H441 monolayers were performed as described previously [35]. Briefly, H441 monolayers were mounted in vertical

Ussing chambers (Physiologic Instruments) and bathed on both sides with solutions containing (in mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.83 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, 10 HEPES, 10 mannitol (apical compartment) and 10 glucose (basolateral compartment). Each solution was iso-osmotic (approximately 300 mmol/Kg), as measured by a freezing depression osmometer (Wescor). The transepithelial I_{sc} 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 above salt solution as designed, bubbled continuously with a 95% O₂-5% CO₂ gas mixture (pH 7.4). The temperature of the bath solution (37°C) was maintained using a water bath. The transmonolayer potential was short-circuited to 0 mV, and I_{sc} level was measured with an epithelial voltage clamp (VCC-MC8, Physiologic Instruments). A 10-mV pulse of 1s duration was imposed every 10s to monitor Rt. Data were collected using the Acquire and Analyse program (version 2.3; Physiologic Instruments). When I_{sc} level reached plateau, drugs were pipetted to the either apical or basolateral compartment.

To determine whether verapamil decreases the amiloride-sensitive I_{sc} level across the apical membrane, 100 μM amphotericin B, a pore-forming antibiotic (Sigma), was added to the basolateral side of Ussing chamber to permeabilize the basolateral membrane [36]. A 145:25 mM Na⁺ ionic gradient (apical to basolateral compartment) was established by replacing 120 mM Na⁺ ions with equal molar N-methyl-D-glucamine, an impermeant cation in the basolateral bath solution. Basolateral permeabilization equilibrates intracellular Na⁺ concentration to 25 mM in the basolateral bath. To exclude any potentially residual Na⁺/K⁺-ATPase activity, 1 mM ouabain was added to the interstitial compartment. Under these experimental conditions, amiloride-sensitive I_{sc} level reflects passive electrogenic Na⁺ movement through ENaC down the Na⁺ concentration gradient [37,38]. When I_{sc} level had attained its stable level, verapamil was applied to the apical side and amiloride-sensitive current component was determined by adding 100 μM amiloride.

To examine the ouabain-inhibitable I_{sc} level across the basolateral membrane, the apical membrane was permeabilized with 10 μM amphotericin B. Apical permeabilization loads the cytosol with Na⁺ ions thereby eliciting the maximal active Na⁺ transport by the Na⁺/K⁺-ATPase [39]. To eliminate any remaining ENaC activity, 100 μM amiloride was included in the apical bath. Under these experimental conditions, ouabain-inhibitable basolateral I_{sc} shall associate with Na⁺/K⁺-ATPase, tightly coupling with K⁺ channels. When the I_{sc} level was stable, verapamil and K⁺ channel modulators were applied. To deter-

mine Na⁺/K⁺-ATPase activity, 1 mM ouabain was added to the basolateral compartment at the end of recording.

Oocyte preparation and voltage clamp analysis

Oocytes were surgically removed from appropriately anesthetized adult female *Xenopus laevis* (Xenopus Express) and cRNAs for human α, β, and γ ENaC were prepared as described previously [40]. Briefly, the ovarian tissue was removed from frogs under anesthesia by ethyl 3-aminobenzoate methanesulfonate salt (Sigma) through a small incision in the lower abdomen. Follicle cells were removed and digested in OR-2 Ca²⁺-free medium (in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl₂, 1.0 Na₂HPO₄, and 10.0 HEPES, pH 7.5) with the addition of 2 mg/ml collagenase (Roche Indianapolis). Defolliculated oocytes were cytosolically injected with ENaC cRNAs (25 ng) per oocyte in 50 nl of RNase free water and incubated in half-strength L-15 medium at 18°C for 48 h. Oocytes were impaled with two electrodes filled with 3 M KCl, having resistances of 0.5-2 MΩ. A TEV-200 voltage clamp amplifier (Dagan) was used to clamp oocytes with concomitant recording of currents. The continuously perfused bathing solution was ND96 medium (in mM: 96.0 NaCl, 1.0 MgCl₂, 1.8 CaCl₂, 2.5 KCl, and 5.0 HEPES, pH 7.5). To prepare a Ca²⁺-free bath solution, CaCl₂ was omitted and 5 mM EGTA was added. To chelate intracellular Ca²⁺ ions, 10 μM BAPTA_AM was added to the Ca²⁺-free bath solution. Experiments were controlled by pCLAMP 10.1 software (Molecular Devices), and currents at -40, -100, and +80 mV were continuously monitored with an interval of 10 s. Data were sampled at the rate of 1,000 Hz and filtered at 500 Hz.

Fluo 4 AM measurements

Intracellular Ca²⁺ signal elicited by ionomycin in epithelial cells was measured as described previously [41-44]. H441 cells were grown on chambered coverglass for 48 h. Culture medium was aspirated and cells were loaded with cell permeable Fluo 4 AM dye (4 μM, Invitrogen, CA) for 1 h. The Fluo 4 AM loaded cells were then incubated with verapamil or culture medium for 10 min. The cells were placed on the specimen stage of an inverted microscope (AxioObserver Z1, Carl Zeiss) equipped with a LSM 510 Meta confocal system (Carl Zeiss, Germany). The argon ion 488 nm laser line was used to excite Fluo 4 AM fluorochrome and the serial live cell images for the emission signal of Fluo 4 AM were captured for a period of 6 min 40 s at an interval of 4 s using a 20 ×/0.8 Plan-apochromat objective lens. Subsequent to a 2 min image acquisition, 15 nM ionomycin was added into the chamber to evoke an increment in cytosolic Ca²⁺ signal. In all cases, a confluent field of cells was chosen for imaging. The relative Ca²⁺ signal was measured as the ratio of fluorescent

intensity (F/F₀) using ZEN 2007 Zeiss imaging software and plotted as a function of recording time.

Statistics

Electrophysiological data from Ussing chamber and voltage-clamp studies were primarily analyzed with the Acquire and Analyze 2.3 (Physiologic Instruments) and Clampfit 10.1 (Molecular Devices), respectively. The measurements were then imported into OriginPro 8.0 (OriginLab) for statistical computation and graphic plot. The IC₅₀ and EC₅₀ values of verapamil and K⁺ channel openers were calculated by fitting the dose-response curves with the Hill equation.

All results are presented as mean ± S.E.M. The unsorted data were examined for the normal distribution using either the Kolmogorov-Smirnov normality test with specified parameters previously published or Lilliefors test. Those without significantly drawn from the normally distributed population were selected for t-test and ANOVA analyses. For the comparison of mean values of repeated measures of short-circuit and whole-cell activities, paired two-tailed Student t-test was used. For unpaired electrophysiological data, one-way ANOVA analysis combined with a post hoc Tukey-Kramer test was used. For analyses of *in vivo* alveolar fluid clearance, mean values between control and CCB challenged groups were compared by the unpaired two-sample Student t-test for both equal variance assumed or not. The mean and SE values of amiloride-sensitive AFC fraction were computed using the following equations:

$$M_t - M_a \pm t_c \cdot SE \cdot \sqrt{\frac{1}{n_t} + \frac{1}{n_a}}$$

and

$$SE = \sqrt{\frac{(n_t-1) \cdot SE_t^2 + (n_a-1) \cdot SE_a^2}{n_t + n_a - 2}}$$

where M_t and M_a are mean values of total and amiloride-resistant fractions; t_c is the $t_{.95}$ value of a freedom of (n_t+n_a-2) in the t-table; SE_t and SE_a are SE values of total and amiloride-resistant AFC. M , SE , and n stand for mean, standard error, and number of mice, respectively. For nonparametric data (*i.e.*, Ca²⁺ signal), the Mann-Whitney U-test was used. The power of sample size was simultaneously evaluated to assure the actual power value > 0.95. $P < 0.05$ was considered statistically significant.

Results

Verapamil reduces murine *in vivo* fluid resolution

To examine the potential deleterious effects of calcium channel blockers (CCB) on fluid resolution in distal lung

air spaces, we measured *in vivo* alveolar fluid clearance (AFC) in anesthetized C57/B6 mice. As plasma verapamil predominately affects cardiovascular function, which may lead to both cardiogenic and noncardiogenic pulmonary edema as reported clinically [1-8], we intratracheally delivered verapamil into lung to avoid any dysfunction beyond air spaces. As shown in Fig. 1A, the normal AFC rate was $23.6 \pm 1.3\%$ ($n = 15$). Intratracheal instillation of verapamil (100 μM) markedly reduced the re-absorption of the 5% BSA instillate ($11.4 \pm 1.2\%$, $P < 0.05$, $n = 12$), which was almost identical to that in the presence of amiloride (1 mM, $12.1 \pm 0.8\%$, $n = 4$, $P < 0.05$ vs Control). In the presence of both amiloride and verapamil, fluid resolution was $10.6 \pm 0.9\%$ ($P < 0.01$ vs Control, $n = 4$), suggesting that verapamil almost completely inhibited amiloride-sensitive fraction of AFC (Fig. 1B). These *in vivo* data clearly demonstrate that CCB impairs transalveolar fluid clearance, which in turn results in fluid accumulation in lung sacs.

K⁺ channel openers profoundly restore verapamil-inhibited alveolar fluid clearance

K⁺ channel openers activated transepithelial ion transport in alveolar monolayers *in vitro* under physiological conditions [25]. It prompted us to hypothesize that K⁺ channel openers may be capable of recovering the verapamil-inhibited fluid resolution *in vivo*. To address this promising pharmaceutical issue, three types of K⁺ channel openers, namely, pyridone-Na (1 mM for K_v), 1-EBIO (1 mM for K_{Ca3.1}), and minoxidil (0.6 mM for K_{ATP}) were intratracheally delivered in the presence (Fig. 1D) and absence of verapamil (Fig. 1C). The K⁺ openers slightly but not significantly altered AFC (Fig. 1C). In sharp contrast, depressed AFC ($10.4 \pm 1.3\%$) in the presence of verapamil was pronouncedly relieved by 1-EBIO ($17.6 \pm 2.5\%$, $n = 4$, $P < 0.05$) and minoxidil ($17.3 \pm 2.3\%$, $n = 4$, $P < 0.05$). These data suggest that augmentation of K⁺ efflux from lung epithelial cytosol facilitates salt/fluid re-absorption in verapamil-injured edematous lungs.

