cAMP-induced changes of apical membrane potentials of confluent H441 monolayers

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Lazrak, Ahmed, and Sadis Matalon. cAMP-induced changes of apical membrane potentials of confluent H441 monolayers. Am J Physiol Lung Cell Mol Physiol 285: L443–L450, 2003. First published April 18, 2003; 10.1152/ajplung.00412.2002.—We recorded apical membrane potentials (Vₐ) of H441 cells [a human lung cell line exhibiting both epithelial Na⁺ (ENaC) and CFTR-type channels] grown as confluent monolayers, using the microelectrode technique in current-clamp mode before, during, and after perfusion of the apical membranes with 10 μM forskolin. When perfused with normal Ringer solution, the cells had a Vₐ of −43 ± 10 mV (means ± SD; n = 31). Perfusion with forskolin resulted in sustained depolarization by 25.0 ± 3.5 mV (means ± SD; n = 23) and increased the number, open time, and the open probability of a 4.2-pS ENaC. In contrast to a previous report (Jiang J, Song C, Koller BH, Matthy MA, and Verkman AS. Am J Physiol Cell Physiol 275: C1610–C1620, 1998), no transient hyperpolarization was observed. The forskolin-induced depolarization of Vₐ was almost totally prevented by pretreatment of monolayers with 10 μM amiloride or by substitution of Na⁺ ions in the bath solution with N-methyl-d-glucamine. These findings indicate that cAMP stimulation of Na⁺ influx across H441 confluent monolayers results from activation of an amiloride-sensitive apical Na⁺ conductance and not from Vₐ hyperpolarization due to Cl⁻ influx through CFTR-type channels.

forskolin; epithelial sodium channel; cystic fibrosis transmembrane conductance regulator; current clamp; patch clamp; single channel currents; amiloride; glibenclamide

THE AMILORIDE-SENSITIVE epithelial sodium (Na⁺) channel (ENaC) plays a fundamental role in the regulation of fluid movement across epithelial cells. In the lungs, the transepithelial transport of Na⁺ ions through ENaC, or ENaC-type channels, generates the osmotic driving force for water movement from the alveolar to the interstitial space, which reduces the amount of fluid in the alveolar space under both physiological and a number of pathological conditions (23, 34).

The existence of amiloride-sensitive Na⁺ absorption across the alveolar epithelium in vivo has been demonstrated in rats, rabbits, hamsters, mice, guinea pigs, sheep, and humans (6, 9, 24, 26, 27, 30). Furthermore, alveolar type II (ATII) cells isolated from the lungs of these species grown to confluence on filters and mounted in Ussing chambers generated a spontaneous potential difference and short-circuit current (Iₛᵣₗ) that was partly inhibited by amiloride with an IC₅₀ of ~0.85 μM (2, 7). On the basis of these findings, it has been proposed that Na⁺ ions diffuse passively across the apical membranes of ATII cells through these channels, down an electrochemical gradient maintained by an Na⁺-K⁺-ATPase pump.

Direct evidence for the existence of an ion channel in the apical membranes of ATII cells was derived from electrophysiological measurements performed on isolated ATII cells. In these cells, three different types of channels were identified: Ca²⁺-activated cation channels (5); Ca²⁺ independent, moderately selective cation channels with unitary conductances between 20 and 25 pS (10, 35, 36); and highly selective (4 pS) ENaC-type Na⁺ channels (11, 22). The basic biophysical properties of these channels depend on the culture conditions: ATII cells grown on air-liquid interface or in the presence of steroids (such as aldosterone) expressed channels with 6.6 pS unitary conductance and with very high selectivity for Na⁺ over K⁺ (P₉₉/Pₖ > 80) where P is permeability. These channels are inhibited by submicromolar concentrations of amiloride (Kᵣ₀.₅ = 37 nM) (11); however, if cells are cultured in the absence of steroids they express either nonselective or poorly selective (P₉₉/Pₖ = 7) 25-pS channels with an amiloride IC₅₀ of ~1 μM (reviewed in Ref. 22). Currently, there is significant controversy as to what type of channel is expressed in alveolar epithelial cells in vivo; the higher-than-expected K⁺ concentration in the alveolar epithelial lining fluid of anesthetized rabbits and the reduction of these values following application of amiloride (28) are consistent with the presence of nonselective or poorly selective amiloride-sensitive channels at the apical side of alveolar epithelial cells. However, it is likely that both types of channels are present at the alveolar epithelium and that their relative expression is regulated by a number of hormones and environmental factors. Furthermore, recent studies indicate that alveolar type I cells, which form >97% of the alveolar epithelium, also have amiloride-sensitive Na⁺ channels, the biophysical properties of which have not been studied at present (14).

