Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium

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Quesnell RR, Han X, Schultz BD. Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium. Am J Physiol Cell Physiol 292: C1739–C1745, 2007. First published January 24, 2007; doi:10.1152/ajpcell.00369.2006.—Mammary epithelia produce an isotonic, low-Na⁺ fluid that is rich in nutrients. Mechanisms that account for the low electrolyte concentration have not been elucidated, although amiloride-sensitive ion transport has been reported in some situations. We hypothesized that corticosteroid exposure modulates epithelial Na⁺ channel (ENaC) expression and/or activity in bovine mammary epithelial cells. BME-UV cells were grown to confluent monolayers on permeable supports with a standard basolateral medium and apical medium of low-electrolyte, high-lactose composition that resembles the ionic composition of milk. Ion transport was assessed in modified Ussing flux chambers. Exposure to glucocorticoids (dexamethasone, cortisol, or prednisolone), but not aldosterone, increased short-circuit current (Isc), a sensitive measure of net ion transport, whereas apical exposure to amiloride or benzamil affected. Exposure to mifepristone (a glucocorticoid receptor antagonist), but not spironolactone (a mineralocorticoid receptor antagonist), precluded both the corticosteroid-induced elevation in amiloride-sensitive Isc close to basal levels. Quantitative RT-PCR indicated a glucocorticoid-induced increase in mRNA for β- and γ-ENaC, whereas α-ENaC mRNA expression was only mildly affected. Exposure to mifepristone (a glucocorticoid receptor antagonist), but not spironolactone (a mineralocorticoid receptor antagonist), precluded both the corticosteroid-induced elevation in amiloride-sensitive Isc and the induced changes in β- and γ-ENaC mRNA. We conclude that Na⁺ movement across mammary epithelia is modulated by corticosteroids via a glucocorticoid receptor-mediated mechanism that regulates the expression of the β- and γ-subunits of ENaC. ENaC expression and activity could account for the low Na⁺ concentration that is typical of milk.

MAMMARY EPITHELIA rely on regulated ion transport processes (4) to produce milk that is low in Na⁺, although the mechanisms for monovalent ion transport remain to be fully characterized. A current model of mammary epithelium shows an unidentified apical Na⁺ conductance and indicates that Na⁺ is distributed across this membrane according to its electrochemical equilibrium (20, 35). In this model, cytosolic, and therefore luminal, Na⁺ concentration is set by the activity of the basolateral components, most notably, Na⁺-K⁺-ATPase. These reviews stress the importance of identifying the transport mechanisms responsible for milk composition and, especially, the identity of apical conductance(s). Recent studies with mammary cells derived from murine (4) and bovine (30) tissues have demonstrated amiloride-sensitive ion transport across this epithelial layer. Thus, we speculated that the epithelial Na⁺ channel (ENaC) may be the unidentified apical path in the epithelial model.

A model that accounts for ion transport requires components other than ENaC. Functional evidence for a number of the requisite components has been demonstrated in the BME-UV cell line (30) as well as other mammary cell systems (35). The large, highly regulated transepithelial ion concentration gradient across the mammary epithelium has been shown to both support the secretory function of the epithelium and be responsible for maintaining milk quality (2, 4, 25, 30, 36–38). Ion transport mechanisms that have been identified in mammary epithelium include Na⁺-K⁺-ATPase, Na⁺-K⁺-2Cl⁻ cotransporter, Ba²⁺-sensitive K⁺ conductance(s) in the basolateral membranes, and a Cl⁻ conductance and Na⁺ channel in the apical membrane (3, 4, 20, 30, 33–36, 38, 41). ENaC and CFTR anion channel activity has been reported in murine mammary epithelium (4), a system in which dexamethasone was included in the medium bathing the cells to promote tight junction formation. Amiloride-sensitive short-circuit current (Isc), suggesting the presence of ENaC, has been demonstrated in bovine mammary epithelium (30) but only after exposure to dexamethasone. Although many distinct ion transport mechanisms have been suggested for mammary epithelium (17, 19, 21, 22, 33, 34), little is known regarding the cation selectivity of the apical membrane, the time course of responses to corticosteroid stimuli, or the conductances that are regulated.

