Differentiation of Epithelial Na\(^{+}\) Channel Function

AN IN VITRO MODEL

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Confluent monolayers of epithelial cells grown on nonporous support form fluid-filled hemicysts called domes, which reflect active ion transport across the epithelium. Clara-like H441 lung adenocarcinoma cells grown on glass supports and exposed to 50 nM dexamethasone developed domes in a time-dependent fashion. Uplifting of small groups of cells occurred within 6–12 h, well formed domes appeared between 24 and 48 h, and after 7 days, individual domes started to merge. Cells inside of domes compared with those outside domes, or with monolayers not exposed to dexamethasone, differed by higher surfactant production, an increased cytotkeratin expression, and the localization of claudin-4 proteins to the plasma membrane. In patch clamp studies, amiloride-blockable sodium currents were detected exclusively in cells inside domes, whereas in cells outside of domes, sodium crossed the membrane through La\(^{3+}\)-sensitive nonspecific cation channels. Cells grown on permeable support without dexamethasone expressed amiloride-sensitive currents only after tight electrical coupling was achieved (transepithelial electrical resistance \((R_{\text{e}})\) > 1 kilohm). In real-time quantitative PCR experiments, the addition of dexamethasone increased the content of claudin-4, occludin, and Na\(^{+}\) channel \(\gamma\)-subunit (\(\gamma\)-ENaC) mRNAs by 1.34-, 1.32-, and 1.80-fold, respectively, after 1 h and was followed by an increase at 6 h in the content of mRNA of \(\alpha\)- and \(\beta\)-ENaC and of \(\alpha\)-1 and \(\beta\)-1 Na,K-ATPase. In the absence of dexamethasone, neither change in gene expression nor cell uplifting was observed. Our data suggest that during epithelial differentiation, coordinated expression of tight junction proteins precedes the development of vectorial transport of sodium, which in turn leads to the fluid accumulation in basolateral spaces that is responsible for dome formation.

Epithelial tissues typically transport ions and water between two compartments. After forming confluent monolayers when grown in vitro, several ion transporting epithelia form fluid-filled hemicysts, or domes (1–3). These domes appear in small areas where cells detach from the underlying glass or plastic surface upon which the cells are plated. Presumably, the apical to basolateral transport of fluid increases the fluid volume underneath the monolayer causing the appearance of domes. This in vitro culture system can be exploited uniquely to study the development of epithelial polarity, junctional formation, transport function, and cell-substrate interactions that are required for fluid accumulation between the monolayer and the underlying surface. The cells found in the domes are polarized, contain markers of epithelial differentiation, including tight junctions and gap junctional intercellular communications, and stain positively for cytokeratins (2–4). Whether morphological differentiation of cells forming domes correlates with the functional maturation of transepithelial ion transport is not known.

The Clara-like H441 lung papillary adenocarcinoma cell line has been used as a glucocorticoid- and cAMP-regulated cellular model of pulmonary epithelial sodium absorption (5–10). These cells produce the Clara cell 10-kDa protein (CC-10) and the surfactant proteins A, B, and D and have ultrastructural characteristics of nonciliated bronchiolar cells (11).

In the present study, we took advantage of the fact that Clara cells form domes in response to dexamethasone (DEX)\(^1\) in a time-dependent fashion, allowing us to compare cells within and outside of domes with regard to cell coupling, Na\(^{+}\)/K\(^{+}\)/ATPase expression, and sodium transport function.

Cells outside of domes, i.e. the cells in the monolayer that remain in contact with the glass surface, express fewer markers of differentiated Clara cells and amiloride-insensitive sodium entry pathways. Highly differentiated dome cells are tightly coupled, Na\(^{+}\) entry is more than twice as high relative to cells outside of domes. This high rate of sodium entry coupled with increased Na\(^{+}\)/K\(^{+}\)/ATPase results in fluid accumulation, whereas the development of tight junctions decreases the transepithelial water permeability that leads to accumulation of fluid and formation of domes. Thus, morphological differentiation during dome formation is coordinated with functional differentiation of vectorial sodium transport.

