cAMP-stimulated Na\(^+\) transport in H441 distal lung epithelial cells: role of PKA, phosphatidylinositol 3-kinase, and sgk1

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Thomas, Christie P., Jason R. Campbell, Patrick J. Wright, and Russell F. Husted. 2004. 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* 287:L843–L851, 2004. First published June 18, 2004; 10.1152/ajplung.00340.2003.—H441 cells, a bronchiolar epithelial cell line, develop a CAMP-regulated benzamil-sensitive Na\(^+\) transport pathway on permeable supports (Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, Thomas CP. *Am J Physiol Lung Cell Mol Physiol* 282:L631–L641, 2002). To understand the molecular basis for the stimulation of Na\(^+\) transport, we delineated the role of specific intracellular pathways and examined the effect of CAMP on \(\alpha\beta\gamma\)-epithelial Na\(^+\) channel (ENaC) and sgk1 expression. Na\(^+\) transport increases within 5 min of CAMP stimulation and is sustained for \(>24\) h. The sustained effect of CAMP on Na\(^+\) transport is abolished by LY-294002, an inhibitor of phosphatidylinositol 3-kinase, by H89, an inhibitor of PKA, or by SB-202190, an inhibitor of p38 MAP kinase. The sustained effect of CAMP on Na\(^+\) transport was associated with increases in enac mRNA and protein but without a detectable increase in \(\beta\gamma\)-ENaC and sgk1. The early effect of CAMP on Na\(^+\) transport is brefeldin sensitive and is mediated via PKA. These results are consistent with a model where the early effect of CAMP is to increase trafficking of Na\(^+\) channels to the apical cell surface whereas the sustained effect requires the synthesis of \(\alpha\)-ENaC.

епителная содовый канал; глюкокортикоиды; воздушная оболочка; p38 mitogen-activated protein kinase

**AMILORIDE-SENSITIVE** Na\(^+\) transport in distal airway and alveolar epithelia provides the driving force for fluid absorption from lumen to interstitial space. This transport process is important at the time of birth for the transition from the fluid-filled air spaces of the fetus in utero to the air-filled airway and alveolar epithelia that are required for efficient gas exchange soon after birth. At birth and throughout life, airway and alveolar fluid transport is tightly regulated to maintain a low lung liquid volume.

In the mammalian lung, Na\(^+\) transport occurs principally via the hetero-multimeric epithelial Na\(^+\) channel (ENaC) complex. Gene deletion experiments have confirmed that in the absence of the \(\alpha\)-ENaC subunit, mice die soon after birth from fluid filling the airway and alveolar lumen (20). In humans, loss of function mutations in \(\alpha\beta\)- or \(\gamma\)-ENaC results in a nonlethal lung phenotype characterized by excess airway liquid, chronic cough, and a tendency to contract frequent respiratory infections (26). Increased airway and alveolar fluid is also seen in many clinical lung disorders, including noninflammatory pulmonary edema and the inflammatory lung injury seen with infectious pneumonitis (2, 9). In at least some of these disorders, airway and alveolar fluid clearance is diminished and therapies that stimulate airway or alveolar Na\(^+\) and fluid transport may reduce the extent of initial injury or facilitate recovery from such injury (19, 60). For example, \(\beta\)-adrenergic agonists increase the rate of airway and alveolar fluid transport in hyperoxic and ventilator-associated lung injury models and in a genetic model of pulmonary edema (28, 44, 52). \(\beta\)-Adrenergic agonists also appear to prevent high-altitude pulmonary edema in susceptible human subjects (53).

Two of the more important physiological regulators of Na\(^+\) transport in the lung are glucocorticoids and catecholamines (9, 36). Glucocorticoids increase transcription and translation of the \(\alpha\)-ENaC subunit and that of a regulatory kinase, sgk1 (23, 55). In turn, sgk1 enhances Na\(^+\) transport by increasing the number of functional Na\(^+\) channels at the cell surface (12, 65). Catecholamines are thought to act via their second messenger, cAMP, because their effects can be reproduced by cAMP analogs (18, 43, 54, 66). The mechanism of the cAMP effect on distal lung epithelial Na\(^+\) transport is less well understood but may include short-term posttranslational effects and long-term transcriptional effects (30, 36, 69).

