

GLUCOCORTICOIDS INDUCE AMILORIDE-SENSITIVE ION TRANSPORT BY  
PATHWAYS THAT ARE TISSUE-SPECIFIC

by

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B.A., Concordia University, 1988

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology  
College of Veterinary Medicine

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

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## Abstract

The goal of this project was to define mechanisms responsible for Na<sup>+</sup> transport in two hormonally-sensitive epithelium, the bovine mammary gland and porcine vas deferens. Glucocorticoid stimulation in these epithelia results in a significant increase in amiloride-sensitive ion transport, suggesting regulation of the epithelial Na<sup>+</sup> channel, ENaC. ENaC has typically been described as a heteromultimeric ion channel with at least three different types of subunits, the most common being  $\alpha$ ,  $\beta$ , and  $\gamma$ . Glucocorticoid-induced regulation of these subunits at the transcriptional level appears to be very different in the porcine vas deferens as compared to the bovine mammary gland.

The aims of the study in mammary epithelium were to elucidate the mechanisms by which apical electrolytes and cytokines compromise barrier function in mammary epithelium. The long term goal is to better understand and manage the interaction between ionic composition of milk and breakdown of the gland epithelium that occurs during mastitis. Our results suggest a causal link between changes in milk electrical conductivity and epithelial barrier breakdown that has not been appreciated previously. Results will provide benefits to dairy farmers by characterizing steps that might prevent the development of mastitis or hasten recovery.

The aims of the study using porcine vas deferens epithelial cells include determining the time course, concentration- and structure-dependency for regulation of amiloride-sensitive ion flux by corticosteroids. Corticosteroids caused a concentration-dependent increase in amiloride-sensitive  $I_{sc}$  with a rank order of potency of dexamethasone > prednisolone > cortisol. Hill analysis indicates steep concentration dependency. The corticosteroid-induced, amiloride-sensitive current is Na<sup>+</sup> absorption as indicated by radiotracer flux measurements. Studies employing selective antagonists (spironolactone, mifepristone) define glucocorticoid receptor mediation. These results suggest that vas deferens epithelia are exquisitely sensitive to corticosteroid exposure. Observed changes in epithelial function in response to corticosteroid exposure would rapidly and chronically affect the luminal environment to which sperm are exposed. Thus, physiological and pharmacological corticosteroid exposure is expected to affect male fertility.

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Approved by:

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## **Dedication**

To my wonderful family (all of you!) without whom none of this would have been possible. All my love.

## CHAPTER 1 - General Introduction

The role of glucocorticoids in regulation of ion transport across epithelial tissues appears to be specific to each tissue. Ion transport across small intestinal epithelium reveals a highly absorptive capacity that gradually declines as an organism ages (Cooke and Dawson, 1978; Dawson and Cooke, 1978; Buddington and Diamond, 1989). Piglets delivered by caesarian section demonstrate lower cortisol levels and exhibit higher intestinal ion transport capacity as compared to piglets delivered vaginally, and corticosteroid administration in these piglets alters intestinal ion transport capacity (Chapple et al., 1989; Bate et al., 1991; Connell et al., 1995). Similarly, ion transport across lung epithelial tissues is drastically altered immediately after delivery as the lung changes from a fluid-filled environment to an air interface responsible for maintaining oxygen delivery to the organism (Frank et al., 1980; Itani et al., 2002). This process has also been correlated to glucocorticoid levels within the organism (Itani et al., 2002; Ramminger et al., 2004). Exogenous glucocorticoid administration affects  $\text{Na}^+$  transport across both intestinal epithelia (Shepherd et al., 1980; James et al., 1987; Rhoads et al., 1988) and lung epithelia (Ramminger et al., 2004). The role of glucocorticoids in some organs, such as the lung and intestine, have been studied extensively, while the effects of glucocorticoids in other glands or organs have not been examined.

This dissertation focuses on glucocorticoid regulation of  $\text{Na}^+$  transport across two very different hormonally-sensitive epithelia: bovine mammary epithelium and porcine vas deferens epithelium. The dissertation does not attempt to compare and contrast the two tissues. Rather, it identifies two very different mechanisms by which glucocorticoids alter amiloride-sensitive  $\text{Na}^+$  transport across these epithelia. Glucocorticoid-induced  $I_{sc}$  is examined at both the functional and molecular levels in each of these tissues. The goal is to determine the mechanistic basis for glucocorticoid-induced alteration of  $\text{Na}^+$  transport across these epithelia.

Regulation of  $\text{Na}^+$  movement across these epithelia has the potential to produce significant changes in the ionic composition of luminal fluids. Bovine mammary epithelia produce and maintain a low  $\text{Na}^+$  fluid that nourishes the offspring. A large ion concentration gradient is maintained across the epithelium in the lactating gland. This gradient assists in supporting fluid secretion across the epithelium and is critical for sustaining the quality and

nutritional content of the milk. Ion transport across the mammary epithelium has been shown through in vitro studies to be a highly regulated process.

The mammary epithelium secretes a high volume of nutrient-rich fluid that differs from serum and other ‘typical’ bodily fluids in its electrolyte composition as well as being high in carbohydrates, proteins, and fat. Typical U.S. dairy cows, depending on the breed, produce 30 to 35 kg/day of milk containing approximately 5% lactose, 3% protein, and 4% fat by weight. Milk is a rich source of  $\text{Ca}^{2+}$  (60 mEq/L), has relatively low  $\text{Na}^+$  (20 mEq/L) and  $\text{Cl}^-$  (30 mEq/L), and high  $\text{K}^+$  (40 mEq/L), resulting in an isotonic fluid (Sorensen et al., 2001; Ontsouka et al., 2003; Miller et al., 2005). Much work has been expended to define mammary epithelial mechanisms associated with carbohydrate, protein, and fat secretion. Substantially less is known regarding the monovalent ion transport mechanisms that generate and maintain these substantial ion gradients across this highly specialized epithelium.

Inflammatory challenge to the mammary gland, commonly called mastitis, results in a rapid and significant change in luminal ionic composition. This change can be measured as an increase in milk electrical conductivity and is used by some in the dairy industry to identify animals with mastitis. Common tenets of mastitis progression suggest that inflammatory mediators, including interleukin-6, interleukin-1 $\beta$ , and TNF- $\alpha$ , incite damage that breaks down the epithelial barrier and allows blood to enter the milk compartment, thus increasing milk electrical conductivity. Our results suggest the need to reconsider this concept. The results included in this dissertation demonstrate a rapid and significant breakdown in barrier function that occurs after a change in luminal electrolyte concentration, and much more rapidly than the breakdown induced by inflammatory mediators.

Dexamethasone exposure has been identified as an effective treatment for mastitis, and the mechanism of action is currently thought to include only suppression of inflammation. This study identifies an alternative or additional explanation for glucocorticoid-induced improvement in gland function. The effect of glucocorticoids on  $\text{Na}^+$  movement across the epithelium would limit or reverse the mastitis-induced change in milk conductivity and thus enhance the epithelial barrier. Thus, we examined the effects of glucocorticoids on epithelial  $\text{Na}^+$  transport via ENaC.

Altered ion transport mechanisms in the vas deferens lead to dysfunction in the organ. Cystic fibrosis (CF) patients express mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR) and have been shown to exhibit abnormal  $\text{Cl}^-$  and  $\text{Na}^+$  movement

across affected epithelia. Ninety-seven percent of adult male CF patients lack a vas deferens. The vas deferens develops normally during gestation in affected individuals. Lack of a vas deferens, a condition termed congenital bilateral absence of the vas deferens (CBAVD), develops sometime between birth and adolescence in CF males.

Non-classical CF patients, those who do not exhibit profound mutations that lead to pancreatic, lung, and intestinal complications, may still present with reproductive complications. Eighty-two percent of males who present at fertility clinics in the United States and are subsequently diagnosed with an obstructive disorder of the vas deferens (like CBAVD), harbor one or two mild CF mutations when genotyped. The importance of optimal ion transport in this tissue is apparent. Because CF is a disease of  $\text{Cl}^-$  transport, and because all CF patients secondarily demonstrate altered  $\text{Na}^+$  transport, the work included in this dissertation is critical to understanding the effects of epithelial ion transport on fertility.

Regulation of ion movement across vas deferens epithelium has the potential to alter luminal fluid composition and volume. A rapid and significant change in luminal fluid composition has the potential to alter sperm motility or function. A better understanding of the ion transport mechanisms responsible for maintaining luminal environment of the vas deferens might lead to new targets for fertility intervention or targets to maintain a patent duct in situations where the duct might become compromised.

The mechanisms of glucocorticoid regulation of channel function in vas deferens epithelium have not been studied previously. The results demonstrate that corticosteroids modulate amiloride-sensitive  $\text{Na}^+$  transport, which would affect the environment to which sperm are exposed. These results are similar to what is known of regulation of channel function in the lung, another tissue significantly affected in cystic fibrosis. Whereas bronchial epithelium is difficult to maintain in primary culture, the primary culture of vas deferens epithelium has been very successful. It is possible that our results might elucidate further pathways to explore within lung tissue in disease states such as cystic fibrosis.

Following is an exploration of glucocorticoid effects on  $\text{Na}^+$  transport across porcine vas deferens epithelium and bovine mammary epithelium. Additional exploration of mechanisms leading to altered  $\text{Na}^+$  absorption and the direct effects of increased luminal  $\text{Na}^+$  ion concentration on the epithelium are included. Each of the next three chapters has been submitted independently for publication in peer reviewed journals, and are independent. Each chapter

addresses an independent examination of Na<sup>+</sup> movement or challenge to epithelial monolayers in these tissues, and each set of experiments explores a unique question. An independent introduction to each project is provided at the beginning of each chapter, and discussion of the project results are included at the end of each chapter, with a separate final discussion following these chapters. The primary goal of this project is focused mainly on examining Na<sup>+</sup> transport via the epithelial Na<sup>+</sup> channel, ENaC, in these two very different tissues. When viewed together, however, the very different regulatory mechanisms that lead to Na<sup>+</sup> absorption through ENaC in these tissues provides a better understanding of the specificity and importance of ion transport regulation within each individual tissue.

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## **CHAPTER 2 – Glucocorticoids upregulate Na<sup>+</sup> transport across porcine vas deferens epithelia**

Manuscript has been submitted for publication under peer review and is currently being revised based upon reviewer's comments.

## **CHAPTER 2 - Glucocorticoids upregulate Na<sup>+</sup> transport across porcine vas deferens epithelia**

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CONDENSED TITLE: Ion transport modulation in porcine vas deferens epithelia

KEYWORDS: short circuit current, amiloride-sensitive epithelial ion transport, ENaC, corticosteroids, human vas deferens.

### **ABSTRACT**

Cystic fibrosis, a disease affecting HCO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup> and Na<sup>+</sup> transport, has been inextricably linked to male infertility that, in most cases, includes anatomical changes to the vas deferens. Hypercortisolemia and psychological stress have also been linked with ion transport anomalies and with infertility. The aim of this study was to determine the basis of amiloride-sensitive ion transport across vas deferens epithelium induced by exposure to corticosteroids with the working hypothesis that epithelial sodium channel (ENaC) expression and/or activity is affected. Cells lining porcine vas deferens were isolated and grown to confluence on permeable supports. Exposure to natural or synthetic corticosteroids (cortisol, dexamethasone, prednisolone) caused a concentration-dependent increase in amiloride-sensitive transepithelial ion transport measured as short-circuit current ( $I_{sc}$ ) in a modified Ussing chamber system. RT-PCR and western blot analyses revealed a glucocorticoid-induced increase in the number of mRNA copies of  $\alpha$ -ENaC and in the amount of immunoreactivity, but no effect on the  $\beta$ - or  $\gamma$ -ENaC mRNA or immunoreactivity. Timecourse studies revealed that  $I_{sc}$  and  $\alpha$ -ENaC mRNA and  $\alpha$ -ENaC

immunoreactivity increases within two hours of ongoing glucocorticoid exposure and remains elevated for the duration of exposure, up to 5 days. Studies employing selective corticosteroid receptor antagonists (spironolactone, mifepristone) indicate mediation by a glucocorticoid receptor. Radiotracer flux measurements suggest that net corticosteroid-induced  $\text{Na}^+$  absorption is greater than  $I_{sc}$ , although both  $I_{sc}$  and net flux were reduced to zero by  $10\mu\text{M}$  amiloride. Observed changes in epithelial function in response to corticosteroids would be expected to rapidly and chronically affect the luminal environment to which sperm are exposed. Thus, physiological and pharmacological corticosteroid exposure is expected to modify the volume and composition of fluid in the lumen of the vas deferens, and may thereby affect male fertility.

## INTRODUCTION

The lining of the vas deferens is a dynamic epithelium that reacts to neurotransmitter stimulation and is involved in active ion transport which modulates the luminal environment (Sedlacek et al., 2001; Carlin et al., 2003; Carlin et al., 2006; Hagedorn T, 2006). Many studies have focused on the morphology of the vas deferens (Hoffer, 1976; Harris and Coleman, 1989; Nistal et al., 1992; Gupta et al., 1994; Regadera et al., 1997; Manivannan et al., 1999), while a few have identified ion transport mechanisms present in the epithelium (Pollard et al., 1991; Bertog et al., 2000; Carlin et al., 2002). Only one, however, has examined steroid modulation of epithelial barrier function and ion transport (Phillips and Schultz, 2002). None have provided an in-depth analysis regarding corticosteroid modulation of specific ion transport mechanisms that affect luminal ionic composition. Regulated anion and cation transport across the epithelium lining the deferent duct is crucial to maintain the appropriate environment for sperm storage and activation. Delineation of the corticosteroid-regulated pathway will identify targets to address infertility and contraception in males, as well as define the progression of ion transport-related diseases in this secretory epithelium.

The epithelial  $\text{Na}^+$  channel, ENaC, and the cystic fibrosis transmembrane conductance regulator (CFTR), which conducts both  $\text{HCO}_3^-$  and  $\text{Cl}^-$ , have been identified in distal reproductive duct epithelium of humans and pigs (Tizzano et al., 1994; Carlin et al., 2006), and in other species (Wong, 1998; Ruz et al., 2004), although CFTR is reportedly absent in rat vas deferens (Tizzano et al., 1994).  $\text{K}^+$  channels and various co-transporters (Pollard et al., 1991; Bertog et al., 2000; Sedlacek et al., 2001; Carlin et al., 2006), as well as a number of  $\text{Na}^+$ -

dependent transport mechanisms ( $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$ , and the  $\text{Na}^+ \text{HCO}_3^-$  cotransporter) have all been identified in this epithelium (Sedlacek et al., 2001). A regulatory link has been suggested between  $\text{Na}^+$  transport via ENaC and  $\text{Cl}^-$  transport via CFTR (Stutts et al., 1997; Donaldson et al., 2002; Huang et al., 2004), which includes evidence that an increase in CFTR expression results in enhanced ENaC activity (Stutts et al., 1997). It is particularly important to understand the regulatory mechanisms for ENaC, which is the only  $\text{Na}^+$  transport mechanism positively identified at the apical membrane of vas deferens epithelia to date. ENaC is a heteromultimeric channel with  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and in some cases  $\delta$ -subunits (Waldmann et al., 1995; Kosari et al., 1998; Alvarez de la Rosa et al., 2000).

Anomalies in epithelial  $\text{Na}^+$  and  $\text{Cl}^-$  transport and/or its regulation contribute to subfertility and infertility. Defects in mechanisms that mediate  $\text{Cl}^-$  secretion and  $\text{Na}^+$  absorption across vas deferens epithelia might be linked to the pathology evident in transport-related diseases like cystic fibrosis (CF; (Jarvi et al., 1998; Sokol, 2001)). CF has classically been considered a disease of anion transport, but also includes symptoms related to  $\text{Na}^+$  hyperabsorption (Aickin and Brading, 1990; Stutts et al., 1997; Donaldson et al., 2002). Cases of CF have been identified in which CFTR expression is normal but genotyping identifies anomalies in ENaC alleles (Sheridan et al., 2005). CFTR mediates movement of both  $\text{Cl}^-$  and  $\text{HCO}_3^-$  (Konig et al., 2002; Kunzelmann and Mall, 2002; Kunzelmann et al., 2002; Irokawa et al., 2004; Sasamori et al., 2004; Melvin et al., 2005; Pena-Munzenmayer et al., 2005; Steward et al., 2005), and the balance of other ion transport, including  $\text{Na}^+$  transport, is intimately related to anion flux (Stutts et al., 1997). CF is intimately and universally linked to male infertility including > 97% of male patients with congenital bilateral absence of the vas deferens (CBAVD) (Anguiano et al., 1992; Hanrahan et al., 1995; Ferrari and Cremonesi, 1996; Ferrari et al., 1996; Padoan et al., 1996; de la Taille et al., 1998). Males with mild CF also present with CBAVD (~97%), and CBAVD is the only symptomatic identifier in some patients in which CFTR mutations are discovered only after reproductive failure (Traystman et al., 1994; Jakubiczka et al., 1999). Additionally, a disproportionately high number of men exhibiting asthenozoospermia and idiopathic infertility harbor at least one mutated CFTR allele (de la Taille et al., 1998; Wong, 1998; Reynaert et al., 2000; Bosard et al., 2005; Hess et al., 2005). Beyond obstructive disorders, these mutations resulting in dysregulated ion transport demonstrate that although sperm are still delivered through the reproductive tract, they are unable to function normally.

Additional disorders that affect ion transport and are associated with infertility include hyper- and hypo-cortisolemia (Tiitinen and Valimaki, 2002; New, 2004; Klimek et al., 2005; Kowal et al., 2006). Also, idiopathic stress-associated infertility is suggested to be linked with elevated cortisol (Oehninger, 2003; New, 2004). Elevated cortisol levels have been associated with increased  $\text{Na}^+$  absorption in many epithelial tissues. Initial studies focusing on porcine vas deferens indicated that the epithelial lining of distal male reproductive duct responds to corticosteroids with increased amiloride-sensitive ion transport (Phillips and Schultz, 2002). However, specific causal links between abnormal epithelial cation transport and infertility remain to be determined.

The primary goal of this work is to examine ion transport, specifically  $\text{Na}^+$  transport, across vas deferens epithelium and the response of these ion transport mechanisms to corticosteroid hormones. This study provides a complete analysis of the time course and concentration-dependency of corticosteroid modulation of  $\text{Na}^+$  absorption across the vas deferens epithelium. Additionally, investigation is provided into the receptor-mechanisms leading to this response. Demonstration of amiloride-sensitive current in human vas deferens (Carlin et al., 2003) validates the porcine vas deferens as a model system for further investigation that will allow inferences to the human reproductive tract. Results indicate that corticosteroids modulate amiloride-sensitive current in primary cultures of porcine vas deferens epithelial cells via ENaC and define mifepristone inhibition of this modulation, suggesting a glucocorticoid receptor-mediated pathway. The outcomes define mechanisms that can be targeted to modulate male fertility, either to address male factor infertility or as a means of contraception.

## **MATERIALS AND METHODS**

***Primary cell culture and corticosteroid treatments.*** Methods for culture of primary porcine vas deferens (PVD) cells have been published previously (Sedlacek et al., 2001; Carlin et al., 2006). Briefly, reproductive tracts from boars were obtained immediately post mortem from a local swine production facility and placed in ice cold Ringer solution (composition in mM; 120 NaCl, 25  $\text{NaHCO}_3$ , 3.3  $\text{KH}_2\text{PO}_4$ , 0.83  $\text{K}_2\text{HPO}_4$ , 1.2  $\text{CaCl}_2$ , and 1.2  $\text{MgCl}_2$ ), packed in ice, and brought to the laboratory for processing. Cells were dissociated from the lumen of the duct and grown in 25  $\text{cm}^2$  plastic culture flasks (Cellstar, Frickenhausen, GE) to 90% confluence. After dissociation, cells were seeded to Costar Snapwell 1.13  $\text{cm}^2$  permeable supports (Cambridge,

MA) and cultured for 2 weeks in growth medium (Dulbecos Modified Eagle Medium; Invitrogen, Baltimore, MD; 10% fetal bovine serum (Invitrogen); penicillin (100 U/ml) and streptomycin (100 µg/ml)) prior to assay. Experiments were paired within treatments such that, within a single 6-well plate, all treatments in a particular study were represented. Monolayers on permeable supports were exposed to both natural and synthetic corticosteroids, including dexamethasone, prednisolone or cortisol in the basolateral medium at varying concentrations (as described for each set of experimental paradigms) or for specific time periods prior to assessment of basal  $I_{sc}$  in modified Ussing chambers. Upon exposure to corticosteroids, cells were fed daily. Mifepristone or spironolactone were included with the corticosteroid treatments as indicated to test for the involvement of glucocorticoid- or mineralocorticoid-receptor mediated processes, respectively. Amiloride, benzamil (selective blockers of the epithelial  $\text{Na}^+$  channel, ENaC), or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; an amiloride analog that preferentially inhibits the  $\text{Na}^+/\text{H}^+$  exchanger, NHE) were added at varying concentrations for pharmacological profiling as described.

***Electrophysiology.*** Transepithelial electrical resistance ( $R_{te}$ ) and short-circuit current ( $I_{sc}$ ), indicators of net  $I_{sc}$ , and therefore ion transport, were assessed in a modified Ussing chamber. Ringer solution was included bilaterally in the Ussing chambers and held at 39° C, continuously bubbled with 5%  $\text{CO}_2$ -95%  $\text{O}_2$ , to achieve constant mixing and to maintain pH, and electrophysiological measurements were made with a voltage-clamp apparatus (model 558C, University of Iowa, Department of Bioengineering, Iowa City, IA). Data were acquired digitally at 1 Hz with a Macintosh computer using an MP100A-CE interface and Aqknowledge software (ver. 3.2.6, BIOPAC Systems, Santa Barbara, CA). Monolayers were exposed to a 5 mV bipolar pulse every 100 seconds and  $R_{te}$  was determined using Ohm's law. Amiloride (Sigma, St. Louis, MO; 10 µM) was added apically or basolaterally to the chambers as described.

***$\text{Na}^+$  flux studies.*** PVD cell monolayers were prepared as described above, in both untreated and corticosteroid-treated conditions, and  $\text{Na}^+$  flux was measured over sequential 20 minute periods under short circuit conditions as described above. Briefly, tracer amounts of  $^{22}\text{Na}^+$  were included in either the apical or basolateral compartment of paired Ussing chambers. Samples were taken from the chamber containing isotope ("hot") chamber to determine specific activity and from the opposite chamber to establish baseline activity. After 20 minutes, a second sample was taken from the chamber that was not initially exposed to isotope ("cold") side to

determine unidirectional flux. Net Na<sup>+</sup> movement was determined by subtraction of unidirectional fluxes in paired monolayers and compared to the integrated  $I_{sc}$ . Amiloride was added apically to all monolayers and again a baseline sample and 20 minute sample were obtained and evaluated to determine unidirectional and net Na<sup>+</sup> fluxes.

**Western blot analysis.** Western blot analysis for glucocorticoid receptor immunoreactivity or ENaC immunoreactivity was performed to test for the presence of the respective epitopes and to determine whether selected treatments affect epitope expression. PVD cell lysates were prepared in Snapwell filters using RIPA lysis buffer including 1% Protease Inhibitor Cocktail (Sigma). Cell monolayers were broken apart by needle aspiration, and cells were rocked 4 hours at 5°C in lysis buffer. Total protein content was determined using a Bicinchoninic Assay (Pierce, Rockford, IL) and 20 µg total protein was loaded in each well of a 10-20% or 8-16% polyacrylamide Tris-HCl Ready Gel pre-cast gel (BioRad, Hercules, CA) for electrophoresis. Proteins were resolved at 160 V for 45 minutes, then transferred to Millipore (Burlington, MA) PVDF-Immobilon membranes for 8.5 hours at 60 V. Transfer was confirmed by staining gels with Gelcode Blue (Pierce). Membranes were blocked in 5% fat-free milk, and probed with anti-α-ENaC, anti-β-ENaC and anti-γ-ENaC antibodies (Ambion, Austin, TX; diluted 1:100), or anti-glucocorticoid-receptor (Affinity BioReagents, Golden, CO; diluted 1:5000) in Tris-buffered saline including 1% Tween 20. Immunoreactivity was visualized using a peroxidase-conjugated secondary antibody (1:12,500) 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.