Modulation of Na⁺ transport by corticosteroids has been delineated in many tissues, including the kidney, gut, and airway (15, 29, 39, 40). Fluid secretion in the mammary gland is affected by corticosteroids as demonstrated by alterations in milk production by stress or corticosteroid exposure. It is postulated that some parallels to mechanisms defined in other tissues may exist in mammary epithelia. In the kidney, corticosteroid regulation of monovalent ion transport is regulated via a mineralocorticoid receptor-mediated process (10, 12). Colon and airway monovalent ion transport processes, on the other hand, have been shown to be dependent on glucocorticoid receptor-regulated pathways (1, 5, 14, 18, 23, 42, 43). Corticosteroid regulation of mammary ion transport processes has not been examined. Since dexamethasone has been shown to affect epithelial transport in both glucocorticoid receptor- or mineralocorticoid receptor-regulated pathways, elucidating the specific receptor-mediated pathway for an effect of corticosteroids in mammary epithelium is critical to formulating an approach to modulate mammary luminal ion concentrations.

The goal of this study was to determine the molecular identity of mechanisms that account for corticosteroid-induced changes in mammary ion transport; specifically, we speculated on the presence and activity of ENaC in the apical membrane. The hypothesis was that corticosteroids alter Na⁺ transport across bovine mammary epithelium via glucocorticoid recep-
tor-mediated modulation of specific ENaC subunits. The outcome demonstrates that mechanisms present in mammary epithelium can account for the low Na⁺ concentration in milk.

MATERIALS AND METHODS

Cell culture. An immortalized bovine mammary epithelial cell line, BME-UV, was obtained from Jeff White at the University of Vermont. The cell line was transformed and first described by Zavizion et al. (45), who reported the synthesis of α-lactalbumin and α₁,₃-casein. BME-UV cells have since been used in studies to assess the regulation of bovine mammary epithelial cell proliferation (6), growth factor expression (44), apoptosis (11), ion transport (30), and barrier function (25). Culture conditions have been described in detail previously (25, 30). Briefly, BME-UV cells were grown to 65–75% confluency in 25-cm² plastic culture flasks (Cellstar, Frickenhausen, Germany) using media of defined composition and lifted using a solution containing trypsin in PBS with 2.6 mM disodium EDTA for 1 min.

Germany) using media of defined composition and lifted using a solution containing trypsin in PBS with 2.6 mM disodium EDTA for 1 min. Osmolarity was 290 mosM in all media. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Media were refreshed daily, including 4–8 h prior to experimental assays.