In this study, we investigate the effects of cAMP on Na\(^+\) transport in a well-characterized human airway epithelial cell line, H441. Our results show that cAMP stimulates an immediate increase in benzamil-sensitive Na\(^+\) current that is brefeldin sensitive and is mediated via PKA. We also show that the sustained stimulation of Na\(^+\) transport involves the activation of PKA, phosphatidylinositol 3-kinase (PI3-kinase), and p38 MAP kinase and is associated with upregulation of \(\alpha\)-ENaC mRNA and protein.

**METHODS**

Materials. Dexamethasone, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). Benzamil was from Research Biochemical International (Natick, MA), LY-294002, H89, SB-202190, U0126, rapamycin, brefeldin, and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were from EMD Biosciences (San Diego, CA), and [\(\alpha\)-32P]UTP was from NEN Life Science Products (Boston, MA). Cultured media were obtained from Life Technologies (Gaithersburg, MD). anti-sgk1 antibody was from Upstate Biotechnology (Charlottesville, VA), the anti-phospho sgk1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), the goat anti-rabbit secondary antibody was from Cell Signaling Technology (Beverly, MA), and the \(\alpha\)-ENaC antibody was a gift from Mark Knepper (NIH, Bethesda, MD).

**Measurement of short-circuit current.** To measure the short-circuit current (Isc), H441 cells were grown for 4–7 days in RPMI 1640 with...
Table 1. Long-term response in H441 monolayers to cAMP and glucocorticoid stimulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Baseline</th>
<th>24-h Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_T$</td>
<td>Total $I_c$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>311±52</td>
<td>3.03±0.56</td>
</tr>
<tr>
<td>Forskolin + IBMX</td>
<td>354±72</td>
<td>3.17±0.36</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>308±71</td>
<td>2.36±0.36</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 experiments per treatment group. $R_T$, transepithelial resistance; $I_{sc}$, short-circuit current; Benz, benzamid; IBMX, 3-isobutyl-1-methylxanthine. *P < 0.005 compared with corresponding baseline $I_{sc}$; †P < 0.005 compared with benzamid-sensitive $I_{sc}$ in vehicle-treated filters.

Table 2. Short-term response in H441 monolayers to cAMP stimulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reading at 15 min</th>
<th>Reading at 45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_T$</td>
<td>Total $I_c$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>667±47</td>
<td>8.44±1.07</td>
</tr>
<tr>
<td>Forskolin + IBMX</td>
<td>663±77</td>
<td>6.79±0.83</td>
</tr>
</tbody>
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Values are means ± SE; n = 11 experiments per treatment group. Baseline reading taken at 15 min and final post-cAMP reading at 45 min. *P < 0.005 compared with corresponding $I_{sc}$ at 15 min; †P < 0.05 compared with corresponding $I_{sc}$ at 15 min; ‡P < 0.005 compared with benz-sensitive $I_{sc}$ in vehicle-treated filters.
stimulate $I_{sc}$. To confirm that the effect of forskolin in H441 cells was primarily on Na$^+$ transport, we examined the effect of NPPB, a potent Cl$^-$ channel blocker. NPPB had a small effect on basal $I_{sc}$ but had no effect on forskolin-stimulated total or benzamil-sensitive $I_{sc}$ after long-term stimulation (Fig. 1C). We then examined the effect of NPPB on the short-term effect of forskolin and IBMX. NPPB appeared to delay the rise in $I_{sc}$ seen but did not diminish the amplitude of current. In both instances, the $I_{sc}$ was completely inhibited by benzamil, which was added at the end of the experiment (Fig. 1C). These results indicate that H441 cells, in contrast to many other airway epithelia, do not appear to have a significant cAMP-stimulated Cl$^-$ current.