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There is also considerable evidence that agents that increase intracellular cAMP upregulate Na+ transport in a number of species, including humans, in vivo and ex vivo, and across isolated ATII cells (reviewed in Refs. 23, 25). Patch-clamp measurements have shown that β-agonists and permeable analogs of cAMP increase the number and/or the open probability (P_o) of the active Na+ channels in ATII cells, depending on the type of channels expressed (3, 36). On the other hand, Jiang et al. (13) and O’Grady et al. (29) have proposed that the cAMP-induced increase of Na+ transport is merely due to an increase of the driving force across the apical membranes, secondary to an activation of CFTR-type Cl− conductance. Thus the mechanism by which an increase in cAMP increases Na+ absorption across lung epithelial cells containing both ENaC and CFTR-type channels is still in dispute.

Herein we cultured H441 cells on transparent membranes until they formed confluent monolayers and measured the apical membrane potential (V_a) and Na+ single channel activity before, during, and after increasing the cytoplasmic cAMP concentration by perfusing these cells with forskolin, an adenylyl cyclase activator. H441 cells are derived from human Clara cells found in the bronchiolar epithelium, which normally lacks mucous cells and produces a mucous-poor, watery proteinaceous secretion. H441 cells express both ENaC (this study) and CFTR channels (16) in the absence of hormone supplementation. Furthermore, in contrast to ATII cells, they exhibit stable recording of V_a when impaled with low-resistance microelectrodes. Our data clearly demonstrate that agents that increase cAMP activate an apical Na+ conductance, by directly increasing the activity and the P_o of an ENaC-type channel, and depolarize V_a.

**MATERIALS AND METHODS**

**Cell culture.** H441 cells were obtained from ATCC and were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES (pH 7.4), and 10% fetal bovine serum. Cells were seeded in 75-cm² flasks and incubated in a humidified atmosphere of 5% CO₂-95% O₂ at 37°C until they reached confluence and then were passaged weekly. For consistency, only cells between passages 82 and 97 were used for the present studies. The cells were subcultured on Millipore membranes (12 mm in diameter, Millicell-CM, Millipore, Bedford, MA), and the medium was replaced every other day. Transepithelial resistance (R_T) was measured daily with an Epithelial Voltohmmeter equipped with chopstick-style electrodes (World Precision Instruments, Sarasota, FL). All measurements were conducted on H441 cells monolayers between the 4th and 6th days after the initial seeding, the time at which they typically formed confluent monolayers. Filters containing H441 cells were rinsed with normal Ringer solution (NRS) and transferred to the recording chamber mounted on the stage of an inverted microscope (Olympus). Both the apical and basolateral sides of the monolayers were perfused continuously with solutions of the same ionic composition at room temperature (20–22°C), using two gravity-driven perfusion systems.

All H441 cells were initially perfused with NRS containing (in mM) 143 NaCl, 5.4 KCl, 1.8 CaCl₂, 2 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). The osmolality of this solution, measured with a Vapor Pressure Osmometer (Vapro Wescor, Logan, UT), was 300 ± 5 mosmol/kgH₂O. In some experiments, cells were perfused with solutions in which NaCl was replaced with equimolar concentrations of Na-glucuronate or N-methyl-d-glucamine (NMDG)-Cl. The osmolality of all solutions was adjusted to 300 mosmol/kgH₂O with mannitol, and the pH was adjusted to 7.4 with 10 mM HEPES and 1 N NaOH. Once stable recordings were obtained, amiloride (10 µM), forskolin (10 µM), or glibenclamide (100 µM) was added into the solutions perfusing the apical side of the monolayers.