Calcium antagonists abrogate transepithelial short-circuit current (I_{sc}) in intact H441 monolayers

Human bronchoalveolar epithelium-derived Clara cells (H441) have been used extensively to study lung epithelial Na⁺ channels, in which ENaC properties are similar to those in primary alveolar type II cells [45-48]. To examine the effects of verapamil on the electrogenic transepithelial Na⁺ transport in lung epithelial cells, confluent H441 monolayers were mounted in an 8-chamber Ussing chamber system. Verapamil inhibited I_{sc} levels when applied to the luminal side of H441 monolayers in a dose-dependent manner (Fig. 2A). The IC₅₀ value was 294.2 μM calculated by fitting the dose-response curve with the Hill equation (Fig. 2B). Nevertheless, verapamil did not

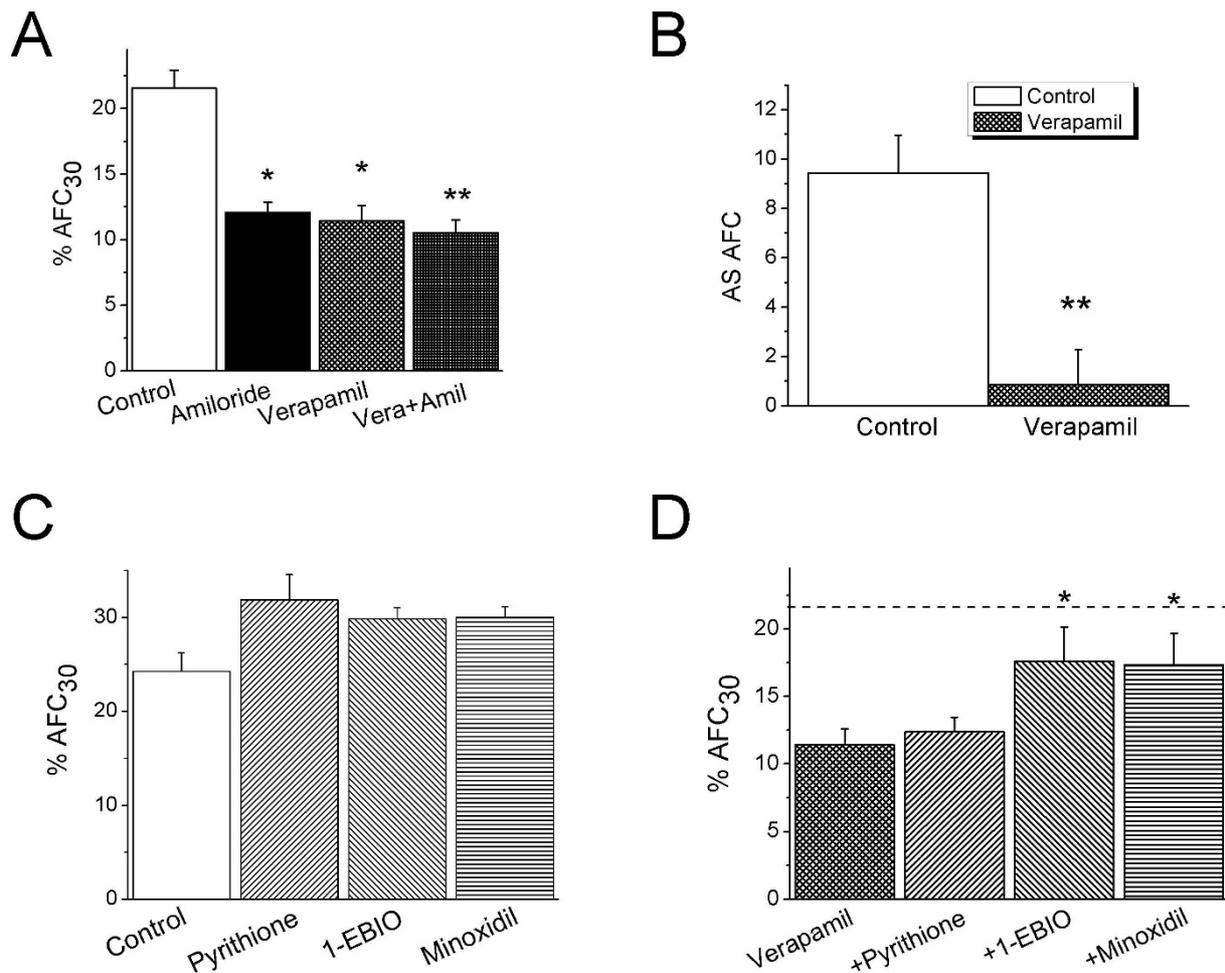
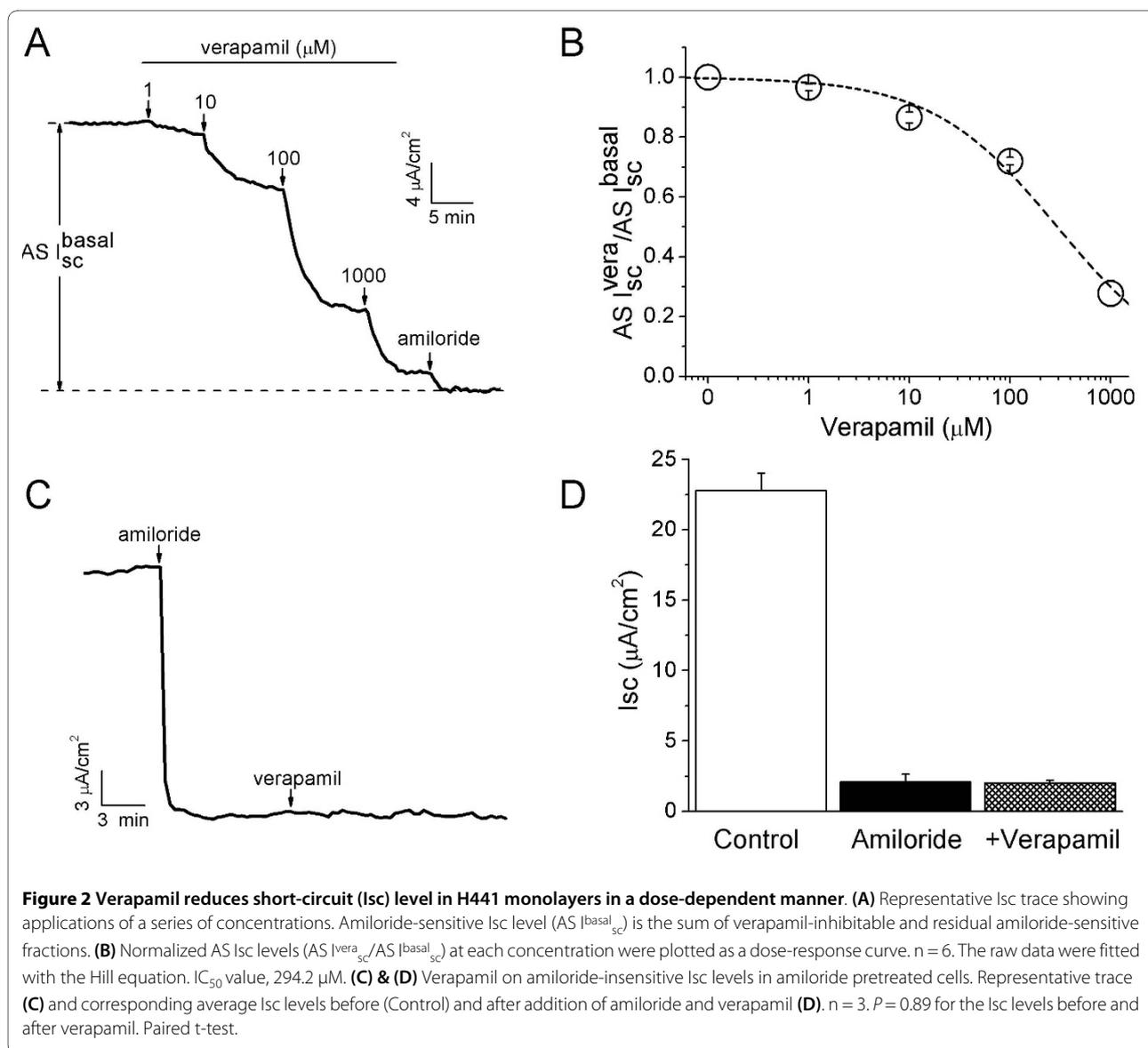


Figure 1 Recovery of verapamil-reduced alveolar fluid clearance (AFC) by K⁺ channel openers *in vivo*. (A) Verapamil intratracheal application reduces alveolar fluid clearance. Verapamil (100 μ M) was intratracheally delivered to mouse lung. Average AFC values in the absence of drugs (Control), in the presence of amiloride (Amiloride), verapamil (Verapamil), and both (Amiloride+Verapamil). Unpaired two-sample two-tailed Student t-test. * $P < 0.05$ and ** $P < 0.01$ when compared with Control. $n = 4-15$. (B) Amiloride-sensitive (AS) AFC. The mean and SE values were computed as described in Methods. Unpaired two-sample two-tailed Student t-test. ** $P < 0.01$. $n = 12-15$. (C) Effects of K⁺ channel openers on basal AFC. Unpaired two-sample two-tailed Student t-test. $n = 5-15$. (D) K⁺ channel openers restore verapamil-reduced AFC. AFC values were measured for Verapamil (100 μ M) alone, + Pyrrithione-Na (1 mM), + 1-EBIO (1 mM), and +Minoxidil (0.6 mM). The dashed line indicates the Control level. Unpaired two-sample two-tailed Student t-test. * $P < 0.05$ vs Verapamil alone. $n = 4-12$.

affect the Isc levels in amiloride-exposed monolayers (Fig. 2C & 2D, before $2.1 \pm 0.6 \mu\text{A}/\text{cm}^2$ and after verapamil $2.0 \pm 0.2 \mu\text{A}/\text{cm}^2$, $P > 0.05$, $n = 3$). These results suggest that verapamil inhibits vectorial transepithelial ion transport in a dose-dependent manner in intact monolayers.

To measure the regulation of ENaC-associated transepithelial Isc levels by representative examples from the other three subgroups of CCB compounds, confluent H441 monolayers were exposed to nifedipine, bepridil, and diltiazem (Fig. 3). As shown by the representative current traces, a reduction in the Isc levels was recorded following bolus addition of nifedipine (200 μ M), bepridil (10 μ M), or diltiazem (50 μ M) (Fig. 3A-C). To compare the inhibitory efficacy of these four subgroups of CCB