We then began to determine the intracellular pathways involved in cAMP-regulated Na$^+$ transport. It is known that cAMP can stimulate several separate pathways in cells or tissues to accomplish various cellular functions, including activation of PKA, and the stimulation of PI3-kinase (11, 16, 40, 51). We tested the role of PKA by using H89, a specific PKA inhibitor and the role of PI3-kinase by using LY-294002, a specific PI3-kinase inhibitor. Both H89 and LY-294002 reduced $I_{sc}$ stimulated by forskolin and IBMX (Fig. 2A). PI3-kinase inhibition also reduced basal $I_{sc}$ and $R_T$ significantly. These results indicate that PI3-kinase is an important modulator of basal current. In addition, inhibiting PI3-kinase activity decreases the transport response to cAMP. The cAMP-stimulated Na$^+$ transport is also inhibited by H89, indicating that the cAMP effect occurs via activation of PKA.

We also tested the effect of H89 and LY-294002 on dexamethasone-stimulated $I_{sc}$. LY-294002 but not H89 reduced basal and dexamethasone-stimulated $I_{sc}$, though both agents reduced $R_T$ (Fig. 2B). Inhibition of PI3-kinase activity reduces the dexamethasone response similar to that seen with elevation of cAMP. In contrast to the cAMP effect, glucocorticoid-stimulated Na$^+$ transport is not inhibitable by H89. Interestingly, although H89 reduces $R_T$ this is not associated with an inhibition of glucocorticoid-responsive $I_{sc}$, indicating that a primary reduction in $R_T$ of that magnitude is not sufficient to reduce $I_{sc}$.

Elevated cAMP levels can also lead to activation of p70 S6 kinase and the regulation of guanine nucleotide exchange factors (GEFs) (16, 48, 51). cAMP-regulated GEFs, in turn,
activate small-molecular-weight GTP-binding proteins, such as Rap1 and Ras, with downstream effects on MAP kinases (25, 63). Activation of PKA and PI3-kinase can also in some instances stimulate MAP kinase activity in a Ras-dependent or -independent fashion (32). We, therefore, examined the effect of the MEK inhibitor U0126, the p38 MAP kinase inhibitor SB-202190, and the p70 S6 kinase inhibitor rapamycin on \( I_{sc} \) in H441 cells. Whereas SB-202190 inhibited cAMP-stimulated \( I_{sc} \) in H441 cells. Whereas SB-202190 inhibited cAMP-stimulated \( I_{sc} \) in H441 cells. However, U0126 and rapamycin had no effect on \( I_{sc} \) (Fig. 3). These results demonstrate that cAMP completely blocks the rise in \( I_{sc} \) seen with cAMP, whereas the effect of LY is more limited (Fig. 7A).

Elevation of cAMP could increase \( \text{Na}^+ \) transport within a few minutes by one of at least three nonexclusive mechanisms. These include increased insertion of channels at the apical membrane, increased retention of channels at the apical membrane, or an alteration in the open probability of channels resident at the cell surface. We tested the effect of brefeldin A, which blocks transllocation of proteins from the endoplasmic reticulum to the Golgi complex and thus prevents newly synthesized channels from reaching the cell surface. Brefeldin A abolished the increase in \( I_{sc} \) seen with cAMP (Fig. 7B), indicating that the cAMP effect is mediated, at least in part, via cAMP for varying periods and \( \alpha\beta\gamma\)-ENaC and sgk1 mRNA, and sgk1 mRNA (Fig. 4), though dexamethasone increased all three transcripts as we have previously described (22). cAMP increased \( \alpha\)-ENaC mRNA expression though this effect was only seen at 24 h and not at earlier time points (Fig. 5). The effect of cAMP was additive to the effect of dexamethasone, suggesting that these agents act through distinct pathways to increase \( \alpha\)-ENaC expression (Fig. 5).

To determine whether the increase in \( \alpha\)-ENaC mRNA was associated with a corresponding increase in protein we measured \( \alpha\)-ENaC protein in H441 cells. Treatment with forskolin + IBMX increased \( \alpha\)-ENaC protein levels at 24 and 48 h (Fig. 6A). We also examined the effect of cAMP on \( \alpha\)-ENaC protein though dexamethasone increased sgk1 protein (Fig. 6B and C).