MATERIALS AND METHODS

Cell Culture—H441 cells were purchased from American Tissue Culture Collection and grown in culture flasks in RPMI 1640 medium (Invitrogen) adjusted to contain 4.5 g/liter glucose and supplemented with 1% penicillin/streptomycin and 5% fetal bovine serum (of South American origin, Bio-Greiner, Belgium). No hormone supplementation was used. The data reported were obtained on cells from passages 58–75. Upon reaching confluence, cells were removed from the flask with trypsin-EDTA and subcultured at a 1:5 ratio. For the experiments, cells were plated either onto glass coverslips (15 mm diameter, Menzel glass, VWR International, Leuven, Belgium) or onto clear polyester Snapwell filters (12 mm diameter, Transwell) at 30% confluence. Unattached cells were removed 24 h later by replacing the medium. At this point differentiating agents were added, and cells were cultured for

\(^1\) The abbreviations used are: DEX, dexamethasone; ENaC, epithelial Na\(^{+}\) channel; RTQ-PCR, real-time quantitative PCR; p\(\text{F}\), picofarads; CNG, cyclic nucleotide-gated; NSC, nonspecific cation.

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Specimens were covered with Aquatex (Merck) and cover-slipped. The reddish water-insoluble product. After the final wash with TBS, the slides were further incubated for 30 min at room temperature for 1 h at a dilution of 1/50, and slides were rinsed for 5 min in TBS. The slides were further incubated for 30 min at room temperature with a secondary antibody (rabbit anti-mouse IgG) and then washed for 5 min in TBS. Cytokeratins and claudin-4 were visualized using the immune complex of alkaline phosphatase-anti-alkaline phosphatase (APAAP Kit System, Dako) and aminoethylcarbazole as the chromogenic reaction (15). In all experiments, 70% compensation of series resistance (15) was used. Whole-cell patches were perfused with bathing medium at a rate of 0.5 ml/min containing (in mM): NaCl 140, KCl 10, CaCl2 1, MgCl2 1, HEPES 10, pH 7.4, adjusted with KOH to 144 mM. Stock solution of nystatin (40 mg/ml in Me2SO) was applied every 15 s. The voltage wave modified by the analysis equation (14). Whole-cell patch clamp configuration induced by nystatin was assessed by evaluating liquid junction potentials were calculated using the conventional procedure (14). Liquid junction potentials were calculated using Microsoft Excel macros based on the generalized Henderson equation (14). Whole-cell patch clamp configuration induced by nystatin permeabilization was achieved after 15–20 min at room temperature. This perforated patch configuration prevents cell dialysis of high molecular weight substances and keeps intracellular Ca2+ ion concentration intact (15). In all experiments, 70% compensation of series resistance (Rc) was used. Whole-cell patches were perfused with bathing solution containing heptanol (1 mM) in order to uncouple cells by blocking intercellular gap-junctional communications. Cells were used within 60 min after being taken from the incubator. The holding potential was -50 mV. Voltage ramp protocols consisted of a -100 mV step following from -100 mV to +100 mV over 1 s were applied every 15 s. The voltage wave modified by the Rc compensation circuit was recorded simultaneously. The stability of the patches was assessed by evaluating R0 (<30 meghs) at the beginning and the end of each experiment, and a >10% difference led us to discard the record from the analysis.