Electrical measurements. Iₑ, a measure of net transepithelial ion transport, was measured continuously with sampling at 1 Hz in a modified Ussing chamber system (model DCV9, Navicate, San Diego, CA) with a voltage-clamp apparatus (model 558C, Department of Bioengineering, University of Iowa, Iowa City, IA). Monolayers grown on Snapwell permeable supports were mounted in the modified Ussing chamber system (model DCV9, Navicate, San Diego, CA) with a voltage-clamp apparatus (model 558C, Department of Bioengineering, University of Iowa, Iowa City, IA). Monolayers grown on Snapwell permeable supports were mounted in the modified Ussing chamber apparatus and bathed in symmetrical Ringer solution [composed of (in mM) 120 NaCl, 25 NaHCO₃, 3.3 K₂HPO₄, 0.8 KCl, 1.2 MgCl₂, and 1.2 CaCl₂]. Fluid was circulated with an airlift system (5% CO₂–95% O₂) using a 5:3:2 mixture of DMEM + Ham’s F-12 RPMI medium–NCTC-135 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS (BioWhittaker, Walkersville, MD), 3% newborn calf serum (GIBCO), 2% iron-supplemented bovine calf serum (GIBCO), 1% insulin-transferrin-sodium selenite media supplement solution (Sigma-Aldrich, St. Louis, MO), and 1% penicillin and streptomycin (GIBCO), L-ascorbic acid, α-lactalbumin, and lactose (targeted ionic composition: 140 meq/l Na⁺, 120 meq/l Cl⁻, 10 meq/l HCO₃⁻, 2.6 meq/l HEPES, 0.1 meq/l Ca²⁺, and 1.0 meq/l Mg²⁺). Transepithelial electrical resistance (Rₑ) measurements were obtained during the typical protocol of data collection by applying a 0.5-mV bipolar pulse across the monolayer at 100-s intervals. Current and voltage measurements were acquired on a Macintosh computer (Apple Computer, Cuppertino, CA) using Aqknowledge software (version 3.2.6, BIOPAC Systems, Santa Barbara, CA). Rₑ was calculated using Ohm’s Law, and monolayers with >150 Ohm·cm² were used for experiments. Corticosteroid treatments and amiloride exposure. To examine the effect of steroid exposure on amiloride-sensitive epithelial Na⁺ absorption, BME-UV monolayers were grown on permeable supports and basolaterally exposed to natural or synthetic corticosteroids, including dexamethasone (0.1 μM), prednisolone (1.0 μM), or cortisol (0.5 μM; Sigma-Aldrich), for 72 h prior to experimental assessment. Apical exposure to either amiloride (10 μM) or benzamil (1 μM) during Ussing chamber experiments was included to quantify the magnitude of ion transport that is sensitive to these agents.

Western blot analysis. To identify the presence of ENaC immunoreactivity and to test for obvious changes in protein levels that occurred concomitant with changes in Iₑ, lysates of untreated and corticosteroid-treated BME-UV cells were prepared in 1.13-cm² Snapwell supports using RIPA lysis buffer including 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Cell monolayers were broken apart using 20-gauge needle aspiration, and cells were rocked overnight at 4°C in lysis buffer. Cells were further processed via 26-gauge needle aspiration and maintained in a frost-free freezer at −20°C until being assayed. Total protein content was determined using a bicinchoninic acid assay (Pierce, Rockford, IL), and 20 μg total protein were loaded in each well of a 10–20% SDS-PAGE prepurged gel (Bio-Rad, Hercules, CA) for electrophoresis. Electrophoretic protein resolution was conducted with 160 mV for 45 min. Proteins were then transferred to Millipore PVF-Immobilon (Millipore, Bedford, MA) membranes for 8.5 h at 60 V. Transfer was confirmed by staining gels with gelcode blue (Pierce). Membranes were blocked in SuperBlock blocking buffer (Pierce) and probed with anti-α-ENaC, anti-β-ENaC, and anti-γ-ENaC antibodies (antibodies for all three subunits were obtained from both Alpha Diagnostics, San Antonio, TX, and Affinity BioReagents, Golden, CO) at the appropriate concentrations for each. Tris-buffered saline including Tween 20 (Sigma-Aldrich) was used as a wash buffer. Immunoreactive protein bands were visualized using a peroxidase-conjugated secondary antibody (Pierce, dilution: 1:12,500) and by enhanced chemiluminescence with Pico-chemiluminescence substrate (Pierce). Membranes were exposed on CL-Xposure film (Pierce) and analyzed using a Kodak RP X-OMAT (model M7B) film analyzer.

RT-PCR for glucocorticoid receptor. RNA was extracted from BME-UV cells using the RNeasy kit (Qiagen, Valencia, CA). The extract was treated with DNase I (Invitrogen, Carlsbad, CA). RT-PCR for the BME-UV glucocorticoid receptor was performed using the One-Step RT-PCR kit (Qiagen) on a Technie Touchgene Thermal Cycler (Krackel Scientific, Albany, NY). The sequences of the primers used were 5’-GGGAAAGATGCAAGGCTC-3’ and 5’-CCCATATGACCATCCTGAA-3’. The reaction components included 1 ng of total RNA from BME-UV cells per 25 μl of reaction mixture, One-Step RT-PCR buffer, dNTP mix, 0.6 μM primers, and One-Step RT-PCR enzyme mix. The RT-PCR cycle parameters were as follows: 50°C for 30 min and 95°C for 15 min and 40 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 40 s, with a final step at 72°C for 5 min. PCR products were examined by 2% agarose gel electrophoresis. The expected product of RT-PCR was 210 bp. Control reactions, which did not include the RT step, were amplified in a parallel reaction to ensure the absence of DNA. The image was captured on a FluorChem 8900 imaging system (Alpha Innotech, San Leandro, CA).