**Quantitative RT-PCR (qPCR).** To test for and quantify mRNA for ENaC subunits, total RNA was isolated from confluent monolayers of PVD cells that had been cultured for 14 days on Snapwell tissue culture inserts using Qiagen Shredder columns and Qiagen Micro RNeasy RNA isolation kit (Qiagen, Valencia, CA) 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 ribosomal RNA. All RNA samples were subjected to DNase 1 treatment (Ambion Inc.) according to the manufacturer's specifications and analysis controls were used to confirm no DNA contamination of samples. Initial quantification of total RNA for loading was performed in an ND-1000 NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington,

DE) using OceanOptics USB2000-EEPROM Load software with the concentration calculation utilizing the Beer-Lambert equation. RT-PCR was carried out (OneStep RT-PCR, Qiagen) using primer pairs specific for coding sequences of  $\alpha$ -ENaC,  $\beta$ -ENaC,  $\gamma$ -ENaC in paired reactions with both untreated and 72-hour dexamethasone-treated monolayers. Quantitative analysis was performed using SYBR-Green (Molecular Probes, Eugene, OR) and run on a Cepheid SmartCycler (Cepheid, Sunnyvale, CA). RT-PCR amplicons were resolved by electrophoresis in a 1.5 % agarose gel and product identity was verified by sequence analysis. Analysis for the presence of  $\delta$ -ENaC mRNA was not included in this study. Reactions with no reverse transcriptase step were included to eliminate the possibility of DNA contamination.

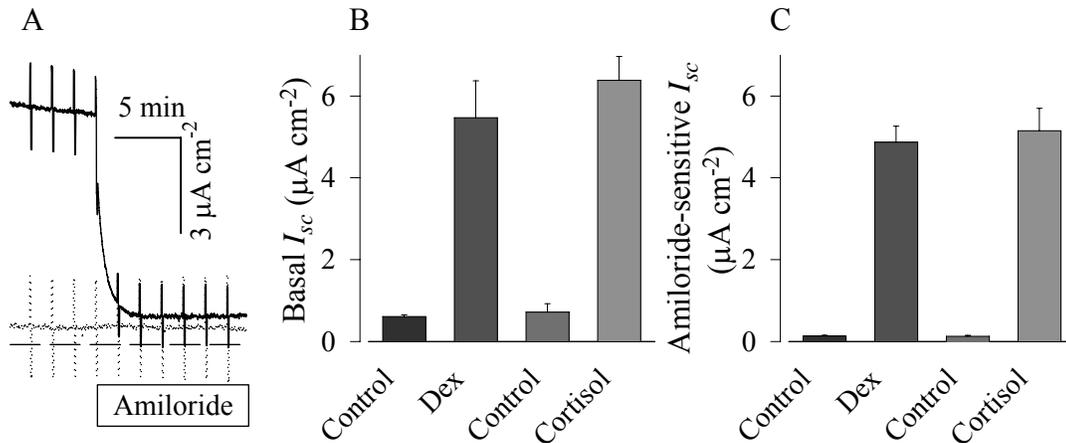
**Data Analysis.** Results were analyzed using a SAS statistical analysis program (SAS Institute Inc., Cary, NC). Data are presented as mean  $\pm$  SEM. The difference between control and treatment data was analyzed using ANOVA and Student's t-test. Differences are considered statistically significant when the probability of a type I error is  $< 0.05$ . Hill analysis was performed with Sigma Plot (Systat Software Inc., Point Richmond, CA). Data sets were fitted by the equation  $y = a * x^b / (c^b + x^b)$ , where  $y$  = measured outcome (e.g.  $\Delta I_{sc}$ ),  $a$  = maximum effect of the treatment (e.g.  $\Delta I_{sc \text{ max}}$ ),  $x$  = concentration,  $b$  = Hill coefficient and  $c$  = apparent  $K_d$  ( $K_{app}$ ), which is the concentration of drug giving a half-maximal response.

## RESULTS

This investigation sought to determine the basis, time course and concentration-dependency for corticosteroid-induced amiloride-sensitive ion transport.

**Corticosteroid exposure elicits amiloride-sensitive elevation in basal  $I_{sc}$ .** Primary culture monolayers of porcine vas deferens epithelial cells were exposed to natural or synthetic corticosteroids for 72 hours prior to assessment of basal  $I_{sc}$ . Elevation of basal  $I_{sc}$  is evident in corticosteroid-treated as compared to vehicle-treated vas deferens epithelial monolayers in a typical  $I_{sc}$  tracing (Fig 2-1A). At the outset of the trace the dexamethasone-treated monolayer (solid line) exhibits much greater ion transport than the vehicle-control monolayer (broken line). Amiloride exposure at the apical membrane elicits a sharp decrease in  $I_{sc}$  across corticosteroid-treated epithelium, whereas there is no effect of amiloride on the vehicle-treated monolayer. In the presence of amiloride, the amount of short circuit current across the corticosteroid-treated monolayer is virtually identical to that of the vehicle-treated control. Data from a total of fifteen

similar experiments are summarized in panels B and C. Dexamethasone and cortisol consistently induced an initial  $I_{sc}$  that was similar in magnitude (Fig 2-1B) and was amiloride sensitive (Fig 2-1C). These results demonstrate that natural or synthetic glucocorticoids induce amiloride-sensitive  $I_{sc}$ , which suggests mediation by a glucocorticoid receptor.



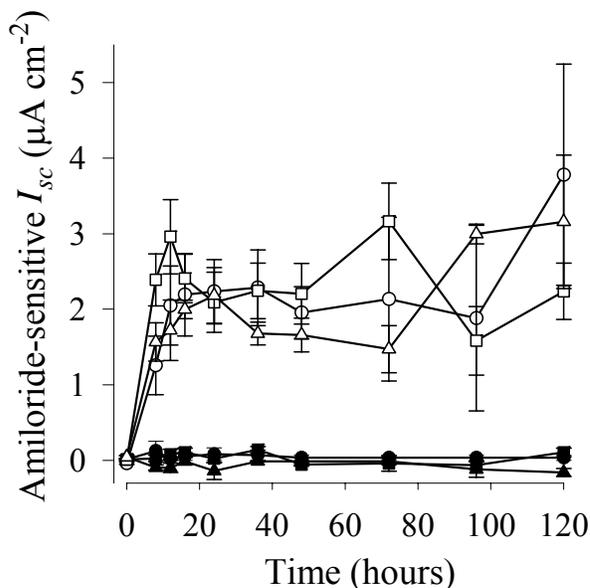
**Figure 2-1 Corticosteroid exposure elicits amiloride-sensitive elevation in basal  $I_{sc}$ .**

Epithelial monolayers were cultured in the presence of cortisol (500 nM) or dexamethasone (Dex; 100 nM) for three days prior to evaluation. A) Typical tracings demonstrate upregulated initial  $I_{sc}$  in a dexamethasone-treated monolayer (solid line) as compared to untreated (broken line). Upregulated current is inhibitable by apical amiloride (10  $\mu\text{M}$ ) exposure as indicated by the bar. Dashed line indicates zero current. B) Summary results indicate that 72 hour exposure to natural or synthetic corticosteroids consistently induces an increase in basal  $I_{sc}$ . C) Subsequent exposure to amiloride (10  $\mu\text{M}$ ) demonstrates inhibition of elevated  $I_{sc}$ . Results are summarized from 15 observations paired within animal, isolation, and snapwell block for each control and experimental condition. Values from the  $I_{sc}$  tracings shown are included in the summary information.

***Corticosteroid-induced change in basal  $I_{sc}$  is dependent on exposure duration.***

To approach the question of the mechanism(s) responsible for basal  $I_{sc}$  elevation and amiloride-sensitivity, a timecourse for elevated  $I_{sc}$  was determined. Elevation in amiloride-sensitive basal  $I_{sc}$  was evident as early as 2 hours after exposure to either natural or synthetic corticosteroids, including dexamethasone, cortisol, and prednisolone (Fig 2-2). Elevation of  $I_{sc}$  began to plateau by 24 hours, and remained elevated in all circumstances for at least 5 days during continuous corticosteroid exposure. This rapid timecourse for induction of transport suggested either a

change in transporter trafficking to/from the membrane, or could have been due to increased transporter transcription or translation.

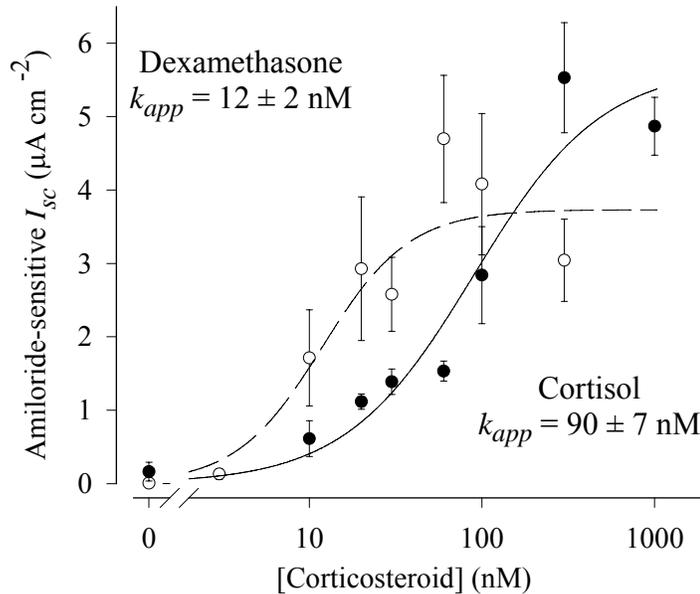


**Figure 2-2 Corticosteroid-induced elevation in basal  $I_{sc}$  that is amiloride-sensitive is dependent on duration of exposure.**

Exposure to natural or synthetic corticosteroids time-dependently induces an increase in amiloride-sensitive  $I_{sc}$  with significant amiloride-sensitive current being observed within two hours of ongoing exposure. Open symbols represent corticosteroid-exposed monolayers, filled symbols represent vehicle exposed observations on tightly paired monolayers (circles = cortisol, 500 nM; squares = dexamethasone, 100 nM; triangles = prednisolone, 1  $\mu$ M). Data points represent the mean and SEM of 5 to 8 observations for each condition. The inset is a magnification of the 0 – 12 hour time period, and demonstrates the rapid response of vas deferens epithelial monolayers to corticosteroid exposure.

**Change in basal  $I_{sc}$  is dependent on corticosteroid concentration.** To evaluate the corticosteroid concentration-dependence of amiloride-sensitive  $I_{sc}$ , and to determine the associated  $K_{app}$  for each, monolayers were exposed to various concentrations of either natural or synthetic corticosteroids. The effect of both dexamethasone and cortisol on amiloride-sensitive  $I_{sc}$  was concentration-dependent (Fig 2-3). Monolayers were exposed to corticosteroids for 72 hours at the indicated concentrations. Significant effects were observed with as little as 10 nM of natural or synthetic glucocorticoids. Aldosterone exposure, on the other hand, demonstrates significant effects by 10 $\mu$ M, which is consistent with aldosterone effects at a glucocorticoid receptor rather than a mineralocorticoid receptor. Analysis indicates that dexamethasone has higher potency with  $K_{app}$  of  $12 \pm 2$  nM (derived from Hill analysis), whereas for cortisol the fit revealed a  $K_{app}$  of  $90 \pm 7$  nM (Fig 3). The Hill coefficient did not differ from unity for either

ligand and the dynamic range is consistent with physiologically and pharmacologically relevant concentrations for corticosteroid-induced upregulation of amiloride-sensitive  $\text{Na}^+$  transport in other tissues (Matalon & O'Brodivich, 1999; Garty, 1994; Kleyman and Cragoe, 1990). The rank order of stimulation and absolute values are consistent with signaling via a glucocorticoid receptor.



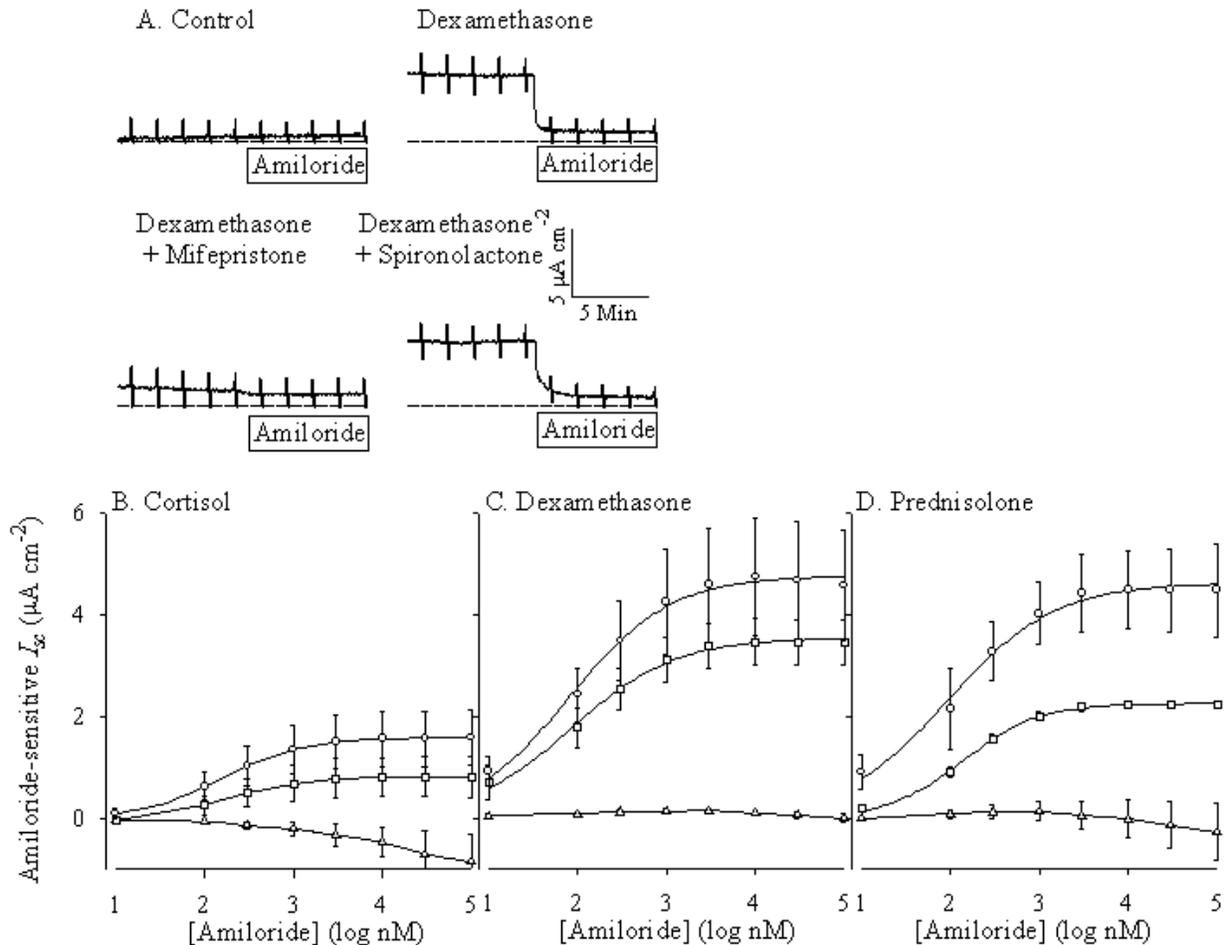
**Figure 2-3 Corticosteroid-induced amiloride-sensitive  $I_{sc}$  is concentration-dependent.**

Chronic exposure to synthetic or natural corticosteroids increases amiloride-sensitive  $I_{sc}$  across porcine vas deferens epithelial cells. Dexamethasone, cortisol, or aldosterone was added to the culture medium for 72 hours prior to evaluation. Aldosterone-induced increase in amiloride-sensitive  $I_{sc}$  is consistent with effects through a glucocorticoid receptor. Data points represent the mean and SEM of 8 or more observations for each condition. Lines represent the best fit of a modified Hill equation to each data set. Hill coefficient = unity.

***Mifepristone, but not spironolactone, inhibits corticosteroid-induced amiloride-sensitive  $I_{sc}$ .*** To test further for the involvement of glucocorticoid- or mineralocorticoid-receptor mediated processes in  $I_{sc}$  elevation, PVD monolayers were exposed to natural or synthetic corticosteroids for 72 hours in conjunction with either mifepristone or spironolactone. Mifepristone, a selective glucocorticoid-receptor inhibitor, precludes corticosteroid-induced elevation in amiloride-sensitive  $I_{sc}$  across PVD monolayers, as can be seen in typical  $I_{sc}$  traces (Fig 2-4A). However, spironolactone, a selective inhibitor of mineralocorticoid receptor, does

not inhibit upregulation of amiloride-sensitive  $I_{sc}$  in these cells. Although spironolactone may demonstrate some abrogation of  $I_{sc}$  at the concentration used (10  $\mu\text{M}$ ), corticosteroid-stimulated amiloride-sensitive  $I_{sc}$  is clearly present. Data are summarized in Fig 2-4B. In all cases, the effect of corticosteroids is absent in monolayers exposed to mifepristone whereas amiloride-sensitive  $I_{sc}$  was observed in all other monolayers exposed to glucocorticoids, regardless of whether spironolactone was present. These results, along with the lack of aldosterone effect reported above, provide strong evidence that glucocorticoid receptors mediate changes in amiloride-sensitive  $I_{sc}$  across vas deferens epithelium.

***Spironolactone does not alter  $K_{app}$  for amiloride in monolayers exposed to either natural or synthetic corticosteroids.*** Exposure to mifepristone abrogates corticosteroid-induced  $I_{sc}$  in PVD monolayers. The partial inhibition by spironolactone in Fig 2-4; panels B-D may reflect the action of dexamethasone or cortisol at the mineralocorticoid receptor, partial inhibition of glucocorticoid receptor by spironolactone, or a non-specific effect of spironolactone on vas deferens ion transport. If the latter alternative is true, one would expect different kinetics to be revealed by spironolactone when compared to monolayers treated with only corticosteroids. Thus, the amiloride sensitivity of  $I_{sc}$  induced by cortisol, dexamethasone, and prednisolone in the absence or presence of mifepristone and spironolactone was assessed (Fig 2-4; panels B-D).  $I_{sc\ max}$  for the three agonists derived from fits of the Hill equation were 1.59, 4.78, and 4.64  $\mu\text{A cm}^{-2}$  respectively. Results again show complete inhibition of corticosteroid-induced  $I_{sc}$  by mifepristone. The magnitude of  $I_{sc}$  was less with spironolactone, with the  $I_{sc\ max}$  determined to be 0.83, 3.56, and 2.26  $\mu\text{A cm}^{-2}$ , respectively. However, the concentration dependence for amiloride inhibition was consistent in the presence and absence of spironolactone. Amiloride exhibits similar concentration dependency for all corticosteroids ( $K_{app}$  range 81-213 nM; Hill coefficient range 0.71 – 1.02), both in the absence and presence of spironolactone. The spironolactone-associated reduction in  $I_{sc\ max}$  may reflect a lack of specificity for mineralocorticoid receptors at the concentration of spironolactone that was used. These results demonstrate a reduction in apparent maximal ion transport rate as opposed to a change in  $K_{app}$ ; a similar kinetic fingerprint for amiloride is observed in the absence and presence of spironolactone. These results are interpreted to indicate that spironolactone at 10  $\mu\text{M}$  is partially inhibiting action of corticosteroids at the glucocorticoid receptor.

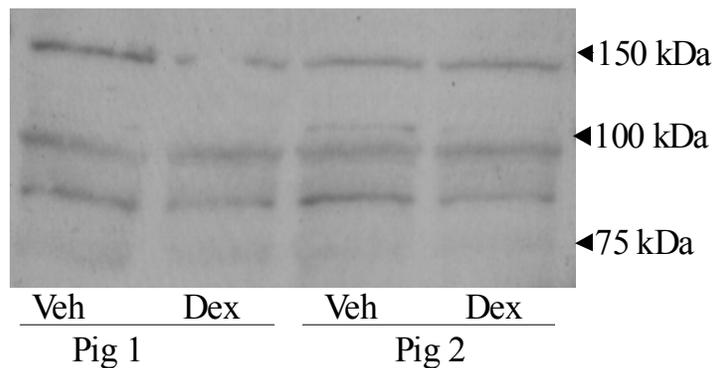


**Figure 2-4 Mifepristone, but not spironolactone, inhibits corticosteroid-induced amiloride-sensitive elevation in basal  $I_{sc}$ , although concentration dependence for amiloride inhibition is unchanged by spironolactone.**

A) Typical tracings from paired monolayers demonstrate no corticosteroid-induced difference, when compared to control, for the mifepristone (Mif; 10 μM)-exposed monolayers. However spironolactone (Spir; 10 μM)-exposed monolayers responded similarly to dexamethasone (Dex; 100nM)-exposed monolayers. B-D) Amiloride-sensitive  $I_{sc}$  is observed across vas deferens epithelial monolayers exposed to corticosteroids (circle; various concentrations), and this  $I_{sc}$  is precluded by mifepristone (triangle; 10 μM), but not spironolactone (square; 10 μM). Data points represent the mean and SEM of 6 observations for each condition. Lines represent the best fit of a modified Hill equation to each data set for corticosteroid- and spironolactone-treated monolayers, and dot to dot lines for mifepristone-treated monolayers. Parameters of each fit are presented in the text.

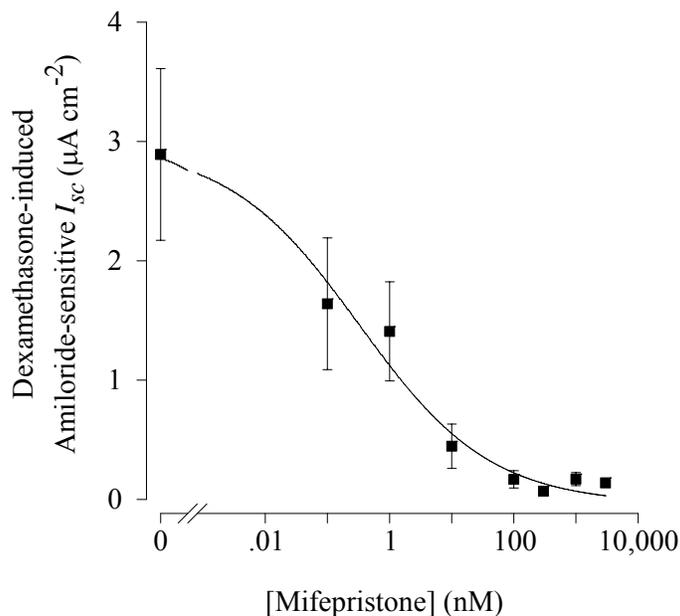
**Glucocorticoid receptor is present in PVD cells at both the transcriptional and translational levels.** RT-PCR and Western blot analysis were performed to test for the presence of glucocorticoid-receptor mRNA and protein in PVD monolayers. Results from this laboratory provide evidence for full length glucocorticoid receptor  $\alpha$  (GR  $\alpha$ ) in PVD cells at the

transcriptional level (Genbank accession #AY779185). Amplicons for GR  $\beta$  and GR  $\alpha\beta$  were also observed and verified by sequence analysis (data not shown). Western blot analysis revealed multiple immunoreactive bands that are at the expected mobility for the GR isoforms, which is in agreement with other publications noting multiple bands for GR via western blot analysis (Yudt and Cidlowski, 2001, 2002; Yudt et al., 2003), indicating the presence of multiple forms of the glucocorticoid receptor at the translational level (Fig 2-5). The evidence presented thus far indicates participation of a GR in the induction of increased  $I_{sc}$ . It is unknown which form(s) of the GR receptor is linked to the induction of amiloride-sensitive  $I_{sc}$ .



**Figure 2-4 Glucocorticoid receptor immunoreactivity is present in vas deferens epithelial cell monolayers.** Western blot analysis of vehicle (Veh)-treated and dexamethasone (Dex)-treated PVD cell lysates demonstrate immunoreactivity when exposed to antibody for glucocorticoid receptor. Multiple bands suggest that several isoforms of the glucocorticoid receptor are present.