**Real-time Quantitative Polymerase Chain Reaction (RTQ-PCR)** - ENaC (a, b, c), claudin-4, occludin, zona occludens 1, and NaK-ATPase (a1, b1) mRNA levels were measured by Sybr® Green RTQ-PCR. Confluent cell cultures, developed 3–4 days after seeding on 1.77 cm2 coverslips, were used to study the effect of DEX on gene expression. Total RNA was extracted from cells using TRIzol® (Invitrogen) as outlined by the manufacturer. Reverse transcription-PCR and RTQ-PCR were performed as described previously (16). To ensure the quality of the measurements, both negative and positive controls were systematically included in duplicate in each plate. The analysis of the RTQ-PCR was done using the ΔCt cycle threshold method (Ct of the gene of interest minus Ct of the reporter gene). Relative gene expression was obtained by using the ΔCt methods (using the time 0 of each culture condition as a calibrator for comparison). The conversion between ΔCt, and relative gene expression levels is: -fold induction = 2 ΔΔCt. (16) Hypoxantine phosphoribosetransferase (HPRT) gene was used as the housekeeping gene. Primers used were as followed: HPRT forward (5′-TCAGCCGAGGATTGTCGG-3′) and reverse (5′-GCCAGGAAGTTGATCGG-3′) (slope: -3.266, L36593); ENaCβ forward (5′-GGCCCTGAAGTTGATCGG-3′) and reverse (5′-CGTGGGAGAATAGTGTCG-3′) (slope: -2.914, L36592); claudin-4 forward (5′-TACCACTCTGGCAGGGATT-3′) and reverse (5′-TCCACGCTGGGTTTCTAGC-3′) (slope: -3.156, BC000671); occludin forward (5′-CCTTAAATACCCGCGGATC-3′) and reverse (5′-CAAAGTTACACCCGCTG-3′) (slope: -3.256, NM_002538); zona occludens forward (5′-ATGAGAAAGATTCCTCATTAGGAC-3′) and reverse (5′-ATAGACGAGAGACCGGTG-3′) (slope: -3.056, NM_002357); NaK-ATPase β1 forward (5′-GCGGGAGGATGACCATGAC-3′) and reverse (5′-CAGACCTTTCCGCTTCG-3′) (slope: -3.358, BC000006).

**RESULTS**

**Cell Morphology** — Monolayers of H441 cells never formed epithelial domes spontaneously (Fig. 1A). The addition of DEX (50 nm) from day 2 after cell seeding promoted the development of numerous hemicysts, which developed from either a single cell (microdome) or from several cells. In phase contrast microscopy, cells forming these fluid-filled blisters presented cytokeratin-8 staining pattern in H441 monolayers. The addition of DEX (50 nm) did not stimulate dome formation, consistent with the view that lung ion transport is not regulated by mineraloestrogens.

**Uptake of Fluorescent Dye** — We next studied uptake of quinacrine by the cultured cells. Quinacrine, an anti-protozoa drug, is a specific marker of lamellar bodies in type II pneumocytes (12, 13). Because of its weak base properties, quinacrine accumulates in lamellar bodies, which have a slightly acidic pH, and becomes fluorescent in their hydrophobic environment. As shown in Fig. 1D, areas of higher fluorescence intensity coincided with dome regions. Although cells from nonstimulated monolayers (Fig. 1B) and cells in the proximity of domes (Fig. 1D) were also fluorescent, this fluorescence was weak and diffuse and lacked the dot-like pattern. Thus, H441 cells forming domes have more surfactant-containing bodies than cells outside the domes.

**Immunocytochemistry** — To assess the degree of cell differentiation within the domes, we performed immunocytochemistry of known epithelia-type markers, including cytokeratin-8 and claudin-4, a specific tight junction protein. Fig. 2A and B, shows the cytokeratin-8 staining pattern in H441 monolayers.

**Maturation of Sodium Transport Needed for Dome Formation**
Consistent with the observation of increased surfactant production, cells inside the domes expressed more cytokeratin (Fig. 2B). We also observed scattered single cells with increased cytokeratin staining, which probably represent microdomes. Cells surrounding domes expressed a similar amount of cytokeratin-8 as cells from nonstimulated monolayers (Fig. 2A).

The development of tight junctions allows functional cell-cell interactions in differentiated cells. Claudin-4 was stained with specific antibodies in nonstimulated and DEX-stimulated H441 monolayers (Fig. 2, C and D). In addition to increased intracellular content of claudin-4 in stimulated cell monolayers, cells within domes expressed claudin-4 in their plasma membrane (Fig. 2D, arrows).

Electrophysiological Characterization of Cells Grown on Nonporous Supports—H441 monolayers cultured in the presence of DEX for 7–10 days were used for ion transport measurements. Currents were measured using the patch clamp technique in the nystatin-perforated whole-cell configuration.