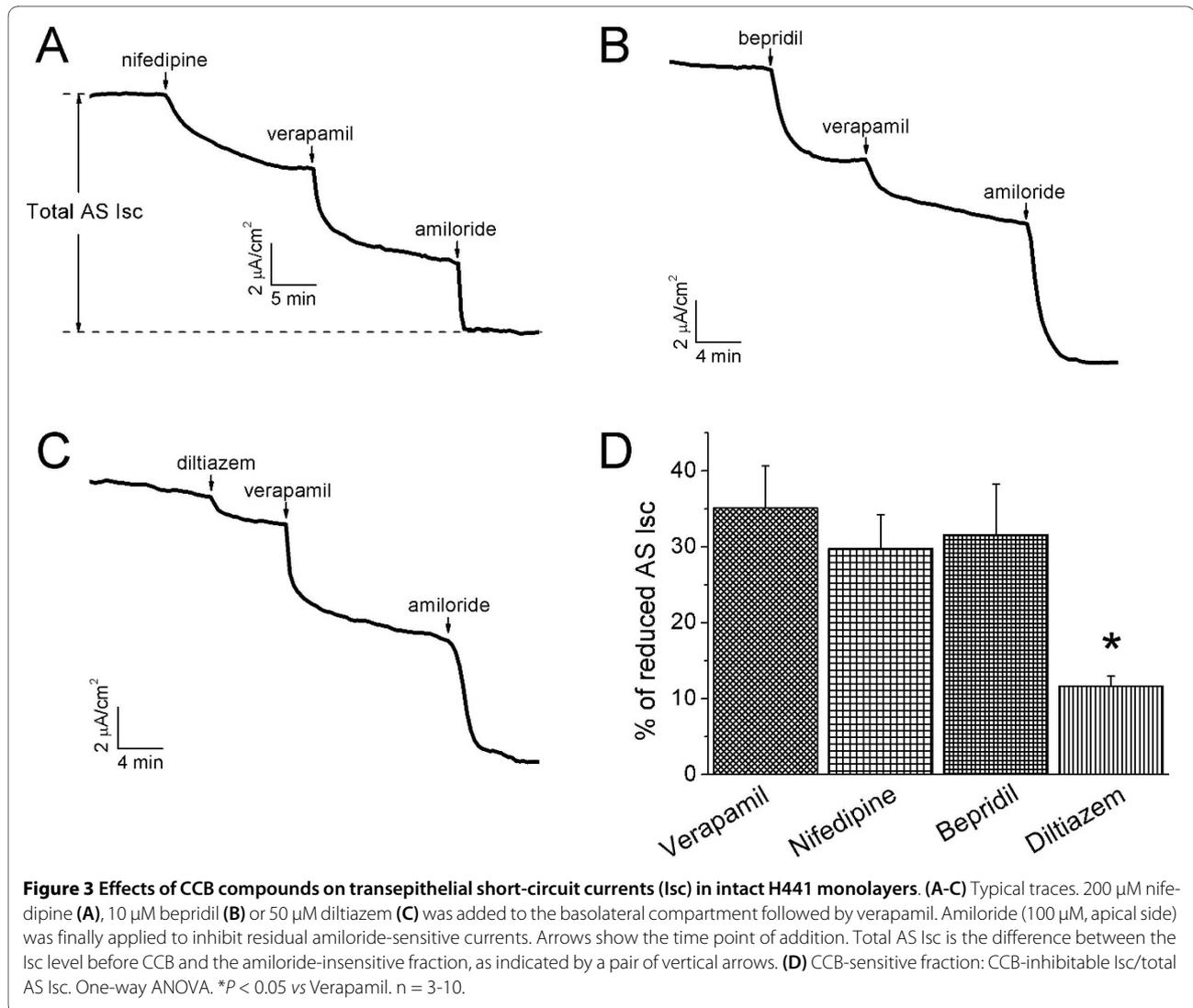
compounds, verapamil (100 μ M) was applied subsequently to these CCB compounds. Interestingly, verapamil resulted in a further decrease in the Isc levels. On average, nifedipine, bepridil, and diltiazem inhibited amiloride-sensitive (AS) Isc levels by $29.8 \pm 4.4\%$ ($P < 0.01$, $n = 4$), $31.6 \pm 6.6\%$ ($P < 0.01$, $n = 3$), and $11.7 \pm 1.3\%$ ($P < 0.01$, $n = 3$), respectively (Fig. 3D). Subsequent addition of verapamil to each group showed a further reduction in the Isc levels to approximately the same level of 70% of total reduction (Fig. 3). Because verapamil displayed potent inhibition on the AS Isc levels in H441 cells, this drug was then used for the follow-up experiments.



Verapamil, as well as other CCB compounds, is cell permeable and therefore may cross the thin alveolocapillary membrane and exhibit its inhibitory effects in the alveolar space. To investigate whether or not verapamil has the same effects on the Isc levels when applied to the basolateral and apical sides, we performed a set of experiments by adding verapamil (100 μM) to either basolateral or apical compartment (Fig. 4). AS Isc levels were inhibited by both basolateral and apical addition of verapamil by $41.4 \pm 2.6\%$ and $38.8 \pm 1.7\%$, respectively (Fig. 4D, n = 4-17). However, addition of the same volume of water did not alter Isc level (Fig. 4A). These data suggest that verapamil reduces AS Na⁺ channels to a similar extent regardless of its application to either luminal or interstitial compartment.

Verapamil inhibits both apical and basolateral Na⁺ conductance in permeabilized H441 monolayers

It has been reported that the total Na⁺ Isc level in polarized lung epithelial monolayers is predominately determined by apical and basolateral vectorial Na⁺ movement [13]. We asked whether verapamil might regulate electrogenic pathways across both apical and basolateral membrane. To examine the effects of verapamil on apical Na⁺ influx, amphotericin B (100 μM) was applied to permeabilize the basolateral membrane (Fig. 5A). A large Na⁺ ion gradient was applied to the permeabilized H441 monolayer to facilitate passive Na⁺ transport predominately through ENaC channels. To confidentially eliminate all of Na⁺/K⁺-ATPase enzymatic activity, ouabain (1 mM) was added to the basolateral compartment. Permeabilization of the basolateral membrane caused a reduction in the Isc



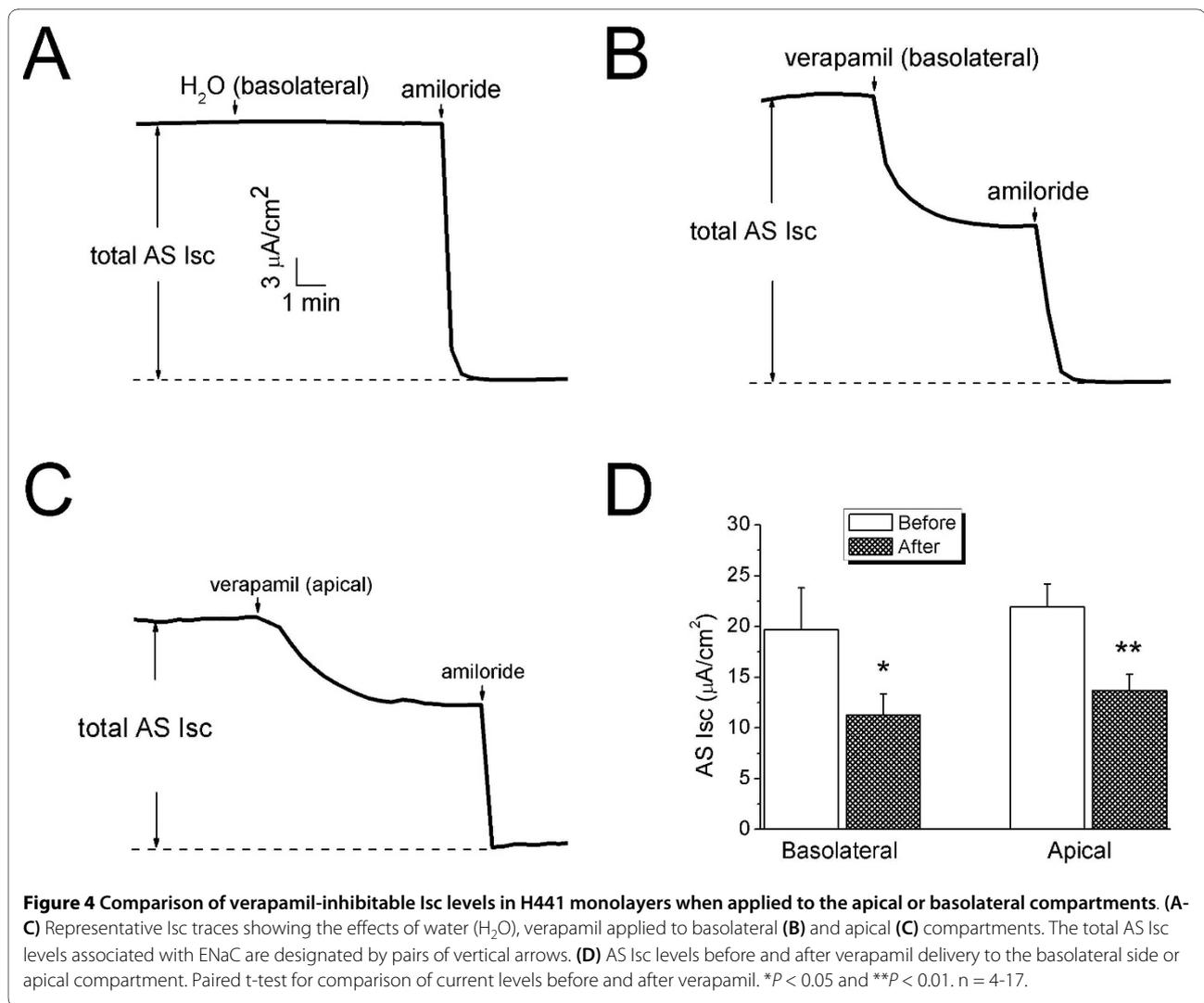
level, suggesting that a relatively larger Na^+ gradient across apical membrane exists in intact cells (apical 145:~10 mM in cytosol) than basolateral permeabilized monoalayers (145:25 mM). Verapamil inhibited transapical AS Isc levels from 9.5 ± 0.9 to 6.7 ± 0.8 $\mu\text{A}/\text{cm}^2$ (paired t-test, $P < 0.001$, $n = 8$, Fig. 5B). Clearly, verapamil regulates AS apical Na^+ conductance in the absence of cytosolic soluble signal elements.

We then examined the effects of verapamil on Na^+/K^+ -ATPase in apically permeabilized confluent H441 monolayers with amphotericin B (10 μM). To eliminate possibility of any AS apical Na^+ channels still remaining in the apically permeabilized cells, amiloride (100 μM) was added to the apical compartment. As shown in Fig. 5C, in the presence of amiloride, apical permeabilization caused a dramatic increase in the Isc level, a hallmark of evoked Na^+/K^+ -ATPase activity following an increment in "cytosolic" Na^+ ions. Verapamil resulted in a marked drop of

the ouabain-sensitive (OS) Isc level from 6.0 ± 1.3 to 3.7 ± 1.1 $\mu\text{A}/\text{cm}^2$ ($P < 0.05$, $n = 4$, Fig. 5D). These experiments provide direct evidence that verapamil inhibits Na^+/K^+ -ATPase in the apically permeabilized H441 cells.

Verapamil serves as a K^+ channel blocker

Verapamil has been known to alter cytosolic Ca^{2+} concentration and to modify a number of K^+ channels [49]. We hence speculated that verapamil might indirectly influence ENaC activity by altering K^+ channels. The basolateral K^+ channels tightly regulate Na^+/K^+ -ATPase activity, by coordinately acting as the K^+ recycling machinery to maintain the negative resting membrane potential. Resultant depolarization of polarized epithelial cells, a consequence of impaired K^+ recycling, weakens the electrochemical driving force for ENaC activity. We thereby attempted to determine the individual contribution of each functional subtype of K^+ channels (K_V , $\text{K}_{\text{Ca}3.1}$