*Mifepristone concentration-dependently inhibits amiloride-sensitive  $I_{sc}$  in monolayers exposed to corticosteroids.* Mifepristone affects both glucocorticoid and progesterone receptors, but with slightly different potencies. Although all results reported above indicate action at a glucocorticoid receptors and a previous report (Phillips and Schultz, 2002) indicates no effect of progesterone on vas deferens ion transport, additional experiments were conducted to quantify the inhibitory effect of mifepristone and to further verify consistency with actions at a glucocorticoid receptor. Monolayers were exposed to 100 nM dexamethasone for 72 hours and additionally exposed to various concentrations of mifepristone. Mifepristone precludes the corticosteroid-induced increase in amiloride-sensitive  $I_{sc}$  in a concentration-dependent manner with a  $K_{app}$  of  $\sim 0.4$  nM (Fig 2-6), which is consistent with previously reported values for inhibition of GR in other epithelial systems (Jung-Testas and Baulieu, 1983; Cadepond et al., 1997).

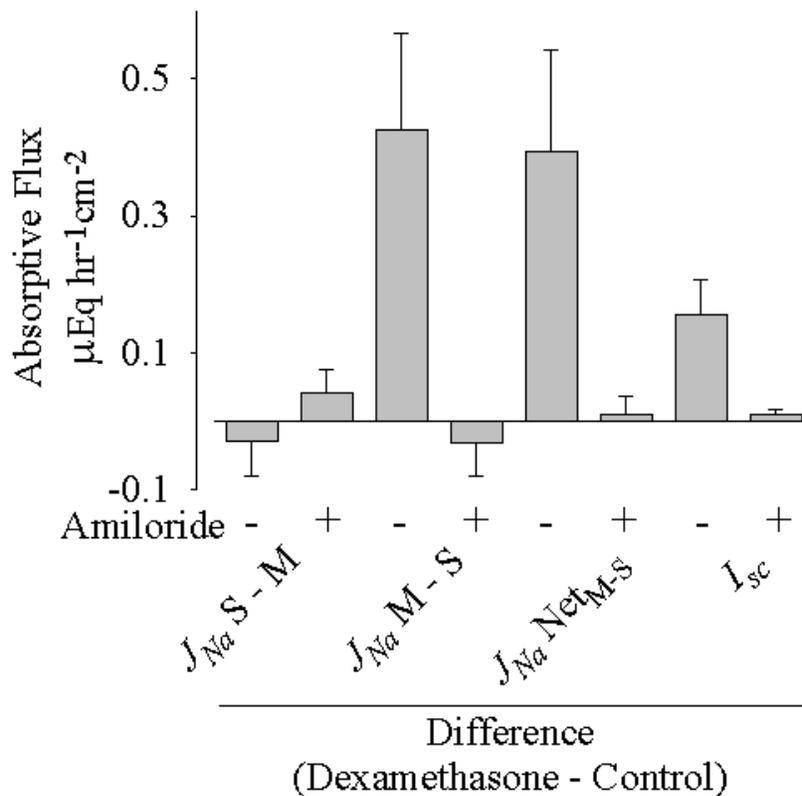


**Figure 2-5 Mifepristone concentration-dependently inhibits amiloride-sensitive  $I_{sc}$  in monolayers exposed to corticosteroids.**

Mifepristone affects both glucocorticoid and progesterone receptors, but with slightly different potencies. Although all results reported above indicate action at a glucocorticoid receptors and a previous report (Phillips and Schultz, 2002) indicates no effect of progesterone on vas deferens  $I_{sc}$ , additional experiments were conducted to quantify the inhibitory effect of mifepristone and to further verify consistency with actions at a glucocorticoid receptor. Monolayers were cultured in the presence of dexamethasone (100 nM) and the indicated concentrations of mifepristone. Mifepristone precludes the corticosteroid-induced increase in amiloride-sensitive  $I_{sc}$  in a concentration-dependent manner with a  $K_{app}$  of  $\sim 0.4$  nM, which is consistent with previously reported values for inhibition of GR in other epithelial systems (Jung-Testas and Baulieu, 1983; Cadepond et al., 1997). Mifepristone concentration-dependently precluded the effect of dexamethasone treatment. Results are the mean and SEM from six tightly paired experiments. Solid line represents the best fit of a modified Hill equation to the data set.

**$\text{Na}^+$  flux exceeds corticosteroid-induced  $I_{sc}$ .**  $^{22}\text{Na}^+$  was employed as a tracer to quantify  $\text{Na}^+$  flux from serosal (basolateral) to mucosal (apical) and from mucosal to serosal compartments with and without previous exposure to corticosteroids. Results were analyzed to determine differences between corticosteroid-treated and untreated monolayers (Fig 2-8). Serosal to mucosal  $\text{Na}^+$  flux was similar in both corticosteroid- and vehicle-treated monolayers (i.e. the reported difference is nearly zero). Absorptive  $\text{Na}^+$  flux, however, was greater in corticosteroid-treated monolayers as compared to controls. This absorptive  $\text{Na}^+$  flux was fully abolished by amiloride exposure (Fig 2-8), whereas there was no effect of amiloride on  $\text{Na}^+$  flux in the

secretory direction. Glucocorticoids induce a large net  $\text{Na}^+$  absorption that, like  $I_{sc}$ , is fully blocked by amiloride.

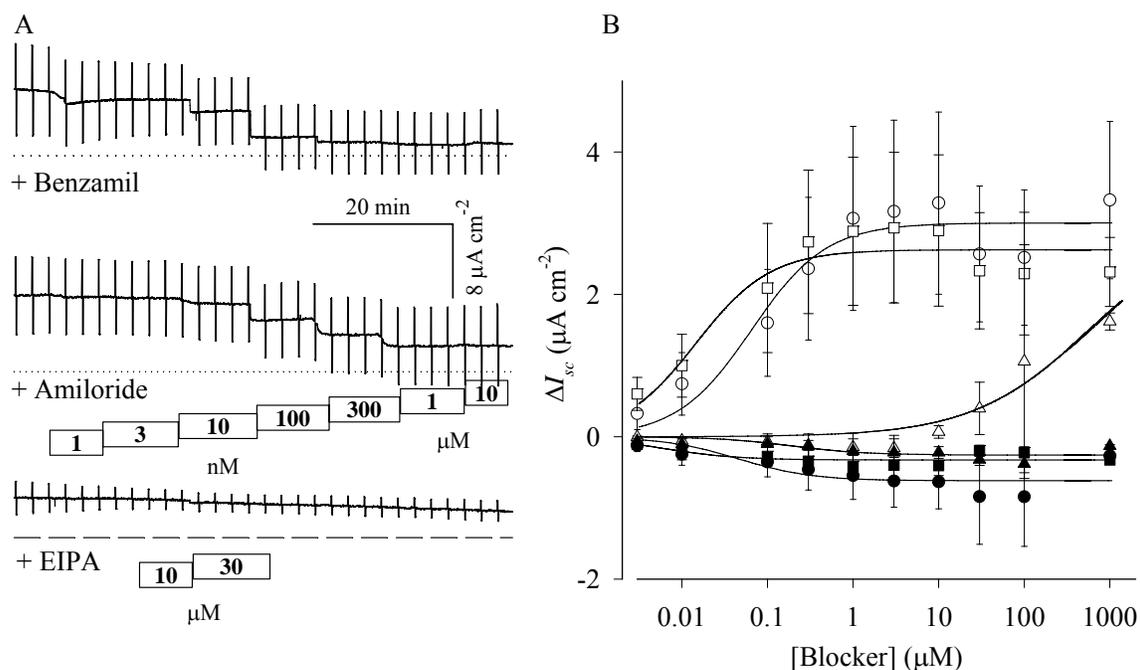


**Figure 2-6 Corticosteroids induce  $\text{Na}^+$  absorption across vas deferens epithelia.**

Unidirectional net  $\text{Na}^+$  fluxes were determined for tightly paired epithelial monolayers that had been cultured in the absence and presence of dexamethasone. Positive values indicate movement of cations in the absorptive direction. Data are summarized from 5 experiments.

***The epithelial sodium channel, ENaC, is indicated by pharmacological analysis.***

Functional analysis of dexamethasone-induced  $I_{sc}$  demonstrated concentration-dependent inhibition by benzamil and amiloride, with weak and incomplete inhibition by EIPA (Fig 2-9). Typical data show stepwise inhibition with increasing blocker concentration. Benzamil and amiloride demonstrate similar maximal effect ( $2.6$  and  $3.0 \mu\text{A cm}^{-2}$  respectively), and the Hill coefficient for all three is not different from unity. The  $K_{app}$  for benzamil is smaller than that of amiloride ( $15 \pm 6 \text{ nM}$  and  $58 \pm 3 \text{ nM}$ , respectively), and the  $K_{app}$  for EIPA is much larger ( $573 \pm 21 \text{ nM}$ ). The rank order of potency, benzamil > amiloride >>>EIPA, indicates a pharmacological fingerprint that is consistent with ENaC activity.



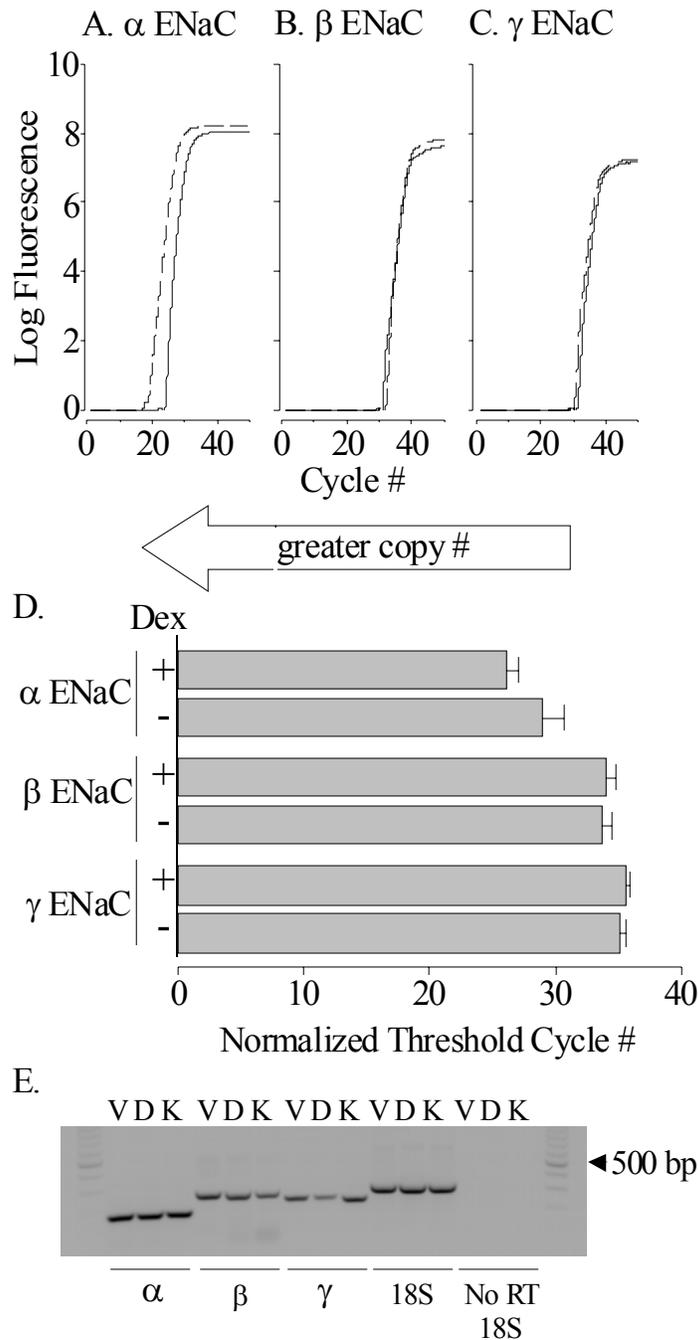
**Figure 2-7 Corticosteroid-induced  $I_{sc}$  is sensitive to amiloride and benzamil.**

A) Typical tracings from a tightly paired experiment in which monolayers that had been cultured in the presence of cortisol were exposed to escalating concentrations of the indicated amiloride-analogs, including amiloride, benzamil, or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) B) Summary data indicate a rank order of potency of benzamil>amiloride>>>EIPA. Open symbols represent corticosteroid-exposed conditions; closed symbols represent paired vehicle controls (squares = benzamil; circles = amiloride; triangles = EIPA). Lines represent the best fit of a modified Hill equation to each data set. Parameters of the fits are presented in the text. Epithelial monolayers were cultured in the presence of cortisol (500 nM) or dexamethasone (100 nM) for three days prior to evaluation. Results are typical of 11 paired observations for each condition.

***ENaC is evident at the mRNA and protein level.*** Total RNA and total proteins were isolated from both vehicle- and dexamethasone-treated PVD epithelial monolayers. RT-PCR for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC was conducted and products were sequenced. The  $\alpha$ -ENaC sequences were identified as 100% homologous with *sus scrofa* ENaC on the NCBI database.  $\beta$ -, and  $\gamma$ -ENaC were highly homologous with  $\beta$ -, and  $\gamma$ -subunits from other species. Western blot analysis indicated a single band of immunoreactivity for  $\alpha$ -,  $\beta$ - or  $\gamma$ -ENaC subunits in untreated and dexamethasone-treated monolayers (data not shown).

***Corticosteroids selectively upregulate  $\alpha$ -ENaC subunit transcription and expression.*** ENaC subunit transcription was evaluated via qPCR analysis, and compared between corticosteroid-treated and untreated monolayers.  $\alpha$ -ENaC transcript demonstrated

significantly greater copy number at the outset as compared to either the  $\beta$ - or  $\gamma$ -subunit, with Ct values of  $26.2 \pm 0.9$  compared to  $34.1 \pm 0.7$  and  $35.6 \pm 0.4$  for  $\beta$  and  $\gamma$  respectively.  $\alpha$ -ENaC transcript was upregulated after corticosteroid exposure (Fig 2-10A). Ct was left shifted by  $2.5 \pm 1.0$ , which represents a 5.6-fold greater number of transcripts in the presence of dexamethasone (Fig 2-10D).  $\beta$ -ENaC and  $\gamma$ -ENaC transcript levels were indistinguishable in dexamethasone-treated monolayers and untreated monolayers (Fig 2-10 B, C, and D). Figure 2-10E is included to demonstrate that qPCR products exhibit the expected mobility for both corticosteroid-treated and untreated monolayers in all cases.  $\alpha$ -ENaC immunoreactivity is greater in cell lysates derived from dexamethasone-treated monolayers when compared to vehicle-treated controls. Immunoreactivity for the  $\beta$ - and  $\gamma$ -ENaC subunits, however, do not appear to be greater in dexamethasone-treated monolayers (data not shown; see also Fig 2-11D).

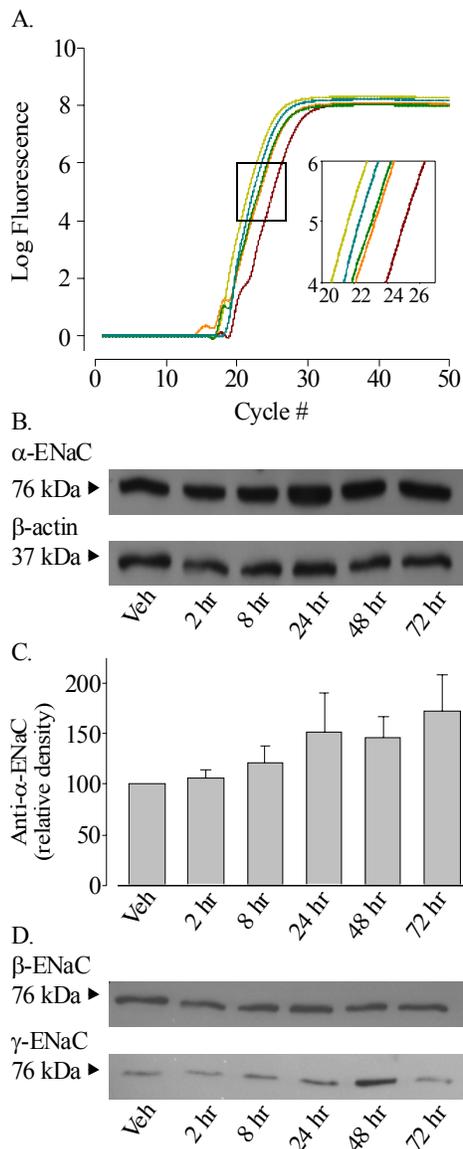


**Figure 2-8 Corticosteroids selectively upregulate  $\alpha$ -ENaC transcription.**

(A-C) PCR fluorescence amplification curves for  $\alpha$ -,  $\beta$ - and  $\gamma$ -ENaC, respectively. Solid lines represent mRNA isolated from vehicle-treated control cells and dashed lines represent mRNA isolated from dexamethasone-treated monolayers. 100ng of total mRNA was included in each reaction, and loading accuracy was verified by including 18S RNA controls for each paired set of experiments. Results are from a single experiment that is typical of five experiments. (D) Summary of quantitative RT-PCR experiments for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC in mRNA isolated from dexamethasone- or vehicle-treated monolayers (experiments from panels A-C are included). All

data in panel D are normalized to 18S RNA as a loading control. (E) Gel electrophoresis of a set of products from a single experiment. mRNA was isolated and resolved on the gel in groups of three for each treatment, including vehicle control (V), dexamethasone-treated (D), and rat kidney as a positive control tissues (K). Lanes 1 and 17 are ladders, lanes 2-16 are products resulting from the indicated primer pairs:  $\alpha$ ,  $\alpha$ -ENaC;  $\beta$ ,  $\beta$ -ENaC;  $\gamma$ ,  $\gamma$ -ENaC.

***Corticosteroid-induced transcriptional and translational upregulation of  $\alpha$ -ENaC is time-dependent.*** Quantitative analysis of mRNA harvested from PVD epithelial monolayers at specific timepoints after corticosteroid exposure indicates an upregulation in the number of  $\alpha$ -ENaC transcripts by as early as 2 hours. For this short time period, Ct was  $1.5 \pm .6$  less indicating a 2.8-fold increase in copy number. Copy number continued to increase through 48 hours, reaching a Ct value that is 2.5 less than at the outset (Fig 2-11A), a  $>5.5$  fold increase in copy number. The inset provides greater resolution of the indicated portion of the qPCR fluorescence curve. Translational upregulation of  $\alpha$ -ENaC is suggested by Western blot analysis of proteins harvested in a similar timecourse as described above (Fig 2-11B). A small increase in  $\alpha$ -ENaC immunoreactivity was evident densitometrically after 2 hours, and further upregulation was demonstrated by 24 hours of dexamethasone exposure (Fig 2-11C). A plateau was apparent after 24 hours. No upregulation of  $\beta$ - or  $\gamma$ -ENaC subunits is evident by either qPCR or Western blot analysis (Fig 2-11D), when densitometrically analyzed with  $\beta$ -actin as a loading control.



**Figure 2-9 Transcriptional and translational upregulation of  $\alpha$ -ENaC, but not  $\beta$ - or  $\gamma$ -ENaC, in response to corticosteroid exposure is time-dependent.**

A) Increase in  $\alpha$ -ENaC mRNA is evident as early as two and eight hours (orange and green lines, respectively) after corticosteroid exposure as compared to control (red) as measured by quantitative RT-PCR analysis. After 24 (blue) and 48 (yellow) hours, further increase in  $\alpha$ -ENaC mRNA is evident. Ct values reported in text. B) Western blot analysis with antibodies for  $\alpha$ -ENaC subunits demonstrated a similar pattern in expression as that seen transcriptionally. Anti- $\beta$ -actin antibody was employed for densitometric analysis. C) Summary results from densitometric analysis of western blot experiments indicate elevation in  $\alpha$ -ENaC protein as early as 2 hours, continuing to increase to 24, 48 and 72 hours of corticosteroid exposure. Summary results represent 5 observations from tightly paired experiments. D) ENaC immunoreactivity for the  $\beta$ - and  $\gamma$ -subunits is clearly evident. Densitometric analysis, with  $\beta$ -actin as a loading control, does not indicate change in  $\beta$ - and  $\gamma$ -ENaC protein levels in corticosteroid-treated PVD monolayers.

## DISCUSSION

Results from the present study demonstrate that, in vas deferens epithelia, corticosteroids modulate  $\alpha$ -ENaC transcription and ultimately the expression of active  $\text{Na}^+$  channels via glucocorticoid receptor-mediated pathway. Significant upregulation of  $\text{Na}^+$  transport, as measured by  $I_{sc}$ , is observed within two hours of exposure to either naturally occurring or clinically used corticosteroids. Transcription and translation of the  $\alpha$ -ENaC subunit are upregulated concomitant with the increase in  $\text{Na}^+$  absorption, which remains elevated for the duration of corticosteroid exposure. Inhibition of corticosteroid-stimulated  $I_{sc}$  is achieved by a glucocorticoid-receptor antagonist at expected concentrations.

ENaC likely plays a major role in regulating fluid volume and composition in the male reproductive duct in vivo. Previous reports indicate amiloride-sensitive ion transport across cultured epithelia derived from human (Carlin et al., 2002), sheep (Bertog et al., 2000), and pig (Phillips and Schultz, 2002) vas deferens and in epithelium derived from rat epididymis (Wong, 1998). Most importantly, a previous report from this laboratory demonstrated that amiloride-sensitive ion transport is present in freshly excised human vas deferens where epithelial cells were not removed from their native substrate (Carlin et al., 2002). While it was shown previously that  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC subunit mRNAs are expressed in PVD epithelial cells (Carlin et al., 2006), the current report is the first to demonstrate expression at the protein level with Western blot analysis and to demonstrate a pharmacological profile that is indicative of ENaC-mediated  $\text{Na}^+$  absorption (Kleyman and Cragoe, 1990; Garty et al., 1994; Matalon and O'Brodivich, 1999). Additionally, it was shown that the amiloride-sensitive ion transport is induced by both natural and synthetic glucocorticoids. Molecular and immunochemical outcomes demonstrate the presence of a glucocorticoid receptor in vas deferens epithelia while the pharmacological outcomes demonstrate that glucocorticoid activation is required to observe amiloride-sensitive current. It was demonstrated further that changes in  $\alpha$ -ENaC transcription and translation occur concurrently with changes in ion transport. Taken together, these results establish a physiological pathway in which ENaC plays a critical role that contributes to male reproductive function.

The current observations suggest that regulation of ENaC expression in this tissue is similar to regulation of ENaC in the airway. Glucocorticoid-stimulated epithelial  $\text{Na}^+$  transport has been demonstrated in the airway (Frank et al., 1980; Itani et al., 2002; Ramminger et al., 2004), and the importance of modulated electrolyte transport in these tissues has been

emphasized (Liggins, 1976; Berger et al., 1991; Berger and Welsh, 1991; Helve et al., 2004). Similarities in the regulation of ENaC activity are important as the implications for both airway and vas deferens in ion transport disorders like CF are considered. Actions of ENaC and CFTR are believed to be intimately linked (Donaldson et al., 2002; Huang et al., 2004), and Na<sup>+</sup> hyperabsorption is reported in CF patients. Thus, reproductive pathologies in CF patients may arise from increased ENaC-mediated Na<sup>+</sup> absorption as well as the lack of anion secretion. The work defined here lays the groundwork for study of Na<sup>+</sup> transport in a tissue that is apparently more sensitive than the airway to ion transport anomalies, as evidenced by the fact that the vas deferens fully regresses in virtually all male CF patients. The integrity and function of both of these organs is affected by the ion transport mechanisms that regulate the composition of the luminal fluids.

Na<sup>+</sup> transport mechanisms likely play a key role in determining luminal volume and composition in the ductus deferens. The focus of this report includes an in depth analysis of Na<sup>+</sup> transport across the apical membrane of porcine vas deferens epithelium. Understanding modulation of this transport is critical because various basolateral transport mechanisms are affected by Na<sup>+</sup> transport across the apical membrane, including Na<sup>+</sup>/K<sup>+</sup>-ATPase, Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> cotransporter, and the Na<sup>+</sup>HCO<sub>3</sub><sup>-</sup> cotransporter. Changes in the rate of Cl<sup>-</sup> or HCO<sub>3</sub><sup>-</sup> secretion has the potential to change the pH of the lumen. ENaC-dependent Na<sup>+</sup> absorption across the apical membrane likely changes the cytosolic composition to affect anion transporters. Na<sup>+</sup> removed from the lumen by ENaC would be expected to raise cytosolic [Na<sup>+</sup>] and depolarize the cell membrane, changes that would affect the activity of the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> co-transporter and the Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> transporter. The decreased electrochemical driving force for Na<sup>+</sup> would be expected to decrease activity of these transport mechanisms in the basolateral membrane. However, depolarization would favor NBCe1b activity leading to luminal alkalization (Devor et al., 1999). Additionally, Na<sup>+</sup>/H<sup>+</sup> exchangers have been identified in rat epididymus (Breton et al., 1996). If this transport mechanism is also present in the vas deferens, ENaC might be expected to reduce acid secretion into the duct. Thus, one might speculate that Na<sup>+</sup> absorption would contribute to an increase in luminal pH, which would activate sperm.