Quantitative RT-PCR. To determine the relative copy numbers of mRNA for ENaC subunits, total RNA was harvested from vehicle- and corticosteroid-treated BME-UV cells, and PCR analysis was performed for α-, β-, and γ-ENaC mRNA expression. Total RNA was isolated from confluent BME-UV monolayers using Shredder columns and Micro RNeasy RNA isolation kits (Qiagen) following the manufacturer’s protocol. High quality of the purified total RNA samples was confirmed by analysis on a RNA Nano LabChip (Agilent Technologies, Palo Alto, CA) as well as detection of expected bands on a denaturing gel corresponding to 18S rRNA. All RNA samples were subjected to DNase I treatment (Ambion, Austin, TX) according to the manufacturer’s specifications. Initial quantification of total RNA for loading was performed in a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RT-PCR was carried out (One-Step RT-PCR, Qiagen) using primer pairs specific for coding sequences of bovine α-ENaC (forward: 5’-GGCGTGTGTTTAACTTCCTCC-3’ and reverse: 5’-TCAGGGGTATGGGTAGTGTA-3’; 243-bp expected product), β-ENaC (forward: 5’-TGCTGTGCCTATCGAGATTG-3’ and reverse: 5’-TGGCAGCGGAGGACTGATTTG-3’; 277-bp expected product), and γ-ENaC (forward: 5’-TCAAGAAAGAATCCTGCCCTTA-3’ and reverse: 5’-CTTTGGGAGAATGGTCAAGTT-3’; 242-bp expected product) as well as 18S primers for normalization (forward: 5’-CGGCTACCACATC-
CAAGGAA-3' and reverse: 5'-CCGCTCCAAATCAGCTTCTA-3'; 248-bp expected product) in paired reactions with RNA derived from dexamethasone- and vehicle-treated monolayers. RT-PCR products were resolved by electrophoresis in a 1.5% agarose gel to confirm a single band of expected mobility. Reactions with no reverse transcriptase step were included to confirm sample purity. Quantitative analysis was performed using SYBR green (Molecular Probes, Eugene, OR) and run on a Cepheid SmartCycler (Cepheid, Sunnyvale, CA). The threshold to determine threshold cycle (Ct) was set to 30 arbitrary fluorescent units, which is in the log linear portion of the fluorescence curve. RT-PCR products were sequenced to verify amplicon identity. Increases/decreases were determined using Ct values taken at the curve. RT-PCR products were sequenced to verify amplicon identity. Increases/decreases were determined using Ct values taken at the same threshold level for each individual experiment, and outcomes were normalized to the Ct values for 18S.

Glucocorticoid/mineralocorticoid receptor inhibitors. To evaluate the receptor mechanism responsible for functional changes in Na+ transport, spironolactone (10 μM) or metipristone (10 μM) was included in the bathing medium for the last 24 h prior to assessment in a subset of the above-described experiments. Analyses performed in conjunction with these experiments included Ussing chamber, Western blot, and quantitative RT-PCR.

Data analysis. Differences between control and treatment data were analyzed using ANOVA and Student’s t-test components of a Unix-based SAS (SAS Institute, Cary, NC) program. Data are presented as means ± SE. Statistical significance was evaluated at P < 0.05.