**Patch-clamp measurements.** The cell-attached mode of the patch-clamp technique (8) was used to detect the discrete activity of the amiloride-sensitive Na+ channels on the apical membranes of H441 confluent cell monolayers. The pipettes were made from LG16-type capillary glass (Dagan, Minneapolis, MN) with a two-stage vertical puller (PIP5; HEKA, Pflaz, Germany). They were back-filled with a solution of the following ionic composition (in mM): 145 Na-glucuronate, 1.8 CaCl₂, 2 MgCl₂, 5.5 mannitol, and 10 HEPES, pH 7.4. Pipette resistance, when filled with this solution, was ~15 MΩ. The offset potential was corrected with an amplifier (Axopatch 200; Axon Instruments, Foster City, CA) just before the giga-seal formation. Before recording channel activity, we perfused the cells with a solution containing (in mM): 134 K-glucuronate, 10 KCl, 5 MgCl₂, 10 HEPES, and 5.5 glucose, pH 7.4, which depolarized V_a to a mean value of ~3 mV (n = 7). The patch potential was then calculated from the following equation: (V_patched = V_a - V_pipette), where V_a = −3 mV and V_pipette is the applied potential. The data were sampled at 2–5 kHz and filtered at 1–2 kHz. During analysis, a 300-Hz low-pass digital filter was used. The amplitude and P_o of the channels were calculated from all event histograms, constructed from at least 10 min of recordings, as previously described (18, 19). Recordings were either continuous or appended to each other to satisfy this condition. Current-voltage (I-V) relationships were constructed from steady-state currents measured at 300 ms from the start of voltage pulses, using Clampfit Program (Axon Instruments) and Origin (Microsoft Software, Northampton, MA). The conductance was measured as the slope conductance of I-V relationships.

**Measurements of V_a.** Microelectrodes were made from 0.5-mm inner diameter glass capillaries (World Precision Instruments) using the P87 micropipette puller (Narishige, Tokyo, Japan) and were filled with 300 mM KCl solution at pH 7.4 (10 mM HEPES). When the pipette was filled with this solution, its tip had a resistance ranging from 125 to 175 MΩ. The cell membrane resistance of single cells (i.e., cells not part of confluent monolayers) was 415 ± 47 MΩ (means ± SD; n = 12). The ground electrode, an Ag-AgCl pellet, against which the membrane potential was measured, was connected to the bath via an agar bridge (2% agar in 150 mM NaCl solution). All measurements were performed in current clamp mode using an IE-251A amplifier (Warner Instruments, Hamden, CT). The data were stored onto the hard drive of a computer equipped with digital/analog and analog/digital converter (Digidata 200) and analyzed using the PCclamp software (Axon Instruments, Union City, CA).

**Chemicals.** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Amiloride was dissolved in water; forskolin was dissolved in ethanol (ethanol's maximal concentration in the bathing solution was <0.1%). Glibenclamide was dissolved in DMSO.

**Statistics.** All data were analyzed by ANOVA, using the Bonferroni method for multiple comparisons or Student's

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RESULTS

All measurements were performed on H441 confluent cell monolayers (\(R_t = 1.9 \pm 0.36 \text{K}\Omega\cdot\text{cm} (\text{means} \pm \text{SD}; n = 15)\) unless stated otherwise. When cells were perfused with NRS, \(V_a\) ranged from \(-30\) to \(-50\) mV (\text{means} \pm \text{SD} = \(-43 \pm 10\) mV; \(n = 31\); see Table 1). Only cells with stable \(V_a\) for at least 3 min were used for further measurements. When the apical side of the cells was perfused with NRS containing 10 \(\mu\)M amiloride, \(V_a\) hyperpolarized by \(-17\) mV and recovered to its initial value when amiloride was washed out (Fig. 1). This finding suggests the presence of a basal \(\text{Na}^+\) influx across the apical membranes of H441 monolayers through amiloride-sensitive pathways.

Single channel recordings using the cell-attached mode showed the presence of an \(\text{Na}^+\) channel with 4.2 pS unitary conductance and long-lasting open states, consistent with the biophysical properties of \(\text{ENaC}\) (Fig. 2). In a number of experiments (\(n = 5\)), the upper part of the pipette (5 \(\mu\)l for a total volume of 10 \(\mu\)l) was filled with a solution containing 4 \(\mu\)M amiloride (for a final concentration of 2 \(\mu\)M). As shown in Fig. 2C, 2 \(\mu\)M amiloride induced the complete cessation of channel activity in the patch. Recordings performed on H441 cell groups forming incomplete monolayers show the presence of two \(\text{Na}^+\) conductances (4.2 and 20 pS; Fig. 3). Although we did not perform single Cl\(-\) channel measurements, H441 cells have been shown to express a CFTR-type chloride channel (16).