Fluid volume, electrolyte concentration and pH of the luminal environment in the male reproductive tract affect sperm function and motility (Okamura et al., 1985; Okamura et al., 1986; Tajima and Okamura, 1990). Testicular and epididymal sperm are not typically capable of

fertilizing an oocyte (Zhou et al., 2004a; Zhou et al., 2004b). Rather, sperm ‘mature’ and become ‘competent’ as they traverse the epididymis and are stored in the cauda epididymis and transitional vas deferens (Jones et al., 1980; Clulow et al., 1994; Jones and Clulow, 1994). The luminal environment to which the sperm are exposed is acidified and fluid volume is reduced while traversing the epididymis (Levine and Marsh, 1971). An acid environment maintains sperm in a quiescent state during storage while exposure to  $\text{HCO}_3^-$  causes sperm to become active and induces the irreversible acrosome reaction that is required to precede fertilization (Okamura et al., 1985; Chen et al., 2000). Epididymal sperm are less active and have decreased motility vigor and trajectory as compared to sperm harvested at the vas deferens (Kawakami et al., 1998; van Der Horst et al., 1999; Zenke et al., 2004; Martinez-Pastor et al., 2006). In the porcine model, after transport through the ductus deferens sperm are active and have fertilization capacity. Results from the present study indicate that ENaC channels are present in vas deferens epithelium and can contribute to changes in luminal volume and electrolyte composition. Thus, ENaC or components of the glucocorticoid receptor-mediated pathway may serve as targets for therapeutic intervention to manage male fertility.

The results in this study clearly document that ENaC is present and that  $\alpha$ -ENaC is acutely regulated by corticosteroids at both the transcriptional and translational levels in vas deferens epithelium, whereas the  $\beta$ - and  $\gamma$ -ENaC expression is not affected. Evidence of glucocorticoid-receptor mediated regulation of ENaC at both the molecular and functional levels, whereas mineralocorticoid-receptor agonists and antagonists were without effect. Changes in ENaC function at the apical membrane are expected to modify volume, ion composition, and pH of luminal fluids and therefore have a profound affect on sperm activity. Functional effects of changes in ENaC activity may be important to the epithelium itself, especially in view of the prevalence of CBAVD in patients with  $\text{Na}^+$  and  $\text{Cl}^-$  transport disorders. The information provided here is not only important for understanding pathologies associated with these disorders, but also identifies possible targets of intervention to affect male fertility.

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## **CHAPTER 3 – Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium**

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# **CHAPTER 3 - Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium**

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RUNNING HEAD: Glucocorticoid-induced ENaC modulation in BME-UV

KEYWORDS: short circuit current, apical cation concentration, corticosteroids, mastitis, ENaC subunits

## **ABSTRACT**

Mammary epithelia produce an isotonic, low Na<sup>+</sup> 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<sup>+</sup> 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 ( $I_{sc}$ ), a sensitive measure of net ion transport while apical exposure to amiloride or benzamil reduced corticosteroid-induced  $I_{sc}$  close to basal levels. Quantitative RT-PCR indicated a glucocorticoid-

induced increase in mRNA for  $\beta$ - and  $\gamma$ -ENaC, whereas  $\alpha$ -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  $I_{sc}$  and the induced changes in  $\beta$ - and  $\gamma$ -ENaC mRNA. We conclude that  $\text{Na}^+$  movement across mammary epithelia is modulated by corticosteroids via a glucocorticoid receptor-mediated mechanism that regulates expression of  $\beta$ - and  $\gamma$ -ENaC. ENaC expression and activity could account for the low  $[\text{Na}^+]$  that is typical of milk.

## INTRODUCTION

Mammary epithelia rely on regulated ion transport processes (Blaug et al., 2001) to produce milk that is low in  $\text{Na}^+$ , although mechanisms for monovalent ion transport remain to be fully characterized. A current model of mammary epithelium shows an unidentified apical  $\text{Na}^+$  conductance and indicates that  $\text{Na}^+$  is distributed across this membrane according to its electrochemical equilibrium (Neville, 1999; Shennan and Peaker, 2000). In this model, cytosolic, and therefore luminal,  $\text{Na}^+$  concentration is set by the activity of the basolateral components, most notably,  $\text{Na}^+/\text{K}^+$  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 (Blaug et al., 2001) and bovine (Schmidt et al., 2001) tissues demonstrated amiloride-sensitive ion transport across this epithelial layer. Thus, we speculated that 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 have been demonstrated in the BME-UV cell line (Schmidt et al., 2001) as well as other mammary cell systems (Shennan and Peaker, 2000). The large, highly regulated transepithelial ionic 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 (Bisbee et al., 1979; Smith et al., 1990; Sjaastad et al., 1993; Silanikove et al., 2000; Blaug et al., 2001; Schmidt et al., 2001). Ion transport mechanisms that have been identified in mammary epithelium include  $\text{Na}^+/\text{K}^+$  ATPase,  $\text{Na}^+/\text{K}^+ / 2\text{Cl}^-$  cotransporter,  $\text{Ba}^{2+}$ -sensitive  $\text{K}^+$  conductance(s) in the basolateral membranes, and a  $\text{Cl}^-$  conductance and  $\text{Na}^+$  channel in the apical membrane (Blatchford and Peaker, 1988; Smith

et al., 1990; Shennan, 1992, 1998; Neville, 1999; Stelwagen et al., 1999; Shennan and Peaker, 2000; Silanikove et al., 2000; Blaug et al., 2001; Schmidt et al., 2001). Epithelial Na<sup>+</sup> channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) anion channel activity have been reported in murine mammary epithelium (Blaug et al., 2001), a system in which dexamethasone is included in the medium bathing the cells to encourage tight junction formation. Amiloride-sensitive  $I_{sc}$ , suggesting the presence of ENaC, has been demonstrated in a bovine mammary epithelium (Schmidt et al., 2001), but only after stimulation with dexamethasone. Although many distinct ion transport mechanisms have been suggested for mammary epithelium (Linzell and Peaker, 1971; Shennan, 1992, 1998; Neville, 2001; Neville and Morton, 2001; Neville et al., 2001) little is known regarding the cation selectivity of the apical membrane, the timecourse of response to corticosteroid stimuli, or the conductances that are regulated.

Modulation of Na<sup>+</sup> ion transport by corticosteroids has been delineated in many tissues, including kidney, gut and airway (Kamynina and Staub, 2002; Snyder, 2002; Snyder et al., 2002; Saxena et al., 2006). Fluid secretion in the mammary gland is, additionally, 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 kidney, corticosteroid regulation of monovalent ion transport is regulated via a mineralocorticoid-receptor mediated process (Gaeggeler et al., 2005; Helms et al., 2005). Colon and airway monovalent ion transport processes, on the other hand, have been shown to be dependent on glucocorticoid-receptor regulated pathways (Bridges et al., 1989; Berger and Welsh, 1991; Venkatesh and Katzberg, 1997; Itani et al., 2002; Mustafa et al., 2004; Thomas et al., 2004; Otulakowski et al., 2006). Corticosteroid regulation of mammary ion transport processes has not been examined. Since dexamethasone has been shown to affect epithelial transport in both glucocorticoid- 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 is to determine the molecular identity of mechanisms that account for corticosteroid-induced changes in mammary ion transport, specifically, we speculate the presence and activity of ENaC in the apical membrane. The hypothesis is that corticosteroids alter Na<sup>+</sup> transport across bovine mammary epithelium via glucocorticoid-receptor mediated

modulation of specific ENaC subunits. The outcome demonstrates that mechanisms present in mammary epithelium can account for the low Na<sup>+</sup> composition of milk.

## MATERIALS AND METHODS

**Cell culture.** An immortalized bovine mammary epithelial cell line obtained from Jeff White at the University of Vermont (BME-UV; (Zavizion et al., 1996)) was used for the present studies. Culture conditions have been described in detail previously (Schmidt et al., 2001; Quesnell, 2006). Briefly, BME-UV cells were grown to 65-75% confluency in 25 cm<sup>2</sup> plastic culture flasks (Cellstar, Frickenhausen, GE) using media of defined composition, and lifted using a solution containing trypsin in phosphate-buffered saline with 2.6 mM disodium ethylenediaminetetraacetic acid for seeding to Snapwell permeable supports (1.13 cm<sup>2</sup>, Costar, Cambridge, MA). Polarized cell monolayers were grown for 2 weeks before assay. Media bathing basolateral aspect of the cells included a 5:3:2 mixture of Dulbecco's Modified Eagle Medium (DMEM) + Ham's F-12: RPMI medium: NCTC-135 medium (Gibco BRL, Rockville, MD) supplemented with 10% heat inactivated fetal bovine serum (FBS, BioWhittaker, Walkersville, MD), 3% newborn calf serum (NCS, Gibco BRL), 2% iron-supplemented bovine calf serum (FeBCS, Gibco BRL), 1% insulin-transferrin-sodium selenite media supplement solution (Sigma-Aldrich, St. Louis, MO) and 1% penicillin and streptomycin (Gibco BRL), L-ascorbic acid, alpha-lactalbumin, and lactose (targeted ionic composition: 140 mEq/L Na<sup>+</sup>). Apical medium (reduced electrolytes with targeted ionic composition of 60 mEq/L Na<sup>+</sup> with 160 mM lactose) was similar in nutrient composition except for reduced NaCl and increased lactose. Details of media composition have been published previously (Schmidt et al., 2001; Quesnell, 2006). Osmolarity was 290 ± 7 mOsm in all media. The cells were incubated at 37° C in a humidified atmosphere containing 5% CO<sub>2</sub>. Media were refreshed daily, including 4-8 hours prior to experimental assays.

**Electrical measurements.** Short circuit current ( $I_{sc}$ ), a measure of net transepithelial ion transport, was measured continuously with sampling at 1 Hz in a modified Ussing chamber system (Model DCV9, Navicyte, San Diego, CA), with a voltage clamp apparatus (Model 558C, University of Iowa, Department of Bioengineering, Iowa City, IA). Monolayers grown on Snapwell permeable supports were mounted in the modified Ussing chamber apparatus and bathed in symmetrical Ringer solution (composition in mM: 120 NaCl, 25 NaHCO<sub>3</sub>, 3.3

KH<sub>2</sub>PO<sub>4</sub>, 0.8 K<sub>2</sub>HPO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, and 1.2 CaCl<sub>2</sub>). Fluid was circulated with an airlift system (5% CO<sub>2</sub>/95% O<sub>2</sub>) and temperature was maintained at 37°C. Transepithelial electrical resistance ( $R_{te}$ ) measurements were included with the typical protocol of data collection, by applying a 0.5 mV bipolar pulse across the monolayer at 100-second intervals. Current and voltage measurements were acquired on a Macintosh computer (Apple Computer, Cupertino, CA) using Aqknowledge software (ver. 3.2.6, BIOPAC Systems, Santa Barbara, CA).  $R_{te}$  was calculated using Ohm's Law, and monolayers with  $>150 \Omega \text{ cm}^2$  were utilized for experiments.

***Corticosteroid treatments and amiloride exposure.*** To examine the effect of steroid exposure on amiloride-sensitive epithelial Na<sup>+</sup> absorption, BME-UV monolayers were grown on permeable supports and basolaterally exposed to natural or synthetic corticosteroids, including dexamethasone (0.1  $\mu\text{M}$ ), prednisolone (1.0  $\mu\text{M}$ ) or cortisol (0.5  $\mu\text{M}$ ) (Sigma-Aldrich), for 72 hours prior to experimental assessment. Apical exposure to either amiloride (10  $\mu\text{M}$ ) or benzamil (1  $\mu\text{M}$ ) 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 determine if change in protein levels occurred concomitant with changes in  $I_{sc}$ , lysates of untreated and corticosteroid-treated BME-UV cells were prepared in the 1.13 cm<sup>2</sup> 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 assay. Total protein content was determined using a Bicinchoninic Assay (Pierce, Rockford, IL) and 20  $\mu\text{g}$  total protein was loaded in each well of a 10-20% SDS-Page pre-poured gel (BioRad, Hercules, CA) for electrophoresis. Electrophoretic protein resolution was conducted with 160 mV for 45 minutes. Proteins were then transferred to Millipore PVF-Immobilon (Millipore Corp., Bedford, MA) membranes for 8.5 hours 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- $\alpha$ -ENaC, anti- $\beta$ -ENaC, and anti- $\gamma$ -ENaC antibodies (antibodies for all three subunits were obtained from both Alpha Diagnostics, San Antonio, TX and Affinity BioReagents, Golden, CO) at 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). Reverse transcription-polymerase chain reaction (RT-PCR) for BME-UV glucocorticoid receptor was performed using the QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA) on a Techne Touchgene Thermal Cycler (Krackeler Scientific, Albany, NY). The sequences of the primers used were 5'- ggaatagatgccaaaggtct-3' and 5'- ccccataatgacatcctgaa-3'. The reaction components included 1 ng total RNA from BME-UV cells per 25  $\mu$ l reaction mixture, Qiagen OneStep RT-PCR buffer, dNTP mix, 0.6  $\mu$ M primers, and QIAGEN OneStep RT PCR enzyme mix. The RT-PCR cycle parameters were: 50 °C 30 min, 95 °C 15 min and 40 cycles at 94 °C for 30 s, 57°C for 40 s and 72 °C for 40 s, 72 °C 5 min. The PCR products were examined by 2% agarose gel electrophoresis. The expected product of RT-PCR was 210 bp. Control reactions that did not include the reverse transcription 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 relative copy numbers of mRNA for ENaC subunits, total RNA was harvested from vehicle- and corticosteroid-treated BME-UV cells and PCR analysis performed for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC mRNA expression. Total RNA was isolated from confluent BME-UV monolayers using Qiagen Shredder columns and Qiagen Micro RNeasy RNA (Qiagen, Valencia, CA) isolation kits following the manufacturer's protocol. High quality of the purified total RNA samples was confirmed by analysis on an RNA Nano LabChip (Agilent Technologies, Palo Alto, CA) as well as detection of expected bands on a denaturing gel corresponding to 18S ribosomal RNA. All RNA samples were subjected to DNase 1 treatment (Ambion Inc., Austin, TX) according to the manufacturer's specifications. Initial quantification of total RNA for loading was performed in an ND-1000 NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). RT-PCR was carried out (OneStep RT-PCR, Qiagen) using primer pairs specific for coding sequences of bovine  $\alpha$ - (forward: 5'- GGCGTGTGTGGTTTCCTTCC-3', reverse: 5'-TCAGGGGGATGGGGTAGTGA-3'; 243 bp expected product),  $\beta$ - (forward: 5'-TGCTGTGCCTCATCGAGTTTG-3', reverse: 5'-

TGCAGACGCAGGGAGTCATAGTTG-3'; 277 bp expected product), and  $\gamma$ -ENaC subunits (forward: 5'-TCAAGAAGAATCTGCCCGTGA-3', reverse: 5'-CTTTTGGGAAGTGGACTTTGATGG-3'; 242 bp expected product), as well as 18S primers for normalization (forward: 5'-cggctaccacatccaaggaa-3', reverse: 5'-ccgctccaagatccaacta-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). Threshold to determine 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 same threshold level for each individual experiment, and outcomes were normalized to the Ct values for 18S.

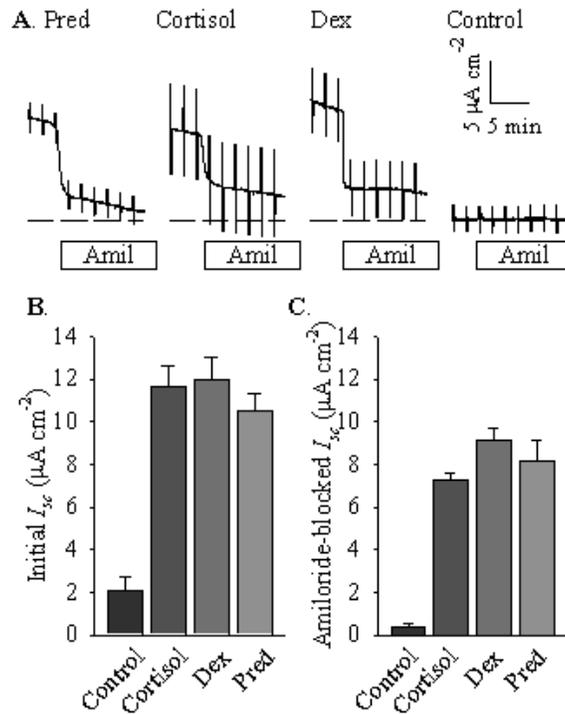
***Glucocorticoid/mineralocorticoid receptor inhibitors.*** To evaluate the receptor mechanism responsible for functional changes in  $\text{Na}^+$  transport, spironolactone (10  $\mu\text{M}$ ) or mifepristone (10  $\mu\text{M}$ ) was included in the bathing medium for the last 24 hours prior to assessment in a subset of the above described experiments. Analysis performed in conjunction with these experiments include: Ussing chamber, Western blot, and quantitative RT-PCR.

***Data Analysis.*** The difference between control and treatment data was analyzed using ANOVA and Student's t-test components of a Unix-based SAS (SAS Institute Inc., Cary, NC) program. Data are presented as mean  $\pm$  SEM. Statistical significance was evaluated at  $p < 0.05$ .

## RESULTS

***Glucocorticoids elevate amiloride-sensitive  $I_{sc}$ .*** Initial experiments were designed to document and extend observations examining the effect of steroid exposure on amiloride-sensitive epithelial ion transport (Schmidt et al., 2001). Typical tracings from paired BME-UV monolayers mounted in a modified Ussing chamber system illustrate the increase in  $I_{sc}$  that is associated with corticosteroid exposure (Fig 3-1A). The dashed line in the tracings represents no net movement of ions. The elevation in  $I_{sc}$  is evident in prednisolone, cortisol and dexamethasone-treated monolayers as compared to  $I_{sc}$  in the control monolayer. In a separate set of experiments, aldosterone was without effect. The size of the periodic deflections is inversely

proportional to  $R_{te}$ . Monolayers that had been exposed to glucocorticoids exhibited lower  $R_{te}$  ( $307 \pm 19 \Omega \text{ cm}^2$ ) than untreated controls ( $362 \pm 36 \Omega \text{ cm}^2$ ). Data summarized from 6 experiments demonstrate that corticosteroid exposure resulted in a significant increase in  $I_{sc}$  (Fig 3-1B) that was of similar magnitude for both naturally occurring and synthetic compounds. Glucocorticoid-induced  $I_{sc}$  was reduced by amiloride exposure in all cases (Fig 3-1A&C). Apical exposure to amiloride ( $10 \mu\text{M}$ ) or benzamil ( $1\mu\text{M}$ ; data not shown), on average reduced  $I_{sc}$  to a level approaching untreated monolayers. Concomitant with the decrease in  $I_{sc}$  was an amiloride-induced increase in  $R_{te}$  ( $15 \pm 3 \Omega \text{ cm}^2$  for all corticosteroids vs.  $-6 \pm 3 \Omega \text{ cm}^2$  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  $R_{te}$ , led to speculation that ENaC expression or activity was enhanced by glucocorticoid exposure.

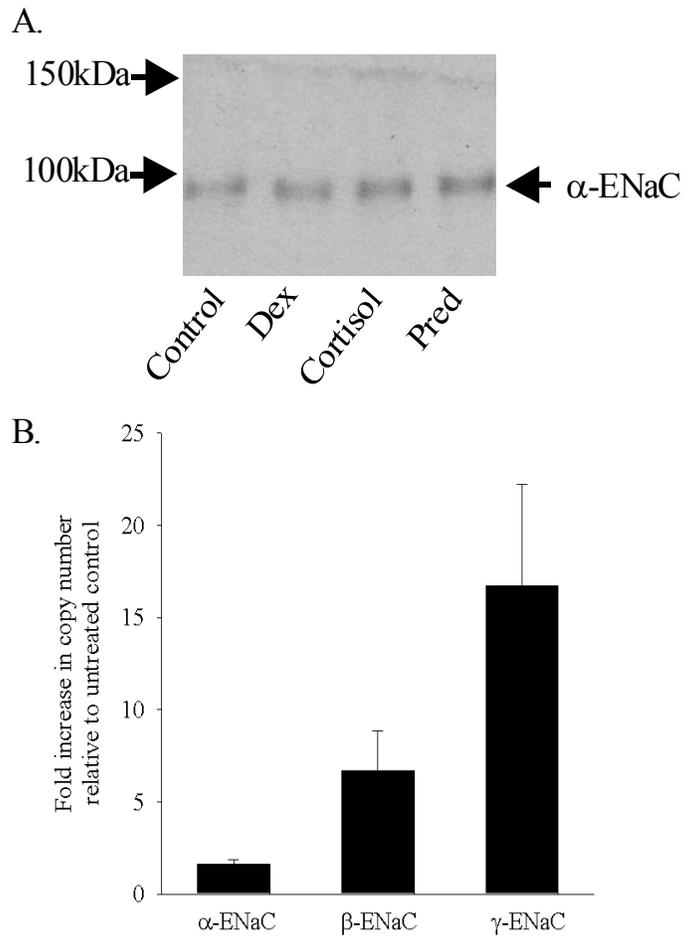


**Figure 3-1 Corticosteroids elevate amiloride-sensitive short circuit current ( $I_{sc}$ ).**

A) Short circuit current tracings taken in a modified Ussing chamber system in tightly paired corticosteroid- or vehicle-treated monolayers demonstrate that the corticosteroid-induced ion transport is inhibited by exposure to the epithelial sodium channel (ENaC)-blocker, amiloride. Data are summarized from tracings in A and 11 additional experiments showing magnitude of basal  $I_{sc}$  (B) and the magnitude of  $I_{sc}$  that is inhibited by amiloride (C). Chronic exposure (72 hours) to prednisolone (Pred), cortisol, or dexamethasone (Dex) in bovine mammary epithelial cell monolayers elevates  $I_{sc}$  (B) which is mostly inhibited by administration of amiloride (C).

***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 3-2A). Presence of α-ENaC was examined in monolayers exposed to either natural or synthetic corticosteroids as compared to lysates of untreated monolayers. Anti-α-ENaC immunoreactivity was evident in all samples tested, although there is no apparent effect of treatment on protein expression level. Commercial antibodies raised against epitopes in rat β- and γ-ENaC gave no signal in bovine cell lysates, although bands of expected mobilities were observed in rat kidney lysates (not shown).

***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, comparatively, only mildly affected by corticosteroid exposure. β-ENaC and γ-ENaC transcript levels, on the other hand were significantly greater in dexamethasone-treated monolayers (Fig 3-2B). 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  $21.7 \pm 0.2$ , whereas threshold for β- and γ-ENaC amplification was not reached until cycle number  $36.0 \pm 1.3$  and  $35.6 \pm 0.8$  respectively. Therefore one can conclude that the copy number for α-ENaC is in great excess ( $\sim 2^{14}$  fold) relative to β- and γ-ENaC. Nonetheless, corticosteroids greatly enhance β- and γ-ENaC expression but have little effect on α-ENaC.