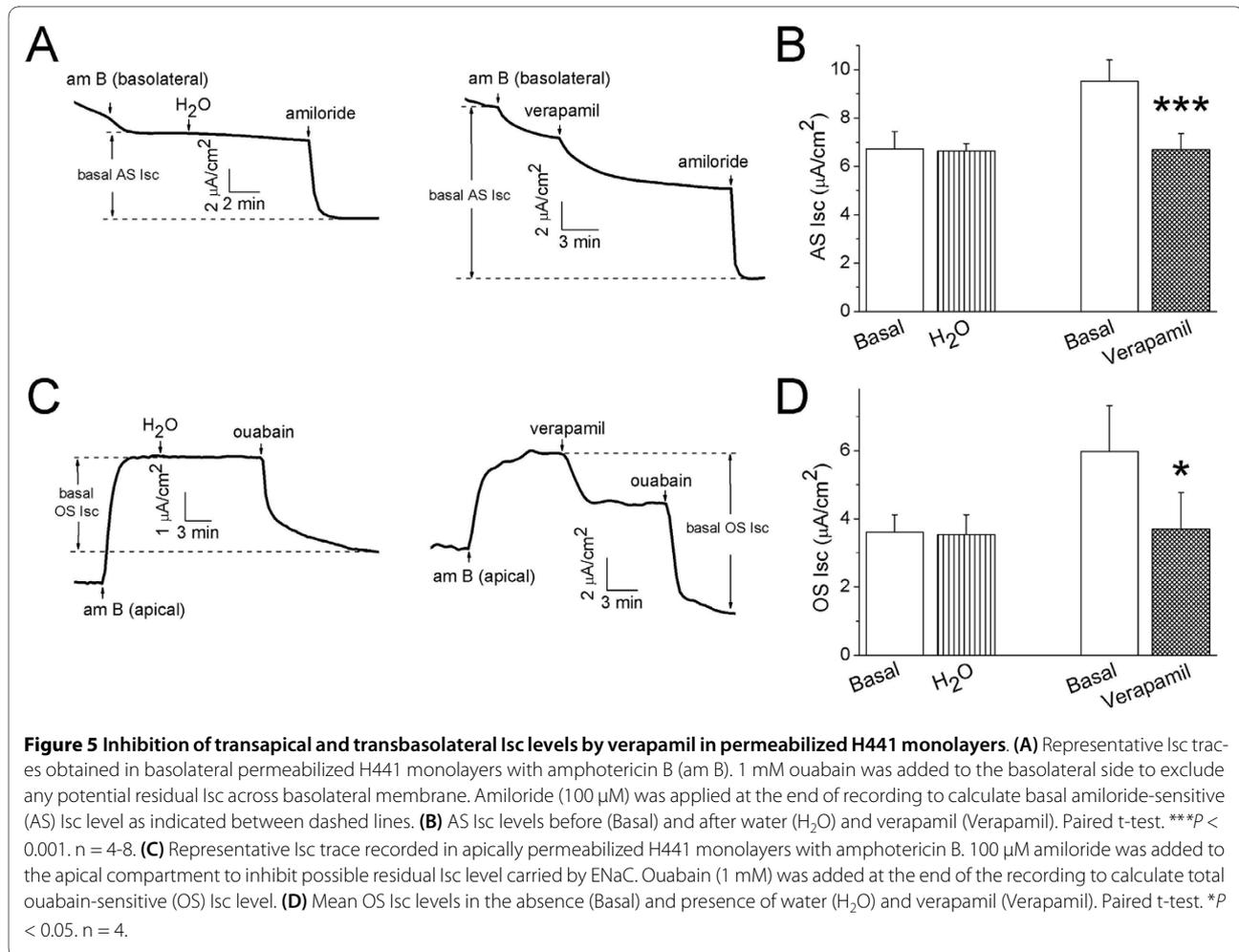


and K_{ATP}) to verapamil-inhibited ENaC activity. The representative Isc traces showed the verapamil-induced decrease in AS Isc subsequent to addition of 100 μM clofilium, 5 μM tram34, and 100 μM glibenclamide, respectively (Fig. 6A). These concentrations were supposed to completely block corresponding K⁺ channels as described previously [21,25]. As summarized in Fig. 6B, clofilium, tram34, and glibenclamide decreased the AS Isc levels by 54.4 ± 4.6% (*P* < 0.05, n = 4), 19.1 ± 1.8% (*P* < 0.001, n = 7), 20.5 ± 1.1% (*P* < 0.01, n = 4), respectively. Subsequent addition of verapamil resulted in a further reduction of the residual AS Isc levels by 23.7 ± 4.3%, 40.3 ± 1.6%, and 36.0 ± 2.8%, respectively. Blockade of KCNQ (3 and 5) [23] but not K_{Ca3.1} and K_{ATP} channels significantly affected the response of AS Na⁺ channels to verapamil (Fig. 6C, *P* < 0.05), when compared to the control (38.8 ± 1.7%, n = 17). Our results showed that these three subtypes of K⁺ channels are functionally expressed in H441 cells at a various levels, in accordance with other studies

[21,25]. Moreover, inhibition of these K⁺ channels by the related specific blockers can influence the inhibitory effects of verapamil on AS Na⁺ channels to various extents.

K⁺ channel openers restore verapamil-inhibited Isc levels in intact H441 cells

Our *in vivo* studies suggest K⁺ channel openers may alter ENaC-like activity. To address this issue, K⁺ channel openers were added basolaterally subsequent to verapamil (100 μM) as shown in Fig. 7A. A set of increasing concentrations for pyrithione-Na (K_{V7.1} opener at 5 μM and KCNQ at larger concentrations), 1-EBIO (K_{Ca3.1} opener), and minoxidil (K_{ATP} opener) were applied to the basolateral compartment. The average concentration-response curves were plotted in Fig. 7B. The half-maximal effective concentrations (EC₅₀) were 2.4 μM, 391.8 μM, and 1.2 μM, respectively, for pyrithione-Na, 1-EBIO, and minoxidil. To maximally activate these K⁺ channels,



the concentration used for each type of K⁺ channels was based on the results of the dose-response studies (Fig. 7A & 7B). As shown in Fig. 7C, pyrithione-Na (10 μ M), 1-EBIO (600 μ M), and minoxidil (10 μ M) significantly increased AS Isc levels from 14.9 ± 1.7 to 17.8 ± 2.4 μ A/cm² (P < 0.01, n = 6), 12.9 ± 1.9 to 18.6 ± 2.6 μ A/cm² (P < 0.01, n = 6), and 14.9 ± 2.4 to 19.4 ± 2.8 μ A/cm² (P < 0.05, n = 3), respectively. These encouraging observations imply that stimulating K⁺ secretion with K⁺ channel openers can reverse, at least partially, verapamil-inhibited AS transepithelial Na⁺ pathways. In fact, this set of experiments was initiated with a low dose of pyrithione-Zn (ZnPy, 10 μ M), which was supposed to specifically open heterologously expressed K_vLQT1 current, one of large K_v family [50]. Interestingly, only a transient increment was observed followed by a continuing decline in an hour (Additional file 1). This is likely due to the non-specific effects of Zn²⁺ ions on transepithelial ion transport systems, including ENaC [51-54]. We thus had to utilize its sodium compound, which has a divergent EC₅₀ value for native K_v channels in H441 cells (Fig. 7B).

We also tried to prevent the inhibitory effects of verapamil on the AS Isc levels by addition of K⁺ channel openers prior to verapamil. The similar transient or sustained elevation in the Isc levels was observed following the application of the K⁺ channel openers but inexplicably the subsequent application of verapamil inhibited Isc levels to the same extent as that of control monolayers in the absence of K⁺ channel openers (data not shown). In sharp contrast to the significant recovery effects of verapamil-inhibited ion transport, the K⁺ channel openers did not prevent the verapamil-induced depression in transepithelial ion transport. These observations indicate that instead of keeping K⁺ channels from the inhibitory of verapamil, K⁺ channel openers are only able to recover impaired K⁺ channel activities.

Diverse stimulating effects of K⁺ channel openers on apical and basolateral ion transport

Recovery of verapamil-inhibited transepithelial Isc levels in H441 cells (Fig. 7) by the K⁺ channel openers raised a new question of what Na⁺ transport systems are regulated by the K⁺ channel openers, apical ENaC or basolateral

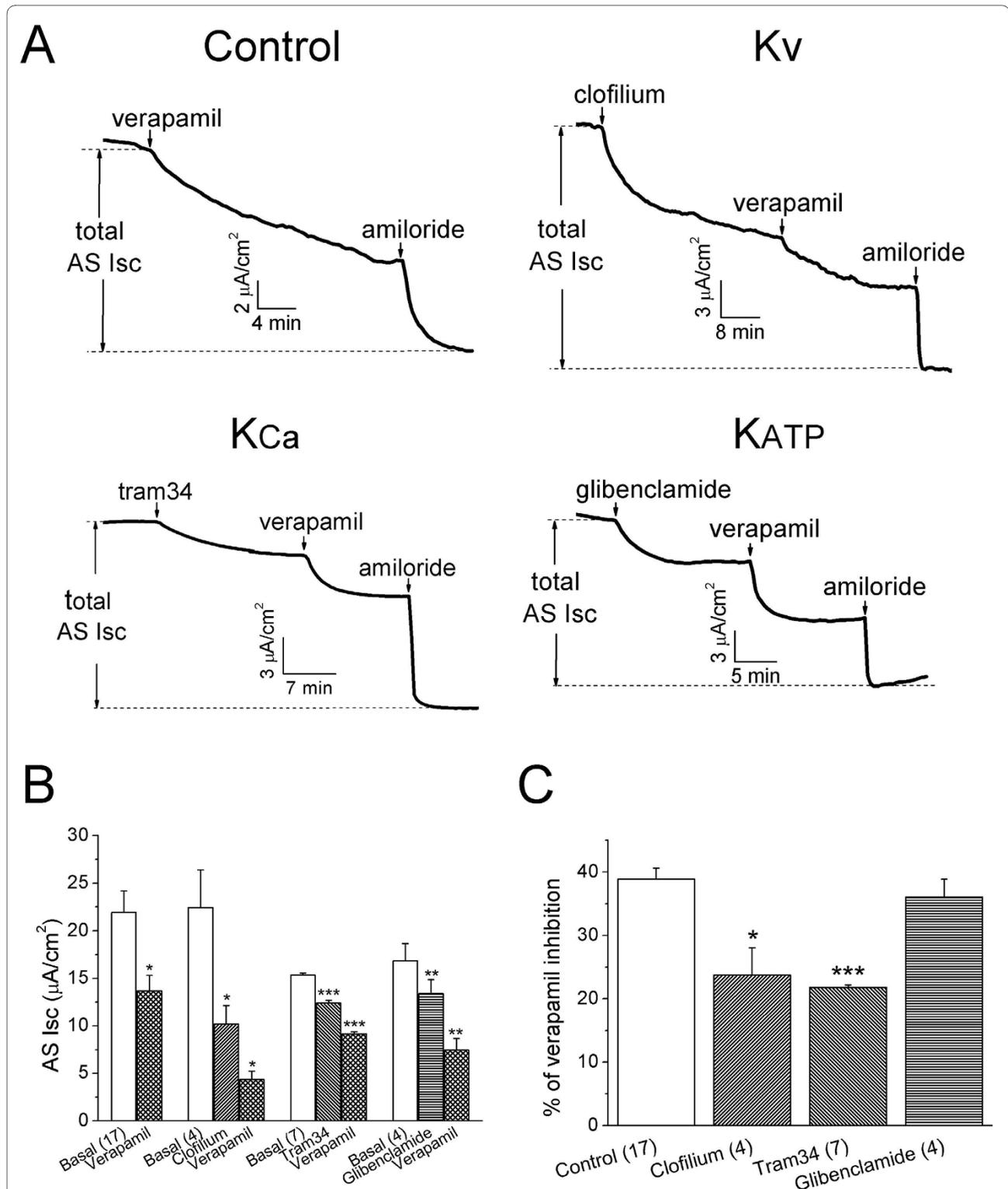
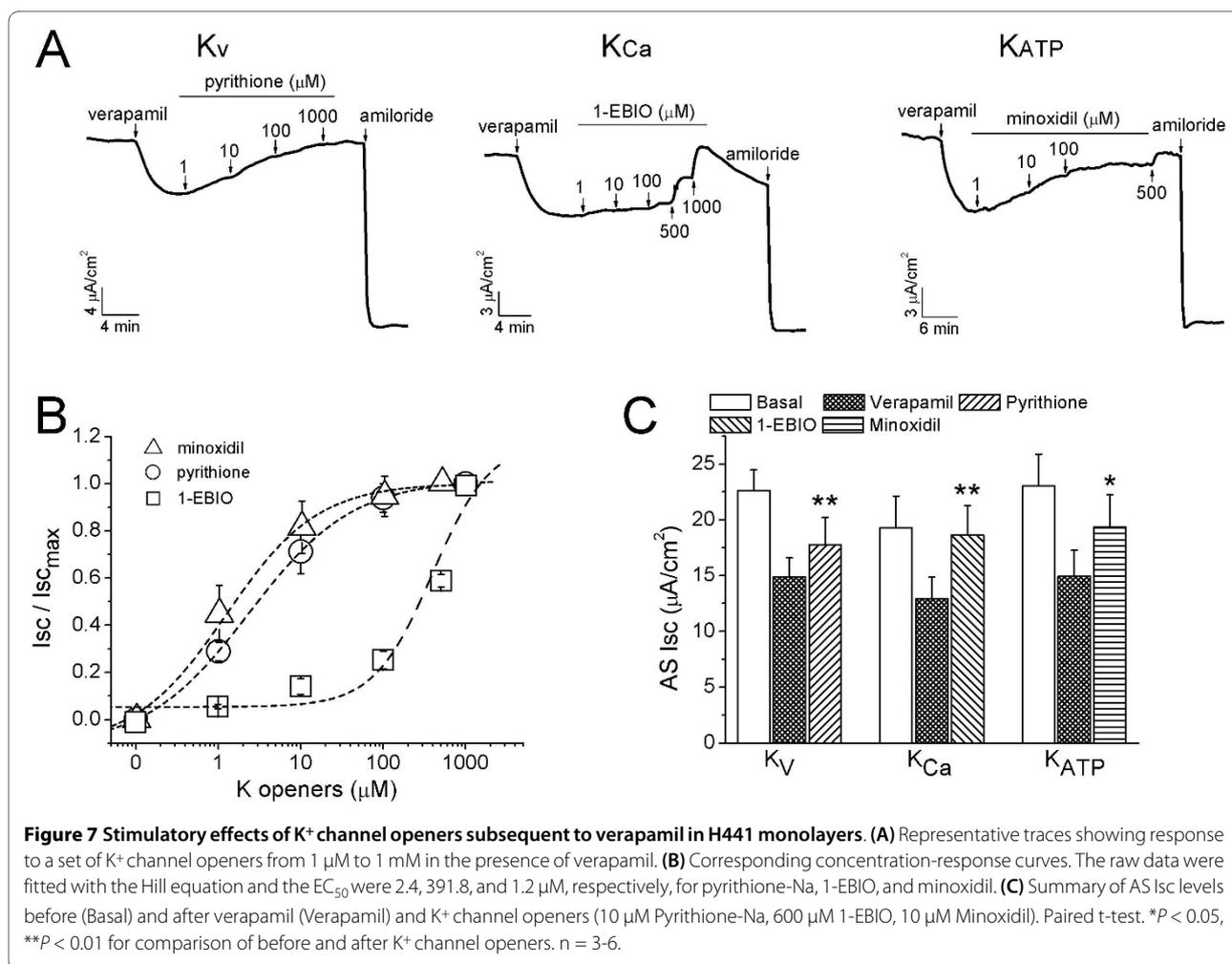