RESULTS

Glucocorticoids elevate amiloride-sensitive Isc. Initial experiments were designed to document and extend observations examining the effect of steroid exposure on amiloride-sensitive epithelial ion transport (30). Figure 1A shows typical tracings from paired BME-UV monolayers mounted in a modified Ussing chamber system illustrating the difference in Isc that is associated with corticosteroid exposure. In Fig. 1, the dashed line in each tracing represents no net ion movement. The elevation in Isc was evident in prednisolone-, cortisol-, and dexamethasone-treated monolayers compared with Isc in the untreated monolayer. In a separate set of experiments, aldosterone was without effect. The size of the periodic deflections is inversely proportional to Rsc. Monolayers that had been exposed to glucocorticoids exhibited lower Rsc (307 ± 19 Ohm·cm²) than untreated controls (362 ± 36 Ohm·cm²). Data summarized from 6 experiments demonstrated that corticosteroid exposure resulted in significantly greater initial Isc (Fig. 1B) that was of similar magnitude for both naturally occurring and synthetic compounds. Glucocorticoid-induced Isc was reduced by amiloride exposure in all cases (Fig. 1, A and C). Apical exposure to amiloride (10 μM) or benzamil (1 μM; data not shown), on average, reduced Isc to a level approaching untreated monolayers. Concomitant with the decrease in Isc was an amiloride-induced increase in Rsc (15 ± 3 Ohm·cm² for all corticosteroids vs. −6 ± 3 Ohm·cm² for untreated controls), indicating the block of a conductive pathway in corticosteroid-treated monolayers. Apical membrane sensitivity to both amiloride and benzamil, along with the increase in Rsc, led to the speculation that ENaC expression or activity was enhanced by glucocorticoid exposure.

α-ENaC is present at the protein level. Western blot analysis to evaluate α-ENaC immunoreactivity confirmed the presence of this protein in lysates of cultured BME-UV cells (Fig. 2A). The presence of α-ENaC was examined in monolayers exposed to natural and synthetic corticosteroids and compared with lysates of untreated monolayers. Anti-α-ENaC immunoreactivity was evident in all samples tested, although there was no apparent effect of treatment on protein expression level. It is noteworthy that control monolayers exhibited no amiloride-sensitive ion transport, yet the α-ENaC immunoreactivity in the control monolayer was indistinguishable from that in glucocorticoid-treated monolayers. Antibodies raised against epitopes in rat β- and γ-ENaC, purchased from two commercial sources, gave no signal in bovine cell lysates, although bands of expected mobilities were observed in rat kidney and porcine vas deferens epithelial cell lysates (not shown). Thus, it was concluded bovine β- and γ-ENaC do not expose the epitope to which these antibodies were raised. A survey of additional antibodies was determined to be outside the scope of the investigation, and alternative approaches were employed to assess ENaC subunit expression.

Quantitative RT-PCR indicates corticosteroid regulation of β- and γ-ENaC mRNA expression. Quantitative analysis of ENaC subunit transcription was compared between corticosteroid-treated and untreated monolayers. α-ENaC transcript was present in all samples and was less than twofold greater (not significant) following corticosteroid exposure. β-ENaC and γ-ENaC transcript levels, on the other hand, were significantly greater in dexamethasone-treated monolayers (Fig. 2B). Copy numbers increased by ~7- and ~15-fold for β- and γ-ENaC subunits, respectively. With amplicons of similar size and reactions that used identical conditions, α-ENaC crossed threshold at cycle 21.7 ± 0.2, whereas thresholds for β- and γ-ENaC amplification was not reached until cycle numbers.
conclude that the copy number for α-ENaC is in great excess (~214-fold) relative to β- and γ-ENaC. Nonetheless, corticosteroids greatly enhanced β- and γ-ENaC expression but had only a small effect on α-ENaC mRNA copy number.

Glucocorticoid receptors are present in BME-UV monolayers. To evaluate the receptor mechanism by which glucocorticoids increase $I_{sc}$ in bovine mammary monolayers, we sought to first identify whether the glucocorticoid receptor was present at the transcriptional or translational levels. Evidence suggested that both were expressed in BME-UV cells. The presence of the receptor alone, however, did not confirm its involvement in the regulatory scheme. Therefore, experiments were conducted to examine functional effects of glucocorticoid-receptor inhibition on upregulated $I_{sc}$ in BME-UV cell monolayers as well as examine the transcriptional effects on ENaC regulatory subunits.