We then examined the early effect of forskolin + IBMX on \( \text{Na}^+ \) transport in H441 cells. As we have previously reported, within 5 min of cAMP stimulation, \( I_{sc} \) begins to increase and peaks by 20–25 min (Fig. 7A). At peak stimulation, we used benzenil, to demonstrate that almost all of the \( I_{sc} \) that is seen with and without cAMP stimulation is inhibitable by benzamil. Our results show that after short-term exposure, cAMP does not stimulate a Cl− current in these cells. We then examined the effect of LY-294002 and H89 on short-term \( I_{sc} \). Our data demonstrates that H89 completely blocks the rise in \( I_{sc} \) seen with cAMP, whereas the effect of LY is more limited (Fig. 7A).

Elevation of cAMP could increase \( \text{Na}^+ \) transport within a few minutes by one of at least three nonexclusive mechanisms. These include increased insertion of channels at the apical membrane, increased retention of channels at the apical membrane, or an alteration in the open probability of channels resident at the cell surface. We tested the effect of brefeldin A, which blocks transllocation of proteins from the endoplasmic reticulum to the Golgi complex and thus prevents newly synthesized channels from reaching the cell surface. Brefeldin A abolished the increase in \( I_{sc} \) seen with cAMP (Fig. 7B), indicating that the cAMP effect is mediated, at least in part, via
an increased delivery of transport proteins to the apical membrane. We also tested the effect of SB-202190, a p38 MAP kinase inhibitor that reduced long-term cAMP stimulation, and showed that SB-202190 did not alter the short-term effects of forskolin + IBMX. Collectively, these data demonstrate that the immediate and the sustained effects of cAMP on Na\(^+\) transport occur via distinct pathways.

The short-term effects of cAMP on Na\(^+\) transport are likely to be secondary to posttranslational effects on the Na\(^+\) channel. Because cAMP is known to phosphorylate sgk1 in other cell systems and because sgk1 can stimulate ENaC-mediated Na\(^+\) transport, we asked whether cAMP would induce the phosphorylation of sgk1 in H441 cells. A p-sgk1-specific antibody detected increasing p-sgk1 in short-term cAMP-stimulated H441 cells with a robust effect seen by 60 min (Fig. 8). We were unable to reliably detect p-sgk at 5 min, the time point at which forskolin-stimulated \(I_{\text{sc}}\) begins to increase. These results are, however, consistent with a model where the early effects of cAMP on Na\(^+\) transport may be mediated via activation of sgk1.

**DISCUSSION**

Active sodium transport in the distal lung is the driving force for iso-osmolar fluid clearance in the distal lung. cAMP is an important second messenger that stimulates Na\(^+\) transport in the lung and is thought to mediate the actions of hormones, like catecholamines and arginine vasopressin in fetal and adult lung epithelia (35, 36). cAMP also stimulates Cl\(^-\) secretion in lung epithelia in vitro and in vivo, especially in the fetal lung, where there is net secretion of fluid (37, 38, 46, 59, 66).

The H441 cell line, established from a papillary adenocarcinoma of the lung, has morphological and phenotypic characteristics of human bronchiolar epithelium. These cells express surfactant proteins and the Clara cell CC-10 protein and have highly selective low-conductance sodium channels with
the biophysical properties of the αβγ-ENaC multimer (22, 45).
Glucocorticoids stimulate benzamil-sensitive Na⁺ transport in these cells associated with the induction of αβγ-ENaC and sgk1. We (22) and others (29) have recently reported that cAMP stimulates Na⁺ transport in these cells.

In this study, we explore the mechanism of the cAMP effect in some detail. We show that after 24 h of exposure to forskolin + IBMX there is a substantial increase in benzamil-sensitive $I_{sc}$, indicative of Na⁺ transport and that this increase occurs in the absence of an increase in the benzamil-resistant

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**Fig. 6.** Effect of cAMP on α-ENaC and sgk1 protein expression. A: H441 cells were treated with F+I or vehicle for various time periods, and cell lysates (40 μg) resolved by PAGE, then α-ENaC protein levels were detected by immunoblotting. There is an increase in α-ENaC protein at 24 and 48 h. B: H441 cells were treated with F+I for various time periods, and cell lysates (400 μg) were resolved by PAGE, then total sgk1 protein levels were detected by immunoblotting. As a positive control, COS-7 lysates overexpressing epitope-tagged sgk1 were run in lane 1. There is no increase in sgk1 with cAMP stimulation. C: dex (100 nM) for 24 h increased sgk1 protein expression in H441 cells. As a positive control, COS-7 lysates overexpressing epitope-tagged sgk1 were run in lane 1.