Figure 6 K^+ channel blockers alter the inhibitory effects of verapamil in H441 cells. **(A)** Typical Isc traces showing the application of 100 μM verapamil alone (control), 100 μM clofilium (K_v inhibitor), 20 μM tram34 ($\text{K}_{\text{Ca}3.1}$ inhibitor), and 100 μM glibenclamide (K_{ATP} inhibitor), respectively. These K^+ channel blockers were applied to basolateral side followed by verapamil and amiloride (apical side) to compute total AS Isc. **(B)** Summary of average AS Isc levels. Paired t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for comparison of pre- and post exposure of CCB. $n = 4-17$. **(C)** Reduced percentages of AS Isc levels by verapamil in H441 cells with and without pretreatment of K^+ channel blockers. Two-sample, two-tailed t-test. * $P < 0.05$ vs Control. $n = 4-17$.



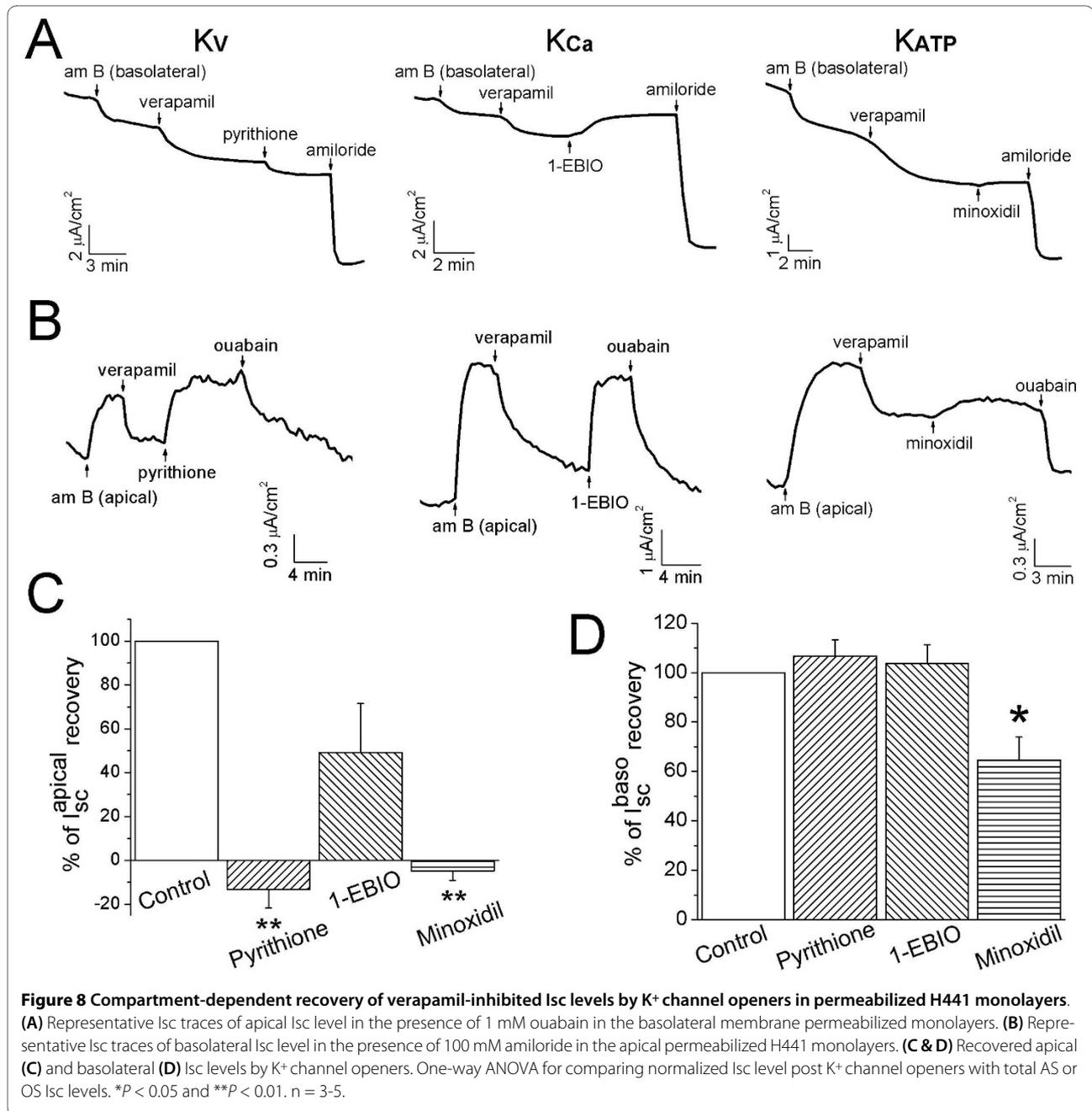
Na⁺/K⁺-ATPase. To address this question, K⁺ channel openers were applied to either apical or basolateral membrane permeabilized monolayers. In basolateral permeabilized cells, neither KCNQ (pyrithione-Na) nor K_{ATP} (minoxidil) channel openers altered AS transapical Na⁺ influx (Fig. 8A & 8C). However, K_{Ca3.1} channel opener (1-EBIO) approximately restored half (49.1 ± 22.3%) of the depressed AS Isc level. Regarding transbasolateral ion transport, pyrithione-Na and 1-EBIO but not minoxidil (64.6 ± 9.2%) completely restored the verapamil-inhibited OS Isc levels in apical membrane permeabilized H441 monolayers (Fig. 8B & 8D). Taken together, these results suggest that K_{Ca3.1} channels are probably located at both apical and basolateral membranes in H441 cells, while pyrithione- and minoxidil-activated channels may be expressed at the basolateral membrane only.

Direct regulation of αβγ ENaC by verapamil in *X. laevis* oocytes

To address the question of whether verapamil directly regulate human ENaC, human α, β, and γ ENaC subunits

were co-expressed in *X. laevis* oocytes, and the effects of verapamil on heterologously expressed ENaC were assessed. Verapamil inhibited ENaC current in an oocyte under physiological conditions (Fig. 9A), which is consistent with the results in H441 cells. An average of 38.2 ± 5.5% ENaC currents was reduced by verapamil (P < 0.05, n = 4, Fig. 9C), suggesting that verapamil may also directly reduce native ENaC channel activity in H441 cells.

If intracellular Ca²⁺ signal mediates the down-regulation of αβγ ENaC by verapamil, one may expect that cell permeable Ca²⁺ chelator could have the same effect. To address this issue, BAPTA-AM was superfused on oocytes bathed in the Ca²⁺-free solution. In an oocyte perfused with the Ca²⁺-free bath solution (5 mM EGTA, 0 mM Ca²⁺), the whole-cell ENaC current declined gradually (Fig. 9B). The residual ENaC currents were no longer sensitive to verapamil under these Ca²⁺-depletion conditions (the ENaC current even increased by 0.4 ± 1.5% after correction of the run-down slope, P > 0.05 compared with basal current, n = 4, Fig. 9D). Obviously, vera-