Upregulation of ENaC expression and/or activity is modulated via a glucocorticoid receptor-mediated process. Mifepristone inhibited the elevation of $I_{sc}$ across BME-UV monolayers that was induced by glucocorticoids. Results from a typical experiment are presented in Fig. 4A. As expected, dexamethasone exposure was associated with an elevated initial $I_{sc}$ that was sensitive to amiloride. Results from a paired monolayer that was cultured in the presence of spironolactone (10 μM), a selective mineralocorticoid receptor antagonist, were indistinguishable from the monolayer that was exposed to dexamethasone only. Most importantly, exposure to mifepristone, a selective glucocorticoid receptor and/or progesterone receptor antagonist, fully abolished the dexamethasone effect. Data from eight experiments are summarized in Fig. 4, B and C. The results are consistent with Fig. 1 and show that dexamethasone-induced $I_{sc}$ was amiloride sensitive, although the magnitude of the responses in these experiments was.

![Western blot analysis of protein harvested from bovine mammary epithelial cell monolayers demonstrated immunoreactivity to primary antibody raised against GRr.](Image)

![RT-PCR executed with primers designed against the bovine GR result in a single, clear band of the expected size.](Image)

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somewhat less. In the presence of spironolactone, results were unchanged from dexamethasone alone, whereas in the presence of dexamethasone and mifepristone, the results were no different than in the absence of dexamethasone.

**Glucocorticoid receptor inhibitors preclude elevation in ENaC subunit transcripts during corticosteroid exposure.** In a separate set of experiments, RNA was isolated from cells that had been cultured with dexamethasone in the absence or presence of mifepristone or spironolactone. Relative levels of mRNA for each ENaC subunit, measured as a percentage of the control (dexamethasone with no antagonist) value, were significantly reduced by concurrent exposure to mifepristone (Fig. 5), although spironolactone was without effect (not shown). Again, it should be noted that, in the presence of dexamethasone, α-ENaC was in much higher copy number than either β- or γ-ENaC, as indicated by the Ct values of 20.7 ± 0.2 (α-ENaC), 31.7 ± 0.04 (β-ENaC), and 28.5 ± 0.9 (γ-ENaC). A comparison of the relative Ct values of dexamethasone-treated and dexamethasone + mifepristone-treated monolayers showed a substantial mifepristone-induced right shift for β- and γ-ENaC expression with a modest right shift for α-ENaC. Differences in copy number for each ENaC subunit are consistent with the data shown in Fig. 2 in that the copy number for α-ENaC was ~2-fold greater with dexamethasone exposure, whereas mifepristone reduced the copy number to ~1/3 of the dexamethasone-induced level. Dexamethasone caused much greater differences in β- and γ-ENaC copy numbers (7- to 15-fold), and mifepristone was associated a reduction in copy number of similarly large magnitudes.

In summary, functional evidence indicates glucocorticoid receptor-mediated modulation of Na⁺ movement across BME-UV cell monolayers during exposure to corticosteroids. This functional evidence is further supported by evidence at a molecular level by the inhibition of corticosteroid upregulation of α-subunit and, especially, β- and γ-subunit expression of ENaC in the presence of mifepristone.

**DISCUSSION**

This study defines a corticosteroid-regulated mechanism that can account for Na⁺ permeation across the apical membrane of mammary epithelium. The results suggest that α-ENaC mRNA and protein are constitutively expressed, but that corticosteroids, acting at a glucocorticoid receptor, increase β- and γ-ENaC transcription substantially, and α-ENaC to a lesser extent, to account for the increase amiloride-sensitive current. Exposure to mifepristone precluded both the corticosteroid-induced elevation in amiloride-sensitive Iₐc and the induced changes in α-, β-, and γ-ENaC mRNA transcription, suggesting that the change in Na⁺ movement across epithelia in the bovine mammary gland is modulated by corticosteroids via a glucocorticoid receptor-mediated mechanism that is linked to ENaC transcription. Changes in ENaC expression and function have the capacity to affect the ion composition within the milk compartment.