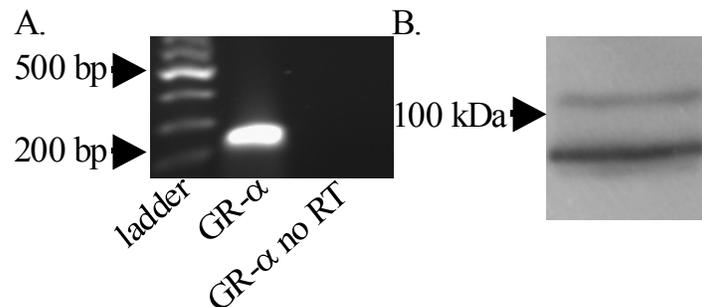


**Figure 3-2 ENaC is present at the protein and transcript level.**

A) Western blot analysis of BME-UV monolayers utilized primary antibody raised against  $\alpha$ -ENaC.  $\alpha$ -ENaC immunoreactivity with the expected mobility of 96 kDa is present in all conditions that were tested. There is no apparent affect of treatment on band density. Swiss 3T3 fibroblast cells were used as a negative control and demonstrated no ENaC immunoreactivity. B) Quantitative analysis using specific primers for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC, normalized to loading quantities of 18S as an indicator of total RNA, indicate greater upregulation in the  $\beta$ -, and  $\gamma$ -ENaC subunits as compared to the modest upregulation in the  $\alpha$ -subunit of ENaC within 24 hours of exposure to 100 nM dexamethasone.  $\alpha$ -ENaC is in much higher copy number ( $Ct = 21.7 \pm 0.2$ ) than either  $\beta$ - ( $Ct = 36.0 \pm 1.3$ ) or  $\gamma$ -ENaC ( $Ct = 35.6 \pm 0.8$ ).

**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 level. Evidence for the presence of mRNA (Fig 3-3A) and protein (Fig 3-3B) for glucocorticoid receptor confirms the presence of this receptor. The PCR primers targeted a

region of the glucocorticoid receptor that is common to both the  $\alpha$ - and  $\beta$ -form of the receptor so that no conclusion regarding receptor subtype expression can be made. However the presence of two immunoreactive bands with mobilities that are appropriate for the  $\alpha$ - and  $\beta$ -form of the receptor in the Western blot suggests that both are expressed in BME-UV cells. The presence of the receptor alone, however, does not confirm its involvement in the regulatory scheme. Therefore, we chose to examine functional effects of glucocorticoid-receptor inhibition on upregulated  $I_{sc}$  in BME-UV cell monolayers, as well as examine transcriptional effects on ENaC regulatory subunits.

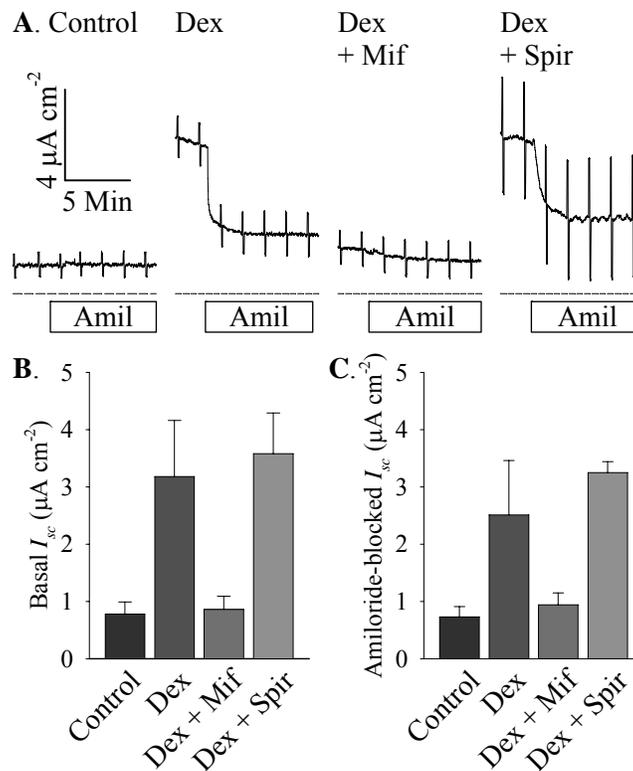


**Figure 3-3 Glucocorticoid-receptor is evident at the molecular level and at the protein level in bovine mammary epithelial cell monolayers.**

A) RT-PCR executed with primers designed against bovine glucocorticoid receptor result in a single, clear band of expected size. Lanes with no RT demonstrate no DNA contamination of the mRNA utilized in the study. Identity was confirmed by sequencing. B) Western blot analysis of protein harvested from bovine mammary epithelial cell monolayers demonstrate immunoreactivity to primary antibody raised against glucocorticoid receptor. The presence of 2 bands with mobilities at  $\sim 90$  and  $\sim 105$  kDa suggests that both the  $\alpha$ - and  $\beta$ -isoforms are present.

***Upregulation of ENaC expression and/or activity is modulated via a glucocorticoid-receptor mediated process.*** Mifepristone inhibits the elevation of  $I_{sc}$  across BME-UV monolayers that is induced by glucocorticoids (Fig 3-4). Results from a typical experiment are presented in Fig 3-4A. As expected, dexamethasone exposure is associated with elevated initial  $I_{sc}$  that is sensitive to amiloride. Results from a paired monolayer cultured in the presence of spironolactone ( $10 \mu\text{M}$ ), a selective mineralocorticoid receptor antagonist, are 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 8 experiments are summarized in Fig 3-4 B&C. The results are consistent with Fig 3-1 showing dexamethasone-induced increase in  $I_{sc}$  that is amiloride-sensitive. In the presence of spironolactone, results are

unchanged from dexamethasone alone whereas in the presence of dexamethasone and mifepristone, the results are no different than in the absence of dexamethasone.

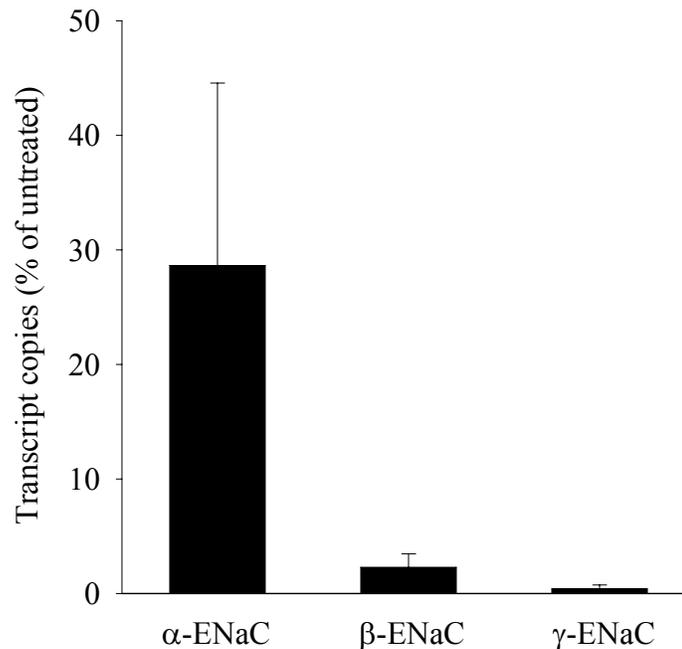


**Figure 3-4 Mifepristone, a glucocorticoid-receptor antagonist, abolishes dexamethasone-induced ion transport.**

A) Typical  $I_{sc}$  tracings demonstrate that mifepristone, but not spironolactone, inhibited the dexamethasone-induced elevation in  $I_{sc}$  as well as amiloride-sensitive current. Cells were incubated in the presence of dexamethasone (0.1  $\mu\text{M}$ ) in the absence or presence of mifepristone (Mif, 10  $\mu\text{M}$ ) or spironolactone (Spir, 10  $\mu\text{M}$ ). Summarized data from tracings in A along with 7 additional experiments indicate that exposure to mifepristone eliminated the dexamethasone-induced increase in initial  $I_{sc}$  (B), that was amiloride-sensitive  $I_{sc}$  as compared to either spironolactone + dexamethasone or dexamethasone-treated monolayers (C).

**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 percent of the control (untreated) value, was significantly reduced by concurrent exposure to mifepristone (Fig 3-5), although spironolactone was without effect (not shown). Again, it should be noted that, in the presence of dexamethasone,  $\alpha$ -ENaC is in much higher copy number than either  $\beta$ - or  $\gamma$ -ENaC as indicated by the Ct of  $20.7 \pm 0.2$  ( $\alpha$ -ENaC),  $31.7 \pm .04$  ( $\beta$ -ENaC) and 28.5

$\pm 0.9$  ( $\gamma$ -ENaC). A comparison of the relative Ct values of dexamethasone-treated and dexamethasone + mifepristone-treated monolayers shows a mifepristone-induced right shift for  $\beta$ - and  $\gamma$ -ENaC expression.



**Figure 3-5 Mifepristone inhibits upregulation of  $\beta$ -, and  $\gamma$ -ENaC subunits by corticosteroids.** Relative levels of mRNA in dexamethasone-treated monolayers were compared to mifepristone + dexamethasone treated monolayers and the percent change in copy number after mifepristone exposure is presented for each ENaC subunit. Data represents mean and standard error from 4 experiments.

In summary, functional evidence indicates glucocorticoid-receptor-mediated modulation of  $\text{Na}^+$  movement across BME-UV cell monolayers during exposure to corticosteroids. This functional evidence is further supported by evidence at a molecular level of the inhibition of corticosteroid upregulation of  $\alpha$ -, and especially  $\beta$ -, and  $\gamma$ -subunit expression of ENaC in the presence of mifepristone.

## DISCUSSION

This study defines a corticosteroid-regulated mechanism that can account for  $\text{Na}^+$  permeation across the apical membrane of mammary epithelium. Results suggest that  $\alpha$ -ENaC mRNA and protein are constitutively expressed, but that corticosteroids, acting at a glucocorticoid receptor, increase  $\beta$ -, and  $\gamma$ -ENaC transcription substantially, and  $\alpha$ -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  $I_{sc}$  and the induced changes in  $\alpha$ -,  $\beta$ - and  $\gamma$ -ENaC mRNA transcription, suggesting that the change in  $\text{Na}^+$  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  $\alpha$ -ENaC, although the presence of GRE for  $\beta$ - or  $\gamma$ -ENaC has not yet been demonstrated. Nonetheless, the relative lack of cortisol on  $\alpha$ -ENaC expression demonstrates that GRE alone is not sufficient to explain glucocorticoid-induced ENaC regulation in mammary gland. Additional co-activator 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 (Linzell and Peaker, 1971; Neville, 1999; Shennan and Peaker, 2000). These authors surmised that  $\text{Na}^+$  moved through a conductive apical pathway and distributed across the membrane according to the electrochemical gradient. In this model, combined activity of  $\text{Na}^+\text{K}^+$  ATPase and  $\text{K}^+$  channels at the basolateral membrane sets a negative membrane potential that drives the absorption of  $\text{Na}^+$  across the apical membrane. The results of this study, which are based on cells derived from bovine tissues and which are consistent with a recent report regarding murine mammary epithelium (Blaug et al., 2001), demonstrate that ENaC is the unidentified conductance in the proposed mammary epithelial model. Furthermore, the results presented herein suggest 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 open probability is likely regulated by neurotransmitters such as norepinephrine (Schmidt et al., 2001; Ramminger et al., 2004). Ion transport mechanisms have been discussed previously, in the context of mammary epithelia from other species (Neville, 1999; Shennan and

Peaker, 2000). The new knowledge identified in this manuscript 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 the  $\beta$ - and  $\gamma$ -subunits and only mildly in the  $\alpha$ -subunit. ENaC expression is regulated by glucocorticoids and/or mineralocorticoids in a tissue-specific pattern. Aldosterone's effects are observed primarily in the kidney with effects also observed in the colon. Glucocorticoids, however, appear to play a central role in regulating ENaC expression in the airway or alveolus and in epithelia lining the male reproductive duct (unpublished observations). Glucocorticoids may also influence ENaC expression in the intestine. 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  $\gamma$ -ENaC compared with their normal littermates, whereas  $\alpha$ - and  $\beta$ -ENaC expression are not different. Preterm human babies have reduced levels for all ENaC subunits in nasal epithelium, and dexamethasone treatment increased  $\alpha$ - and  $\beta$ -ENaC expression. H441 cells, which are of bronchiolar origin, respond to dexamethasone with increased mRNA for all ENaC subunits, although  $\beta$ -ENaC appears to be affected most. Primary cultures of rat alveolar epithelial cells responded to dexamethasone with a fourfold increase in  $\alpha$ -ENaC mRNA, whereas less than a twofold change in  $\beta$ - and  $\gamma$ -ENaC mRNA was observed. Likewise, our laboratory has observed that glucocorticoids increase the mRNA and protein expression of  $\alpha$ -ENaC while having no appreciable effect on  $\beta$ - or  $\gamma$ -ENaC expression in pig vas deferens epithelia (unpublished observations). Thus, bovine mammary epithelium appears to be unique in that glucocorticoids regulate the expression of  $\beta$ - and  $\gamma$ -ENaC while having little effect on  $\alpha$ -ENaC. The basis for the differences that have been noted could reflect both the species and tissue of origin. The human  $\alpha$ -ENaC gene harbors a GRE in the 5' flanking region. Whether similar response elements regulate  $\beta$ - or  $\gamma$ -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 pattern of modulation is similar to that found in the lung (Itani et al., 2002; Cole et al., 2004; Helve et al., 2004; Ramminger et al., 2004), but different from the most commonly studied  $\alpha$ -subunit-

mineralocorticoid-receptor regulated modulation found in renal tissues (Kamynina and Staub, 2002; Snyder, 2002; Gaeggeler et al., 2005; Shane et al., 2006). In fact, Na<sup>+</sup> reabsorption across lung tissue is radically altered at parturition, as is mandated by the tissue changing very quickly from a fluid to an air interface with immediate necessity for functional transitions (Liggins, 1976; Frank et al., 1980; Berger and Welsh, 1991). The observations that glucocorticoids affect mammary ion transport would suggest a similarly drastic alteration of mammary function at parturition as the gland changes from minimal secretion to colostrum-filled to milk-producing secretory epithelium with a significant ionic gradient being developed rapidly. Corticosteroids are increased at parturition, the time when both the airway of the offspring and the mammary system of the mother are undergoing great change in function. It is possible that glucocorticoid-induced ENaC expression contributes to the transition in mammary function.

This study has provided both functional and molecular evidence for the presence of the amiloride-sensitive apical sodium 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<sup>+</sup> reabsorption from the apical (i.e. milk) compartment. Cortisol is required for the transition to lactation. Colostrum is high in Na<sup>+</sup> and milk is low in Na<sup>+</sup> (Neville and Morton, 2001). The results presented in this paper may suggest that cortisol induces the transition in milk composition by increasing ENaC expression. Clearly, additional studies are required to test this hypothesis.

The ionic composition of the milk can be reflected as milk electrical conductivity (Sheldrake et al., 1983; Fernando et al., 1985). 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. It was recently reported that elevated apical electrolyte concentrations contribute to a breakdown of bovine mammary epithelium (Quesnell, 2006). The current work defines a mechanism whereby milk electrolytes could be rapidly reduced.

These results suggest that corticosteroids set or modulate the ion composition of milk by inducing ENaC expression. In light of a previous report that showed a causal relationship

between reduced apical  $\text{Na}^+$  concentration and increased mammary epithelial barrier integrity (Quesnell, 2006), 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  $\text{Na}^+$  absorption. The results suggest that within 24 hours after corticosteroid administration, mammary ENaC expression could be heightened and  $\text{Na}^+$  reabsorption could be substantial. If one makes a conservative assumption for bovine mammary epithelial surface area of about  $100 \text{ M}^2$  (equal to the estimated surface area for human lungs) and a  $\text{Na}^+$  transport rate of  $\sim 10 \mu\text{A cm}^{-2}$  (see Fig. 1), one computes a maximal  $\text{Na}^+$  absorption rate of 360 millimoles per hour. If one further conservatively assumes a milk volume of  $\sim 20 \text{ L}$  (which is nearly the maximum in a high producing dairy cow) and a milk  $\text{Na}^+$  concentration of  $\ll 50 \text{ mEq L}^{-1}$ , the total  $\text{Na}^+$  in the bovine udder would be  $\ll 1$  mole. Alternatively, colostrum or mastitic milk may be as high as  $100 \text{ mEq L}^{-1} \text{ Na}^+$  and total  $\text{Na}^+$  could approach 2 moles. Nonetheless, these calculations show that the mammary epithelium is capable of turning over the entire milk  $\text{Na}^+$  load in a few hours with corticosteroid induction. The authors appreciate that, as milk  $\text{Na}^+$  concentration is reduced, the rate of  $\text{Na}^+$  transport would decline and would ultimately be limited by the electrochemical gradient for  $\text{Na}^+$  across the apical membrane. Regardless, the results from this research project demonstrate that the mammary epithelium is capable of rapidly reducing  $\text{Na}^+$  concentration to the levels that are typically observed in healthy mature milk.

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

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**CHAPTER 4 – Apical electrolyte concentration modulates barrier function and tight junction protein localization in bovine mammary epithelium**

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# **CHAPTER 4 - Apical electrolyte concentration modulates barrier function and tight junction protein localization in bovine mammary epithelium**

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RUNNING HEAD: Electrolytes/cytokines affect mammary epithelial barrier

KEYWORDS: transepithelial electrical resistance, apical cation concentration, paracellular permeability, mastitis, inflammatory cytokines, occludin.

## **ABSTRACT**

*In vitro* mammary epithelial cell models typically fail to form a consistently tight barrier that can effectively separate blood from milk. Our hypothesis was that mammary epithelial barrier function would be affected by changes in luminal ion concentration and inflammatory cytokines. BME-UV cells were grown to confluence on permeable supports with a standard basolateral medium and either high electrolyte (H-elec) or low electrolyte (L-elec) apical medium for 14 days. Apical media were changed to/from H-elec at predetermined times prior to assay. Transepithelial electrical resistance ( $R_{te}$ ) was highest in monolayers continuously exposed to apical L-elec. A time-dependent decline in  $R_{te}$  began within 24 hours of H-elec exposure. Change from H-elec to L-elec time-dependently increased  $R_{te}$ . Permeation by FITC-conjugated dextran was elevated across monolayers exposed to H-elec, suggesting compromise of a paracellular pathway. Significant alteration in occludin distribution was evident concomitant with the changes in  $R_{te}$ , although total occludin was unchanged. Neither substitution of  $\text{Na}^+$  with N-methyl-D-glucosamine (NMDG<sup>+</sup>) nor pharmacological inhibition of transcellular  $\text{Na}^+$  transport

pathways abrogated the effects of apical H-elec on  $R_{te}$ . Tumor necrosis factor alpha, but not interleukin-1 $\beta$  nor interleukin-6, in the apical compartment causes significant decrease in  $R_{te}$  within 8 hours. These results indicate that mammary epithelium is a dynamic barrier whose cell-cell contacts are acutely modulated by cytokines and luminal electrolyte environment. Results not only demonstrate that BME-UV cells are a model system representative of mammary epithelium, but also provide critical information that can be applied to other mammary model systems to improve their physiological relevance.

## INTRODUCTION

Mammary epithelium is responsible for both secretion of milk and the barrier to separate milk from blood, and therefore has unique pathophysiological challenges that are difficult to address. When the barrier function of this secretory epithelium is compromised, permanent damage to the gland can occur. Change in milk electrical conductivity, related to ionic composition, is one of the earliest indicators of pathological changes in epithelial function in the bovine mammary gland (Sheldrake et al., 1983; Fernando et al., 1985). The mammary epithelial barrier breaks down during gland inflammation, milk production is reduced and the lack of separation of the milk compartment from the blood compartment is associated with a cascade of events that can cause permanent damage to the gland (Kitchen, 1981; Oshima et al., 1990; Leitner et al., 2004). However, any causal relationship between the change in epithelial barrier function and the change in milk ion content remains incompletely defined. Current tenets suggest that breakdown of the epithelial barrier allows electrolyte-rich plasma to enter the milk compartment, thus causing the changes in milk electrical conductivity associated with clinical or preclinical mastitis. An alternative explanation is that a pathologically-induced elevation in electrolyte concentration contributes to the breakdown of mammary epithelial integrity.

Inflammatory mediators are recruited to the mammary gland under pathological conditions, although direct effects of cytokines on mammary epithelium have not been fully delineated. During mammary infection, cytokines interleukin-1 (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- $\alpha$ ) are elevated in the milk within 6-8 hours following bacterial inoculation of the gland (Persson Waller et al., 2003; Alluwaimi, 2004; Zhu et al., 2004; Pareek et al., 2005). It is speculated, from work in other tissues, that these chemicals signal changes in milk composition by directly or indirectly affecting the epithelium (Marano et al., 1998; Schmitz

et al., 1999; Heyman and Desjeux, 2000; Bruewer et al., 2003; Hanada et al., 2003). It is possible that cytokine and chemokine recruitment may directly affect the barrier function of the epithelium. Thus, it is critical to determine if these cytokines directly affect the epithelium and to determine which aspects of epithelial function are affected.

Mammary epithelial barrier function has been difficult to quantify [reviewed in (Shennan and Peaker, 2000; McManaman and Neville, 2003)]. A previous report from this laboratory suggested that luminal composition, which is dependent on epithelial ion transport, could affect the epithelial barrier integrity as measured by transepithelial electrical resistance ( $R_{te}$ ) (Schmidt et al., 2001). Nonetheless, little is known regarding the timecourse over which this barrier breakdown occurs, whether a paracellular pathway is affected, or the target proteins that are involved in increased epithelial permeability. Examining the effects of changes in ionic composition on the epithelia is a critical step to understanding the progression of the breakdown of cell-cell contacts, as well as illuminating subsequent options for treatment of mastitis.

Many *in vitro* mammary epithelial cell lines are currently being used to study the dynamics of the gland and to examine a variety of pathologies, including breast cancer (Gable et al., 2006; Juan et al., 2006; Paszkiewicz-Gadek et al., 2006; Saarinen et al., 2006; Wang et al., 2006). In most cases mammary cancer cell lines of epithelial origin are being grown on solid supports, which precludes full membrane polarization and the opportunity to assess physiological function. Also, these systems have typically utilized the same high electrolyte medium bathing both the apical and basolateral aspects of the epithelium. Attempts are being made to grow mammary epithelial cells on permeable supports (Cowley, 2006). However, Schmidt et al (Schmidt et al., 2001) provide the only publication to date in which transformed mammary epithelial cells were cultured on permeable supports in asymmetrical media that approximates the *in vivo* environment and indicates a culture-dependent change in barrier function.

The primary goal of this study was to test mammary epithelial integrity following changes in apical ionic composition (to simulate changes in milk electrical conductivity) using an *in vitro* bovine mammary epithelial model. This attempt to determine whether luminal electrolytes affect epithelial integrity is the first to concomitantly measure  $R_{te}$ , permeation by uncharged particles, and the distribution of proteins associated with tight junctions directly at the level of the epithelium in an *in vitro* system. A secondary goal for this study was to identify and characterize effects of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) on epithelial barrier

function in this *in vitro* model. We hypothesize that exposure to increased luminal ionic concentration or inflammatory cytokines would rapidly decrease epithelial barrier function. The results indicate that mammary epithelial tight junctions are dynamic structures that can be rapidly modified by TNF- $\alpha$  and by changes in the fluid electrolyte composition in the milk compartment. More importantly, the cytokine effects on mammary epithelial barrier function pale in comparison to the rapid and significant compromise of barrier function during exposure to elevated electrolytes in the luminal compartment. The decrease in barrier function is due, at least in part, to changes in localization of the tight junction protein occludin. This information brings into focus the need to re-evaluate current paradigms in progression of mammary pathology.

## MATERIALS AND METHODS

**Cell culture media.** An immortalized bovine mammary epithelial cell line, BME-UV, was used for the present studies, and two media were used for cell culture. The medium used to bathe the basolateral aspect of the monolayers was described along with the BME-UV cells (Zavizion et al., 1996b). This medium contains little lactose, has concentrations of electrolytes that closely mirror serum, and in the context of the current experiments will be termed ‘high electrolyte’ (H-elec) to differentiate it from the low-electrolyte medium (L-elec) that is closer in electrolyte composition to milk (Schmidt et al., 2001), containing less than half the Na<sup>+</sup> and Cl<sup>-</sup> and including 160 mM lactose (see Table 4-1 for analysis). The L-elec medium in the current studies is similar in composition to the alternative medium described by Schmidt et al (Schmidt et al., 2001). Osmolarity was  $290 \pm 5$  mOsm in both H-elec and L-elec media, pH was adjusted to 7.3, and included an HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> buffer system.