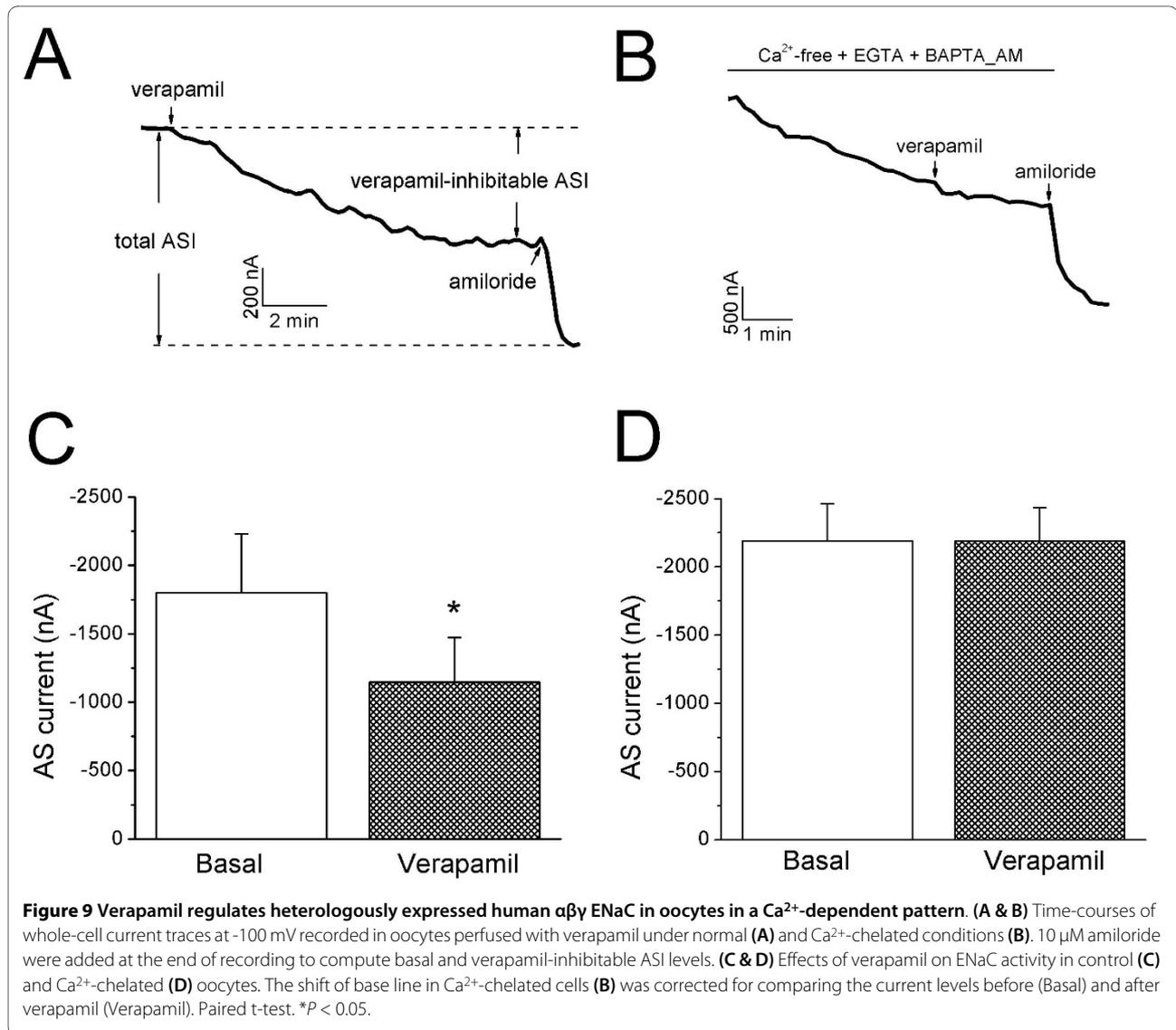


pamil down-regulates human $\alpha\beta$ ENaC in a cytosolic Ca^{2+} -dependent fashion in oocytes. We also tried to repeat these experiments in Ca^{2+} depleted H441 monolayers, unfortunately, the resistance and current levels post BAPTA-AM exposure were too low to detect due to impaired gap junctions and ion transport (data not shown).

Verapamil alters cytosolic Ca^{2+} signal

The regulation of alveolar ENaC by Ca^{2+} signal has been documented by the well-designed *in vivo* and *in vitro* studies [55,56]. We reason that verapamil may interfere

with transepithelial Na^+ transport by altering cell Ca^{2+} signal, which is regulated by mechanic stress associated with breath. The intracellular Ca^{2+} signal was measured with Fluo 4AM in real time using confocal microscopy (Additional files 1 & 2) in H441 cells in the absence or presence of verapamil. Ionomycin was added to the chamber to mimic the Ca^{2+} wave caused by breath. An approximately three-fold increment in fluorescent intensity was observed following the exposure to ionomycin (Fig. 10A & 10B, Additional file 2). In comparison, this increment was significantly diminished in the presence of verapamil (Fig. 10A & 10B, Additional file 3). In addition,



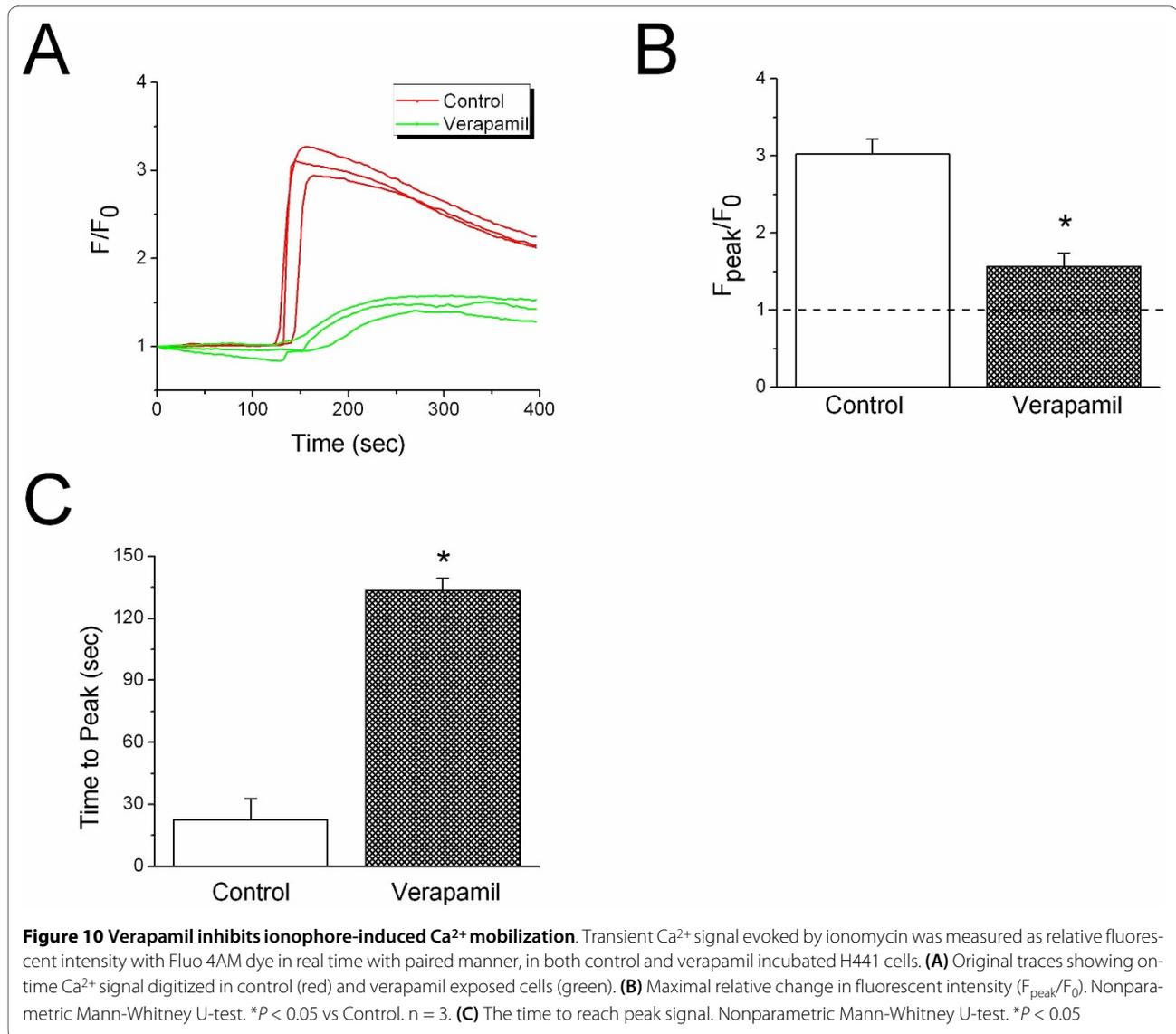
the time required to reach the maximal value of fluorescent intensity was considerably prolonged in verapamil exposed cells (Fig. 10C, $P < 0.05$).

Discussion

We aimed to study the cellular mechanistic pathogenesis of the CCB-induced noncardiogenic defect in lung fluid clearance. Ussing chamber studies suggest that transepithelial Na^+ transport is inhibited by four structurally distinct subgroups of CCB compounds in human lung epithelial cells (H441 cells). Verapamil reduces amiloride-sensitive (AS) I_{sc} levels in a concentration-dependent manner. Ca^{2+} signal is involved in the down-regulation of AS Na^+ transport by verapamil. Furthermore, verapamil alters K^+ recycling via stimulating the apical and basolateral K^+ channels as well as Na^+/K^+ -ATPase activity. K^+ channel openers restore the suppressed ENaC activity *in*

vitro to a significant extent. Of note, our *in vivo* alveolar fluid clearance (AFC) studies show that K^+ channel openers restore the verapamil-inhibited fluid resolution.

A Ca^{2+} signal has been shown to up-regulate alveolar fluid clearance and epithelial Na^+ channel activity [55,57]. Depletion of intracellular Ca^{2+} by thapsigargin in late-gestational guinea pig lungs completely inhibited amiloride-sensitive AFC [55]. On the other hand, elevation of intracellular Ca^{2+} concentration by β -adrenergic agonists and other AFC-enhancing reagents, for example, terbutaline, has been confirmed [58]. CCB partially blocked terbutaline-stimulated Na^+ absorption via amiloride-sensitive channels in primary rat alveolar type II cells [57,59]. Our results that show BAPTA-AM completely abolishes verapamil-induced inhibition of $\alpha\beta$ ENaC activity in oocytes suggest that the regulation of ENaC by CCB is at least partially mediated by an alteration in cytosolic Ca^{2+}

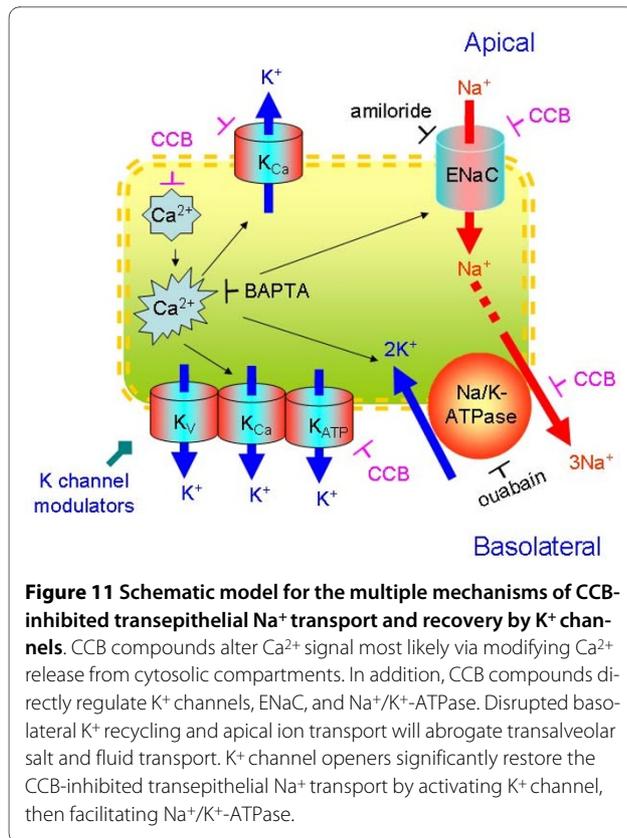


signal (Fig. 10). The observation that verapamil inhibits ionophore-induced Ca^{2+} mobilization supports this notion. The direct inhibitory effects of Ca^{2+} ions on ENaC *in vitro* [60,61] were, perhaps, overwhelmed by the stimulatory effects of Ca^{2+} downstream signals on ENaC and other transporters in these cell models and *in vivo* studies.