The basis of tissue specific differences in ENaC subunit expression is not known. Glucocorticoid response elements (GRE) are present upstream of genes for all ENaC subunits in humans. A GRE is present upstream of bovine α-ENaC, although the presence of a GRE for β- or γ-ENaC has not yet been demonstrated. Nonetheless, the relative lack of a corticosteroid effect on α-ENaC expression demonstrates that the GRE alone is not sufficient to explain glucocorticoid-induced ENaC regulation in mammary gland. Additional coactivator factors, such as NCOa1, may be differentially expressed between tissues. Alternatively, RNA stability may be regulated differently in these tissues. Clearly, additional studies must be conducted to test these hypotheses.

Placing ENaC in the apical membrane enhances the current models proposed for mammary epithelial ion transport (17, 20, 35). The authors of these reviews surmised that Na⁺ moved through a conductive apical pathway and distributed across the apical membrane according to the electrochemical gradient. In this model, combined activity of Na⁺-K⁺-ATPase and K⁺ channels at the basolateral membrane sets a negative membrane potential that drives the distribution of Na⁺ across the apical membrane. The results of this study, which are based on cells derived from bovine tissues and are consistent with a recent report (4) regarding murine mammary epithelium, demonstrate that ENaC is the unidentified conductance in this mammary epithelial model. Furthermore, the results presented herein demonstrate that the apical conductance is dynamic. It is possible that the number of ENaC channels in the apical membrane is regulated by hormones such as cortisol and/or that the channel open probability is likely regulated by neurotransmitters such as norepinephrine (27, 30). There is evidence for all components of a minimal model to achieve Na⁺ absorption across BME-UV cells, ouabain-inhibitable Na⁺-K⁺-ATPase, bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter, and Ba²⁺-sensitive K⁺ conductance. These ion transport mechanisms have been discussed previously, in the context of mammary epithelia from other species (20, 35). The new knowledge presented in the current study has many implications for our understanding of mammary physiology and the treatment of pathological situations.

Corticosteroid regulation of ENaC at the molecular level in bovine mammary epithelium takes place in β- and γ-subunits and only mildly in the α-subunit. ENaC expression is regulated...
by glucocorticoids and/or mineralocorticoids in a tissue-specific pattern. Aldosterone’s effects are observed primarily in the kidney (Ref. 10; for a review, see Ref. 31) with effects also observed in the colon (28). Glucocorticoids, however, appear to play a central role in regulating ENaC expression in the airway or alveolus (7, 8, 13, 14, 27) and in epithelia lining the male reproductive duct (unpublished observations). Glucocorticoids may also influence ENaC expression in the intestine (28). There appears to be differences in the underlying mechanism(s) by which glucocorticoids stimulate ENaC expression in these tissues. Glucocorticoid receptor knockout mice exhibit reduced lung γ-ENaC compared with their normal littermates, whereas α- and β-ENaC expression are not different (7). Preterm human babies have reduced levels for all ENaC subunits in nasal epithelium, and dexamethasone treatment increased α- and β-ENaC expression (13). H441 cells, which are of bronchiolar origin, respond to dexamethasone with increased mRNA for all ENaC subunits (14), although β-ENaC appears to be affected most (27). Primary cultures of rat alveolar epithelial cells responded to dexamethasone with a fourfold increase in α-ENaC mRNA, whereas less than a twofold change in β- and γ-ENaC mRNA was observed (8). Likewise, our laboratory has observed that glucocorticoids increase the mRNA and protein expression of α-ENaC while having no appreciable effect on β- or γ-ENaC expression in pig vas deferens epithelia (unpublished observations). Thus, bovine mammary epithelium appears to be unique in that glucocorticoids regulate the expression of β- and γ-ENaC while having little effect on α-ENaC. The basis for the differences that have been noted could reflect both the species and tissue of origin. The human α-ENaC gene harbors a GRE in the 5’ flanking region (16). Whether similar response elements regulate β- or γ-ENaC expression is not yet known. Coactivators or repressors could also be expressed in a tissue-specific pattern, which might account for the differences that are reported across tissue sources and even with tissues derived from lungs or airways of a single species. Clearly, additional studies must be conducted to delineate the underlying mechanisms that account for the unique regulation of ENaC expression in mammary epithelium.