Total inward sodium currents were estimated by lowering the sodium ion concentration in the bath to 10 mM by replacement with N-methyl-D-glucamine (+), thus reducing the Na\(^+\) gradient across the apical cell membrane. Perfusion this with low sodium solution decreased inward Na\(^+\) current within a few seconds and induced cell hyperpolarization as evidenced from the shift in the $I/V$ curve reversal potential (Fig. 3, A, C, and E). Cells located outside of domes did not express significant amiloride-sensitive currents (amiloride(−) cells, Fig. 3, A and B). By contrast, within the domes, cells exhibited inward sodium currents that were either partially inhibited by amiloride (Fig. 3, C and D, amiloride(+/−) cells) or completely inhibited by amiloride (Fig. 3, E and F, amiloride(+) cells). Mean whole-cell current values were calculated by averaging the voltage ramp $I/V$ curve difference between control and either low sodium or normal sodium in the presence of amiloride in the interval of −95 to −90 mV, where the driving force for sodium entry is maximal. These data are summarized in Fig. 4.
In the presence of amiloride (+) cells, a significant fraction of the inward sodium current was amiloride-insensitive (p = 0.002, paired t test). This cell population was slightly larger in size, as evidenced from the values of cell capacitance (55.1 ± 6.5 pF as compared with 34.5 ± 5.6 pF and 31.9 ± 6.2 pF in amiloride(−) and amiloride(+) cells, respectively; p = 0.045 unpaired t test). A significantly greater amiloride-sensitive current was observed in amiloride(+/−) cells compared with amiloride(−) cells (−0.66 ± 0.21 pA/pF versus −0.04 ± 0.02 pA/pF; p = 0.002 unpaired t test). In amiloride(+) cells, amiloride-sensitive currents were even larger compared with amiloride(+/−) cells (−1.92 ± 0.50 pA/pF versus −0.66 ± 0.21 pA/pF; p = 0.048 unpaired t test). Assuming a membrane capacitance of 1 pF/µm², we calculated the Na⁺ flux via nonspecific channels in amiloride(−) cells to be 0.47 pmol/min/µm² and via ENaC in amiloride(+) cells to be 1.22 pmol/min/µm², i.e., 2.6-fold higher.

The reversal potentials of amiloride-sensitive currents were +52.3 ± 4.2 and 65.4 ± 5.1 mV for amiloride(+/−) and amiloride(+) cells, respectively. These values are close to the equilibrium sodium potential under the ionic conditions used (E_{Na}^0 = +68 mV). This observation indicates high Na/K selectivity of channels expressed in cells located within domes. The low magnitude of amiloride-sensitive currents in amiloride(−) cells precluded a correct determination of the reversal potential.

Monolayers that were not exposed to DEX expressed conductance properties similar to amiloride(−) cells (data not shown). Inward sodium currents in amiloride(−) cells from DEX-treated cell monolayers were characterized using several ion transport inhibitors. We tested for the possible contribution to the amiloride-insensitive sodium current of several transport proteins expressed in the apical membranes of airway epithelial cells, including Na/Ca-exchanger, sodium-glucose and sodium-phosphate cotransporters, as well as cyclic nucleotide-gated (CNG) channels and nonspecific cation (NSC) channels. The pharmacological profile of these currents is shown in Fig. 5. There was no difference in inhibition in the presence of 10 µM and 1 mM amiloride, which indicated a lack of Na/Ca-exchanger activity. Likewise, phlorizin (50 µM), an inhibitor of sodium-glucose cotransporters, and phosphonoformic acid (1 mM), an inhibitor of "slippage" electrogenic currents through sodium-phosphate cotransporters, had no effect. Although Mg²⁺ (10 mM) did not inhibit sodium currents, other known blockers of the CNG channels, pimozone (10 µM) and Zn²⁺ (100 µM), reversibly inhibited 8.5% and 22.7% of inward sodium currents, respectively. By contrast, more than 80% of these currents were sensitive to 1 mM lanthanum, an inhibitor of NSC channels. This inhibition was fast and reversible. Inhibited currents had a reversal potential of zero millivolts, consistent with the activity of nonspecific cation channels in these amiloride(−) cells.