Accumulating evidence demonstrates that the regulation of epithelial K^+ channels by the Ca^{2+} signal. The expression of various K^+ channels has been detected in alveolar and bronchial epithelial cells [20]. Ca^{2+} signal may regulate those K^+ channels by both directly affecting the gating kinetics and serving as a second messenger for signal transduction. On the other hand, the relationship between a Ca^{2+} signal and Na^+/K^+ -ATPase is not known. Control of endoplasmic reticulum (ER) Ca^{2+} release by Na^+/K^+ -ATPase has been recently confirmed in "knock-

out" cultured renal epithelial cells [62]. It raises the possibility that CCB may directly inhibit Na^+/K^+ -ATPase and in turn alter the intracellular Ca^{2+} content. Nevertheless, our data clearly confirm that impaired K^+ ion transport across alveolar basolateral membrane is an essential mechanism for CCB to inhibit ENaC function. Interruption of K^+ ion recycling may be a critical mechanism for CCB-induced inhibition of ENaC activity (Fig. 11).

What are the underlying mechanisms for the diverse regulation of apical and basolateral conductance by K^+ channel openers? If the K^+ channel openers restore the depressed ENaC and Na^+/K^+ -ATPase by stimulating K^+ influx which facilitates Na^+/K^+ -ATPase in intact cells, no effects on ENaC should be observed in basolateral permeabilized monolayers. Intriguingly, $\text{K}_{\text{Ca}3.1}$ channel opener still activated ENaC. It is possible that K_{Ca} channels are expressed in apical membrane [29]. Increased



extrusion of K⁺ ions in the presence of 1-EBIO may locally build up an electrical gradient resulting in elevated ENaC activity. Another possibility is that 1-EBIO directly stimulates ENaC. The less effect of minoxidil in permeabilized monolayers, by comparison to the results in intact H441 cells, *in vivo* fluid clearance, and previous publication [29], may be due to loss of cell ATP.

CCB compounds may not act on the same Ca²⁺ entry/exit pathways due to their divergent pharmaceutical properties [63]. Lung epithelial Ca²⁺ content is determined by Ca²⁺ influx/efflux pathways, endoplasmic reticulum (ER) Ca²⁺ release, and concentration of Ca²⁺-binding proteins. Ca²⁺ ions may enter epithelia through, for example, L-type Ca²⁺ channels, ECaC, and other TRP channels; while Ca²⁺-ATPase and Ca²⁺/Na⁺ exchanger are major transporters to extrude Ca²⁺ ions. Our results indicate that verapamil may not alter basal Ca²⁺ content, instead, the Ca²⁺ wave, possibly due to Ca²⁺ release from cytosolic compartments, was abolished (Fig. 11).

The potent efficacy of K⁺ channel openers to recover transepithelial Na⁺ reabsorption and fluid clearance may be a promising therapeutic approach to mitigate drug-induced as well as other deleterious agents-induced noncardiogenic lung edema. On the other hand, inhalation CCB compounds will definitely bring life-threatening noncardiogenic lung edema to patients. Reasonably, any

medicines capable of depleting lung epithelial Ca²⁺ ions may have the same fatal side-effect when delivered either intravenously or intratracheally. Indeed, nifedipine cannot prevent lung edema in mountain sickness [64].

As supported by our observations in permeabilized H441 monolayers and Ca²⁺-depleted cells, verapamil inhibited basolateral and apical K⁺ conductance directly. Verapamil inhibits IK (K_{Ca3.1}) channels with an IC₅₀ value of 72 μM, various K_V channel subtypes between 10-200 μM and K_{ATP} channels at ~10 μM [65-67]. Moreover, concentrations typically used to achieve nearly complete inhibition of voltage-gated Ca²⁺ channels is less than 100 μM. Therefore, it is most likely that verapamil alters ENaC activity via multiple mechanisms, for example, through Ca²⁺-mediated regulation, direct inhibition of ENaC, and interrupting K⁺ recycling (Fig. 11). In summary, CCB reagents decrease vectorial transepithelial Na⁺ transport directly by inhibiting apical ENaC and indirectly by altering cytosolic Ca²⁺ signal and K⁺ recycling at the basolateral membrane. Recovery of the CCB-depressed edema resolution by K⁺ channel openers indicates that pharmaceutical augmentation of K⁺ recycling may be a potent strategy to combat CCB-induced noncardiogenic lung edema.

Additional material

Additional file 1 Pyrrhione zinc on K_V in H441 cells. The specific blocker for heterologously expressed K_V channels, pyrrhione zinc transiently activates AS Isc followed by a pronounced decline. The remaining Isc level is approximately 0 for AS Isc fraction.

Additional file 2 Video clip in a control H441 cell. Fluo 4AM fluorescent intensity was monitored in real time before and after addition of ionomycin.

Additional file 3 Video clip in a verapamil incubated H441 cell. Fluo 4AM fluorescent intensity was monitored in real time before and after addition of ionomycin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DYH and HGN performed Ussing chamber and voltage clamp studies and analyzed data. XG carried out *in vivo* alveolar fluid clearance. DYH and RCN detected the intracellular Ca²⁺ intensity. XFS and YC prepared cRNA and voltage clamp recording. HLJ, JF, and VR designed experiments, analyzed data, and prepared manuscript. All authors have read and approved the final manuscript.

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References

1. Sami Karti S, Ulusoy H, Yandi M, Gunduz A, Kosucu M, Erol K, Ratip S: **Non-cardiogenic pulmonary oedema in the course of verapamil intoxication.** *Emerg Med J* 2002, **19**(5):458-459.
2. Nassar AH, Ghazeeri G, Usta IM: **Nifedipine-associated pulmonary complications in pregnancy.** *Int J Gynaecol Obstet* 2007, **97**(2):148-149.
3. Vaast P, Dubreucq-Fossaert S, Houfflin-Debarge V, Provost-Helou N, Ducloy-Bouthors AS, Puech F, Subtil D: **Acute pulmonary oedema during nicardipine therapy for premature labour; Report of five cases.** *Eur J Obstet Gynecol Reprod Biol* 2004, **113**(1):98-99.
4. Abbas OM, Nassar AH, Kanj NA, Usta IM: **Acute pulmonary edema during tocolytic therapy with nifedipine.** *Am J Obstet Gynecol* 2006, **195**(4):e3-4.
5. Janower S, Carbonne B, Lejeune V, Apfelbaum D, Boccarda F, Cohen A: **Acute pulmonary edema during preterm labor: role of nicardipine tocolysis (three cases).** *J Gynecol Obstet Biol Reprod (Paris)* 2005, **34**(8):807-812.
6. Chapuis C, Menthonnex E, Debaty G, Koch FX, Rancurel E, Menthonnex P, Pons JC: **Acute pulmonary edema during nicardipine and salbutamol therapy for preterm labor in twin pregnancy.** *J Gynecol Obstet Biol Reprod (Paris)* 2005, **34**(5):493-496.
7. Stanek EJ, Nelson CE, DeNofrio D: **Amlodipine overdose.** *Ann Pharmacother* 1997, **31**(7-8):853-856.
8. Humbert VH Jr, Munn NJ, Hawkins RF: **Noncardiogenic pulmonary edema complicating massive diltiazem overdose.** *Chest* 1991, **99**(1):258-259.
9. Brass BJ, Winchester-Penny S, Lipper BL: **Massive verapamil overdose complicated by noncardiogenic pulmonary edema.** *Am J Emerg Med* 1996, **14**(5):459-461.
10. Matthay MA, Folkesson HG, Clerici C: **Lung epithelial fluid transport and the resolution of pulmonary edema.** *Physiol Rev* 2002, **82**:569-600.
11. Eaton DC, Helms MN, Koval M, Bao HF, Jain L: **The Contribution of Epithelial Sodium Channels to Alveolar Function in Health and Disease.** *Annu Rev Physiol* 2009, **71**:403-423.
12. Matalon S, Lazrak A, Jain L, Eaton DC: **Invited review: biophysical properties of sodium channels in lung alveolar epithelial cells.** *J Appl Physiol* 2002, **93**(5):1852-1859.
13. Zemans RL, Matthay MA: **Bench-to-bedside review: the role of the alveolar epithelium in the resolution of pulmonary edema in acute lung injury.** *Crit Care* 2004, **8**(6):469-477.
14. Matthay MA, Robriquet L, Fang X: **Alveolar epithelium: role in lung fluid balance and acute lung injury.** *Proc Am Thorac Soc* 2005, **2**(3):206-213.
15. Hummler E, Barker P, Gatzky J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC: **Early death due to defective neonatal lung liquid clearance in α -ENaC-deficient mice.** *Nat Genet* 1996, **12**(3):325-328.
16. Chen L, Song W, Davis IC, Shrestha K, Schwiebert E, Sullender WM, Matalon S: **Inhibition of Na^+ transport in lung epithelial cells by respiratory syncytial virus infection.** *Am J Respir Cell Mol Biol* 2009, **40**(5):588-600.
17. Rossier BC, Pradervand S, Schild L, Hummler E: **Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors.** *Annu Rev Physiol* 2002, **64**:877-897.
18. O'Grady SM, Lee SY: **Molecular diversity and function of voltage-gated (Kv) potassium channels in epithelial cells.** *Int J Biochem Cell Biol* 2005, **37**(8):1578-1594.
19. Inglis SK, Brown SG, Constable MJ, McTavish N, Olver RE, Wilson SM: **A Ba^{2+} -resistant, acid-sensitive K^+ conductance in Na^+ -absorbing H441 human airway epithelial cells.** *Am J Physiol Lung Cell Mol Physiol* 2007, **292**(5):L1304-1312.
20. O'Grady SM, Lee SY: **Chloride and potassium channel function in alveolar epithelial cells.** *Am J Physiol Lung Cell Mol Physiol* 2003, **284**(5):L689-700.
21. Leroy C, Dagenais A, Berthiaume Y, Brochiero E: **Molecular identity and function in transepithelial transport of K(ATP) channels in alveolar epithelial cells.** *Am J Physiol Lung Cell Mol Physiol* 2004, **286**(5):L1027-1037.
22. Bardou O, Trinh NT, Brochiero E: **Molecular diversity and function of K^+ channels in airway and alveolar epithelial cells.** *Am J Physiol Lung Cell Mol Physiol* 2008.
23. Greenwood IA, Yeung SY, Hettiarachi S, Andersson M, Baines DL: **KCNQ-encoded channels regulate Na^+ transport across H441 lung epithelial cells.** *Pflugers Arch* 2009, **457**(4):785-794.
24. Bernard K, Bogliolo S, Soriani O, Ehrenfeld J: **Modulation of calcium-dependent chloride secretion by basolateral SK4-like channels in a human bronchial cell line.** *J Membr Biol* 2003, **196**(1):15-31.
25. Leroy C, Prive A, Bourret JC, Berthiaume Y, Ferraro P, Brochiero E: **Regulation of ENaC and CFTR expression with K^+ channel modulators and effect on fluid absorption across alveolar epithelial cells.** *Am J Physiol Lung Cell Mol Physiol* 2006, **291**(6):L1207-1219.
26. Szkotak AJ, Ng AM, Sawicka J, Baldwin SA, Man SF, Cass CE, Young JD, Duszyk M: **Regulation of K^+ current in human airway epithelial cells by exogenous and autocrine adenosine.** *Am J Physiol Cell Physiol* 2001, **281**(6):C1991-2002.
27. Monaghan AS, Baines DL, Kemp PJ, Olver RE: **Inwardly rectifying K^+ currents of alveolar type II cells isolated from fetal guinea-pig lung: regulation by G protein- and Mg^{2+} -dependent pathways.** *Pflugers Arch* 1997, **433**(3):294-303.
28. Berthiaume Y, Folkesson HG, Matthay MA: **Lung edema clearance: 20 years of progress: invited review: alveolar edema fluid clearance in the injured lung.** *J Appl Physiol* 2002, **93**(6):2207-2213.
29. Sakuma T, Takahashi K, Ohya N, Nakada T, Matthay MA: **Effects of ATP-sensitive potassium channel opener on potassium transport and alveolar fluid clearance in the resected human lung.** *Pharmacol Toxicol* 1998, **83**(1):16-22.
30. Peth S, Karle C, Dehnert C, Bartsch P, Mairbaurl H: **K^+ channel activation with minoxidil stimulates nasal-epithelial ion transport and blunts exaggerated hypoxic pulmonary hypertension.** *High Alt Med Biol* 2006, **7**(1):54-63.
31. Briot R, Frank JA, Uchida T, Lee JW, Calfee CS, Matthay MA: **Elevated levels of the receptor for advanced glycation end products, a marker of alveolar epithelial type I cell injury, predict impaired alveolar fluid clearance in isolated perfused human lungs.** *Chest* 2009, **135**(2):269-275.
32. Factor P, Mutlu GM, Chen L, Mohameed J, Akhmedov AT, Meng FJ, Jilling T, Lewis ER, Johnson MD, Xu A, *et al.*: **Adenosine regulation of alveolar fluid clearance.** *Proc Natl Acad Sci USA* 2007, **104**(10):4083-4088.
33. Lazrak A, Nita I, Subramaniyam D, Wei S, Song W, Ji HL, Janciauskiene S, Matalon S: **α 1-antitrypsin inhibits epithelial Na^+ transport *in vitro* and *in vivo*.** *Am J Respir Cell Mol Biol* 2009, **41**(3):261-270.
34. Gu X, Wang Z, Xu J, Maeda S, Sugita M, Sagawa M, Toga H, Sakuma T: **Denopamine stimulates alveolar fluid clearance via cystic fibrosis transmembrane conductance regulator in rat lungs.** *Respirology* 2006, **11**(5):566-571.
35. Chen L, Patel RP, Teng X, Bosworth CA, Lancaster JR Jr, Matalon S: **Mechanisms of cystic fibrosis transmembrane conductance regulator activation by S-nitrosoglutathione.** *J Biol Chem* 2006, **281**(14):9190-9199.
36. Thome U, Chen L, Factor P, Dumasius V, Freeman B, Sznajder JI, Matalon S: **K, K-ATPase gene transfer mitigates an oxidant-induced decrease of active sodium transport in rat fetal ATEC cells.** *Am J Respir Cell Mol Biol* 2001, **24**(3):245-252.
37. Thome UH, Davis IC, Nguyen SV, Shelton BJ, Matalon S: **Modulation of sodium transport in fetal alveolar epithelial cells by oxygen and corticosterone.** *Am J Physiol Lung Cell Mol Physiol* 2003, **284**(2):L376-385.
38. Guo Y, DuVall MD, Crow JP, Matalon S: **Nitric oxide inhibits Na^+ absorption across cultured alveolar type II monolayers.** *Am J Physiol* 1998, **274**(3 Pt 1):L369-377.
39. Kirk KL, Halm DR, Dawson DC: **Active sodium transport by turtle colon via an electrogenic Na-K exchange pump.** *Nature* 1980, **287**(5779):237-239.
40. Ji HL, Su XF, Kedar S, Li J, Barbry P, Smith PR, Matalon S, Benos DJ: **δ -subunit confers novel biophysical features to $\alpha\beta$ -human epithelial sodium channel (ENaC) via a physical interaction.** *J Biol Chem* 2006, **281**(12):8233-8241.
41. Musa-Aziz R, Oliveira-Souza M, Mello-Aires M: **Signaling pathways in the biphasic effect of ANG II on Na^+/H^+ exchanger in T84 cells.** *J Membr Biol* 2005, **205**(2):49-60.
42. Espelt MV, Estevez AY, Yin X, Strange K: **Oscillatory Ca^{2+} signaling in the isolated *Caenorhabditis elegans* intestine: role of the inositol-1,4,5-**