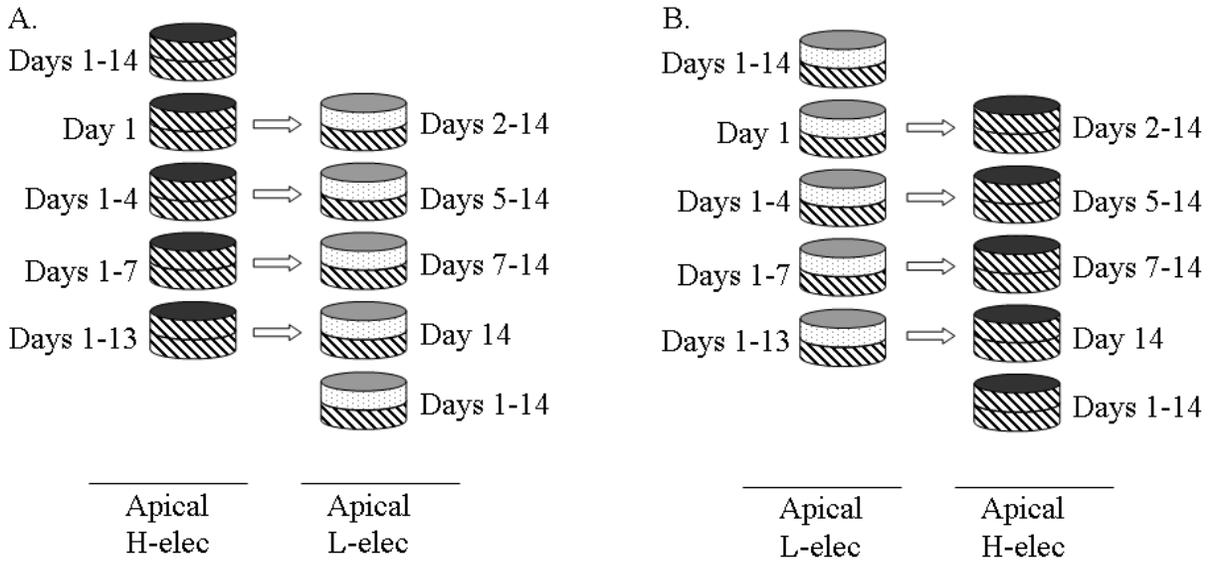
Component	H-elec	L-elec
Sodium	129 mM	56 mM
Potassium	5.0 mM	19.8 mM
Chloride	107 mM	45 mM
Bicarbonate	21 mM	23 mM
Calcium	5.2 mg/dl	5.6 mg/dl
Magnesium	1.5 mg/dl	1.6 mg/dl
Glucose	194 mg/dl	200 mg/dl

**Table 4-1 H-elec and L-elec media component analysis**

Analysis conducted at the Department of Diagnostic Medicine/Pathobiology Clinical Pathology Laboratory in the Kansas State University College of Veterinary Medicine. A single preparation of each medium was analyzed using a Hitachi 911 selective analyzer.

**Cell culture protocols.** Stock cultures of BME-UV cells (provided by Jeff Smith, University of Vermont) were grown in H-elec medium on 25 cm<sup>2</sup> plastic culture flasks (Cellstar, Frickenhausen, GE) to a maximum of 65-75% confluency. Cells were dissociated for passage using a solution containing 0.25% v/v trypsin and 2.65 mM disodium ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline for cell culture (PBSc; composition in mM: 140 NaCl, 2 KCl, 1.5 KH<sub>2</sub>PO<sub>4</sub>, 15 Na<sub>2</sub>HPO<sub>4</sub>). Dissociated and dispersed cells were seeded to permeable tissue culture supports (Snapwell Clear, Corning-Costar, Acton, MA) as described previously (Schmidt et al., 2001). Cells were maintained in culture on permeable supports for 15 days prior to assay during which growth to a confluent, polarized, electrically tight monolayer has been documented (Schmidt et al., 2001). H-elec medium bathed the basolateral aspect of the cells throughout all experiments. The apical aspect of the cells was exposed to either H-elec or L-elec medium as described below. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Media on both the apical and basolateral aspects of the cells was refreshed daily, with a final change occurring 4-8 hours prior to experimental analysis.

Two sets of experiments were conducted to test for effects of changing the apical medium from, or to, H-elec. In both experiments, Snapwell trays were seeded with BME-UV cells in symmetrical H-elec medium. Twenty-four hours post seeding is considered “day 1” in culture. In the first experiment, the apical media of five Snapwells in each tray was changed to L-elec on day 1 in culture. Beginning on day 4, 7, 10, and 13, successive monolayers were apically exposed to H-elec medium for the remaining duration of the culture period (Fig. 4-1A). One monolayer was exposed to H-elec apical medium throughout the entire culture period. In the second experiment, the H-elec apical medium was maintained for 1, 4, 7, 10, and 13 days before changing to apical L-elec medium on successive monolayers (Fig. 4-1B). In all cases, monolayers were evaluated for barrier integrity on post-seeding day 15. At this time, monolayers were also fixed for assessment of tight junction-associated proteins by immunocytochemistry, or harvested for Western blot analysis as described below.



**Figure 4-1** Experimental protocols for figures 2 – 8 include apical medium transition from A) H-elec to L-elec or B) L-elec to H-elec on the days indicated.

Media were changed daily, and once the transition to a new apical medium was made, the same medium was used throughout the remainder of the experimental timeframe. Basolateral medium was H-elec through the duration of all experiments.

**Transepithelial electrical resistance.** Epithelial cell monolayers were mounted in modified Ussing flux chambers (Model DCV9, Navicyte, San Diego, CA) in symmetrical Ringer solution (composition in mM: 120 NaCl, 25 NaHCO<sub>3</sub>, 3.3 KH<sub>2</sub>PO<sub>4</sub>, 0.8 K<sub>2</sub>HPO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>) maintained at 37°C and continually circulated with an air-lift system using 95% O<sub>2</sub>/5% CO<sub>2</sub>. After recording open circuit transepithelial electrical potential, each monolayer was clamped to zero mV with a multichannel voltage clamp apparatus (Model 558C, University of Iowa, Department of Bioengineering, Iowa City, IA). At 100 second intervals the monolayers were exposed to a 5-sec, 0.5 mV bipolar pulse. Voltage and current measurements were made continuously and data were acquired digitally at 1 Hz using a Macintosh computer (Apple Computer, Cupertino, CA), an MP100A-CE interface (BIOPAC Systems, Santa Barbara, CA), and Aqknowledge software (ver. 3.2.6, BIOPAC Systems). Ohms law (resistance =  $\Delta$  voltage/ $\Delta$  current) was applied to the resulting current deflections to determine  $R_{te}$ .

**Transepithelial dextran permeation.** Movement of large, uncharged molecules across epithelial monolayers was assessed with FITC-conjugated dextran. Methods were similar to those used by Broughman et al (Broughman et al., 2004). Briefly, BME-UV monolayers were grown on Snapwell permeable supports with changes from or to H-elec apical medium on the

days post-seeding as indicated. Monolayers were washed with PBScc and then bathed in Ringer solution, both apically and basolaterally. Two different sizes of FITC-conjugated dextran, 9.5 kDa and 77 kDa (Sigma-Aldrich, St. Louis, MO), were utilized to assess epithelial permeability. FITC-conjugated dextran was introduced into the apical compartment of all wells at the outset of the assay. Monolayers were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 60 minutes after which samples of basolateral solution were obtained for analysis. Filter supports lacking cells were included to verify that the culture support did not provide a significant barrier to dextran permeation and cell monolayers exposed to Ringer solution in the absence of dextran were employed to establish that no components of the assay system contributed to the fluorescence measurements. Fluorescence in the basolateral compartment was quantified in a Fluoroskan *Ascent* FL plate reader (Labsystems, Helsinki, Finland). Known dilutions of each dextran stock were included in each 96-well assay plate to construct a standard curve after background subtraction.

***Immunocytochemistry.*** After exposure to the indicated culture conditions, BME-UV cell monolayers were washed in PBScc and fixed in 4% paraformaldehyde (Fisher Scientific International Inc., Hampton, NH), and stored less than 24 hours in phosphate buffered saline for cytochemistry (PBS; composition in mM; 150 NaCl, 5 KH<sub>2</sub>PO<sub>4</sub>, 15 K<sub>2</sub>HPO<sub>4</sub>, pH 7.2). Monolayers were permeabilized with 0.1% Triton X-100 in PBS, blocked for 1 hour with goat serum (Gibco-BRL, Rockville, MD; 5% in PBS). Primary antibodies to zonula occludens-1 (rat anti-ZO-1, Chemicon, Temecula, CA), and occludin (rabbit anti-occludin, Zymed, Carlsbad, CA), diluted 1:200 with PBS were incubated with the monolayers for 2 hours at room temperature. Cells were washed 3 X 10 minutes in PBS and subsequently incubated for 2 hours at room temperature with a rhodamine-conjugated goat anti-rat (for ZO-1; Vector Laboratories, Burlingame, CA) or Alexa 488-conjugated goat anti-rabbit (for occludin; Molecular Probes, Eugene OR) secondary antibody. Once again, cells were washed 3 X 10 minutes in PBS. Cells were further incubated with 10 µM TO-PRO3 (Invitrogen, Eugene, OR) for 1 hour and cells were washed briefly with PBS immediately prior to mounting on slides with Fluormount G (SouthernBiotech, Birmingham, AL). Images were assessed by both standard (Leica Microsystems AG, Wetzlar, Germany), and confocal (Carl Zeiss MicroImaging, Inc., Thornwood, NY) microscopy using appropriate filters for each fluorophore. Identical settings were used to obtain and process images derived from paired monolayers. Negative controls

included protocols with the primary antibody omitted, the secondary antibody omitted, or, when available, peptide-adsorbed primary antibody.

**Western blot analysis.** BME-UV cell lysates were prepared in the 1.13 cm<sup>2</sup> Snapwell culture substrates using Phosphosafe lysis buffer (Novagen, San Diego, CA) including 1% Protease Inhibitor Cocktail (Sigma-Aldrich) and maintained in a frost-free freezer at -20°C until assay. Total protein content was determined using a Bicinchoninic Assay (Pierce, Rockford, IL) and 20 µL total protein was loaded in each well of a 10-20% SDS-Page pre-poured gel (BioRad, Hercules, CA) for electrophoresis. Gels were run at 160 mV for 45 minutes, then transferred to Millipore PVF-Immobilon membranes for 8.5 hours at 90 mV. Transfer was confirmed by staining gels with Gelcode Blue (Pierce). Membranes were blocked in SuperBlock Blocking Buffer (Pierce), and probed with rat anti-ZO-1 and rabbit anti-occludin diluted in SuperBlock Blocking Buffer. Protein was visualized using a peroxidase-conjugated secondary antibody (1:5000) by enhanced chemiluminescence with Pico-chemiluminescence substrate (Pierce). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce), and stripping was confirmed with visualization using Femto-chemiluminescence substrate (Pierce), a more sensitive chemiluminescence substrate to preclude any residual labeling. Membranes were re-probed with mouse monoclonal primary antibody for either GAPDH (Abcam, Cambridge, MA) or beta-actin (Sigma-Aldrich) as a loading control for densitometric analysis. Membranes were exposed on CL-Xposure film (Pierce) and analyzed using a Kodak RP X-OMAT (Model M7B) film analyzer. Imaging and densitometric analysis was performed on a Fluor Chem 8900 Alpha Innotech Imaging System with Alpha Ease FC StandAlone Software (Alpha Innotech, San Leandro, CA).

**Amiloride/EIPA-exposure experiments.** BME-UV cells were seeded onto Snapwell permeable supports (in groups of six) as described above. The apical medium of three Snapwells in each tray was changed to L-elec on day 1, and additional wells were exposed apically to H-elec conditions for the duration of the 15 day culture period. Beginning on day 13, one L-elec and one H-elec monolayer was cultured in the presence of amiloride (10 µM; a selective blocker of the epithelial Na<sup>+</sup> channel, ENaC), and one L-elec and one H-elec monolayer was cultured in the presence of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 1 µM; an amiloride analog that preferentially inhibits the Na<sup>+</sup>/H<sup>+</sup> exchanger, NHE) in the apical culture media for 24 hours prior to evaluation of  $R_{te}$  in the Ussing chamber.

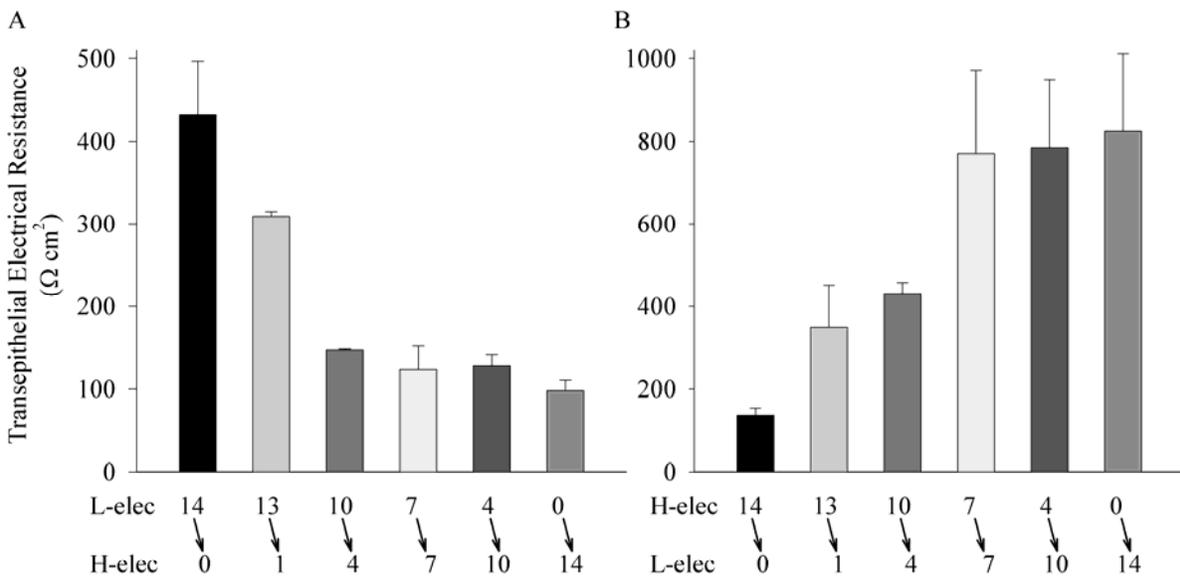
***Alternative solutions for Ussing chamber experiments.*** Reduced Na<sup>+</sup> and/or Cl<sup>-</sup> solutions were employed for certain experiments by equimolar replacement of 120 mM NaCl with N-methyl-D-glucosamine•HCl (NMDG-Cl), Na-gluconate, or NMDG-gluconate. Alternatively, in some experiments 60 mM NaCl was replaced by 120 mM lactose to test for effects of electrolyte reduction.

***Cytokine treatments.*** BME-UV cell monolayers, cultured in the presence of a L-elec apical medium, were exposed to cytokines that have been associated with mammary inflammation (Persson Waller et al., 2003; Zhu et al., 2004; Pareek et al., 2005) [reviewed in (Alluwaimi, 2004)], including TNF- $\alpha$  (0.5  $\mu$ g/mL), IL-1 $\beta$ , (0.1  $\mu$ g/mL) or IL-6 (1  $\mu$ g/mL) in the apical medium for 8 or 12 hours prior to assessment of  $R_{te}$  in modified Ussing chambers.

***Data Analysis.*** Quantitative results were analyzed via ANOVA using SAS (SAS Institute Inc., Cary, NC). Data are presented as mean  $\pm$  SEM. Differences are considered statistically significant when the probability of a type I error is  $< 0.05$ .

## RESULTS

***H-elec apical medium decreases transepithelial electrical resistance.*** Initial experiments document and extend observations (Schmidt et al., 2001) regarding the relationship of apical electrolyte concentration to  $R_{te}$  by systematically assessing the time-course over which differences in  $R_{te}$  can be induced by changes in apical electrolyte concentration. Results presented in Fig. 4-2 demonstrate that L-elec apical medium is consistently associated with the greatest  $R_{te}$ . In the first set of experiments (Fig. 4-2A), monolayers exposed only to L-elec for the entire culture period exhibited more than 4-fold greater  $R_{te}$  than monolayers exposed to H-elec throughout. More importantly, exposure to H-elec medium for only one day was associated with greater than 25% reduction in  $R_{te}$  ( $432 \pm 64 \Omega\text{cm}^2$  vs.  $309 \pm 6 \Omega\text{cm}^2$ ). In fact, within 1 hour of continuous exposure to H-elec, barrier function, as measured by  $R_{te}$ , began to decline (data not shown). Four-days exposure to H-elec showed further incremental reduction in  $R_{te}$  to less than 30% of the L-elec value. Although a trend toward further reduction in  $R_{te}$  was observed with additional exposure to H-elec, the incremental changes did not achieve statistical significance. These results demonstrate that short-term apical exposure to H-elec compromises the mammary epithelial barrier function as assessed by electrical means.



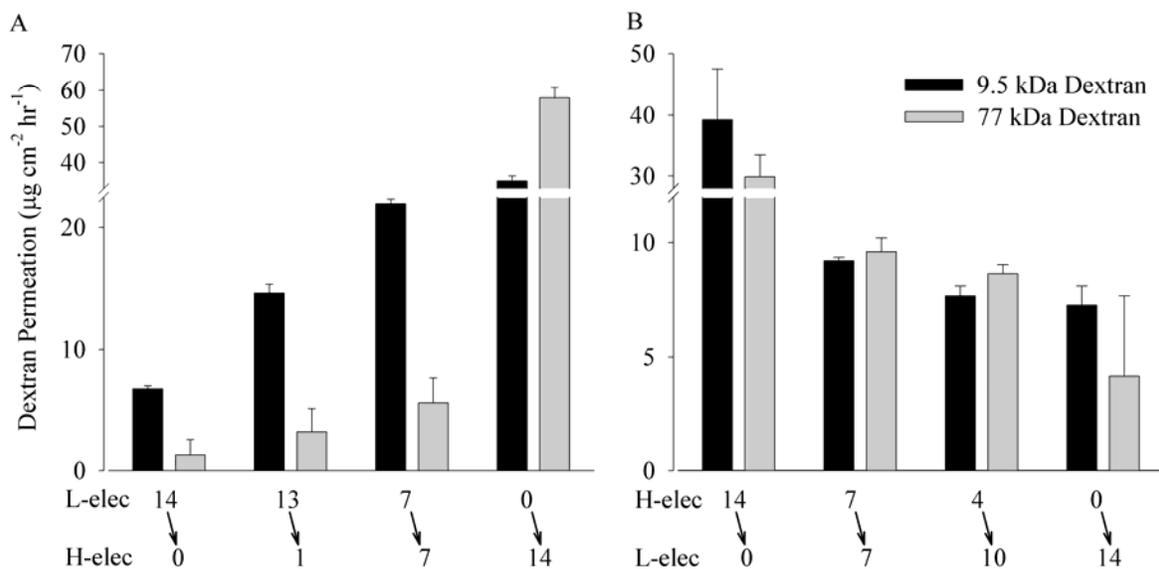
Days of apical exposure to initial medium followed by days of apical exposure to alternative medium  
**Figure 4-2 Apical medium composition affects epithelial barrier integrity as assessed by electrical measurements.**

A) Transition from L-elec to H-elec apical medium is associated with reduced  $R_{te}$ . BME-UV cell monolayers were cultured with L-elec apical medium (Schmidt et al., 2001) for the number of days indicated, and then cultured in apical H-elec for the remainder of the 14 day culture period (see methods). B) Transition from H-elec to L-elec apical medium is associated with elevated  $R_{te}$ . BME-UV cell monolayers were cultured with H-elec apical medium for the number of days indicated, and then cultured in apical L-elec for the remainder of the 14 day culture period. In every case, the greatest  $R_{te}$  is associated with the most recent and most prolonged exposure to L-elec. Results are summarized from 15 (A) or 13 (B) tightly paired experiments.

***L-elec apical medium enhances transepithelial electrical resistance.*** A transition of apical medium from H-elec to L-elec is associated with greater  $R_{te}$  (Fig. 4-2B) and results provide a virtual mirror image of Fig. 4-2A. Monolayers apically exposed to H-elec throughout the 14-day culture period exhibited the lowest  $R_{te}$  of all wells in each block ( $138 \pm 17 \Omega \text{ cm}^2$ ). Monolayers exposed to H-elec for 13, 10, and 7 days before an apical transition to L-elec for 1, 4, and 7 days, respectively, exhibited incrementally greater  $R_{te}$ . Differences in  $R_{te}$  were not associated with longer periods (i.e., up to 14 days) of apical exposure to L-elec. These results suggest that a reduction in apical electrolyte concentration, to a value that more closely approximates that of typical (i.e., healthy) milk, rapidly promotes the enhancement of the mammary epithelial barrier and an electrically tight monolayer.

***Apical H-elec increases paracellular permeability.*** Reductions in  $R_{te}$  might reflect an increase in transcellular permeability, paracellular permeability, or some combination of these

two permeation pathways. Thus, gradient-driven permeation of 9.5 and 77 kDa dextran was employed to assess changes in the paracellular pathway resulting from differences in the apical solution. As shown in Fig. 4-3A, monolayers exposed apically to L-elec medium for the entire culture period exhibited the lowest permeation rates (i.e., most substantial barrier) to diffusion when compared to other time points for both 9.5 and 77 kDa dextran. Apical exposure to H-elec for as little as 1 day, following 13 days of L-elec, was associated with significantly greater permeation of both sizes of dextran. Incrementally greater permeation was observed with 7 days exposure to apical H-elec. The greatest permeation was observed when monolayers were exposed apically to H-elec throughout the entire culture period. These observations demonstrate that short-term exposure to a serum-like medium on the apical face of bovine mammary epithelial cells promotes a loss of the barrier integrity that is associated with the opening of the paracellular pathway to allow the permeation of high molecular weight solutes; an observation that is consistent with the reduction in  $R_{te}$  (Fig. 4-2A).



Days of apical exposure to initial medium followed by days of apical exposure to alternative medium  
**Figure 4-3 Apical medium composition affects the permeation rate across BME-UV cell monolayers for 9.5 kDa and 77 kDa dextran conjugated to FITC.**

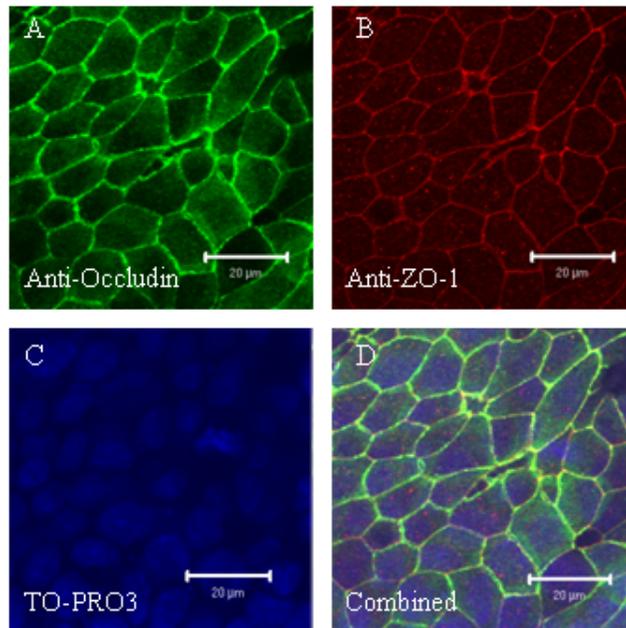
A) Transition from L-elec to H-elec apical medium is associated with increased paracellular permeability to both 9.5 kDa and 77 kDa dextran. BME-UV cell monolayers were cultured with L-elec apical medium for the number of days indicated, and then cultured in apical H-elec for the remainder of the 14 day culture period. B) Transition from H-elec to L-elec apical medium is associated with a decrease in paracellular permeability to both 9.5 kDa and 77 kDa dextran. BME-UV cell monolayers were cultured with H-elec apical medium for the number of days indicated, and then cultured in apical L-elec for the remainder of the 14 day culture period.

Experiments were conducted in symmetrical Ringer solution with FITC-dextran added to the apical compartment and the appearance of FITC-dextran in the basolateral compartment was quantified after one hour. Results are summarized from 10 (A) or 9 (B) tightly paired experiments.