**Cell Morphology and Abundance of Gene Transcripts during Dome Formation**—To gain further insight into the mechanism of dome formation, we studied the effect of DEX on the appear-
Maturation of Sodium Transport Needed for Dome Formation

Fig. 5. Pharmacological profile of sodium currents seen in amiloride(-) cells. Values express the percentage of current inhibited normalized to the value of inhibition obtained with the low sodium medium (100%).

Fig. 6. Cell monolayer morphology during dome formation. Nomarsky pictures were taken at the times indicated. Monolayers received either the vehicle Me2SO (A–D) or 50 nM DEX (E–H). Arrows indicate areas of uplifted cells. In H, the dome occupies most of the field.

ance of epithelial uplifting and on the time course of coordinated expression of selected genes, including α-, β-, and γ-subunits of ENaC, α1 and β1 subunits of Na,K-ATPase, claudin-4, occludin, and zona occludens1. As shown in Fig. 6, uplifting of small groups of cells started between 6 and 12 h after DEX treatment, and well formed domes were observed after 24 h. After 7 days in the presence of DEX individual domes started to merge. When cells were exposed to amiloride, domes collapsed overnight. The results of RTQ-PCR, consistent with previous studies (6, 18–21), are presented in Table I. We observed substantial up-regulation of mRNA content of claudin-4, occludin, and α1- and β1-ENaC after only 1 h of exposure to DEX (1.34-, 1.32-, and 1.80-fold, respectively). At the time of uplifting (6 h), α1- and β1-ENaC mRNA had increased by 40, 20, and 203%, respectively, and α1- and β1-Na,K-ATPase mRNA levels had reached 1.90- and 2.36-fold increases, respectively.

Some authors have suggested that dome formation is facilitated in zones of relatively weak attachment of cells to the substrate (22, 23). This was examined in H441 cells grown on coverslips covered with a thin layer of Matrigel, which enhances cell attachment and promotes differentiation (24). Despite better anchorage, the addition of 50 nM of DEX induced dome formation with the same time course and magnitude as in monolayers grown on bare glass supports. Therefore, stronger attachment does not seem to inhibit dome formation, but it is possible that certain groups of cells are more adhesive than others. In addition, the removal of DEX caused an almost complete disappearance of domes within 48 h (data not shown). Therefore, it seems unlikely that release from the substrate triggers differentiation. Because there must be fluid transport for a dome to appear, it seems plausible that regional areas of Na+ channel differentiation leading to regional vectorial fluid flow combine with regional areas of relatively lower adherence. If there were no differences in adherence, domes should continuously grow larger unless back-leak starts to match fluid transport rate in the dome.

Electrophysiological Characterization of Cells Grown on Porous Supports—We also studied H441 cells grown on porous supports, a condition in which, as in domes, the cells have basolateral access to fluid and may be considered “uplifted” albeit with no increase in pressure. Sodium currents were measured on cells grown on permeable polyester snap-well filters. Transepithelial electrical resistance ($R_{te}$) was used as a measure of cell differentiation. Consistent with the literature, differentiation of these monolayers was variable despite several manipulations including treatment of filters with matrix proteins, addition of DEX, or culturing with an air-liquid interface. Thus, cultures were divided into low resistance (mean $R_{te}$ = 323 ± 48 ohms cm$^{-2}$, n = 36) or high resistance groups (mean $R_{te}$ = 1240 ± 321 ohms cm$^{-2}$, n = 9). Fig. 7 summarizes patch clamp results obtained in these two groups. Inward sodium currents in cells from low resistance monolayers were not amiloride-sensitive, in contrast to the high resistance monolayers, in which sodium entry was completely blocked by amiloride.

Thus, during epithelial cell differentiation, the sodium entry pathway undergoes both qualitative and quantitative functional modifications. The increased sodium entry, together with the coordinated development of tight junction and pump proteins, causes vectorial fluid transport. This fluid is trapped at the basolateral side of the monolayer and is responsible for epithelial uplifting from the nonporous culture support.