- triphosphate receptor and phospholipases C β and γ . *J Gen Physiol* 2005, **126**(4):379-392.
43. Pendurthi UR, Ngyuen M, Andrade-Gordon P, Petersen LC, Rao LV: **Plasmin induces Cyr61 gene expression in fibroblasts via protease-activated receptor-1 and p44/42 mitogen-activated protein kinase-dependent signaling pathway.** *Arterioscler Thromb Vasc Biol* 2002, **22**(9):1421-1426.
44. Praetorius HA, Leipziger J: **Released nucleotides amplify the cilium-dependent, flow-induced $[Ca^{2+}]_i$ response in MDCK cells.** *Acta Physiol (Oxf)* 2009, **197**(3):241-251.
45. Lazrak A, Matalon S: **cAMP-induced changes of apical membrane potentials of confluent H441 monolayers.** *Am J Physiol Lung Cell Mol Physiol* 2003, **285**(2):L443-450.
46. Thomas CP, Campbell JR, Wright PJ, Husted RF: **cAMP-stimulated Na^+ transport in H441 distal lung epithelial cells: role of PKA, phosphatidylinositol 3-kinase, and sgk1.** *Am J Physiol Lung Cell Mol Physiol* 2004, **287**(4):L843-851.
47. Nie HG, Chen L, Han DY, Li J, Song WF, Wei SP, Fang XH, Gu X, Matalon S, Ji HL: **Regulation of epithelial sodium channels by cGMP/PKGII.** *J Physiol* 2009, **587**(Pt 11):2663-2676.
48. Woollhead AM, Baines DL: **Forskolin-induced cell shrinkage and apical translocation of functional enhanced green fluorescent protein-human α ENaC in H441 lung epithelial cell monolayers.** *J Biol Chem* 2006, **281**(8):5158-5168.
49. Zhang S, Zhou Z, Gong Q, Makielski JC, January CT: **Mechanism of block and identification of the verapamil binding domain to HERG potassium channels.** *Circ Res* 1999, **84**(9):989-998.
50. Gao Y, Chotoo CK, Balut CM, Sun F, Bailey MA, Devor DC: **Role of S3 and S4 transmembrane domain charged amino acids in channel biogenesis and gating of $KCa_{2.3}$ and $KCa_{3.1}$.** *J Biol Chem* 2008, **283**(14):9049-9059.
51. Bancila V, Cens T, Monnier D, Chanson F, Faure C, Dunant Y, Bloc A: **Two SUR1-specific histidine residues mandatory for zinc-induced activation of the rat KATP channel.** *J Biol Chem* 2005, **280**(10):8793-8799.
52. Swaminath G, Lee TW, Kobilka B: **Identification of an allosteric binding site for Zn^{2+} on the β_2 adrenergic receptor.** *J Biol Chem* 2003, **278**(1):352-356.
53. Amuzescu B, Segal A, Flonta ML, Simaels J, Van Driessche W: **Zinc is a voltage-dependent blocker of native and heterologously expressed epithelial Na^+ channels.** *Pflugers Arch* 2003, **446**(1):69-77.
54. Bossy-Wetzel E, Talantova MV, Lee WD, Scholzke MN, Harrop A, Mathews E, Gotz T, Han J, Ellisman MH, Perkins GA, *et al.*: **Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K^+ channels.** *Neuron* 2004, **41**(3):351-365.
55. Norlin A, Folkesson HG: **Ca^{2+} -dependent stimulation of alveolar fluid clearance in near-term fetal guinea pigs.** *Am J Physiol Lung Cell Mol Physiol* 2002, **282**(4):L642-649.
56. Swystun V, Chen L, Factor P, Siroky B, Bell PD, Matalon S: **Apical trypsin increases ion transport and resistance by a phospholipase C-dependent rise of Ca^{2+} .** *Am J Physiol Lung Cell Mol Physiol* 2005, **288**(5):L820-830.
57. Marunaka Y, Niisato N: **Effects of Ca^{2+} channel blockers on amiloride-sensitive Na^+ permeable channels and Na^+ transport in fetal rat alveolar type II epithelium.** *Biochem Pharmacol* 2002, **63**(8):1547-1552.
58. Niisato N, Nakahari T, Tanswell AK, Marunaka Y: **β_2 -agonist regulation of cell volume in fetal distal lung epithelium by cAMP-independent Ca^{2+} release from intracellular stores.** *Can J Physiol Pharmacol* 1997, **75**(8):1030-1033.
59. Marunaka Y, Niisato N, O'Brodovich H, Eaton DC: **Regulation of an amiloride-sensitive Na^+ -permeable channel by a β_2 -adrenergic agonist, cytosolic Ca^{2+} and Cl^- in fetal rat alveolar epithelium.** *J Physiol* 1999, **515**(Pt 3):669-683.
60. Ismailov II, Berdiev BK, Shlyonsky VG, Benos DJ: **Mechanosensitivity of an epithelial Na^+ channel in planar lipid bilayers: release from Ca^{2+} block.** *Biophys J* 1997, **72**(3):1182-1192.
61. Schild L, Schneeberger E, Gautschi I, Firsov D: **Identification of amino acid residues in the α , β , and γ subunits of the epithelial sodium channel (ENaC) involved in amiloride block and ion permeation.** *J Gen Physiol* 1997, **109**(1):15-26.
62. Chen Y, Cai T, Yang C, Turner DA, Giovannucci DR, Xie Z: **Regulation of inositol 1,4,5-triphosphate receptor-mediated calcium release by the Na^+/K^+ -ATPase in cultured renal epithelial cells.** *J Biol Chem* 2008, **283**(2):1128-1136.
63. DeWitt CR, Waksman JC: **Pharmacology, pathophysiology and management of calcium channel blocker and β -blocker toxicity.** *Toxicol Rev* 2004, **23**(4):223-238.
64. Hohenhaus E, Niroomand F, Goerre S, Vock P, Oelz O, Bartsch P: **Nifedipine does not prevent acute mountain sickness.** *Am J Respir Crit Care Med* 1994, **150**(3):857-860.
65. Ninomiya T, Takano M, Haruna T, Kono Y, Horie M: **Verapamil, a Ca^{2+} entry blocker, targets the pore-forming subunit of cardiac type KATP channel (Kir6.2).** *J Cardiovasc Pharmacol* 2003, **42**(2):161-168.
66. Pancrazio JJ, Viglione MP, Kleiman RJ, Kim YI: **Verapamil-induced blockade of voltage-activated K^+ current in small-cell lung cancer cells.** *J Pharmacol Exp Ther* 1991, **257**(1):184-191.
67. Waldegger S, Niemeyer G, Morike K, Wagner CA, Suessbrich H, Busch AE, Lang F, Eichelbaum M: **Effect of verapamil enantiomers and metabolites on cardiac K^+ channels expressed in *Xenopus oocytes*.** *Cell Physiol Biochem* 1999, **9**(2):81-89.

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