***Apical L-elec medium decreases paracellular permeability.*** To test further the hypothesis that shifts in the apical medium composition modulate paracellular permeability, experiments were conducted in which the apical medium was changed from H-elec to L-elec at specified time points. Results presented in Fig. 4-3B demonstrate that the ‘leakiness’ of the mammary epithelium is reduced by apical exposure to L-elec medium incrementally, depending upon the duration of exposure. As predicted, the greatest permeation was observed in monolayers continually exposed to H-elec. However, exposure to L-elec medium for the shortest time tested in these experiments, 7 days, was associated with a decrement of >70% in the magnitude of both 9.5 and 77 kDa dextran permeation. Also as predicted, based upon the results presented in Fig. 4-2B, longer periods of exposure to L-elec were not associated with further reductions in permeation. Taken together, results presented in Figs. 4-2 and 4-3 demonstrate that the mammary epithelial barrier, whether quantified by either  $R_{te}$  or by the permeation of large, uncharged solutes, is sensitive to the medium composition in the apical compartment.

***Occludin, but not ZO-1, distribution is altered by apical medium.*** To test whether reduced integrity of the epithelial barrier was related to changes in junctional proteins, and to rule out the possibility that apical H-elec was causing a portion of the cells to ‘release’ from the culture substrate and/or the epithelium, experiments were conducted to visually evaluate the epithelium and to assess the distribution of the tight junction proteins ZO-1 and occludin. Examination of H-elec exposed BME-UV cell monolayers revealed no evidence for the loss of cells. In every case, a survey of at least 5 objective fields per filter revealed a confluent monolayer with a cobblestone appearance throughout. Monolayers exposed to L-elec medium for 14 days were co-labeled with anti-occludin primary antibody that was visualized using an Alexa 488-conjugated secondary antibody, and with anti-ZO-1 primary antibody that was visualized using either Alexa 594- (confocal microscopy) or rhodamine- (light microscopy; data not shown) conjugated secondary antibody. TO-PRO3 staining of nuclear material was included to provide an additional reference point within each cell. Occludin (Fig. 4-4A) and ZO-1 (Fig. 4-4B) immunoreactivities were identified in the apical-lateral portions of all cells in the epithelial monolayer. Fig. 4-4 provides the image at a single focal plane that is near the apical aspect of the

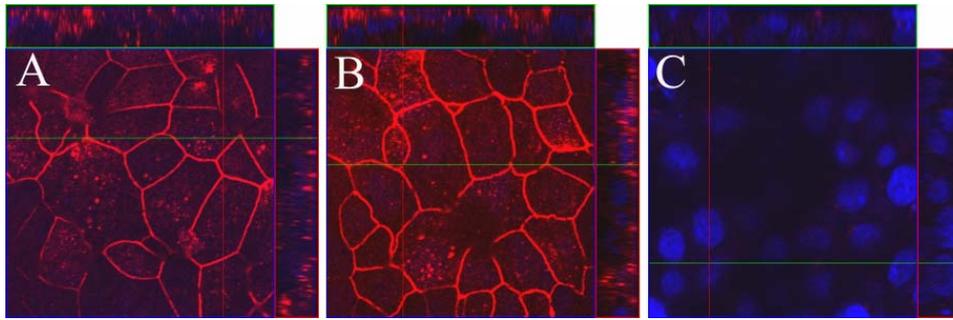
cells and was selected from a stack of images through the BME-UV cell monolayer. In the BME-UV cell monolayer, each cell is fully circumscribed by immunoreactivity for each of the epitopes. The combined image (Fig. 4-4D) clearly shows that the distribution of these two tight junction-associated proteins is virtually identical in these conditions.



**Figure 4-4 ZO-1 and occludin immunoreactivity circumscribes all cells in the BME-UV cell monolayer.**

The image of a single focal plane from a tri-labeled monolayer is presented. All cells are circumscribed by occludin (A) and ZO-1 (B) immunoreactivity that can be readily superimposed (D). That each cell is circumscribed becomes apparent by visualizing nuclei with TO-PRO3 (C) and observing that each nucleus is surrounded by immunoreactivity (D) Results are typical of 5 separate experiments.

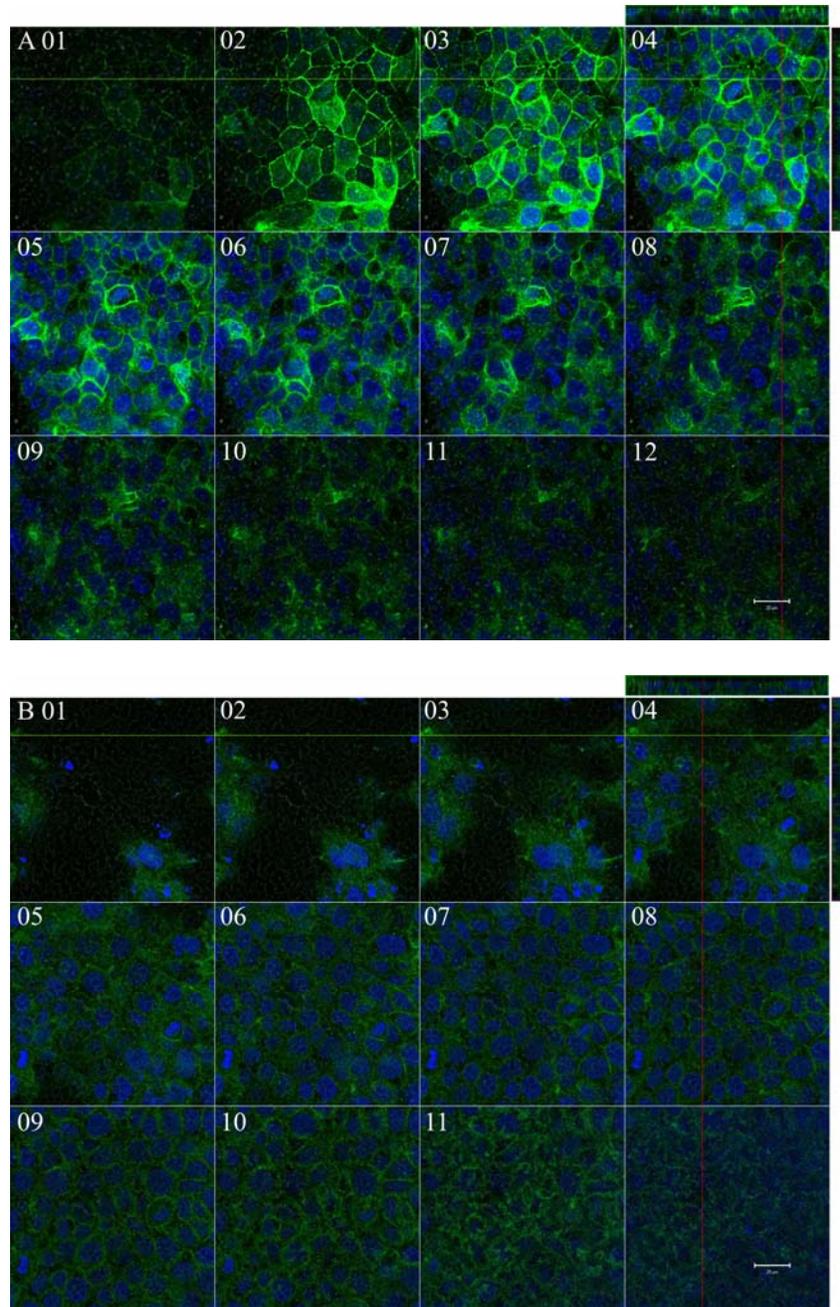
Experiments were conducted to determine whether distribution or intensity of these tight junctional proteins correlated with the differences noted in the  $R_{te}$  and dextran permeability studies. No differences in apparent quantity or localization of ZO-1 immunoreactivity were identified via light or confocal microscopy between monolayers exposed to H-elec or L-elec for the entire 14 day culture period (Fig 4-5).



**Figure 4-5** No differences in localization of ZO-1 immunoreactivity were identified in confocal images of anti-ZO-1/rhodamine-labeled cells in 14-day L-elec-exposed monolayers (A) as compared to 14-day H-elec-exposed monolayers (B).

Labeling by secondary antibody in the absence of primary antibody, as well as TOPRO-3 nuclear stain were included in (C) to demonstrate the specificity of the secondary antibody association with the primary antibody, and to preclude any concerns about non-specific secondary antibody labeling.

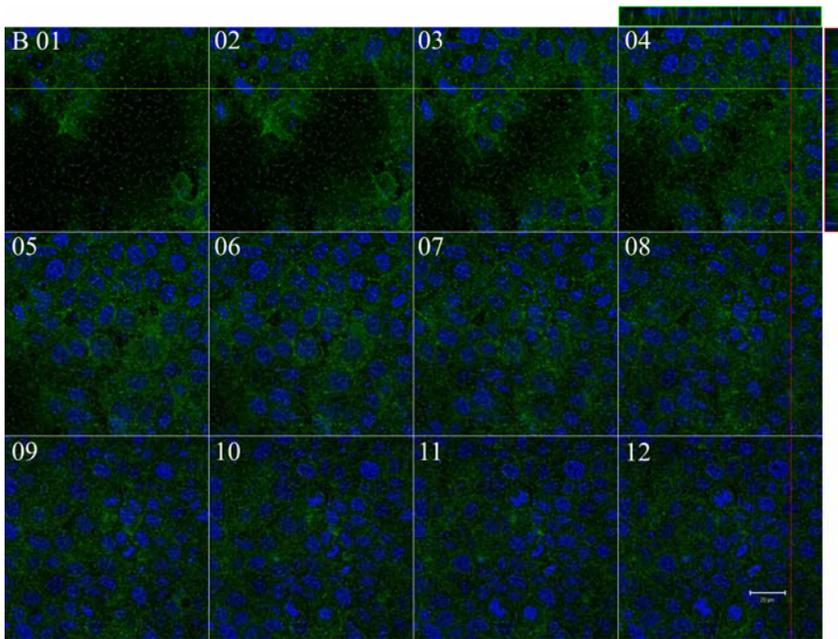
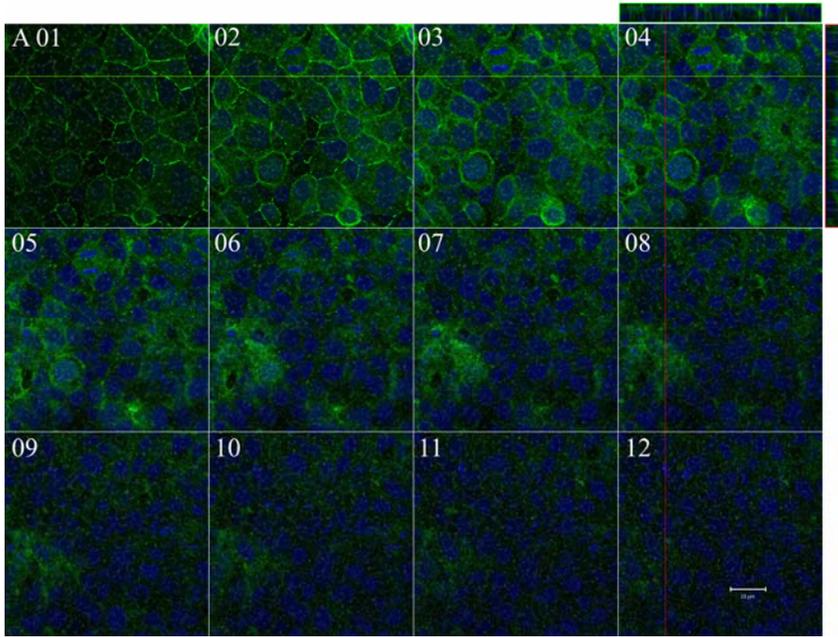
However, significant differences in occludin immunoreactivity were identified between monolayers exposed to L-elec and H-elec throughout the culture period (Fig. 4-6). Fig. 4-6A includes 12 individual plane images (z-stack) from a 14-day-L-elec-treated monolayer. The stacked images were taken at 1  $\mu\text{m}$  intervals sagittally through the monolayer and are presented in sequential order as indicated. Occludin immunoreactivity is clearly present in the apical portion of each cell and is particularly concentrated at the cell margins (panels 2 & 3). Some immunoreactivity is also present in the cytosol at the level of the nucleus (panels 4 through 7) and little immunoreactivity is present in the basal portion of the cells that is near to the filter substrate (panels 9 through 12). In contrast, occludin immunoreactivity is substantially less in BME-UV cells that were apically exposed for 14 days to H-elec (Fig. 4-6B). The layout of the confocal images is similar to that presented for the paired filter in Fig. 4-6A with the upper left image at the apical aspect of the monolayer and the lower right panel nearest to the culture substrate. In this case, progressive optical sections through the monolayer demonstrate that the pattern of occludin immunoreactivity is distinctly different in BME-UV cell monolayers cultured in apical H-elec as compared to apical L-elec-exposed monolayers. These results are consistent with observations presented in Figs. 4-2 and 4-3, and suggest that changes in occludin expression or distribution might account for the changes in  $R_{te}$  and dextran permeation. Thus, we next sought to test for redistribution of occludin that could occur within different time frames.

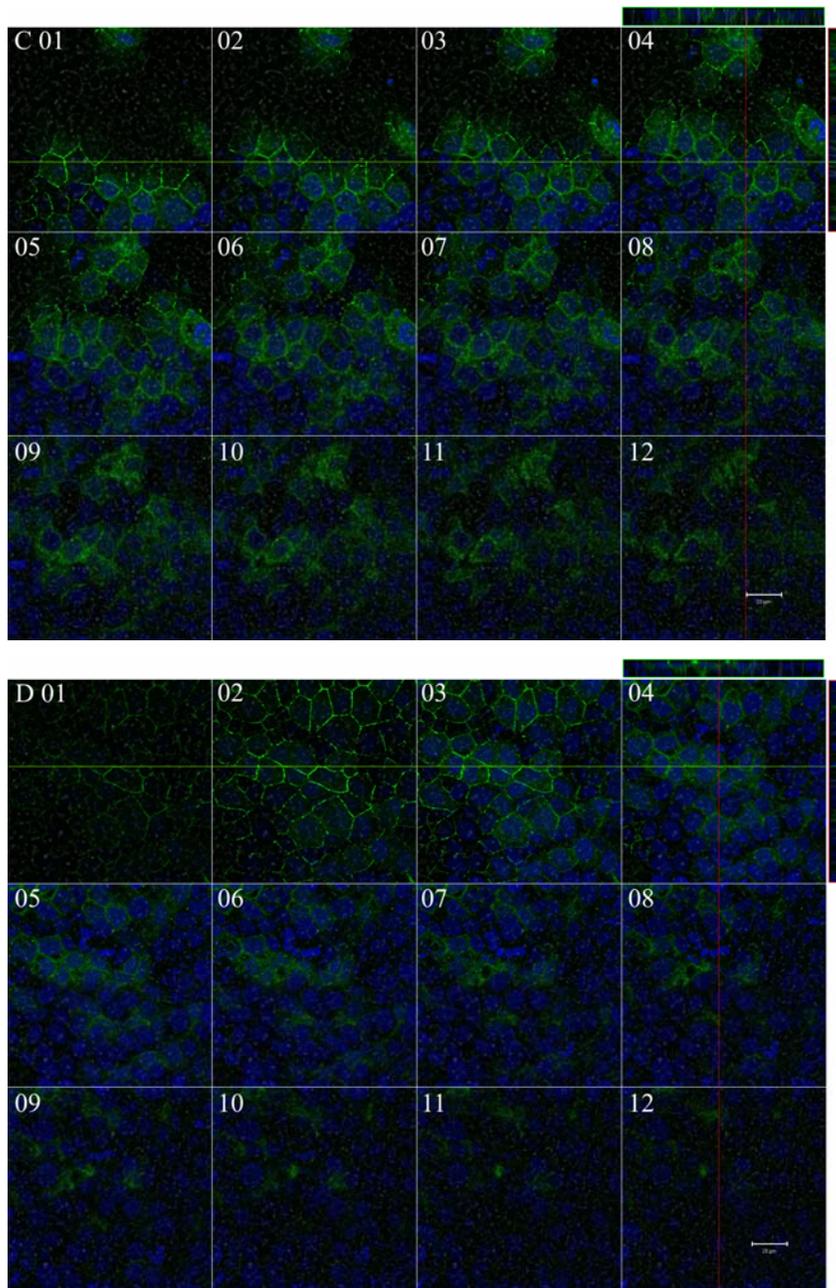


**Figure 4-6** Localization of occludin immunoreactivity is modified by apical media composition as shown in stacked confocal microscope images of anti-occludin/Alexa 488-labeled cells in 14-day L-elec-exposed monolayers (A) as compared to 14-day H-elec-exposed monolayers (B). After a two week exposure to L-elec in the apical compartment (A), occludin immunoreactivity clearly circumscribes all cells in the monolayer. After a two week exposure to H-elec composition in the apical compartment, minimal occludin localization is evident at the expected level of the tight junctions as compared to monolayers exposed to low apical electrolyte concentration. Monolayers were exposed to experimental treatments in tightly paired units (in conjunction with corresponding images in Figure 4-7), and processed in parallel. Confocal images represent sequential planes taken at 1  $\mu$ m intervals from apical (top left) to more

basolateral (bottom right) through the cell monolayer. Results are typical of 5 separate experiments. Panels to the top and right of the image represent a horizontal (top) and vertical (right) slice through the monolayer from apical to basolateral aspect at the indicated red or green line on the image. Scale bar indicates 20  $\mu\text{m}$ .

In an experiment tightly paired with the images in Fig. 6, occludin immunoreactivity was assessed in monolayers grown in L-elec and switched to H-elec for either 1 or 7 days, as well as monolayers grown in H-elec and switched to L-elec for 4 and 7 days. An apparent redistribution of occludin immunoreactivity was evident with 24-hours of apical H-elec exposure (Fig. 4-7A). When compared to Fig. 4-6A, occludin immunoreactivity is punctate, less intense and less defined, but still predominantly at the apical margins (panels 2 and 3). With 7 days exposure to H-elec (Fig. 4-7B) no occludin immunoreactivity is observed at the expected location of the tight junction. There is little difference between occludin distribution following 7 or 14 days exposure to H-elec (compare Fig. 4-7B to Fig. 4-6B). Alternatively, occludin immunoreactivity is apparent at the apical cell margins with 4 days exposure to apical L-elec after 10-days exposure to H-elec (Fig. 4-7C) although occludin immunoreactivity remains obvious in the cytosol in the nuclear and basal portions of the cells. Results presented in Fig. 4-7D show that, following 7-days of apical L-elec exposure, intense occludin immunoreactivity is present at the apical cell margins and lesser immunoreactivity is present in the cytosol. Taken together, data presented in Figs. 4-2 through 4-7 demonstrate that apical exposure of BME-UV cell monolayers to a 'blood-like' medium is associated with an apparent loss of barrier integrity as measured by electrical resistance, solute permeation, and protein distribution. Alternatively, barrier integrity is enhanced with exposure to a 'milk-like' medium. The mechanism(s) by which the epithelial cells sense the apical composition and the signaling pathway leading to the response, however, remain to be determined.



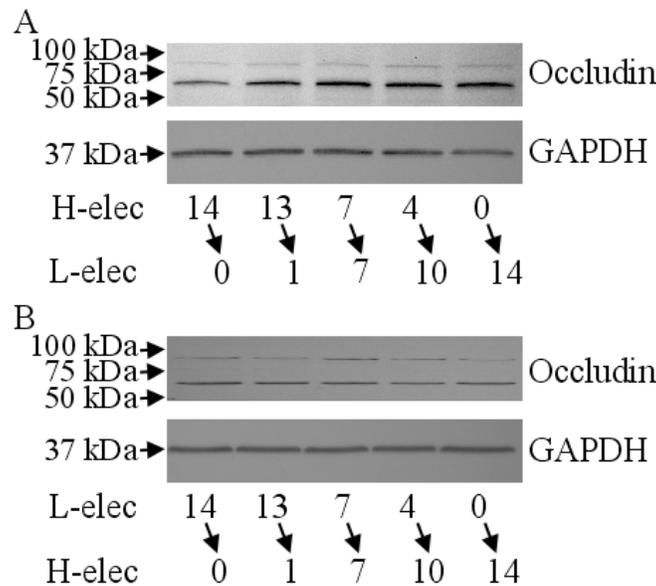


**Figure 4-7 Occludin localization is changed rapidly after apical medium changes.**

Changes in occludin localization were evident as early as 24 hours after exposure to high electrolyte apical medium. (A) More punctuate or diffuse labeling of occludin was evident (as compared to the correlating image in Fig. 6A). (B) After 7 days exposure to high electrolyte medium in the apical compartment, occludin localization was similar to 14 day high electrolyte images (see Fig. 6B), where there is no indication of accumulation at tight junctions. (C) Occludin appears to begin reappearing at the level of the tight junctions by 4 days of exposure to low electrolyte medium. (D) Occludin is organized at the level of the tight junctions after 7 days of exposure to low electrolyte apical medium. Images in Figures 6 and 7 represent cell monolayers exposed to experimental treatments in tightly paired units and processed in parallel.

Confocal images represent sequential planes taken at 1  $\mu\text{m}$  intervals from apical (top left) to basal (bottom right) through the cell monolayer in each set of images. Results are typical of 5 separate experiments. Secondary-antibody-only controls, to rule out evidence for non-specific labeling, for each monolayer are included as Supplementary data Figs 2-7, and include goat anti-rabbit Alexa 488 secondary antibody (for occludin) and goat-anti-rat rhodamine secondary antibody (for ZO-1) treatments. TO-PRO3 nuclear staining demonstrates cell locale, as does the superimposed brightfield image. Panels to the top and right of the image represent a horizontal (top) and vertical (right) slice through the monolayer from apical to basolateral aspect at the indicated red or green line on the image. Scale bar indicates 20  $\mu\text{m}$ .

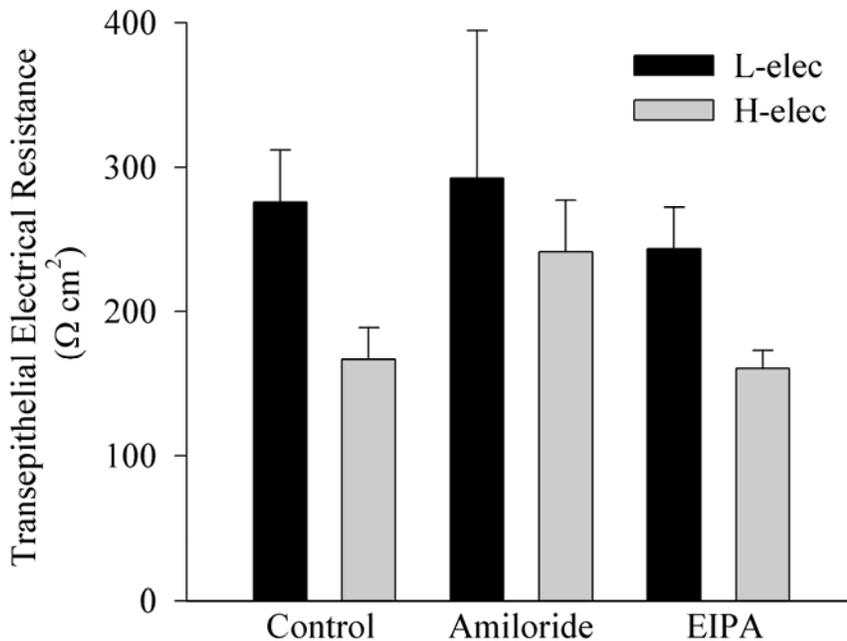
**Occludin expression is not altered by apical medium.** BME-UV cell monolayers exhibited similar levels of occludin expression regardless of apical culture conditions over the timecourse of the experiment. Cell lysates from each treatment, containing similar amounts of total protein, were applied in sequential lanes of a gel for Western blot analysis and densitometric analysis, including GAPDH and  $\beta$ -actin controls for normalization of total protein loading, was performed. Two clear bands were evident for occludin protein. Although it is possible that these multiple mobilities are due to different phosphorylation states for the occludin protein, experiments were not performed to test this possibility. No significant differences in immunoreactive occludin protein were identified in monolayers exposed to different apical conditions (Fig 4-8). These observations indicate that occludin redistribution, rather than expression, is one of the defining factors for loss of barrier function across this epithium.



**Figure 4-8 Western blot analysis of total protein lysates identified no significant change in overall amounts of anti-occludin immunoreactivity in H-elec-treated monolayers as compared to L-elec-treated monolayers, or over the timecourse of treatment.**

Monolayers were exposed to experimental treatments in tightly paired units, and results are typical of 6 experiments performed in duplicate (12 total blots).