**DISCUSSION**

The distal airway and alveolar epithelium absorb sodium ions from the lining fluid, water then follows the osmotic gradient passively. Our data are consistent with previous observations of sodium absorption by the H441 lung cells (5–10). In
addition, we provide strong evidence for the existence of a link between the level of cell differentiation and their transport properties. In fact, we observed several populations of cells within the same monolayer with qualitative and quantitative differences in sodium entry properties. Although in highly differentiated dome-forming cells the sodium ion absorption occurs mainly through amiloride-sensitive channels, in poorly differentiated flat cells most of the sodium conductance is amiloride-insensitive. Nonetheless, a small amiloride-sensitive conductance is also observed in flat cells. Amiloride-insensitive sodium entry in H441 cells was also observed by Clunes et al. (8), in cells cultured for 24 h in the absence of dexamethasone.

Several lines of evidence indicate that within a single H441 cell monolayer, cells forming epithelial domes are different from cells that are not uplifted. These differences include elevated surfactant production, increased cytokeratin expression, and localization of claudin-4 proteins to the plasma membrane (Figs. 1 and 2). These observations imply that the changes involve timed gene expression. Zucchi et al. (4, 25) have studied the coordinated activity of genes and proteins involved in epithelial dome formation and found that the expression of αβγ1 integrin, which promotes cell-cell adhesion, is followed by the expression of E-cadherin and cytokeratin 8. This protein synthesis precedes the detachment of the monolayer and dome formation and is almost absent in cells surrounding domes (4, 25). It has also been shown that epithelial differentiation and dome formation is under the positive/negative control of many factors, including ENaC and tight and gap junctions (4, 21, 25, 26). In our RTQ-PCR experiments, we observed a substantial increase in the content of tight junction protein mRNA followed by an increase in the mRNA of pumps, which preceded the appearance of cell uplifting. It is known that dome formation requires the continuous stimulation by differentiating agents, the most widely used of those being dexamethasone, Me2SO, and cAMP analogs (4, 27), and that withdrawal of dexamethasone leads to dome disappearance within 48 h (Ref. 27 and this study).

There is a growing body of evidence supporting the view that cell-cell communications play a significant role in the regulation of alveolar epithelial cell phenotype and function (28, 29). Cell that form domes in culture are polarized, possess tight junctions, and contain markers of differentiated epithelial cells (Refs. 2–4 and this study). In the present study, we also observed that the expression of the claudin-4 tight junction protein preceded changes in the cell ion transport properties, which is consistent with the view that the tight junctional contribution to tissue architecture is essential for transepithelial transport and dome formation. In this regard, A6 cells, a cellular model of the distal nephron epithelium, exhibit between 0 and 100% amiloride-inhibitable sodium transport depending on whether cultured on nonpermeable or permeable supports (30, 31). Furthermore, Nakahari and Marunaka (32) showed that in A6 monolayers preincubated in nominally calcium-free medium to uncouple cells, the majority of sodium entry appeared to be amiloride-insensitive albeit still regulated by vasopressin and cytosolic calcium, as observed for the normal amiloride-sensitive Na⁺ channels. Their results, like ours, point to the importance of cell polarization and coupling in maintaining differentiated ion transport function.

A significant fraction of amiloride-insensitive sodium conductance has been attributed to cyclic nucleotide-gated channels in freshly isolated type II pneumocytes (33) and A549 lung epithelial cell line (34). In our experiments, blockers of CNG channels, pimozide and Zn ions, but not Mg ions, inhibited some of the amiloride-insensitive sodium conductance. By contrast, La³⁺, a known inhibitor of NSC channels, blocked the majority of these currents.