In subsequent experiments, we evaluated the effects of cAMP on \(V_a\) on H441 \(V_a\). As shown in Fig. 4 and Table 1, perfusion of the apical sides of monolayers with NRS containing forskolin (10 \(\mu\)M) resulted in a sustained and fully reversible depolarization of \(V_a\) (\(\Delta V_a = 25.0 \pm 3.5\) mV; mean \pm SD; \(n = 23\)). Furthermore, when amiloride (10 \(\mu\)M) was added into the solution at the plateau of the forskolin response, \(V_a\) rapidly hyperpolarized and returned to a baseline that was less negative than when perfused with NRS alone (Fig. 4). The forskolin-induced \(V_a\) depolarization was attenuated significantly when amiloride was added in the apical perfusion solution (Fig. 5) or when we replaced \(\text{Na}^+\) in the apical and basolateral baths with equimolar concentrations of NMDG (Fig. 6). In the latter case, addition of glibenclamide into the perfusion medium totally abolished the forskolin-induced depolarization. Thus the resulting depolarization was most likely due to \(\text{Cl}\)-secretion through cAMP-stimulated CFTR channels. However, significant depolarization was observed when cells were reperfused with NRS containing forskolin (Fig. 7). On the other hand, the amplitude of the forskolin-induced depolarization was not affected when \(\text{Cl}\)-in the perfusion solution was mostly replaced with equimolar concentrations of gluconate (Fig. 8). In this case, the depolarization was preceded by a transient hyperpolarization most likely due to the efflux of \(\text{K}^+\) ions. Because previous studies have shown that agents that increase cAMP may also increase intracellular \(\text{Ca}^{2+}\) in lung epithelial cells (21), this \(\text{K}^+\) efflux may have occurred through \(\text{Ca}^{2+}\)-activated \(\text{K}\) channels.

As an aggregate, our findings show that the forskolin-induced depolarization in H441 monolayers results from the entry of \(\text{Na}^+\) ions through newly activated amiloride-sensitive \(\text{Na}^+\) or cation channels. Indeed, as shown in Fig. 9, B and C, perfusion of H441 cells with forskolin increased the number of active channels in the patches from two to three and their \(P_o\) from 0.23 \pm 0.03 to 0.55 \pm 0.05 (mean \pm SD; \(n = 7\); \(P < 0.05\)) without affecting the unitary conductance. Because the number of channels in the patches was not determined, it is possible that forskolin activated existing quiescent channels. In any case, our results differ from those of Chen et al. (3), who report that stimulation of ATII cells with terbutaline increased the number but not the \(P_o\) of 4-pS \(\text{Na}^+\)-selective channels.

Table 1. \textit{Mean apical membrane potential values across confluent monolayers of H441 cells}

<table>
<thead>
<tr>
<th>Condition</th>
<th>(V_a \pm \text{SD})</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS</td>
<td>(-43 \pm 10)</td>
<td>31</td>
</tr>
<tr>
<td>NRS + Amiloride (10 (\mu)M)</td>
<td>(-59 \pm 63^{*})</td>
<td>11</td>
</tr>
<tr>
<td>NRS + Forskolin (10 (\mu)M)</td>
<td>(-18 \pm 5.5^{*})</td>
<td>23</td>
</tr>
<tr>
<td>Na(^{-})-Gluconate</td>
<td>(-38 \pm 6^{*})</td>
<td>7</td>
</tr>
<tr>
<td>Na(^{-})-Gluconate + Forskolin (10 (\mu)M)</td>
<td>(-22 \pm 5.8^{*})</td>
<td>7</td>
</tr>
<tr>
<td>NMDG(^{-})-Cl</td>
<td>(-57 \pm 5)</td>
<td>8</td>
</tr>
<tr>
<td>NMDG(^{-})-Cl + Forskolin</td>
<td>(-50 \pm 5.6)</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are \textit{means} \pm \text{SD}. \(V_a\), apical membrane potential; NRS, normal Ringer solution; NMDG, \(N\)-methyl-d-glucamine; \(n\), number of recording. \(^{*}\) \(P < 0.05\) compared with NRS, \(P < 0.05\) compared with the corresponding value without forskolin.
Fig. 2. Single channel currents in a H441 cell confluent monolayer. Single channel currents recorded in a cell-attached patch mode from the apical membrane of an H441 cell that was part of a confluent monolayer, at a holding potential of −100 mV (V ≈ V + P). The pipette was filled with 145 mM Na\(^+\); the cell was perfused with a solution containing 145 mM K\(^+\), which depolarized the membrane potential to about −3 mV (see MATERIALS AND METHODS). The recording (A) and amplitude distribution histogram (B), constructed by appending single channel events of 3 different recordings (10 min total), point to the presence of a channel characterized by a unitary conductance of 4.2 ± 0.43 pS (mean ± SD; n = 1,010 events for a 10-min recording). The single channel activity was totally blocked by 2 μM amiloride in the pipette solution (C). Results from typical experiments reproduced at least 13 times under both experimental conditions. C, closed.