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**Fig. 7.** Effect of kinase inhibitors and brefeldin A on cAMP-stimulated early Na⁺ transport. Kinase inhibitors and brefeldin A were added to monolayers 60 min prior to mounting filters in Ussing chambers. $I_{sc}$ was measured every 5 min, and at 15 min forskolin + IBMX (cAMP) or vehicle was added, and measurements were continued for 30 min. The $I_{sc}$ for each condition was plotted relative to the value at 15 min, which was arbitrarily set to unity. A: cAMP stimulates $I_{sc}$ within 5 min, which peaks within 30 min. H89 (10 μM) but not LY-294002 (LY; 50 μM) inhibits cAMP-stimulated $I_{sc}$ in H441 cells. Benzamil almost completely abolishes basal and stimulated $I_{sc}$ indicating that Na⁺ transport accounts for virtually all of the $I_{sc}$. B: brefeldin A (1 μg/ml) but not SB-202190 (10 μM) inhibits cAMP-stimulated $I_{sc}$ in H441 cells.
show that the effect of cAMP and glucocorticoids on α-ENaC expression are additive. This, together with the discordant effect of H89 on long-term cAMP- and glucocorticoid-regulated Na\(^+\) transport, indicates that these agents activate parallel and distinct pathways.

As has been reported by others in alveolar and airway epithelia, we noted a rapid effect of forskolin + IBMX on \(I_{sc}\) in H441 cells. These early effects are also not associated with an increase in Cl\(^-\) current. The early effect of cAMP on Na\(^+\) transport is mediated via PKA, is sensitive to brefeldin, and is associated with phosphorylation of sgk1. In recently published studies terbutaline was shown to increase the abundance of βγ-ENaC protein at the cell surface of rat alveolar type II cells exposed to hypoxia (49). In other studies, cAMP was shown to increase the number of highly selective Na\(^+\) channels in rat alveolar type II cells and in H441 cells by patch-clamp analysis performed in cell-attached mode (7, 29). On expressing ENaC subunits in Fisher rat thyroid and Madin Darby canine kidney cells or in Xenopus oocytes, cAMP stimulates Na\(^+\) transport by increasing cell surface expression of ENaC (15, 41, 57). Our findings with brefeldin A are thus consistent with a model where cAMP stimulates the translocation of preexisting ENaC subunits to the cell surface.

Sgk1 is fairly widely expressed in mammalian tissues with the highest levels seen in the ovary, thymus, and lung (68). In situ hybridization studies in human lung confirm that sgk1 is expressed in alveolar type II cells and in bronchiolar epithelium (31, 65). Sgk1 significantly enhances Na\(^+\) current when coexpressed with αβγ-ENaC mRNA in Xenopus oocytes and in Fisher rat thyroid epithelia (6, 42, 58, 64, 65). This effect of sgk1 is achieved by increasing the number of ENaC channels assembled at the cell surface (12, 65). The effect of sgk1 may not be limited to trafficking of ENaC subunits. Recent data (5, 14, 56, 70, 71) suggest that sgk1 may also increase the cell surface expression of a number of ion transport proteins, including Na\(^+\)-K\(^+\)-ATPase, the K\(^+\) channels Kir1.1 and KCNE, the sodium channel SCN5A, and the sodium proton exchanger NHE3. Because cAMP can increase the activity of the basolateral Na\(^+\)-K\(^+\)-ATPase, our findings are also consistent with a primary effect of cAMP to increase Na\(^+\) pump number or activity in H441 cells (3).