Sodium entry through amiloride-insensitive pathways has also been described in surface cells of rectal colon (35), esophageal epithelia (36), and keratinocytes (37). In patch clamp studies of rectal colon cells in situ, distinct populations of cells with either amiloride-sensitive or amiloride-insensitive sodium currents were described (35). Thus, our in vitro culture model mimics the naturally occurring coexistence of regional differences in of amiloride sensitivity, and various stages of functional properties or states of differentiation may be present in apparently identical cell types. Likewise, Kunzelmann et al. (38) reported that 16HBE14o bronchiolar cells in culture did not exhibit amiloride-sensitive sodium conductance unless exposed to DEX overnight. Finally, it is also possible that spontaneous ENaC activity triggers cell differentiation, as shown for normal epidermal differentiation (37).

The lung, skin, and digestive tract are lined by an epithelium that undergoes substantial proliferation. Results from the literature as well as from the present study indicate that amiloride-insensitive sodium absorption is the Na⁺ transport pathway found in cells that have not achieved complete maturation. The proteins involved in amiloride-insensitive Na⁺ transport include NSC channels, CNG channels, and possibly immature...
or "misassembled" ENaC (36). It was recently shown that immature ENaCs have very low activity at the cell membrane (39), whereas cells with amiloride-insensitive sodium currents express α, β, and γ ENaC subunits (36). Whether ENaC subunits can misassemble or bind with other proteins to form conductive channels, has never been shown. Thus, the mechanism as well as the significance of the gradual shift from nonselective or amiloride-insensitive currents to ENaC type currents is still unclear. As we cannot test tissues in which development of tight junctions and pumps are dissociated from the differentiation of the sodium entry pathways, it is difficult to assess the importance of ENaC versus NSC channels in the formation of domes, except for the increased rate of sodium transport. A priori, any sodium transport pathway could lead to dome formation provided that this transport is vectorial, due to the presence of basolateral pumps, and that tight junctions are present to prevent back-leak of fluid. However, because inhibition of ENaC-type sodium transport by either amiloride or antiseense mRNA leads to the collapse of domes (25), we conclude that development of a differentiated Na⁺ entry pathway, along with tight junctions and pumps, is a necessary maturation step for vectorial sodium transport in epithelia.