DISCUSSION

Our results clearly demonstrate that perfusion of confluent monolayers of H441 cells with forskolin, an agent known to increase intracellular cAMP levels in epithelial cells, resulted in significant depolarization of their apical membrane potential; this effect was decreased either by adding amiloride into the perfusate or by replacing Na\(^+\) ion with equimolar concentrations of NMDG\(^+\), a large impermeant positive ion. Furthermore, perfusion with forskolin increased both the number of active channels and the P\(_o\) of a 4-pS conductance of an ENaC-type channel. Previously, Jiang et al. (13) proposed that exposure of confluent monolayers of ATII cells to either β-adrenergic agents or cell-permeable analogs of cAMP stimulates an apical CFTR-type Cl\(^-\) conductance, resulting in the hyperpolarization of the ATII cell V\(_a\). These authors speculate that the observed increase in the Na\(^+\) component of the I\(_{sc}\) across these monolayers following β-adrenergic stimulation was the result of an increased driving force for Na\(^+\) ions across the apical membranes instead of a direct effect on Na\(^+\) conductance. It should be stressed that Jiang et al. (13) provide no direct evidence for the development of membrane hyperpolarization following increases of intracellular cAMP levels. Our direct measurement of V\(_a\) in H441 cells shows no evidence of transient V\(_a\) hyperpolarization during perfusion of the cell monolayers with NRS containing forskolin. Instead, our measurements of V\(_a\) and the biophysical properties of Na\(^+\) single channels are strong evidence that perfusion of H441 cells with forskolin increased the total Na\(^+\) conductance, in agreement with both measurements of I\(_{sc}\) and Na\(^+\) single channel activity in ATII cells (3, 17, 19, 35, 36). Our findings are also in agreement with theoretical analysis using the Nerst equation. It shows that the driving force for Na\(^+\) ions across the apical membrane (V\(_a\) − E\(_{Na}\)) where E\(_{Na}\) is the sodium reversal potential of H441 or ATII cells is about −100 mV, and thus, as suggested (33), a significant degree of hyperpolarization, unlikely to be achieved by the influx of Cl\(^-\) ions, would be needed to explain the large increase in Na\(^+\) current following an increase in intracellular cAMP. On the contrary, as shown in Fig. 5, perfusion of H441 cells with forskolin in the presence of amiloride-depolarized V\(_a\), consistent with Cl\(^-\) secretion and not absorption.

Admittedly, our measurements were conducted in a different cell type than those of Jiang et al. (13) (H441 vs. cultured ATII cells). The reason for choosing this system was the biophysical properties of Na\(^+\) channels.
in ATII cells vary according to the culture conditions. For example, ATII and A549 cells cultured in the presence of steroids express 4-pS ENaC-type channels (11, 20); however, in the absence of steroids, they express mainly nonselective 20-pS cation channels (11, 35). We were concerned that steroids may alter the response of these cells to agents that increase cAMP and thus opted to use H441 cells, which express both

![Fig. 4. Effects of forskolin (Forsk) on $V_a$. Initially, both the apical and basolateral compartments of the cells shown in A and B were perfused with NRS. Once stable, a recording of $V_a$ was obtained ($-35$ and $-45$ mV, respectively), the apical compartments were perfused with NRS containing 10 μM forskolin, which resulted in a significant and sustained depolarization. $V_a$ returned to its control value when the apical solution was switched to NRS alone (A) or when amiloride (10 μM) was added into the perfusate (B). In both cases, the membrane depolarization during forskolin perfusion is consistent with the activation of a Na$^+$ conductance in these cells. Results are of typical experiments, which were reproduced at least 23 and 11 times, respectively.](#)