How might cAMP affect activation and function of sgk1? When heterologously expressed in COS-7 cells, cAMP analogs activate sgk1 by phosphorylation of Thr369 and Ser422 in a PKA- and PI3-kinase-dependent process (47). In ovarian granulosa cells, cAMP activation leads to phosphorylation of sgk1, in a complex process that appears to involve PKA, PI3-kinase, and p38 MAP kinase (17). In H441 cells we demonstrate that cAMP-stimulated Na\(^+\) transport is associated with phosphorylation of sgk1. Thus cAMP-mediated activation of sgk1 in airway epithelia could result in enhanced trafficking of ENaC to the cell surface and increased Na\(^+\) transport within a few minutes of stimulation. We were, however, unable to detect phosphorylation of sgk before 15 min and preceding the increase in Na\(^+\) transport. On the basis of these data, we cannot conclude that activation of sgk is required for the cAMP effect on Na\(^+\) transport. The sustained effect of cAMP, on the other hand, is associated with the synthesis of new αENaC subunits. These results are reminiscent of the effect of aldosterone on Na\(^+\) transport, where an increase in sgk1 in seen first, which

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I_{sc}, \text{ suggesting that there is very little if any cAMP-stimulable Cl}^-\text{ current. We confirmed the lack of Cl}^-\text{ current by demonstrating that the forskolin-stimulated response was largely preserved in the presence of NPPB. These results are in agreement with a recent report that forskolin increased the number and open probability of ENaC-type channels in H441 cells (29). The reported increase in Na}^+\text{ transport in that study was not associated with Cl}^-\text{ influx or hyperpolarization of the membrane. H441 cells have been shown to express CFTR mRNA and protein, though functional activity has not been previously reported (27). Our results suggest that the little benzamil-resistant \(I_{sc}\) seen in H441 cells may be secondary to a CFTR or non-CFTR Cl\(^-\) channel.}

Earlier studies in airway cells had indicated that cAMP-stimulated Na\(^+\) transport may be secondary to the activation of an apical Cl\(^-\) channel, which hyperpolarizes the cell and provides the driving force for Na\(^+\) movement (24). However, cytosolic cAMP can increase the activity of Na\(^+\) channel in patch-clamp experiments, demonstrating an effect independent of Cl\(^-\) entry (30, 69). The increase in cAMP-mediated Na\(^+\) transport can also be seen when Cl\(^-\) entry is blocked by glibenclamide in fetal rat distal lung epithelia and is not seen when Na\(^+\) is substituted with N-methyl-D-glucamine (8, 29). Our studies thus support the conclusion that cAMP stimulation of Na\(^+\) entry does not require sustained Cl\(^-\) transport.

The long-term effect of cAMP and dexamethasone on Na\(^+\) transport is substantially reduced by LY-294002, whereas H89 abolishes cAMP-stimulated, but has no effect on dexamethasone-stimulated, current. The importance of the PI3-kinase pathway to Na\(^+\) transport has previously been reported in A6 cells, an amphibian cell line that is used as a model of the renal collecting duct. In these cells, LY-294002 inhibits insulin, arginine vasopressin, and aldosterone-stimulated Na\(^+\) transport (4, 13, 50, 67). Our data indicate that the PI3-kinase pathway is required for regulated basal and cAMP-stimulated Na\(^+\) transport in human lung epithelia as well. We noted that PKA and p38 MAP kinase activation is also important for the sustained cAMP response.

The sustained effect of cAMP on Na\(^+\) transport is associated with an increase in the steady-state levels of α-ENaC mRNA and protein with no effect on other ENaC subunits or on sgk. cAMP has also been shown to stimulate α-ENaC mRNA expression in adult rat alveolar epithelial cells (10). In these cells, the stimulation by cAMP was abolished by actinomycin D, an effect that we have also seen (data not shown), suggesting that cAMP stimulates transcription of the α-ENaC gene. However, we and others have not been able to demonstrate a functional cAMP-responsive cis-element in the proximal 5' flanking region of α-ENaC gene (10). This may indicate that the regulatory elements required for cAMP stimulation are elsewhere in the gene or flanking regions. In our studies, we
increases surface expression of preexisting channels, followed by an increase in transcription of ENaC subunits (33).

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