***Na<sup>+</sup> transport blockers fail to affect medium-associated changes in epithelial barrier function.*** It was speculated that the effect of H-elec on the epithelial barrier might result from the relatively high Na<sup>+</sup> concentration that could affect cellular metabolism by changes in membrane potential, cytosolic ion concentration, or cytosolic pH. To investigate these possibilities, cells were cultured in conditions to minimize Na<sup>+</sup> entry via conductive or exchange pathways. In the first experiment, amiloride, EIPA, or vehicle control were included in the apical growth medium for 24 hours prior to assay. As shown in Fig. 4-9, the same general trend of lower  $R_{te}$  in the presence of H-elec was observed in the presence of amiloride or EIPA. There was, however a modest, although not statistically significant, trend toward an amiloride-associated increase in  $R_{te}$  in the presence apical H-elec. These results might be construed to indicate that an amiloride-sensitive ion channel (e.g., ENaC) is present in these cells. However, it must be pointed out that the basal ion transport was not different between these treatment groups (not shown). Additionally, it was reported previously (Sjaastad et al., 1993; Schmidt et al., 2001) that amiloride-sensitive current is readily observed in corticosteroid-treated monolayers, but not in untreated monolayers, as were used in the current study. Thus, the results suggest that the effect of apical H-elec to decrease  $R_{te}$  is not mediated by a cytosolic event that depends on the entry of Na<sup>+</sup> into the cell by either ENaC or NHE.

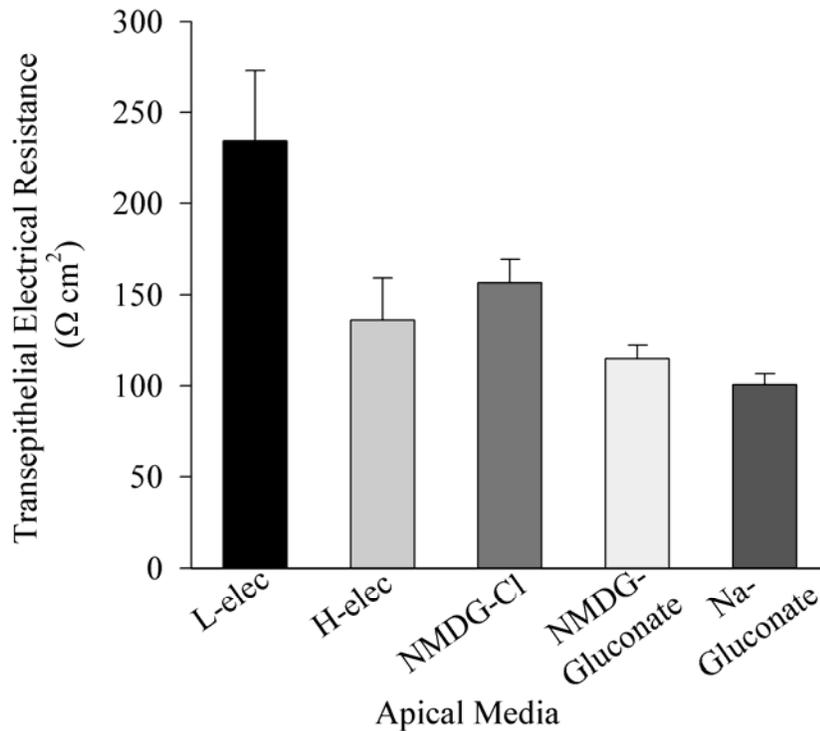


**Figure 4-9 Effects of H-elec medium to reduce epithelial barrier integrity are not changed by amiloride or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA).**

Pharmacological inhibition of Na<sup>+</sup> movement via the channel blocker, amiloride, or the transport blocker, EIPA, demonstrate the same pattern of decreased  $R_{te}$  across the monolayers treated apically with high electrolyte medium. Paired cell monolayers were cultured for 14 days in the presence of H-elec or L-elec medium (see METHODS) and, for the final 24 hours, in the absence or presence of apical amiloride or EIPA. Results are summarized from 5 experiments that included all treatments.

***Substitution of impermeant ions fails to affect medium-associated changes in epithelial barrier function.*** The hypothesis that electrolyte permeation into the epithelial cells contributed to the change in  $R_{te}$  was tested further by conducting ion substitution experiments. Monolayers were cultured in the presence of apical L-elec until the day before assay when the apical solution was changed to reduce apical lactose concentration and increase apical electrolyte concentrations with permeant monovalent ions (Na<sup>+</sup>, Cl<sup>-</sup>) or monovalent ions (N-methyl-D-glucosamine [NMDG<sup>+</sup>], gluconate) that are typically considered to be impermeant. Results presented in Fig. 4-10 demonstrate that the  $R_{te}$  is significantly lower in all monolayers exposed to reduced lactose/increased electrolytes in the apical medium. The decrement in  $R_{te}$  at first appears greater in H-elec than in the presence of NMDG-Cl<sup>-</sup>, although this difference is not statistically significant. Furthermore, NMDG-gluconate replacement of both Na<sup>+</sup> and Cl<sup>-</sup>, is associated with a

greater decrement in  $R_{te}$ . Taken together, results presented in Figs. 4-9 and 4-10 suggest that either an increase in extracellular apical electrolyte concentration or a decrease in lactose concentration is associated with a significant change in  $R_{te}$  although permeation of electrolytes into the cells is not required for the effect to be observed. Thus, alternative experiments must be designed and conducted to identify stimuli and/or signaling pathways that modulate the mammary epithelial barrier.

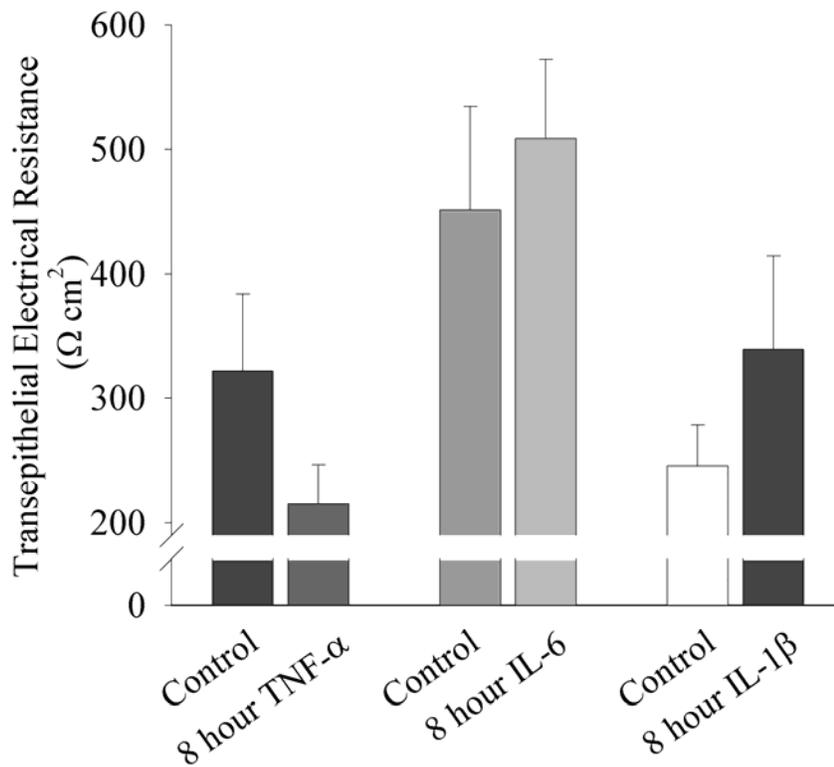


**Figure 4-10 High concentrations of impermeant or permeant monovalent ions similarly affect epithelial integrity.**

NMDG, an impermeant cation, was substituted for  $\text{Na}^+$ , and gluconate, an impermeant anion, replaced the  $\text{Cl}^-$  in various combinations. Overall,  $R_{te}$  was reduced in the monolayers exposed to higher electrolyte concentrations in the apical compartment as compared to those exposed to low electrolyte apical composition. Results are summarized from 5 experiments that included all treatments.

***TNF- $\alpha$  decreases BME-UV monolayer barrier function.*** Chemicals derived from inflammatory cells, including the cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, are increased under pathological conditions in the bovine mammary gland and may directly or indirectly affect epithelial cell function. Experiments were conducted to test for effects of these inflammatory cytokines on mammary epithelial integrity via analysis of  $R_{te}$ . Exposure to TNF- $\alpha$  for 8 hours, a

time point at which these inflammatory cytokines have been detected to be elevated *in vivo*, was associated with a 30% decrease in  $R_{te}$  (Fig. 4-11). Twelve hour exposure to TNF- $\alpha$  was associated with more variable results that were not significantly different from the control (not shown). No significant effect on  $R_{te}$  was noted after exposure to IL-1 $\beta$  or IL-6 (Fig 4-11). Likewise, increased time of exposure, at 24 and 30 hours, demonstrated no effect on  $R_{te}$  (data not shown). These observations demonstrate the importance of examining each of these effects both separately and together to determine the overall effect of challenge to the gland within the optimal physiological context.



**Figure 4-11 TNF- $\alpha$  directly alters  $R_{te}$  in bovine mammary epithelial cells.**

Confluent BME-UV monolayers were exposed to 0.5  $\mu\text{g/mL}$  TNF- $\alpha$ ,  $\mu\text{g/mL}$  IL-6 or 20  $\mu\text{g/mL}$  IL-1 $\beta$  for 8 hours prior to assay. A 30% decrease in  $R_{te}$  was evident in monolayers exposed to the inflammatory cytokine TNF- $\alpha$  for 8 hours, whereas no significant difference was identified in the IL-6 or IL-1 $\beta$ -treated monolayers. Results represent 5-6 experiments for each cytokine and separate control monolayers accompanied each cytokine.

## DISCUSSION

Mammary epithelial cells respond to differences in the environment in the apical (milk) compartment with rapid changes in the barrier function of the epithelium. To our knowledge, we were the first to demonstrate an association between apical solute composition and mammary epithelial barrier function with an *in vitro* system (Schmidt et al., 2001). These observations have been systematically extended to characterize the timecourse over which the changes occur and to determine that the barrier can be modulated to either higher or lower resistivity shown in three ways:  $R_{te}$ , dextran permeation, and occludin localization. In addition, the new data show that there can be direct modulation of the mammary epithelial barrier by certain cytokines. These results indicate that mammary epithelial tight junctions can be modulated by a variety of solutes expected to be present in bodily fluids bathing the apical and/or the basolateral aspect of these cells. Inferences can be drawn that relate to nutrition, child nurturing, dairy production/management, and perhaps to breast cancers. Much work has been done to examine the modulation of junctional proteins in small animal models and in mammary carcinoma cells *in vitro*, however the mechanisms of tight junction modulation in the healthy gland are less defined (Firestone and Yamamoto, 1983; Zettl et al., 1992; Nguyen and Neville, 1998; Neville et al., 2001; Stelwagen and Callaghan, 2003). Additionally, the *in vitro* systems that have been used previously are suboptimal representations of the physiological environment because they employed solid supports and/or symmetrical buffers. This project provides a model system representative of mammary epithelium, and critical information that can be extrapolated to other mammary model systems to improve their physiological relevancy to the study of both normal and compromised mammary epithelial integrity.

BME-UV cells provide the basis for an *in vitro* system to characterize mammary epithelial cell function. The original description included the claim that these immortalized cells exhibited many characteristics of mammary epithelia including a cobblestone appearance when grown on a solid supports, cytokeratin production, and synthesis of  $\alpha$ -lactalbumin and  $\alpha_{s1}$ -casein (Zavizion et al., 1996b). It has since been reported that BME-UV cells respond to a variety of hormones and pharmacological agents with changes in cell signaling, cellular metabolism, proliferation, and apoptosis (Zavizion et al., 1996a; Zavizion et al., 1998; Cheli et al., 2003; Gajewska and Motyl, 2004; Zarzynska et al., 2005). It has also been shown that BME-UV cells

grow on a permeable support system to form a confluent polarized monolayer that is capable of regulated ion transport (Schmidt et al., 2001). The fact that these cells form a polarized and electrically tight epithelial monolayer provides opportunities to study both barrier function and ion transport across the mammary epithelium in a way that is not possible in the native gland, with freshly isolated cells, or in a 3-dimensional culture system.

Leaders in the field have noted that the mammary epithelium is a structure with complex geometry and, therefore, have suggested that an *in vitro* model such as this would be amenable to study with powerful techniques such as the Ussing chamber (Shennan and Peaker, 2000). The BME-UV cell system is amenable to tightly controlled systematic experimentation. Optimization of the system by Schmidt et. al. (Schmidt et al., 2001) demonstrated the benefit of a milk-like apical medium. The current report utilizes the polarized monolayer configuration and demonstrates that BME-UV cells exhibit functional responses to changes in electrolyte composition and to cytokines that would be predicted based upon *in vivo* observations. The timecourse of these changes is important to understanding the progression of epithelial barrier modulation within the mammary gland.

The results indicate that mammary epithelial integrity is modulated by changes in the electrolyte or carbohydrate concentration in the apical medium. Three separate and complementary techniques were employed to document differences between the treatments. First,  $R_{te}$  was employed as a rapid and sensitive indication of the ease with which small charged solutes, especially monovalent ions, move through the epithelium. Since the lactating mammary gland secretes a high volume (30 - 35 kg/d in dairy cows) of isosmotic solution, one might expect the epithelium to exhibit a relatively low resistance. However the composition of milk is distinctly different from serum, which might suggest the presence of a 'tight' epithelium. Furthermore, it was reported that cultures of freshly isolated mammary epithelial cells exhibited greater than  $1000 \Omega\text{cm}^2$  (Smits et al., 1996), although the contribution of the substrates were not reported. Thus, the previous report (Schmidt et al., 2001) that BME-UV achieved a maximal  $R_{te}$  of  $<200 \Omega\text{cm}^2$  was somewhat surprising and suggested that optimal culture conditions had not been fully defined. The hypothesis that a 'milk-like' medium on the apical face of the monolayer would enhance resistance was tested and determined to be correct. The goal was to create a medium that was low in total electrolytes, relatively high in  $\text{K}^+$ , relatively high in lactose, and isosmotic with the basolateral medium. There were, however, certain constraints that guided the

apical medium construction. First, one cannot remove a solute and maintain osmolarity. Hence, there is not an experiment to test for effects of changing electrolyte concentrations without a concomitant change in carbohydrate to maintain osmolarity. A previous report shows that similar results are obtained when either mannitol or lactose are used as the compensating osmolyte when electrolytes are reduced (Schmidt et al., 2001). Additionally, the optimal medium defined by Zavizion et al. (Zavizion et al., 1996b), included 15% serum, which includes a substantial amount of  $\text{Na}^+$  and  $\text{Cl}^-$ . It was determined that a systematic evaluation of serum withdrawal was beyond the scope of the current studies. Rather, the current study focused on the timecourse over which medium-induced differences in  $R_{te}$  could be observed.

In every experimental block, recent duration of L-elec exposure is associated with the greatest electrical resistance. Although block to block and experiment to experiment variation in the maximal resistance was observed, continuous exposure to H-elec is associated with the lowest  $R_{te}$  in every block. It was previously reported that BME-UV cell monolayers reach a maximal plateau in  $R_{te}$  between 7 and 10 days of culture that is maintained consistently to at least day 14 (Schmidt et al., 2001). Thus, the observations that the transition from apical L-elec to H-elec for as little as one day at the end of the 14 day culture period results in a >25% reduction in  $R_{te}$  is striking. The results suggest that high electrolyte/low carbohydrate at the apical membrane causes a change in the epithelium that permits gradient-driven flux of small charged solutes, either through transcellular or paracellular routes. Similarly, a one day transition to L-elec can result in a doubling of  $R_{te}$ . The pathway(s) that are affected by the change in apical composition can be readily modulated in either direction. Furthermore, the response appears to be complete or nearly complete within four days. There is a four-fold reduction in  $R_{te}$  with four days of apical H-elec exposure and there is a four-fold increase in  $R_{te}$  with four days of apical L-elec exposure. This time frame for changes in barrier function is important because it parallels the period over which substantial changes are seen in milk composition and in mammary function at the initiation of lactation, at the initiation of involution, and in the course of mastitis.

Dextran permeation across the BME-UV monolayer indicates flux through the paracellular pathway. The electrical parameters described above are blind to transcellular versus paracellular movement and are insensitive to the movement of any non-ionic solute. Numerous mechanisms, both transcellular and paracellular, have been described to account for solute movement across mammary epithelium (Broughman et al., 2004). Thus, a protocol was

conducted to assess flux through the paracellular pathway. The experimental design tested for changes in paracellular permeation that paralleled the changes observed in electrical measurements and assessed the approximate size of the solutes that could readily traverse the epithelium. The results show that the BME-UV cells can be a formidable barrier to the diffusional movement of large solutes, with basal permeation rates that are similar to a canine kidney epithelial cell line (MDCK), and to T84 (colonic origin) and Calu-3 (airway origin; (Broughman et al., 2004) and unpublished observations). The experimental results provide compelling evidence that changes in the apical medium affect the paracellular pathway with a timecourse that is similar to that observed for the effects on  $R_{te}$ . One day of exposure to H-elec is associated with a doubling in the rate of permeation by both the small (9.5 kDa) and large (77 kDa) dextran molecules. Paralleling the changes observed for  $R_{te}$ , dextran permeation was incrementally greater after longer periods of H-elec exposure. The results with 7 or more days exposure to L-elec are consistent with the  $R_{te}$  measurements in that 7 days exposure to L-elec produces a significantly greater barrier to solute flux and that, within these experiments, no additional incremental changes are associated with additional exposure to L-elec. Seventy-seven kDa dextran was selected because of its similarity in size to serum albumin (~66 kDa). Thus, one can infer from these results that changes in the paracellular pathway that are caused by the modified apical medium are sufficient to allow for the permeation of albumin into the milk. Both the appearance of albumin and the elevation in electrolytes have been used as clinical measures of mastitis (Linzell and Peaker, 1975; Fernando et al., 1985; Nielen et al., 1995). The fact that milk conductivity is used as an early indicator of mastitis suggests, but does not demonstrate, that changes in milk electrolyte composition precede changes in the epithelial barrier integrity. Thus, the current results provide impetus to conduct systematic measurements in milk electrolyte concentration and serum albumin content at the onset of mastitis.

Immunocytochemistry revealed that occludin, but not ZO-1, was absent from the expected apical lateral location following exposure to H-elec. Systematic metabolic assays were not conducted, so no inferences can be drawn regarding the underlying mechanisms that are affected. However, there appears to be a consistent amount of overall occludin immunoreactivity in the epithelial cells following H-elec exposure when examined via Western blot analysis, but less localization of occludin at the level of the tight junctions. The possibility that the H-elec treatment caused a general redistribution of proteins or modified overall cell structure is ruled out

by the observation that ZO-1 distribution and intensity were unaffected by the H-elec treatment, whereas specific redistribution of occludin protein was apparent. It is intriguing that a similar kinetic profile is observed for the decrease in resistance, increase in permeability, and decrease in occludin immunoreactivity. The results clearly show that these modifications in epithelial function occur concurrently during exposure to H-elec. Additionally, it is shown that transition to L-elec affects all three parameters ( $R_{te}$ , dextran permeation, occludin distribution) in the opposite direction of that seen with H-elec, and with similar timecourse.

The magnitude and duration of exposure to altered apical medium composition required to cause a change in epithelial integrity or occludin distribution remains to be determined. The current results show that an abrupt and substantial change in apical medium composition causes a change in barrier  $R_{te}$ , dextran permeation and occludin distribution that can be readily observed within one day. However, one would not necessarily expect to observe such abrupt changes in milk composition in a physiological or clinical setting, although mammary saline infusion has been employed in a research setting (Shennan and Peaker, 2000). Furthermore, ion transport mechanisms that are present in mammary epithelium and account for electrolyte movement into or out of the milk compartment remain to be defined along with their associated regulatory cascades. Knowledge of these mechanisms is required to establish pharmacological targets that can be used to modulate milk electrolyte composition *in vivo*. Evidence has been provided for the anion channel that is mutated in cystic fibrosis (CFTR) and for ENaC (Blaug et al., 2001; Schmidt et al., 2001) in mammary epithelial cells along with regulation by both hormones and neurotransmitters. The magnitude or rate by which milk electrolyte composition can change, however, has not been determined. Clearly, additional experiments are required to test for effects of more subtle changes in apical composition on epithelial function.

A mechanistic link between apical medium composition and tight junction integrity has not been ascertained. The initial supposition was that either  $\text{Na}^+$  or  $\text{Cl}^-$  in the apical medium might affect membrane electrical potential or, by movement through channels or transporters, cytosol composition to precipitate a change in cell function. This hypothesis was tested both pharmacologically and by substitution with impermeant ions. The results, however, were not particularly instructive except to indicate that these hypotheses were inadequate. Likewise, an osmotic effect is ruled out by meticulous care to insure that all media were isosmotic (290 mOsm). The possibility that a high apical lactose concentration is required to enhance barrier

integrity is ruled out by a previous report, which showed that similar results were obtained with mannitol as the compensating osmolyte (Schmidt et al., 2001). That a mechanism is not currently revealed does not diminish the impact of the observations that changes in apical composition affect epithelial integrity. Rather, these observations provide additional impetus to identify players in the regulatory cascade.

Times when luminal electrolyte composition would be expected to change, including parturition and involution, are associated with increased incidence of infection, inflammation, and cytokine recruitment (Convey, 1974; Kitchen, 1981; Buse et al., 1995; Heyman and Desjeux, 2000; Neville et al., 2001; Alluwaimi, 2004). Cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, have been reported to modulate the mammary epithelial barrier *in vivo* (Zhu et al., 2004). Intramammary TNF- $\alpha$  infusion is associated with the recruitment of neutrophils, decreased milk proteins, and the appearance of serum proteins in milk (Watanabe et al., 2000). TNF- $\alpha$  has also been shown to downregulate occludin expression in other cell systems (Wachtel et al., 2001), and to alter Na<sup>+</sup> and Cl<sup>-</sup> movement across epithelial barriers (Marano et al., 1998). Whether the effects are direct or indirect, however, is still open to question. The results presented in this manuscript indicate that TNF- $\alpha$  has a direct effect on mammary epithelial cells to cause a breakdown in the barrier function, with the same time course that has been observed *in vivo*. Alternatively, the results failed to support a direct role for IL-1 $\beta$  or IL-6 in modulation of  $R_{te}$  in mammary epithelium. It is important to note that the conditions used in the present assays were not selected to mimic any particular clinical study. Thus, the discordance may reflect differences in the health status of the mammary gland that was used relative to the *in vitro* cell system. The results demonstrate that the BME-UV cell system is sensitive to selected cytokines and will provide an excellent system to delineate the cellular mechanisms that are involved in the response.

The current results provide a new factor, luminal electrolyte composition, that must be considered as one investigates mammary epithelial function. Changes in epithelial function can occur rapidly and do not require ion permeability into the cells. Rather, mammary epithelial monolayers are extremely sensitive to changes in luminal fluid composition, which is one of the earliest measurable indicators of mastitis. This evidence is complimented by observations that lowering the apical ion concentration leads to an increase in  $R_{te}$ , indicating enhanced barrier function. These results identify new factors that contribute to the progression of mastitis damage

in the mammary gland, and offer hope for new targets to circumvent (prevent) or treat inflammatory disease within the mammary gland. Reducing direct damage to the epithelium via altering cytokine effects, decreasing electrolytes, or modulating ion transport mechanisms may provide new options for investigation and ultimately for intervention. Work toward understanding the mechanism(s) of action that leads to changes in luminal ion concentration, cytokines, and epithelial remodeling will provide novel targets for prevention and treatment of mastitis at the earliest stages of the disease.

In summary, the current results demonstrate, with an *in vitro* model, that cytokines and apical electrolytes rapidly affect the epithelium that separates milk from blood. The epithelial barrier function is directly modulated by the local environment and some cytokines. These data provide information for those working with other *in vitro* mammary models to improve the quality of those systems by more closely representing the *in vivo* environment. Most importantly, the data indicate that there can be a feedback mechanism whereby the composition of milk that is secreted by the epithelium can affect barrier integrity. This observation provides impetus to define the cellular mechanisms that account for the low electrolyte content of milk and to seek interventions to therapeutically target the activity of these mechanisms.

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