![Fig. 5. Amiloride prevents the forskolin-induced depolarization of H441 $V_a$. Initially, both the apical and basolateral compartments of this H441 monolayer were perfused with NRS. After establishment of a stable $V_a$ (around $-30$ mV), the apical compartment was perfused with NRS containing 10 μM amiloride, which caused an immediate hyperpolarization of $V_a$ to about $-57$ mV. Subsequent perfusion with NRS containing both forskolin and amiloride (10 μM each) resulted in very modest depolarization, which was completely reversed when glibenclamide (100 μM) was added into the solution perfusing the apical compartment. Result of a typical experiment that was reproduced at least 6 times.](#)

![Fig. 6. Sodium ions are necessary for the forskolin-induced depolarization in H441 cells. Both the apical and basolateral compartments of an H441 cell were perfused with solutions in which Na$^+$ ion was substituted with equimolar amounts of N-methyl-D-glucamine (NMDG$^+$, an impermeant cation). When a new stable $V_a$ value was attained, the apical compartment was perfused with a solution containing NMDG-Cl and 10 μM forskolin. This resulted in a very modest $V_a$ depolarization, which was completely reversed when glibenclamide (100 μM) was added into the solution perfusing the apical compartment. Result of a typical experiment that was reproduced at least 5 times.](#)
ENaC and CFTR-type channels without being treated with steroids. It should be kept in mind that the biophysical properties of Na\(^+\)/H\(^+\) ion channels of alveolar epithelial cells in vivo have not been determined: as mentioned above, ATII cells express a variety of cation channels (23), and recent data are consistent with the presence of amiloride-sensitive Na\(^+\)/H\(^+\) influx across isolated alveolar type I cells (14). In other systems, Uyekubo et al. (32) show that forskolin tripled fluid absorption across open-circuited primary cultures of bovine tracheal epithelial cells and that the effect was inhibited by CFTR blockers. Microelectrode studies suggest that the magnitude of the absorptive response to forskolin in bovine cells depends on the size of an inwardly directed electrochemical driving force for Cl\(^-\) movement across the apical membrane. However, this effect was not seen in human tracheal cells, perhaps due to the maximum stimulation of CFTR under control conditions.

There is considerable evidence that increases in cAMP and cAMP-dependent protein kinase A (PKA) increase both the \(P_o\) and the number of channels in alveolar epithelial cells. Addition of terbutaline or PKA into the bath solution of ATII cells patched in the cell-attached and inside-out mode, respectively, doubles the \(P_o\) of a 27-pS nonselective Na\(^+\) channel without affecting its single channel conductance (4.2 pS). Result of a typical experiment that was successfully repeated 13 times.

![Figure 7](image-url)  
**Fig. 7.** Sodium ions are necessary for the forskolin-induced depolarization in H441 cells monolayers. After perfusion with NMDG-Cl, a monolayer was perfused with NRS containing 10 \(\mu\)M forskolin. It resulted in a significant and sustained depolarization of \(V_{a}\). Result of a typical experiment that was reproduced successfully at least 4 other times.

![Figure 8](image-url)  
**Fig. 8.** Chloride ions are not necessary for the forskolin-induced \(V_{a}\) depolarization. Both the apical and basolateral compartments of an H441 cell monolayer were perfused with solutions in which Cl\(^-\) ion was replaced with equimolar concentrations of gluconate (gluc). Once a stable \(V_{a}\) value (−44 mV) was obtained, the apical compartment was perfused with a Na-glucuronate solution containing 10 \(\mu\)M forskolin. This resulted in a transient hyperpolarization, followed by a sustained depolarization. The amplitude of \(V_{a}\) depolarization was similar to that seen when monolayers were perfused with NaCl containing 10 \(\mu\)M forskolin (Fig. 4 and Table 1). Result of a typical experiment that was reproduced 7 times.