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REFERENCES
1. Mason, R. J., Williams, M. C., Widdicombe, J. H., Sanders, M. J., Misfeldt, D. S., and Berry, L. C., Jr. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6033–6037
2. Shen, S. S., Hamamoto, S. T., and Pitelka, D. R. (1976) J. Membr. Biol. 29, 373–382
3. Rothen-Rutishauser B., Kramer, S. D., Braun, A., Gunther, M., and Wunderli-Allenspach, H. (1998) Pharm. Res. (N Y) 15, 964–971
4. Zucchi, I., Montagna, C., Susani, L., Montesano, R., Aifer, M., Zanotti, S., Redolfi, I., Vezzoni, P., and Dulbecco, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13766–13770
5. Sayegh, R., Auerbach, S. D., Li, X., Loftus, R. W., Husted, R. F., Stokes, J. B., and Thomas, C. P. (1999) J. Biol. Chem. 274, 12431–12437
6. Itani, O. A., Auerbach, S. D., Husted, R. F., Volk, K. A., Ageloff, S., Knepper, M. A., Stokes, J. B., and Thomas, C. P. (2002) Am. J. Physiol. 282, L631–L641
7. Laarak, A., and Matalon, S. (2003) Am. J. Physiol. 285, L1453–L1459
8. Clunes, M. T., Butt, A. G., and Wilson, S. M. (2004) J. Physiol. 557, 809–819
9. Ramminger, S. J., Richard, K., Inglis, S. K., Land, S. C., Oliver, R. E., and Wilson, S. M. A. (2004) Am. J. Physiol. 287, L837–L841
10. Thomas, C. P., Campbell, J. B., Wright, P. J., and Husted, R. F. (2004) Am. J. Physiol. 287, L843–L851
11. Gazdar, A. F., Linnola, R. I., Kurita, Y., Oie, H. K., Mulshine, J. L., Clark, J. C., and Whitsett, J. A. (1990) Cancer Res. 50, 5481–5487
12. Chander, A., Johnson, R. G., Reicherter, J., and Fisher, A. B. (1986) J. Biol. Chem. 261, 6126–6131
13. Wadsworth, S. J., Spitzer, A. R., and Chander, A. (1997) Am. J. Physiol. 273, L427–L436
14. Barry, P. H., and Lynch, J. W. (1991) J. Membr. Biol. 121, 101–117
15. Akaike, N., and Harata, N. (1994) Jpn. J. Physiol. 44, 433–473
16. Winer, J., Jung, C. K., Shackell, I., and Thomas, C. P. (1999) Anol. Biochem. 270, 41–49
17. Cuta, E., and Conen, P. E. (1971) J. Pathol. 62, 127–141
18. Barquin, N., Ciccolella, D. E., Ridge, K. M., and Sznajder, J. I. (1997) Am. J. Physiol. 273, L245–L250
19. Chalakia, S., Inghar, D. H., Sharma, R., Zhou, Z., and Wende, H. C. (1999) Am. J. Physiol. 277, L197–L203
20. Gonzales, L. W., Guttenart, S. H., Wade, K. C., Postle, A. D., and Ballard, P. L. (2002) Am. J. Physiol. 283, L940–L951
21. Hao, H., Wende, C. H., Sandhu, G., and Inghar, D. H. (2003) Am. J. Physiol. 285, L593–L601
22. Rabito, C. A., Téhao, R., Valentich, J., and Leighton, J. (1980) In Vitro 16, 461–468
23. Sugahara, K., Caldwell, J. H., and Mason, R. J. (1984) J. Cell Biol. 99, 1541–1544
24. Rannels, S. E., and Rannels, D. E. (1989) J. Mol. Cell. Cardiol. 21, 151–159
25. Zucchi, I., Hini, L., Valaperta, R., Ginestra, A., Albani, D., Susani, L., Sanchez, J. C., Liberatori, S., Magi, B., Raggiassi, R., Hochstrasser, D. F., Pallini, V., Vezzoni, P., and Dulbecco, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5608–5613
26. Tonoli, H., Flachon, V., Audebet, C., Calle, A., Jarry-Guichard, T., Statuto, M., Rouset, B., and Munari-Silem, Y. (2000) Endocrinology 141, 1403–1413
27. Lever, J. E. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1323–1327
28. Abraham, V., Chou, M. L., George, P., Pooler, P., Zaman, A., Savani, R. C., and Koval, M. (2001) Am. J. Physiol. 280, L1085–L1093
29. Schneeberger, E. L., and Lynch, R. D. (1997) The Lung. Scientific foundation, pp. 505–515, Lippincott-Raven, Philadelphia
30. Sariban-Sohraby, S., Burg, M. B., and Turner, R. J. (1984) Am. J. Physiol. 245, C167–C171
31. Hamilton, K. L., and Eaton, D. C. (1985) Am. J. Physiol. 249, C200–C207
32. Nakahari, T., and Marunaka, Y. (1996) J. Membr. Biol. 154, 35–44
33. Kemp, P. J., Kim, K. J., Borok, Z., and Crandall, E. D. (2001) J. Physiol. 536, 693–701
34. Xu, W., Leung, S., Wright, J., and Guggino, S. E. (1999) J. Membr. Biol. 171, 117–126
35. Inagaki, A., Yamaguchi, S., and Ishikawa, T. (2004) Am. J. Physiol. 286, C380–C389
36. Ayawda, M. S., Bengrine, A., Tobey, N. A., Stockand, J. D., and Orlando, R. C. (2004) Am. J. Physiol. 287, C395–C402
37. Mauro, T., Guitard, M., Behne, M., Oda, Y., Crumrine, D., Komuves, L., Rassner, U., Elus, P. M., and Hummer, E. (2002) J. Investig. Dermatol. 118, 589–594
38. Kunzelmann, K., Kathofe, S., Hipper, A., Gruenert, D. C., and Gregner, R. (1996) Pflugers Arch. Eur. J. Physiol. 431, 578–586
39. Hughey, R. B., Prus, J. B., Kinleigh, C. L., and Kleyma, T. R. (2004) J. Biol. Chem. 279, 48491–48494