This study has provided both functional and molecular evidence for the presence of the amiloride-sensitive apical Na+ channel ENaC in BME-UV monolayers. This absorption pathway could play a fundamental role in regulation of milk composition. It is evident from the present study that corticosteroid exposure increases Na+ absorption from the apical (i.e., milk) compartment. Cortisol is required for the transition to lactation that occurs in the mammary gland at parturition. Colostrum is high in Na+, and milk is low in Na+ (21). The results presented in this article suggest that cortisol induces the transition in milk composition by increasing ENaC expression. Additional studies are required to test this hypothesis.

The ionic composition of milk can be reflected as milk electrical conductivity (9, 32). Milk conductivity is elevated in the instances of mastitis and can be one of the earliest measurable indicators of infection in the bovine mammary gland. Current tenets suggest that breakdown and wholesale loss of cells from the epithelial barrier separating the blood and milk creates this increase in milk conductivity when plasma is allowed to enter the milk compartment. An alternative possibility, however, is that a change in conductivity reflects an early change in epithelial ion transport, for example, a reduction in ENaC activity. Indeed, it was recently reported that TNF-α reduced both the basal and dexamethasone-stimulated expression of α-, β-, and γ-ENaC mRNA in rat lung epithelial cells (8). TNF-α is elevated in mastitic conditions (24) and has been shown to reduce the barrier integrity of BME-UV cell monolayers (25). It was concurrently reported that elevated apical electrolyte concentrations contribute to a breakdown of bovine mammary epithelium (25). The current work defines a mechanism whereby milk electrolytes could be rapidly reduced to promote recovery.

These results suggest that corticosteroids set or modulate the ion composition of milk by inducing ENaC expression. In light of a previous report (25) that showed a causal relationship between reduced apical Na+ concentration and increased mammary epithelial barrier integrity, the current results suggest that a portion of the therapeutic effects associated with synthetic corticosteroids in the treatment of mastitis can be attributed to enhanced Na+ absorption. The results suggest that within 24 h after corticosteroid administration, mammary ENaC expression could be heightened and Na+ reabsorption could be substantial. If one makes a conservative assumption for the bovine mammary epithelial surface area of ~100 m² (equal to the estimated surface area for human lungs) and a Na+ transport rate of ~10 μA·cm⁻² (see Fig. 1), one can compute a maximal Na+ absorption rate of 360 mmol/h. If one further conservatively assumes a milk volume of ~20 liters (which is nearly the maximum for a high-producing dairy cow) and a milk Na+ concentration of 50 meq/l, the total Na+ in the bovine udder would be ~1 mol. Alternatively, colostrum or mastitic milk may be as high as 100 meq/l Na+, and total Na+ could approach 2 mol. Nonetheless, these calculations show that the mammary epithelium is capable of turning over the entire milk Na+ load in a few hours with corticosteroid induction. The authors appreciate that, as milk Na+ concentration is reduced, the rate of Na+ transport would decline and would ultimately be limited by the electrochemical gradient for Na+ across the apical membrane. Regardless, the results from this research project demonstrate that the mammary epithelium is capable of rapidly reducing Na+ concentration to the levels that are typically observed in healthy mature milk.

In summary, this work has provided evidence that corticosteroids modulate Na+ transport across mammary epithelium via a glucocorticoid receptor-mediated process. Changes in specific subunits of ENaC are implicated in this Na+ transport modulation, but additional mechanisms in the pathway have yet to be confirmed or studied. Identifying and exploring these mechanisms are critical steps toward the understanding of mammary gland development, involution, and recovery from pathological challenge.

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