![Figure 9](image-url)  
**Fig. 9.** Effects of forskolin on single channel activity of H441 cells. Single channel currents recorded in cell-attached mode from 2 H441 monolayers at a holding potential of −100 mV (\(V_{holding} = V_{apical} - P_{pipette}\)). The pipettes were filled with 145 mM Na\(^+\), and the cell monolayers were perfused with a solution containing 145 mM K\(^+\). **A:** the monolayer was perfused with NRS; **B:** the monolayer was perfused with NRS containing 10 \(\mu\)M forskolin. **C** and **D:** an expanded view of **B** and the associated current amplitude distribution histogram. The histogram was constructed from a 10-min recording. As can be seen, forskolin induced an increase of both the number of the active channels in the patches and their open probability without affecting their unitary conductance (4.2 pS). Result of a typical experiment that was successfully repeated 13 times.
planar bilayers containing a putative immunopurified ATII Na\(^+\) channel protein (31). Berdiev et al. (1) showed that PKA phosphorylates both the 135-kDa and the 70-kDa polypeptides of the immunopurified ATII Na\(^+\) channel protein. Finally, perfusion of A549 cells with forskolin significantly increased the whole-cell amiloride-sensitive Na\(^+\) current and the \(N_P\) of an 8.6-pS Na\(^+\) channel in cell-attached patches (20) where \(N\) refers to the number of channels. These data support the hypothesis that phosphorylation of the Na\(^+\) channel complex (or of cytoskeletal proteins interacting with this complex) is involved in cAMP activation.

On the other hand, there is also significant evidence showing that cAMP may promote insertion of new channel protein from a cytoplasmic pool to the apical membranes. Chen et al. (3) report that exposure of ATII cells to agents that increase cAMP upregulated \(P_N\) of the 20–25-pS nonselective cation channel while it increased the numbers but not the \(P_N\) of a 4-pS ENaC channel. These results are consistent with insertion of new ENaC channels in the apical membrane via cAMP-dependent processes. These observations are also consistent with the findings of Kleyman et al. (15), who report that exposure of A6 cells to increasing intracellular cAMP doubled the amount of ENaC protein in the apical membrane of A6 cells. However, as mentioned previously, our data indicate significant increases in the \(P_N\) of a 4-pS channel in H441 cells. In any event, these data provide strong evidence that an increase in intracellular cAMP activates existing Na\(^+\) channel in the apical membranes by a variety of mechanisms.

Movement of Na\(^+\) ions from the alveolar to the interstitial space necessitates the simultaneous movement of an anion (such as Cl\(^-\)) to preserve the electroneutrality. A number of in vivo studies suggest the movement of Cl\(^-\) ion occurs via transcellular vs. paracellular pathways. Nielsen et al. (27) found that the substitution of Cl\(^-\) ions with methanesulfonate leads to the total block of the basal Na\(^+\)-dependent fluid clearance across rabbit lungs. Under these conditions, forskolin induced Cl\(^-\) secretion instead of Na\(^+\) absorption. However, addition of methanesulfonate into the alveolar space may have depolarized the apical membrane (due to the efflux of Cl\(^-\) ions), decreasing the driving force for Na\(^+\). Fang et al. (4) show that perfusion of mouse lungs in situ with solutions containing 50% of the normal Cl\(^-\) resulted in a significant inhibition of Na\(^+\)–dependent alveolar fluid clearance. Interestingly, \(\Delta F_{508}\) mice, lacking surface expression of CFTR, had normal levels of AFC, indicating that transcellular movement of Cl\(^-\) ions may occur via a variety of Cl\(^-\) channels. On the other hand, stimulation of clearance by isoproterenol upregulated AFC and Cl\(^-\) absorption in wild-type mice but not in \(\Delta F_{508}\). On the basis of these findings, the authors propose that functional CFTR is necessary for the cAMP-induced stimulation of Na\(^+\) transport in vivo. However, recently published findings (12) show that β-adrenergic stimulation increased Cl\(^-\) transport across the alveolar epithelium in 50% of CFTR\((-/-)\) mice, indicating the involvement of complex mechanisms in fluid clearance. In any event, these studies show that transcellular Cl\(^-\) movement through CFTR and other types of Cl\(^-\) channels plays an important role in the vectorial transport of Na\(^+\) ion in vivo; however, they provide absolutely no evidence of membrane hyperpolarization as the critical factor as suggested by Jiang et al. (13).

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**DISCLOSURES**

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